The migration and developmental remodeling of intrinsic interneurons in visual thalamus and the role of retinal signaling.

Naomi E. Charalambakis
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THE MIGRATION AND DEVELOPMENTAL REMODELING OF INTRINSIC INTERNEURONS IN VISUAL THALAMUS AND THE ROLE OF RETINAL SIGNALING

By

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B.S., University of Kentucky, 2013
M.S., University of Louisville, 2017

A Dissertation
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DEDICATION

This dissertation is dedicated to my parents,

John and Fedra Charalambakis
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First and foremost, I would like to thank my mentor Dr. William Guido, my biggest advocate, and someone who has truly made me a better scientist and person over the last five years. I am tremendously grateful that he made both my research and career development a top priority. A big thank-you to the members of my committee: Dr. Martha Bickford, Dr. Robin Krimm, Dr. Aaron McGee, and Dr. Michael Fox. Your advice and support throughout this process has pushed me to be a stronger thinker. I would not be here today without the help and support from all the members of Dr. Guido’s lab: Peter Campbell, Guela Sokhadze, Govin Govindaiah, and Barbara O’Steen. Working together over these years has made it clear that I not only learned important techniques from these wonderful people, but I also made life-long friends. I will treasure all the laughs and memories. Thank you to Donna Bottorff in the department office, someone who has seen me laugh, cry, and panic within mere hours; thank you for all your help and support. Finally, I would like to thank my parents, John and Fedra Charalambakis, for loving me when I least deserve, and supporting me through thick and thin. You are my rock. To my siblings, Joel, Debbie, and Cassandra, I love you all dearly and am undeserving of your love and patience all these years. It’s been a joy to walk this life with my family by my side.
ABSTRACT

THE MIGRATION AND DEVELOPMENTAL REMODELING OF INTRINSIC INTERNEURONS IN VISUAL THALAMUS AND THE ROLE OF RETINAL SIGNALING

Naomi E. Charalambakis

October 16, 2018

The dorsal lateral geniculate nucleus (dLGN) of the mouse is a model system to study the development of thalamic circuitry. While most studies focus on relay neurons of dLGN, little is known about the factors regulating the development of the other principal cell type, intrinsic interneurons. To date, the targeting and migratory path of dLGN interneurons as well as their morphological development remains unclear. Here we examined whether the migration, structure, and function of interneurons relies on retinal signaling. We took a loss-of-function approach and crossed GAD67-GFP mice, which express green fluorescent protein (GFP) in dLGN interneurons, with math5 nulls (math5−/−), mutants that lack retinofugal projections. Tracing studies with BDA show that interneurons migrate from the third ventricle, and arrive in dLGN at early postnatal ages. By P4, neurons complete the first phase of a multi-staged developmental process, as interneuron number and distribution reaches an
adult-like state. *In-vitro* recordings and 3-D reconstructions of biocytin-filled interneurons at different ages showed that remodeling begins during the second week, and includes a period of exuberant branching, where arbors grow in number, complexity, and field size. Such growth is followed by pruning and stabilization, as interneurons adopt their adult-like bipolar architecture and form functional connections with relay cells. However, the absence of retinal signaling disrupts these processes. Fewer interneurons occupy dLGN, and cells fail to disperse. Estimates of interneuron number in thalamic nuclei along the migratory path in math5−/− mice suggest that the absence of retinal input causes misrouting of dLGN interneurons into the ventrobasal complex (VB). In fact, studies in Fibroblast growth factor 15 null (Fgf15−/−) mice, a neurotrophic factor responsible for GABAergic differentiation, show Fgf15 regulates interneuron targeting to dLGN. Arbor remodeling is also disrupted in the absence of retinal signaling, as interneurons maintained a sparse architecture at all ages. DHPG experiments in math5−/− mice, the metabotropic glutamate receptor 1,5 (mGluR1,5) agonist that targets F2 terminals on interneuron processes, revealed interneurons exhibited fewer/and or weaker synapses with relay neurons in dLGN. Taken together, these data suggest that retinal signaling is needed to support the targeting, arbor elaboration, and synaptic connectivity of dLGN interneurons.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS................................................................. iv
ABSTRACT....................................................................................... v
LIST OF FIGURES................................................................. viii

INTRODUCTION.................................................................................. 1

CHAPTER I – Retinal Signaling Regulates the Targeting and Migration of Intrinsic Interneurons to the Dorsal Lateral Geniculate Nucleus
Introduction ................................................................................ 8
Materials and Methods................................................................. 12
Results....................................................................................... 17
Discussion..................................................................................... 52

CHAPTER II – Developmental Remodeling of Thalamic Interneurons Requires Retinal Signaling
Introduction ................................................................................ 58
Materials and Methods................................................................. 61
Results....................................................................................... 66
Discussion..................................................................................... 91

SUMMARY AND CONCLUSIONS .................................................. 95

REFERENCES................................................................................ 99

CURRICULUM VITA................................................................. 107
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Green fluorescent protein (GFP) expressing interneurons in mouse thalamus of WT and math5⁺ mice</td>
<td>31</td>
</tr>
<tr>
<td>2. BDA injections near the third ventricle (V3) and in superior colliculus (SC) to target the presumed migratory streams of interneurons</td>
<td>32</td>
</tr>
<tr>
<td>3. Pattern of interneuron migration and distribution in dLGN of WT and math5⁺ mice</td>
<td>34</td>
</tr>
<tr>
<td>4. Estimates of the number of interneurons and their distribution in dLGN of WT and math5⁺ mice</td>
<td>36</td>
</tr>
<tr>
<td>5. Observation of caspase-3 labeling in interneurons of WT and math5⁻ mice</td>
<td>37</td>
</tr>
<tr>
<td>6. Pattern of interneuron migration in the ventrobasal complex (VB) of WT and math5⁺ mice</td>
<td>38</td>
</tr>
<tr>
<td>7. Interneuron distribution in vLGN of WT and math5⁺ mice</td>
<td>40</td>
</tr>
<tr>
<td>8. Estimates of the number and distribution of lateral posterior nucleus (LPN) interneurons</td>
<td>42</td>
</tr>
<tr>
<td>9. Organization of thalamic interneurons in WT and math5⁺ mice</td>
<td>44</td>
</tr>
<tr>
<td>10. Interneuron number and dispersion in WT and Fgf15⁺ mice</td>
<td>46</td>
</tr>
<tr>
<td>11. Estimates of the number of VB interneurons in Fgf15⁺ mice</td>
<td>48</td>
</tr>
</tbody>
</table>
12. Fewer vLGN interneurons in Fgf15−/− mice .......................................................... 50
13. Green fluorescent protein (GFP) expressing interneurons in dorsal lateral geniculate nucleus (dLGN) of WT and math5−/− mice ........................................ 73
14. Biocytin filled interneurons in dLGN of WT and math5−/− mice ...................... 75
15. Morphological development of interneurons in WT and math5+/− mice ........... 77
16. Intrinsic membrane properties WT and math5+/− interneurons .................... 79
17. Passive and active membrane properties of WT and math5+/− interneurons .... 81
18. Pattern of spontaneous inhibitory activity in relay neurons in WT and math5+/− mice ........................................................................................................ 83
19. IPSC frequency and amplitude response before and after mGluR5 activation in WT and math5−/− relay neurons .............................................. 85
20. Development of interneurons in WT and math5+/− mice .............................. 87
21. Frequency distribution of soma size, $V_{sag}$, and spike firing of WT interneurons ........................................................................................................ 89
INTRODUCTION

In the past decade, the mouse has surfaced as the model system of choice to examine the establishment and functional processes of visual thalamic circuits. One reason is because the mouse retinogeniculate pathway is highly conserved. Despite low acuity, the mouse exhibits parallel visual pathways that are well-separated, similar to higher mammals such as the cat and macaque (Reese, 1988). Secondly, the recent surge in new genetic tools such as transgenic mouse lines has provided unmatched access to specific cell types and circuits. For the first time, we are able to isolate and manipulate individual cells within the visual pathway to understand their development, structure, and function. Finally, the mouse affords us the ability to study vision in combination with behavioral function. Now with molecular tools and genetically-labeled mouse lines, studies can conceivably link particular behaviors and functions with the circuits and cells actively involved. Taken together, the mouse model has transformed the field of neuroscience, and will continue to advance our understanding of the visual thalamus.

In the mouse, the axons of retinal ganglion cells (RGCs) form the optic nerve/tract to exit the eye and project to the dorsal lateral geniculate nucleus (dLGN). These projections are highly organized to deliver an accurate
representation of the contralateral visual hemifield (Grubb and Thompson, 2003; Pfeiffenberger et al., 2006). Although mice do not have distinct laminae separating contralateral and ipsilateral eye projections as seen in carnivores and primates, retinal afferents in rodent are still organized into separate eye-specific domains (Reese, 1988). Another example of such conserved circuitry exhibited in the mouse is cell-type specificity and regional preferences in dLGN. Similar to all higher-order species, mice exhibit 2 primary cells: excitatory thalamocortical relay cells and inhibitory interneurons. Relay neurons are responsible for directly transferring visual signals from the retina to visual cortex (Martin, 1986), whereas interneurons remain intrinsic to dLGN and function as the neural stop-signs of the nucleus, ensuring retinogeniculate transmission remains balanced and linear by operating through feedforward connections (Williams et al., 1996; Sherman, 2004). Currently, we understand more about the development of relay neurons, whereas many more unanswered questions remain about dLGN interneurons. However, for both cell types, recent research has revealed numerous examples of the mouse’s conserved visual circuitry, and has demonstrated why the mouse serves as a tractable experimental platform to understand the underlying mechanisms of vision.

Relay cells of the dLGN are characterized by their type I/Class A morphology, bearing a thick axon and multiple, complex dendrites emanating from its large, round soma. Additionally, analysis of their dendritic architecture reveal relay neurons are classified into three classes based on morphology—X-, Y-, and W-cells—similar to what’s been found in the cat (Friedlander et al., 1981;
Stanford et al., 1981; Krahe et al., 2011). In fact, each class occupies specific regions within dLGN (Krahe et al., 2011). For example, W-cells exhibit a hemispheric structure, and reside along the dorsolateral, “shell” region of the nucleus (Bickford et al., 2015), the homologue of the C-laminae in cat and where most W-cells are found (Stanford et al., 1981; Sur and Sherman, 1982). Y-cells possess a concentric dendritic architecture, and occupy the central core region of dLGN that is binocularly innervated. Finally, X-cells have a biconical dendritic tree, and primarily reside in the ventral sector of dLGN. Such region specificity is a reflection of the projection patterns of difference RGC types. For example, direction-selective ganglion cells (DSGCs) largely project to the dorsolateral shell onto W-cells, and OFF-alpha transient ganglion cells connect with Y-cells in the core (Martin, 1986; Dhande et al., 2015).

Developmentally, relay neurons experience two growth spurts (Parnavelas et al., 1977; El-Danaf et al., 2015). The first takes place during the first postnatal week when dendritic branching and complexity increases. In fact, by the end of week 1, neurons have adopted their adult-like morphology and class specification. The second growth spurt occurs during the second and third postnatal week when the size of the dendritic field and the area of dLGN increases, with little change in branch number or complexity. There is also strong evidence suggesting that such growth is, in part, regulated by retinal innervation. One report studied relay cell development in a mouse that is devoid of retinal innervation, and discovered that their dendritic growth and maturation is disrupted. Although neurons maintain cell-class specificity, they also experience
an exuberant branching period, followed by branch elimination and a decline in dendritic field size (El-Danaf et al., 2015).

Our understanding about the other primary cell type, intrinsic interneurons, is an emerging story. Exhibiting a Type II/Class B morphology with spindle-shaped somata, dLGN interneurons form connections only with other dLGN neurons, and function to modulate excitatory signals from the retina by providing feedforward inhibition. Interneurons accomplish this by refining the center-surround receptive field properties of relay cells, sharpening the timing of excitatory responses, and shaping the spatial and temporal features of the visual scene (Sherman, 2004; Blitz and Regehr, 2005; Hirsch et al., 2015). Moreover, interneurons are equipped with unique structural features that allow them to operate as local, independent processors. Similar to other higher mammals, in addition to a typical axonal output (F1 terminals), embedded within interneuron dendritic trees are distal processes with grape-like appendages, the presumed site of F2 terminals and location of dendro-dendritic synapses. Oftentimes, F2 terminals participate in unique, triadic synaptic arrangements that involve a relay cell dendrite, F2 terminal, and retinal axon in contact with one another (Sherman, 2004; Bickford et al., 2010). Such inhibitory activity is electrically isolated from the soma, thus affording interneurons with the ability to integrate synaptic inputs locally (via dendrites) and/or globally (via axon) (Sherman, 2004; Govindaiah and Cox, 2006). This feedforward inhibition becomes apparent around the time of eye-opening (~P14), and is comprised of a GABA_A and GABA_B component. Such activity corresponds to the synaptic development of the mouse dLGN, as its
ultrastructure reaches adult-like patterns by P14 (Bickford et al., 2010).

Furthermore, the synaptic organization of the mouse is highly conserved when compared to the make-up of other species such as rat, cat, and monkey (Wilson and Hendrickson, 1981; Gabbott et al., 1986; Montero, 1991).

There is evidence to suggest that there are two classes of interneurons, similar to relay cell class specification. In rat, interneurons have been classified based on NADPH Diaphorase immunoreactivity. Those that stained positive only for GABA are called B1 neurons, while cells that were both diaphorase and GABA positive are called B2 neurons (Gabbott and Bacon, 1994). Similarly, a study in cat demonstrates that dLGN interneurons can be classified based on brain nitric oxide synthase (BNOS). BNOS-stained interneurons were consistently found in the C-laminae (the homologue to the mouse dorsolateral tier), and possess larger somas compared to BNOS-negative interneurons. Additionally, ultrastructure analyses revealed that BNOS-positive interneurons participate in dendrodendritic synapses in extraglomerular neuropil, whereas their counterparts function within synaptic glomeruli (Hamos et al., 1985; Bickford et al., 1999). A recent study in mouse also suggests that dLGN interneurons are comprised of two classes. Interneurons are classified based on soma diameter, with smaller ones having a higher density of I_H channels and higher rates of firing compared to larger ones (Leist et al., 2016). While the issue of interneuron classification remains open to debate, information thus far provides support for the use of the mouse as a model system to study visual thalamic development.
As research continues to evolve about dLGN interneurons, it is worth emphasizing that compared to relay neurons, far less is known about their development, or the factors that influence their maturation. Such investigation has been hampered by the lack of available tools. In the past, researchers had difficulty visualizing interneurons, especially given the fact that in rodents, these cells comprise only 5-10% of neurons in dLGN (Arcelli et al., 1997; Evangelio et al., 2018). However, the mouse model has moved to the forefront of neuroscience research, and the advent of mouse transgenics affords us the ability to accomplish what we could not before. The discovery of the GAD67-GFP strain, a transgenic mouse line that expresses GFP in dLGN interneurons, allows us to visualize and target these cells for anatomical and electrophysiological examination. Because this expression is seen as early as embryonic age 15 (E15) (Tamamaki et al., 2003) it also permits for tracking progenitors as they differentiate into interneurons and migrate to target structures (Golding et al., 2014). Loss-of-function mouse models further provide the ability to isolate the cells and circuitry of different inhibitory pathways, and learn about their development and underlying mechanism (Seabrook et al., 2013; Golding et al., 2014; Jager et al., 2016). Thus, to address the remaining unknowns about dLGN interneurons, we took advantage of development of sophisticated genetic tools, and used the GAD67-GFP mouse to study the migratory path, distribution, morphological development, and synaptic connectivity of dLGN interneurons. Utilizing genetic knockout mice with the math5 null (math5\(^{-}\)) and Fgf15 null (Fgf15\(^{-}\)) mice allowed us to assess how retinal signaling influences these
developmental processes, and its underlying mechanism, respectively. Taken together, these studies will shed light on the establishment and integration of intrinsic interneurons into thalamus, and the factors that influence its timing and patterning.
CHAPTER I

RETINAL SIGNALING REGULATES THE TARGETING AND MIGRATION OF INTRINSIC INTERNEURONS TO THE DORSAL LATERAL GENICULATE NUCLEUS

Introduction

Much of our understanding about the development, form, and function of thalamic circuits is derived from work done in the dorsal lateral geniculate nucleus (dLGN) of the mouse (Jaubert-Miazza et al., 2005; Huberman et al., 2008; Krahe et al., 2011; Hong et al., 2014). The dLGN serves as a relay of visual signals between the retina and visual cortex, and is comprised of two primary cell types, excitatory thalamocortical relay cells and intrinsic inhibitory interneurons. While both types are innervated by the retina, only relay neurons have axons that exit dLGN and project to visual cortex. Interneurons and their processes are intrinsic to dLGN and form feedforward inhibitory connections with relay cells (Guillery and Sherman 2002; Seabrook et al., 2013; Hirsch et al., 2015). This inhibitory circuit serves to limit the spatial and temporal spread of retinally-evoked excitatory activity in dLGN. Such activity results in the sharpening of receptive field properties of relay neurons, and improving the
contrast-gain of retinogeniculate signal transmission (Norton and Godwin 1992; Sherman, 2004; Blitz and Regehr, 2005; Hirsch et al., 2015).

To date, relay neurons have been the principal focus for understanding thalamic operations, but the generation of a transgenic GFP mouse line that labels thalamic interneurons has led to a number of important discoveries about this cell type (Acuna-Goycolea et al., 2008; Seabrook et al., 2013; Golding et al., 2014). The biggest advances involve their migration to dLGN, and the assembly of inhibitory circuits (Munsch et al., 2005; Seabrook et al., 2013; Golding et al., 2014). dLGN interneurons originate from the neuroepithelium of the third ventricle, and travel through ventral thalamus to arrive in dLGN (Golding et al., 2014). However, a recent report has challenged these findings, asserting that interneurons arise exclusively from the dorsal midbrain, and course rostrally before entering dLGN (Jager et al., 2016).

While their migratory path to dLGN is open to debate, their dispersion throughout the nucleus and the establishment of feedforward connections seems more firmly established (Sherman and Guillery, 2002; see chapter II). In part, these events depend on retinal signaling (Golding et al., 2014). Early postnatal optic nerve section models show an abnormal clustering of interneurons, and decreased inhibition in dLGN. However, what remains unanswered is whether their initial migration and targeting to dLGN also relies on retinal signaling or innervation. The molecular mechanism(s) underlying how retinal input guides interneuron migration is also unknown. Recent unpublished observations in the Fox lab suggest that fibroblast growth factor 15 (Fgf15) plays an important role.
Fgf15, a neurotrophic factor involved in GABAergic differentiation (Martinez-Ferre et al., 2016), is expressed in RGCs at ages when retinal axons innervate dLGN (Kurose et al., 2004). Preliminary data from Fox and colleagues show that in the absence of retinal input, there is a 3-fold reduction of Fgf15 in both dLGN and vLGN, suggesting this factor may provide a signal to help guide interneurons to dLGN.

To address these issues, we used the GAD67-GFP strain, mice that express green fluorescent protein (GFP) in thalamic interneurons, to visualize the location of interneurons as they make their way to dLGN. Moreover, by making biotinylated dextran amine (BDA) injections at perinatal ages into presumed sites of interneuron origin, we tracked the location of double-labeled GFP-containing cells, to trace the migratory path of dLGN interneurons. Secondly, we employed a loss-of-function approach by using a form of genetic deafferentation to evaluate if retinal signaling plays a role in the targeting of interneurons to dLGN. A genetic approach provides considerable advantages over postnatal manipulations such as optic nerve section or enucleation. Since the latter are done well after the time retinal axons innervate dLGN (Godement et al., 1984; Guido, 2008) and during the peak period of interneuron migration, it is difficult to rule out if results are caused by trauma or degeneration. By contrast, the math5+ mouse, mutants that lack an optic nerve and central projections (Brown et al., 1998), provides a way to directly test the relationship between retinal axon innervation and dLGN development.
Finally, we began to explore the potential link between retinal signaling and Fgf15 during interneuron migration and distribution by utilizing the Fgf15 knockout mouse (Fgf15\(^{-/-}\)). Mutants show disruptions in the organization of thalamic progenitors (Martinez-Ferre et al., 2016), which allowed us to test how developmental processes such as interneuron migration are impacted by the loss of this neurotrophic factor.
Materials and Methods

Animals

All experiments were conducted in mice of either sex ranging in age between P0-P45. Three strains were used: GAD67-GFP (line G42; Jax stock no. 007677) established on a C57/B6 x CB6F1/J), math5<sup>+</sup> crossed onto GAD67-GFP, and Fgf15<sup>-</sup> mice crossed onto GAD67-GFP. The math5<sup>+</sup> strain was established on a mixed background of C57/B6 and 129/SvEv (Wang et al., 2001; El-Danaf et al., 2015), and Fgf15<sup>-</sup> mice were donated from the Mike Fox lab (VCU), derived from Dr. Steven Kliewer (University of Texas Southwestern) (Wright et al., 2004). All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Tissue harvest

Brain tissue was harvested from mice that were deeply anesthetized by hypothermia (<P5) or 4% isoflurane vapors (>P5). All animals were transcardially perfused with phosphate-buffered saline (PBS, 0.01 M phosphate buffer with 0.9% NaCl) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were excised and post-fixed overnight in 4% PFA, then transferred to PBS.

Interneuron imaging and analysis

Using a vibratome (Leica VT1200S), thalamic sections of 70 µm that included vLGN, dLGN, LPN, and VB were collected, mounted with Prolong Gold (with
DAPI; Invitrogen, catalog no. P36931), and coverslipped for confocal microscopy. Sections were imaged throughout the rostral-caudal extent of thalamus using a multi-photon laser scanning confocal microscope (Olympus, model no. FV1200BX61). A HeNe laser (488 nm) was used to excite GFP fluorescence and visualize interneurons under 10x (0.3 NA) or 20x (0.75 NA) objective lenses at a scanning resolution of 1600x1600 resolution. Optical slices were collected sequentially, with the optimal step size through the Z axis (8.51 µm, 10x; 1.26 µm, 20x). Z-stacked datasets were uploaded into Imaris software (Bitplane, version 8.4.1) and ImageJ (version 1.50h, NIH) for analysis. DAPI was used to delineate the borders of thalamic nuclei, and we observed the migratory path of interneurons through thalamus.

To examine the number of interneurons in different thalamic nuclei, we obtained estimates of cell counts in dLGN, vLGN, LPN, and VB. For each nucleus, we utilized ImageJ to first outline (freehand selection tool) the region of interest. Using the Cell Counter tool, we counted and averaged the number of interneurons in 3 sections through the middle of the nucleus from 3 different mice at several different postnatal ages ranging from P0 to P45.

To determine the distribution of interneurons in dLGN and LPN, we divided each nucleus into “top” and “bottom” tiers. Three straight lines (Straight, Segmented tool) were drawn across the width of the nucleus: the bottom border, the widest point, and the top (Fig. 3A, 9A). The midpoints of each line were then connected. Using the cell counter, the total number of interneurons in each sector was calculated and used to generate a ratio: 

$$\frac{\text{# of cells in top}}{\text{# of cells in bottom}}$$
In order to examine interneurons density in dLGN, first we measured the area of dLGN (analyze tab) \( (\mu m^2) \) and calculated: 

\[
\text{Total \# of interneurons} / \text{dLGN Area.}
\]

To convert these values to whole numbers, we created a proportion to calculate values as a function of 2500 um2: 

\[
\text{total \# of interneurons} / \text{dLGN Area} = x / 2500 \quad \text{(Seabrook et al., 2013)}.
\]

---

**Biotinylated Dextran Amine (BDA) injections**

To identify the migratory path of dLGN interneurons, intracranial unilateral injections of biotinylated dextran amine (BDA) were made in the superior colliculus \( (n = 7 \text{ mice}) \) and along midline near the dorsal aspect of the third ventricle \( (V3) \) \( (n = 4 \text{ mice}) \) at postnatal day zero \( (P0) \) in GAD67-GFP mice. Before injections, all animals were given Carprofen \( (5mg/kg) \), then deeply anesthetized by hypothermia. A glass pipette \( (20-40 \mu m \text{ tip diameter}) \) connected to a picospritzer \( (\text{General Valve Corporation}) \) \( (1-2 \mu l, 2-4 \text{ pulses}) \) containing a 5% solution of BDA \( (\text{Invitrogen}) \) in saline was lowered into V3 \( (\text{from bregma: 3.75 mm posterior, 0.5 mm lateral, 2 mm ventral}) \) or SC \( (\text{from bregma: 4.09 posterior, 0.68 lateral, 1.7 ventral}) \) \( (\text{Atlas of the Developing Mouse Brain, Paxinos}) \). After removal of glass pipette, scalp skin was sealed with tissue adhesive \( (n\text{-butyl cyanoacrylate}) \), and animals were placed on a heating pad until mobile. Carprofen was administered once every 12 hours for 24 hours, and we assessed and administered analgesic need for each animal up to 48 hours after surgery.

At P7, mice were perfused and brains excised as previously described. To visualize BDA-labeled cells, 40 \( \mu m \) sections were collected for streptavidin-546 \( (\text{Invitrogen}) \) immunohistochemistry. First, sections were placed in blocking
medium (0.3% Triton X-100 in filtered PBS) for 30 minutes, and incubated for 1 hour with SA-546 (1% SA546 in filtered PBS). Sections were washed 3 times for 15 minutes each in filtered PBS, and then mounted and coverslipped using ProLong Gold mounting medium containing DAPI (Invitrogen, catalog no. P36931) for confocal microscopy. To image BDA-labeled and double-labeled interneurons, the lasers activating wavelengths 488nm (GFP) and 546 nm (BDA; red) were included in imaging settings. Optical slices were collected sequentially though the entire rostral-caudal extent of dLGN (~6 40 µm sections) using the “Line” mode to prevent channel bleed-through during acquisition.

Caspase-3 immunohistochemistry
To stain for cleaved caspase-3 (Cell Signaling Technology, product #9661), 40 µm sections were placed in blocking medium (10% normal goat serum (NGS) and 0.3% Triton X-100 in filtered PBS) for 30 minutes, and then incubated overnight (at least 18 hours) in primary antibody (1:400 caspase 3 and 1% NGS in filtered PBS). The next day, treated sections were washed 3x in filtered PBS, 15 minutes each. All sections were incubated for 1 hour with the secondary antibody (1:100 goat anti-rabbit AF546 (Invitrogen) and 1% NGS in filtered PBS). Sections were washed 3 times (15 minutes each) before mounted and coverslipped using ProLong Gold with DAPI. Sections from WT and math5−/− were imaged on the confocal using the 546 and 488 lasers to excite red, caspase-3 expressing neurons and GFP fluorescence, respectively. Sections were collected sequentially using “Line” mode to avoid bleed-through with the 10x and 20x
lenses. For each case (n = 1-2 mice per age), 6-8 sections through the rostral-caudal extent of thalamus were imaged, allowing us to estimate the number of double-labeled (GFP+ and caspase-3+) interneurons at different ages.

**Experimental design and statistical analysis**

All statistical tests were conducted using Prism 7.0 (Graphpad Software, La Jolla, CA). A two-way ANOVA was used to examine age-related different within group (GAD67-GFP or math5\(^{-}\) or Fgf15\(^{-}\)) and between groups (GAD67-GFP vs. math5\(^{-}\); GAD67-GFP vs. Fgf15\(^{-}\)) across the various criteria for migration/distribution (i.e. total number of cells, area, ratio of cells, density, etc.). The *post-hoc* Holm-Sidak test was applied twice to allow for multiple comparisons within and between group across postnatal weeks. To compare the ratios of cells between dLGN and other thalamic nuclei in WT and math5\(^{-}\) (Fig. 9), a Chi-Square analysis was performed.
**Results**

We investigated the migratory path of thalamic interneurons that are destined to populate the dorsal lateral geniculate nucleus (dLGN). To accomplish this, we utilized the GAD67-GFP mouse, a transgenic strain that expresses green fluorescent protein (GFP) in dLGN interneurons (Munsch et al., 2005; Seabrook et al., 2013; Golding et al., 2014; Leist et al., 2016). This allowed us to track their perinatal migration to dLGN, and assess how they enter and disperse themselves throughout the nucleus. Additionally, to examine whether retinal signaling influences migration, targeting, and distribution in dLGN, we employed a genetic form of retinal deafferentation by crossing the GAD67-GFP strain with the math5 null (math5\textsuperscript{−/−}) mouse.

**Migratory path of interneurons in WT and math5\textsuperscript{−} mice**

Figure 1 reveals the migratory path of GFP-containing interneurons in the dorsal thalamus of GAD67-GFP (A) (i.e. WT) and GAD67 x math5\textsuperscript{−} (B) (i.e. math5\textsuperscript{−}) mice. In both groups, interneurons followed a path that originates from the ventral wall of the third ventricle (see Fig. 1, P0). In WT, at perinatal ages neurons can be seen coursing the ventral border of the somatosensory ventrobasal (VB) complex, to then gather in the ventral lateral geniculate nucleus (vLGN). By P2, interneurons innervate the dLGN and occupy the region just beneath the optic tract, along the dorsolateral edge of the nucleus (see also Fig. 3). However, in math5\textsuperscript{−}, fewer interneurons were seen along this path and the trajectory, targeting, and dispersion of cells were disrupted. These observations are consistent with previous findings that show in WT mice, interneurons
destined for dLGN follow a ventrolateral stream from 3rd ventricle to travel through thalamus before innervating dLGN, and that this migratory path is disrupted by early postnatal optic nerve section. (Golding et al., 2014). However, the presence of this ventrolateral thalamic stream has been challenged. A recent report argues that the exclusive migratory path of interneurons originates from a region in the dorsal midbrain. Ex-vivo time lapse imaging of a subset of genetically labeled interneurons identified a presumptive stream originating in superior colliculus (SC), migrating along a rostrolateral path through pretectum before entering the dorsolateral edge of dLGN (Jager et al., 2016). To address this discrepancy, we injected a neuronal marker, biotinylated dextran amine (BDA), to label GFP-containing interneurons near the third ventricle (V3) and superior colliculus (SC) of P0 GAD67 mice. This allowed us to examine the location of labeled interneurons after the first postnatal week when migration is complete (Golding et al., 2014). Following the injections, we conducted streptavidin-546 immunohistochemistry to visualize and track BDA-labeled cells (red), and assess whether interneurons doubled-labeled with GFP and BDA (see Fig. 2) migrated into dLGN.

The top panels in Figure 2 show examples of BDA injections made near V3 (left) or SC (right). The bottom panels show representative sections through the middle of dLGN at P7 for three different cases. For injections near V3, in all 4 cases there were at least 1-3 double-labeled interneurons found in section through dLGN (Fig. 2, bottom left). By contrast, in all 7 cases of SC injections, we failed to detect any double-labeled interneurons in dLGN (Fig. 2, bottom right).
Taken together, our results are consistent with earlier reports showing that interneurons follow a ventral-dorsal pattern of migration from the neuroepithelium of V3 (Golding et al., 2014).

**Number and distribution of interneurons in dLGN of WT and math5<sup>−/−</sup> mice**

To determine how and when interneurons fully populate dLGN, we obtained estimates of their numbers and distribution throughout the nucleus at different ages for WT and math5<sup>−/−</sup> mice. Figure 3A displays representative coronal sections through WT dLGN at different postnatal ages between P0-P30. During the first week, we observed a rapid increase in the number of interneurons populating dLGN. Initially, interneurons clustered within the dorsolateral region of dLGN just beneath the optic tract, then gradually migrated in a ventral manner to become evenly distributed throughout the nucleus. However, as shown in Figure 3B, in math5<sup>−/−</sup> mice, far fewer interneurons were found in dLGN, and those that entered failed to disperse themselves, remaining clustered in the dorsolateral sector. These age-related changes in WT (black symbols), and the disruptions noted in math5<sup>−/−</sup> (red symbols) are summarized in Figure 4A-B.

Figure 4A plots the mean number of interneurons found in a section through the middle of dLGN as a function of age (n = 3 mice and 3 sections per age). For WT, between P0-P4, the mean number of neurons increased substantially (Fig. 4A) (Two-Way ANOVA, F<sub>(9, 178)</sub> = 198.2, p < 0.001; Sidak post-hoc test, all p values < 0.0001), and stabilized at P4 to approximately 110-120 cells per section. As noted in Figure 3, interneurons seem to follow a dorsal-to-ventral trajectory as they disperse themselves throughout dLGN. To track this
progression, for each section through dLGN, we divided the nucleus in half, and simply counted the number of cells in each tier to generate a top-to-bottom ratio (see Methods, Fig. 3, P7). The mean ratios in a section of dLGN plotted by postnatal age are shown in Figure 4B. Between P0-P3, there were twice as many interneurons in the top vs. bottom tier of dLGN (i.e., 2:1 ratio) (Two-Way ANOVA, \(F_{(9, 181)} = 53.29, p < 0.001\); Holm-Sidak post hoc test, all p values < 0.004). However, by P4, both tiers had equal numbers and a 1:1 ratio was maintained at all ages thereafter (\(p = 0.998\)). Taken together, these results indicate that interneuron migration to, and dispersion within dLGN are linked processes that are complete by P4.

As shown in Figure 4, math5\(^{-}\) mice interneuron cell counts and distribution were altered. Between P0-7, the number of interneurons in dLGN increased significantly (Two-Way ANOVA, \(F_{(9, 178)} = 198.2, p < 0.0001\); Holm-Sidak post-hoc test, all p values < 0.0001), with mean values stabilizing after P7 (\(p = 0.904\)) to 50-70 interneurons per section (Fig. 4A). During this time, math5\(^{-}\) interneurons experienced significant changes in the way they arranged themselves within the nucleus (Fig. 4B) (Two-Way ANOVA, \(F_{(9, 181)} = 53.29, p < 0.0001\); Holm-Sidak post hoc test, all p values < 0.0001). Between P0-P3, math5\(^{-}\) showed ratios as high as 7:1 and 4:1. When interneuron distribution stabilized (\(p = 0.965\)) by the end of week 1, twice as many neurons were found in the top compared to the bottom of dLGN (i.e., 2:1).

Although math5\(^{-}\) interneurons arrive and disperse within dLGN, comparisons with WT revealed a number of differences. At all ages after P4,
math5\(^1\) mice contained significantly fewer dLGN interneurons (Fig. 4A) (Two-Way ANOVA, \(F_{(1, 178)} = 454.9, p < 0.001\); Holm-Sidak post-hoc test, all p values < 0.0001). Moreover, at all ages there were significantly more interneurons occupying the superficial tiers of dLGN (Fig. 4B) (Two-Way ANOVA, \(F_{(1, 181)} = 268, p < 0.001\); Holm-Sidak post-hoc test, all p values < 0.0001). These results suggest that retinal innervation is needed for interneuron dispersion after arrival in dLGN.

Estimates of WT interneuron density, which take into account the age-related changes in dLGN size (Seabrook et al., 2013; El-Danaf et al., 2015), suggest that the overall size of dLGN is a regulating factor of interneuron number in dLGN. As shown in Figure 4C, there were significant age-related changes in interneuron density found in a section of dLGN (Two-Way ANOVA, \(F_{(9, 181)} = 59.99, p < 0.001\); Holm-Sidak post-hoc test, all p values < 0.001). During week 1, density increased from ~1 to 2-2.5 cells (per 2500 \(\mu\)m\(^2\) area), peaking at P5. Density then dropped back to 1 cell (per 2500 \(\mu\)m\(^2\)) between P5 and P14 (Holm-Sidak post-hoc test, all p values < 0.004) and stabilized thereafter (p = 0.724). Figure 4D plots mean changes in dLGN size as a function of age, and reveals that dLGN area progressively increased during the first two postnatal weeks, as interneuron density fluctuated (Two-Way ANOVA, \(F_{(9, 183)} = 213.544, p < 0.0001\); Sidak post-hoc test, all p values < 0.0001). By P14, the area of dLGN reached its adult-like size (Fig. 4D, black) (p = 0.992), similar to when interneuron density stabilized (Fig. 4C, black).
In math5−, density estimates revealed steady increases during the first postnatal week, as density increased from ~1 to 2 cells (per 2500 um² area) between P0 and P5 (Fig. 4C, red) Two-Way ANOVA, $F_{(9,181)} = 59.99$, $p < 0.0001$; Holm-Sidak post-hoc test, all $p$ values < 0.001). Density significantly decreased between P5-P7 ($p < 0.0009$) and stabilized thereafter to 1 cell (per 2500 um² area) (Holm-Sidak post-hoc, all $p$ values > 0.720). The area of dLGN also increased significantly during week 1 (Fig. 4D) (Two-Way ANOVA $F_{(1,183)} = 213.544$, $p < 0.0001$; Holm-Sidak post-hoc test, all $p$ values < 0.0001), reaching an adult-like size by P7 (Holm-Sidak post-hoc test, all $p$ values > 0.821).

Interestingly, despite math5− dLGN containing fewer interneurons, density estimates were similar to WT (Two-Way ANOVA $F_{(1,181)} = 1.84$, $p = 0.177$; all $p$ values > 0.373) because of a ~2-fold reduction in dLGN area (Two-Way ANOVA, $F_{(1,183)} = 509.9$, $p < 0.001$; Sidak post-hoc test, all $p$ values < 0.0001) (El-Danaf et al., 2015). Taken together, these results suggest that the size of dLGN (Fig. 4C) regulates the number of interneurons present in the nucleus as a means to maintain constant density patterns.

There are two possible reasons for why there are fewer dLGN interneurons in math5− mice. First, mutants may undergo increased rates of apoptotic activity. Another may be that because of the interdependence between dLGN area and number of dLGN interneurons (Fig. 4C-D), interneurons are misrouted into nearby thalamic nuclei.
Apoptotic activity of interneurons in WT and math5\(^{-}\) mice

To test for these possibilities, first we conducted caspase-3 immunohistochemistry on thalamic sections containing dLGN from WT and math5\(^{-}\). Caspase-3, a protein responsible for disassembling cells into apoptotic bodies, serves as a reliable marker to visualize cells destined for cell death (McIlwain et al., 2013). Figure 5 demonstrates representative sections of dLGN that were processed for cleaved caspase-3 (red) from WT (A) and math5\(^{-}\) (B) mice at young (P0-6), middle (P11-14) and adult (P24-25) ages (n = 1 mouse per age and 4 sections per age). In both cases, we detected neurons positive for cleaved caspase-3 in different thalamic nuclei, (see Fig. 5) but neither WT or math5\(^{-}\) exhibited caspase-3+ dLGN interneurons at any age.

Number and distribution of interneurons in neighboring thalamic nuclei of WT and math5\(^{-}\) mice

To assess whether interneurons are misrouted in the absence of retinal signaling, we obtained estimates of the number and distribution of interneurons in three other thalamic nuclei in math5\(^{-}\) mice: ventrobasal complex (VB) and the ventral lateral geniculate nucleus (vLGN), both of which are part of the migratory stream, and the lateral posterior nucleus (LPN), a higher order visual nucleus that also contains interneurons.

First, we examined the ventrobasal complex (VB) which consists of the ventral posteromedial nucleus (VPM) and ventral posterolateral nucleus (VPL), that together serve to transfer nociceptive information to the primary somatosensory cortex (Welker, 1973; Harris and Hendrickson, 1987). Presently,
it is widely believed that the adult VB contains few, if any, interneurons (Harris and Hendrickson, 1987; Spreafico et al., 1994; Arcelli et al., 1997). However, as shown in Figure 6, many seem to pass through this region before arriving in dLGN.

Examples of cell count estimates in sections of VB in WT and age-matched math5^-/- mice at different postnatal ages (P0-P30) (n = 3 animals and 3 sections per age) are shown in Figure 6A-B. Interestingly, in WT mice, interneurons were found throughout VB and were especially prevalent between P7-14. There was a significant increase (Two-Way ANOVA, F(9,134) = 12.71, p < 0.001; all p values < 0.0001) in the number of interneurons within the first two weeks of postnatal life, with as many as 30 cells found in a given section through VB (Fig. 7C, black). After P11, the number of interneurons in a section decreased (Holm-Sidak post-hoc test, p = 0.004), but stabilized (Holm-Sidak post-hoc test, p = 0.998) to ~15-20 interneurons per section. These observations are consistent with other observations revealing a sparse population of interneurons residing in mouse VB. (Cavdar et al., 2014).

As shown in Figure 6B, math5^-/- mice showed a similar trend for VB. During week 1, the number interneurons greatly increased from ~10 to ~45 per section. Values then decreased after P7, with sections averaging between 25-35 for the remaining ages (Holm-Sidak post-hoc test, all p values > 0.317). However, math5^-/- mice exhibited far more interneurons in VB compared to WT (Fig. 6C, red) (Two-Way ANOVA, F(1,134) = 94.43; p < 0.0001), particularly between the ages P4-P21, where as many as 35-45 cells were found in a section
of VB (Holm-Sidak post-hoc test, p < 0.001). These results suggest that in the absence of retinal innervation, interneurons destined for dLGN are misrouted, resulting in increased numbers in VB.

We also examined the number of interneurons in another retino-recipient nucleus, the ventral lateral geniculate nucleus (vLGN). This structure plays an important role in visuomotor function and photo-entrainment of circadian rhythms (Brauer and Schober, 1982; Harrington, 1997), and is located just ventral to the dorsal dLGN. As shown in Figure 7A, a large population of GFP-positive interneurons reside primarily in the lateral magnocellular external sector of vLGN (Monavarfeshani et al., 2017).

Figure 7A-B provides representative coronal sections through vLGN at P0, P7, P14, and P21 for WT and math5−/− mice. As shown in the summary plot in Figure 7C, the mean number of vLGN interneurons in a section (n = 3 mice and 3 sections per age) significantly decreased (Two-Way ANOVA F(3, 64) = 114, p < 0.001; all p values < 0.001) until P14 when values stabilized to ~75 cells (p values > 0.130). The number of vLGN cells in math5−/− followed a similar pattern, showing a decrease between P0 and P14 (Two-Way ANOVA F(3,64) = 114, p < 0.001; all p values < 0.001), when cell count estimates then stabilized to ~25 neurons (p values > 0.058). However, at all ages, math5−/− mice maintained significantly fewer interneurons compared to WT (Fig. 7C, red) (Two-Way ANOVA, F (1,64) = 70.24, p < 0.001 P0-P14; p = 0.013 P21). These results support the hypothesis that interneurons in math5−/− mice are misrouted, with many failing to arrive in vLGN and dLGN, remaining instead in VB.
We also examined the distribution of interneurons in LPN, a higher order thalamic nucleus that receives input from the superior colliculus, striate cortex, lateral extrastriate cortex, as well as the retina from melanopsin-expressing RGCs (Kaas and Lyon, 2007; Allen et al., 2016; Zhou et al., 2017). Though retino-LPN projections reduce to sparser levels in adult, it is possible that these retinal projections serve to coordinate the circuitry necessary for its visually-guided functions (Warner et al., 2012). This includes the arrangement of interneurons.

Figure 8A-B displays representative coronal sections through LPN at different postnatal ages (P0-P30) in WT and math5⁻/⁻ mice. In a given section (n = 3 mice and 3 sections per age) we measured area of LPN, total number of interneurons, and their distribution. These findings are summarized in Figure 8C-E. In WT, within the first two weeks, the area of LPN significantly grew (Two-Way ANOVA, F(8,108) = 73.82, p < 0.0001, all p values < 0.001), until P11 when its size stabilized (Fig. 8C, red) (p = 0.965). Estimates based on the cell counts per section showed that total number of LPN interneurons significantly increased until P4 (Two-Way ANOVA, F(8,108) = 12.47, p < 0.0001, all p values < 0.001), when values stabilized thereafter to ~40 cells (Fig. 8D) (p = 0.998). During this time, estimates of the distribution of interneurons between the top and bottom tiers showed several significant changes. Between P0-P3, the majority of interneurons in a section populated the upper sector as much as 4:1 and 5:1 (Fig. 8E) (Two-Way ANOVA F(8,108) = 10.05, p < 0.05; Holm Sidak test, all p
values < 0.005). However, by P4 these ratios decreased and stabilized to a value of 2:1 (Holm-Sidak post-hoc test, p = 0.875).

As shown in Figure 8B, the LPN of math5\(^{\dagger}\) mice demonstrated similar age-related changes to WT. The size of the nucleus shows a steady increase until P11 (Fig. 8C, red) (Two-Way ANOVA, F\(_{(8,108)}\) = 218.7, p < 0.0001, all p values < 0.001; (Holm-Sidak post-hoc test, p = 0.989). However, compared to WT, after week 1, LPN remained notably smaller at all ages (Fig. 8C) (Two-Way ANOVA, F\(_{(1,108)}\) = 73.82, p < 0.0001; all p values < 0.001). Estimates of the numbers of interneurons populating LPN in math5\(^{\dagger}\) mice revealed a steady increase from P0-4, then stabilized to values that averaged ~30 cells per section (Fig. 8D) (Two-Way ANOVA, F\(_{(8,108)}\) = 12.47, p = 0.864). Additionally, between P0-P3, each section showed a large portion of interneurons occupying the top tiers compared to the bottom of LPN, having ratios as high as 8:1. At P4, LPN interneurons shifted to 5:1 ratio (Two-Way ANOVA, F\(_{(8,108)}\) = 10.05, p < 0.001), and remained relatively steady thereafter (Fig. 8E) (Holm-Sidak post-hoc test, p = 0.728). However, compared to WT, math5\(^{\dagger}\) LPN displayed significantly fewer interneurons at ages P3, P11, and P14 (Fig. 8D) (Two-Way ANOVA, F\(_{(1,108)}\) = 22.73, p = 0.0006; Holm-Sidak post-hoc test, P3 p = 0.049, P11 p = 0.003, P14 p = 0.006), and had a substantially higher proportion of cells residing in the top tier between P0 and P14 (Fig. 8E) (Two-Way ANOVA, F\(_{(1,108)}\) = 73.57; p < 0.0001; Holm-Sidak post-hoc test, all p values < 0.04).

Taken together, our data suggests that the targeting of interneurons into visual thalamus is synchronized. Furthermore, the absence of retinal signaling
disrupts the targeting and organization of interneurons, as math5^{-} mice exhibited fewer and more clustered spacing. These disruptions are summarized in Fig 9A-B, which compares the number of interneurons residing in VB, vLGN, dLGN, and LPN for WT (A) and math5^{-} (B) mice. From these estimates, we generated ratios (at either P21 or 30) that compared the number of interneurons residing in dLGN, with those found in LPN, VB and vLGN (Fig. 9). Of these nuclei, the greatest shift was found in the proportion of interneurons in dLGN compared to VB, where WT mice displayed a 10:1 distribution (Fig. 9C, E), and math5^{-} mice showed a substantially smaller ratio, 2.5:1 (Fig. 9D, E) (Chi-square test 14.81, p = 0.0001). Taken together these data suggest that thalamic interneurons are misrouted and remain largely in VB, with many failing to complete their trajectory to dLGN.

**Number and distribution of interneurons in dLGN, VB and vLGN in Fgf15^{-} mice**

The results in math5^{-} mice suggest that migration and distribution of interneurons in dLGN relies on retinal signaling. However, the underlying molecular mechanism remains unclear. Here we explored Fgf15 as a potential candidate. This molecule is part of a large family of Fibroblast Growth Factors (FGFs) involved in cell differentiation, proliferation, and guidance (Borello et al., 2008; Hagemann and Scholpp, 2012; Martinez-Ferre et al., 2016). Indeed, Fgf15 is expressed in RGCs, the optic tract, retino-recipient nuclei, and ventricular zones (Gimeno et al., 2003; Kurose et al., 2004). Recent *in-situ* work from the Fox laboratory demonstrates a strong link between Fgf15 expression and retinal
signaling. Math5\(^{−}\) mice exhibit a 3-fold reduction in Fgf15 expression in dLGN and vLGN at ages when interneuron migration is actively occurring (~P1-P3). To explore this further, we employed a loss-of-function approach by utilizing the Fgf15 knockout mouse (Fgf15\(^{−}\)), crossed onto the GAD67-GFP strain to evaluate how the absence of Fgf15 influences interneuron migration to and spacing within dLGN.

We estimated the size of dLGN, as well as the total number and distribution of intrinsic interneurons within dLGN of Fgf15\(^{−}\) mice (n = 3 mice and 3 sections per age). Fig. 10A provides examples of coronal sections through the dLGN of Fgf15\(^{−}\) and age-matched WT mice. Mutant mice displayed GFP-positive interneurons at P7, P14, and P21. Interestingly, the area of dLGN was similar to WT (Two-Way ANOVA \(F_{(1,48)} = 12.76, p = 0.087\), Holm-Sidak post-hoc test, all \(p\) values > 0.343), with both showing a progressive increase in area between P7 and P14, before stabilizing to adult-like sizes by the end of the second postnatal week (Fig. 10B) (WT, \(F_{(2,48)} = 0.599\); Fgf15\(^{−}\), \(F_{(2,48)} = 12.75, p = 0.066\)). However, at all ages, Fgf15\(^{−}\) mice exhibited significantly fewer number of interneurons in a section of dLGN compared to WT (Fig. 10C, red) (\(F_{(1,48)} = 38.8, p < 0.0001\); Holm-Sidak post-hoc test, all \(p\) values < 0.006). Despite fewer cells, interneurons were evenly dispersed at all ages (Fig. 10D, red) (\(F_{(2,48)} = 3.77, p = 0.301\); Holm-Sidak post-hoc test, all \(p\) values > 0.552), and with no difference from WT (\(F_{(1,48)} = 3.998, p = 0.512\); Holm-Sidak post-hoc test, all \(p\) values > 0.378). Thus, the absence of Fgf15 led to reduced numbers of interneurons arriving in dLGN, but had no impact on the size of dLGN or the
manner by which interneurons arranged themselves within the nucleus. To determine if the loss of interneurons was due to misrouting, we examined the number of interneurons along the migratory of dLGN interneurons, including VB and vLGN.

Figure 11A shows examples of coronal sections through VB at different ages (P7, P14, P21) of WT and Fgf15¼ mice, and estimates of the number of interneurons in VB (n = 3 mice and 3 sections per age) are shown in Figure 11B. At ages P7 and P14, mutants displayed significantly more interneurons per section of VB compared to WT (Fig. 11B, red) (F(1,48) = 19.97, p < 0.001; P7, p < 0.0001; P14, p = 0.033). Figure 12A provides examples through vLGN with corresponding estimates of interneuron numbers for the two groups summarized in Figure 12B (n = 3 mice and 3 sections per age) at ages P7, P14, and P21. At all ages, Fgf15¼ showed significantly fewer vLGN interneurons (Fig. 12B, red) compared to WT (F(2,72) = 64.45, p < 0.0001; Holm-Sidak post-hoc test, p < 0.001).

Taken together these data suggest that loss of interneurons noted in dLGN of Fgf15 mutants is likely due to a misrouting of interneurons, with many failing to arrive in vLGN and instead settling in VB.
Figure 1: Green fluorescent protein (GFP) expressing interneurons in mouse thalamus of WT and math5<sup>-/-</sup> mice.

**A:** Representative coronal sections through the dorsal thalamus of GAD67-GFP (i.e. WT) mouse showing the migratory path of dLGN interneurons.  
**B:** Coronal sections through dorsal thalamus of GAD67 x math5<sup>-/-</sup> (i.e. math5<sup>-/-</sup>) mouse depicting the trajectory, targeting, and distribution of dLGN interneurons at early ages. Scale bar: 150 µm.
Figure 2: BDA injections near the third ventricle (V3) and in superior colliculus (SC) to target the presumed migratory streams of interneurons. (Top): Representative coronal sections through dorsoventral thalamus (left) and SC (right) depicting the site of biotinylated dextran amine (BDA) injections in WT
mice at P0 (red; conjugated to Streptavidin-546) Scale bar = 150 µm. (Bottom): Examples of sections through the middle of dLGN at P7 taken from 3 mice that had either V3 (left) or SC (right) BDA injection. For each case, BDA-labeled interneurons (left) and the corresponding GFP-labeled interneurons in dLGN are shown. Double-labeled interneurons are illustrated (right) and found in dLGN of mice after V3 injections. Scale bar = 70 µm.
Figure 3: Pattern of interneuron migration and distribution in dLGN of WT and math5⁻ mice.

Representative coronal sections through dLGN in WT (left) and math5⁻ (right) depicting distribution and density of interneurons at different postnatal ages (P0,
P3, P7, P9, P14, P16, P18, P21, P30) (3 mice and 3 sections per age). In WT, interneuron migration is complete by P4. In math5<sup>+</sup> mice, there are fewer interneurons that reach dLGN, and those that do, appear clustered in the dorsolateral region of dLGN. At P7 WT, dotted lines illustrate how top and bottom tiers of dLGN were determined. Scale bar = 70 µm.
Figure 4: Estimates of the number of interneurons and their distribution in dLGN of WT and math5^−/− mice.

A-D: Summary plots depicting age-related changes in interneuron number (A), distribution (B), density (C) as well as estimates of the area of dLGN (D). Each point represents mean values +/- SEMs for WT (black) and math5^−/− (red) as a function of postnatal age (3 mice and 3 sections per age). Asterisks (*) represent significance (<0.05) between WT and math5^−/−. Dotted line in (B) delineates equal distribution between top and bottom (1:1).
Figure 5: Observation of caspase-3 labeling in interneurons of WT and math5⁻/⁻ mice.

A: Representative coronal sections through thalamus of WT mice at various postnatal ages (P0, P1, P4, P6, P11, P24) demonstrating the lack of caspase-3+ (red, Alexa-Fluor 546) interneurons in dLGN. B: Similarly, at all ages (P0, P2, P3, P6, P10, P24) math5⁻/⁻ interneurons did not stain positive for caspase-3. For both groups and all ages: 1-2 animals, 5-7 sections per age. Scale bar = 70 µm, except for P10, P11, P24 = 150 µm.
Figure 6: Pattern of interneuron migration in the ventrobasal complex (VB) of WT and math5<sup>−/−</sup> mice. A: Representative coronal sections through VB in WT mice at different postnatal ages (P0, P3, P7, P14, P21, P30). Dotted line
delineates bottom boundary of VB; dLGN is outlined. B: Representative sections of VB in age-matched math5\(^{−}\) mice. C: Summary plot demonstrating age-related changes in the number of VB interneurons in WT (black) and math5\(^{−}\) (red) (3 mice and 3 sections per age) animals. At all ages, math5\(^{−}\) mice show an increased number of VB interneurons. Asterisks (*) represent significance (<0.05) between WT and math5\(^{−}\). Scale bar = 150 \(\mu\)m.
Figure 7: Interneuron distribution in vLGN of WT and math5<sup>−/−</sup> mice.

A: Coronal sections through vLGN in WT at postnatal ages 7, 14, and 21. B:
Representative coronal sections through vLGN in age-matched math5<sup>−/−</sup> mice. C:
Scatter plot displaying age-related changes in number of interneurons in WT
(black) and math5<sup>−/−</sup> (red) (3 mice and 3 sections per age). At all ages, math5<sup>−/−</sup>
exhibit fewer interneurons. Asterisks (*) represent significance (<0.05) between WT and \textit{math5}⁺. Scale bar = 70 µm.
Figure 8: Estimates of the number and distribution of lateral posterior nucleus (LPN) interneurons. A: Example coronal sections through middle thalamus in WT, demonstrating number and distribution of cells in LPN (outlined).
at various postnatal ages (P0, P3, P5, P7, P11, P14, P21, P30). At early ages, LPN interneurons occupy the dorsolateral sector before distributing ventrally. **B:** Representative sections through thalamus in math5\(^{-}\), mice showing altered interneuron distribution in LPN at different postnatal ages (P0, P3, P5, P7, P11, P14, P21, P30). **C-E:** Summary scatter plots demonstrating the age-related changes in LPN area (C), number of interneurons (D), and ratio between cells in the top and bottom sectors (E) in WT (black) and math5\(^{-}\) (red) (3 mice and 3 sections per age). Overall, math5\(^{-}\) exhibited reduced number of LPN interneurons, and more cells clustered in the top sector of LPN compared to WT. Asterisks (*) represent significance (<0.05) between WT and math5\(^{-}\). Scale bar = 70 µm.
Figure 9: Organization of thalamic interneurons in WT and math5−/− mice. A-B: Scatter plots summarizing changes in interneuron number across different thalamic nuclei and at postnatal ages ranging from P0-P30: dLGN (black), LPN (red), VB (green), and vLGN (purple) in WT (A) and math5−/− (B) mice (3 mice...
and 3 sections per age). Mutants show disruptions in total number and organization of thalamic interneurons. **C-D:** Same plots as shown in A-B, but only showing estimates from dLGN (black) and VB (green). **E:** Summary table demonstrating the discrepancy in interneuron number between dLGN and surrounding thalamic nuclei at an example age, P30 (VB, LPN) and P21 (vLGN). The yellow column and asterisk (*) highlights how the largest difference in interneuron number was between dLGN and VB (p = 0.0001).
Figure 10: Interneuron number and dispersion in WT and Fgf15⁻/⁻ mice.

A-B: Representative coronal examples through dLGN of WT (left) and Fgf15⁻/⁻ (right) mice at postnatal ages 7, 14, and 21 (3 mice and 3 sections per age). B-D:
Scatter plots demonstrating age-related changes in area (B), number (C), and distribution (D) of interneurons in WT (black) and Fgf15⁺ (red). Each point represents mean values +/- SEMs arranged by postnatal day (3 mice and 3 sections per age). B: Both WT and Fgf15⁺ show similar area measurements. C: At all ages, Fgf15⁺ displayed decreased number of cells. D: However, interneurons in Fgf15⁺ mice maintained an even distribution at all ages, similar to WT. Asterisks (*) represent significance (<0.05) between WT and Fgf15⁺. Scale bar = 70 μm.
Figure 11: Estimates of the number of VB interneurons in Fgf15<sup>−/−</sup> mice.

**A:** Examples of coronal sections through thalamus showing number of VB interneurons in WT (left) and Fgf15<sup>−/−</sup> (right) at ages P7, P14, and P21. **B:** Scatter
plot displaying age-related changes in interneuron number in WT (black) and Fgf15
(red) (3 mice, 3 sections per age). Fgf15
mice exhibited increased number of neurons in VB, particularly at P7 and P14. Asterisks (*) represent significance (<0.05) between WT and Fgf15
. Scale bar = 150 µm.
Figure 12: Fewer vLGN interneurons in Fgf15\(^{-/-}\) mice.

A: Representative examples of the number of intrinsic interneurons in vLGN in WT (left) and Fgf15\(^{-/-}\) (right) at ages P7, P14, and P21. B: Summary plot
depicting changes in vLGN interneuron number in WT (black) and Fgf15⁻/⁻ (red) (3 mice and 3 sections per age). At all ages, Fgf15⁻/⁻ mice displayed fewer interneurons than WT counterparts. Asterisks (*) represent significance (<0.05) between WT and Fgf15⁻/⁻. Scale bar = 70 µm.
Discussion

Our studies provide new insight about the migration, targeting, and dispersion of thalamic interneurons to, and within dLGN. Tracing studies with biotinylated dextran amine (BDA) injections confirm that dLGN interneurons follow a migratory path originating from the third ventricle (V3), and course through thalamus to gather in vLGN prior to their arrival in dLGN. We found that when interneurons were labeled with BDA at P0 in and around the midline of V3, they were distributed throughout dLGN at postnatal day 7. These findings are consistent with a previous study that showed dLGN interneurons follow a ventro-lateral stream from the neuroepithelium of V3, through VB and vLGN, before arriving to dLGN (Golding et al., 2014). This trajectory for interneurons has been challenged by a recent report that claims the exclusive source of dLGN interneurons is the midbrain structure superior colliculus (SC), that traverse in a rostral manner through pre-tectum to dLGN (Jager et al., 2016). However, we failed to identify such a path; BDA injections into SC at P0 did not reveal labeled interneurons in dLGN. One reason for such discrepancy may be due to the timing of injections, which were designed to focus on early postnatal migration, whereas the presumed rostral stream from SC involved perinatal ages (Jager et al., 2016). As a consequence, the timing of our early postnatal injections (P0) may have simply missed the time-frame when the bulk of migration along this path occurred. Thus, it is possible that there are two sources of interneurons, with two streams travelling in different directions and arriving in dLGN at successive times. The first migratory stream may occur largely at late embryonic ages and
includes interneurons traveling rostrally from SC. The second begins postnatally with interneurons taking a ventro-lateral path through thalamus to dLGN.

Our experiments in WT mice reveal that dLGN interneurons first arrive in the dorsolateral region of the nucleus just beneath the optic tract, then traverse ventrally to become evenly dispersed by postnatal day 4. This progression is similar to what others observe (Golding et al., 2014), but our analysis also showed that migration to and dispersion within dLGN is complete earlier than previously recognized. Moreover, work in math5+/ reveal that fewer interneurons migrate to dLGN, and those that do arrive remain clustered in the dorsolateral sector. These results suggest that retinal signaling plays an important role in the migration and dispersion of interneurons. This abnormal clustering associated with math5+/ is consistent with a previous report showing that after early postnatal optic nerve section, interneurons fail to disperse evenly throughout dLGN, and instead collect along the dorsolateral border (Golding et al., 2014). However, our data reveals that retinal innervation also influences the targeting and migration of interneurons to dLGN. Estimates reveal a ~50% reduction in the number of interneurons present in dLGN in math5+/ mice compared to WT. Interestingly, despite these reduced numbers, the density of interneurons in dLGN of math5+/ mice is similar to WT. The similarity in density between WT and these mutants is likely related to the highly reduced size of dLGN noted in math5+/ mice (El-Danaf et al., 2015). Indeed, we found math5+/ mice exhibit a ~2-fold decrease in dLGN area compared to WT (Fig. 4D). Thus, it appears that the overall size of dLGN is a major determinant of the total number of interneurons
residing in dLGN. Such a relationship is found in other brain structures such as the hippocampus, where studies have shown that a reduction in size of the CA1 region brought about by degeneration/aging, leads to substantially fewer neurons (Simic et al., 1997; Harding et al., 1998).

Although the density is the same, there are still far fewer interneurons in dLGN of math5^−/−mice. To account for this loss, we explored whether this was a result of increased apoptotic activity, or the misrouting of migrating interneurons to other nearby nuclei. Caspase-3 labeling in math5^−/− showed no evidence of postnatal interneuron cell death either in dLGN, or other thalamic and midbrain nuclei. Instead, estimates of interneuron numbers in nearby thalamic nuclei suggest that the absence of retinal input associated with math5^−/− mice, leads to misrouting of interneurons destined for dLGN. In these mice, VB contained far more interneurons compared to WT (Fig. 6,9). Normally, the ratio of interneurons between dLGN vs. VB is 10:1, whereas in math5^−/− mice, this value becomes 2.5:1 (Fig. 9E). In fact, when comparing the proportion of interneurons between dLGN and the surrounding nuclei, only VB was significantly different between WT and math5^−/− (Fig. 9E). Taken together, these results support the hypothesis that the absence of retinal input disrupts the migration and targeting of dLGN interneurons at the level of VB. Neurons are misrouted and fail to reach their first destination (i.e. vLGN) prior to arrival in dLGN.

We also note that the absence of retinal signaling disrupted the number and pattern of interneurons in the lateral posterior nucleus (LPN). Similar to our results in dLGN of math5^−/− mice, LPN showed fewer interneurons that were
clustered along the dorsolateral border. This structure is a higher-order visual thalamic nucleus that receives its primary, excitatory drive from cortical layer V (Roth et al., 2016; Zhou et al., 2017). Nonetheless, the link between retinal signaling and interneuron fate likely exists given that LPN receives direct retinal innervation from a subset of RGCs. A recent in-vivo study indicates that at least 25 percent of all LPN neurons can be driven by their retinal input (Allen et al., 2016). Moreover, the density of retinal innervation is particularly high at early postnatal ages (Warner et al., 2012), the time when interneurons are actively migrating to their respective nuclei. While little is known about the migratory path of interneurons destined to reside in LPN, our results in math5\textsuperscript{5} suggest that retinal signaling plays a role in their targeting and dispersion.

Finally, to address the underlying molecular mechanism for how retinal signaling regulates interneuron migration and dispersion within dLGN, we explored the role of the neurotrophic factor Fgf15. This signaling protein is a member of the Fibroblast Growth Factor (FGF) family, known to play a role in embryogenesis, cellular proliferation, and migration (Ornitz and Itoh, 2015). Fgf15 in particular is involved in GABAergic neuron differentiation and proliferation, operating through endocrine, paracrine, and autocrine signaling pathways ((Borello et al., 2008; Ornitz and Itoh, 2015; Martinez-Ferre et al., 2016). Moreover, Fgf15 is regulated by Otx2 expression, a transcription factor implicated in directing interneuron migratory streams throughout many subcortical structures (Vue et al., 2007; Golding et al., 2014). Previous studies have also shown that Fgf15 is present within the neuroepithelium of the third
ventricle, the optic tract, as well as retino-recipient nuclei such as vLGN and dLGN (Gimeno et al., 2003). In fact, recent evidence from the Fox lab utilized in-situ, microarray, and quantitative-PCR to reveal that Fgf15 is expressed transiently at early postnatal ages, during the period of active interneuron migration (~P1-3). Most importantly, the Fox lab demonstrates that Fgf15 expression is linked to retinal innervation. In math5\(^{-}\) mice, microarray and RNA-seq analysis showed a 3-fold decrease in Fgf15 expression within vLGN and dLGN compared to WT (Fox lab, unpublished observations).

Our experiments in Fgf15\(^{-}\) mice seem to support the link between retinal innervation and Fgf15, as the number of interneurons in dLGN and vLGN were markedly decreased compared to WT (Fig.10, 12). Indeed, much like math5\(^{-}\) mice, more neurons were found in VB (Fig. 11), suggesting that the migratory path of dLGN interneurons was disrupted in Fgf15\(^{-}\) mice. Given that the loss of retinal axon innervation leads to decreased Fgf15 within the LGN complex (Fox lab, unpublished observation), these results indicate that the misrouting of dLGN interneurons is brought about by the loss of Fgf15. While both Fgf15 and math5\(^{-}\) mice show fewer interneurons in dLGN, the losses were more severe in math5\(^{-}\). These comparisons suggest that Fgf15 is a contributing factor, but not the exclusive one. It is likely that retinal signaling provides trophic support that influences the overall growth of dLGN, and the maturation of its neuronal components (El-Danaf et al., 2015; see Chapter II).

It is also important to note that while there were fewer interneurons within dLGN of Fgf15 mice, their dispersion within the nucleus was similar to WT (Fig.
These findings imply that Fgf15 plays a distinct role during interneuron migration, regulating their targeting and arrival but not dispersion. Thus, our data suggests that retinal signaling supports two molecular mechanisms, one for interneuron targeting and migration to dLGN (i.e. Fgf15 pathway), and the other within the nucleus that regulates their dispersion. Presently, we are exploring whether Down Syndrome Cell Adhesion Molecule (DSCAM) is involved in the spacing of interneurons within dLGN. This gene is expressed in several retinal cell types, and is required for a number of developmental processes including dendritic stratification, laminar patterning, and neuronal spacing (Fuerst et al., 2008; Yamagata and Sanes, 2008; Fuerst et al., 2012). Notably, DSCAM functions as a repulsive signal for proper neuronal spacing, and its loss leads to disruptions in the mosaic patterning of different retinal cell types (Fuerst et al., 2012). Additionally, a recent report showed that DSCAM is needed for proper segregation of eye-specific retinal terminal domains (Blank et al., 2011), and in WT, this gene is expressed along retinal axons that project to dLGN (Fuerst lab, unpublished observations). Thus, its loss in math5Δ mice may lead to abnormal dispersion of interneurons within dLGN.

Taken together, our results demonstrate the first comprehensive analysis of interneuron development, and shed light on the potential mechanisms that regulate the migration and dispersion of interneurons destined for dLGN. While retinal signaling plays an important role, it is clear that a number of molecules are affected, and that each may control a distinct aspect of interneuron targeting, migration, and dispersion.
Introduction

The dorsal lateral geniculate nucleus (dLGN) of the mouse has become the leading model system to understand many aspects of thalamic circuit development (Huberman et al., 2008; Hong and Chen, 2011; Seabrook et al., 2017; Guido, 2018). The advance of sophisticated genetic tools and widespread use of genetically modified mouse strains permit remarkable experimental access to study how specific cell types and circuits are established during early postnatal life. However, our understanding of thalamic circuit development has been largely limited to thalamocortical neurons, which serve as the exclusive relay of retinal information to the visual cortex. These neurons undergo substantial remodeling during the first few postnatal weeks, developing complex and stereotypic dendritic architecture that defines their cell class specificity (Krahe et al., 2011; El-Danaf et al., 2015). Such growth is accompanied by the synaptic pruning of retinal connections whereby the number of functional inputs onto dLGN relay neurons are greatly reduced (Chen and Regehr, 2000; Jaubert-Miazza et al., 2005; Cheadle et al., 2018; Tschetter et al., 2018). Many of these
changes rely on retinal signaling. For example, the elimination of retinal input to dLGN leads to dystrophic dendritic growth (El-Danaf et al., 2015), and the blockade of early retinal activity results in synaptic weakening and a failure to prune excess retinal inputs onto developing dLGN relay neurons (Hooks and Chen, 2006).

By comparison, our knowledge about the development of the other dLGN cell type, intrinsic interneurons, remains rudimentary. These local circuit neurons which receive retinal input comprise <10% of total dLGN cell population (Evangelio et al., 2018). Despite their small numbers, they play a key role in retinogeniculate signal transmission by sharpening the spatio-temporal receptive field structure of dLGN relay neurons through a feedforward inhibitory circuit (Cox and Sherman, 2000; Bickford et al., 2010; Hirsch et al., 2015).

The recent utilization of the GAD67-GFP transgenic strain, which expresses green fluorescent protein (GFP) in dLGN interneurons, has led to a number of important discoveries about their genesis, migration, and assembly of inhibitory synaptic connections (Seabrook et al., 2013; Golding et al., 2014; Jager et al., 2016). Thalamic interneurons migrate to dLGN at perinatal ages, distributing themselves evenly throughout the nucleus by the end of the first postnatal week (Golding et al., 2014; Jager et al., 2016; see Chapter I). While interneurons receive retinal input at early postnatal ages, the formation of inhibitory connections occurs much later, well after the maturation of dLGN relay neurons and the initial phase of retinogeniculate synapse remodeling (Bickford et al., 2010; Seabrook et al., 2013). What is lacking is an understanding about how
these milestones relate to the morphological and functional development of interneurons, and whether retinal signaling is needed.

To examine these issues, we used the GAD67-GFP mouse to target developing dLGN interneurons during *in vitro* intracellular recordings. To assess whether retinal signaling plays a role in the development of their morphological and functional properties, we used a genetic form of retinal deafferentation by utilizing the math5 null (math5<sup>−/−</sup>) mouse and crossing it onto the GAD67-GFP strain. These mutants lack the math5 gene that is required for retinal progenitors to differentiate into retinal ganglion cells (RGCs) (Brown et al., 1998). Consequently, math5<sup>−/−</sup> mice display a >95% loss of RGCs, fail to form an optic nerve, and thus leave dLGN devoid of retinal innervation (Brown et al., 2001; Wang et al., 2001; El-Danaf et al., 2015). This loss of function approach is advantageous to postnatal manipulations such as enucleation or optic nerve section. While “eyeless” phenotypes exist in the mouse, they are problematic because they involve a polygenic form of inheritance and additional mutations that likely have a deleterious impact on neuronal development (Cullen and Kaiserman-Abramof, 1976; Tucker et al., 2001; Verma and Fitzpatrick, 2007). Thus, we used math5<sup>−/−</sup> along with age-matched wildtypes GAD67-GFP to understand whether retinal signaling influences interneuron structure, function, and establishment of synaptic connectivity with dLGN relay neurons.
Materials and Methods

Animals
Experiments were conducted in mice of either sex ranging in age between P0-45. Two strains were used: GAD67-GFP (line G42; Jax stock no. 007677) established on a pigmented background (C57/B6 x CB6F1/J), and math5+ crossed onto GAD67-GFP. The math5+ strain was on a mixed C57/B6 and 129/SvEv background (Wang et al., 2001; El-Danaf et al., 2015). All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

Acute in vitro thalamic slice preparation and whole-cell recording procedures
We used an acute thalamic slice preparation and conducted whole cell recordings on dLGN neurons using methods described in detail elsewhere (Govindaiah and Cox, 2006; Seabrook et al., 2013; El-Danaf et al., 2015). Briefly, mice were anesthetized with isoflurane, decapitated, and excised brains were placed in 4°C oxygenated (95% O2/5% CO2) slicing solution (in mM: 26 NaHCO3, 234 sucrose, 10 MgSO4, 11.0 glucose, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2). A vibratome (Leica VT1200S) was used to make 270 μm-thick coronal sections containing dLGN. Slices were placed for at least 30 minutes in a 32°C oxygenated artificial cerebral spinal fluid (ACSF) (in mM: 126 NaCl, 26 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 2 MgCl2, 2 CaCl2, and 10 glucose) and then maintained at room temperature. Throughout the recording, slices were continuously
perfused in an oxygenated solution of ACSF (2.5 ml/min, 95% O₂/5% CO₂) and maintained at 32°C.

Whole-cell recordings were obtained in both current-clamp and voltage-clamp mode using a chamber that was mounted on an upright microscope (Olympus BX51WI) equipped with DIC and florescence optics to visualize GFP-expressing interneurons. Recordings were made using a Multiclamp 700B amplifier (Molecular Devices), filtered at 3-10 kHz, and digitized (Digidata 1440A) at 10 kHz. Patch electrodes made of borosilicate glass were pulled using a vertical puller (Narshige PC-10) with a final tip resistance between 4-7 MΩ.

For current clamp recordings, electrodes were filled with an internal solution containing (in mM): 117 K-gluconate, 13.0 KCl, 1 MgCl₂, 0.007 CaCl₂, 0.1 EGTA, 10 HEPES, 2 Na-ATP, and 0.4 Na-GTP. To examine the passive and active membrane properties of interneurons, we recorded the voltage responses evoked by square wave current pulses (-120 to +150 pA) of varying duration and intensity (600 and 1000 ms, 15 pA increments). Spike firing frequency (Hz) was based on maximal firing rate evoked by a large depolarizing current step. The degree of I_H-dependent inward rectification (V_sag) was determined by measuring the relative change between the maximal (V_max) and steady state (V_ss,) voltage response: (V_max − V_ss / V_max) * 100 (Leist et al., 2016).

To record inhibitory postsynaptic currents (IPSCs), we conducted voltage clamp recordings from dLGN relay neurons at 0 mV using a cesium-based internal solution containing (in mM): 117 Cs-gluconate, 11 CsCl, 1 MgCl₂, 1 CaCl₂, 0.1 EGTA, 10 HEPES, 2 Na₂-ATP, 0.4 Na₂-GTP. Concentrated stock
solution of (RS)-3,5-dihydroxyphenylglycine (DHPG) (Tocris catalog no. 0342) was prepared and stored as recommended. The agonist was diluted in ACSF to its final concentration (50 µM) prior to use, and applied via a short bolus into the input line of the recording chamber using a syringe pump (Govindaiah and Cox, 2006).

IPSCs were detected and analyzed with MiniAnalysis software (Synaptosoft, Leonia, NJ) using threshold (10 pA) and waveform detection protocols established by (Govindaiah and Cox, 2006). To quantify sIPSCs, the average frequency and amplitude were averaged from 120 second windows before mGluR5 agonist administration (pre), and after agonist administration (post).

For all recordings, biocytin (0.5%) was included in the internal solution to allow for intracellular filling and subsequent 3-D reconstruction using confocal microscopy. Following the completion of the recording session, slices were fixed overnight with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered-saline (PBS) (pH = 7.2). To visualize biocytin-filled interneurons, slices were washed with PBS (3x, 10 min. each), and incubated overnight in PBS solution containing 0.1% Triton X-100 and 0.1% Alexa Fluor 647-conjugated streptavidin (Invitrogen, catalog no. S21374). The following day, slices were washed in PBS (3x, 10 min each), mounted with Prolong Gold with DAPI (Invitrogen, catalog no. P36931), and coverslipped for confocal imaging.
3-D reconstructions of biocytin-filled interneurons

Biocytin-filled interneurons were imaged using a multi-photon laser scanning confocal microscope (Olympus, model no. 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 1.26 µm (20x/0.75 NA lens) created a 3-D, Z-stack dataset. Three-dimensional reconstructions and analysis were done using Imaris software (Bitplane, version 8.4.1). Soma area was measured by using the “Surface” function tool, and highlighting the soma in the X, Y, and Z planes. Branch reconstruction was generated in the Z-plane by using the “Filament tracer” function and verified by visual inspection. The total number of branches was determined by using the “Total Number of Terminal Points” tab in the Filament function. To evaluate the degree of arbor complexity, we labeled the filament-generated arbor tree using the “Statistics Coded Color” tab. This automated tool uses the soma as the starting point, and traces the arbor tree according to n<sup>th</sup> order branch. The “Statistics” tab was used to calculate the average and maximal branch order for each interneuron. To measure the volume of the arbor field, we started with the filament reconstruction, and used the “Convex Hull XTension” function in the Tools tab. This feature creates a new surface object superimposed on the filament. The convex polyhedron envelops all distal points (i.e. distal processes) of the selected filament and is used to calculate volume.
Experimental design and statistical analysis

All statistical tests were performed using either SigmaPlot 11.0 (Systat Software, San Jose, CA) or Prism 7.0 (Graphpad Software, La Jolla, CA). To test for normality, we utilized the Shapiro-Wilk Normality and Equal Variance Test. A Two-way ANOVA was used to examine age-related differences within a group (WT or math5^-/-) and between groups across the various morphological features (e.g., soma area, arbor complexity, arbor field volume) and membrane properties (V_sag, firing frequency). The post-hoc Holm-Sidak method allowed for pairwise multiple comparisons both within group and across postnatal weeks. For pharmacology experiments, we conducted a two-way ANOVA repeated measures to assess pre- vs post-drug differences in IPSC frequency and amplitude. The post-hoc Holm-Sidak method was applied to make multiple comparisons between groups. To compare percent changes in mGluR5 agonist-mediated responses ((Post–Pre / Pre) * 100) we used the non-parametric Mann-Whitney rank sum test to determine whether median values of frequency and amplitude were different between WT and math5^-/-. For all statistical tests, p values <0.05 were considered significant.
Results

Morphology of developing interneurons in WT and math5<sup>−/−</sup> mice

Figure 1 shows examples of coronal sections through the dLGN of P5 (A) and P21 (B) GAD67-GFP (WT) and GAD67 x math5<sup>−/−</sup> (math5<sup>−/−</sup>) mice. In both strains, we observed GFP labeling in dLGN interneurons even at early postnatal ages. In WT, interneurons are evenly distributed throughout the nucleus (Seabrook et al., 2013; Golding et al. 2014). However, in math5<sup>−/−</sup>, interneurons failed to migrate properly and clustered in the upper sector of the dLGN. This observation is consistent with previous reports showing that early postnatal elimination of retinal signaling disrupts the migratory path and spacing of dLGN interneurons (Golding et al., 2014). Nonetheless, in both strains we were able to readily identify and target interneurons for recording and biocytin filling.

We generated 3-D reconstructions for a total of 130 biocytin-filled interneurons (WT, n = 64; math5<sup>−/−</sup>, n = 66) across the first four weeks of postnatal life. Figure 2 provides representative examples of interneuron reconstructions from WT and age-matched math5<sup>−/−</sup> mice arranged by postnatal week. At early ages and in both groups, interneurons had Type II morphology, displaying expansive and complex arbors that arise from opposite poles of fusiform-shaped soma (Guillery, 1966). Arbors are comprised of both dendrites and axons. Not all interneurons have conventional axons, and there is no clear way to distinguish them from dendrites (Lieberman, 1973; Wilson, 1986; Sherman, 2004). Additionally, bead-like protrusions on distal processes (Fig. 2, inset) were especially apparent in WT after the second postnatal week. It is
presumed these grape-like appendages represent pre-synaptic dendritic terminal sites (F2 profiles) that form part of a triadic arrangement with a retinal terminal and relay cell dendrite (Szentagothai, 1963; Guillery, 1966; Hamos et al., 1985; Sherman, 2004). Despite the similarities in overall morphology between WT and math5⁻ interneurons, the absence of retinal signaling had a dystrophic effect on their growth and these defects are summarized in Fig. 3.

WT interneurons experienced significant growth in soma size between weeks 1 and 2 (Fig. 3A black, $F_{(3,120)} = 3.722; p = 0.013$), displaying ~2-fold change in area ($p = 0.026$). After the second postnatal week, soma size stabilized. By contrast, math5⁻ somata did not undergo an age-related change, remaining constant between weeks 1-4 (Fig. 3A red, $F_{(3,120)} = 3.722; p = 0.884$). Comparisons with WT revealed math5⁻ interneuron somata were significantly smaller between weeks 2-4 ($F_{(1,120)} = 36.54; week 2 p < 0.0001$, week 3 $p = 0.0015$, week 4 $p = 0.0066$).

To assess the development of arbor architecture, we examined the total number of processes, branch order, and size of the arbor field. (Fig. 3B-D). When considering these features, all branching elements (presumed axons and dendrites) were included in the analyses. WT interneurons undergo a number of age-related changes in arbor architecture (Fig. 3B-D, black). For example, there was a significant increase in the total number of processes (Fig. 3B, $F_{(3,122)} = 18.65, p < 0.0001$). Between weeks 1-2 the total number of processes increased ~3-fold ($p < 0.0001$). However, this increase was transient, and following the second postnatal week, the number of processes showed a significant decrease
by nearly 40% (p < 0.001). Despite this pruning, the number of arbors during week 4 was still 1.6-fold greater than week 1 (p = 0.003). These changes were accompanied by a significant increase in branch order (Fig. 3C, F (1, 120) = 19.09, p < 0.001). For instance, between weeks 1-2, branch order nearly doubled from 4th order to 9th order, with some cells exhibiting as high as 12th order branching (p < 0.001). WT branch order also decreased during weeks 2-3, from 9th to 6th order (p < 0.001), and at week 4 remained higher than week 1 (p = 0.029). Finally, dLGN interneurons experienced a significant change in arbor field size (Fig. 3D, F (3, 137) = 3.307; p = 0.012). Between weeks 1-2, arbor volume doubled (p = 0.022) and then stabilized (p = 0.998). Overall, these data suggest that interneuron arbors undergo an exuberant branching phase between weeks 1-2, then prune back during week 3 when their architecture stabilizes to resemble their adult-like form.

Unlike WT, math5β interneurons failed to show many of these changes (Fig. 3B-C, red). The number of processes and branch complexity remained stable across age (total processes, F (3, 122) = 18.65, p = 0.9666; branch complexity, F (3, 120) = 19.09, p = 0.580), and after week 1, these features were significantly reduced compared to WT (total processes, F (1, 122) = 104.87; p < 0.001; branch complexity, F (1, 120) = 83.17, p < 0.001). Interestingly, math5β interneurons undergo a 2.5-fold retraction in arbor field size between weeks 3-4 (p = 0.022) and were significantly smaller than WT (Fig. 3D, F (1, 137) = 16.348; week 2 p = 0.006, week 4 p = 0.023). Thus, these results demonstrate that
math5\(^{-}\) interneurons fail to remodel and maintain a simple, sparse morphology throughout development.

**Intrinsic membrane properties of WT and math5\(^{-}\) interneurons**

To assess the development of intrinsic membrane properties, we evaluated resting membrane potential (RMP), input resistance, the hyperpolarization-activated mixed cation conductance \(I_H\), and the frequency of spike firing frequency. Figure 4 provides representative examples of the voltage responses of interneurons evoked by square wave hyperpolarizing (Fig. 4, top) and depolarizing (Fig. 4, bottom) current pulses for WT (P3-30, n = 68 cells) and math5\(^{-}\) (P4-30, n = 75 cells). Overall, neurons exhibited a relatively high membrane input resistance, which led to a strong voltage response to current injection. A prominent feature of interneurons is the presence of an inward rectification (i.e. depolarizing sag, \(V_{sag}\)) that occurs during membrane hyperpolarization, a response attributed to the activation of the mixed cation conductance (\(I_H\)) (Williams et al., 1996; Seabrook et al., 2013; Leist et al., 2016). Membrane depolarization also gave rise to a train of action potentials that showed little to no frequency accommodation. These properties were apparent in both WT and math5\(^{-}\) interneurons, and began to emerge between weeks 1-2.

Figure 5A-B summarizes the age-related changes in RMP and input resistance of dLGN interneurons. In WT, RMP and input resistance remained relatively stable with age, averaging -56 mV and 450-460 M\(\Omega\), respectively (Fig. 5A-B, black; RMP, \(F_{(1,137)} = 1.132, p = 0.854\); input resistance, \(F_{(1,130)} = 2.178, p = 0.702\)). A similar pattern was apparent in math5\(^{-}\) interneurons (Fig. 5A-B,
red; RMP, -50 mV and input resistance, 660-670 MΩ) (RMP, $F_{(1, 137)} = 1.132$, $p = 0.259$; input resistance, $F_{(1, 130)} = 2.178$, $p = 0.472$). However, these values were different from WT. In math5\(^+\), RMPs were more depolarized between weeks 1-2 (Fig. 5A, $F_{(1,137)} = 18.01$; week 1 $p = 0.003$, week 2 $p = 0.035$), and overall input resistance was higher at all ages (Fig. 5B, $F_{(1,130)} = 17.65$; all $p$ values < 0.038) a result likely due to the their smaller soma size (Fig. 3A). We also examined $I_H$ by measuring the amplitude of the depolarizing sag as a percent change from steady state ($V_{\text{sag}}$). In WT, interneurons exhibited a 1.5-fold change between weeks 1-2 (Fig. 5C, $F_{(2,127)} = 5.648$; $p = 0.044$), and stabilized thereafter ($p = 0.589$). Maximal spike firing to strong depolarizing current pulses (-120-150 pA) showed a 2-fold increase between weeks 1-2 (Fig. 5D, $F_{(2,138)} = 8.254$; $p = 0.037$), and then stabilized ($p = 0.607$).

By contrast, in math5\(^+\), $I_H$ remained relatively stable with age (Fig. 5C, $F_{(2,138)} = 2.47$, $p = 0.351$), along with a 6-fold increase in spike firing between weeks 1-2 ($F_{(2,138)} = 8.254$, $p = 0.005$). Compared to WT, math5\(^+\) showed significantly lower values for $V_{\text{sag}}$ and maximal spike firing (Fig. 5C-D, $I_H$, $F_{(1,127)} = 14.537$, $p < 0.001$; firing frequency $F_{(1,138)} = 14.764$, $p < 0.001$), due largely to the differences seen during week 2 ($I_H$, $p = 0.002$; firing frequency, $p = 0.015$). Thus, these results reveal that math5\(^+\) interneurons remain functionally immature.

**Synaptic connectivity between dLGN interneurons and relay neurons**

To study the synaptic connectivity between interneurons and relay cells, we measured the frequency and amplitude of inhibitory postsynaptic currents
(IPSCs) in relay neurons that were pharmacologically evoked by the activation of F2 terminals. These processes express the Group I metabotropic glutamate receptor 1,5 (mGluR1,5), and the bath application of the agonist (RS)-3,5-
dihydroxyphenylglycine (DHPG) evokes F2-mediated GABA_A receptor mediated
inhibition onto relay neurons (Cox et al., 1998; Cox and Sherman, 2000;
Govindaiah and Cox, 2006). Figure 6 shows examples of biocytin-filled relay cells
in WT (A) and math5^- (B) along with their corresponding IPSC activity (5 min)
recorded before, during, and after DHPG application. As summarized in Figure
7A-B, during pre-drug conditions there were no differences in IPSC activity
between WT and math5^- (frequency, \( F_{(1,54)} = 35.03; p = 0.950 \); amplitude, \( F_{(1,54)}
= 0.623, p = 0.106 \)). However, WT neurons (n = 23 cells, n = 59,061 IPSCs)
showed a significant increase in IPSC frequency and amplitude following DHPG
treatment that persisted for several seconds (120-420 s, Fig. 7A, frequency \( F_{(1,54)}
= 35.03, p < 0.0001 \); Fig. 7B amplitude, \( F_{(1,54)} = 0.623, p = 0.0004 \)). IPSC
frequency more than doubled with amplitude showing a 1.3- fold change after
agonist application. In math5^- relay cells (n = 33 cells, n = 50,676 IPSCs)
showed a modest increase in IPSC frequency (Fig. 7A, \( F_{(1,54)} = 35.03, p = 0.018 \))
and no change in amplitude (Fig. 7B, \( F_{(1,54)} = 0.623, p = 0.995 \)) following DHPG
application. Moreover, post-drug math5^- IPSC frequency as well as amplitude
were significantly lower than WT (p < 0.0001).

In order to demonstrate the differences in magnitude of the DHPG-evoked
increase in IPSCs between the two groups, for each neuron we calculated the
percent change in IPSC frequency (Fig. 7A, right) and amplitude (Fig. 7B, right)
for each neuron. Overall, WT relay neurons displayed far greater changes (95% confidence interval of the mean; frequency 120-260%, amplitude 10-50%), showing significantly higher values than math5+ (95% confidence interval of the mean; frequency 20-40%; amplitude -5-10%; Mann-Whitney Rank Sum Test, p < 0.0001). Taken together, these data suggest that the absence of retinal signaling disrupts the synaptic connectivity between intrinsic interneurons and relay neurons.
Figure 13: Green fluorescent protein (GFP) expressing interneurons in dorsal lateral geniculate nucleus (dLGN) of WT and math5\(^{-}\) mice.

A: Coronal sections of dLGN from a P5 GAD67 WT mouse (left) and math5\(^{-}\) (right) crossed onto the GAD67 strain. Top: In WT, interneurons are evenly
distributed throughout dLGN, however in math5−/−, migration is disrupted and they cluster in the dorsolateral region (Golding et al., 2014) Scale bar = 70 um.

**Bottom:** Examples of GFP-expressing interneurons that were filled with biocytin during *in-vitro* intracellular recordings and used for 3-D reconstructions. Scale bar = 40 um. **B:** Coronal sections of dLGN from P21 WT and math5−/− mice. **Top:** Distribution of GFP-expressing interneurons in dLGN. **Bottom:** Examples of biocytin-filled interneurons.
Figure 14: Biocytin filled interneurons in dLGN of WT and math5<sup>−/−</sup> mice.

Examples of interneurons arranged by postnatal week (1-4) for WT (top row) and
math5\(^i\) (Bottom row). Reconstructions are based on Z-stacked confocal images of biocytin-filled interneurons. Scale bar = 50 \(\mu\)m; inset scale bars = 5 \(\mu\)m.
Figure 15: Morphological development of dLGN interneurons in WT and math5<sup>−/−</sup> mice.

Summary plots depicting age-related changes in soma area (A), number of processes (B), branch complexity (C), and arbor field size (D) of interneurons in
WT (black) and math5+/− (red). Each point represents mean values +/- SEM plotted by postnatal week. A: Between weeks 1-2, WT soma area increased ~2-fold (*, p = 0.026). Math5+/− soma areas are reduced compared to WT between weeks 2-4 (*, week 2 p < 0.001, week 3 p = 0.0015, week 4 p = 0.0066). B: WT interneurons show increased number of arbor processes between weeks 1-2 (*, p < 0.0001), followed by a reduction in weeks 3-4. Math5+/− interneurons show no age-related changes, and are reduced compared to WT during weeks 2-4 (*, weeks 2-4 p < 0.001). C: WT interneurons display increased higher-order branching between weeks 1-3, (*, week 2 p < 0.0001, week 3 p = 0.029). Math5+/− interneurons display decreased complexity compared to WT between weeks 2-4 (*, week 2 p < 0.001, week 3 p = 0.014, week 4 p = 0.002). D: Math5+/− interneurons displayed smaller arbor field sizes during weeks 2 and 4 (*, week 2 p = 0.006, week 4 p = 0.023), and experience field retraction during week 4 (*, p = 0.022).

For all analyses, WT: week 1, n = 3 mice, n = 10 cells; week 2, n = 7 mice, n = 24 cells; week 3, n = 4 mice, n = 17 cells; week 4, n = 3 mice, n = 14 cells. Math5+/−: week 1, n = 4 mice, n = 12 cells; week 2, n = 5 mice, n = 24 cells; week 3, n = 5 mice, n = 18 cells, week 4, n = 3 mice, n = 12 cells.
Figure 16: Intrinsic membrane properties WT and math5⁻/⁻ interneurons.

Examples of voltage traces showing responses to hyperpolarizing (-120 pA) and
depolarizing (150 pA) current step pulses from interneurons at different postnatal weeks (1-4) for WT (left) and math5^\textsuperscript{\textdagger} (right); WT RMP: -55-60 mV; math5^\textsuperscript{\textdagger} RMP: -45-50 mV. For WT, interneurons exhibit a strong inward rectification ($V_{\text{sag}}$) to hyperpolarizing pulses following the termination of the current pulse. Membrane depolarization evokes a train of action potentials. In math5^\textsuperscript{\textdagger}, interneuron responses remain relatively immature.
Figure 17: Passive and active membrane properties of WT and math5<sup>i</sup> interneurons. Scatter plots displaying age-related changes in resting membrane potential (A), input resistance (B), I<sub>H</sub> amplitude (C), and firing frequency (D) of interneurons in WT (black) and math5<sup>i</sup> (red). Each point represents mean values
+/− SEM arranged by postnatal week. **A:** Math5\(^{−}\) interneurons exhibit more depolarized RMPs at weeks 1 and 2 (\(^{*}\), week 1 \(p = 0.003\), week 2 \(p = 0.035\)). **B:** At all ages, math5\(^{−}\) interneurons have a higher input resistance (\(^{*}\), week 1 \(p = 0.038\), week 2 \(p = 0.013\), weeks 3-4 \(p = 0.027\)). **C:** WT interneurons show increases in \(I_H\) conductance between weeks 1-2 (\(^{*}\), \(p = 0.029\)), whereas math5\(^{−}\) interneurons show no change and are decreased compared to WT (\(^{*}\), \(p = 0.002\)). **D:** In WT, interneurons experience a 2-fold increase in frequency between weeks 1-2 (\(^{*}\), \(p = 0.005\)). Between weeks 1-2, math5\(^{−}\) interneurons also show increases in frequency (\(^{*}\), \(p = 0.0006\)), but remain decreased compared to WT (\(^{*}\), \(p = 0.015\)). Numbers of cells same as Figure 3.
Figure 18: Pattern of spontaneous inhibitory activity in relay neurons in WT and math 5<sup>−/−</sup> mice. Examples of WT (A) and math5<sup>−/−</sup> (B) dLGN relay neurons (Scale bar = 40 um) and their corresponding spontaneous inhibitory postsynaptic
current (IPSC) responses before and after bath application of mGluR$_5$ DHPG (50 µM). Arrows depict the time of drug delivery. Adjacent to the responses are the corresponding frequency histograms that plot the number of inhibitory events as a function of time. Horizontal bars reflect the adjacent current responses. Bin width is 30 sec. Activation of metabotropic glutamate receptor 1,5 (mGluR$_{1,5}$) leads to a sustained increase in IPSC activity in WT and little or no change in $\text{math5}^\dagger$. 
Figure 19: IPSC frequency and amplitude response before and after mGluR5 activation in WT and math5^−/− relay neurons.

A: Left: Scatter plot showing DHPG-induced changes in IPSC frequency for WT (black) and math5^−/− (red). Solid lines and dots depict pre- and post-DHPG
measurements from individual relay neurons (WT, n = 23; math5<sup>−/−</sup>, n = 33). WT cells display higher post-DHPG frequencies compared to math5<sup>−/−</sup> (*, p < 0.0001). Right: Scatterplot illustrating the percent change frequency for each relay cell. Horizontal bars are means and 95% confidence intervals. B: Left: Scatter plots showing the DHPG-induced changes in IPSC amplitude. Only WT relay neurons showed an increase (*, p = 0.033). Right: Summary plot of drug effect expressed as percent change. WT cells showed increased amplitude percent change compared to math5<sup>−/−</sup> (*, p = 0.0002).
Figure 20: Development of interneurons in WT and math5⁻/⁻ mice. Schematic summarizing the timeline of WT (top) and math5⁻/⁻ (bottom) interneuron development. Boxes indicate various WT milestones: migration (Golding et al., 2014), arbor remodeling (i.e., exuberant branching, pruning, and stabilization),
arbor field growth and stabilization, maturation of membrane properties, and synapse formation and connectivity. For math5\( \text{math5}^{5} \), the absence of retinal signaling results in disrupted migration, arrested arbor branching, dystrophic arbor field growth, decreased firing frequency/\( V_{\text{ sag}} \), and reduced patterns of synaptic connectivity.
Figure 21: Frequency distribution of soma size, $V_{sag}$, and spike firing of WT interneurons. A: Frequency distribution of soma areas of GFP-expressing interneurons measured from coronal slices of dLGN at ages when soma size has stabilized (P14+, $n = 3$ animals, 3 sections per animal). Red solid line represents
single Gaussian fit generated from nonlinear regression analysis to fit all data points ($\mu = 92.08$, $\sigma = 24.71$). **B:** Examination of data from Fig. 5 to determine if there is a bimodal distribution of percent changes in $I_H$. Nonlinear regression analysis revealed single Gaussian curve best fits the data ($\mu = 10.22$, $\sigma = 6.831$).

**C:** Same set of cells in (B) to examine distribution in firing frequency, which also followed a single Gaussian function ($\mu = 12.63$, $\sigma = 17.56$).
Discussion

A number of studies in mouse describe how and when interneurons migrate to dLGN and make feedforward inhibitory connections with relay neurons (Bickford et al., 2010; Seabrook et al., 2013; Golding et al., 2014; Jager et al., 2016). Here we provide additional details about their morphological growth and the emergence of adult-like membrane properties to present a comprehensive timeline of interneuron development (Fig. 8, top). In addition, our experiments in math5+ mutants reveal that many of these features require retinal signaling (Fig. 8, bottom).

At perinatal ages, interneurons begin to migrate to dLGN, and become evenly dispersed throughout the nucleus by the end of the first postnatal week (Fig. 8, migration) (Golding et al., 2014; Jager et al., 2016). Once migration is complete, arbor elaboration and remodeling begins. By the second postnatal week, the number of processes, arbor complexity, and arbor field size exhibit substantial increases (Fig. 8, exuberant branching and arbor field growth). During this time, intrinsic membrane properties are maturing, as $I_H$ ($V_{sag}$) and firing frequency reach peak values (Fig. 8, maturation of membrane properties). Interestingly, this phase of exuberant growth is followed by a brief period of arbor pruning in week 3, where the number of processes and complexity decrease (Fig 8, branch pruning). By week 4 the arbor architecture stabilizes and interneurons assume a stereotypic, Type II morphology (Fig 8, branch and field stabilization). Moreover, during the active period of arbor remodeling (exuberant branching and
pruning) feedforward inhibitory synapses emerge (Fig. 8, *synapse formation and connectivity*) (Bickford et al., 2010).

According to a recent report, dLGN interneurons are comprised of two classes based on soma diameter, with smaller ones having a higher density of \( I_H \) channels and higher rates of firing compared to larger ones (Leist et al., 2016). However, in our study we failed to find such distinctions. For example, in WT soma area (\( \mu m^2 \)) measurements of interneurons were normally distributed and best fit by a single Gaussian function. Similar relationships were found for the amplitude of \( I_H V_{sag} \) and spike firing frequency (Fig. 8-1, Extended Data). The reasons for such discrepancy remain unclear but may be related to differences in the measurement of soma size, the primary distinguishing factor (e.g. 3-D area vs. 2-D diameter).

Our results in math5 mutants reveal that the absence of retinal innervation disrupts many aspects of interneuron development (Fig. 8, bottom). Consistent with other reports, the elimination of retinal signaling leads to abnormal clustering of interneurons, and a failure to distribute evenly throughout dLGN (Fig. 8, *disrupted migration*) (Golding et al., 2014). Additionally, based on our 3-D reconstructions, math5\(^-\) interneurons do not undergo arbor remodeling during postnatal development. Instead, they maintain simple, sparse arbors at all ages (Fig. 8, *arrested arbor branching*). The size of their arbor field is also restricted (Fig. 8, *dystrophic arbor field growth*), and during the fourth postnatal week fields actually retract (Fig. 8, *field retraction*). Functionally, the intrinsic membrane properties of math5\(^-\) interneurons fail to mature, and exhibit decreased spike
firing rates and weaker $V_{\text{sag}}$ (Fig. 8, *decreased firing frequency and $I_H$*).

Furthermore, the elimination of retinal signaling also leads to a disruption in feedforward inhibition. Recordings in math5$^{-}$ relay cells show little change in IPSC frequency after pharmacological activation of F2 terminals. By contrast, WT relay neurons exhibit a 2 to 4-fold increase in IPSC frequency (Cox and Sherman, 2000; Govindaiah and Cox, 2006). These findings suggest that math5$^{-}$ contain fewer and/or weaker inhibitory synapses (Fig. 8, *reduced synaptic connectivity*). These results are consistent with previous studies that employ early postnatal elimination of retinal signaling (Golding et al., 2014). However, the utility of a genetic form of deafferentation (i.e., math5$^{-}$) rules out the possibility that these disruptions were brought about by trauma or degeneration. Instead they are likely a consequence of stunted arbor growth or the abnormal clustering of interneurons that occurs after the removal of retinal innervation. Interestingly, WT and math5$^{-}$ relay neurons display similar baseline IPSC activity, suggesting inhibitory input from extrinsic sources (e.g., pretectum, thalamic reticular nucleus) are intact and may act to compensate for the loss of intrinsic inhibition. Such compensatory activity may reflect a form of homeostatic plasticity designed to maintain proper E/I balance (Turrigiano and Nelson, 2004; Maffei et al., 2006).

The current study elucidates the importance of retinal signaling, and its role in supporting the morphological development and emergence of feedforward inhibitory connections in dLGN. These findings are consistent with previous studies from our lab demonstrating that retinal input provides vital trophic support for the morphological development of dLGN relay cells (El-Danaf et al., 2015).
Furthermore, our results imply that retinal signaling has a greater impact on interneurons than on relay cells. Although math5<sup>−/−</sup> relay neurons experience dystrophic dendritic growth, they retain relatively complex dendritic architecture, cell-class identity, and the full complement of voltage gated membrane properties (El-Danaf et al., 2015). By contrast, dLGN interneurons in math5<sup>−/−</sup> fail to remodel, and instead remain morphologically and functionally immature.

While it is evident that retinal axons provide some form of trophic support for dLGN interneurons, the underlying molecular mechanism remains unknown. One prospect is brain-derived neurotrophic factor (BDNF). This growth factor is synthesized in RGCs and released by retinal axons, where it binds to receptor tyrosine kinase (trkB) located on the dendrites of relay cells and interneurons (Avvenagha et al., 2006). Another intriguing candidate is fibroblast growth factor 15 (Fgf15), a signaling molecule involved in widespread developmental processes including GABAergic differentiation, cell survival, and synapse formation (Hagemann and Scholpp, 2012; Martinez-Ferre et al., 2016). At perinatal ages Fgf15 is expressed along the presumed the migratory path of interneurons, including ventricular layer of the 3<sup>rd</sup> ventricle, vLGN and dLGN (Gimeno et al., 2003). More importantly, developing RGCs express Fgf15 at perinatal ages during the time when retinal axons innervate dLGN (Kurose et al., 2004). Overall these studies provide an in-depth analysis of dLGN interneuron development and underscore the importance of retinal signaling in the developmental remodeling of their arbors and feedforward inhibitory synapse formation.
SUMMARY AND CONCLUSIONS

These studies addressed the outstanding questions pertaining to the development, form, and function of thalamic interneurons. Here we primarily focused on dLGN, and the manner by which interneurons arrive, distribute, and remodel throughout postnatal development. While the mouse model has provided extraordinary information about the establishment of inhibitory circuits, the migratory path by which dLGN interneurons travel, as well as their morphological development has remained unclear. Furthermore, while it is understood that retinal signaling contributes to some aspects of interneuron dispersion (Golding et al., 2014), we addressed whether retinal axon innervation regulates their targeting, morphology, and synaptic connectivity. To accomplish this, we used the GAD67-GFP transgenic mouse line that labels thalamic interneurons with GFP to track their migration, and conduct in-vitro patch-clamp recordings with biocytin-filled pipettes throughout postnatal life to examine their structure and function. In order to determine how retinal signaling influences these developmental events, we used a genetic deafferentation model and utilized the math5 null mouse (math5⁻/) to compare with age-matched controls.
In Chapter I, we examined the migratory path by which interneurons follow to arrive in dLGN and addressed the debate about the source of these neurons (Golding et al., 2014; Jager et al., 2016). BDA injections near V3 and into SC confirm that interneurons travel from the neuroepithelium of V3, across the ventral border of VB, and into vLGN. At early ages, interneurons gradually migrate dorsally into dLGN and finish evenly dispersing themselves throughout the nucleus by P4. Similarly, LPN migration and distribution also reaches stable levels by this time. However, in the absence of retinal innervation, fewer interneurons arrive in visual thalamus (vLGN, dLGN, LPN) and neurons remain clustered in the top sectors of nuclei. By contrast, the somatosensory VB complex contained substantially more interneurons, which implies that interneurons are misrouted en-route to their final destination. Together, our findings indicate that retino-recipient nuclei rely on retinal signaling for proper recruitment and integration of inhibitory neurons.

To determine the underlying molecular mechanisms for these defects, we used the Fgf15 knockout transgenic mouse (Fgf15\(^{−/−}\)) to assess how the loss of this growth factor impacts interneuron migration and distribution. This neurotrophic factor was a plausible candidate because it is involved in GABAergic differentiation (Ornitz and Itoh, 2015; Martinez-Ferre et al., 2016) and is downregulated in math5\(^{−/−}\) mice (Fox lab, unpublished observations). Our studies showed that Fgf15\(^{−/−}\) regulates interneuron migration to dLGN, but not dispersion within the nucleus. In Fgf15\(^{−/−}\) mice, interneurons are misrouted, as there were fewer interneurons in vLGN and dLGN, but VB contained far more.
However, the interneurons that arrive at their destination maintained normal distribution patterns. These results signify the likely possibility that multiple signaling pathways are involved in the establishment of inhibitory circuitries in thalamus that are independent of each other, yet all dependent on retinal axons.

In Chapter II, we discussed how the development of dLGN interneurons is a multi-step process that involves migration (see Chapter I), arbor remodeling, and synapse formation. Arbor remodeling consists of an exuberant branching period, whereby interneuron branch number and complex increase. Such growth is transient however, as interneurons then undergo pruning and stabilization to assume their adult-like architectures. Meanwhile, the emergence of feedforward inhibition occurs around this time (~P14) (Bickford et al., 2010), after intrinsic membrane properties have fully matured. Our results in math5 mutants revealed that the absence of retinal signaling causes numerous developmental disruptions. Structurally, interneurons in math5\textsuperscript{-/-} mice did not experience arbor remodeling, but rather maintained simple architectures at all ages. Functionally, their intrinsic membrane properties remained immature, and interneurons made fewer and/or weaker inhibitory synapses onto relay neurons.

Taken together, our studies demonstrate the essential role of retinal innervation in interneuron targeting, migration, distribution, as well as their morphological and synaptic development. These studies provide a foundation for future experiments that can address the issues that remain unknown regarding the development of thalamic interneurons. For example, although our caspase-3 studies showed negative results during postnatal ages, it is necessary to confirm
whether interneurons in math5−/+ mice experience increased apoptotic activity during embryonic development. This will clarify if the loss of dLGN interneurons is caused by misrouted neurons and/or cell death in the absence of retinal signaling. Additionally, the underlying mechanism for interneuron distribution through dLGN is unclear. While our studies show that Fgf15 is vital for their arrival, future studies should explore the role of DSCAMs in the spacing of thalamic interneurons because this cell adhesion molecule is needed for appropriate retinal organization (Fuerst et al., 2012) and its role may extend beyond the retina. Finally, it would be worthwhile for future research to examine the link between the reported phenotypes and behavior. An alteration in inhibitory circuitry such as fewer cells in LPN and increased interneurons in VB may disrupt visuomotor and/or escape behaviors, and/or increase sensitivity to somatosensory stimuli, respectively. The mouse has proven to be an invaluable model system to understand the development of visual thalamus, and building on the results outlined here will further illuminate how thalamic interneurons contribute to functional visual circuits.
REFERENCES


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EDUCATION:

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October 2018
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M.S./Ph.D, Anatomical Sciences & Neurobiology
GPA: 3.95

University of Kentucky  
2010 – 2013
Lexington, KY
B.S.: Pre-Medicine & Psychology
Minor: Mathematics
GPA: 3.80

POLICY/ADVOCACY/LEGISLATIVE EXPERIENCE:

Director and Policy Coordinator, Science Policy  
Fall 2016 – Present
And Outreach Group (SPOG)

• Co-founder and current director of the student science engagement organization at the University of Louisville
• Oversee policy and outreach activities throughout the community
• Organize monthly meetings, and maintain communication with state lawmakers, guest speakers, and local science teachers
• Build and manage relationships with outside partners, including other student science policy groups

Research Lab Tour w/ Sen. Rand Paul’s October 2016
Field Representative
• Hosted Field Representative Whitney Meadows for a tour of the labs throughout the department
• Discussed the importance of federal research funding

Funding Your Future Advocacy Forum w/ Keynote August 2016
Speaker Rep. John Yarmuth (KY-03)
• Prepared a presentation for attendees about how the federal appropriations process operates and provided resources for students/faculty to advocate for increased biomedical research funding
• House Budget Committee Ranking Member Rep. John Yarmuth (KY-03) discussed the appropriations process from his perspective and encouraged attendees to continue advocating for scientific research funding

Society for Neuroscience Capitol Hill Day March 2016
• Represented SfN as their Early Career Science Policy Ambassador
Society for Neuroscience (SfN) Early Career  January – December 2016

Science Policy Ambassador
- Served on behalf of SfN as leader for science advocacy in my community
- Organized three local advocacy events and presented on these events at the annual national meeting

Research!America, Science Policy Intern  Summer 2015
- Met with members of Congress to discuss science agency appropriations for Fiscal Year 2016
- Provided research and analysis on federal health agency funding trends and other issues salient to science communication/advocacy; presented data to colleagues and board members
- Attended and briefed staff members on congressional committee hearings
- Drafted and disseminated fact sheets focused on the value of research through the prism of specific diseases, including eating disorders, glaucoma, and tuberculosis
- Conducted research for Know Your Candidates Project Election 2016, providing information on each candidate’s views regarding health, science, and biomedical research.


Legislative Director
- Hosted Legislative Director Zack Marshall for a tour of the neuroscience labs
Society for Neuroscience Capitol Hill Day  
March 2015

- Participated in meetings with members of Congress including: Sen. Mitch McConnell (KY), Sen. Rand Paul (KY), Sen. Lamar Alexander (TN), and Sen. Tom Cotton (AR)

RESEARCH AND CLINICAL EXPERIENCE

University of Louisville School of Medicine  
Fall 2013 – Present

Doctoral Candidate, Anatomical Sciences & Neurobiology

Mentor: Dr. William Guido, Ph.D.

- Studied the morphological and synaptic development of dorsal lateral geniculate nucleus (dLGN) interneurons using transgenic mouse lines
- Developed protocol for 3-D neuronal reconstruction with Imaris (Bitplane) software following confocal microscopy
- Performed anatomical/electrophysiological experiments, including pharmacological and whole-cell patch-clamping
- Evaluated data with statistical analysis using SigmaPlot, MiniAnalysis, and Prism GraphPad
- Prepared scientific abstracts, publications, and posters for presentations at professional conferences

University of Kentucky  
Fall 2011 – May 2013

Research Assistant, Department of Psychology

Mentor: Dr. Christia Brown, Ph.D.

- Researched the impact of male/female stereotypes on middle-school students
- Performed statistical (SPSS) analysis to examine the influence of peer group interactions on negative behavior/discrimination in schools
University of Kentucky Alcoholism Lab  
Research Assistant, Department of Psychology  
Summer 2012
- Assisted with data collection for a research study that observed the physical and mental effects of alcohol among adolescents

University of Kentucky Clinic  
Research Assistant, Department of Pediatric Endocrinology  
Mentor: Dr. Irene Hong-McAtee, M.D.  
Summer 2011
- Compiled patients’ A1c levels between the years 2008-2011
- Observed and assisted with neonatal thyroid surgeries

COMMUNITY/PROFESSIONAL ACTIVITIES:

Co-organizer, Louisville Science Pathways  
Summer 2017
- Created a summer research internship program for students interested in science, but without prior hands-on laboratory experience

Louisville March for Science  
April 2017
- Served on the organizing committee to create a sister march to the national March for Science
- Worked to sustain nonpartisan nature of march and keep focus on the value of science
- Recruited speakers, supplies, city permits, and deployed social media to communicate event details

Louisville Regional Science & Engineering Fair  
March 2016 – 2017
Advocacy Day
Helped organize Student Advocacy Day at the Capitol in Frankfort, KY for regional science fair winners

- Students met with state policymakers, including the Governor and Lt. Governor, and explained their respective science projects

**MEMBERSHIPS:**

- Society for Neuroscience
  - 2013 – Present
- Career Research Advancement Focused
  - Training Seminar Series Committee
  - 2016 – Present
- Kentucky Academy of Sciences (KAS) Science Education & Advocacy Committee
  - Fall 2015 – Present
- Louisville Regional Science & Engineering Fair Board Member
  - Fall 2015 – 2018
- School of Medicine Graduate Student Council, Graduate Student Representative
  - 2016 – 2017

**HONORS/AWARDS/APPEARANCES:**

- Guest Lecturer, Bellarmine University, IDC 101-26: Science and Society
  - April 2018
- Gordon Research Conference Travel Award
  - February 2018
- Society for Neuroscience Public Advocacy Forum “Advocating for Basic Science in a Disease-Focused World,” Panel Member
  - November 2017
- University of Louisville “Focused” Video Interview and Article “Focused: PhD student wants to ‘be that voice in the science community’”
  - April 2017
• Presenter, Girls STEM Collaborative Conference            April 2017
• Kentucky Science Center Award to SPOG                    October 2016
  Outstanding Service & Volunteer Work
• 3rd Place Research Poster Winner                          April 2016
  SfN Louisville Chapter, Neuroscience Day
• Video Interview, SfN Capitol Hill Day                     March 2015
  “Why Advocacy is Important to Me”
• Dean’s List, University of Kentucky                       Fall 2011 – 2013
• America’s Top Chemistry/Physics Students                  Fall 2010
• Presidential Scholarship, University of Kentucky          2009 – 2013
• 1st Place Speech Contest, University of Kentucky          March 2011

PUBLICATIONS:

• Abstract, Poster Presentation – The absence of retinal input affects the targeting, dendritic architecture, and synaptic connectivity of intrinsic interneurons in mouse dLGN, Gordon Research Conference, February 2018. Lucca, Italy.

• Letter to the Editor, The New York Times – “Kentucky and Medicaid,” January 21st, 2018


• Letter to the Editor, The Lexington Herald Leader – “House tax bill risks brain drain” November 22nd, 2017


• Abstract, Poster Presentation – *The absence of retinal input affects the targeting and morphological development of intrinsic interneurons of dLGN*. Society for Neuroscience Conference, Fall 2016. San Diego, CA.

• Abstract, Poster Presentation – *The importance of student advocacy at the science policy intersect: my journey as an early career neuroscientist*. Society for Neuroscience Conference, Fall 2016. San Diego, CA.


• Abstract, Poster Presentation – *Optogenetic activation of inhibitory synapses in the dorsal lateral geniculate nucleus of the mouse*; Society for Neuroscience Conference, Fall 2014, Washington, DC

• Video Interview, Society for Neuroscience Capitol Hill Day 2014, “Why Advocacy is Important to Me.”

• Medical Student Curriculum, University of Kentucky School of Medicine 2012, “Waisting Time: An Outlook on Eating Disorders.”