Lipophilic Tracer DiI Used for Neuronal Tracing in the Fixed Hippocampal Formations of Mice

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Abstract

Both the hippocampus and the entorhinal cortex are involved in memory formation. It is thought that they work together to create a loop that is involved in the formation of long term memories. The entorhinal cortex sends projections to the CA1 field of the hippocampus and the CA1 field sends projections back to the entorhinal cortex. How exactly they communicate is unknown. In order to do electrophysiological studies it is necessary to know in which orientation to cut the brain to preserve the most connections between these two areas. Here we show that DiI crystal application can be used to successfully label known fiber paths from the dentate gyrus to the CA3 field. Then we begin with sagittal and horizontal slices of the brain to see how well connections between the CA1 field and the EC are preserved.

Key words: hippocampus, entorhinal cortex, memory, fiber paths

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Introduction

Lesion studies, EEG studies fMRI studies, and electrophysiology studies indicate that the medial temporal lobe (MTL) plays a large role in learning and memory (Johnston, 1998; O'Keefe, 1979; Eldridge, 2000; Buzsaki, 1989; Eichenbaum, 2007). There is some debate as to how different types of memory are created within certain regions of the MTL.

It is understood that the hippocampus, part of the MTL, plays a central role in forming new memories. The observation of patient H.M., who suffered from epilespy in his MTL, best exemplifies the importance of the hippocampus in forming new memories. Due to the severity of the epilepsy, his hippocampi were surgically removed, which damaged the entohinal cortex (EC). Following the surgery, H.M. experienced anterograde amnesia, he could no longer store any new long term memories. However, the rest of his cognitive abilities stayed intact. He still had full control over his working (short-term) and procedural memories (Johnston, 1998). The results of the H.M. study have been replicated in other studies in which hippocampal damage also results in anterograde amnesia (Johnston, 1998; Zola-Morgan, 1986).

Alzheimer's disease, a disease in which people are unable to make new memories, has been found to target the hippocampus and the EC (Johnston, 1998). It is hypothesized that the hippocampus becomes functionally disconnected from the rest of the brain in Alzheimer's disease, perhaps suggesting the importance of the EC as a relay center and a connective intermediate (Hyman, 1984).

Previous studies in rats have shown that a major source of input to the hippocampus is from the cortical layer of the EC through the perforant pathway (PP) and that outputs of the hippocampal areas CA1 and subiculum project to the EC. This means that there are many major connections traveling from the EC to the hippocampus and then many connections from the hippocampus to the EC (Johnston, 1998; van Groen, 2003). This connectivity is thought to contribute to processing of sensory information. Neurons in layer II of the EC comprise the PP and terminate in the dentate gyrus and CA3 region of the hippocampus, and neurons in layer III project to CA1 and the subiculum. Axons from CA1 and the subiculum project back to the EC, returning to the original cortical area that relayed the information to the EC (Johnston, 1998; Witter, 2000; de Curtis, 1991). One study in rats proposed that dye injection in CA1 resulted in terminal fibers labeled in layers V-VI of the EC (Naber, 2001). These loops are thought to be essential for the formation of long-term memories (Johnston, 1998; Witter, 2000; de Curtis, 1991).

Although the mouse brain is similar to the well-characterized rat brain, there are some differences (van Groen, 2002). In order to further explore the formations and circuitry in mice, we intend to label neurons from the EC to the DG and CA3, as well projections

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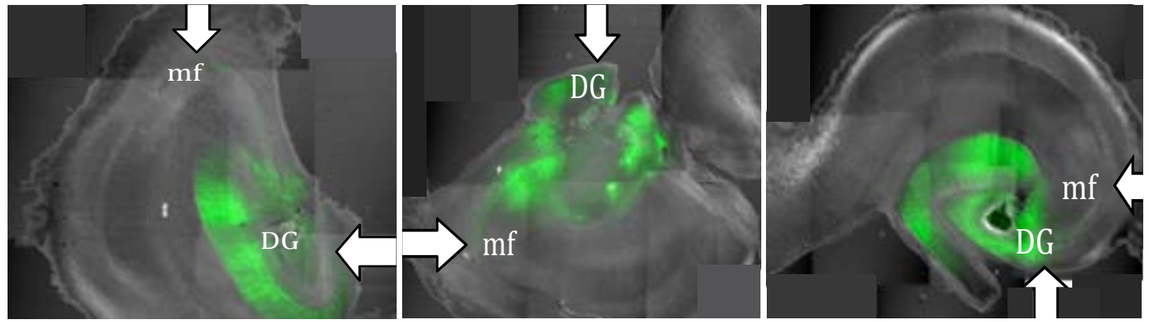

Figure 1A **Figure 1B** **Figure 1C**

Figure 1 *1A, 1B, and 1C are all transverse slices stained with a DiI crystal, imaged under a confocal microscope, and stitched together with Adobe Photoshop. The arrows point to the DG and the mossy fibers traveling up to CA3. The slices in 1A and 1B had dye applied with the needle syringe method and were incubated for three days at 30 degrees C and then placed at 4 degrees C until being mounted. The slice in 1C had dye applied with microfil tip method and was incubated at 30 degree C for 2 days and then placed in a 4 degree C room until mounting.*

from the EC to CA1 and then back to the EC. Knowing how this connectivity is preserved in in vitro mouse models (fixed brain slices) will allow for the development of a protocol for future electrophysiological experiments on the connectivity between the entorhinal cortex and hippocampus. These studies require acute, unfixed slices that contain preserved loops between the EC and hippocampus, so understanding how to slice the brain to preserve connections is essential.

To inspect the connectivity in the hippocampus and EC specifically, we used the lipophilic tracer DiI. It was first introduced in 1987 by Godement et al. as a new fluorescent tracer. It was originally used to label neurons in fixed slices from mouse brains and chicken embryos (Godement, 1987). DiI is an especially reliable dye since it has no discernable effect on the survival of neurons and causes them to fluorescence for long periods of time (Honig, 1985; Vindal-Sanz, 1985).

It seems that, DiI has not previously been used to label circuitry in the hippocampal formation of mice. A similar experiment in Japan at the Fukui Medical School examined the entorhinal-hippocamus-entorhinal circuit of rats by means of in vivo injections of DiI into the rhinal sulcus. This study proposes that the CA1 projection, rather than the subicular projection, is the main projection that feeds back information from the hippocampus to the enthorhinal cortex. Their data support the idea that the connections between the subiculum, CA1 field, and EC is reciprocal and that the entorhinal input to the CA1 field is fed back to the same cortical column directly through the CA1 field or by the subiculum (Tamamaki, 2003).

In this study, we show that the application of DiI crystals can be used to successfully label known fiber paths from the DG to the CA3 field. We also show that orienting the slices sagitally and horizontally is useful for demonstrating how well connections between the CA1 field and the EC are preserved. These findings have great potential to benefit future studies of brain circuitry and neuronal pathways.

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Materials and Methods

Obtaining Brain Samples

Adult male mice were anesthetized with a mix of ketamine (40 mg/ml) and xylosine (2.8 mg/ml), 50 microliters/10g of mouse. A pump was used to deliver 25 ml of filtered PBS and then 20 to 25 ml of 4% PFA to the heart and blood vessels. The excised brain was fixed for a minimum of one night in 15 to 20 ml of 4% PFA and kept at 4°C.

A series of washes were carried out to clear each brain of PFA. First a 10 minute wash with 0.1M glycine and then 3x10 minute washes with filtered PBS.

Sectioning the Brain

A Vibratome Series 1000 sectioning system was used to slice the brain in the orientation of choice. The brain was sliced in several different orientations: dissected hippocampi were sectioned transversely along the temporal axis, whole brains were cut coronally, sagittally, and horizontally. The first slices were transverse slices of only the hippocampus 400 microns thick. Later, sagittal slices of the entire brain were sectioned, at 50, 200 and 300, and 400 microns thick. Horizontal and coronal sections were cut at a thickness of 400 microns.

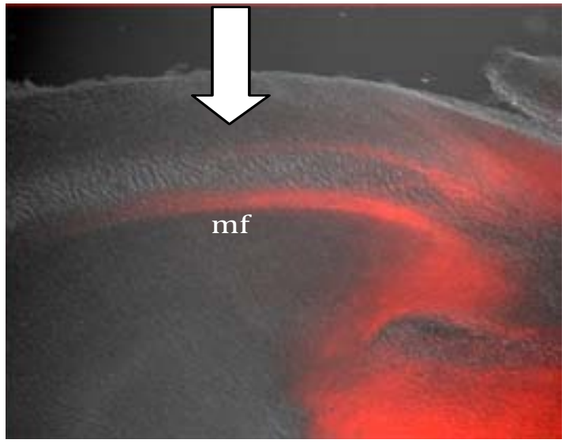

Figure 2 *This is a section of a transverse slice. The mossy fibers from the dentate gyrus (DG) clearly project to the CA3 field. The arrow points to the mossy fibers. The DiI crystals were applied using the syringe needle method. This slice was incubated for 4 days at 30 degrees C and then placed at 4 degrees until being mounted.*

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Application of the Dye

A Wild Heerbrugg stereo-dissection microscope was used in the application of the DiI to the DG in the transverse slices and CA1 in the sagittal slices and to the CA1 in whole brain mounts. After the dye was applied, these slices were incubated at 30°C for a period between 2 and 4 days and then placed in at 4°C before mounting. In whole mounts, the brain was coronally cut from the rostral side until -2.30 mm rostral from Bregma.17 Then under the dissection microscope, DiI was applied to the CA1 region of one side of the brain. The brain was then fixed to a silicon plate, which was glued down to the bottom of a beaker. The beaker was filled with PBS with 0.1% azide and incubated at 30 °C for 1-2 weeks. After incubation the brain was sectioned at 50 or 400 microns and then placed in a 4°C room before mounting.

Methods for dye application previously have not been well developed. The first method we applied was to use the tips of two syringe needles to push the DiI into the slice. One needle was used to pick up the crystal from the dye jar and the other was used to slide the crystal off the first needle and onto the slice. Then one or both of the needles were used to push the crystal underneath the surface of the slice.

A second method employed was to use the tip of an eyelash to apply the dye. Two small hairbrushes were made, consisting of a single eyelash applied to the end of a glass tube with superglue.

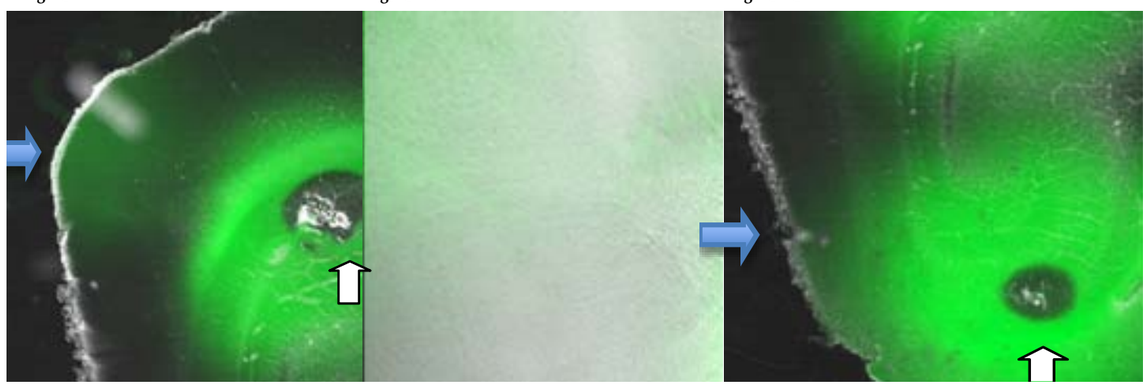
The last and most successful method was to use the tips of microfils, which are nonmetalic syringe needles usually used to fill micropipettes, to apply the dye. The microfils were cut down so that they were approximately 1 cm away from their base and then they were attached to syringes to make them easier to use.

Imaging the Slices

The slices were mounted under coverslips onto microscope slides with mounting media. Images were obtained using the Ziess LSM 700 scanning confocal microscope with 1.25x, 5x, 10x, and 20x objectives. Slices were imaged in sections, and these sections were then pieced together using Image J and Adobe Photoshop software.

Figure 3 *These are images of a 200 mircon sagittal slice with two DiI crystals. Figure 3A is an image of the top of the slice. The white arrow is where the DiI crystal was placed in CA1 and the blue arrow point to labeling in the top of CEnt. Figure 3B is a close up of the connection between the two areas. Figure 3C is the lower half of the slice and a white arrow shows where the DiI crystal was placed and the blue arrow points to the CEnt.*

Figure 2A Figure 2B Figure 2C



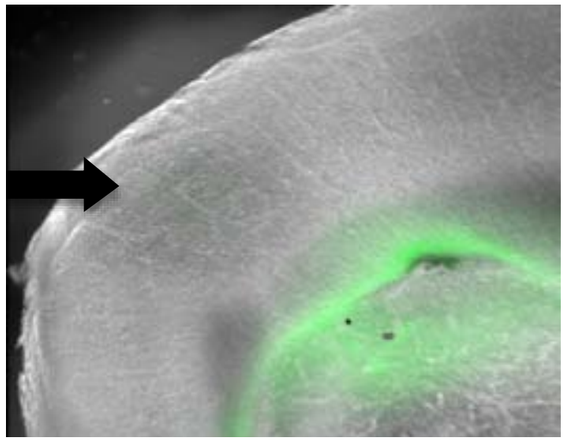


Figure 4A

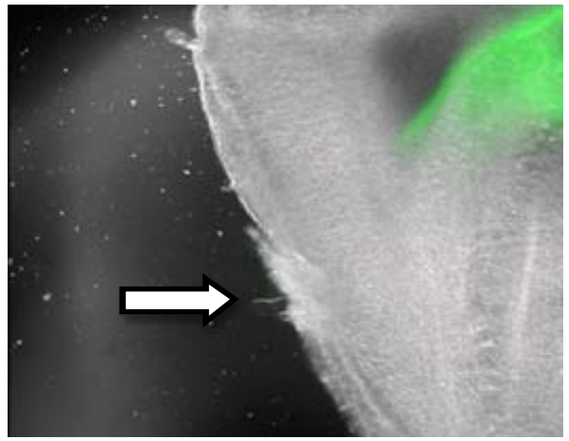


Figure 4B

Figure 4 *These images are of 400 micron thick sagittal slices and show labeling in the CEnt. Figure 4A shows labeling in the upper region of the CEnt and figure 4B shows labeling farther down in the CEnt. Both labeling is light and a little hard to see, but it is clear under the microscope. The arrows point to where the dye is labeled in the CEnt.*

New Method

The final method of labeling the circuitry was stereotaxically to inject the live mouse in the CA1 field with a lentivirus expressing GFP, at +/-1.9 (x), -2.2 (y), -1.5 (z1), -1.3 (z2) at least two weeks before being perfused and stored in 4% PFA. These slices were cut at 50 mm.

Results

The mossy fibers are clear and well known projections that arise from the DG and run to the CA3 field. Two of the three dye application methods successfully labeled these projections in transverse slices of the fixed mouse hippocampus. The first method used syringe needles and the second method used the microfil tips. Both application of the dye and preservation of the slice were easier with the second method, but either method would successfully stain the mossy fibers, as long as the crystal was successfully submerged in the dentate gyrus under the surface of the slice.

Figures 1A-1C show images of transverse slices. Only the mossy fibers originating from the DG are labeled, along with the DG itself. Therefore, the dye can be used to label specific projections. Consistent with other experiments, it appears that the dye travels both retrogradely and anterogradely, but not transynaptically (Herredia, 1991). Figures 1A and 1B both display slices in which dye was applied with the needle syringe method. These slices were incubated for three days at 30 degrees C and then placed at 4 degrees C until being mounted. Figure 1C shows a slice in which the dye was applied with microfil tip method. This made applying the dye easier and more accurate. This slice was incubated at 30 degree C for 2 days and then placed in a 4 degree C room until mounting.

Figure 2 shows a magnification of the mossy fibers traveling from the edge of the DG to field CA3. The DiI crystals were applied using the syringe needle method. This slice was incubated for 4 days at 30 degrees C and then placed at 4 degrees until being mounted.

The images show that both methods of applying the dye work well. The results also support the hypothesis that incubation time can vary without greatly impacting the effect of the dye. The fibers labeled after 2 days of incubation do not vary significantly from the fibers labeled after 4 days. The successful labeling of these projections allows these transverse slices to serve as controls.

Sagittal slices of the whole brain were cut in an attempt to preserve connections between the hippocampus and the EC. To test if and where these connections were preserved and where, DiI labeling was used. In most sagittal sections, there were two CA1 sites, therefore, dye was placed in two locations in the hippocampus of each slice. These slices were 200 and 300 microns thick, had DiI applied with the microfil tip to both the upper (dorsal) and lower (ventral) CA1 regions, and were incubated for 4 days at 30 degrees. One of the 200 microns slices, corresponding to image 127/128 (lateral 3.12mm/3.25mm) of the brain atlas, is

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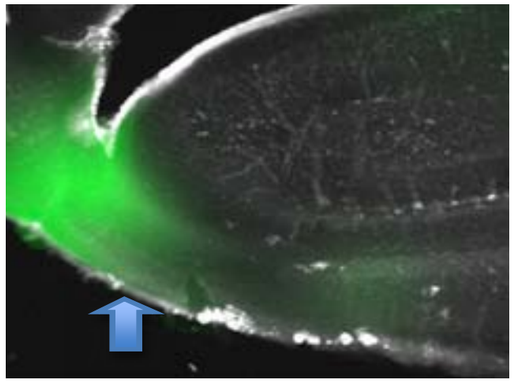


Figure 5A

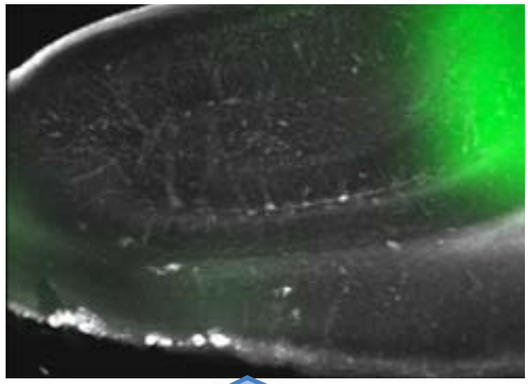


Figure 5B

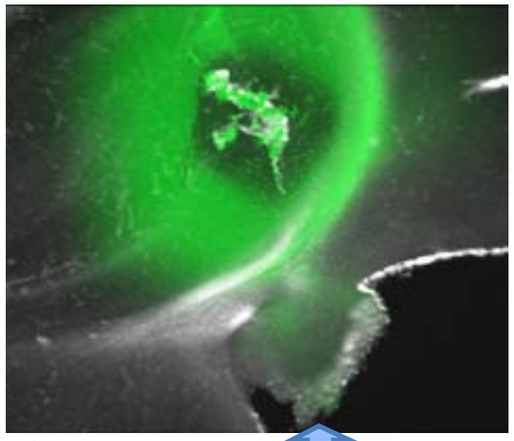


Figure 5C

Figure 5D

Figure 5 *This sagittal slice had one DiI crystal inserted in the upper CA1 region, this is seen in figure 5C. Figure 5C shows some labeling in the upper CEnt region marked by the arrow. Figure 5A and 5B show labeling in the mid region of the CEnt as well as in the DLEnt (both places are marked by arrows). There were no crystals placed in the lower region in CA1 so this dye must have come from the dye put in the upper CA1 region. Figure 5D is a close up of the connections between projections from the upper CA1 region and the mid region of the CEnt.*

shown in **Figure 3** (Franklin 2007). This slice clearly has dye traveling out of the CA1 region and labeling in the EC. There is terminal labeling in the very upper region (most dorsal) of the caudomedial entorhinal cortex (CEnt) traveling from the top (dorsal) CA1 region as well as some light labeling from the lower (ventral) CA1 region to the dorsolateral entorhinal cortex (DLEnt) up into the CEnt.

Two other sagittal slices that were 400 microns thick also showed similar labeling. **Figure 4a** shows one of these two slices, which had dye crystals placed in the upper (dorsal) CA1 region only and had an incubation time of 7 days. Both slices had labeling in the same region as the slice previously mentioned. A third 400 micron slice had slightly different labeling that is more ventral in the CEnt shown in **Figure 4b**. **Figure 5** shows labeling in the upper (most dorsal) region of the CEnt close to where the dye was placed in the upper (dorsal) CA1 region and also near the lower (ventral) CA1 region in the DLEnt and in the adjacent CEnt. Since there were no crystals placed in the lower (ventral) region in CA1, this labeling must have come from the dye put in the upper (dorsal) CA1 region.

In the first whole mount, dye was placed on the left side of the brain and the brain was cut sagittally into 100 micron thick slices. Light labeling can be seen not only on the same side the dye was on, but also on the other side of the brain, confirming that the connections projecting from CA1 travel contralaterally. Projections on the same side of the brain appear to travel to two places, the upper region of the CEnt as in previous sagittal slices, as well as the lower region of the DLEnt as also seen in previous sagittal slices. **Figure 6** displays this connectivity in a slice that is comprable to figure 130 in the Mouse Brain Atlas (lateral 3.44mm). Contralaterally, the projections terminate only in the upper region of the CEnt, as shown in **Figure 7**, which approximately correlates to figure 128/129 in the atlas (lateral 3.25mm/3.36mm).

In a second whole mount, dye was placed on the left side of the brain and the brain was cut horizontally into slices that were 100 microns thick. Labeling can be clearly seen on the same side of the brain in the dorsolateral entorhinal cortex (DLEnt) in at least five of the slices. This labeling is shown in **Figure 8**, which appears to correlate to altas figures 145 to 147 (bregma -3.96mm to -3.60mm).

In the third whole mount, dye was placed on the left side of the brain and the brain was cut sagittally into slices that were 400 microns thick. In these slices, it is very clear that the fibers from the corpus callosum are turning in to the EC. It appears as though subiculum fibers from CA1 use the same track as fibers of the corpus callusum. After the corpus callosum ends, there appear to be tiny bright dots and thick fibers on the surface of the slice. Because the corpus collusum connects the two halves of the brain, this data indicate that fibers are traveling contralaterally. This data can be seen in **Figure 9**.

In **Figure 10**, a sagittal section from a GFP stereotaxic injection in CA1 is shown. In this figure, labeling

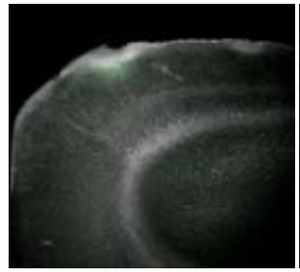


Figure 7A

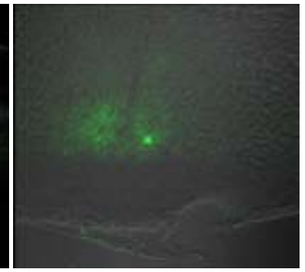


Figure 7B

Figure 7 *These are both 100 micron thick sagittal slices from a whole mount from the opposite side that the dye was put on. This shows there are contralateral connections from CA1 to the EC. Both slices are labeled in the upper CEnt region.*

Figure 6 *This is a 100 micron thick sagittal slice from a whole mount where the dye was inserted into CA1 while the brain was still intact. Here there is labeling in the upper region of the CEnt and the DLEnt. This is a slice from the same side of the brain that the dye was placed on.*

Figure 6A

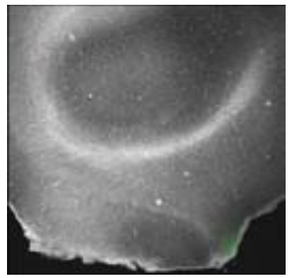
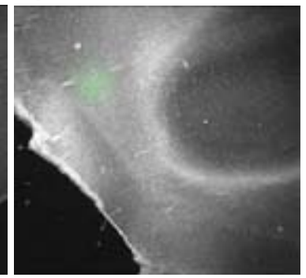


Figure 6B



is very clear in layer II/III and V/VI of the EC. However, no axonal projections can be seen, only small dots.

Discussion

Mossy fibers of the hippocampus were clearly labeled using DiI, running from the dentate gyrus to the CA3 field in 400 micron thick transverse slices. This labeling of well-defined and studied projections served to show how to best apply the dye and treat the slices to develop clear staining of pathways. Only mossy fibers originating from the DG are labeled, which is significant because it indicates that the dye can be used to label specific projections.

Experimentation with sagittal slices was carried out in order characterize the relatively undefined and unstudied pathways running from the CA1 field of the hippocampus to the EC and from the EC to the CA1 field. So far, the dye has been placed in the CA1 field to observe how the projections run to the EC. Several of the 300 micron sagittal slices appear to be promising, but further analysis of the slices is needed. After this analysis, the brains will be cut at a variety of different angles in an attempt to preserve the most connections between CA1 and the EC.

Applying dye with microfil tips worked better than the dye application using needles or the eyelash brush. The shortened microfils were rigid enough to apply the dye and also thinner and therefore more accurate than the needles. The microfils also proved to be less electrostatic and were less destructive to the slice. The eyelash brush proved to be completely ineffective at applying crystals underneath the surface of the slice, as it was not rigid enough.

Thirteen 400 micron thick transverse slices had DiI crystal applied with needles. Four of the slices were in-

cubated for 4 days and nine were incubated for 3 days. The first four slices all showed mossy fiber staining from the DG to CA3. Of the nine, four showed some mossy fiber staining, but only two of those showed mossy fibers running all the way to CA3. All of the slices showed staining of the DG. Four slices had DiI applied with the eyelash brush. One was incubated for three days and the others for two days. Because it was hard to get the crystal beneath the surface with this method, not one of the slices displayed mossy fiber staining. Because the crystals stayed on top of the surface of the slice and could easily move around when the slice was put back into a well of PBS, the staining was random and diffuse. Four slices had DiI applied with the microfil tip and were incubated for two days. Of these one was completely unsuccessful, one was very successful sending stained projections from the DG to CA3, and two were partially successful, clearly staining the DG and the beginning of the mossy fibers. To ensure that the dye had enough time to travel, it was important to leave the slices in incubation for as long as possible, though a significant difference between the slices incubated for two days and those incubated for four days was not seen. The clear labeling of the mossy fibers seems to be more dependent on getting the dye underneath the surface of the slice and into the correct location in the DG.

Although we originally wanted slices to be as thin as 50 microns in order to see as much detail as possible, such slices were too thin to apply DiI crystals. Any attempt at application caused these slices to tear immediately.

Applying the DiI crystals to the 200-400 micron thick sagittal slices proved to be fairly successful. Some-

Figure 8

These are images from a whole mount cut horizontally in 100 micron thick slices. Labeling is seen in the CEnt. Figure 8B is a magnification of 8A. Figures 8D and 8E are magnifications of 8C.

Figure 8A

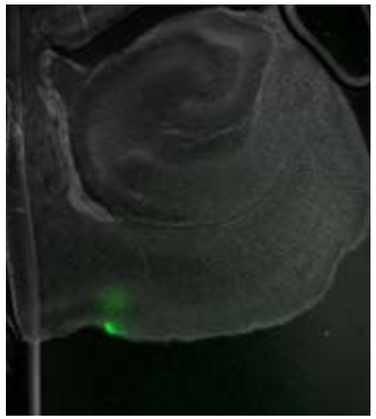


Figure 8B

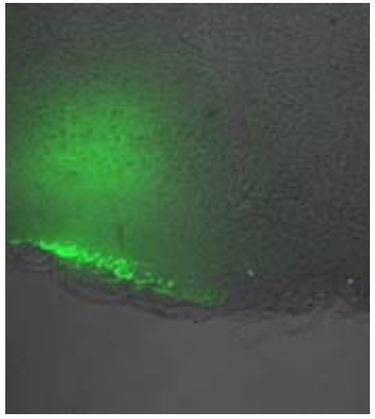


Figure 8C

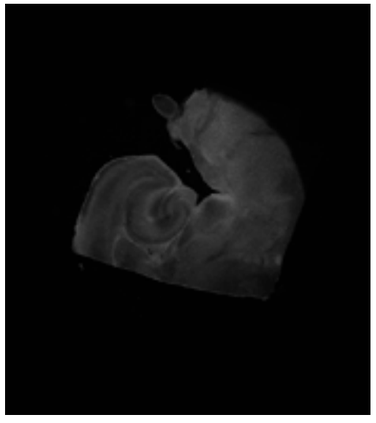


Figure 8D

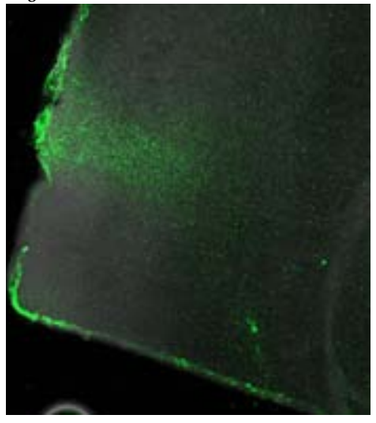
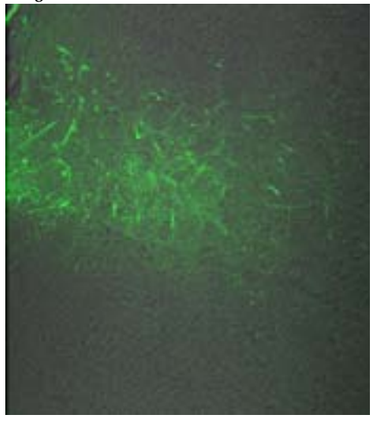


Figure 8E



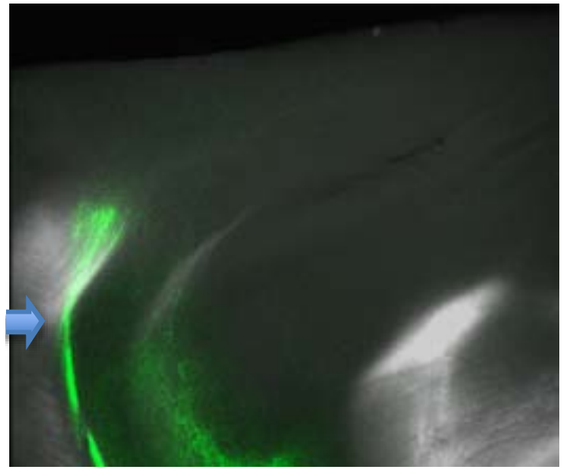


Figure 9 *This image is of a 400 microns thick sagittal slice. This image displays labeling of the corpus callosum (arrow points to this). These are fibers that connect the two hemispheres on the brain. This is interesting because it means that fibers projecting from CA1 are going through the corpus collosum meaning*

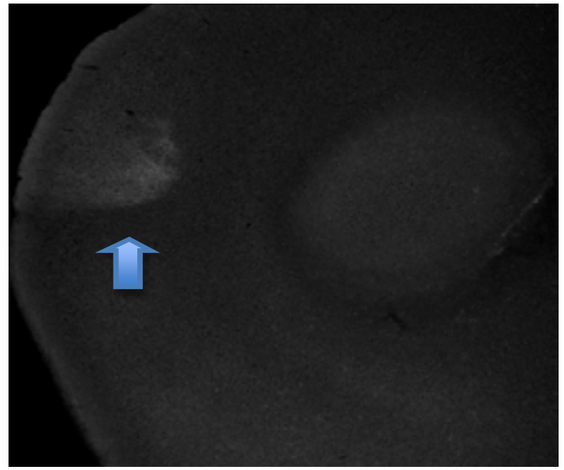


Figure 10 *This is an image of a sagital slice of a GFP injected brain. This slice also shows labeling in the upper CEnt region, consistent with DiI labeling findings.*

times it was difficult to observe the exact pathway of the projections because the diffusion area of the dye was so large. Clear projections can be seen to go from the upper CA1 region to the upper region of the CEnt. Additionally, dye in the lower CA1 region appears to travel through the DLEnt and up into the CEnt.

In total, five successful whole mounts were made. Two were cut horizontally and three were cut sagittally. The first sagittal one appeared to have ipsilateral and contralateral projections, the ipsilateral projections went to the DLEnt and CEnt and the contralateral projections only went to the CEnt. In the horizontal orientation, whole mount light labeling can be seen in the ipsilateral DLEnt. More analysis of the whole mount slices and images still remains to be carried out. The whole mount method, though more technically challenging, can hopefully provide clearer results than applying the dye after slicing because it allows the dye to run throughout the brain and does not severe connections.

Another method of labeling connections was used to show connections between the CA1 field and the entorhinal cortex. A GFP lentivirus was injected in the CA1 field of a live mouse stereotaxically, at +/-1.9 (x), -2.2 (y), -1.5 (z1), -1.3 (z2) relative to Bregma. Preliminary analyses of the GFP labeling of this brain to be promising. There seems to be labeling in layers II/III and V/VI; however, it is unclear which projections are contralateral because both sides of the mouse's brain were injected with virus. The GFP method might prove more useful and clearer because there should be better accuracy when injecting virus into CA1 and not as much random diffusion as with the dye. The illumination of GFP appears as tiny dots rather than long axon fibers. We hypothesize that this is because the virus collected in the bouton at the end of the axons. To test this we will carry out antibody staining specific to the bouton.

DiI application in the DG of 400 micron thick transverse slices proved to be very successful and showed DiI application in thick slices to label projections well. These projections ran from the DG to the CA3 field, showing the path of the well studied mossy fibers. Application of the DiI crystals to the sagittal slices has produced some positive results, but further investigation must be conducted. Experimentation with the angle of sectioning will be performed to assess which plane is best for preserving the most connections from CA1 to the EC. The ultimate aim of this project is to develop methodology to carry out successful electrophysiological studies of acute slices, with a focus on the communication between the EC and the CA1 field of the hippocampus in order to further the investigation of memory storage.

Future Directions

In the future, we will focus more on studying the connections between CA1 and the EC by stereotaxically injecting GFP virus. This method appears to be more promising as it labels more selectively than the dye, which somtimes diffuses too much and the causes

the connections to appear unclear. By stereotaxically injecting virus we can also be more accurate and consistent with future injections into the mouse brain. Different orientations of slicing must be tested in order to find the optimal orientation for preserving connections. Once the connection from CA1 to the EC is clearly established, we also plan to examine the connections projecting from the EC to CA1. This anatomical data will contribute to the success of future electrophysiological studies.

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