Cellular and Axonal Constituents of Neocortical Molecular Layer Heterotopia

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Key Words

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Abstract

Human neocortical molecular layer heterotopia consist of aggregations of hundreds of neurons and glia in the molecular layer (layer I) and are indicative of neuronal migration defect. Despite having been associated with dyslexia, epilepsy, cobblestone lissencephaly, polymicrogyria, and Fukuyama muscular dystrophy, a complete understanding of the cellular and axonal constituents of molecular layer heterotopia is lacking. Using a mouse model, we identify diverse excitatory and inhibitory neurons as well as glia in heterotopia based on molecular profiles. Using immunocytochemistry, we identify diverse afferents in heterotopia from subcortical neuromodulatory centers. Finally, we document intracortical projections to/from heterotopia. These data are relevant toward understanding how heterotopia affect brain function in diverse neurodevelopmental disorders.

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Introduction

Dyslexia, epilepsy, cobblestone lissencephaly, polymicrogyria, and Fukuyama muscular dystrophy are very different neurodevelopmental disorders with highly dissimilar clinical manifestations. These diverse disorders surprisingly share one important neuroanatomical phenotype in common. Specifically, postmortem analyses of individuals affected by these disorders have revealed the presence of neocortical heterotopia consisting of aggregations of hundreds of neurons and glia in the molecular layer (layer I) that are indicative of neuronal migration defect [1–8]. Despite the potential clinical importance of understanding how heterotopia affect neocortical function in such disparate disorders, much remains to be revealed regarding the anatomy, physiology and etiology of heterotopia.

Spontaneous neocortical molecular layer heterotopia (MLH) are present in several inbred mice [9, 10], including C57BL/6 mice [11–13]. MLH have also been observed in several knockout (KO) mice [14–23]. MLH in mice are indistinguishable from heterotopia observed in humans. Consistent with a model of altered brain function associated with MLH, mice with heterotopia have impaired

learning of spatial and nonspatial memory tasks [24–28]. Mice with MLH also exhibit sensory processing deficits demonstrated by both behavioral measures in sensory discrimination tasks as well as with sensory-evoked potential recording [29–32]. Thus, findings in mice with MLH mimic the cognitive defects seen in humans with MLH. Furthermore, we have shown that mice with MLH also have lower seizure thresholds and shorter latency to seizures following chemo-convulsant treatment [33, 34], which mimic the brain excitability changes observed in epileptic humans with MLH. Thus, behavioral changes in mice with MLH closely resemble those observed in humans with MLH, demonstrating the utility of mice as a model of human MLH.

In the present report, we describe the cellular axonal anatomy of MLH in mice. We document the presence of astrocytes, oligodendrocytes and microglia in heterotopia. Furthermore, we identify the presence of serotonergic, cholinergic, catecholaminergic, and glutamatergic axons in heterotopia. These data are relevant toward an understanding of the mechanisms of neuronal migration and axonal targeting during normal and abnormal neocortical development.

Materials and Methods

We used C57BL/6J (stock No. 000664) and Thy1-yellow fluorescent protein mice (Thy1-YFP; H line; stock No. 003782 [35]) bred in academic vivaria from founders obtained from The Jackson Laboratory, as previously described [36, 37]. Laminin $\beta 2/\gamma 3$ double-KO mice were also examined, as well as laminin $\beta 2/\gamma 3$ double-KO mice crossed with Cx3Cr1/eGFP transgenic mice, as previously described [14]. The mice were housed in temperature-controlled facilities maintained between 20 and 22.5°C, with 12-hour light/dark cycles, in standard plastic cages (11.75 × 7.5 × 5 inches) with no more than 5 mice per cage, and with food and water available ad libitum. Mice of either sex aged between 14 and 90 days were used, since by 7 days of age all neocortical neuronal migration has ended. As previously described [13, 36], breeding schemes at New York Institute of Technology were mixed and included both sibling and nonsibling matings. All measures were taken to minimize pain or discomfort in the mice and experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23). All procedures were approved by the Institutional Animal Care and Use Committee at the New York Institute of Technology.

We followed histological methods identical to those described previously for mice [12, 36, 38]. Following perfusion and fixation (0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer), brains were cryopreserved in a 30% sucrose solution and then sectioned on a cryostat (40 μ m) and collected in 0.1 M PBS. Unstained sections were first screened with a dissection microscope and oblique illumination. Heterotopia and associated myelinated bundles are visible with this method [13]. Once heterotopia

were identified, sections were further processed for histochemistry or immunocytochemistry. Sections from 2–4 different brains containing heterotopia were examined for each stain described in the present report.

Immunocytochemistry was used to reveal the presence of different axonal phenotypes in heterotopia using methods previously described [12, 38]. Briefly, free-floating sections were collected into different wells and washed with PBS (3 times). The sections were permeabilized and blocked in 5% normal goat serum and 0.2% Triton X-100 for 1 h. They were then incubated in primary antibodies with 0.1% Triton X-100 and 2.5% normal goat serum in PBS at 4°C overnight. In the present study the following primary antibodies were used, as previously described [38]: serotonin transporter (5-HTT; 1:1,000; ImmunoStar, Hudson, Wisc., USA), tyrosine hydroxylase (TH; 1:1,000; Millipore, Billerica, Mass., USA), oligodendrocyte transcription factor 2 (Olig2; 1:1,000; Millipore), choline acetyltransferase (ChAT; 1:1,000; Millipore), glial fibrillary acidic protein (GFAP; 1:1,000; Sigma, St. Louis, Mo., USA) and ionized calcium-binding adapter molecule 1 (Iba1; 1:1,000; Wako, Richmond, Va., USA).

Following incubation in primary antibodies for use with bright-field microscopy, the sections were rinsed in PBS and then incubated in biotinylated secondary antibodies (goat anti-mouse, goat anti-rabbit; 1:200, Vector Labs, Burlingame, Calif., USA) for 2 h at room temperature. They were then rinsed in PBS and incubated for 1 h in an avidin-horseradish peroxidase mixture. Again, the sections were rinsed in PBS and then reacted with 0.05% diaminobenzidine/0.0015% H₂O₂. For epifluorescence imaging, Alexa 488- or 568-conjugated secondary antibodies were used (1:200; Life Technologies, Grand Island, N.Y., USA). Fluorescent counterstaining was performed with DAPI, propidium iodide or SYTOX Green (Life Technologies).

Stained sections were collected onto gelatin-coated slides and coverslipped. Digital photomicrographs were taken at varying magnification on an Olympus BX41 or Leica SP5 confocal microscope. Figures were prepared in Adobe Photoshop.

Virtual Data Collection from the Allen Brain Atlas

The Allen Brain Atlas (ABA; www.brain-map.org) was used to document malformations in C57BL/6J mice according to methods previously described [12, 36, 38, 39]. The ABA is a public database containing photomicrographs of brains from C57BL/6J mice (obtained from Jackson Laboratories) processed for in situ hybridization according to methods described by Lein et al. [40]. Serial coronal and/or sagittal cryostat sections are found in the ABA (25- μ m thickness) for each of over 20,000 genes.

We used the search features of the ABA to generate lists of genes/histological cases from which we searched for heterotopia. For example, the ABA has preset lists of genes/cases that show differential expression in particular regions of interest (including distinct layers of the neocortex) that are easily accessible at the 'Gene Search' homepage of the ABA (<http://mouse.brain-map.org/search/index>). We directly examined the cases found in the following preset lists under the 'Browse by Differential Expression' tab: cerebral cortex, layer 2–3; cerebral cortex, layer 4; cerebral cortex, layer 5, and cerebral cortex, layer 6a. For a search of genes in layer VIb neurons, we used the list found in the 'Fine Structure Search' tab for the search term 'layer 6B, isocortex' (http://mouse.brain-map.org/search/show?page_num=0&page_size=20&no_paging=false&search_term=16&search_type=fine_structure). We

also used published articles for layer-specific genes to include in our search [41–45]. For genes differentially expressed in different glial cell types, we used the list found in the study by Lein et al. [40]. A list of genes differentially expressed in GABAergic neurons was created based on the interneuron subtypes described by DeFelipe et al. [46].

From among thousands of images examined, a very small number of cases were archived that contained one of the following: (1) largely-unequivocal differential expression in a given neocortical layer or (2) expression in a given cell type. All of the cases meeting these criteria are either displayed in part of a figure or mentioned in the text with the corresponding ABA experiment number listed. The raw ABA data for each of the cases described/illustrated can be easily accessed by typing the experiment number on the ABA website search tab. Cases that did not have heterotopia were not recorded because we cannot be certain that heterotopia were not present, since not every section is stained and imaged for a given gene. In addition, we did not record cases where heterotopia were present but the staining pattern was either uninformative about cell type or where the expression pattern was not layer specific.

Having identified genes with cell type- or layer-specific expression, we examined additional material of the ABA, including the Developing Mouse Brain database (<http://developing-mouse.brain-map.org/>), which contains data from embryonic (E) and postnatal (P) C57BL/6J mice. Data from mice aged 18 and 24 months (M) are also present in this database. The Sleep (<http://sleep.alleninstitute.org/>), Mouse Connectivity (<http://connectivity.brain-map.org/>) and Mouse Diversity (<http://mousediversity.alleninstitute.org/>) databases found in the ABA were also examined in the present report. According to the technical white paper regarding the in situ hybridization and tissue processing from the ABA (<http://help.brain-map.org/download/attachments/2818169/ABADataProductionProcesses.pdf?version=1&modificationDate=1319477154403>), each brain was used in the production of approximately 6–8 cases/tissue series, which were used to hybridize for multiple genes or replicates of similar genes.

Photomicrographs containing the section with the largest extent of heterotopia were digitally archived. Representative photomicrographs taken from the ABA were imported into Photoshop for the preparation of figures.

Virtual Data Collection from the Mouse Brain Architecture Project and Mouse Connectome Project

Our laboratory has established that internet databases like the ABA [12] and the Mouse Brain Library [13] that feature C57BL/6 mouse brain histology can be used to determine the cell types present in MLH. With this in mind, we performed detailed searches through databases with histological data from neuronal tracer studies performed in C57BL/6 mice, including the following: (1) the Mouse Connectome Project (MCP, mouseconnectome.org), (2) the Mouse Brain Architecture Project (MBAP, brainarchitecture.org/mouse) and (3) the Mouse Connectivity database (connectivity.brain-map.org) of the ABA. In each of these databases, several dozen digital micrographs are available for each brain following unilateral neuronal tracer injection. Thus, thousands of digital micrographs from these databases were examined for the presence of MLH. Cases with MLH and labeled axons or neurons from anterograde and retrograde (respectively) tracing experiments were digitally archived.

Results

MLH Contain Glutamatergic Neurons from All Neocortical Lamina

With the continued addition of new data to the ABA, we were able to identify numerous neuronal classes in heterotopia not previously described. For example, as shown in figure 1, neurons expressing reprimo (*Rprm*; P56; ABA 78525701; fig. 1a, b), protein phosphatase 1, regulatory (inhibitor) subunit 1B (*Ppp1r1b*; P4; ABA 100055033; fig. 1c), and connective tissue growth factor (*Ctgf*; 18M; ABA 100098244; fig. 1d) were observed in MLH. *Ppp1r1b*-expressing neurons were not only found in heterotopia but also scattered throughout a number of other lamina. Given that the normal laminar profile of neurons expressing these genes is exclusive to layer VI, these data are indicative of defective migration among a limited number of glutamatergic neurons destined for layer VI. Furthermore, because cohorts of newly generated neurons migrate radially past earlier-born neurons during corticogenesis [47–49], these results indicate that even the earliest-born neocortical neurons are present in MLH.

ABA searches of tissue hybridized for genes found exclusively in layer V also indicated the presence of layer V neurons in MLH. For example, as shown in figure 1, we also observed neurons in MLH that express ets variant gene 1 (*Etv1*; P14; ABA 100026968; fig. 1e), fezf-family zinc-finger 2 (*Fezf2*; P28; ABA 100022558; fig. 1f) and *Ctip2* (also known as *Bcl11b*; P4; ABA 100055034; data not shown). In light of the fact that *Fezf2*-, *Ctip2*- and *Etv1*-expressing cells are known to send projections to the spinal cord [41, 42, 45], these data suggest that MLH contain neurons with subcortical projections.

Based on the above observations and our previous results that nearly all substrains of C57BL/6 mice have MLH [13], we predicted that Thy1-YFP transgenic mice would also have MLH, since these mice have been backcrossed onto a C57BL/6 background [35]. As described previously [35, 50], YFP expression in the neocortex of these mice is specific to thick-tufted layer V neurons with corticospinal projections. Not surprisingly, following examination of primary histological material generated in our laboratory, we observed MLH in these transgenic mice (with a prevalence of about 10%) and identified YFP-expressing cells in 100% of heterotopia as well as scattered in superficial lamina immediately below MLH (fig. 1g, h). Together with data from the ABA described above, these data demonstrate that layer V neurons with subcortical projections are found in MLH and that transgenic mice backcrossed onto a C57BL/6 background can exhibit MLH.

Fig. 1. Neurons born throughout corticogenesis are present in heterotopia. ISH = In situ hybridization. **a–d** Neurons belonging in layer VI are present in MLH. The Nissl-stained section in **a** is adjacent to the section shown in **b**. **e–h** Neurons belonging in layer V are present in MLH. Propidium iodide counterstaining is shown in **h** and is the same section shown in **g**. **i, j** Neurons belonging in

layer VI are present in MLH. The Nissl-stained section in **i** is adjacent to the section shown in **j**. **k, l** Neurons belonging in layer II–III are present in MLH. Arrows in **a–l** point to heterotopia. Data in **a–f** and **i–l** are from the ABA and all are from C57BL/6J mice. Data in **g** and **h** (Thy1-YFP tissue) are from primary histological samples from our laboratory. Scale bars: **a–f, i–l** = 421 μ m; **g, h** = 400 μ m.

Neurons expressing RAR-related orphan receptor- β (*Rorb*; P28; ABA 100039966; fig. 1i, j) were found in MLH as well as neurons expressing sperm-associated antigen 5 (*Spag5*; P56; ABA 72081516; fig. 1k) and inhibin- β -A (*Inhba*; P4; ABA 100056072; fig. 1l). We also observed *Satb2*-expressing neurons in heterotopia in P1 (ABA 100092087; data not shown) and P4 (ABA 100092539; data not shown) brains as well as *Nlk*-expressing neurons at P4 (ABA 100056055; data not shown). These observations demonstrate defective migration of neurons normally fated to be in layer II/III (e.g. *Inhba*, *Spag5*) and IV (e.g. *Rorb*), respectively. In light of previous reports that *Inhba*-expressing cells include neurons that make inter-hemispheric projections [44], these data suggest that MLH contain callosal neurons. Thus, MLH contain misplaced neurons that belong in all layers and therefore indicate a migration defect of neurons born during early (layer VI), mid (layers V, IV) and late (layers III, II) stages of corticogenesis.

MLH Contain Diverse GABAergic Interneuron Subtypes

Different neocortical GABAergic neuron subtypes are generated from progenitor cells located in different spatial locations of the embryonic forebrain. For example, GABA neurons expressing parvalbumin (*Pvalb*) are born in the ventral division of the medial ganglionic eminence, while those expressing somatostatin (*Sst*) are born in the dorsal division [51]. In contrast, GABA neurons expressing vaso-

active intestinal polypeptide (*Vip*) and calretinin (*Calr*) derive from progenitors located in the caudal ganglionic eminence [51]. Different subtypes of GABAergic interneurons also differ in morphological and electrophysiological features as well as their preferred synaptic targets [52]. For example, *Pvalb* interneurons have multipolar morphology, preferentially synapse on the soma and proximal dendrites of pyramidal cells and are capable of very high-frequency action potential discharge [53]. In addition, *Pvalb* neurons form electrical and chemical synapses onto other *Pvalb*-expressing cells [54, 55] and form chemical synapses on the soma and proximal dendrites of *Vip* neurons [56]. Based on these functional and developmental characteristics of GABAergic neurons, identifying different subtypes in MLH has important implications for understanding the morphological and physiological phenotypes as well as the developmental origins of GABAergic cells in heterotopia.

Three major subgroups of interneurons account for nearly 100% of neocortical GABAergic neurons in the mouse brain and can be distinguished by the following molecular markers: *Pvalb*, *Sst* and the serotonin 3a receptor (*5HT3aR* [52]). ABA searches revealed that numerous GABAergic subtypes are indeed present in MLH, including neurons expressing glutamic acid decarboxylase 1 (*Gad1*; P28; ABA 100039955; fig. 2a, b), *Vip* (P4; ABA 100074480; fig. 2c), *Pvalb* (P28; ABA 100039969; fig. 2d), and *5HT3aR* neurons (P56; ABA 74724760; fig. 2e). We also observed additional interneuron subclasses, including those expressing *Tac1* (P28; ABA 100056865; fig. 2f), *Erb4*

Fig. 2. Diverse GABAergic interneurons are present in heterotopia. ISH = In situ hybridization. **a–h** In situ hybridization of GABAergic interneuron subtypes in heterotopia. The Nissl-stained section in **a** is adjacent to the section shown in **b**. Arrows in **a–h** point to heterotopia. All data (**a–h**) are from the ABA and all are from C57BL/6J mice. Scale bars: **a–h** = 421 μm.

(P56; ABA 72472797; fig. 2g) and *Kcnc2* (P56; ABA 73512364; fig. 2h). In addition, we identified *Pdyn*-expressing cells (P28; ABA 100042562; data not shown) in heterotopia, which were recently shown to be a large subpopulation of *Sst* interneurons [57]. These data indicate that many, if not all, subtypes of GABA neurons migrate into MLH in mice, including those born in the caudal (*Vip*) and medial (*Pvalb*) ganglionic eminences [51]. These data also suggest that somatic (*Pvalb*) and dendritic (*Vip*) GABAergic synapses are present in heterotopia [52].

MLH Contain Diverse Glial Cell Types

Three major subtypes of glial cells found in the neocortex include astrocytes, oligodendrocytes, and microglia. We searched the ABA for genes exclusively found in astrocytes and confirmed that astrocytes are present in MLH. As shown in figure 3a–c, we found that cells expressing *Glast* (P28; ABA 100083582), *Gfap* (P56; ABA 79160968) and *Id3* (P56; ABA 74724763) were in MLH and none of these expression profiles indicated increased numbers of astrocytes associated with heterotopia. Moreover, when we performed immunostaining against GFAP (data not shown) on sections of C57BL/6J brains with MLH, we did not observe astrocytes with activated morphologies, indicating no reactive astrogliosis.

Myelinated axons have been previously identified in MLH [12, 13], indicating that oligodendrocytes are also present. Not surprisingly, we performed immunostaining against the oligodendrocyte-specific molecule *Olig2* [58–60] on sections of C57BL/6J brains with MLH and observed that oligodendrocytes are indeed present in MLH (data not shown). Moreover, as shown in figure 3d–f, ABA searches for oligodendrocyte-specific genes revealed the presence of cells expressing *Mbp* (18M; ABA 100082169), *Plp1* (P28; ABA 100039826) and *Gpr37* (P28; ABA 100039821) in heterotopia.

In order to identify microglia in heterotopia, we performed immunostaining on sections of C57BL/6J brains with MLH against *Iba1*, a microglia-specific marker which is particularly useful in determining the activation of microglia via changes in staining intensity, soma size and shape, and ramification of processes [61, 62]. *Iba1*-expressing microglia were present in heterotopia but microglial morphology did not indicate an activated phenotype (data not shown). In order to confirm these results, we examined primary histological material in our laboratory of brains of laminin $\beta 2/\gamma 3$ double-KO mice which develop numerous MLH throughout most of the neocortex [14]. Similar to our observations in C57BL/6J mice, we found that *Iba1*-expressing microglia were present in heterotopia in laminin $\beta 2/\gamma 3$ double-KO mice (data not shown). Moreover, as shown in figure 3g, when laminin $\beta 2/\gamma 3$ double-KO mice were crossed to reporter mice expressing enhanced GFP in microglia (Cx3Cr1/eGFP), numerous microglia were evident in heterotopia, though activated morphologies were not observed, suggesting that heterotopia are not associated with inflammatory or macrophagic responses. As shown in figure 3h and i, similar results were found following ABA searches for microglia-specific markers such as *CD11b/Itgam* (P14; ABA 100075158) and *C1qa* (18M; ABA 100082352).

Fig. 3. Diverse glial cell types are present in heterotopia. **a–c** Astrocytes are present in MLH. **d–f** Oligodendrocytes are present in MLH. **g–i** Microglia are present in MLH in laminin $\beta 2/\gamma 3$ -Cx3Cr1/eGFP triple transgenic mice. Arrows in **a–i** point to heterotopia. Nissl-stained sections in left-side panels of **a–f**, **h** and **i** are adjacent to sections shown in right-side panels. Propidium iodide counter-

staining shown in the left-side panel in **g** is the same section shown in the right-side panel. Data in **a–f**, **h** and **i** are from the ABA. Data in **g** are from primary histological samples from our laboratory. All data are from C57BL/6J mice except in **g–i**. Scale bars: **a**, **d**, **f**, **h**, **i** = 209 μ m; **b**, **e** = 262 μ m; **c** = 196 μ m; **g** = 125 μ m.

Taken together, these results indicate that all three glial cell types are present in MLH without apparent reactive astrocytes or activated microglia.

MLH Contain Diverse Axons from Subcortical Afferents

Identifying the afferent and efferent projections of MLH has remained elusive due to the fact that only a fraction of mice exhibit heterotopia [12, 13], and the location of MLH is not known a priori to microinjection of tracers. However, afferents from specific subcortical nuclei to MLH can be identified by immunostaining for specific neurotransmitter phenotypes, as we have previously described for projections to cerebellar malformations [38]. As shown in figure 4, following the examination of primary histological material generated in our laboratory, we identified a dense plexus of axons expressing the serotonin transporter (5HTT) in MLH in C57BL/6J mice. The density of labeled axons within heterotopia appeared largely similar to the density present in adjacent regions of the molecular layer. Moreover, 5HTT-labeled axons were not found to form bundles immediately below heterotopia (fig. 4e, f), as we have observed previously for myelinated axons [12, 13]. These data indicate that serotonergic neurons project to neurons in heterotopia.

ChAT and TH immunostaining were also performed to determine whether cholinergic and catecholaminergic axons are in MLH, respectively. As shown in figures 5 and 6 (respectively), following the examination of primary histological material generated in our laboratory of C57BL/6J mouse brains with heterotopia, a dense plexus of ChAT- and TH-expressing axons was also observed in heterotopia. However, neither axon type was found to form bundles immediately below the heterotopia, as has been observed for myelinated axons [12, 13]. ChAT-expressing interneurons were also found in heterotopia. These data indicate that diverse axonal afferents from several subcortical nuclei are present in MLH.

MLH Contain Callosal Neurons, Callosal Axons and Local Intracortical Axons

We identified the Mouse Connectivity database, MCP and MBAP as three online databases we could use to further examine the afferent and efferent projections to heterotopia in C57BL/6J mice. Results from detailed searches of the data on the MCP and MBAP are only reported here as these two databases have Nissl counterstaining which allowed us to unequivocally identify MLH. We did indeed identify cases with heterotopia in both the MCP and MBAP; however, only a small fraction of cases had anterograde or retrograde labeling. This is to be expected

Fig. 4. Serotonergic axons project to heterotopia. **a** Low magnification of the propidium iodide-stained section with visible heterotopia (arrows). **b, c** High magnification of heterotopia shown in **a**, revealing neurons (**b**) and a dense plexus of 5HTT immunoreactive axons (**c**). **d** Merged image of **b** and **c**. Arrowheads in **b** and **c** point to pial surface. **e, f** High magnification of the region shown in **a** by arrowheads, indicating that 5HTT fibers are not part of the axon bundle associated with MLH. All data (**a–f**) are from primary histological samples from our laboratory and all are from C57BL/6J mice. Scale bars: **a** = 220 μ m; **b–f** = 35.5 μ m.

Color version available online

Fig. 5. Cholinergic axons project to heterotopia. **a** Low magnification of the propidium iodide-stained section with visible heterotopia (arrows). **b** Same section as shown in **a** with visible ChAT-labeled axons in the heterotopia and ChAT-labeled neurons immediately beneath the heterotopia. **c** Higher magnification of the section shown in **b**. **d** Example of ChAT-labeled neurons and axons present in heterotopia. All data (**a–d**) are from primary histological samples from our laboratory and all are from C57BL/6J mice. Scale bars: **a, b** = 200 μ m; **c** = 66 μ m; **d** = 50 μ m.

Color version available online

as the presence or location of MLH are not known a priori to tracer injection.

A total of 85 cases found in the MCP were rigorously screened for MLH based on the locations of tracer injections made into regions where MLH are most commonly found, including the frontal, motor, and somatosensory cortices. From this group, 18 cases (21%) were identified containing MLH. Only 2 cases were found that contained any tracer labeling; however, the labeling present in these

cases did indicate that heterotopia send and receive afferent and efferent (respectively) callosal projections. This small number of cases is expected given that the location of heterotopia are not known before tracer injection. Figure 7 contains photomicrographs from a case (SW110210-03A) where the retrograde tracer, cholera toxin β -subunit (Ctb), was injected unilaterally into the primary motor cortex. As shown in figure 7a–d (arrows), a heterotopia was identified contralateral to the Ctb in-

Fig. 6. Catecholaminergic axons project to heterotopia. **a** Low magnification of the TH-stained (**a**) and propidium iodide-stained (**inset**) section with visible heterotopia (arrows). Arrowhead points to the unstained region corresponding to the myelinated axon bundle. **b, c** High magnification of heterotopia shown in **a**. **d** High magnification of the unstained axon bundle region in **a** demonstrating sparse TH-labeled axons. All data (**a–d**) are from primary histological samples from our laboratory and all are from C57BL/6J mice. Scale bars: **a, inset** = 800 μm ; **b** = 80 μm ; **c, d** = 160 μm .

jection site and retrogradely labeled neurons were present in the malformation (fig. 7d, arrowhead and inset). In this same case, the anterograde tracer, *Phaseolus vulgaris* leucoagglutinin (PhaL), was injected unilaterally into the same site as Ctb. As shown in figure 7e–h (arrows), anterogradely labeled axons were present in the heterotopia (fig. 7h, arrowhead and inset).

A total of 380 cases found in the MBAP were rigorously screened for MLH based on the locations of tracer injections made into regions where MLH are most commonly found, including the frontal, motor and somatosensory cortices. From this group, 27 cases (7%) were identified containing MLH and 11 had labeling in heterotopia, which was indicative of callosal or local innervation into heterotopia. Figure 8 shows 2 representative cases of ipsilateral anterograde labeling in heterotopia. In the first example (fig. 8a–d), a heterotopia (fig. 8a, b, black arrow) can be seen lateral to a biotinylated dextran amine (BDA) injection site (fig. 8a, white arrow) found in the ipsilateral cingulate cortex along the same rostrocaudal plane. Higher magnification of the heterotopia (fig. 8c, d), reveals BDA-labeled axons present within, suggesting that axons from ipsilateral/medial sites innervate the heterotopia. In the second example (fig. 8e–i), a heterotopia (fig. 8e, f, arrow) can be seen ipsilateral and rostral to the injection tracer site (fig. 8g, arrow). Higher magnification of the heterotopia (fig. 8h, i) reveals labeled ax-

ons present within, suggesting that axons from ipsilateral/caudal sites innervate the heterotopia.

Figure 8 also shows 2 representative cases of callosal axons in heterotopia. In the first example (fig. 8j–n), a heterotopia (fig. 8j, k, black arrow) can be seen rostral to a BDA injection site (fig. 8l, arrow) found in the contralateral somatosensory cortex at a more caudal position. Higher magnification of the heterotopia (fig. 8m, n) reveals BDA-labeled axons present within, suggesting that axons from contralateral/caudal sites innervate the MLH. In the second example (fig. 8o–s), a heterotopia (fig. 8o, p, arrow) can be seen rostral to the tracer injection site (fig. 8q, arrow) found in the contralateral somatosensory cortex at a more caudal position. Higher magnification of the heterotopia (fig. 8r, s) reveals BDA-labeled axons present within, suggesting that axons from contralateral/caudal sites innervate the MLH.

Together, data from the MCP and MBAP demonstrate that callosal afferent and efferent projections are associated with MLH.

Discussion

Despite having been associated with dyslexia, epilepsy, cobblestone lissencephaly, polymicrogyria, and Fukuyama muscular dystrophy [1–8], a complete understand-

Fig. 7. Data found in the MCP indicate that MLH send and receive contralateral projections. **a, b** Injection of Ctb into the motor cortex (arrowhead in **b**) results in retrogradely labeled neurons in the heterotopia found in the contralateral hemisphere (arrows in **a, b**). **a** Nissl-stained adjacent section to that shown in **b**. **c, d** High magnification of the heterotopia shown in **a** and **b**, respectively. Arrowhead in **d** points to individual labeled neurons. **Inset** in **d** shows higher magnification of retrogradely labeled neurons in MLH. **e, f** Injection of PhaL into the motor cortex (arrowhead in **f**) results in anterogradely labeled axons in the heterotopia found in the contralateral hemisphere (arrows in **e, f**). **e** Nissl-stained adjacent section to that shown in **f**. **g, h** High magnification of the heterotopia shown in **e** and **f**, respectively. **Inset** in **h** shows higher magnification of labeled axons. All data (**a–h**) are from MCP and all are from C57BL/6J mice. Scale bars: **a, b, e, f** = 1,000 μ m; **c, d, g, h** = 15 μ m.

ing of the cellular and axonal constituents of MLH is lacking. In the present report we further document the cellular and axonal anatomy of MLH in mice, as a tool toward understanding MLH in humans.

Using the ABA, we show that excitatory neurons from all neocortical layers are present in MLH, suggesting that neurons born during all periods of corticogenesis are present in heterotopia. These data are consistent with the observation of glutamatergic postsynaptic potentials recorded from neurons in MLH [33]. In addition, we demonstrate that numerous GABAergic neuron subtypes are present in MLH, including those born in the caudal (*Vip*) and medial (*Pvalb*) ganglionic eminence as well as those making somatic and dendritic inhibitory synapses. These data are consistent with the observation of GABAergic postsynaptic potentials recorded from neurons in MLH [33]. Taken together, these data indicate that there exists a diverse complement of neurons in MLH and suggest that these neurons may participate in a synaptic network that contributes to the cortical dysfunction manifested by patients with MLH. Consistent with this hypothesis, mice with MLH have impaired learning of spatial and nonspatial memory tasks [24–28], sensory processing deficits [29–32] and increased seizure susceptibility [34]. Future studies should be aimed at characterizing the synaptic circuitry and physiological profile of neurons in C57BL/6J mice with MLH, toward gaining a better understanding of how heterotopia cause cortical dysfunction.

Radial glial fibers are disrupted in areas of developing MLH [11]. Surprisingly, an analysis of adult mice with MLH did not reveal significant changes in glial cell distribution or morphology. In particular, neither reactive astrocytes nor activated microglia were identified by GFAP or Iba1 immunostaining. These observations were confirmed with data from the ABA as well as in laminin β 2/ γ 3 double-KO mice that exhibit numerous and large MLH throughout most of the neocortex [14]. Consistent with earlier findings of myelinated axons in MLH [12, 13], we identified oligodendrocytes in MLH using immunostaining for Olig2 as well as data from the ABA. Taken together, these data demonstrate that all three subtypes of glia are present in MLH. An important caveat of our findings is that only a limited number of ages/time-points were examined for any given glial cell marker, leaving open the possibility that changes in glial cell number or activity occur in older ages than those examined.

Using immunocytochemistry for neurotransmitter-specific phenotypes, we identified ChAT, 5HTT and TH-labeled axons in heterotopia. We propose that cholinergic axons in the MLH likely arise from basal forebrain nuclei [63, 64], while it is likely that those expressing 5HTT originate in the median raphe [65]. The catecholaminergic fibers that were present probably arise from noradrenergic neurons in the locus coeruleus [66, 67] and dopami-

Fig. 8. Data found in the MBAP indicate that MLH receive ipsilateral and contralateral projections. **a** Injection of BDA into the cingulate cortex (white arrow in **a**) results in labeled axons in the adjacent heterotopia (black arrow in **a**). **b** Nissl-stained adjacent section to that shown in **a**. **c, d** High magnification of the heterotopia shown in **a** and **b**, respectively. **e, g** The heterotopia in the frontal motor cortex (arrows in **e, f**) contains anterogradely labeled axons after injection in the ipsilateral motor cortex (arrow in **g**) at a more caudal level. **f** Nissl-stained adjacent section to that shown in **e**. **h, i** High magnification of the heterotopia shown in **e** and **f**, respectively. **j–l** The heterotopia in the frontal motor cortex (ar-

rows in **j, k**) contains anterogradely labeled axons after injection in the contralateral somatosensory cortex (arrow in **l**) at a more caudal level. **k** Nissl-stained adjacent section to that shown in **j**. **m, n** High magnification of the heterotopia shown in **j** and **k**, respectively. **o–q** The heterotopia in the motor cortex (arrows in **o, p**) contains anterogradely labeled axons after injection in the contralateral somatosensory cortex (arrow in **q**) at a more caudal level. **p** Nissl-stained adjacent section to that shown in **j**. **r, s** High magnification of the heterotopia shown in **o** and **p**, respectively. All data (**a–s**) are from MBAP and all are from C57BL/6J mice. Scale bars: **a, b, e–g, j–l, o–q** = 1,000 μ m; **c, d, h, i, m, n, r, s** = 100 μ m.

nergic neurons of the ventral tegmental area [68, 69]. Though definitive electron microscopic data confirming synaptic contacts within MLH are lacking, these data suggest that axons from subcortical neuromodulatory centers innervate neurons in MLH.

In the present report, we identify cortical afferent and efferent projections of MLH, which include local ipsilateral afferents, callosal afferents, and callosal efferents. Exactly how these connections participate in the cortical dysfunction associated with heterotopia (learning and memory deficits, increased seizure susceptibility) is not

known. However, due to the excitatory/glutamatergic profile of these circuits, epileptiform activity initiated by heterotopic neurons could propagate via one or more of these pathways. There are likely to be additional circuitry changes present in the 'normatopic' cortex which remain unidentified but that participate in the cortical dysfunction associated with MLH. Furthermore, an important caveat to our axonal immunohistochemical data and the virtual tracer data is that a limited age range of brains was examined and afferent/efferent connections associated with MLH could change over time.

We believe that most, if not all, of the molecular markers demonstrated above would be present in most, if not all, heterotopia. First, YFP-labeled neurons were found in 100% of heterotopia in Thy1-YFP mice with MLH, suggesting that there are always layer V neurons in heterotopia. Second, we show multiple layer-specific molecular markers (layer II–III, IV, V, VI), which supports our observations in Thy1-YFP mice. In addition, we show 7 different molecular markers for GABAergic neurons which are found in heterotopia. Given the fact that *Gad1* is found in all of the interneuron subtypes shown, this would argue strongly for the fact that 100% of heterotopia will have some complement of GABAergic neurons. We also demonstrate several examples of glial cell markers with largely overlapping expression profiles within a given glial cell type (astrocyte, oligodendrocytes, etc.), lending support to our interpretation of data for layer-specific neurons and GABAergic subtypes in heterotopia. Third, our immunocytochemical studies examining axonal afferents (cholinergic, serotonergic, catecholaminergic) in heterotopia yielded similar staining profiles in multiple brains with heterotopia examined. Fourth, we have previously shown that MLH are associated with myelinated axon bundles across different substrains of C57BL/6 mice and in both males and females [13], suggesting that the axonal constituents described above are also likely to be present in most heterotopia. Taken together, these data suggest that the cellular and axonal constituents of MLH are consistent and that it is likely that each of the given cell types illustrated above would be present in most, if not all, heterotopias.

MLH almost identical to that observed in C57BL/6J mice are found in numerous transgenic and KO mice. In transgenic mice generated on a C57BL/6J background or backcrossed onto this strain, we believe that MLH formation results from the C57BL/6J genotype, as we have shown with Thy1-YFP mice in the present report, and in BXD and AXB recombinant inbred mice, which we demonstrated previously [13]. Unpublished results from an ongoing study using the ABA Transgenic Characterization database (<http://connectivity.brain-map.org/transgenic>) also indicate that Cre-Lox transgenic mice on a C57BL/6J (or congenic) background exhibit MLH, which is consistent with the finding that Cre-Lox transgenic mice can exhibit malformations of the posterior cerebellum [70].

Although heterotopia can be significantly larger and more prevalent in mutant and KO mice, we believe that heterotopia in mutant/KO mice share many similarities to MLH seen in C57BL/6J mice. First, the general cytoarchi-

tectonic profile of heterotopia in several KOs is strikingly similar, with the characteristic feature of cells in the molecular layer. Second, there is evidence that the cellular constituents of heterotopia in mutant/KO mice are similar to those seen in MLH in C57BL/6J mice. For example, in the following mutant/KO mice: *Fak* [22], *Gpr56* [15], *Col3a1* [71], *Dag1* [72] and *Foxc1* [73], heterotopia were found to contain layer V neurons that express CTIP2 and/or ER81, which is similar to our findings in C57BL/6J mice. Moreover, heterotopia in *Zic1/3* mutants [74] were found to contain GAD-expressing neurons similar to our observations. Third, in NXSMD/EiJ mice, which are a recombinant inbred line derived from NZB/BinJ and SM/J progenitors, heterotopia contains oligodendrocytes, GFAP-expressing astrocytes and several GABAergic neuron subtypes, including PVALB-expressing neurons [75], which is also similar to our observations in C57BL/6J mice. Fourth, preliminary analysis of layer-specific neuronal expression in MLH found in Cre-Lox transgenic mice (ABA Transgenic Characterization database) confirms our observation of *Etv1* (P56; ABA 100126208)-expressing cells and *Pvalb* (P147; ABA 100144576)-expressing cells in heterotopia. Taken together, these data indicate that heterotopia in KO, mutant and transgenic mice share a similar cellular composition to that seen in C57BL/6J heterotopia.

In the present report we describe the cellular and axonal composition of MLH in C57BL/6J mice, placing MLH among the most anatomically well-described neocortical malformations in a rodent model. Nevertheless, to date, little is known regarding the underlying molecular mechanisms that cause heterotopia or exactly how the cells and circuits associated with heterotopia result in cortical dysfunction. This is an important area for future investigation. More broadly, our results demonstrate the importance of careful consideration as to how MLH may affect anatomical, physiological and behavioral studies using C57BL/6J mice. Transgenic and KO mice generated on a C57BL/6J background presenting with a heterotopia phenotype must also be carefully evaluated in light of the known prevalence of MLH in C57BL/6J mice.

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