Thalamic spontaneous activity coordinates the timing of corticothalamic innervation in the visual system

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Summary

Sensory processing relies on the correct development of circuits connecting thalamus and cortex. Visual corticothalamic axons (CTAs) invade the thalamic dorsolateral geniculate nucleus (dLGN) of the thalamus following an early postnatal time-regulated programme in mice. Retinal spontaneous activity influences the thalamic incursion of CTAs, however, the perinatal thalamus also generates intrinsic patterns of spontaneous activity whose role in modulating afferent connectivity remains unknown. Here, we found that patterned spontaneous activity in the dLGN is critical for the proper spatial and temporal innervation of CTAs. When the spontaneous dLGN activity is disrupted *in vivo*, CTA innervation is severely delayed until eye-opening. Indeed, visual input influenced the temporal development of CTAs by modulating thalamic activity, as embryonic enucleation enhanced thalamic calcium waves and accelerated the entrance of CTAs into the dLGN. Our results show that patterned spontaneous activity in the perinatal thalamus is a key element driving the wiring of visual circuits.

Keywords

Thalamus, development, corticogeniculate, corticothalamic, thalamocortical, spontaneous activity, retinal input.

22 Introduction

23 The formation of sensory circuits relies on the highly regulated spatial and temporal
 24 organization of axon connectivity. The visual system has been used extensively as a
 25 model to decipher the processes that control the development of neuronal networks,
 26 such as axon guidance and/or targeting. Indeed, retinofugal projections have been
 27 employed to study in some detail how topographic organization is established in the
 28 visual pathway (Fox & Guido, 2011; Huberman et al., 2008; Shatz & Rakic, 1981a;
 29 Zipursky & Sanes, 2010). The development of the visual system in mice, depends on
 30 feed-forward input from the retina through the retinothalamic axons (RTAs) that reach
 31 the dorsolateral geniculate nucleus (dLGN) of the thalamus on embryonic day 15 (E15),
 32 ensuring that ipsilateral and contralateral axon segregation is completed by postnatal
 33 day 8 (P8: Godement, Salaün, & Imbert, 1984a). By contrast, corticothalamic axons
 34 (CTAs) from layer 6 (L6) of the primary visual cortex (V1) reach the dorsal border of
 35 the dLGN at E18 and they begin to invade this nucleus around P4. Indeed, after the first
 36 postnatal week, CTAs have colonized the entire dLGN (Brooks et al., 2013; Grant,
 37 Hoerder-Suabedissen, & Molnár, 2016; Seabrook, El-Danaf, Krahe, Fox, & Guido,
 38 2013).

39 Numerically, CTAs represent the largest input to the dLGN and it has been
 40 demonstrated that they are key regulators of dLGN cell firing (Erişir, Bickford, Van
 41 Horn, & Sherman, 1997; Godwin et al., 1996; McCormick & Krosigk, 1992). However,
 42 the feedback from the V1 to dLGN develops relatively late with respect to the retinal
 43 inputs (Dräger & Hubel, 1975; Godement, Salaün, & Imbert, 1984b; Grant et al., 2016;
 44 Jacobs et al., 2007; Seabrook et al., 2013; Shatz & Rakic, 1981b). It has been suggested
 45 that RTAs and CTAs might interact to ensure the correct development of both
 46 projections during the second postnatal week (Bickford et al., 2009; Seabrook et al.,

2013). More recent insights into this process demonstrate that the invasion of the dLGN by cortical and retinal inputs relies on their mutual interactions at the thalamus (Brooks et al., 2013; Grant et al., 2016; Jurgens, Bell, McQuiston, & Guido, 2012; Seabrook et al., 2013; Thompson, Gribizis, Chen, & Crair, 2017). These interactions seem to be activity-dependent, as disrupting postnatal retinal waves by injecting the cholinergic agonist epibatidine accelerates CTA entry into the dLGN (Grant et al., 2016). Indeed, postnatal enucleation of mice leads to earlier innervation of the dLGN by CTAs (Brooks et al., 2013; Grant et al., 2016; Seabrook et al., 2013). These results suggest that early peripheral input and the electric messages conveyed are key elements for the timely innervation and correct development of CTAs in the visual pathway. However, retinal waves are not the only activity that trains the developing thalamus. It was recently shown that the perinatal thalamus generates an intrinsic pattern of spontaneous activity that is known to regulate the organization of sensory systems (Antón-Bolaños et al., 2019; Castillo-Paterna et al., 2015; Mire et al., 2012; Moreno-Juan et al., 2017), suggesting this activity may possibly regulate the innervation of the dLGN. Here, we tested this hypothesis using an *in vivo* approach in which thalamic spontaneous activity is modified in the dLGN. We found that alterations to the patterns of spontaneous thalamic activity in the dLGN during prenatal and postnatal stages impair the timing of CTA innervation of this nucleus. Remarkably, the absence of CTAs in the dLGN is rescued by eye opening, strongly suggesting that both intrinsic spontaneous activity and experience-dependent programmes play a role in the development of the corticothalamic circuits associated with the visual system.

Results

Disrupting the pattern of spontaneous activity in the developing visual thalamus

To determine whether innervation of the dLGN by visual CTAs requires thalamic spontaneous activity, we disrupted the activity of the thalamus by overexpressing the inward rectifier potassium channel 2.1 (Kir2.1) in all the principal thalamic sensory nuclei. As such, we administered tamoxifen to pregnant *Gbx2^{CreER};R26^{Kir2.1-floxed}* mice (referred herein as *Th^{Kir}*) when embryos were at E10.5, inducing the overexpression of Kir2.1 (fused to mCherry) in embryonic thalamic neurons (Antón-Bolaños et al., 2019). As expected, the increase in Kir2.1 suppressed the patterned spontaneous activity in the dLGN of new-born *Th^{Kir}* mice, this activity normally evident as calcium waves (**Figure 1A-D**; Moreno-Juan et al., 2017). Calcium imaging of *ex vivo* brain slices from *Th^{Kir}* mice at P0 demonstrated that neurons from the dLGN only activated asynchronously, with no large and propagating co-activation witnessed, such as that recorded in control slices. Therefore, and as shown previously for the somatosensory and auditory nuclei of the thalamus (Moreno-Juan et al., 2017; Antón-Bolaños et al., 2019), overexpression of Kir2.1 transforms the pattern of dLGN activity from synchronous to asynchronous at perinatal stages. Towards the end of the first postnatal week, an additional change in the pattern of spontaneous activity was recorded in the *Th^{Kir}* mice *in vivo*. Extracellular recordings with multichannel electrodes in the thalamus revealed that the firing rate of dLGN neurons decreased significantly in the *Th^{Kir}* mice at P6 (**Figure 1E-H**). Therefore, there is *ex vivo* and *in vivo* evidence that the overexpression of Kir2.1 alters the properties of spontaneous activity in the visual thalamus during the initial days post-partum, the time when CTAs reach and invade the dLGN.

Thalamic spontaneous activity mediates the entry of CTAs into the dLGN

In light of the above, we determined whether an abnormal intrinsic pattern of spontaneous activity in the thalamus could affect the progression of CTAs into the dLGN. As such, we visualized CTAs in the dLGN through their expression of the vGlut1 transporter expressed at the terminals of corticothalamic neurons (Grant et al., 2016; Piñon, Jethwa, Jacobs, Campagnoni, & Molnár, 2009; Varoqui, Schäfer, Zhu, Weihe, & Erickson, 2002), as well as in the transgenic mouse line *Golli-tau-eGfp* that labels cortical L6 neurons and their axons (Grant et al., 2016; Jacobs et al., 2007). At P3, control axons from L6 appeared adjacent to the ventromedial border of the dLGN, progressively invading the nucleus over successive days (**Figure S1**: Brooks et al., 2013). While control CTAs had already entered the dLGN by P4, they remained at the border in the *Th^{Kir}* mice (**Figure 2A**). In addition, we also measured the invasion of CTAs in the dLGN in terms of the vGlut1 fluorescence in the dLGN area of control and *Th^{Kir}* mice at P4, P6 and P9. The invasion of the dLGN by CTAs was severely perturbed in the *Th^{Kir}* mice and while CTAs covered more than half of the nucleus in control animals at P9, the invasion of this territory by CTAs was reduced by more than 90% when thalamic spontaneous activity was disturbed (**Figure 2B**). This result was confirmed by labelling L6 neurons from the V1 with a lentivirus expressing *Gfp*, showing that virtually no CTAs had invaded the dLGN in *Th^{Kir}* mice at P9 (**Figure 2C**). This effect was not due to a developmental delay in the arrival of CTAs in these *Th^{Kir}* mice since L6 axons appear to be waiting at the border of the dLGN in both the control and *Th^{Kir}* mice at P0 (**Figure 2D**). Together these results indicate that spontaneous activity in the dLGN regulates the invasion of this structure by CTAs.

Disrupting the patterned thalamic activity leads dLGN neuron death

When analysing CTA invasion in the *Th^{Kir}* mice, we noticed that perturbing spontaneous thalamic activity provoked a decrease in the size of the dLGN. While the dLGN size was normal in *Th^{Kir}* mice from P0 to P3, it remained stationary from P4 onwards, a period in which the size of the dLGN normally increased in control mice and when the CTAs invaded the thalamus (**Figure 3A**). To determine whether this lack of growth of the dLGN is due to an increase in cell-death and/or the enhanced compaction of dLGN cells, we analysed caspase-3a activity (Ahern et al., 2013; Lossi, Castagna, & Merighi, 2015) and the cell density in the dLGN of control and *Th^{Kir}* mice. There was a significant increase in caspase-3a immunostaining from P3 to P6 in the dLGN of the *Th^{Kir}* mice (**Figure 3B and 3C**) but no increase in cell density in this nucleus (**Figure S2A and S2B**). Hence, these data suggest that the failure to increase the size of the dLGN in the *Th^{Kir}* mice might be the result of enhanced cell-death in this nucleus, in conjunction with an absence of CTAs in the visual thalamus.

The lack of innervation of the dLGN by CTAs is independent of RTAs

It was previously suggested that a hierarchy exists in the temporal invasion of the dLGN by afferent axons, with RTAs preceding CTAs (Brooks et al., 2013; Guido, 2018; Jacobs et al., 2007; Seabrook et al., 2013). Furthermore, it was suggested that cortical input from L6 neurons is required for the correct targeting of RTAs to the dLGN and for their eye-specific segregation (Shanks et al., 2016; Thompson, Picard, Min, Fagiolini, & Chen, 2016). Therefore, the mechanisms defining axon interactions might regulate the invasion of the dLGN by these two sets of projections, whereby RTAs are reorganized and segregated once CTAs start to innervate the dLGN. Indeed, Cholera-Toxin subunit B (CTB) injections into the eyes of *Golli-tau-eGfp* mice revealed that RTAs preceded CTA innervation of the dLGN (**Fig. 4A and 4B**), supporting previous findings

(Seabrook et al., 2013). As perturbed thalamic spontaneous activity strongly affects the innervation of the dLGN by CTAs, we tested whether the absence of CTAs in the *Th^{Kir}* mouse is a consequence of an early defect in RTA innervation. As such, we performed anterograde labelling of RTAs by injecting Alexa-647 conjugated CTB into the retina. Remarkably, we found that the timing of contralateral and ipsilateral RTA invasion of the dLGN is normal at P2 in *Th^{Kir}* mice (**Figure 4C and 4D**). Hence, retinal axons target and invade the dLGN independently of thalamic activity and the absence of CTAs in the dLGN is not due to a defect of the RTAs.

Retinal inputs modify the spontaneous thalamic activity in the dLGN

Our previous results demonstrated that normal spontaneous activity in the dLGN is required for CTAs to invade this nucleus. Interestingly, the pattern of the spontaneous activity in the somatosensory nucleus of the thalamus is thought to be modified after the removal of retinal input due to embryonic bilateral enucleation (embBE; Moreno-Juan et al., 2017). Hence, we cannot rule out that the presence of RTAs might somehow also influence spontaneous activity in the visual thalamus and thereby affect the development of CTAs. In fact, postnatal deprivation of the visual periphery input provokes an aberrant development of the CTAs in the dLGN (Grant et al., 2016; Jacobs et al., 2007; Seabrook et al., 2013). To test this possibility, we performed embryonic bilateral enucleations at E14.5 to ensure that visual input never reached the thalamus, as retinal axons reach the dLGN at E15.5 in mice (Moreno-Juan et al., 2017). To detect the spontaneous activity in the dLGN, we performed calcium imaging *ex vivo* in control and embBE slices at P1, prior to CTA invasion of the dLGN (**Figure 5A and Figure 5E**). At this stage we found a higher frequency of spontaneous synchronous calcium events in the dLGN of embBE mice, which included the appearance of more thalamic calcium

waves than in control mice (**Figure 5B and 5C**). As such, these experiments show that despite the emergence of thalamic waves that are independent of retinal input, RTAs exert a modulatory role on the patterned activity of the dLGN that is revealed in enucleated animals.

We next determined whether innervation of the dLGN by CTAs is affected in embBE mice by performing enucleations at E14.5 in wild-type and *Golli-tau-eGfp* mice (**Figure 5D**). In control animals, CTAs began to invade the dLGN from P4 and they have covered the entire nucleus by around P12, close to the time of eye-opening (Brooks et al., 2013; Seabrook et al., 2013). By contrast, in embBE mice CTAs expressing GFP or labelled for vGlut1 immunostaining already covered a significantly higher proportion of the dLGN (8.85%) at P1 than in their control littermates (3.25%). This acceleration of dLGN invasion was maintained at all the developmental stages studied (P2, P3 and P7: **Figure 5E and 5F, Figure S3**), and it was in accordance with data found previously in mice enucleated at P0 (Brooks et al., 2013; Grant et al., 2016; Seabrook et al., 2013).

These results show that the arrival of retinal axons has strong impact on the developing dLGN. First, they regulate the pattern of spontaneous activity and secondly, they influence the postnatal innervation of the dLGN by CTAs. However, it remains unclear whether there is a causal link between these events. In order to address this question, we performed bilateral embryonic enucleations in the *Th^{Kir}* mice (**Figure 5G**). Remarkably, we found that CTA innervation of the dLGN is rescued when RTAs are eliminated in *Th^{Kir}* mice. The relative invasion of the dLGN by CTAs in the embBE *Th^{Kir}* mice was similar to that of embBE mice (**Figure 5H and 5I**), demonstrating the existence of a hierarchical organization in the invasion of the dLGN whereby the arrival

of RTAs switches the pattern of spontaneous activity in the nucleus, in turn, delaying the entry of CTAs.

Visual experience rescues CTA innervation of the dLGN in the *Th^{Kir}* mice

Our results demonstrated that altering the patterns of early thalamic spontaneous activity affects the development of the corticothalamic connections to the dLGN. The reduction in spontaneous dLGN activity due to the absence of thalamic calcium waves induced a severe defect in CTA innervation, which were virtually absent from the dLGN during the first postnatal week, and at least until P9. Nevertheless, as *Th^{Kir}* mice survive until adulthood, we wondered whether this effect might persist. Notably, at P30 CTAs had innervated the full extent of the dLGN in *Th^{Kir}* mice as in control littermates (**Figure S4A**), indicating that the aberrant CTA innervation detected early in development is reversed at later stages.

Eye-opening occurs in mice at around P13, an event that is crucial for the final maturation and refinement of the visual system (Hensch, 2005; Hofer, Mrsic-Flogel, Bonhoeffer, & Hübener, 2009; Hooks & Chen, 2006; Pecka, Han, Sader, & Mrsic-Flogel, 2014). As CTA invasion of the dLGN is rescued after P9, we assessed whether eye-opening might play a role in this recovery. We analysed the innervation of the dLGN by CTAs in both control and *Th^{Kir}* mice at P10, P13 and P14, just before and after eye-opening. We found that while at P10 when the eyes are still closed, CTAs poorly innervated the dLGN in *Th^{Kir}* mice relative to the controls (**Figure 6A**).

However, after eye-opening, the CTA invasion in *Th^{Kir}* mice at P13-P14 was indistinguishable from that of the control mice (**Figure 6B and S4B**).

Visual stimulation through close eyelids evokes glutamatergic retinal waves, and light deprivation in mice from P8 to P14 provokes deficient retinogeniculate

segregation, demonstrating that activity derived by light stimulus just prior to eye opening influences the topographic development of the visual system (Tiriac, Smith, & Feller, 2018). Thus, we hypothesised that visual experience may alter the network properties of dLGN neurons, allowing CTAs to enter the dLGN. To test this possibility, we maintained control and *Th^{Kir}* mice in a dark rearing (DR) environment from P5 until P15 and we then analysed the CTA innervation of the dLGN (**Figure 6C**). We did not find major modifications in CTA innervation at P15 in control animals subjected to DR (**Figure 6D and 6E**). However, in contrast to the *Th^{Kir}* mice, there was a significant reduction in CTA invasion of the dLGN in DR *Th^{Kir}* mice after eye opening (**Figure 6D and 6E**). Together these results demonstrate that early visual experience is necessary for the plastic recovery of CTA invasion of the dLGN that occurs in juvenile *Th^{Kir}* mice.

Discussion

Here we describe a novel aspect in the coordination of afferent connectivity to the dLGN by intrinsic thalamic spontaneous activity. Using a model in which spontaneous dLGN activity is perturbed, we show that the timing of CTA entry into this structure depends on this thalamic activity. In a scenario of impaired thalamic spontaneous activity, CTA invasion of the dLGN is severely delayed. In addition, the lack of calcium waves in the dLGN leads to a decrease in its size and a concomitant increase in cell death, as occurs after embBE or in *Math5* mutant mice where retinal input is absent from embryonic stages (El-Danaf et al., 2015; Moreno-Juan et al., 2017). Hence, in addition to the trophic factors produced by retinal innervation, spontaneous thalamic activity may directly regulate dLGN neuronal survival, as in the developing cortex (Blanquie et al., 2017; Luhmann & Khazipov, 2018). However, it is also possible that

the effect on cell death is indirect and due to the lack of a proper corticogeniculate innervation, although this will require further study. While CTAs invade the dLGN after RTAs, it was suggested that CTAs regulate the initial targeting of the visual thalamus by the retina. In transgenic mice in which the V1 was eliminated and where CTAs therefore fail to develop, some RGCs did not project to the dLGN (Diao et al., 2018; Shanks et al., 2016). Moreover, when the activity of L6 neurons was silenced in the V1, functional modifications were apparent in visually-evoked activity in the thalamus (Denman & Contreras, 2015). Despite these findings, our work on the *Th^{Kir}* mice in which CTAs are absent in the dLGN and RTAs normally innervate this nucleus suggest the retinothalamic afferents from the CTAs initial develop following an independent programme.

Retinal waves are transmitted to the dLGN and the superior colliculus, and this transmission is crucial for the correct development of visual topographic maps (Ackman, Burbridge, & Crair, 2012; Colonnese & Khazipov, 2010; Mooney, Penn, Gallego, & Shatz, 1996; Siegel, Heimel, Peters, & Lohmann, 2012). During prenatal development, type I retinal waves coexist with the other patterns of spontaneous activity in the thalamus. Although the role of type I retinal waves in visual system development remains poorly understood, here we demonstrate that embryonic ablation of the retinal input, before the retinal axons reach the dLGN, modulates thalamic wave activity. We found that the frequency of thalamic calcium waves augments in the dLGN of embBE mice, which leads to a premature invasion of the dLGN by CTAs. Although the functional synapses between CTAs and dLGN neurons are established during the second postnatal week (Seabrook et al., 2013; Bickford et al., 2010), vGlut1 is already present in the terminals of the CTAs that invade the dLGN of embBE mice at P1. This transporter has previously been associated with functional excitatory transmission

(Koch et al., 2011; Marcucci, Zou, & Firestein, 2009; Nakamura, Hioki, Fujiyama, & Kaneko, 2005; Sherry, Wang, Bates, & Frishman, 2003; Wilson et al., 2005). Thus, it is possible that eliminating retinal input accelerates corticogeniculate maturation in the dLGN.

Our data from *Th^{Kir}* mice indicate that spontaneous thalamic activity is crucial for the timely invasion of the dLGN by CTAs but also, they helped us unravel the hierarchical organization of RTAs, CTAs and thalamic spontaneous activity. When retinal input is eliminated in *Th^{Kir}* mice the lack of CTAs in the dLGN was completely rescued, as not only the absence of thalamic waves but also, the presence of RTAs in *Th^{Kir}* mice prevented the entry of CTAs into the dLGN. As such, it would appear that RTAs and CTAs compete for dLGN innervation, with RTAs arriving first, and that spontaneous thalamic activity represents a permissive phenomenon in this context.

The analysis of CTAs in postnatal *Th^{Kir}* mice shows that the delay in CTA invasion of the dLGN is reversed at eye-opening, when visually evoked activity commences and spontaneous retinal waves subside (Demas, Eglén, & Wong, 2003; Huberman et al., 2008). At this stage, CTAs innervate the dLGN despite the disturbed thalamic activity, suggesting that visual evoked inputs are sufficient to drive CTA innervation of the dLGN. Indeed, visual evoked activity is necessary for this recovery as CTAs did not fully recover in DR *Th^{Kir}* mice.

In summary, our data suggest patterned spontaneous activity in the perinatal thalamus provides a fundamental signal that regulates the timing of CTA invasion of the dLGN, indicating that the thalamus is as a key structure in the development of visual circuits. Furthermore, these results also suggest the existence of an experience-dependent mechanism that helps to recover from early defects in circuits within the visual system.

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Author Contributions

V.M-J. and G.L-B. conceived the idea and designed the experiments. V.M-J. conducted the experiments and analyzed the calcium imaging data, corticothalamic innervation, cell death, CTB tracing, embryonic enucleations and dark-reared experiments. L.P-S performed the *in vivo* recordings in the *Th^{Kir}* mice, F.J.M generated the Matlab codes and F.J.M and L.P-S analyzed this data. A.H-N performed the lentiviral injections in *Th^{Kir}* mice. G.L-B. acquired the funding; V.M-J, F.J.M, M.V and G.L-B. wrote the paper.

Declaration of Interests

The authors have no competing interests to declare

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Main Figures

Figure 1. Overexpression of Kir2.1 gene in the dLGN disrupts the patterns of spontaneous activity. (A) Scheme of the experimental design used for calcium imaging of *ex vivo* slices from control and *Th^{Kir}* mice at P0. (B) *Left*, maximal projection of an *ex vivo* thalamic spontaneous calcium wave in the dLGN of a control mouse at P0. *Right*, quantification of the relative active Region Of Interest (ROIs) in the dLGN over a 15 minute recording (top), and the corresponding raster plot of the active ROIs per frame (bottom). (C) *Left*, maximal projection of *ex vivo* thalamic spontaneous calcium activity in the dLGN of *Th^{Kir}* mouse at P0. *Right*, quantification of the proportion of active ROIs in the dLGN over 15 minutes recording (top) and the corresponding raster plot of active ROIs per frame (bottom). (D) *Left*, graph showing the mean event frequency per ROI per minute in the dLGN of control and *Th^{Kir}* mice. *Right*, graph showing the mean frequency of the waves in the dLGN of control and *Th^{Kir}* mice at P0 (control *n* = 7, *Th^{Kir}* *n* = 5). (E) Scheme of the experimental design used for the *in vivo* recording of spontaneous activity in control and *Th^{Kir}* mice at P6. (F) Coronal sections of control and *Th^{Kir}* mice revealing the position of the recording electrodes in the dLGN. Red fluorescence corresponds to the mCherry fused to the Kir2.1 gene (labelled by RFP

518 immunostaining). (G) Sample traces that illustrate the spiking activity in control and
 519 *Th^{Kir}* mice at P6. (H) Quantification of the firing rate in the dLGN at P6 (control $n = 6$,
 520 *Th^{Kir}* $n = 8$). Graphs represent the mean $\pm$ SEM, each dot corresponding to a single
 521 value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μm .
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Figure 2. Correct spontaneous activity in the dLGN is necessary for the invasion of CTAs. (A) Coronal sections of *Golli-tau-eGfp* in control and *Th^{Kir}* mice at P4 showing the expression of vGlut1 in the dLGN. Insets show GFP immunostaining. (B) Quantification of the relative dLGN area occupied by vGlut1 in control and *Th^{Kir}* mice at different postnatal stages (P4 control $n = 5$, *Th^{Kir}* $n = 5$; P6 control $n = 5$, *Th^{Kir}* $n = 5$; P9 control $n = 5$, *Th^{Kir}* $n = 3$). (C) Coronal sections of control and *Th^{Kir}* mice at P9 showing vGlut1 and Gfp expression in CTAs. A lentivirus expressing *Gfp* was injected into V1. (D) Coronal sections of *Golli-tau-eGfp* control and *Th^{Kir}* mice at P0 showing the expression of Gfp in CTAs (see arrowheads) in the dLGN (control $n = 3$, *Th^{Kir}* $n = 6$). Graphs represent the mean $\pm$ SEM and each dot correspond to a single value:

*P<0.05, **P<0.01, ***P<0.001. Scale bars, 100 μ m.

Figure 3. Significant increase in cell death in the dLGN of *Th^{Kir}* mice. (A)

Quantification of the dLGN area in controls and *Th^{Kir}* mice (P0 control $n = 4$, *Th^{Kir}* $n = 6$; P3 control $n = 3$, *Th^{Kir}* $n = 5$; P4 control $n = 4$, *Th^{Kir}* $n = 4$; P6 control $n = 5$, *Th^{Kir}* $n = 5$). (B) Coronal sections of control and *Th^{Kir}* mice showing caspase3a positive cells and mCherry fused to Kir2.1 (labelled by RFP immunostaining) at P0, P3, P4 and P6. Note that no caspase3a positive cells were found in P0 *Th^{Kir}* mice. (C) Quantification of caspase3a positive cells in the dLGN of control and *Th^{Kir}* mice at P3, P4 and P6 (P3 control $n = 3$, *Th^{Kir}* $n = 5$; P4 control $n = 4$, *Th^{Kir}* $n = 4$; P6 control $n = 5$, *Th^{Kir}* $n = 5$).

The graphs represent the mean $\pm$ SEM and each dot corresponds to a single value:

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μm .

Figure 4. RTAs correctly invade the dLGN of *Th^{Kir}* mice. (A) Scheme of the experimental design used to label CTAs and RTAs in control P3 and P7 mice. Injections of CTB-555 and CTB-647 were performed in each eye of *Golli-tau-eGFP* mice at P0, and they were analysed at P3 and P7 by immunostaining for GFP. (B) Coronal sections of *Golli-tau-eGFP* mice at P3 and P7 showing GFP expression in CTAs (green), and the contralateral (CTB-647) and ipsilateral RTAs (CTB-555). (C) Scheme of the experimental design used to label RTAs in control and *Th^{Kir}* mice at P2. Injection of CTB-647 was performed in one eye in control and *Th^{Kir}* mice at P0 and analysed at P2. (D) *Left:* Coronal sections showing CTB-647 labelling in the contralateral and ipsilateral retinal axons in the dLGN of control and *Th^{Kir}* mice at P2. *Right:* Quantification of the percentage of contralateral and ipsilateral RTAs in the dLGN at P2 (control $n = 3$, *Th^{Kir}* $n = 4$). The graphs represent the mean $\pm$ SEM and each dot corresponds to a single value. Scale bars, 100 μ m.

Figure 5. Innervation of the dLGN by CTAs is dictated by RTAs and thalamic spontaneous activity plays a permissive role. (A) Scheme of the experimental design used for *ex vivo* calcium imaging of the dLGN in control and embBE mice at P1. (B) *Left*, quantification of the event frequency of spontaneous calcium activity in the dLGN in controls and embBE mice at P1. *Right*, quantification of the frequency of thalamic calcium waves in the dLGN of control and embBE mice at P1 (control $n = 5$, embBE $n = 5$). (C) *Upper panel*, quantification of the percentage of active ROIs over 15 minutes recordings in control and embBE mice. *Lower panel*, raster plot of the number of active ROIs per frame. (D) Scheme of the experimental design used to reveal L6 cortical axons in the *Golli-tau-Gfp* mice that were embryonically enucleated at E14.5. (E) Coronal sections of control and embBE mice showing GFP immunostaining in CTAs at P1, P3

579 and P7. (F) Quantification of the percentage of the dLGN area occupied by GFP-
580 positive axons (P1: control $n = 3$, embBE $n = 3$; P3: control $n = 4$, embBE $n = 6$; P7
581 control $n = 5$, embBE $n = 5$). (G) Scheme of the experimental design used to reveal
582 CTAs in embBE Th^{Kir} mice. Tamoxifen was administered at E10.5 and embBE was
583 performed at E14.5 in control and Th^{Kir} mice. (H) Coronal sections showing vGlut1
584 immunostaining in the dLGN in control ($n = 5$), Th^{Kir} ($n = 3$), embBE ($n = 5$), and embBE
585 Th^{Kir} ($n = 4$) mice at P7. (I) Quantification of the relative vGlut1 immunostaining in the
586 dLGN of control, Th^{Kir} , embBE and embBE Th^{Kir} mice at P7. Note that CTAs invade
587 the dLGN of the Th^{Kir} embBE mice. Graphs represent the mean $\pm$ SEM and each dot
588 correspond to a single value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μm .

Figure 6. Eye-opening rescues the entry of CTAs in Th^{Kir} mice. (A) *Left*: Coronal sections from P10 control and Th^{Kir} mice showing vGlut1 immunostaining in the dLGN at P10, before eye opening. *Right*: Quantification of the relative dLGN area occupied by vGlut1 labelling at P10 (control $n = 7$, Th^{Kir} $n = 6$). (B) *Left*: Coronal sections from P13 control and Th^{Kir} mice showing vGlut1 immunostaining at P13, after eye opening. *Right*: Quantification of the relative dLGN area occupied by vGlut1 labelling at P13 (control $n = 5$, Th^{Kir} $n = 5$). (C) Scheme of the experimental design used for the dark rearing (DR) experiments. Control and Th^{Kir} mice were place in a dark room from P5 to P15. (D) Coronal sections showing the vGlut1 immunostaining in the dLGN of control, control DR, Th^{Kir} and Th^{Kir} DR mice. (E) Quantification of the relative dLGN area occupied by vGlut1 labelling in these four conditions: Control ($n = 3$), Th^{Kir} ($n = 3$), control DR ($n = 5$), and Th^{Kir} DR ($n = 6$). The graphs represent the mean $\pm$ SEM and each dot corresponds to a single value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μ m.

Supplementary Figures

Figure S1. Distribution of CTAs and vGlut1 expression in the dLGN. Coronal sections showing CTAs labelled by *Golli-tau-eGfp* and immunostaining against vGlut1 in the dLGN of P3 ($n = 6$), P6 ($n = 3$) and P7 ($n = 3$) mice. CTAs start to invade the dLGN around P3. Scale bars, 100 μ m.

Figure S2. The density of dLGN cells remains normal in *Th^{Kir}* mice. (A) Coronal sections showing Nissl staining of control and *Th^{Kir}* mice at P4. (B) Quantification of the data shown in A. No significant changes in cell density were observed between the control ($n = 4$) and *Th^{Kir}* mice ($n = 4$). The graph represents the mean $\pm$ SEM and each dot correspond to a single value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μ m.

Figure S3. Early CTA invasion of the dLGN in embBE mice. (A) *Left*, Coronal sections of the dLGN showing CTAs labelled with GFP in control and embBE *Golli-tau-eGfp* mice at P2. *Right*, Quantification of the data shown on the left (control $n = 4$, embBE $n = 4$). (B, C and D) *Left*, Coronal sections of the dLGN showing vGlut1 immunostaining in control and embBE mice at P2 (control $n = 3$, embBE $n = 3$), P3 (control $n = 3$, embBE $n = 3$) and P7 (control $n = 3$, embBE $n = 3$). *Right*, Quantification of the data shown in the images on the left. Note that in all embBE mice a higher percentage of CTAs invaded the dLGN. The graphs represent the mean $\pm$ SEM and each dot corresponds to a single value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars, 100 μm .

Figure S4. Normal pattern of dLGN invasion by CTAs after eye opening. (A) *Left*, Coronal sections showing vGlut1 immunostaining in control and Th^{Kir} mice at P30. *Right*, Quantification of the dLGN area occupied by vGlut1 expression at P30 (control $n=3$, Th^{Kir} $n=3$). No significant differences were found between both conditions. (B) *Left*, Coronal sections showing vGlut1 immunostaining in control and Th^{Kir} mice at P14. *Right*, Quantification of the dLGN area occupied by vGlut1 expression at P14 (control $n=3$, Th^{Kir} $n=3$). No significant differences were found between both conditions. The graphs represent the mean $\pm$ SEM and each dot corresponds to a single value. Scale bars, 100 μ m.

Materials and Methods

Mouse strains

Wild type mice maintained on an ICR/CD-1 background, were used for the *in vivo* experiments, tracing studies and Ca²⁺ imaging. The day on which the vaginal plug was detected was designated as E0.5. The *R26^{Kir2.1-mCherry}* mouse line has been described previously (Moreno-Juan et al, 2017) and it was crossed with an inducible thalamic-specific *Gbx2^{CreER}* line (L. Chen, Guo, & Li, 2009) to generate *Gbx2^{CreER/+}; R26^{Kir2.1-mCherry}* double mutant embryos, referred to as *Th^{Kir}*. Tamoxifen induction of Cre recombination in the double mutant embryos was performed by gavage administration of tamoxifen (5 mg dissolved in corn oil: Sigma) at E10.5 to specifically target the principal sensory thalamic nucleus. The *Golli-tau-Gfp* mouse line was described previously (Jacobs, 2007) and was crossed with the *Th^{Kir}* animals. Mutants of either sex were analyzed randomly at stages from P0 to P30. is

Histology

For immunohistochemistry, mice were perfused with 4% paraformaldehyde (PFA) in PBS (0.01M), and their brains were removed and post-fixed overnight in the same fixative. Immunohistochemistry was performed on 80 µm vibratome brain sections that were first incubated for 1h at room temperature in a blocking solution containing 1% BSA (Sigma), 2% Donkey Serum (Biowest) and 0.25% Triton X-100 (Sigma) in PBS 0.01M. Subsequently, the sections were incubated overnight at 4 °C with the primary antibodies: guinea-pig anti-vGlut1 (1:5000, Synaptic Systems, #135304), chicken anti-GFP (1:3000; Aves Labs, #Gfp-1020) and rat anti-RFP (1:1000 Chromotek, #5F8), rabbit anti-Capasa3a (1:150; Abcam Ab2302). The sections were then rinsed three times in PBS (0.01M) and incubated for 2h at room temperature with secondary antibodies:

Alexa-546 donkey anti-rabbit (1:500, ThermoFisher, #A10040), Alexa-488 goat anti-chicken (1:500, ThermoFisher, #A11039), Alexa-594 donkey anti-rat (1:500, ThermoFisher, #A21209), Alexa-488 donkey anti-rabbit (1:500, ThermoFisher, #A21206), Alexa-647 donkey anti-Guinea Pig (1:500, ThermoFisher). Finally, the sections were counterstained with the fluorescent nuclear dye DAPI (Sigma-Aldrich).

Tracing of retinal axons

For axonal tracing at postnatal stages, P0 pups were anesthetized under hypothermia. An incision was made over the eyelid of one or both eyes and they were injected with CTB conjugated to Alexa-647 or Alexa-594 (Mol. Probes C34778 or C22842) using a nanoliter injector (WPI). Two, three or seven days later, the animals were perfused with 4% PFA in PBS (0.01M), and their brain was dissected out and post-fixed overnight in the same fixative. Vibratome sections (80 μ m) were then counterstained with the fluorescent nuclear dye DAPI (Sigma-Aldrich).

***In utero* enucleation**

For *in utero* enucleation, pregnant females (E14.5) from wildtype colonies or *Th^{Kir}* mice were administered tamoxifen at E10 and deeply anesthetized with isoflurane to perform laparotomies. The embryos were exposed and both eyes were cauterized in half of the embryos of each litter. The surgical incision was closed and the embryos were allowed to develop until postnatal stages.

Calcium imaging in slices

The brains of postnatal mice (P0 and P1) were used for calcium imaging experiments, obtained after they were sacrificed by decapitation. The brains were immediately

dissected out and 300 μ m coronal vibratome sections (VT1200 Leica Microsystems Germany) were obtained in slicing solution (95% O₂ and 5% CO₂) containing (mM): 2.5 KCl, 7 MgSO₄, 0.5 CaCl₂, 1 NaH₂PO₄, 26 NaHCO₃, 11 glucose and 228 sucrose. The slices were placed at room temperature in standard aCSF (119 NaCl, 5 KCl, 1.3 MgSO₄, 2.4 CaCl₂, 1 NaH₂PO₄, 11 glucose) in an atmosphere of 95% O₂ and 5% CO₂.

To load the calcium indicator, the slices were incubated at 37 °C for 30 min in artificial CSF (aCSF) containing 5 μ l of Cal520 AM (AAT Bioquest), 1 mM DMSO and 20% pluronic acid. The slices were then placed in a recording chamber with 90% O₂ and 5% CO₂ at 34 °C and recorded under a Leica DMI8 microscope. We acquired images with Hamamatsu ORCA-Flash 4.0LT camera every 300 ms for 15 minutes. All the calcium imaging data was analyzed as described extensively elsewhere (Moreno-Juan et al., 2017; Antón-Bolaños et al., 2019).

In vivo extracellular recordings

P6 mice were anesthetized with isoflurane (2.5 %), their scalp was removed and the skull was cleaned. A 3D-printed holder was glued to the skull with cyanoacrylate adhesive and dental cement, and attached to a stereotaxic apparatus. A 2 x 2 mm craniotomy was made over the left hemisphere leaving the dura mater intact. The craniotomy revealed a window above the area of the dLGN (2.0-2.2 mm anterior to lambda and 2.0 mm from the midline) allowing the perpendicular insertion of the multi-channel electrodes. During the recordings, the mice were kept warm (36-37 °C) with a heated blanket and lightly anesthetized with isoflurane (0.5%). Multi-unit activity (MUA, >300Hz) was recorded with a linear 16 channel electrode separated by 50 μ m (E16+R-50-S1-L6NT, ATLAS). Shank trajectories were stained with Dil (1,1'-

dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate: Invitrogen) diluted in 70% alcohol. Probes were inserted perpendicularly, 2800-3000 μm deep for dLGN recordings. The electrical signal activity was sampled at 20 kHz with a filter amplifier and visualized using MC_RACK software (Multi Channel Systems). After the recording sessions, the animal's brain was dissected out and fixed overnight in 4% PFA. In order to determine the position of every channel, 100 μm thick coronal sections were counterstained with the fluorescent nuclear dye DAPI (Sigma-Aldrich) and the data was analyzed using MatlabTM routines.

Dark reared experiments

For the dark reared experiments, *Th^{Kir}* animals and their control littermates were put into a completely dark environment with their mother from P5 to P15. After this time the animals were rapidly perfused with 4% PFA in PBS (0.01M). The animal's brain was dissected out and post-fixed overnight in the same fixative. Vibratome sections (80 μm) were then subjected to immunohistochemistry.

Data analysis and statistics

The analysis of the calcium imaging experiments was described in detail elsewhere (Moreno-Juan et al., 2017; Antón-Bolaños et al., 2019). ImageJ software was used to quantify CTA and RTA invasion of the dLGN. Coronal slices (80 μm) were obtained from the animals subjected to the different conditions and immunostained for vGlut1 or GFP. At least three dLGN sections were analyzed from each animal, correcting for the fluorescence background. Threshold images of the fluorescence were analyzed by ImageJ software, analyzing the particles of the pixels that cover the dLGN and measuring the proportion of pixels occupied by CTAs in the dLGN.

Caspase3a positive cells were quantified in coronal sections (80 μ m) and normalized to the area of each dLGN. The whole dLGN was measured from P3, P4 and P6 control and *Th^{Kir}* mice, and the results were normalized to the mean of the controls, representing the fold-change in Caspase3a positive cells in the *Th^{Kir}* mice.

Statistical analysis was carried out in GraphPad Prism6TM and MatlabTM, and the data are presented as the mean and SEM. Statistical comparison between the groups was performed using an unpaired two-tailed Student's *t* test and when the data failed a Kolmogorov-Smirnov or a Shapiro Wilk test of normality, a two-tailed, non-parametric Mann-Whitney U-test was used. A Two-way ANOVA test was performed on the embryonic enucleation in *Th^{Kir}* mice and for the DR experiments. No statistical methods were used to predetermine the sample size, but our sample sizes are considered adequate for the experiments and consistent with the literature (Brooks et al., 2013; Grant et al., 2016; Seabrook et al., 2013). The mice were not randomized.

Figure 1

Figure 1D Event frequency: control 0.27 ± 0.03 , $n = 7$; *Th^{Kir}* 0.02 ± 0.005 , $n = 5$ (**P<0.001 Two-tailed student's *t*-test). Wave frequency in dLGN: control 0.34 ± 0.04 , $n = 7$; *Th^{Kir}* 0 ± 0 , $n = 5$ (**P=0.0025, Mann-Whitney U-test). Figure 1H Firing rate dLGN: control 0.16 ± 0.05 , $n = 6$; *Th^{Kir}* 0.07 ± 0.12 , $n = 8$ (*P=0.04 Mann-Whitney U-test).

Figure 2

Figure 2B vGlut1 at P4: control $10 \pm 1\%$, $n = 5$; *Th^{Kir}* $1.67 \pm 0.34\%$, $n = 5$ (**P<0.001 Two-tailed student's *t*-test). At P6: control $35.96 \pm 4.47\%$, $n = 5$; *Th^{Kir}* $1.24 \pm 0.51\%$, $n = 5$ (**P<0.001, Two-tailed student's *t*-test). At P9: control $62.4 \pm 4.49\%$, $n = 5$; *Th^{Kir}* $12.15 \pm 0.74\%$, $n = 3$ (**P<0.001, Two-tailed student's *t*-test).

Figure 3

Figure 3A dLGN size at P0: 84326 ± 2872 , $n = 4$; Th^{Kir} 83720 ± 2498 , $n = 6$ ($P=0.88$, Two-tailed student's t -test). At P3: 89800 ± 4558 , $n = 3$; Th^{Kir} 80403 ± 2300 , $n = 5$ $P=0.14$, Mann-Whitney U -test. P4: 118410 ± 6262 , $n = 4$; Th^{Kir} : 79748 ± 4351 , $n = 4$ ($*P=0.02$, Two-tailed student's t -test). At P6: 193136 ± 5603 , $n = 5$; Th^{Kir} 92022 ± 3380 , $n = 5$ ($***P<0.001$, Two-tailed student's t -test).

Figure 3C Caspase3A⁺ cells normalized to the control data at P3: control $100 \pm 14.97\%$, $n = 3$; Th^{Kir} $816.3 \pm 122.3\%$, $n = 5$ ($*P=0.03$, Mann-Whitney U -test). At P4 control $100 \pm 30.98\%$, $n = 4$; Th^{Kir} $684.4 \pm 139.2\%$, $n = 4$ ($*P=0.028$, Mann-Whitney U -test). At P6 control $100 \pm 7.71\%$, $n = 6$; Th^{Kir} $762.6 \pm 87.69\%$, $n = 6$ ($***P<0.001$, Two-tailed student's t -test).

Figure 4

Figure 4D Percentage of RTAs in dLGN at P2. Contralateral RTAs in dLGN, control: $69.94 \pm 1.56\%$, $n = 3$; Th^{Kir} : $66.5 \pm 1.74\%$, $n = 4$. $P=0.22$ Two-tailed student's t -test.

Ipsilateral RTAs in dLGN control: $20.45 \pm 2.27\%$, $n = 3$; Th^{Kir} : $24.34 \pm 2.3\%$, $n = 4$. $P=0.29$ Two-tailed student's t -test.

Figure 5

Figure 5B Event frequency: control 0.08 ± 0.02 , $n = 5$; embBE 0.23 ± 0.02 , $n = 5$ ($***P<0.0005$, Two-tailed student's t -test). Wave frequency in dLGN: control 0.08 ± 0.03 , $n = 5$; embBE 0.31 ± 0.03 , $n = 5$ ($*P=0.01$, Mann-Whitney U -test).

Figure 5F % of CTAs in the dLGN at P1: control $3.26 \pm 0.2\%$, $n = 3$; embBE $8.85 \pm 1.44\%$, $n = 3$ $*P=0.018$, Two-tailed student's t -test). At P3: control $12.86 \pm 1.59\%$, $n = 4$; embBE $53.92 \pm 5.05\%$, $n = 6$ ($***P<0.001$, Two-tailed student's t -test). At P7: control $43.89 \pm 2.46\%$, $n = 4$; embBE $74.82 \pm 1.68\%$, $n = 5$ ($*P=0.01$, Mann-Whitney U -test).

Figure 5I % of vGlut1 in the dLGN at P7: control $n = 5$, embBE $n = 5$, Th^{Kir} $n = 3$, Th^{Kir} embBE $n = 4$. A two-way ANOVA test with Tukey's post hoc analysis indicates that the interaction between all the conditions are significant, comparisons: control vs Th^{Kir} *** $P < 0.001$; control vs embBE *** $P < 0.001$; control vs Th^{Kir} embBE * $P = 0.01$; Th^{Kir} vs embBE *** $P < 0.001$; Th^{Kir} vs Th^{Kir} embBE *** $P < 0.001$; embBE vs Th^{Kir} embBE ** $P = 0.0025$.

Figure 6

Figure 6A %vGlut1 in the dLGN of P10: control $79.28 \pm 2.31\%$, $n = 7$; Th^{Kir} $18.85 \pm 1.85\%$, $n = 6$ (*** $P < 0.001$, Two-tailed student's t -test).

Figure 6B %vGlut1 in the dLGN of P13: control $76.62 \pm 1.23\%$, $n = 5$; Th^{Kir} $76.69 \pm 0.8\%$, $n = 5$ ($P = 0.96$, Two-tailed student's t -test).

Figure 6E % of vGlut1 in the dLGN at P15: control $n = 3$, control DR $n = 5$, Th^{Kir} $n = 3$, Th^{Kir} DR $n = 6$. A two-way ANOVA test with Tukey's post hoc analysis, comparisons: control vs Th^{Kir} $P = 0.97$; control vs control DR $P = 0.99$; control vs Th^{Kir} DR *** $P < 0.005$; Th^{Kir} vs control DR $P = 0.99$; Th^{Kir} vs Th^{Kir} DR *** $P < 0.001$; control DR vs Th^{Kir} DR *** $P < 0.001$.

Figure S2

Figure S2B Nissl⁺ cells/100 μm^2 : control 110.5 ± 4.29 , $n = 4$; Th^{Kir} $124.8 \pm 6.34\%$, $n = 4$ ($P = 0.11$, Mann-Whitney U -test).

Figure S3

Figure S3A %CTAs in the dLGN of P2 mice: control $7.28 \pm 2.24\%$, $n = 4$; embBE $39.3 \pm 3.1\%$, $n = 4$ (* $P = 0.02$, Mann-Whitney U -test).

Figure S3B % of vGlut1 in the dLGN at P2: control $7.1 \pm 9.92\%$, $n = 3$; embBE $44.32 \pm 2.35\%$, $n = 3$ (*** $P < 0.001$, two-tailed student's t -test).

818 Figure S3C % of vGlut1 in the dLGN at P3: control $10.68 \pm 1.92\%$, $n = 3$; embBE

819 $53.06 \pm 4.41\%$, $n = 3$ (**P<0.001, two-tailed student's *t*-test).

820 Figure S3D % of vGlut1 in the dLGN at P7: control $39.01 \pm 3.03\%$, $n = 3$; embBE

821 $71.45 \pm 1.11\%$, $n = 3$ (**P<0.001, two-tailed student's *t*-test).

822 **Figure S4**

823 Figure S4A %vGlut1 at P30: control $87.26 \pm 3.36\%$, $n = 3$; *Th^{Kir}* $87.16 \pm 3.28\%$, $n = 3$

824 (P=0.98, two-tailed student's *t*-test).

825 Figure S4B %vGlut1 at P14: control $93.17 \pm 1\%$, $n = 3$; *Th^{Kir}* $89.31 \pm 1.16\%$, $n = 3$

826 (P=0.1, two-tailed student's *t*-test).