Ontogenetic establishment of order-specific nuclear organization in the mammalian thalamus

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The thalamus connects the cortex with other brain regions and supports sensory perception, movement, and cognitive function via numerous distinct nuclei. However, the mechanisms underlying the development and organization of diverse thalamic nuclei remain largely unknown. Here we report an intricate ontogenetic logic of mouse thalamic structures. Individual radial glial progenitors in the developing thalamus actively divide and produce a cohort of neuronal progeny that shows striking spatial configuration and nuclear occupation related to functionality. Whereas the anterior clonal cluster displays relatively more tangential dispersion and contributes predominantly to nuclei with cognitive functions, the medial ventral posterior clonal cluster forms prominent radial arrays and contributes mostly to nuclei with sensory- or motor-related activities. Moreover, the first-order and higher-order sensory and motor nuclei across different modalities are largely segregated clonally. Notably, sonic hedgehog signaling activity influences clonal spatial distribution. Our study reveals lineage relationship to be a critical regulator of nonlaminated thalamic development and organization.

The thalamus, with its intricate cortical, subcortical, and cerebellar connections, is a pivotal network node in relaying and modulating sensory and motor signals to the cortex as well as supporting higher-order cognitive functions such as attention and consciousness1–3. It receives inputs from diverse brain regions, including the retina, medial lemniscus, inferior colliculus, basal ganglia, spinal cord, cerebellum, and cortex, and projects to multiple brain structures, especially the cortex4. The extensive reciprocal connections between the cortex and thalamus allow the travel of different sensory and motor information along separate pathways, as well as effective information integration.

The thalamus is composed of more than 30 cytoarchitectonically and functionally distinct nuclei, each of which has a different pattern of anatomical connectivity1,5–7. In particular, every sensory system (with the exception of olfaction) relies on a thalamic nucleus that receives sensory signals and sends them to the corresponding primary cortical area. Furthermore, each thalamic sensory relay nucleus also receives feedback connections from the cortex. The distinct sources and properties of inputs to the thalamus have led to the concept of first-order (FO) and higher-order (HO) thalamic nuclei linked to different sensory modalities5. FO nuclei relay peripheral or subcortical information of a particular type to a primary cortical area, whereas HO nuclei relay information from one cortical area to another cortical area. For example, visual inputs from the retina are sent to the lateral geniculate nucleus (LGN) of the thalamus, which in turn projects to the primary visual cortex. In comparison, the pulvinar (lateral posterior (LP) in rodents) nucleus relays information between the primary and HO visual cortical areas, or between two HO cortical areas. Similarly, peripheral somatosensory inputs reach the primary and HO somatosensory cortical areas largely via the ventral posterior (VP) and posterior (PO) thalamic nuclei, respectively. Peripheral auditory inputs reach the primary and HO auditory cortical areas mostly via the ventral division (vMG) and dorsal division (dMG) of the medial geniculate nucleus in the thalamus, respectively. Although the order-specific nuclear organization across different modalities provides an influential framework for understanding thalamic structure and function, very little is known about the mechanisms responsible for its establishment.

The thalamus emerges from the embryonic diencephalon9,10. It consists of glutamatergic excitatory neurons and GABAergic inhibitory interneurons. In rodents, the vast majority of thalamic nuclei contain exclusively excitatory neurons; exceptions are the LGN, which contains both excitatory and inhibitory neurons, and the thalamic reticular nucleus (TRN), which harbors exclusively GABAergic interneurons that provide inhibition to all other thalamic nuclei11. Previous genetic mapping studies in mice have demonstrated that the caudal progenitor domain of the developing thalamus generates all thalamic excitatory neurons12, whereas the rostral progenitor domain of the developing thalamus and pre-thalamus produces GABAergic interneurons in the LGN and TRN12,13. Although the progenitor domains of thalamic neurons have been delineated, the principles underlying complex nuclear formation and organization of the mammalian thalamus remain largely elusive.

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Previous studies have demonstrated that lineage relationship has an instructive function in guiding the structural and functional assembly of the cortex\(^{14–18}\), a laminated structure closely associated and reciprocally interconnected with the thalamus\(^{8,19,20}\). It has been suggested that cell lineages in the chick diencephalon exhibit diverse migration routes in general\(^{21,22}\). However, it remains largely unclear whether lineage relationship influences the complex nuclear formation and organization of the generally nonlaminated thalamus. To address this, we performed a systematic clonal analysis of the progenitor behavior and progeny organization in the developing mouse thalamus using mosaic analysis with double markers (MADM)\(^{23,24}\) and Cre recombinase–dependent retroviral labeling\(^{25}\).

**RESULTS**

**MADM labeling of thalamic clones**

To label individual neural progenitors lining the third ventricle in the developing mouse thalamus, we introduced the Nes-Cre\(^{ERT2}\) transgene\(^{26}\), in which a tamoxifen (TM)-inducible Cre recombinase is selectively expressed in neural progenitors, including thalamic radial glial progenitors (RGPs) expressing oligodendrocyte transcription factor 3 (OLIG3)\(^{12}\) and brain lipid-binding protein (BLBP) (Supplementary Fig. 1a), into the chromosome 11–targeted MADM system (MADM\(^{11}\))\(^{27}\). In MADM, Cre recombinase–mediated interchromosomal recombination in dividing progenitors followed by X-segregation (G\(_2\)-X) restores one fluorescent marker—EGFP (green)

![Figure 1](https://example.com/figure1.jpg)

**Figure 1** Labeling of thalamic clones using MADM. (a) Confocal image of a MADM-labeled E12 clone labeled by TM treatment at E10 and stained for EGFP (green), tdTomato (red), and OLIG3 (gray) and with DAPI (blue). Ep, epithalamus; Thal, thalamus; PTh, prethalamus; Ncx, neocortex; Hip, hippocampus; GE, ganglionic eminence; 3rd V, third ventricle. Scale bar, 100 µm. (b) 3D reconstructed image of the hemisphere in a. Blue lines indicate the contours of brain structures; white lines indicate OLIG3+ thalamic domain, and dots represent the cell bodies of labeled neurons. A, anterior; P, posterior; D, dorsal; V, ventral; M, medial; L, lateral. Scale bar, 100 µm. (c) High-magnification confocal images of the clone in a. Arrows indicate bipolar RGPs and white arrowheads indicate progeny arrayed along radial glial fibers (open arrowheads). Broken lines indicate the VZ surface. Right, magnified images of RGPs (area 1) and a progeny located near the pia with numerous branches (area 2). Asterisks indicate the ventricular end-feet of RGPs. Scale bars, 20 µm (left) and 10 µm (right). (d) Percentage of thalamic hemispheres with indicated numbers of green/red clones or yellow clones.
or tdTomato (red)—in each daughter cell (Supplementary Fig. 1b). This results in permanent labeling of the two daughter cells and their descendnt lineages in two distinct colors. In addition, upon G2-Z recombination and segregation or G1/G0 recombination events, EGFP and tdTomato are restored simultaneously in the same daughter cell, resulting in double-labeled (yellow) lineages.

We induced Cre recombinase activity to trigger recombination and labeling via a single dose of TM administered to timed pregnant female mice at embryonic day 9 (E9), E10, E11, or E12 and analyzed the brains at embryonic stages or postnatal days 21–24 (P21–P24) (Supplementary Fig. 1c). We found no labeling in the absence of TM treatment (n = 3 mice). To ensure unequivocal clonal analysis, we titrated the TM dose to achieve very sparse labeling—on average, 1–2 progenitors (i.e., clones) per thalamic hemisphere (n = 120 from 60 embryonic brains) (Fig. 1). We sometimes observed labeled cells in other brain regions, such as the cortex (data not shown). Notably, Olig3-expressing progenitors in the developing thalamus produced neurons exclusively located in the thalamus (Supplementary Fig. 2a), as previously shown. To recover all labeled cells in the thalamus, we performed serial sectioning, immunostaining, and three-dimensional (3D) reconstruction of individual thalami (Online Methods).

We observed individual clusters of cells showing green or red (Fig. 1a–c) or yellow (Supplementary Fig. 1d) fluorescence in the embryonic thalamus. These clusters were radially (i.e., mediolaterally) organized and contained bipolar RGDs, with their defining morphological characteristics including a cell body in the ventricular zone (VZ), a short process reaching the VZ surface with a large end-foot, and a long fine radial glial fiber pointing toward the pial surface, as well as a number of cells with short processes arrayed along long radial glial fiber (Fig. 1c). There were no scattered fluorescent cells or clusters of green or red fluorescent cells mixed with yellow fluorescent cells (Fig. 1d). Notably, similar discrete radial clusters in the embryonic thalamus were also reliably labeled by in utero intraventricular injection of low-titer Cre recombinase-dependent retroviruses expressing EGFP into Olig3-Cre mouse embryos (Supplementary Fig. 2b), which selectively infected individual Olig3-expressing thalamic progenitors dividing at the VZ surface and thereby labeled their progeny. The consistent observation of radial clusters with two distinct clonal labeling methods confirmed the clonal nature of individual radial clusters in the developing thalamus.

Cellular composition of embryonic thalamic clones

We next assessed the cell identity of individual radial clonal clusters and found that the bipolar RGDs were positive for neural progenitor marker nestin (Fig. 2a, area 1) and proliferation marker Ki-67 (Fig. 2b, area 1). We also observed short-processes cells outside of the VZ positive for Ki-67 (Fig. 2b, area 2), suggesting the existence of intermediate progenitors (IPs, or basal progenitors), as previously suggested. Besides RGDs and IPs, individual radial clonal clusters also contained cells located farther away from the VZ that grew relatively long processes with numerous branches (Fig. 1c, area 2) and expressed the neuron-specific marker TUJ1 (Fig. 2c, area 3). Together, these results suggest that individual thalamic RGDs actively divide to produce IPs and postmitotic neurons that are spatially organized along the mother radial glial fiber into radial clusters.

RGDs divide either symmetrically, to generate two RGDs, or asymmetrically, to give rise to an RGP and a differentiating progeny such as an IP or a neuron. Consistent with this, we observed two types of G2-Z green and red fluorescent clones. One type contained both green and red fluorescent RGDs as well as a cohort of green and red fluorescent progeny distributed radially along the radial glial fibers (Fig. 1b), representing the symmetrically dividing clone. The other type contained a ‘majority’ population including RGP(s) in one color and a ‘minority’ population in the other color (Fig. 2a–c), representing the asymmetrically dividing clone. We found that as development proceeded, we observed a decrease in the frequency of symmetric clones and a concurrent increase in the frequency of asymmetric clones (Fig. 2d). Related to this, we observed a progressive decrease in the average neuronal number in clones labeled between E10 and E12 (Fig. 2e). There

![Figure 2](image-url)
was a small but significant ($P = 0.03$; Mann–Whitney test) decrease in the average neuronal number in clones labeled at E10 and examined at E15 and P21–P24 (Supplementary Fig. 3a), indicating that some neurons are pruned away during development. We also occasionally observed small local clusters without RGPs (Supplementary Fig. 3b), probably representing dividing IP-derived clones.

**Spatial clustering of thalamic clones**

The diverse functions of the thalamus are carried out by a large number of nuclei. To test whether progenitor origin and lineage relationship influence nuclear formation and organization, we systematically examined the spatial distribution of individual E9-, E10-, E11-, and E12-labeled clones at P21–P24 (Supplementary Table 1), when the nuclei are formed and functional connections are largely established. The vast majority of clones spanned several consecutive sections and consisted of NeuN-positive neurons with the characteristic morphological features of thalamic excitatory neurons (Fig. 3a and Supplementary Fig. 4a,b,g), as shown previously. Moreover, within sections, neurons in individual clones were not broadly distributed but restricted to a defined region (Fig. 3a,b). Notably, the overall spatial localization of progenitors labeled at different embryonic stages correlated well with the overall regional localization of clones observed at P21–P24 (Supplementary Fig. 3c), suggesting that clones located in different regions of the mature thalamus are probably derived from progenitors in the corresponding regions of the embryonic thalamus.

To quantitatively assess the spatial distribution and clonal relationship of labeled neurons, we applied nearest-neighbor distance (NND) analysis to all the reconstructed P21–P24 data sets labeled with anterograde tracers revealing the specific nuclear occupation of neurons in individual clones (Fig. 4a–d). Additional immunohistochimistry analyses revealed that cells with small soma and nucleus and short bushy neurites typically expressed the astrocyte marker S100 (Supplementary Fig. 4c,d,g) and that cells with a small soma and nucleus and short parallel neurites characteristically expressed the oligodendrocyte marker Olig2 (Supplementary Fig. 4e–g). These results suggest that clonally related neurons originating from sparsely labeled progenitors do not randomly mix but form spatially segregated clusters in the thalamus. Therefore, each neuronal cluster represents a clone that arises from a single dividing thalamic progenitor.

We also observed NeuN-negative cells with small soma and nucleus and short neurite processes in nearly half (34 out of 77) of the labeled clones (Fig. 4a–d). Additional immunohistochimistry analyses revealed that cells with a small soma and nucleus and short bushy neurites typically expressed the astrocyte marker S100 (Supplementary Fig. 4c,d,g) and that cells with a small soma and nucleus and short parallel neurites characteristically expressed the oligodendrocyte marker Olig2 (Supplementary Fig. 4e–g). These results suggest that NeuN-negative cells are glial cells. The labeled glia were typically in close proximity to the labeled neurons (Fig. 4c); however, there was no obvious correlation between the numbers of glia and neurons at the clonal level (Fig. 4e). Clonally related glia also showed a clear clustering feature in spatial distribution (Fig. 4f). The fraction of glia-containing clones decreased progressively between E9 and E11 (Supplementary Fig. 3e), probably reflecting the progressive decrease in the RGP number in individual clones as a result of the temporal switch of symmetric proliferative to asymmetric neurogenic division by RGPs (Fig. 2d).

**Progenitor origin of thalamic nuclei**

To reveal the specific nuclear occupation of neurons in individual clones, we systematically aligned individual experimental 3D data sets to the reference thalamus with a defined nuclear boundary based on the Allen Brain Atlas (ABA) by adopting a previously established method (Supplementary Fig. 5). Notably, the labeled clones as a whole covered a vast majority of thalamic nuclei, representing more than 98% of the total volume. Moreover, consistent with a regional restriction in spatial distribution, individual clones generally occupied a few nuclei (~4.2 ± 0.3 out of a total of 34 nuclei).

The regional occupation of individual clones raised the possibility that different nuclei may have different progenitor origins. To test this, we performed an unsupervised agglomerative hierarchical clustering analysis of all neurons in individual RGP clones labeled at E9–E12 (n = 77 clones) on the basis of their distribution in different nuclei (Fig. 5a). Notably, we observed two major clonal clusters, corresponding to excitatory neuron clones and inhibitory interneuron clones, respectively (Fig. 5a). Clones occupying the TRN and zona incerta (ZI) were exclusively GABAergic interneurons (Supplementary Fig. 6a,b) and segregated from the clones located in other thalamic nuclei except the LGN (Fig. 5a), the only other rodent thalamic nucleus besides the TRN that contains inhibitory interneurons, suggesting a clear lineage separation of excitatory and inhibitory neurons in the thalamus at the clonal level. This lineage separation is consistent with previous genetic mapping studies and strongly supports the reliability of our clonal labeling and clustering analysis. The remaining excitatory neuron clones fell into three main subclusters (Supplementary Fig. 7). They were located predominantly in the anterior, medial dorsal, and ventral posterior regions, respectively. We thus termed them the ‘a’, ‘md’, and ‘mvp’ clonal clusters.

We then quantitatively analyzed the fraction of individual neuronal clones in these four major clonal clusters located in different thalamic nuclei (Fig. 5b). As expected, the GABAergic neuron clonal cluster was exclusively located in the TRN and LGN, including both the ventral and dorsal (dLG) regions, as well as the ZI (Fig. 5b). The three excitatory neuron clonal clusters occupied largely nonoverlapping nuclei, as reflected by the top 6–7 dominant nuclei, which harbored more than 80% of neurons in each clonal cluster (Fig. 5b). Specifically, the ‘md’ clonal cluster largely occupied the mediodorsal (MD), PO, LP, central medial (CM), paracentral (PCN), and intermediodorsal (IMD) nuclei. The ‘mvp’ clonal cluster occupied mainly the ventral posteromedial (VPM), ventral posterolateral (VPL), ventral anterior lateral (VAL), dLG, vMG, ventral medial (VM) and reuniens (RE) nuclei. The ‘a’ clonal cluster predominantly occupied the anteromedial (AM), RE, anteroventral (AV), rhomboid (RH), paraventricular (PVT), VM, and lateral dorsal (LD) nuclei. These results suggest that individual progenitors produce neurons preferentially located to distinct sets of nuclei. Notably, the nuclei occupied by the ‘mvp’ clonal cluster largely overlap with the nuclei harboring the excitatory neuron progeny of Olig2-positive progenitors in the caudal progenitor domain of the developing thalamus (Supplementary Fig. 8), indicating a linear correlation between them. The four sets of a total of 20 dominant nuclei (not including ZI) account for >80% of the entire thalamic volume. Moreover, clonally labeled neurons occupied all 34 nuclei, including nondominant nuclei that are generally small (Fig. 5). These results suggest that our clonal labeling and clustering analysis effectively covers the majority, if not all, of the thalamus and that the small nondominant nuclei are probably marginally formed by the three excitatory neuron clonal clusters.
Figure 3  Spatial clustering of clonally related neurons in the thalamus at the mature stage. (a) Confocal images of EGFP, tdTomato, and DAPI staining in consecutive sections from a green/red G2-X clone in a P21 brain treated with TM at E10. Dotted lines indicate contours of the thalamus and landmark nuclei including the medial and lateral habenula (MH and LH), LGN, and TRN. Arrowheads indicate labeled neurons. Bottom, high-magnification images of representative labeled NeuN+ neurons (white) (areas 1 and 2). Scale bars, 500 µm (top), 20 µm (bottom, areas 1 and 2), and 10 µm. (b) 3D reconstructed images of the thalamic hemisphere containing the clone shown in a. Blue lines indicate contours of the thalamus and landmark nuclei; dots represent the cell bodies of labeled neurons. Scale bar, 500 µm. (c) NND analysis of MADM-labeled neuronal clones (n = 77) in the P21–P24 thalamus. (d,e) 3D reconstructed images (d) and NND analysis of MADM-labeled green/red and yellow neuronal clones (e) from a thalamic hemisphere containing a green/red G2-X clone and a yellow clone. Scale bar, 500 µm. n = 11 clones per group. Data are mean ± s.e.m. n.s., not significant. ****P = 10^-15 (unpaired t-test with Welch's correction).
Ontogenetic segregation of nuclei

Among the major nuclei occupied by excitatory neuron clones, VM and RE were partially occupied by both the ‘mvp’ and ‘a’ clonal clusters, raising the question of whether the labeled neurons between these clonal clusters in these overlapping nuclei were the same or different populations. We found that neurons labeled in the ‘a’ clonal cluster were predominantly located in the anterior half of VM and RE, as well as RH, whereas neurons labeled in the ‘mvp’ clonal cluster were located mostly in the posterior half of these three nuclei (Supplementary Fig. 9a), suggesting lineage-based segregation of the anterior and posterior parts of VM, RE, and RH.

Figure 4 Spatial clustering of clonally related glial cells in the thalamus at the mature stage. (a) Scanned images of EGFP, tdTomato, and DAPI staining in consecutive sections from yellow G2-Z clone in a P21 brain treated with TM at E9. Open arrowheads indicated the labeled NeuN-negative cells and arrowheads indicate labeled neurons. Scale bar, 500 µm. (b) High-magnification images of the representative labeled NeuN+ (white, area 1) and NeuN− glial cells (areas 2 and 3). Scale bars, 20 µm (top) and 10 µm (bottom). (c) 3D reconstructed images of the thalamic hemisphere containing the clone in a. Scale bar, 500 µm. (d) Quantification of the fraction of P21–P24 clones labeled at E9–E12 containing neuron only (N) or neuron and glia (N+G). (e) No correlation between the number of neurons and the number of glia at the clonal level (n = 77 clones). (f) NND analysis of MADM-labeled glial clones (n = 25) in the P21–P24 thalamus. Data are mean ± s.e.m. ****P = 10−15 (unpaired t-test with Welch’s correction). Abbreviations as in Figure 3.
Notably, our in-depth analysis of the previous thalamocortical axonal tracing data showed that the anterior parts of VM, RE, and RH (aVM, aRE, and aRH, respectively) projected predominantly to cognition-related HO areas of the cortex, such as the anterior cingulate cortex (ACC) and prelimbic cortex (PrL) (Supplementary Fig. 9b). In contrast, the posterior parts of VM, RE, and RH (pVM, pRE, pRH, TRN, VPL, VPM, PO, MD, LP, dLG-i, SMT, AV, PVT, AD, IAM, PT, IAD, SPF, PP, vMG, dMG, SGN, vLG, POL, PF, IMD, CL, LD, PCN, CM, RH, AM, VM, dLG, VAL, ZI) projects to other areas of the cortex.
respectively) projected mostly to the sensory and motor areas of the cortex, consistent with the idea that they are involved in multimodal sensory processing and motor activity.\(^{32,33}\) Moreover, the remaining cells in the respective clones (n = 18) were located in regions sharing a similar cognition-related HO versus a sensory- or motor-related cortical projection pattern (Supplementary Fig. 9b), indicating a clonal but not nuclear level of correlation in functionality. Together, these results suggest that lineage relationship influences the organization of thalamic structures associated with distinct functionality, even at a resolution beyond the conventional individual nucleus level.

To further test this, we systematically analyzed the spatial configuration and nuclear occupation of individual clones in the ‘mvp’ and ‘a’ clonal clusters. We found that clones in the ‘mvp’ cluster were predominantly arrayed radially along the mediolateral axis with a dorso-posterior bend (Fig. 6a.b and Supplementary Movie 1). In contrast, clones in the ‘a’ cluster were relatively more tangentially dispersed (Fig. 6a.b and Supplementary Movie 2). This distinction in clonal configuration appeared to be an intrinsic feature of the respective lineages, as we observed similar differences in clonal organization at the embryonic stage (Fig. 6c.d).

There was also a tight association between the ‘mvp’ and ‘a’ clonal clusters and distinct functionalities. The ‘a’ clonal cluster occupied mostly the nuclei associated with cognition-related HO functions (for example, AM; AV; aRE, aRH, and aVM; and interanteromedial (IAM)) (Fig. 6a.e,f), whereas the ‘mvp’ clonal cluster occupied predominantly the nuclei associated with sensory- and motor-related activities (for example, VPL, VPM, VAL, dLG, vMG, and pRE, pRH, and pVM) (Fig. 6a.e,f). These results suggest that HO cognitive nuclei and sensory- and motor-related nuclei are ontogenetically segregated with distinct progenitor origins. These results also further indicate that lineage relationship not only affects spatial configuration of thalamic neurons but also contributes to a global nuclear organization of the thalamus related to functionality.

**Lineage distinction between FO and HO nuclei**

Clones in the ‘md’ clonal cluster appeared to be associated with either cognition-related (for example, MD, CM, PCN, and IMD) or sensory or motor (for example, PO and LP) activities (Fig. 7a.b). Notably, the sensory and motor activities mediated by the ‘md’ clonal cluster appeared to be fundamentally different from those mediated by the ‘mvp’ clonal cluster (Fig. 7c.d). Sensory- and motor-related thalamic nuclei are well organized in a modality- and hierarchy-based manner.\(^{1,8}\) Notably, the clones occupying the sensory- and motor-related FO and HO nuclei were largely segregated (Fig. 7c.d and Supplementary Movies 3 and 4). The ‘mvp’ clonal cluster contributed predominantly to the FO sensory- and motor-related nuclei such as VPL, VPM, VAL, dLG, and vMG as well as a small fraction in pRE, pRH, and SMT, whereas the ‘md’ clonal cluster mainly contributed to the HO sensory- and motor-related nuclei such as PO and LP (Fig. 7d.f). These results suggest a strong lineage-related separation of the sensory- and motor-related nuclei in hierarchy (i.e., in order). However, individual clones were frequently found to occupy the FO or HO nuclei across different modalities (Fig. 7e.f). Together, these results support a progenitor origin of a cross-modal hierarchical framework of sensory and motor pathways in the thalamus.

**Shh signaling influences organization of thalamic clones**

Previous studies suggested that sonic hedgehog (Shh) signaling regulates thalamic progenitor identity and nuclear specification.\(^{28,34}\) To explore the influence of Shh signaling on the development and organization of thalamic clones, we examined the size and spatial distribution of clones with a higher level of Shh signaling than normal. To achieve this, we performed MADM analysis in R26SmoM2-EYFP\(^{+}\) (SmoM2) mice, in which SmoM2 with a constitutively active point mutation encoding a W539L substitution is fused to enhanced YFP (EYFP)\(^{35}\). Notably, the R26SmoM2-EYFP and MADM\(^{11}\) transgenes are located in different chromosomes, which prevents a genetic link between EGFP or tdTomato expression and SmoM2-EYFP expression for a strict mosaic analysis. Nonetheless, in Nes-CreER\(^{12}\), MADM\(^{11}\); R26SmoM2-EYFP\(^{+}\) mice, a single dose of TM would trigger MADM labeling upon interchromosomal recombination and expression of SmoM2 upon intrachromosomal recombination, as confirmed by the expression of SmoM2-EYFP in tdTomato-labeled RGPS (Supplementary Fig. 10).

Within the diencephalon, Shh is expressed in the basal plate and zona limitans intrathalamica, which are ventral to the developing thalamus.\(^{26,36}\) As a result, the embryonic thalamus is exposed to graded Shh activity. Coinciding with this Shh signaling gradient, clones in the anterior and medial dorsal regions were more tangentially distributed than clones in the medial ventral or posterior region (Fig. 6a-d), indicating that high Shh signaling suppresses tangential distribution of clonally related neurons. Consistent with this notion, clones in the anterior region of the SmoM2 thalamus became less tangentially and more radially distributed than similarly located wild-type control clones (Fig. 8a.b), whereas clones located in the medial ventral and posterior regions did not show a significant change in spatial distribution (Supplementary Fig. 11a.b). In addition, we observed an expansion of the expression of retinoic acid receptor–related orphan receptor α (ROR\(\alpha\)), a marker predominantly expressed in VP and dLG,\(^{37}\) to the anterior region as well as dorsally (Supplementary Fig. 11d), indicating a change in nuclear boundary and functional organization of the thalamus, as previously shown.\(^{28}\) We did not observe significant changes in average neuronal number in individual clones between wild-type and SmoM2 thalamus (Supplementary Fig. 11c).

However, although the average glial number in individual clones was not significantly different between wild-type and SmoM2 thalam.

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**Figure 6** Distinct spatial configuration and functionality of the clonal clusters. (a) 3D rendered images of a representative ‘mvp’ or ‘a’ clonal clone that predominantly occupies sensory- and motor-related nuclei and cognition-related HO nuclei, respectively. Dotted lines indicate the radial (white) versus tangential (black) dispersion of the clone. Scale bar, 500 μm. (b) Average ratios of radial versus tangential dispersion normalized to the total number of neurons in individual clones at P21–P24 (‘mvp’, n = 33; ‘a’, n = 9). \(\alpha\) = 0.008 (Mann–Whitney test). (c) Confocal images of a representative E15 medial ventral posterior (top) or anterior dorsal (bottom) clone labeled at E10. Dotted lines indicate the contours of the thalamus. Right, high-magnification images. Arrows indicate radial glial progenitors with a long radial glial fiber (open arrowheads); white arrowheads indicate progeny. Scale bars, 200 μm (left) and 50 μm (right). (d) Average ratio of the radial versus tangential dispersion normalized to the total number of neurons in individual clones at E15–E16 (medial ventral posterior, n = 20; anterior, n = 5). \(\alpha\) = 0.012 (Mann–Whitney test). (e) Percentage of individual ‘mvp’ or ‘a’ clonal clones located in sensory- and motor-related, cognition-related, or unclear function nuclei. Each bar represents a clone. (f) Percentage of all ‘mvp’ or ‘a’ clonal clones located in sensory- and motor-related, cognition-related, or unclear function nuclei (‘mvp’: sensory- and motor-related, 83.7 ± 3.6%; cognition-related, 11.9 ± 3.3%; unclear function, 4.4 ± 1.6%; ‘a’: sensory- and motor-related, 16.3 ± 11.0%; cognition-related, 80.7 ± 11.4%; unclear function, 3.0 ± 1.7%). Data are mean ± s.e.m. \(\alpha\) = 5 × 10\(^{-5}\) (sensory- and motor-related); \(\alpha\) = 2.5 × 10\(^{-5}\) (cognition-related) (Mann–Whitney test). In b and d, center line, median; box, interquartile range; whiskers, minimum and maximum.
(Supplementary Fig. 12a), there was a substantial increase in the fraction of clones with glia in the SmoM2 thalamus (Supplementary Fig. 12b), indicating that Shh signaling promotes gliogenesis in the thalamus. The overall distribution of clonally related neurons and glia remained spatially clustered in the SmoM2 thalamus (Supplementary Figs. 11e and 12c). Together, these results suggest that Shh signaling...
influences the spatial organization of thalamic neurons at the clonal level, which is likely to contribute to distinct thalamic nuclear configuration and organization.

**DISCUSSION**

Our findings reveal a previously unknown ontogenetic logic for structural development and functional organization of the mammalian thalamus (Fig. 8c and Supplementary Fig. 12d). RGPS in the VZ of the developing thalamus actively divide to produce a cohort of neuronal progeny. At the early embryonic stage, individual RGPS and their progeny are organized radially along a mediolateral axis. As development proceeds, clones in the medial ventral posterior region remain tightly arrayed radially along the radial glial fiber, with the early born cells moving progressively outward (i.e., laterally) and dorso posteriory, contributing mostly to the FO sensory- and motor-related nuclei, whereas clones in the anterior and medial dorsal regions become more tangentially dispersed, occupying predominantly the HO cognitive and HO sensory- and motor-related nuclei.

Diverse migratory patterns of lineages have been suggested in the embryonic chick diencephalon. It has also been suggested that migrating cells in the developing rat thalamus follow both radial and nonradial glia pathways. However, it is unclear how different migratory patterns may be related to distinct nuclear formation and functionality in the thalamus. In this study we carried out a systematic clonal analysis of mouse thalamic progenitor cells using MADM, which provides a fine-resolution view of progenitor behavior and lineage progression in a temporal and spatial manner. MADM enables labeling of progeny throughout development, starting from TM treatment. Our focus on E9–E12 coincides with the beginning of thalamic neurogenesis, thereby providing a comprehensive grasp of neuronal production and migration and nuclear formation in the thalamus.

Notably, our unsupervised agglomerative hierarchical clustering analysis of the nuclear occupation of neurons in individual clones at the relatively mature stage (P21–P24) not only reliably distinguished excitatory and inhibitory neuronal clones but also identified three main excitatory neuron clonal subclusters that occupy largely nonoverlapping sets of thalamic nuclei. These results suggest that the spatial configuration and nuclear occupation are robust and reliable features of thalamic progenitors and their neuronal lineages. Our finding of co-distribution of clonal clusters to certain thalamic nuclei at the single progenitor level is consistent with the previous population-level observation. Notably, additional subclusters may be revealed by further dissection of the hierarchical dendrogram. For example, in the 'md' clonal cluster, there appear to be two subclusters largely corresponding to the clones preferentially occupying the HO cognition-related nuclei (for example, MD, PVT, and CM) and HO sensory- and motor-related nuclei (for example, MD, PVT, and CM) and HO sensory- and motor-related nuclei.
motor-related nuclei (for example, PO, LP, and dMG), respectively. On the other hand, lineage-related organization is not absolute but preferential, as reflected in our quantitative analyses. Whereas individual clonal clusters show a strong propensity for distinct nuclei, clones within individual clonal clusters show some variability.

The differences in clonal behavior and neuronal organization are probably related to morphogen gradients and transcriptional regulations that affect thalamic progenitor specification and neuronal differentiation. Consistent with this, distinct spatial configurations of clones can be observed at the embryonic stage. Previous studies have shown that Shh, Fgf, and Wnt act together to orchestrate the development and regionalization of the thalamus\textsuperscript{9,10,28,39–42}. We found that neuronal clones in the medial ventral and posterior region with high levels of Shh activity were more radially organized, whereas neuronal clones in the anterior and medial dorsal regions with low levels of Shh activity were more tangentially organized. Moreover, enhanced Shh activity by SmoM2 expression suppressed tangential organization and promoted radial organization of clones in the anterior and dorsal regions, suggesting that Shh signaling influences the spatial organization of thalamic clones. Therefore, it is likely that the precise localization of progenitors, and the corresponding signaling environment, regulates lineage progression and clone organization.

Figure 8 Shh signaling regulates the spatial distribution of thalamic clones depending on the localization. (a) Representative 3D reconstructed images of the thalamic hemispheres containing a MADM-labeled clone in the anterior region of wild-type control (top) and SmoM2 (bottom) mice. Scale bars, 500 μm. (b) Average ratio of radial versus tangential dispersion normalized to the total number of neurons in individual P21–P24 clones in the anterior and medial ventral posterior region of the control and SmoM2 mouse (anterior: control, \(n\) = 9 clones; SmoM2, \(n\) = 4 clones; \(P = 0.02\); medial ventral posterior: control, \(n\) = 33 clones; SmoM2, \(n\) = 20 clones; n.s., not significant, \(P = 0.2\); Mann–Whitney test). Center line, median; box, interquartile range; whiskers, minimum and maximum. (c) Illustration of ontogenetic origin and organization of thalamic nuclei and function. S/M, sensory and motor.
Besides spatial regulation by a combination of signaling pathways, there are also important temporal regulations. Individual RGPAs are capable of producing a cohort of neuronal progeny in a sequential manner. The temporal dynamics of RGP and IP divisions of the corresponding clones probably dictate the size and configuration of thalamic nuclei in which the clones reside, in conjunction with neuronal migration. Notably, besides the relative radial versus tangential dispersion difference, the average neuronal number of the 'mvp' clonal cluster occupying the FO sensory- and motor-related nuclei appears to be higher than that of the A clonal cluster occupying the HO cognitive nuclei, which may contribute to the overall structural differences between these two general groups of nuclei. Therefore, although sharing close lineage relationship, neurons in the same clone probably also have temporal differences in maturation and properties, which may contribute to neuronal diversity and individual nuclear distinction in the thalamus.

The clonal relationship is not only coupled to structural formation but also related to functional organization of the thalamus. The 'a' and 'md' clones principally contribute to the nuclei of cognition-, sensory-, and motor-related FO functions, whereas the 'mvp' clones mostly contribute to the nuclei of sensory- and motor-related FO functions. The thalamus is commonly demarcated into a large number of distinct nuclei with defined functions. However, little is known about the general principles underlying the complex structural and functional organization of thalamic nuclei. Moreover, accumulating evidence suggests that, although useful, these nuclear divisions may not always be the relevant functional unit. Our data showed that cognition-related HO nuclei in the anterior region share a similar progenitor origin, and sensory- and motor-related FO nuclei in the medial ventral posterior region share a similar progenitor origin. In addition, sensory- and motor-related HO nuclei share a close but largely distinguishable lineage relationship with cognition-related HO nuclei in the medial dorsal region. These results suggest that lineage relationship influences functional organization of the thalamus.

This lineage-related functional organization can manifest at the resolution beyond the conventional individual nuclear boundaries. For example, we found that neurons in the VM, RE, and RH have two distinct progenitor (i.e., clonal) origins. Neurons in the anterior portion of these nuclei are largely derived from the 'a' clonal cluster, whereas neurons in the posterior portion are predominantly originated from the 'mvp' clonal cluster. Notably, the anterior and posterior regions of VM, RE, and RH exhibit distinct thalamocortical axonal projection patterns. Whereas the anterior regions predominantly innervate cognitive cortical areas such as the ACC and PLC, the posterior regions largely project to sensory- and motor-related cortical areas. Taken together, these results demonstrate that clonal relationship predicts functionality of neurons both across nuclei and within the same nucleus.

We also observed a clonal segregation between the FO and HO sensory- and motor-related nuclei across different modalities. Notably, previous studies have shown that thalamic nuclei of the same order across different modalities are composed of neurons with extremely similar axonal arborization architectures. Consistent with the anatomical connectivity difference, the FO and HO nuclei also show distinct properties in information processing. The distinct progenitor origins of neurons in the FO and HO nuclei probably contribute to their different morphologies, anatomical connections, and functional properties. In addition, we observed a lineal segregation of GABAergic interneurons in the TRN and LGN, which may have critical functional implications.

Limited intrathalamic connectivity has been found to structurally or functionally organize the complex thalamic nuclei. Our findings suggest that lineage-related development and organization provides a fundamental blueprint for the assembly and function of the thalamus. Consistent with this, the overall topography of thalamocortical axonal projections appears to be normal in the absence of any evoked synaptic transmission. Moreover, our observation of a strong lineage segregation of the sensory- and motor-related FO and HO nuclei across different modalities points to a model of the development of thalamic sensory and motor pathways in distinct phases. The developmental origin promotes the establishment of a generic hierarchical framework that is further specified into different modalities, likely dependent on distinctive inputs from the periphery and other brain regions. This is consistent with a recent study suggesting a cross-modal genetic framework for the development of sensory pathways in the thalamus. Additionally, changes in the periphery sensory input can also lead to changes in the functional subdivisions and the relevant corticothalamic connectivity of different thalamic nuclei, indicating a degree of rewiring and plasticity of thalamic organization.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.S. and S.-H.S. conceived the project; W.S. collected, reconstructed and analyzed MADM and retroviral labeling data with help from A.X.; Z.H., X.T., and K.H. performed NND analysis; Z.L. generated retrovirus; A.X., Z.H., and K.H. performed alignment and nuclear identity inference and clustering analysis with input from H.Z. and T.M.; H.Z. and T.M. provided thalamocortical axonal projection data; W.S. and S.-H.S. wrote the paper with input from all other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Animals and in utero intraventricular injection.** MADM11\textsuperscript{GT} (JAX Stock No. 013749) and MADM11\textsuperscript{TG} (JAX Stock No. 013751) mice were produced as previously described\textsuperscript{27}. MADM11, Nes-Cre\textsuperscript{ER\textsuperscript{2}} (ref. 26), R26-Smo\textsuperscript{M2-EYFP}\textsuperscript{53}, and Olig3-Cre\textsuperscript{28} mice were kindly provided by S. Hippenmeyer, R. Kageyama, A.L. Joyner, and Y. Nakagawa, respectively. Mice were bred and maintained according to guidelines established by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center. For MADM labeling, Nes-Cre\textsuperscript{ER\textsuperscript{2}}/\textsuperscript{TG} mice were crossed with MADM11\textsuperscript{GT}/\textsuperscript{TG} mice, and the time of pregnancy was determined by the presence of the vaginal plug (E0). For clone induction, pregnant females were injected intraperitoneally with tamoxifen (T5648, Sigma) dissolved in corn oil (C8267, Sigma) at E9, E10, E11, and the time of pregnancy was determined by the presence of the vaginal plug (E0). For clone induction, pregnant females were injected intraperitoneally with tamoxifen (T5648, Sigma) dissolved in corn oil (C8267, Sigma) at E9, E10, E11, and E12 at a dose of 5–25 µg/g body weight. In utero intraventricular injection of retrovirus was performed as previously described\textsuperscript{28}. In brief, uterine horns of retrovirus were injected into the third ventricle through a beveled, calibrated glass micropipette (Drummond Scientific). After injection, the uterus was placed back in the abdominal cavity and the wound was surgically sutured. After surgery, the animal was placed in a recovery incubator under close monitoring until it fully recovered.

**Serial sectioning, immunohistochemistry, and 3D reconstruction.** Both male and female mice were perfused intracardially with 4% paraformaldehyde (PFA) in PBS (pH 7.4). Brains were removed and post-fixed overnight at 4 °C. Serial coronal sections of individual brains were prepared using a vibratome or cryostat (Leica Microsystems) and subjected to immunohistochemistry. The following primary antibodies were used: chicken anti-GFP (1:1,000 dilution, GFP-1020, Aves Lab)\textsuperscript{51}, rabbit anti-RFP (1:1,000 dilution, 600–401–379, Rockland)\textsuperscript{31}, guinea pig anti-RFP (1:20,000 dilution, gift from J. Nicholas Betley, University of Pennsylvania), mouse anti-OLIG2 (1:200 dilution, MAB2456, RD Systems)\textsuperscript{32}, rabbit anti-BLB (1:200 dilution, ab12423, Abcam)\textsuperscript{32}, mouse anti-Ki-67 (1:500 dilution, 556003, BD Pharmingen)\textsuperscript{32} mouse anti-TUJ1 (1:500 dilution, MMS-435P, Covance)\textsuperscript{32}, mouse anti-nestin (1:500 dilution, rat-401, Developmental Studies Hybridoma Bank)\textsuperscript{25}, mouse anti-NeuN (1:500 dilution, MAB377, Millipore)\textsuperscript{31}, rabbit anti-S100 (1:500 dilution, Z0311, Dako)\textsuperscript{32}, rabbit anti-OLIG2 (1:500 dilution, AB9610, Millipore)\textsuperscript{32}, and mouse anti-PARVALBUMIN (1:500 dilution, MAB1572, Millipore)\textsuperscript{32}. Sections were mounted on glass slides, imaged using confocal microscopy (FV1000, Olympus or LSM700, Zeiss) and slide scanner (NanoZoomer 2.0-HT; Hamamatsu Photonics), and reconstructed using Neurolucida and StereoInvestigator (MBF Bioscience). Neurons, astrocytes, and oligodendrocytes were distinguished on the basis of their morphology (for example, nuclear size and neurite length) and marker expression.

For 3D reconstruction, each section was analyzed sequentially in anterior to posterior order. The boundaries of the entire thalamus as well as the landmark nuclei including MH, LH, LGN, TRN, and parts of MGN and VM were traced in each section and aligned. Individually labeled neurons were represented as colored dots (~4 times the size of the cell body at P21–P24 and ~2 times at E12), respectively. The anterior region of the thalamus was defined as the part before the anterior limit of cortical projections. A V, CM, IAD, IAM, IMD, MD, LD, PT, PVT, aRE, aRH, and aVM. For quantification of the order-versus-modality segregation, only clones with more than half of the neurons located in the well-characterized sensory- and motor-related nuclei (VPM, VPL, PO, dLG, vMG, VMG, AL, and pVM) were included in the analysis. Specifically, VAL and pVM are motor-related nuclei; VPM, VPL, and PO are somatosensory-related nuclei; dLG and LP are visual-related nuclei; vMG and dMG are auditory-related nuclei. Among them, VPM, VPL, dLG, VMG, VAL, and pVM are FO nuclei, and PO, LP, and dMG are HO nuclei.

Thalamic correlative axonal projection labeling and analysis was done as previously described\textsuperscript{26}. In brief, P14–P18 C57BL/6J mice were anesthetized and stabilized in a custom stereotaxic apparatus. Recombinant adeno-associated viruses expressing either EFGP or tdTomato were injected into the thalamus using a beveled sharp glass micropipette. Coordinates for injections ranged from: 0.5 to −1.6 mm anterior to posterior, 0.1–1.6 lateral, and 2.8–4.2 deep from the pia (in mm from bregma). Animals were perfused transcardially with PBS followed by 4% PFA 14 d after surgery. The brains were collected, post-fixed and sectioned coronally on a cryostat at 50 µm thickness. All sections were imaged on the NanoZoomer slide scanner (Hamamatsu) and Zeiss Axio Imager. A suite of custom algorithms using MATLAB (MathWorks) was developed to analyze and compare thalamic injections across animals. Each thalamus was manually traced and each injection site was marked, to generate the binary thalamic masks and injection masks. All thalamic masks were normalized, corrected for variability in cutting angle, and aligned together to obtain the averaged model thalamus. Then, each injection site was mapped onto the model thalamus. Finally, injection and target information for the injections were combined to localize the precise thalamic origin of the cortical projections.

**RNA in situ hybridization.** Cryostat sections (18 µm) of P4 brains were prepared and used for RNA in situ hybridization, as previously described\textsuperscript{56}. In brief, the primer sequences for the ROR\textalpha\textsuperscript{α} probe were obtained from Allen Brain Database. T7 or SP6 promoter sequences were added to the 5’ end of the reverse or forward primer sequences, respectively, which were then used for PCR amplification to generate the probe template. In vitro transcription of the resultant PCR product was performed to generate digoxigenin (DIG)-labeled antisense RNA probes. Hybridization was performed at 62 °C overnight, followed by post-hybridization washes. Sections were then incubated with alkaline phosphatase-coupled anti-DIG antibody (Roche, diluted 1:3,000) at 4 °C overnight. For the visualization of the reaction product, sections were incubated in the dark at room temperature with freshly prepared NTMT buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl\textsubscript{2}, 0.1% Tween-20, 0.5 mg/ml NaCl).
levamisole) containing NBT/BCIP (Roche). Once the color was fully developed, sections were washed, post-fixed with 4% PFA, dehydrated, and mounted with Permount (Fisher) for image acquisition using a slide scanner (NanoZoomer 2.0-HT, Hamamatsu Photonics).

Statistics. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications.24,51 Data collection and analysis were not randomized nor performed blind to the conditions of the experiments. No data points were excluded. Data are presented as median with interquartile range and whiskers as the minimum and maximum, or as mean ± s.e.m., and statistical differences were determined using nonparametric Mann–Whitney test, unpaired t-test with Welch’s correction (unequal variances), chi-square test, or linear regression analysis. Data distribution was not formally tested. Statistical significance was set at $P < 0.05$. A Supplementary Methods Checklist is available.

Data and code availability. The data and scripts that support the findings of this study are available from the corresponding author upon request.

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