Brainstem projections to molecular layer heterotopia of the cerebellar vermis: Evidence from the Allen Mouse Brain Connectivity Database

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Abstract

Molecular layer heterotopia of the cerebellar vermis are a characteristic feature of C57BL/6 mice. Heterotopia consist of neurons and glia in the molecular layers between folia VIII and IX in regions lacking pia. Previously, we described the cellular composition of heterotopia which includes granule cells, Purkinje cells, Golgi cells, etc. However, the axonal constituents and afferent connections of these malformations remain poorly understood. In the present report axonal projections to heterotopia are documented from diverse brainstem nuclei such as the spinal vestibular nucleus, dorsal cochlear nucleus, and nucleus prepositus. These findings are relevant toward understanding the mechanisms of normal and abnormal cerebellar development and the establishment of cerebellar circuits.

Introduction

Neurodevelopmental defects of the human cerebellum can affect posture, balance, movement kinematics, and motor learning. Compared to the cerebellar hemispheres, the vermis is particularly vulnerable to malformation, hypoplasia, or agenesis, and reduced vermis size has been observed in children with autism spectrum disorders [1] as well as Fragile X syndrome [8]. Thus, understanding the normal and abnormal development of the cerebellar vermis has important clinical implications.

We recently described a malformation of cerebellar development affecting the posterior vermis in C57BL/6 mice, which may serve as a model of human cerebellar malformations [3]. Cerebellar molecular layer heterotopia (MLH) in this strain are characterized by collections of neurons and glia in the molecular layer between folia VIII and IX, in regions lacking pia. The majority of cells in MLH were Calb2- and Zic1-expressing granule cells suggestive of migration defect by these cells exiting the external granule cell layer during early postnatal periods (Mangaru 3]. Consistent with a model of neuronal migration defect as the cause
of molecular layer heterotopia (MLH), Glast-expressing Bergmann glia in heterotopia exhibit morphological abnormalities with radial fibers that fail to reach the pial surface or with fibers that inappropriately cross into the adjacent molecular layer [3, 4].

Using immunocytochemistry and Thy1-yellow fluorescent protein (Thy1-YFP) transgenic mice which also have MLH, we previously demonstrated that brainstem glutamatergic, cholinergic, serotonergic, and catecholaminergic neurons project to MLH [5]. However, the exact nuclei supplying these afferent projections remained unknown. In the present report, we identify afferents to MLH from diverse brainstem nuclei including the spinal vestibular nucleus, dorsal cochlear nucleus, nucleus prepositus, and paragigantocellular nucleus. Our results provide greater knowledge of the axonal afferents to MLH of the posterior vermis and have important implications toward our understanding of normal cerebellar development as well as cerebellar disorders with defective neuronal migration and altered lamination.

Material and Methods

C57BL/6 mice contain both cerebellar and neocortical malformations [3-8]. In light of the fact that the Allen Brain Atlas (ABA) contains data from this same strain, our laboratory has exploited this powerful and freely-available internet resource to identify of cellular constituents of cerebellar MLH [3, 5-6] as well as neocortical malformations [8]. Using an approach similar to that described previously, we used the ABA *Mouse Connectivity* database to identify the brainstem projections to cerebellar MLH.

The *Mouse Connectivity* database (MCD), contains high-resolution serial sections of C57BL/6 mouse brains following unilateral anterograde tracer injection. This database was created as part of a broader project to map neural connections in the mouse brain. A smaller number of cases are also present in the MCD of tracer-injected Cre transgenic mice. Note that we recently demonstrated that transgenic mice on a C57BL/6 background also have cerebellar MLH [6].

Details of the mice, tracers (biotinylated dextran amine; recombinant adeno-associated virus), stereotaxic injection procedure/sites, brain preparation for histology (intracardia perfusion), high-resolution imaging (serial two-photon tomagraphy), and data annotation/processing can be found on the ABA website

(http://help.brainmap.org/display/mouseconnectivity/Documentation). Generating the kind of data found in the MCD in most laboratories would be extremely challenging for several reasons. First, injection in a large number of mice is necessary because the presence of MLH in any given mouse is not known *a priori*. Second, the number of brainstem nuclei with known cerebellar projections is numerous thereby further increasing the number of injections and mice needed. Finally, the high throughput tissue processing facilities and high-resolution photomicroscopy and digital archiving available at the AIBS is not found at most institutions. Thus, our use of the MCD represents a novel *virtual* approach to describing neuronal connections present in a mouse model of human cerebellar malformation.

Search tools available on the MCD website were used to identify histological cases where injections of tracer were made into brainstem nuclei known to project to the cerebellar vermis. In particular, cases were identified according to injection site into brainstem nuclei using the *"Source Search"* and *"Target Search"* tools of the MCD. Digital photomicrographs were uploaded, and the presence or absence of a cerebellar MLH was identified. Next sections were rigorously reviewed for the presence of axons labeled by the anterograde tracer. In this report data is presented only from cases where 1] a heterotopia was identified and 2] labeled axons were evident. Figures were prepared in Abode Photoshop and reflect the rostro-caudal sections corresponding to those shown in Figure 1.

Results

The MCD contains >1000 cases of photomicrographs of serial-sections from tracerinjected mice in nearly all parts of the brain. Furthermore, each case contains >100 digital photomicrographs encompassing the entire rostral-caudal extent of the brain. From this large database cases were examined where tracer injection had been made into brainstem nuclei known to have cerebellar projections such as the cochlear nuclei, abducens nucleus, cuniform nucleus, dorsal raphe nucleus, facial motor nucleus, gracilis nucleus, gigantocellular reticular nucleus, paragigantocellular nucleus, hypoglossal nucleus, inferior colliculus, inferior olive, tegmental reticular nuclei, nucleus prepositus, vestibular nuclei, sensory nucleus of the trigeminal, and spinal nucleus of the trigeminal. A complete list of all the cases found in the MCD can be found on the ABA website (http://help.brainmap.org/download/attachments/2818171/InjectionSites_and_StereotaxicCoordinates.pdf?version=1&modificationDate=1370996355068). Data described below come from more than one thousand photomicrographs examined.

From among the many cases found in the MCD where injections were localized to brainstem nuclei, labeled axons present in heterotopia could be documented in several cases. Table 1 provides a detailed list of all of the cases and injection targets where labeled axons where observed in heterotopia. Representative examples of cases identified are also shown in Figures 2-5. These data confirm previous observation of immuno-labeled and YFP-labeled axons in MLH but add important additional specificity of the nuclei contributing axonal afferents. The following text describes in more detail some of the observations of labeled projections present in heterotopia.

The vestibular group of nuclei of the brainstem play an important role in postural control and oculomotor reflexes. Vestibular nuclei projections to the cerebellar vermis have also been extensively studied and are characterized by mossy fiber-type projections targeting the granule cell layer [9-14]. The MCD contains numerous cases of injections targeting vestibular nuclei. As

shown in Figure 2A-C, following injection into the spinal vestibular nucleus (SVN), labeled axons were visible in the granule cell layers of both folia VIII and IX in a case without heterotopia and with clear/intact pial borders. However, labeled axons in a malformation were also observed in a case in the MCD following injection in the SVN. As shown in Figure 2D-F, both axonal fibers and large terminal swellings/rosettes were clear among heterotopic neurons in the molecular layer in a region lacking pia. No axons were observed in the molecular layer adjacent to the malformations. These data indicate that neurons from the SVN project to cerebellar MLH.

The cochlear group of nuclei constituent an important part of the auditory brainstem system which is critical for sound localization/processing and has been implicated in hearing disorders such as tinnitus [15]. Projections from the dorsal cochlear nuclei (DCN) in particular, have been demonstrated after retrograde tracer injection into the posterior vermis [16]. As shown in Figure 3A-C, anterogradely-labeled axons present in the granule cell layers of folia VIII and IX were identified in a normal case in the MCD where an injection was made into the DCN. As shown in Figure 3D-F, a malformation was also identified in a corticotrophin releasing hormone Cre transgenic mouse (Crh-IRES-Cre (BL) that was injected into the DCN with a virus specifically infecting Cre-expressing neurons (Table 1). Labeled axons and terminal swellings/rosettes were evident in the cell-dense region of the malformation in a region clearly lacking pia separating folia VIII and IX. These data indicate that corticotrophin releasing hormone neurons from the dorsal cochlear nucleus project to MLH.

The paragigantocellular reticular nucleus (PGN) is found at the ventral base of the medulla and plays an important part role in the central regulation of the sympathetic autonomic function. The projections from the PGN to the cerebellar vermis in the rat were described by [17 Newman & Ginsbuerg] and include connections to the posterior vermis. As shown in Figure 4AC, following injection into the PGN, labeled axons were visible in the granule cell layers of both folia VIII and IX in a case without heterotopia and with clear/intact pial borders. However, labeled axons in a malformation were also observed in a case in the MCD following injection in the PGN. As shown in Figure 4D-F, both axonal fibers and large terminal swellings/rosettes were clear among heterotopic neurons in the molecular layer in a region lacking pia. These data indicate that PGN neurons project to cerebellar heterotopia.

The pontine serotonergic nuclei modulate the function of diverse brain regions and have been implicated in sleep and mood disorders such as depression [18]. The nuclei supplying serotonergic axons to the cerebellar have been previously described [19]. As shown in Figure 5A-C, following injection into the dorsal raphe nucleus (DRN) of a serotonin transporter Cre transgenic mouse (Slc6a4-Cre_ET33), labeled axons were visible in the granule cell layers of both folia VIII and IX in a case without heterotopia and with clear/intact pial borders. However, labeled axons in a malformation were also observed in a case in the MCD following injection in the DRN of a different serotonin transporter Cre transgenic mouse (Slc6a4-CreERT2_EZ13). As shown in Figure 4D-F, sparse and thin axonal fibers and putative terminals were clear among heterotopic neurons in the molecular layer in a region lacking pia. These data indicate that serotonergic axons from the DRN project to heterotopia.

Nucleus prepositus (NPP) is made up of a column of neurons in the medulla adjacent to the hypoglossal and abducens nuclei which plays an important role in oculomotor behavior [20 McCrea]. Projections from the NPP to the cerebellum including the posterior vermis have been extensively described in numerous species using neuronal tracers [21-26]. As shown in Figure 6A-C, anterogradely-labeled axons present in MLH were identified in a case in the MCD where

an injection was made into the NPP. In particular, labeled axons could be seen traversing from folia VIII to folia IX through a region lacking pia. Given that NPP neurons with cerebellar projections have been previously shown to express choline acetyltransferase [20, 24], these data indicate that cholinergic neurons of the NPP project to heterotopic neurons in cerebellar vermis malformations.

The main goal of the present study was to identify brainstem projections to cerebellar heterotopia. However, one case was observed were an injection was made directly into the cerebellum containing a malformation. As shown in Figure 6D-F, following tracer injection into granule cell layer of folia VIII, numerous parallel fibers were labeled in the molecular layer. Remarkably, labeled axons from folia VIII could be observed entering folia IX though a breach in the pia between the two folia. Labeled axons entering folia IX were restricted to the molecular layer. These data demonstrate that cerebellar malformations are associated heterotopic parallel fiber projections from granule cells from adjacent folia.

Discussion

MLH of the cerebellar vermis are found in C57BL/6 mice and emerge from disruption of pial development and impaired migration of granule cell neurons [3-6]. In addition to granule cells, heterotopia consist of several other neuronal classes (such as Golgi cells, Purkinje cells, and GABAergic interneurons) as well as glial cell-types (astrocytes, oligodendrocytes, microglia) [3-4]. Using immunocytochemistry and Thy1-YFP transgenic mice, we previously demonstrated that diverse axonal classes are present in heterotopia, however, the exact origins of these projections were unknown [5].

In the present report, brainstem projections to heterotopia were identified using a novel *virtual* approach. By searching the MCD, projections from the DCN, PGN, NPP, and SVN were identified in heterotopia. Despite not having electron microscopic evidence, these data suggest that potentially all brainstem nuclei with cerebellar projections send axons into heterotopia. Exactly how developing brainstem axons target heterotopic granule cells in malformations is an important subject for future research and has implications for understanding the development of synaptic connections in the cerebellum.

Previously, we demonstrated that axons expressing the serotonin transporter are found in heterotopia using immunocytochemistry [5]. In the present report GFP-labeled axons were present in heterotopia after injection into the DRN in a Cre serotonin transporter transgenic mouse (Slc6a4-CreERT2_EZ13) where virus infection only occurs in serotonin transporter expressing neurons. These observations confirm the presence of serotonergic axons in heterotopia and now reveal the source of these afferents which include the DRN. Previously, we also demonstrated that axons expressing choline acetyl-transferase (ChAT) are found in heterotopia using immunocytochemistry [5]. In the present report GFP-labeled axons were present in heterotopia after injection into the NPP which is known to contain ChAT-expressing neurons with cerebellar projections [27]. These data confirm the presence of cholinergic axons in heterotopia and now reveal the source of these afferent which include the NPP. Axons in heterotopia were observed after virus injection into the DCN in a corticotrophin releasing hormone-Cre transgenic mouse indicating that corticotrophin releasing hormone is another neurochemical present in heterotopia together with those we previously described including glutamate, GABA, catecholamines, acetylcholine, and serotonin.

Axons from brainstem nuclei were present in heterotopia including those involved in diverse sensory-motor functions. In light of the presence of axons from the DCN, SVN, SNT, etc, heterotopia in C57BL/6 mice may be associated with deficits in auditory, vestibular, and somatosensory processing. Exactly how heterotopia affect cerebellar function and possibly manifest in behavioral changes of sensorimotor integration, postural control, motor learning, etc, remains an open question. One prediction is that mice with heterotopia might be impaired on behavioral tests such as the balance beam task [28] or the ladder rung task [29] – a finding that would also shed new light on the importance of folia VIII and IX on cerebellar control of sensorimotor function. Thus, when considering that C57BL/6 mice also have neocortical malformations affecting sensory and motor cortices [7-8], MLH of the cerebellar vermis may affect interpretation of results of a wide range of behavioral studies where sensory and motor function is involved in task performance.

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Table 1. Table of the cases identified in the MCD where GFP labeled axons were observed in the vermis of unaffected mice and mice with heterotopia.

Case ID	Primary Injection Site	Vermis Anatomy	Mouse Strain	Virus used for tracing
146794439	Dorsal cochlear nucleus	Normal	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
167213641	Dorsal cochlear nucleus	Heterotopia	Crh-IRES-Cre (BL)	rAAV2/1.CAG.FLEX.EGFP.WPRE.bGH
128055110	Dorsal raphe nucleus	Normal	Slc6a4-Cre_ET33	rAAV2/1.CAG.FLEX.EGFP.WPRE.bGH
114155190	Dorsal raphe nucleus	Heterotopia	Slc6a4-CreERT2_EZ13	rAAV2/1.CAG.FLEX.EGFP.WPRE.bGH
127908173	Nucleus prepositus	Heterotopia	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
175142304	Paragigantocellular reticular nucleus	Normal	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
181058463	Paragigantocellular reticular nucleus	Heterotopia	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
127042540	Paragigantocellular reticular nucleus	Heterotopia	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
125831616	Pyramus, cerebellar folia VIII	Heterotopia	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
129567943	Spinal vestibular nucleus	Normal	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH
128056535	Spinal vestibular nucleus	Heterotopia	C57BL/6J	rAAV2/1.hSynapsin.EGFP.WPRE.bGH

Figure Legends

Figure 1. A, C, E, Nissl-stained sequential sections of a C57BL/6 mouse cut along the coronal plane. Folia VIII and IX are visible without heterotopia and with clear pial boundaries between these folia. B, D, F, High magnification of region in A, C, E highlighted by arrows. G, Nissl-stained section with a visible heterotopion between folia VIII and IX. H, High magnification of region in G highlighted by an arrow. Section in G-H is at a similar rostral-caudal position as in A. Calibration in microns: A, C, E = 2010; B, D, F = 419; G = 1049; F = 420. Abbreviations: ML = molecular layer; GCL = granule cell layer. All data from the Allen Brain Atlas.

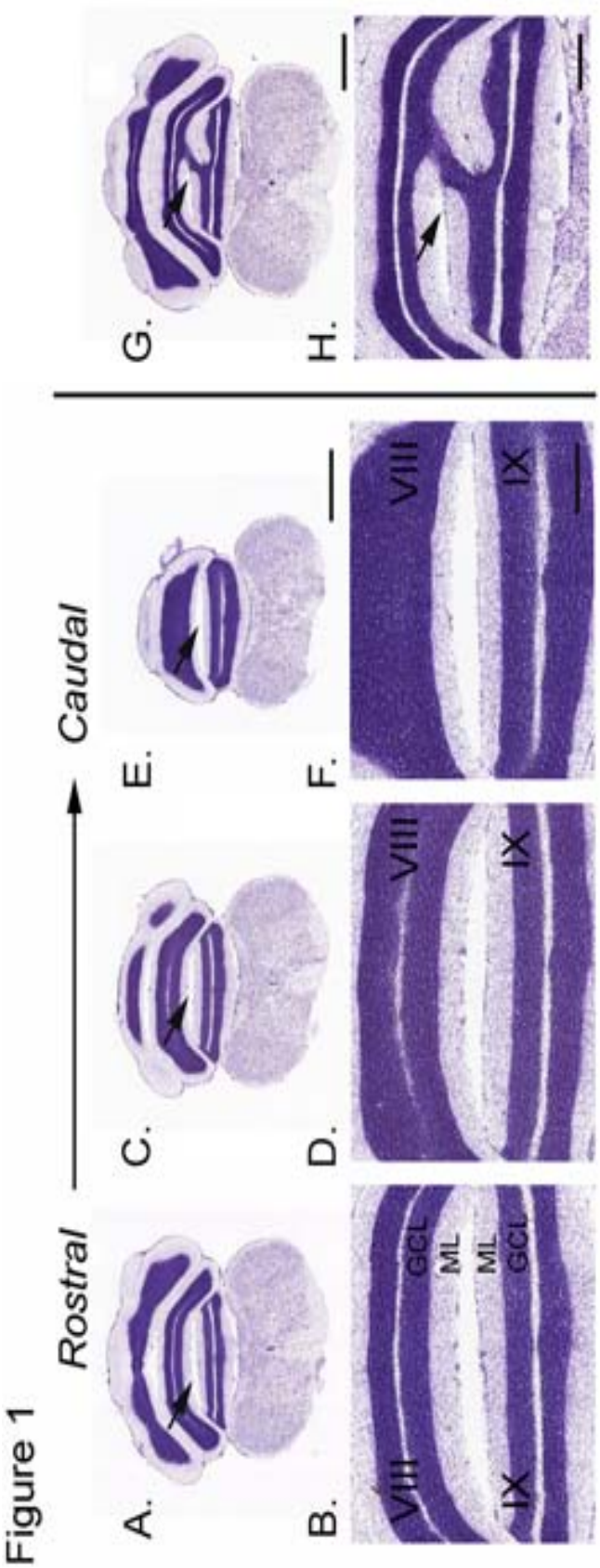
Figure 2. A-C, Sequential sections demonstrating GFP-labeled axons in the normal folia VIII and IX after virus injection in the spinal vestibular nucleus. D-F, Sequential sections demonstrating GFP-labeled axons in a malformation between folia VIII and IX (arrow) after virus injection in the spinal vestibular nucleus. Calibration in microns: A-F = 140. Abbreviations: ML = molecular layer; GCL = granule cell layer. All data from the Allen Brain Atlas.

Figure 3. A-C, Sequential sections demonstrating GFP-labeled axons in the normal folia VIII and IX after virus injection in the dorsal cochlear nucleus. D-F, Sequential sections demonstrating GFP-labeled axons in a malformation between folia VIII and IX (arrow) after virus injection in the dorsal cochlear nucleus. Calibration in microns: A-F = 140. Abbreviations: ML = molecular layer; GCL = granule cell layer. All data from the Allen Brain Atlas.

Figure 4. A-C, Sequential sections demonstrating GFP-labeled axons in the normal folia VIII and IX after virus injection in the paragigantocellular reticular nucleus. D-F, Sequential sections demonstrating GFP-labeled axons in a malformation between folia VIII and IX (arrow) after virus injection in the paragigantocellular reticular nucleus. Calibration in microns: A-F = 140. Abbreviations: ML = molecular layer; GCL = granule cell layer. All data from the Allen Brain Atlas.

Figure 5. A-C, Sequential sections demonstrating GFP-labeled axons in the normal folia VIII and IX after virus injection in the dorsal raphe nucleus. D-F, Sequential sections demonstrating GFP-labeled axons in a malformation between folia VIII and IX (arrow) after virus injection in the dorsal raphe nucleus. Calibration in microns: A-F = 140. Abbreviations: ML = molecular layer; GCL = granule cell layer.

Figure 6. A-C, Sequential sections demonstrating GFP-labeled axons in a malformation between folia VIII and IX after virus injection in the nucleus prepositus. D-F, Sequential sections demonstrating GFP-labeled axons in folia IX (arrow) after virus injection in folia VIII nucleus. Axons course through a breach in the pia. Calibration in microns: A-F = 140. Abbreviations: ML = molecular layer; GCL = granule cell layer. All data from the Allen Brain Atlas.



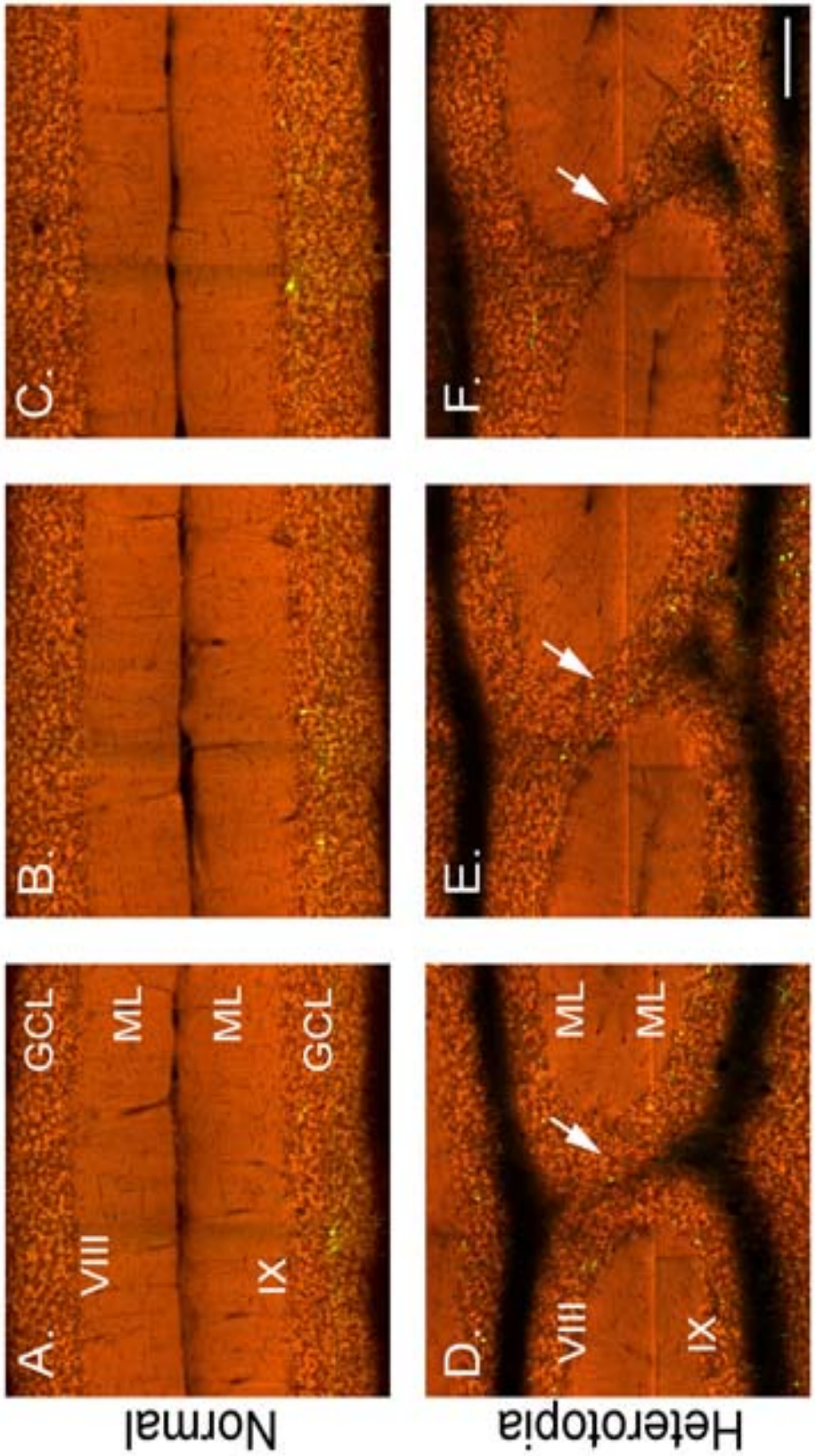


FIGURE 2.

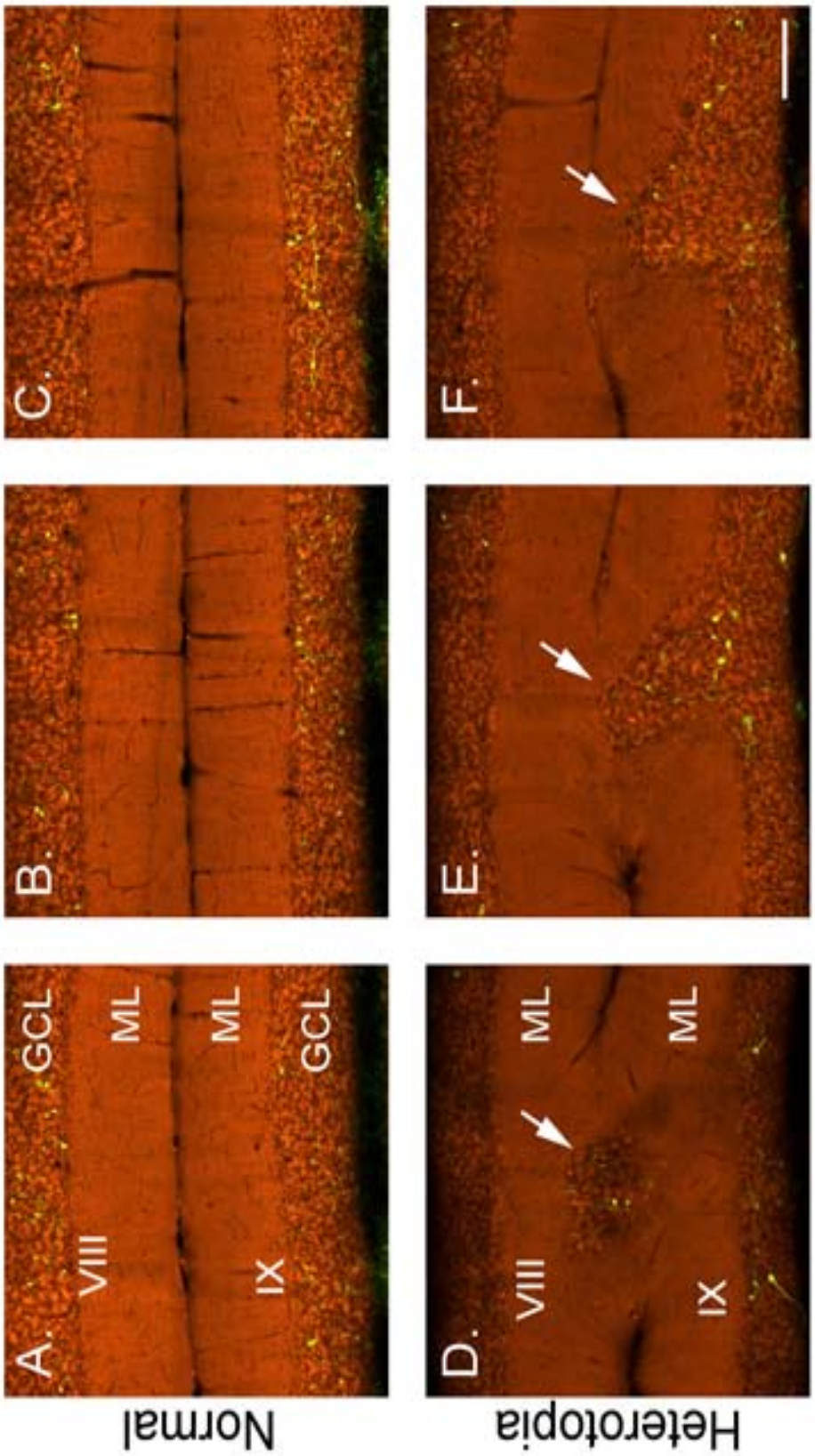


FIGURE 3.

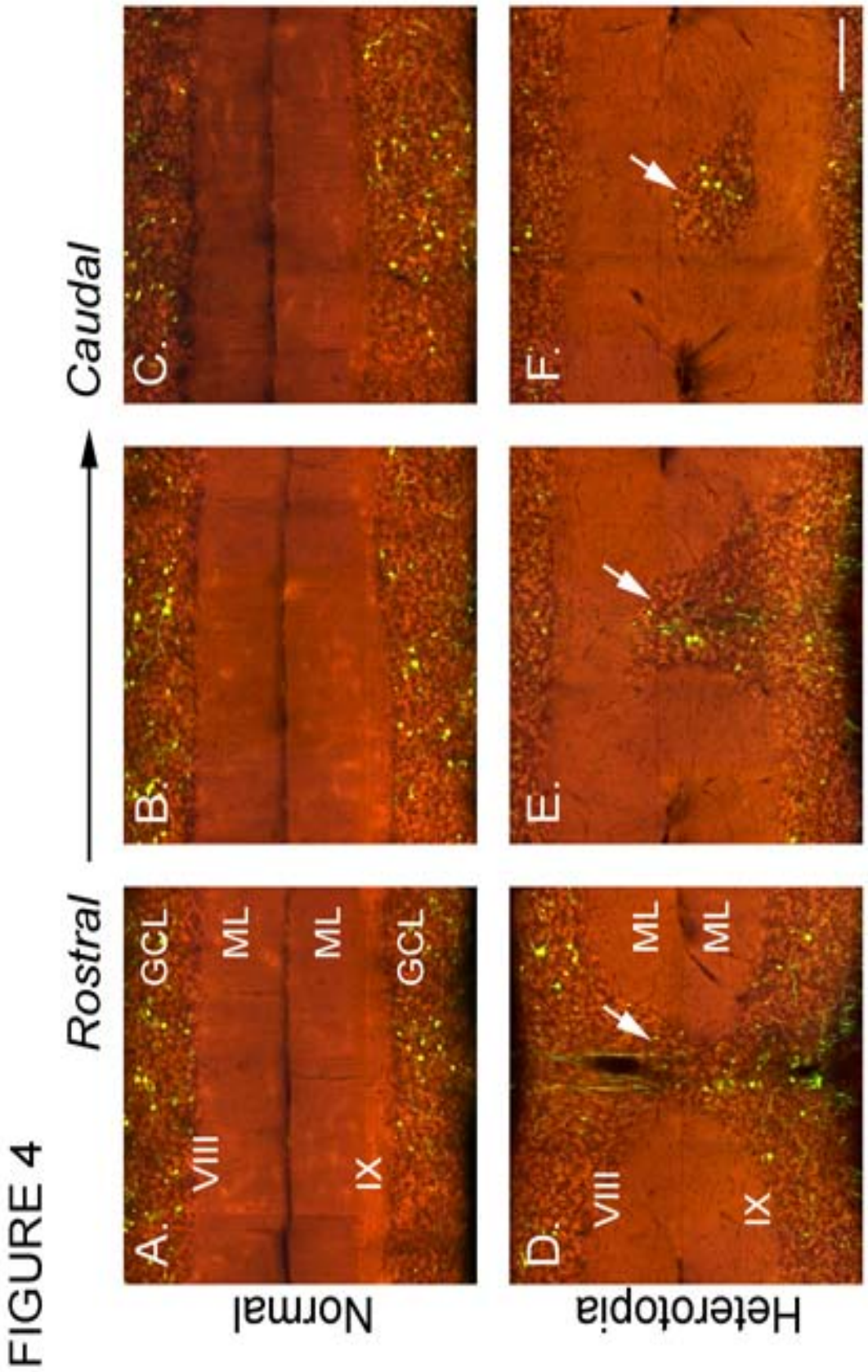


FIGURE 4

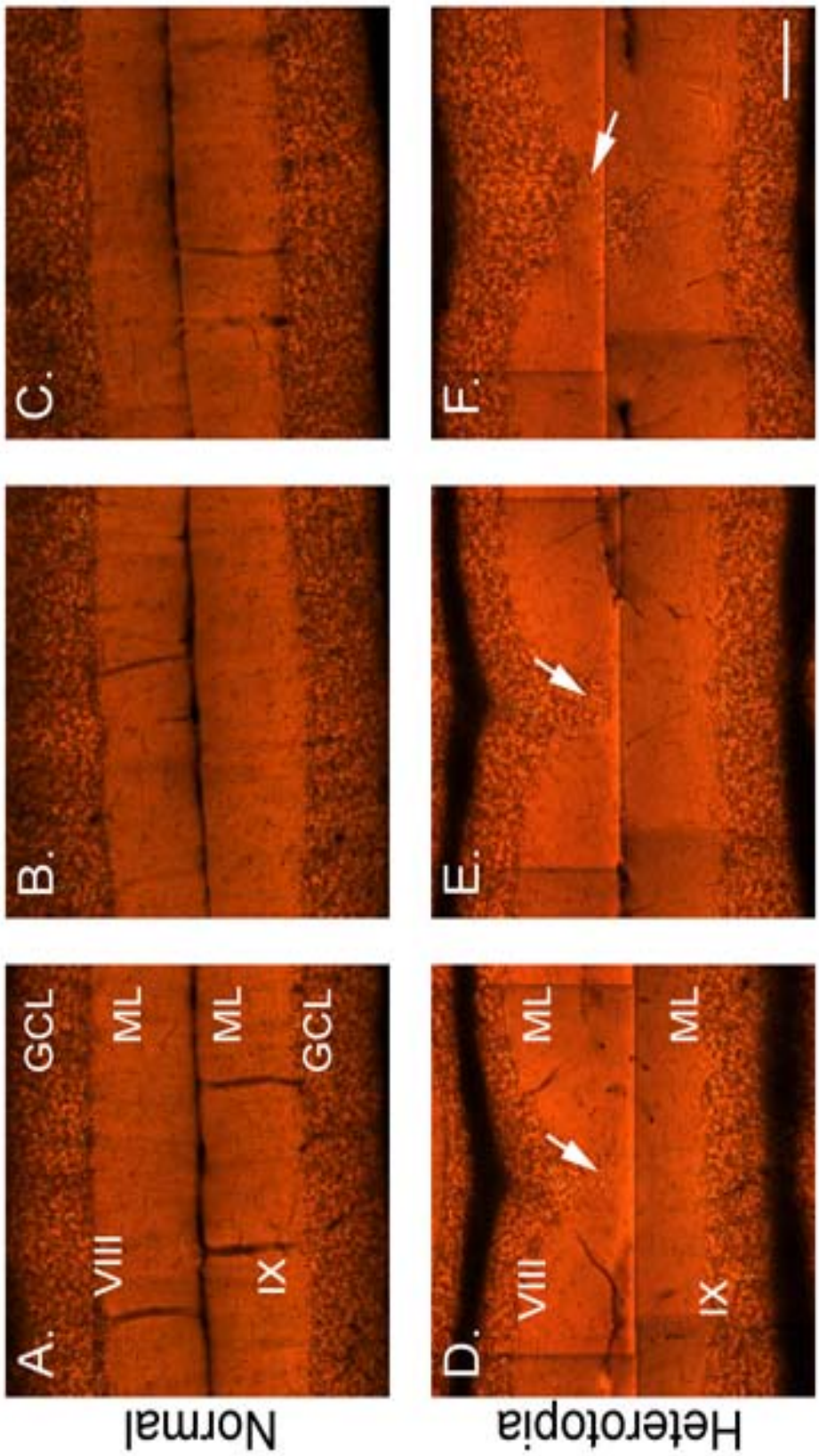


FIGURE 5.

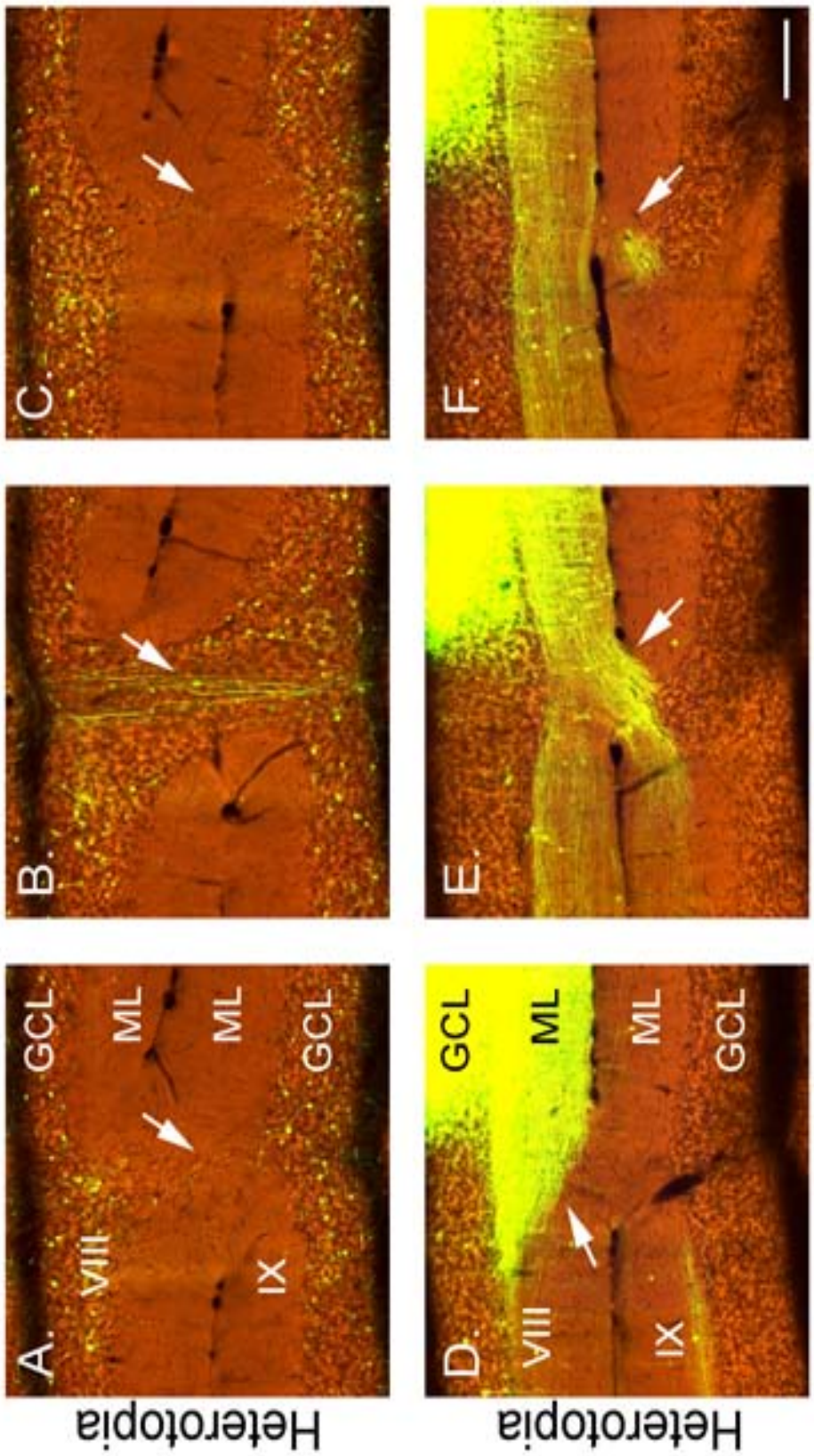


FIGURE 6