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Postnatal development of cholinergic input to the thalamic reticular nucleus of the mouse

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Abstract

The thalamic reticular nucleus (TRN), a shell-like structure comprised of GABAergic neurons, gates signal transmission between thalamus and cortex. While TRN is innervated by axon collaterals of thalamocortical and corticothalamic neurons, other ascending projections modulate activity during different behavioral states such as attention, arousal, and sleep-wake cycles. One of the largest arise from cholinergic neurons of the basal forebrain and brainstem. Despite its integral role, little is known about how or when cholinergic innervation and synapse formation occurs. We utilized genetically modified mice, which selectively express fluorescent protein and/or channelrhodopsin-2 in cholinergic neurons, to visualize and stimulate cholinergic afferents in the developing TRN. Cholinergic innervation of TRN follows a ventral-to-dorsal progression, with non-visual sensory sectors receiving input during week 1, and the visual sector during week 2. By week 3, the density of cholinergic fibers increases throughout TRN and forms a reticular profile. Functional patterns of connectivity between cholinergic fibers and TRN neurons progress in a similar manner, with weak excitatory nicotinic responses appearing in non-visual sectors near the end of week 1. By week 2, excitatory responses become more prevalent and arise in the visual sector. Between weeks 3–4, inhibitory muscarinic responses emerge, and responses become biphasic, exhibiting a fast excitatory, and a long-lasting inhibitory component. Overall, the development of cholinergic projections in TRN follows a similar plan as the rest of sensory thalamus, with innervation of non-visual structures preceding visual ones, and well after the establishment of circuits conveying sensory information from the periphery to the cortex.

Graphical Abstract

We examined the postnatal development of cholinergic projections to the mouse thalamic reticular nucleus (TRN) and found that both anatomical and functional patterns of connectivity follow a ventral to dorsal gradient, with nonvisual sectors innervated prior to the visual sector of TRN.

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Author contributions

GS and WG were involved in all aspects of the research and writing of the manuscript. PC participated in anterograde tracing experiments and contributed to the writing of the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Data accessibility

The datasets for the current study are available upon request.

These results also indicate that modulation of TRN activity by cholinergic input emerges during postnatal week 2, with adult-like biphasic responses seen during week 4.

Keywords

acetylcholine; basal forebrain; brainstem; nicotinic; muscarinic

Introduction

The thalamic reticular nucleus (TRN) is a thin sheet of GABAergic neurons that surrounds the dorsolateral aspect of the mammalian thalamus (Jones, 1975 & 2007; Guillery & Harting, 2003). While providing inhibitory input to thalamocortical relay neurons, TRN receives its primary excitatory drive from ascending thalamocortical (TC) and descending corticothalamic (CT) axon collaterals (Guillery et al., 1998; Pinault, 2004; Halassa & Acsády, 2016). Additionally, TRN receives input from several subcortical structures, including a large cholinergic projection from the brainstem and basal forebrain (Woolf & Butcher, 1986; Steriade et al., 1987; Hallanger et al., 1987; Jourdain et al., 1989). Together, these projections work in concert to influence many aspects of sensory and motor processing, and play a critical role in the establishment of different cognitive states ranging from sleep and wakefulness, to attention and decision making (Crick, 1984; Pinault, 2004; Fogerson & Huguenard, 2016; Halassa & Acsády, 2016).

The study of adult TRN circuitry has been the topic of intense investigation, however remarkably little is known about when these elements are assembled during postnatal life. While both TC and CT projections begin to course through TRN at perinatal ages (Mitrofanis & Baker, 1993), the question of how and when other subcortical inputs innervate TRN and become operational remains unanswered. To address this, we focused on the development of cholinergic projections to the sensory sectors of TRN. In rodents, these inputs arise from two regions, the brainstem tegmental nuclei (i.e., Laterodorsal tegmentum, LDTg, and Pedunculopontine tegmentum, PPTg), and the cholinergic basal forebrain groups (i.e., nucleus of the horizontal diagonal band, HDB, and substantia innominata, SI) (Hallanger et al., 1987; Jourdain et al., 1989). When activated in sensory sectors of TRN, cholinergic projections evoke biphasic postsynaptic responses (Hu et al., 1989; Pinault & Deschenes, 1992; Sun et al., 2013; Pita-Almenar et al., 2014), modulating the signal transfer of TRN neurons in a state-dependent manner (Ni et al., 2016). Here we adopted a mouse model and utilized genetically modified strains (Madisen et al., 2010 & 2012) to assess the time course of cholinergic innervation and circuit formation within the sensory sectors of TRN.

Materials and Methods

Subjects

All breeding and experimental procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. Cre recombinase expressing mouse lines ChAT-IRES-Cre (Jackson Labs, stock # 006410, strain B6;129S6-Chat^{tm2(cre)Low1/J}) and Crh-Cre (MMRRC, stock # 030850-UCD, strain Tg(Crh-cre)^{KN282Gsat/Mmucd}) were bred to Cre-dependent reporter lines Ai9 (tdTomato; Jackson Labs, stock # 007905 B6;129S6-Gt(ROSA)^{26Sortm9(CAG-tdTomato)Hze/J}), or Ai32 (channelrhodopsin-2-eYFP; Jackson Labs, stock #012569, strain B6;129S-Gt(ROSA)^{26Sortm32(CAG-COP4*H134R/EYFP)Hze/J}). A total of 77 mice aged P1-P120 of either sex were used in experiments and included Crh-Cre^{+/+} ($n = 3$), Crh-Cre^{+/-} x Ai9^{+/-} ($n = 1$), ChAT-Cre^{+/-} x Ai9^{+/-} ($n = 18$), and ChAT-Cre^{+/-} x Ai32^{+/-} ($n = 55$).

Histology

Brain tissue was harvested from mice that were deeply anaesthetized by hypothermia (<P5) or isoflurane vapors (4%). 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 removed and postfixed overnight in 4% PFA, then transferred to PBS. Brains were then blocked, and a vibratome (Leica VT1000S) was used to cut 70 μ m-thick coronal sections containing TRN, dorsal thalamus, brainstem, and basal forebrain.

To amplify the inherent tdTomato (tdT) fluorescence in cholinergic processes within TRN, and to prevent photobleaching during confocal imaging, tissue was incubated in DsRed (Clontech) antibody. Sections were placed in blocking medium (10% normal goat serum (NGS), and 0.3% Triton X-100 in PBS) for 1 hour, and then incubated for 12 hours in rabbit anti-DsRed (1:1000) with 1% NGS in PBS. Treated sections were then incubated for 1 hour in 1:100 biotinylated goat anti-rabbit IgG antibody (Vector Labs) with 1% NGS in PBS, followed by 1 hour in 1:100 streptavidin Alexa Fluor (AF) 546 (Life Technologies) in PBS. NeuN antibody was used to visualize TRN neurons and outline the cytoarchitectural landmarks. Tissue was blocked (10% NGS 0.3% Triton X-100 in PBS) for 1 hour, incubated overnight in mouse monoclonal NeuN antibody (Chemicon, 1:100), then for one hour in 1:100 biotinylated goat anti-mouse IgG antibody (Vector Labs) in PBS, followed by 1 hour in 1:100 streptavidin AF-488 (SA AF-488; Life Technologies). All tissue was mounted onto gelatin-subbed glass slides using ProLong mounting medium containing DAPI (Life Technologies).

Viral tracing

To delineate sensory sectors of TRN, intracranial injections of a Cre-dependent adeno-associated viral tracer Flex-rev-oChIEF-tdTomato (Addgene plasmid #30541, serotype 9) were made in the thalamic relay nuclei of adult (P60-P120) Crh-Cre mice. Animals were deeply anesthetized using a ketamine (50mg/kg) /xylazine (10 mg/kg) mixture and placed in a stereotaxic frame. An incision was made along the scalp, and a hole was drilled in the skull above the injection site. A 2% lidocaine gel was applied to the scalp incision. A Hamilton

syringe attached to a nanopump was used to deliver 40 nL of the virus into vMGN (−3.4 AP, −2.0 ML, −3.2 DV; mm from Bregma), VB (−2.2 AP, −1.7 ML, −3.5 DV), or dLGN (−2.3 AP, −2.2 ML, −2.7 DV) of the left hemisphere. The scalp incision was sealed with Vetbond. Heart rate and breathing were continuously monitored and body temperature was maintained at 38 °C throughout the procedure. After completion of the surgery, mice were monitored for 48 hours and given an analgesic (Carprofen, 5 mg/kg) every 12 hours. Thereafter, mice were monitored daily for a 14–17 day survival period. Mice were then euthanized, and sections containing thalamus and TRN were harvested using methods described above.

Slice preparation and in vitro recording

For *in vitro* electrophysiological recordings, ChAT-Cre x Ai32 mice aged P4–60 were deeply anesthetized with isoflurane (4%) and rapidly decapitated. The brains were removed and placed into cold (4 °C) oxygenated sucrose cutting solution containing (in mM): Sucrose 234, glucose 11, KCl 2.5, CaCl₂ 0.5, MgSO₄ 10, NaH₂PO₄ 1.25, NaHCO₃ 26. A vibratome was used to make 270 µm-thick coronal sections containing TRN. Slices were then incubated in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 126, NaHCO₃ 26, glucose 10, KCl 2.5, MgCl₂ 2, CaCl₂ 2, NaH₂PO₄ 1.25 at 32 °C for 30 minutes and then transferred to a recording chamber maintained at 32 °C and perfused continuously at a rate of 2–3 ml/min with oxygenated ACSF.

During recordings, the tissue was visualized using DIC optics on a microscope equipped with a 10X objective, a 60X water immersion objective (Olympus), and a CCD camera (Olympus Y-150), using previously described methods (Dilger et al., 2011). EYFP was visualized with a fluorescent light source through a Chroma filter using a 10X or 60X objective. Borosilicate glass patch electrodes were pulled in two steps using a vertical puller (Narashige PC-10). Patch electrodes were filled with internal recording solution containing (in mM): K-gluconate 117, KCl 13, MgCl₂ 1, CaCl₂ 0.07, EGTA 0.1, HEPES 10. Biocytin (0.1–0.2% w/v; Molecular Probes) was incorporated in the recording solution and allowed to diffuse into neurons to allow for morphological reconstructions. After completion of recordings, slices containing biocytin-filled neurons were postfixed and incubated overnight in SA AF-647. The final tip resistance of patch electrodes was 8–12 MΩ. Whole-cell current and voltage clamp recordings were made using Multiclamp 700B amplifier (Molecular Devices), filtered at 3–10 kHz, and digitized (Digidata 1440A) using techniques described in detail elsewhere (Dilger et al., 2011; Jurgens et al., 2012; Seabrook et al., 2013). Recordings were conducted in neurons with resting membrane potential between −55 and −75 mV and series resistance between 10 and 25 MΩ. Pipette capacitance, series resistance, input resistance, and whole-cell capacitance were monitored throughout the recording session. To photoactivate ChR2-expressing cholinergic neurons or their processes, a light emitting diode (LED) (Prizmatix) was used to deliver blue light through the 60X objective. The illuminated spot was 0.45 mm in diameter, with light power of 300 mW/mm².

All light evoked responses were recorded in the presence of glutamate receptor antagonists (AMPA: 20 µM DNQX; NMDA: 10 µM (RS)-CPP). In some instances, (*n* = 3), GABA blockers (GABA_A: 10 µM SR-95531; GABA_B: 10 µM CGP-54626) were used to assess the pharmacology of light-evoked hyperpolarizing responses. To isolate nicotinic responses,

recordings were done in the presence of the muscarinic blocker atropine (5 μ M) or the M₂ muscarinic receptor antagonist, AF-DX 116 (5 μ M). To examine muscarinic responses, recordings were conducted with the α 4 β 2 nicotinic receptor antagonists Dh β E (300 nm-5 μ M) or the α 7 antagonist MLA (5 μ M). All drugs were dissolved in ACSF and bath-applied using methods described previously (Jurgens et al., 2012).

To quantify the incidence and amplitude of nicotinic and muscarinic receptor-mediated postsynaptic responses (Fig. 8 & 9), whole-cell recordings were conducted in ChAT-Cre x Ai32 mice between P4-P28 in voltage-clamp mode and at a holding potential of -70 mV. For photactivation, a short pulse (3 ms.) of blue light was presented over 5 consecutive trials. An inter-stimulus interval of 40 seconds between trials was used to avoid desensitization of cholinergic receptors and to account for long-lasting metabotropic muscarinic receptor-mediated currents. ClampFit analysis software (Molecular Devices) was used to measure the peak amplitude for postsynaptic nicotinic excitatory postsynaptic currents (nEPSC) or muscarinic inhibitory postsynaptic currents (mIPSC) on a trial-by-trial basis. Only trials where the peak amplitude of the nEPSC or mIPSC was above 2 standard deviations of root mean square (RMS) peak-to-peak noise were considered as a response. For each neuron a mean peak amplitude was calculated that was based on 3–5 trials.

Image acquisition and analysis

All images were acquired using a laser scanning confocal microscope (FV 12000BX61) equipped with a 20X objective (0.75 NA). For clarity, the anatomical images displayed in Figs. 1–3, and 5–6 were digitally adjusted by inverting and enhancing their contrast. The images portrayed in Figs. 1B, 2C, 3, 5A–B were comprised of 2 or more sections that were digitally aligned and stitched together.

To quantify the degree of cholinergic innervation in TRN at different postnatal ages, three coronal sections through the rostro-caudal extent of sensory TRN were used (Figs. 1,3; AP -1.7 mm, -1.5 mm, and -1.3 mm from bregma; Paxinos & Franklin, 2004) (70 μ m thickness, 1.14 μ m optical sections). In order to maximize fluorescent signal detection while avoiding oversaturation, parameters for image acquisition were calibrated using the brightest and dimmest samples, and held constant for all analyzed sections. The images were collapsed in the Z-plane and imported into Photoshop (Adobe), where the area of TRN was outlined using DAPI staining. A threshold value (pixel intensity of 60) was chosen on the histogram that provided a clear distinction between fluorescent signal and background, thereby generating a binarized image (Jaubert-Miazza et al., 2005; Demas et al., 2006; Seabrook et al., 2013; Dilger et al., 2015). ImageJ (NIH) was used to count the number of pixels comprising the signal and the total area of visual and nonvisual sectors of TRN (see Fig. 1D). Based on anterograde tracing experiments, the visual sector of TRN (visTRN) was defined as the area above the apex and included the “head” of TRN, while nonvisual sector of TRN (non-visTRN) was defined as the area below the apex, and included the tail of TRN (see Results & Fig. 1C). This delineation is consistent with the divisions used by others (Kimura, 2014; Wimmer et al., 2015; Chen et al., 2015; Clemente-Perez et al., 2017). For each sector, the values were expressed as a percentage of the fluorescence signal in relation to the total area. Thus, in Figure 4 the degree of innervation is the area containing detectable

fluorescence in relation to the total area of visTRN and non-visTRN. The percentages simply reflect an estimate of the density of cholinergic fibers innervating TRN.

In slices containing biocytin-filled TRN neurons, the location of responsive and non-responsive neurons to photoactivation of cholinergic terminals were mapped and plotted on a composite reconstruction of TRN (Figs. 7–8). For postnatal weeks 2–4, each TRN was digitally aligned using the apex as the reference point. For postnatal weeks 3 and 4, the size and shape of slices containing TRN were comparable. However, during postnatal week 2 (P7–13) TRN expanded in size, especially in the dorso-ventral extent. Thus, the slices comprising the composite plot for week 2 were calibrated to reflect the size and shape of TRN measured at P13.

Results

Previous studies in rat demonstrate that sensory thalamic nuclei project to TRN in a modality-specific, sectorial manner (Montero et al., 1977; Ohara & Lieberman, 1985; Coleman & Mitrofanis, 1996; Kimura et al., 2005). To confirm that such an arrangement exists in mouse, we used anterograde tracing techniques to label thalamocortical projections and their collaterals in TRN. To accomplish this, we utilized a mouse line that expresses Cre recombinase under the control of a promoter for corticotropin releasing hormone (Crh-Cre). In this strain, Cre is expressed in the thalamocortical neurons of first order sensory thalamic nuclei but not in higher order nuclei (e.g., ventral lateral geniculate nucleus, lateroposterior nucleus, posterior nucleus, etc), TRN, or the neocortex (Fig. 1A). When crossed with a tdTomato reporter line (Ai9), somatic labeling is present in ventral medial geniculate nucleus (vMGN), ventrobasal nucleus (VB; includes ventral posterolateral nucleus, VPL, and ventral posteromedial nucleus, VPM), and dorsal lateral geniculate nucleus (dLGN). The tdT-labeled axons of thalamocortical relay neurons can be seen coursing through TRN, internal capsule, and ultimately terminating in primary sensory areas of cortex, including auditory (A1), somatosensory (S1), and visual (V1) areas. By utilizing a Cre-dependent anterograde viral tracer FLEX-AAV-ChIEF-tdT, and making targeted injections into dLGN, VB, or vMGN of Crh-Cre mice, we were able to label thalamocortical projections in TRN. Staining with a neuronal marker NeuN (Fig. 1B) also reveals the cytoarchitecture and general shape of TRN in the coronal plane, including the dorsal “head”, the lateral “apex”, and the ventral “tail” (Clemente-Perez et al., 2017). The viral tracing of thalamic inputs to TRN is summarized in Figure 1C. The left panels provide locations of targeted viral injections which resulted in tdT expression restricted to vMGN, VB, or dLGN. Corresponding panels on the right depict the location of tdT-labeled axonal projections arising from each sensory relay nucleus at three caudo-rostral levels of sensory TRN. In each case projections were arranged largely in a non-overlapping, sectorial manner. Projections from vMGN were found in the caudal-most regions of the TRN, and occupied a limited area of the “tail” in more rostral sections (Fig. 1 C1, right). Whereas, VB projected to more rostral areas of sensory TRN compared to vMGN, targeting regions below the apex and the ventral “tail” (Fig. 1 C2). Projections from dLGN were found in a similar rostral-caudal plane as those from VB, but innervated a region dorsal of the apex, including the “head” of the nucleus (Fig. 1 C3). Because vMGN and VB projections occupy regions below the apex, it is difficult to distinguish the caudo-rostral arrangement in the coronal plane. Therefore, we

considered the region ventral to the apex, including the tail, as non-visual TRN (non-visTRN), and regions above the apex, including the head, as visual TRN (visTRN) (Fig. 1D; Kimura 2004; Wimmer et al., 2015; Clemente-Perez et al., 2017). Overall, these tracing experiments underscore the sectorial arrangement of sensory TRN, (Fig. 1D). Moreover, we used these demarcations to assess whether separate sensory sectors exhibit age-related differences in the progression of cholinergic innervation.

To visualize cholinergic neurons and their processes we crossed a ChAT-Cre mouse, which expresses Cre recombinase under the control of choline acetyltransferase (ChAT) promoter, to a tdTomato (tdT) reporter line (Ai9) (Madisen et al., 2010). Figure 2 shows the pattern of tdT labeling in the brainstem, basal forebrain, and TRN of an adult ChAT-Cre x Ai9 mice. In the brainstem (Fig. 2A-B), somatic tdT labeling was evident in TRN-projecting cholinergic neurons of the pedunculopontine tegmentum (PPTg) and laterodorsal tegmentum (LDTg) (Woolf & Butcher, 1986). Similarly, tdT labeling was present in cholinergic neurons of the basal forebrain (Fig. 2A, B top), including nucleus of the horizontal diagonal band (HDB), and substantia innominata (SI), which innervate TRN and the cortex (Hallanger et al., 1987). Together, brainstem and basal forebrain cholinergic nuclei give rise to a dense labeling pattern throughout the sensory regions of TRN (Fig. 2C). Moreover, tdT-labeled neurons in brainstem and BF were present at birth (Fig. 2B, bottom), indicating that their projections could be followed throughout early postnatal development.

To determine the time course of cholinergic innervation of sensory TRN, we examined ChAT-Cre x Ai9 mice at different postnatal ages from birth to adulthood. Figure 3 depicts examples of TRN sections of ChAT-Cre x Ai9 mice at selected ages between P1-P21. This series suggests that cholinergic innervation begins around the time of birth, proceeds in a ventral-to-dorsal manner, and becomes adult-like by the end of the third postnatal week. Between P1-P3, thin unbranched cholinergic axons were seen coursing in a medial-lateral direction within the thalamus and entering the ventral, non-visTRN. At P5, a sparse field of cholinergic arbors was evident in non-visTRN. More dorsal regions corresponding to visTRN began to receive cholinergic input at P7, with the fibers extending to the dorsal tip of the head of TRN by P11. During the second and third week, cholinergic processes throughout TRN increased in density, and by P21, exhibited a reticular appearance that was especially prominent below the apex in non-visTRN. This progression is summarized in Figure 4, which quantifies the degree of cholinergic innervation in visual and non-visual regions of TRN throughout the first postnatal month. The scatter plot depicts the values of cholinergic innervation within visTRN and non-visTRN, expressed as the percentage of area containing tdT fluorescence. In non-visTRN, innervation increased rapidly during the first postnatal week, reaching maximal values by P11. However, cholinergic innervation of visTRN lagged considerably, showing a slower rate of innervation and reaching maximal values at P21. Both visTRN and non-visTRN showed a significant age-related increase in cholinergic innervation (two-way ANOVA, $F(8,54) = 80.4$, $p = 0.0001$). Moreover, between P1-P9, the degree of innervation of non-visTRN was significantly greater than visTRN (Bonferroni *post hoc* test, P1, $p = 0.035$; P3, $p = 0.021$; P5, $p = 0.015$; P7, $p = 0.002$; P9, $p = 0.003$). Between P11–14 values for the two regions were comparable. However, at P21 and P30, visTRN continued to show a substantial increase, whereas non-vis TRN stabilized (Bonferroni *post hoc* test, P21, $p = 0.001$; P30, $p = 0.001$). This difference is likely brought

about by the formation of the reticular pattern of cholinergic processes within ventral regions of TRN (Figs. 2C, Fig. 3).

To assess the synaptic properties of cholinergic transmission in TRN, we conducted *in vitro* whole-cell recordings from TRN neurons of acutely prepared thalamic slices while stimulating the release of Ach from synaptic terminals. To accomplish this, we took an optogenetic approach and selectively expressed channelrhodopsin-2 (ChR2) in cholinergic neurons by crossing ChAT-Cre mice to a Cre-dependent ChR2-eYFP reporter line (ChR2-eYFP; Ai32). Figure 5 portrays an acutely prepared coronal slice containing TRN from an adult ChAT-Cre x ChR2 mouse, as well as examples of light-evoked evoked cholinergic responses recorded throughout the visual and non-visual regions of TRN. In TRN, ChR2-eYFP fusion protein was present in cholinergic arbors, and resembled the pattern of tdT labeling pattern shown above (Fig. 5A vs. Figs. 2–3). Figure 5B depicts a typical recording experiment, where biocytin-filled electrodes were used to track the location of TRN neurons and their light-evoked responses. In adult TRN neurons (P30–45; n = 7) blue light stimulation (single pulse, 3 ms.) of cholinergic terminals evoked a biphasic, excitation-inhibition (E-I) response (Fig. 5B & C, left; see also Sun et al., 2013; Pita-Almenar et al., 2014). The E-I response consisted of a fast initial depolarization, followed by a long-lasting hyperpolarization. In some neurons, the initial excitatory response evoked spike firing (Fig. 5C, right). Figure 5D provides additional examples of light evoked responses and their underlying pharmacology. Bath application Dh β E (5 μ M), a nicotinic antagonist abolished the excitatory component of the E-I response, while atropine (5 μ M), a muscarinic antagonist blocked the inhibitory component. Taken together, these data confirm previous reports indicating that the biphasic E-I response is mediated by nicotinic and muscarinic receptor activation, respectively (Hu et al., 1989; Sun et al., 2013; Pita-Almenar et al., 2014; Ni et al., 2016).

To explore the development of cholinergic synaptic activity in TRN we conducted recordings in ChAT-Cre x ChR2 mice at early postnatal ages. To confirm that ChR2 is present in cholinergic neurons, we first recorded somatic light-evoked responses directly from cholinergic neurons of neonatal ChAT-Cre x ChR2-eYFP mice. Figure 6 depicts examples of responses from biocytin-filled cholinergic neurons of basal forebrain (Fig. 6 A1; HDB) and brainstem (Fig. 6 A2; PPTg) of a P4 ChAT-ChR2-eYFP mouse. Repetitive presentation of blue light pulses (1 ms., 1 or 5 Hz) evoked a train of depolarizations with spikes riding their peaks (Fig. 6 B1&B2). Stimulation with a sustained light pulse (100 ms.) evoked fast-onset, long-lasting, stable excitatory currents, showing little if any desensitization (Fig. 6 C1&C2). However, for TRN neurons recorded during the first postnatal week (P4–5), light evoked cholinergic responses were extremely rare (2/9, 22.2%), purely excitatory, small in amplitude (2–3 mv), and found only in the non-visTRN. During week 2 cholinergic responses were more reliably evoked (29/39; 74.4%) with most exhibiting a biphasic profile.

To examine the development of each E-I component separately, we conducted voltage-clamp recordings during postnatal weeks 2–4, in the presence of muscarinic (5 μ M atropine) or nicotinic antagonists (300 nM Dh β E). The results for excitatory nicotinic responses are summarized in Figure 7. Figure 7A depicts the location of TRN neurons during blue light

stimulation (3 ms. pulse) of cholinergic terminals for postnatal weeks 2, 3 and 4. Each neuron was designated as residing in visTRN or non-visTRN, and as responsive or non-responsive to blue light stimulation. The location of each neuron within TRN was determined by biocytin reconstructions ($n = 117$; plotted in Fig. 7A), or through visualization of the pipette tip during recordings ($n = 33$, not shown but included in the analysis). Figure 7B summarizes the percentages of responsive and non-responsive neurons in visTRN and non-visTRN. Overall, there was an age-related increase in the incidence of responsive neurons (Chi square, week 2: 32/73, 43.8% vs. week 3: 34/51, 66.7%, $\chi^2(1) = 6.26$, $p = 0.01$; week 3: 34/51, 66.7% vs. week 4: 25/26, $\chi^2(1) = 8.21$, $p = 0.004$). During week 2, a significantly greater proportion of responsive neurons were located in non-visTRN, compared to visTRN (Chi square, visTRN 27.3%, $n = 33$ vs. non-visTRN 57.5%, $n = 40$, $\chi^2(1) = 6.62$, $p = 0.01$). For weeks 3 and 4, the proportions of responsive and non-responsive neurons were comparable between the two sectors (Chi-square, week 3: visTRN 70.4%, $n = 27$ vs. non-visTRN 62.5%, $n = 24$, $\chi^2(1) = 0.35$, $p = 0.55$; week 4: visTRN 90.9%, $n = 11$ vs. non-visTRN 100%, $n = 15$, $\chi^2(1) = 1.36$, $p = 0.24$). Examples of nicotinic responses recorded at different postnatal weeks are shown in Figure 7A, and the average response amplitudes for neurons located in visTRN and non-visTRN are summarized in Figure 7C. Overall, the amplitude of nicotinic responses increased significantly between postnatal weeks 2 and 4, (Kruskal-Wallis, $H = 27.6$, $p = 0.001$, Dunn's comparison, week 2 vs. 3, $Q = 3.1$, $p = 0.005$, week 2 vs. week 4, $Q = 5.19$, $p = 0.001$). While a 3-fold increase in amplitude was noted between weeks 2 and 4, there were no significant differences between visTRN and non-visTRN (Mann-Whitney, week 2: $U = 99$, $p = 0.87$; week 3: $U = 89$, $p = 0.11$; week 4: $U = 56$, $p = 0.30$).

A similar analysis was conducted on muscarinic inhibitory responses of 71 neurons and the results are summarized in Figure 8. The plots in Figure 8A depict the locations of TRN neurons for each week ($n = 66$), along with representative examples of muscarinic responses in visTRN and non-visTRN. Overall, an age-related increase in the incidence of muscarinic responses occurred between week 2 and 4 (Chi-square, week 2 30.8%, $n = 26$ vs. week 4 90.9%, $n = 22$, $\chi^2(1) = 17.4$, $p = 0.001$). The incidence of responsive neurons located in visTRN and non-visTRN (Fig. 8B) was similar across each postnatal week (Chi square, week 2: visTRN 38.5%, $n = 13$ vs. non-visTRN 33.1%, $n = 13$, $\chi^2(1) = 0.69$, $p = 0.40$; week 3: visTRN 53.8%, $n = 13$ vs. non-visTRN 60%, $n = 10$, $\chi^2(1) = 0.10$, $p = 0.76$; week 4: visTRN 90%, $n = 10$ vs. non-visTRN 91.7%, $n = 12$, $\chi^2(1) = 0.02$, $p = 0.89$). The amplitude of muscarinic responses increased significantly between postnatal weeks 2 and 3 (Kruskal-Wallis, $H = 17.32$, $p = 0.001$, Dunn's comparison, week 2 vs. 3, $Q = 2.68$, $p = 0.022$; week 2 vs. 4, $Q = 4.16$, $p = 0.001$), and there were no significant differences between visTRN and non-visTRN values (Mann-Whitney, week 2: $U = 6$, $p = 0.79$; week 3: $U = 17$, $p = 0.63$; week 4: $U = 48$, $p = 0.94$).

Discussion

We took advantage of genetically modified mouse lines to examine the development of cholinergic projections to the sensory sectors of TRN. To fully understand the patterning of cholinergic input to TRN and to test whether there was a systematic progression of innervation across different sensory regions of TRN, we first conducted anterograde tracing

experiments to delineate the location of these regions. While the sectorial arrangement of sensory regions in TRN has been established in a number of mammals, including rodents (Jones, 1975; Montero et al., 1977; Ohara & Lieberman, 1985; Conley et al., 1991; Harting et al., 1991; Crabtree 1992a & 1992b; Coleman & Mitrofanis, 1996; Kimura et al., 2005; Fitzgibbon et al., 2007), studies in the mouse have been limited largely to the analysis of a single modality (Wimmer et al., 2015; Chen et al., 2015; Clemente-Perez et al., 2017). Here we used a mouse line that expresses Cre recombinase in the thalamocortical neurons of first-order thalamic sensory nuclei (Crh-Cre), and performed Cre-dependent anterograde viral tracing to confirm that like other rodents, the TRN of the mouse exhibits a modality-specific sectorial arrangement. In the coronal plane, auditory and somatosensory sectors are located ventral to the apex and in the tail of TRN, while the visual sector resides dorsal to the apex and within the head of TRN. While TC projections from sensory thalamic nuclei are largely segregated within TRN, it does not preclude the possibility of cross-modal interactions (Crabtree & Isaac, 2002; Kimura, 2014). Indeed, TRN neurons are not bound by cytoarchitectural laminae, have dendritic arbors that span across multiple sectors (Pinault, 2004; Kimura, 2014; see Fig. 5B) and are themselves, electrically and synaptically coupled (Landisman et al., 2002; Long et al., 2004).

In assessing the progression of cholinergic projections within sensory sectors of TRN, we compared visual and non-visual (auditory & somatosensory) sectors, as these regions could be reliably defined in a coronal plane by conventional designations that outline its shape (e.g., apex, head, and tail). Using a Cre-dependent mouse line to visualize cholinergic projections, we found that cholinergic axons follow a ventral to dorsal progression during development, with non-visual sectors receiving innervation earlier than visual ones. During the first week, axons target the non-visual ventral sectors of TRN, with processes growing into the visual sector over the course of the second postnatal week. Thereafter, the density of innervation increases throughout the TRN and forms a reticular profile by the third postnatal week, which is especially conspicuous in the non-visual regions of TRN. The timing and progression of cholinergic innervation in sensory TRN seems consistent with observations made in other sensory thalamic nuclei. For example, cholinergic projections seem to innervate somatosensory thalamic structures (e.g., VB, and posterior nucleus) before visual ones (e.g., dLGN, and lateroposterior nucleus). In ChAT-Cre x Ai9 mice, we found that brainstem cholinergic projections are evident throughout all of VB by P0, yet dLGN remains devoid of fibers until the end of the first postnatal week (unpublished observations, see also Ballesteros et al., 2005). Perhaps this sequence is related to a general principle of sensory thalamic development whereby the somatosensory system and develops sooner than the visual system (Schlaggar & O'Leary, 1994; O'Leary et al., 1994; Molnár & Blakemore, 1995; Bayer & Altman, 2001; Hevner et al., 2002; Molnár et al., 2003; Fox & Wong, 2005; Jacobs et al., 2007). Moreover, projections that convey sensory information seem to be established prior to those that serve a modulatory role. While TRN does not receive direct sensory inputs from the periphery, thalamocortical projections from first-order nuclei pass through the TRN at early perinatal ages (Mitrofanis and Baker, 1993), before the arrival of cholinergic projections. A similar sequence occurs in dLGN, in which the arrival and establishment retinofugal projections occurs well before the emergence of modulatory inputs from brainstem and layer VI of visual cortex (Ballesteros et al., 2005; Seabrook et al., 2013).

Together, these observations suggest highly orchestrated development plan for thalamic circuit development in which nonvisual structures mature before visual ones, and where sensory projections are established before modulatory ones.

Our optogenetic experiments reveal that the emergence of functional cholinergic connections also follows the same ventral-to-dorsal progression. Light-evoked postsynaptic responses first emerge in non-visTRN during week 1, becoming more prevalent and stronger during week 2. By contrast, a comparable proportion of responsive cells was not detected in visTRN until week 3. It is also important to note that adult TRN, responses are biphasic in nature, having both a fast nicotinic excitatory component and a slower muscarinic inhibitory one (Hu et al., 1989; Sun et al., 2013). Typically, activation of metabotropic receptors requires repetitive stimulation, however we and others show that muscarinic responses in TRN are reliably evoked with single electrical or optogenetic pulses. (Sun et al., 2013; Pita-Almenar et al., 2014). In thalamus, such an arrangement is unique to TRN neurons, and helps shape oscillatory activity during different behavioral states (Pita-Almenar et al., 2014; Ni et al., 2016). Our recordings suggest these elements develop in sequential fashion. Initially, responses are purely excitatory, with biphasic excitation-inhibition responses emerging later. Indeed, inhibitory responses were rarely encountered though week 2 and were not prevalent until week 4.

Cholinergic inputs to the TRN play an important role in sleep, including the regulation of oscillatory rhythms during slow wave sleep (e.g., sleep spindles), and sleep-wake transitions (Steriade, 2004; Han et al., 2014; Ni et al., 2016; Kroeger et al., 2017). During development, the emergence of distinct sleep-wake patterns of cortical activity appears to coincide with formation of functional cholinergic synapses in TRN. While state-dependent modulation is largely absent before P10, rodents exhibit distinct patterns of cortical activity by the end of the second postnatal week (Shen & Colonnese, 2016; Khazipov & Luhmann, 2006; Cirelli & Tononi, 2015). This is characterized by an alternation between synchronous low frequency activity during slow wave sleep, and asynchronous high frequency activity during rapid eye-movement (REM) sleep and wakefulness. While an adult-like pattern of cortical activity is already evident by P17, further changes in the duration of REM and nREM sleep occur as late as the end of the first postnatal month (Jouvet-Mournier et al., 1970; Daszuta & Gambarelli, 1985; Mirmiran et al., 2003). Thus the protracted development of cholinergic inputs to TRN, which occurs largely after the circuits mediating thalamocortical transmission are established, likely contributes to the modulation of sleep-wake states.

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Abbreviations

A1 Primary auditory cortex

AAV	Adeno-associated virus
ACSF	Artificial cerebrospinal fluid
AF	Alexa Fluor
ANOVA	Analysis of variance
AP	Antero-posterior
ChAT	Choline acetyltransferase
ChR2	channelrhodopsin-2
CT	Corticothalamic
dLGN	Dorsal lateral geniculate nucleus
DV	Dorso-ventral
E-I	Excitation-inhibition
eYFP	enhanced yellow fluorescent protein
HDB	Nucleus of the horizontal diagonal band
IPSC	Inhibitory postsynaptic current
LDTg	Laterodorsal tegmentum
LED	Light-emitting diode
LP	Lateral posterior nucleus
ML	Medio-lateral
NGS	Normal goat serum
non-visTRN	Non-visual TRN
P	Postnatal
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
Po	Posterior nucleus
PPTg	Pedunculopontine tegmentum
RMS	Root mean squared
S1	Primary somatosensory cortex
SA	Streptavidin
SI	Substantia innominata

TC	thalamocortical
tdT	tdTomato
TRN	Thalamic reticular nucleus
V1	Primary visual cortex
VB	Ventrobasal complex
visTRN	visual TRN
vLGN	Ventral lateral geniculate nucleus
vMGN	ventral medial geniculate nucleus
VPM	ventral posteromedial nucleus
VPL	Ventral posterolateral nucleus

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Figure 1:

Delineation of sensory sectors in TRN. **A.** Coronal sections depict the pattern of tdTomato (tdT) labeling in sensory regions of thalamus and cortex of an adult Crh-Cre x Ai9 mouse. TdT labeling is expressed in thalamocortical (TC) neurons of 1st order thalamic relay nuclei ventral medial geniculate nucleus (vMGN; A1), ventrobasal complex (VB; A2 & A3), dorsal lateral geniculate nucleus (dLGN; A2), and their axonal projections coursing through the thalamic reticular nucleus (TRN) and terminating in sensory regions of cortex. Dotted lines delineate the borders of vMGN, VB, dLGN, and TRN. Scale = 500 μ m. **B.** NeuN staining in a coronal section of TRN illustrates its cytoarchitectural boundaries and salient landmarks including the head, apex, and tail. Scale = 100 μ m. **C1–3.** Representative sections of thalamus in different Crh-Cre mice after targeted injections of the Cre-dependent anterograde viral tracer FLEX-AAV-ChIEF-tdT into vMGN, VB, or dLGN. Adjacent panels depict the sensory-specific sectorial pattern of labeled projections in TRN. vMGN (C1) and VB (C2) injections labeled TC afferents that were segregated in the caudo-rostral plane, in

ventral TRN below the apex and within the tail of TRN. dLGN injections (C3) labeled projections above the apex in the head of TRN. Scale = 500 μm (left panels), 100 μm (right panels). Images of TRN (C1-C3) are in the same antero-posterior plane. **D.** Summary diagram illustrating the sectorial arrangement of TC projection within TRN. The horizontal line through the apex is used to separate dorsal visual TRN (visTRN) from ventral non-visual TRN (non-visTRN).

Figure 2.

Pattern of tdT labeling in coronal sections of the brainstem, basal forebrain, and TRN of ChAT-Cre x Ai9 mouse. **A.** Coronal sections through the brainstem (left) and basal forebrain (right) showing tdT labeling of cholinergic neurons in an adult (P60) mouse. Low power views depict cholinergic neurons of the laterodorsal tegmentum (LDTg) and pedunculopontine tegmentum (PPTg) (left), and nucleus of the horizontal diagonal band (HDB) of the basal forebrain (BF) (right). TdT labeling is also present within cholinergic neurons of the caudate putamen (CPu), and ChAT-containing cortical interneurons (right). Scale = 500 μ m. Dotted lines outline the approximate borders of brainstem and basal forebrain nuclei. **B.** High power views of tdT labeling in brainstem and basal forebrain cholinergic neurons of an adult (top, P60) and neonate (bottom, P0) mouse. Scale = 50 μ m. **C.** Pattern of cholinergic projections in adult TRN, showing a prominent reticular pattern of innervation. Scale = 100 μ m. Dotted lines show the borders of TRN. VB: Ventrobasal nucleus.

Figure 3.

Cholinergic innervation of TRN. Examples of coronal sections through TRN of ChAT-Cre x Ai9 mice at different postnatal ages (P1, P3, P5, P7, P9, P11, P14, and P21). Between P1–5, cholinergic axons emerge within the ventral non-visTRN. Between P7–P11, cholinergic innervate the dorsal, visTRN. At P21, innervation resembles an adult-like pattern. For clarity, images were inverted and contrast-enhanced. Dotted lines outline the boundaries of TRN. Scale = 100 μ m.

Figure 4.

Age-related increase in the cholinergic innervation of TRN. Shown is a scatterplot that depicts the degree of cholinergic innervation in visTRN (blue), and non-visTRN (red) as a function of postnatal age in ChAT-Cre x Ai9 mice. Each point represents a single hemisphere, expressed as percentage of fluorescent signal compared to the total area of visTRN or non-visTRN. Horizontal lines depict mean and $\pm$ SEM values that are based on 4 hemispheres (three sections/hemisphere). Asterisks represent significant differences between visTRN and non-visTRN (* $p < 0.05$, ** $p < 0.01$).

Figure 5.

Optogenetic activation of cholinergic projections in TRN. **A.** Example of an acutely prepared coronal slice taken from an adult (P120) ChAT-Cre x ChR2-eYFP mouse showing the expression of ChR2-eYFP fusion protein in cholinergic arbors within TRN. **B.** Example of a typical slice recording experiment in TRN of a P23 ChAT-Cre x ChR2-eYFP mouse. *Left:* Reconstructions of biocytin-filled TRN neurons recorded along the dorso-ventral extent of TRN. *Right:* Corresponding whole-cell voltage recordings showing postsynaptic responses evoked by blue light stimulation (single 3 ms. pulse). Light-evoked responses

were biphasic, consisting of an initial rapid depolarization, followed by long-lasting hyperpolarization. **C. Left:** Example of light-evoked, biphasic excitation-inhibition response in TRN. **Right:** Example of light-evoked depolarization leading to the firing of action potentials. **D.** Underlying pharmacology of light-evoked cholinergic responses in TRN. Examples of light-evoked responses recorded before (black) and after (red) bath application of cholinergic receptor antagonists. The excitatory component was blocked by nicotinic antagonist DH β E (5 μ M; left) and the inhibitory component was abolished by muscarinic antagonist atropine (5 μ M; right). All responses were recorded at resting membrane potential (–60 to –70 mV) and in the presence of bath-applied glutamate receptor antagonists DNQX (20 μ M, AMPA antagonist) and (RS)-CPP (10 μ M, NMDA antagonist). Vertical blue lines represent the timing of blue light pulses.

Figure 6.

Light-evoked responses in cholinergic brainstem and basal forebrain neurons of neonatal (P4) ChAT-Cre x Chr2-eYFP mice. **A1–2.** Reconstructions of a biocytin-filled neurons in HDB of basal forebrain (A1), and PPTg of brainstem (A2). **B1–2.** Corresponding voltage responses evoked by blue light stimulation. Repetitive stimulation (1 ms. / 1 or 5 Hz) leads to a train of depolarizations with a spike riding their peaks. **C1–2.** A single 100 ms. pulse evokes a large, sustained inward current that shows little desensitization. Recordings were performed in current clamp (B1–2, $V_M = -50$ mV, -65 mV) or voltage clamp (C1–2, $V_H = -70$ mV), and in the presence of bath-applied glutamate receptor antagonists DNQX and (RS)-CPP.

Figure 7.

Development of light-evoked nicotinic responses in visTRN and non-visTRN. **A.** Composite plots depicting the relative location of light responsive (green dots) and non-responsive (yellow dots) neurons in TRN at postnatal weeks 2, 3, and 4. Adjacent to each plot are representative light-evoked (3 ms. pulse) nicotinic EPSCs in visTRN (top) and non-visTRN (bottom). Recordings were done in voltage clamp ($V_H = -70$ mV) and in the presence of glutamate (DNQX, (RS)-CPP) and muscarinic (atropine) receptor antagonists. **B.** Bar graphs depict the percentage of responsive and nonresponsive visTRN (blue) and non-visTRN (red) neurons at each postnatal week. Asterisks represent significant differences between visTRN and non-visTRN (** $p < 0.01$). **C.** Scatter plots showing the average peak response amplitude for neurons in visTRN and non-visTRN at each postnatal week. Each point represents the response of a single neuron. Horizontal lines depict mean values and 95% confidence intervals. Asterisks represent significant age-related differences (* $p < 0.05$, ** $p < 0.01$).

Figure 8.

Development of light-evoked muscarinic responses in visTRN and non-visTRN. Composite plots depicting the relative location of light responsive (green dots) and non-responsive (yellow dots) neurons in TRN at postnatal weeks 2, 3, and 4. Adjacent to each plot are representative light-evoked (3 ms. pulse) muscarinic IPSCs in visTRN (top) and non-visTRN (bottom). Recordings done in voltage clamp ($V_H = -70$ mV) in the presence of glutamate (DNQX, (RS)-CPP) and nicotinic (DH β E) receptor antagonists. **B.** Bar graphs depict the percentage of responsive and nonresponsive visTRN (blue) and non-visTRN (red) neurons at each postnatal week. **C.** Scatter plots showing the average peak response amplitude for neurons in visTRN and non-visTRN at each postnatal week. Each point represents the response of a single neuron. Horizontal lines depict mean values and 95% confidence intervals. Asterisk represents significant age-related differences (* $p < 0.05$, ** $p < 0.01$).