Video Article

Modification of a Colliculo-thalamocortical Mouse Brain Slice, Incorporating 3-D printing of Chamber Components and Multi-scale Optical Imaging

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Abstract

The ability of the brain to process sensory information relies on both ascending and descending sets of projections. Until recently, the only way to study these two systems and how they interact has been with the use of *in vivo* preparations. Major advances have been made with acute brain slices containing the thalamocortical and cortico-thalamic pathways in the somatosensory, visual, and auditory systems. With key refinements to our recent modification of the auditory thalamocortical slice[1], we are able to more reliably capture the projections between most of the major auditory midbrain and forebrain structures: the inferior colliculus (IC), medial geniculate body (MGB), thalamic reticular nucleus (TRN), and the auditory cortex (AC). With portions of all these connections retained, we are able to answer detailed questions that complement the questions that can be answered with *in vivo* preparations. The use of flavoprotein autofluorescence imaging enables us to rapidly assess connectivity in any given slice and guide the ensuing experiment. Using this slice in conjunction with recording and imaging techniques, we are now better equipped to understand how information processing occurs at each point in the auditory forebrain as information ascends to the cortex, and the impact of descending cortical modulation. 3-D printing to build slice chamber components permits double-sided perfusion and broad access to networks within the slice and maintains the widespread connections key to fully utilizing this preparation.

Video Link

The video component of this article can be found at http://www.jove.com/video/53067/

Introduction

In the auditory system, although there is substantial processing of information between the sensory periphery and the inferior colliculus, there is considerable additional processing before it reaches the auditory cortex. We know very little about how that processing is done and therefore little about how that transformation allows the brain to interpret incoming sensory information. With the exception of olfaction, each of the senses has a very similar organization with peripheral signals initially being relayed with high fidelity which declines as the signal ascends to the cortex. The cortex then sends projections to the lower structures to further modulate the incoming information. This complex system has been studied in a variety of ways *in vivo* as well as in a number of *in vitro* preparations. In the former, all connections are intact, enabling the researcher to probe any set of connections, while controlling the sensory input and measuring output in any given area. With this approach, there is little to no control of the large variety of other inputs, including other sensory inputs, arousal, and attention, giving rise to an intensely complex output. *In vitro*, brain slices have been cut to capture either a single set of projections, or two connected brain areas, which allow researchers to stimulate and evaluate various afferents or brain areas. These are often either thalamocortical or tectothalamic slices where either the input to the thalamus or the thalamus and its output to the cortex are preserved[2-5]. These preparations allow for a wide variety of pharmacological, electrical, and optogenetic manipulations. However with only two brain regions, they primarily evaluate the transfer of information and lack the ability to evaluate the transformation of information as it passes through the thalamus. Also the reticulo-thalamic projection, which may play a role in attention modulation[6-9] is present in this slice. Here we demonstrate improvements upon our previous preparation[1], which allows the investigator control of various inputs to the thalamus to give a unique perspective of how the thalamus gates and filters information. We couple this novel slice preparation with flavoprotein autofluorescence imaging for assessing slice connectivity and large-scale activation analysis, calcium imaging in the thalamus for neuronal population analysis, and single cell recording to measure the impact of the various inputs on a single cell level.

To assist in maintaining these widespread connections we have also developed a number of modifications of the normal slice anchor (a.k.a. “harp”) for holding the brain slice in place and a bridge to elevate the slice for enhanced perfusion. The harp is designed in a modified horseshoe shape to surround the slice and allow for customizable attachment points for the harp strings. Three strings are attached such that i) one lies horizontally along the medial edge of the slice, ii) one extends from the caudal edge of the IC to the caudal edge of the AC and iii) one extends diagonally from the medial edge of the slice to an area rostral to the AC (see **Figure 1A**). Small indentations in the frame for gluing (with cyanoacrylate glue) of the harp strings allow for a decreased amount of pressure on the slice to help maintain slice integrity (see **Figure 1B**). By using three dimensional printing, we are able to custom design harps to our unique specifications, as well as bridges which allow for ideal flow of

artificial cerebrospinal fluid (aCSF) above and below the tissue. This also maintains large areas for light to penetrate the tissue for patch clamp electrophysiology.

Protocol

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois. All animals were housed in animal care facilities approved by the American Association for Accreditation of Laboratory Animal Care. Every attempt was made to minimize the number of animals used and to reduce suffering at all stages of the study.

1. Preparation for and Removal of Brain from Mouse for Slicing

1. Prepare for perfusion and slice incubation.
    1. Approximately 30 min before slicing, prepare high sucrose cutting solution and low calcium aCSF for incubation of the slice prior to recording or imaging.
    2. Lay out all necessary tools (large blunt end scissors, small spring scissors, anatomic forceps, large scissors or guillotine, iris scissors, jewelers forceps, 10 ml syringe with 27G x 1/2 inch needle, small piece of 1 cm x 2 cm filter paper, and a broken tipped Pasteur pipette for slice transfer) for perfusion and slicing, and prepare perfusion tray.
    3. Set up incubating chamber with oxygenated aCSF in a 32 ^{o}C water bath and a culture dish filled with cutting solution.

2. Prepare cutting stage for slicing.
    1. Cut a small piece of 3% agar approximately 1 cm^{3} to use as a backstop for the brain and 1.5 cm x 1.5 mm x 3 mm for a bump to be used to prop up the IC.
    2. Glue the backstop onto the stage with cyanoacrylate adhesive as well as the bump on the right side of the backstop such that they form an 80^{o} angle.

3. Remove the brain.
    1. Deeply anesthetize a p12-p20 (ideally p14-18) mouse with ketamine 100 mg/kg and xylaxine 3 mg/kg (or comparable ethics committee approved procedure).
    Note: Confirm proper anesthesia level via lack of response to toe pinch. As the animal is under anesthesia briefly, ophthalmic ointment is unnecessary.
    2. Using blunt end scissors, expose the ribcage from the xiphoid process to the neck.
        1. Find the xiphoid process, and make a horizontal cut of approximately 1.5 cm.
        2. Make two vertical cuts from the ends of the previous horizontal cut to the shoulders, approximately 1.5 cm each.
    3. Cut through the diaphragm and the costochondral junctions to expose the heart.
    4. With small spring scissors, make a small, 2-5 mm cut in the right atrium, from the ventral to dorsal side of the heart.
    5. Using a 10 ml syringe with a 27G x 1/2 inch needle, inject the left ventricle and quickly perfuse the animal with high sucrose cutting solution.
    6. Once the blood runs clear, use larger scissors to remove the head.
    Note: We have not systematically assessed the utility of perfusion. However, in our experience, transcardiac perfusion in mice this young can be done with nearly 100% success, and does have the benefit of eliminating red blood cells, which can fluoresce and interfere with imaging.
    7. Cut the skin down the midline to expose the skull.
    8. Using bent iris scissors, cut the skull between the eyes, then starting from the cut between the eyes, carefully cut from anterior to posterior along the midline suture taking care not to damage the brain underneath.
    Note: The dura usually comes off with the skull. If necessary, remove the dura before carefully removing the brain.
    9. Using jewelers forceps, pry open the skull and carefully remove the brain, then place the brain in cutting solution. Take care to avoid damaging the cortices.

2. Preparing Brain for Slicing

1. Preparing brain for mounting on vibratome stage.
    1. Using a slide marked with two lines at 90^{o} and a diagonal line at 17^{o} from the top left to bottom right, intersecting where the two lines meet, place the brain dorsal side up, using a razor blade, remove 2-4 mm of the rostral end brain creating a flat surface.
    2. Place the brain caudal side up on the newly created flat surface, and align the dorsal surface of the brain with the horizontal and the midline of the brain with the vertical line.
    3. Align the razor blade with the 17^{o} line, tilt the razor at a 30^{o} angle and remove approximately 3 mm of the right cortex in a double diagonal cut (17^{o} from horizontal plane and 60^{o} from the coronal).

2. Mounting brain on vibratome stage.
    1. Place a small piece of filter paper 1 cm x 2 cm on the ventral side of the brain so that the long dimension is perpendicular to the midline.
    2. Carefully apply a small amount of cyanoacrylate adhesive to the area in front of the backstop (and to the left of the bump).
    3. Place the double diagonally-cut brain face on to the glue so that the caudal part of the brain and hindbrain are propped upon the bump and the right side of the brain is against the backstop.
    4. Delicately press down on the brain, ensuring that the entire surface is in contact with the slicing chamber.

3. Obtaining the Colliculo-thalamocortical Slice

1. Quickly take the cutting stage and place in the vibratome, fill the stage with cutting solution.
2. Align the blade with the top (ventral side) of the brain.
3. Remove 1-1.5 mm from the top of the brain, remove 300-500 µm slices and assess depth after removal.
   Note: When the IC, the MGB, and the lateral geniculate nucleus (LGN) are all visible, and the dentate gyrus forms a 'C' shape take one to two 600 µm slices. See **Figure 3A.**
4. Place slices in heated (32 ^{o}C) holding chamber.

4. Imaging of the Slice

1. Preparation for flavoprotein autofluorescence imaging.
   1. Prepare aCSF for perfusion of slice in a standard electrophysiological recording chamber, bubble aCSF with 95% O_2/5% CO_2.
   2. Pull glass electrode for stimulation. Note that multiple different stimulating electrodes and configurations (glass, tungsten, carbon fiber, monopolar, bipolar) all work very well in conjunction with flavoprotein autofluorescence imaging.
   3. Turn on computer, camera, micromanipulator, light source, stimulation software, image capture software.
   4. Perfuse the recording chamber with oxygenated aCSF, place custom bridge (design available in Supplemental Materials) to elevate slice in chamber.
      Note: Perfusion rate can vary with any individual set up; 5-15 ml/min is used here.
   5. Place slice in chamber, lower the liquid level in the chamber to prevent the slice from lifting off the bridge while placing specially constructed harp over slice, taking care to avoid placing harp strings over pathways of interest (**Figure 1A**). Increase liquid level to approximately 1-2 mm above slice to maintain aCSF flow above and below slice.
      Note: If repositioning is necessary, use the broken end Pasteur pipette or allow the aCSF level to rise and use forceps with extreme care.
   6. Insert silver chloride electrode into glass electrode filled with aCSF, insert the glass electrode into micromanipulator and connect to stimulus isolator, and carefully place glass electrode in IC/white matter leading to thalamus(see **Figure 1A**).

2. Flavoprotein autofluorescence image acquisition.
   1. Collect images at 4 Hz (using an infinity-corrected 2X macro objective (NA 0.13)) and a camera for 105 sec while electrically stimulating (each stimulation consists of 1 sec of 40 Hz 2 msec pulses tissue at 0.05 Hz five times) while illuminating the tissue with blue light 470-490 nm and capturing above 515 nm. Ensure that the image is neither too dark, nor blown out see **Figure 3** left column for reference.
      Note: Collection time, collection frequency, and stimulation frequency can be changed to suit the experiment, the custom program included in supplementary materials can be modified as such.
   2. Export images to Matlab, and using the custom written program available in supplementary materials to analyze spectral power of the images at 0.05 Hz. The resulting image will show connected pathways.
      Note: The custom program uses a fast Fourier transform of the pixel values in the time series to produce the image.

3. Preparation for calcium imaging.
   1. Prepare small incubation chamber using a 3 cm culture dish and raised culture membrane to allow aCSF to reach both the top and bottom of the slice, and place on a heating pad to maintain temperature at approximately 32 ^{o}C.
   2. Fill small incubation chamber with warm aCSF and slowly bubble with 95% O_2/5% CO_2.
   3. Mix 2 µl of pluronic F-127 acid and 50 µg of Fura-2AM dissolved in 48 µl of DMSO.
   4. Place slice in small incubation chamber on raised membrane and carefully using a micropipette add staining mixture directly above the MGB (or other structure of interest).
   5. Cover slices to prevent bleaching prior to imaging, and allow slices to incubate for 45 min - 1 hr.
   6. Remove slices to heated holding chamber for 10-15 min to wash off excess material.
   7. Follow steps 4.1.1 to 4.1.6 for preparation to stimulate and image the colliculo-thalamocortical slice.

4. Calcium image acquisition.
   1. Collect images at 10 Hz (using an 20X water immersion objective) for 25 sec while electrically stimulating (each stimulation consists of one 2 msec pulse) tissue at 0.2 Hz five times while illuminating the tissue with 365 nm light and capturing fluorescence above 510 nm.
   2. Export images to Matlab and using the custom written program available in supplementary materials to analyze spectral power of the images at 0.2 Hz. The resulting image will show active cells.

Representative Results

An example of colliculo-thalamocortical mouse brain slice obtained in P15 mouse is shown in **figure 2**. The ideal slice will contain the four major midbrain and forebrain auditory structures IC, MGB, TRN, and AC, which are all activated when the IC is stimulated (**Figure 2A**). Using Fourier analysis, the spectral power is measured at the electrical stimulation frequency, with connected brain regions showing activity that is periodic and entrained at the stimulation frequency[10]. This protocol allows for study of the ascending flow of information particularly in the thalamus as a nexus between the midbrain and the cortex. While this protocol aids in the production of the connected colliculo-thalamocortical slice as well as providing a more easily customizable set of hardware with the incorporation of 3D printing, included are an example of a slice not connected from the thalamus to the cortex (**Figure 2B**), as well as a connectivity pattern where the thalamus does not show activity with flavoprotein autofluorescence imaging (**Figure 2C**). This is likely due to the connection being internal to the slice such that the activation is not on the surface of the slice and therefore unable to be seen with the flavoprotein autofluorescence imaging. It is possible though unlikely that this is antidromic activation. We have not seen antidromic activation when stimulating the white matter of the thalamocortical projections[11], and with this

stimulation paradigm synaptic blockade in the thalamus causes a reversible elimination of the cortical signal[1]. Using this slice, it is also possible to look at cell population activity in the thalamus with electrical stimulation of the IC. A sample calcium imaging experiment shows the spiking activity of a small population of thalamic neurons in response to a midbrain stimulus (**Figure 3**).

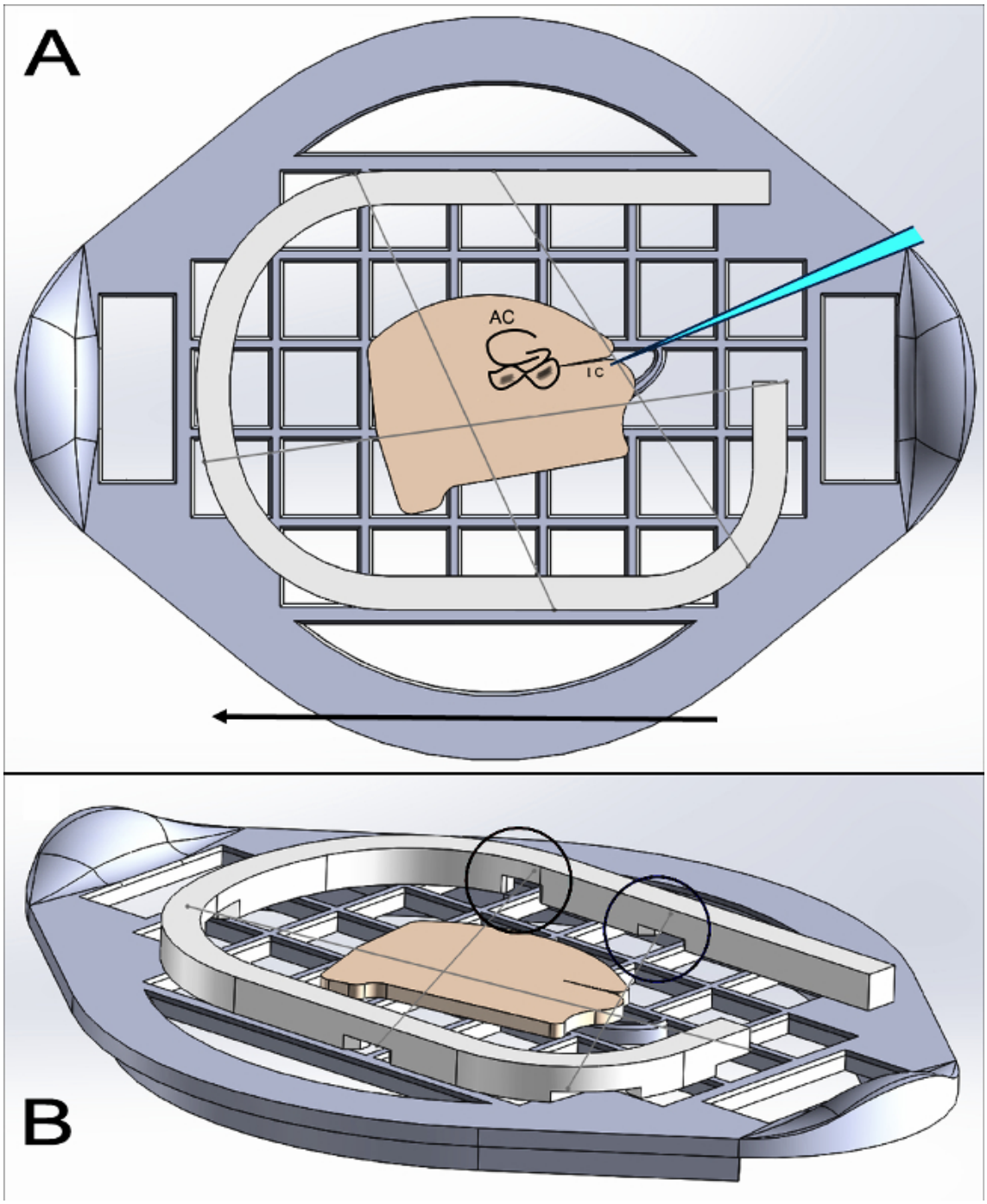

Figure 1. Rendering of 3D printed materials. (A) Rendered image of the slice sitting on the bridge with the printed harp in place. Arrow indicates flow of aCSF. **(B)** Rendered image highlighting grooves for the strings of the harp allowing for the thicker slice (circles).

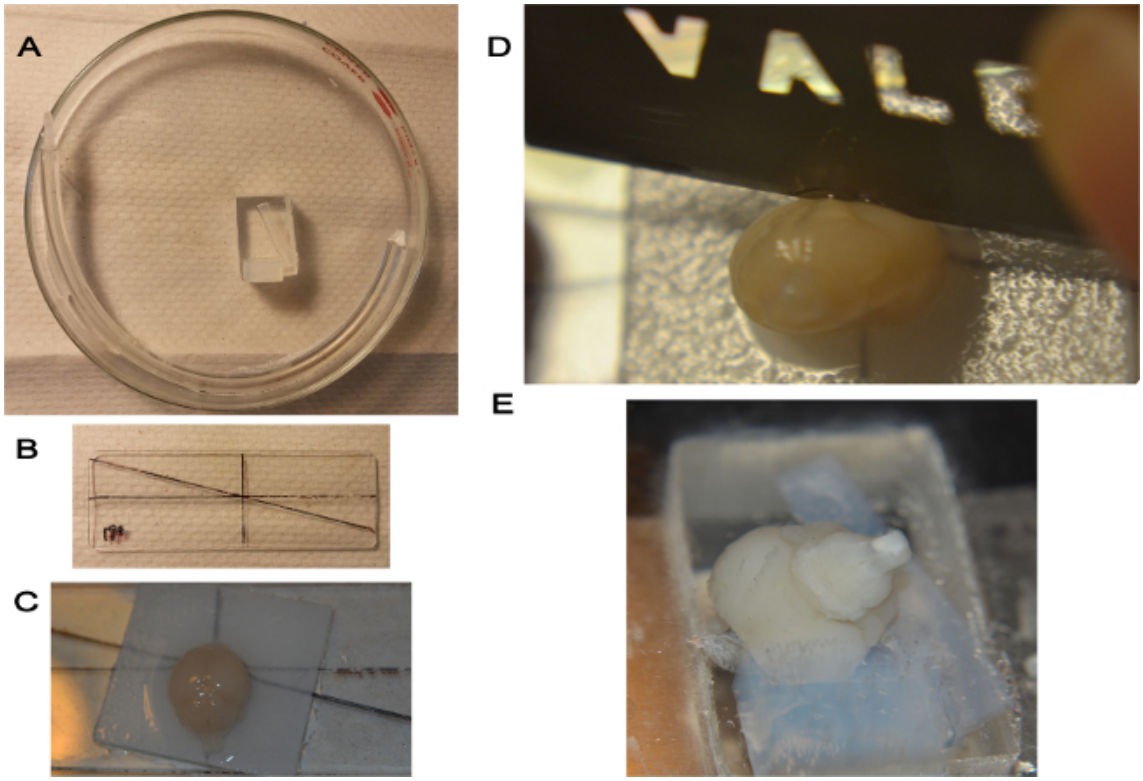

Figure 2. Cutting the brain for slicing. (A) Agar positioning on the cutting stage. **(B)** Slide for brain positioning. **(C)** Position of brain for anterior cut. **(D)** Position of brain and razor for double angled cut. **(E)** Final blocking of brain for slicing.

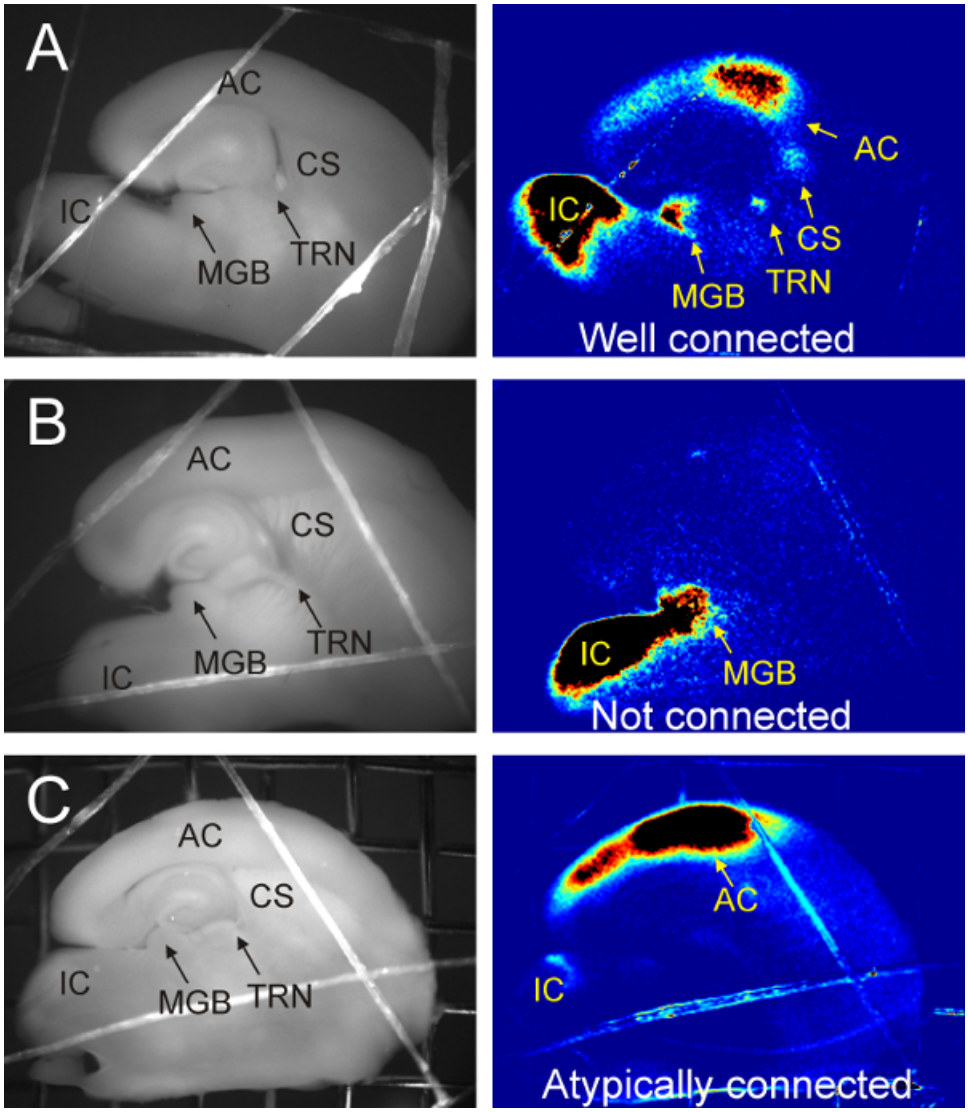

Figure 3. Flavoprotein autofluorescence imaging of colliculo-thalamocortical brain slice. (A) Connected colliculo-thalamocortical brain slice as confirmed by flavoprotein autofluorescence. Electrical stimulation at 0.05 Hz of IC imaged at 4 Hz and Fourier processed to show power at stimulation frequency. Note activation of MGB, TRN and, AC as well as the corpus striatum (CS). **(B)** Unconnected slice. Electrical stimulation of IC with only activation of MGB. Entire pathway was not captured likely due to 17^{o} angle being slightly too steep. **(C)** Atypical activation of AC. Electrical stimulation of IC with activation of AC without visible signal in the MGB or TRN. Pathway likely intact however the active cells in the MGB are likely on the other side of the tissue.

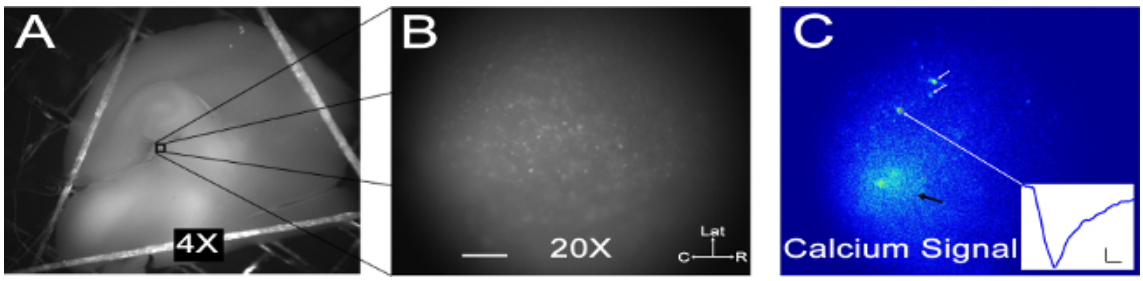

Figure 4. Calcium imaging. (A) Raw image of example slice illuminated for calcium imaging. **(B)** Raw 20X image of the MGB, bright points are cells. Scale bar 100µm. **(C)** Calcium signal with electrical stimulation of the IC. Fourier processing shows activation of both cells white arrows and neuropil black arrowhead. Inset, time course of averaged signal (inset scale bar 0.5% change in fluorescence, 1 sec).

Discussion

This protocol describes improvements upon a previously described colliculo-thalamocortical brain slice in p12-20 mouse to study information flow in the auditory system[1]. This method has a number of advantages over other, similar, brain slice preparations by retaining connections between more brain areas in a single slice, which gives investigators new tools to understand the interaction and interplay between auditory nuclei in the forebrain. There have been a few key modifications in this protocol, compared to our previous work[1], which increase the yield of connected slices. These modifications include a slightly different cutting angle in the horizontal plane 17^{o} compared to 20^{o}, an added angle in the coronal plane, 60^{o} from coronal and a smaller bump under the IC 1.5 mm compared to 2 mm. While small, these modifications greatly improve the yield and reliability of the method, with practice slices should be obtained 80-100% of the time. Along with obtaining the colliculo-thalamocortical slice, the protocol also describes rapid assessment of the connectivity of the slice ensuring that the slice is connected before further experimentation is done. With the enhanced reliability of the slice, it will be easier to use in conjunction with disease models to understand changes in the auditory system in the diseased state.

There are a few critical steps to optimize obtaining the colliculo-thalamocortical slice. While it is possible to use mice from postnatal day 12 through postnatal day 22, the highest rates of obtaining full connectivity in the slice are between postnatal day 14 and 18. Another critical step is rapid removal of the mouse brain, this will help ensure the health of the tissue providing for a better experiment. After removing the brain from the mouse and removing the frontal cortex, the double angled cut is the most critical step; a clean, fluid motion of the cut as well as maintaining the appropriate angles is necessary to align the appropriate brain structures. If the angled cuts are performed incorrectly, it is likely the connections from the MGB to the AC will not be maintained. If the 17 ^{o} angled cut is off by more than a few degrees, the connections from the IC to the MGB will be lost as well. Similarly, if the step underneath the IC is too large, the IC and MGB will be out of alignment. Using flavoprotein autofluorescence imaging, it is easy to quickly determine slice connectivity. If the slice is not fully connected, the stimulator can be moved around the slice to quickly determine which parts of the slice are connected so corrections can be made in future experiments.

While this slice provides a method to explore information flow through the auditory system, there are some limitations. One of the major strengths of this method is the control of inputs to the nuclei of study, however many connections are severed in the slice. The functions are more complex *in vivo* so the modeling of this system would be incomplete. Similarly, the stimulation paradigms currently available will be different than the signals generated from sensory perception. Though these limitations are inherent in this *in vitro* system, the preparation is useful to understand information flow in the auditory system. Though onset of hearing in mice is approximately p12[12,13]the central auditory system is not yet fully developed at the time that these slices are prepared. This lack of full maturity of these slices may limit the generalizability of the findings derived from this slice.

The use of three-dimensional printing for specialized hardware for preserving and maintaining the slice's connections allows the experimenter to customize these pieces to their needs. Advances in three-dimensional printing allow for a highly customizable set of harps and bridges using materials better suited for placement of strings to hold down the slice at more precise tensions as well as holding up the slice and allowing for better fluid perfusion. These designs are easily customizable to the individual setup without the need for expensive or difficult to make pieces of equipment.

Disclosures

The authors have nothing to disclose.

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