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# In Vitro Wedge Slice Preparation for Mimicking In Vivo Circuit Connectivity

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Cover letter

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and Other Communication Disorders

DATE: May 15, 2020

FROM: Catherine Weisz, Ph.D.
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SUBJECT: Submission of manuscript to JoVE

Dear Dr. Werth,
Enclosed please find our manuscript titled Mimicking *in vivo* circuit connectivity with a novel *in vitro* slicing technique: the wedge slice, by Matthew J. Fischl and Catherine J. C. Weisz.

Our group recently published a paper titled "Synaptic inhibition of medial olivocochlear efferent neurons by neurons of the medial nucleus of the trapezoid body", by Lester Torres Cadenas, Matthew J. Fischl, and Catherine J.C. Weisz, in the Journal of Neuroscience. This study detailed newly-discovered inhibitory synaptic inputs to the medial olivocochlear (MOC) neurons, which are critical for gain control and protection against noise trauma in the cochlea. We were invited to submit a JoVE manuscript based on this work by Dr. Aaron Berard, a former editor at JoVE. The current submission details a new technique recently developed in the lab to extend our result from the Torres Cadenas et al 2020 paper to a more *in vivo*-like preparation in order to study integration of excitatory and inhibitory synaptic inputs to the MOC neurons. This technique, which we call a wedge slice, involved a novel slicing method to include the majority of the presynaptic circuitry of the MOC neurons in a slice that is thick on one side to mimic *in vivo* neuron connectivity, but thin on the other side where MOC neurons are located to allow powerful *in vitro* techniques. The manuscript details the process of creating the slice, histological methods for confirming slice contents, and some results showing an increased diversity of responses when the full auditory circuit is stimulated from the auditory nerve, relative to direct stimulation at the axons of the pre-synaptic neurons.

We believe that the wedge slice technique will be applicable to individuals studying neurons of the auditory brainstem, where *in vivo* experimentation is extremely difficult due to the location of neurons deep in the brain, but also to other deep brain regions such as the thalamic projections to cortical neurons.

Thank you for your consideration of this work,

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**TITLE:**

# In Vitro Wedge Slice Preparation for Mimicking In Vivo Circuit Connectivity

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**KEYWORDS:**

medial olivocochlear neurons, in vitro slice electrophysiology, synaptic integration, auditory nerve, auditory brainstem, cochlear nucleus, inhibitory neurotransmission

**SUMMARY:**

Integration of diverse synaptic inputs to neurons is best measured in a preparation that preserves all pre-synaptic nuclei for natural timing and circuit plasticity, but brain slices typically sever many connections. We developed a modified brain slice to mimic in vivo circuit activity while maintaining in vitro experimentation capability.

**ABSTRACT:**

In vitro slice electrophysiology techniques measure single-cell activity with precise electrical and temporal resolution. Brain slices must be relatively thin to properly visualize and access neurons for patch-clamping or imaging, and in vitro examination of brain circuitry is limited to only what is physically present in the acute slice. To maintain the benefits of in vitro slice experimentation while preserving a larger portion of presynaptic nuclei, we developed a novel slice preparation. This “wedge slice” was designed for patch-clamp electrophysiology recordings to characterize the diverse monaural, sound-driven inputs to medial olivocochlear (MOC) neurons in the brainstem. These neurons receive their primary afferent excitatory and inhibitory inputs from neurons activated by stimuli in the contralateral ear and corresponding cochlear nucleus (CN). An asymmetrical brain slice was designed which is thickest in the rostro-caudal domain at the lateral edge of one hemisphere and then thins towards the lateral edge of the opposite hemisphere. This slice contains, on the thick side, the auditory nerve root conveying information about auditory stimuli to the brain, the intrinsic CN circuitry, and both the disynaptic excitatory and trisynaptic inhibitory afferent pathways that converge on contralateral MOC neurons. Recording is performed from MOC neurons on the thin side of the slice, where they are visualized using DIC optics for typical patch-clamp experiments. Direct stimulation of the auditory nerve is performed as it enters the auditory brainstem, allowing for intrinsic CN circuit activity and synaptic plasticity to occur at synapses upstream of MOC neurons. With this technique, one can

mimic in vivo circuit activation as closely as possible within the slice. This wedge slice preparation is applicable to other brain circuits where circuit analyses would benefit from preservation of upstream connectivity and long-range inputs, in combination with the technical advantages of in vitro slice physiology.

**INTRODUCTION:**

Observation of activity of neural circuits is ideally performed with native sensory inputs and feedback, and intact connectivity between brain regions, in vivo. However, performing experiments that give single-cell resolution of neural circuit function is still limited by technical challenges in the intact brain. While in vivo extracellular electrophysiology or multiphoton imaging methods can be used for investigating activity in intact nervous systems, interpreting how different inputs integrate or measuring subthreshold synaptic inputs remains difficult. In vivo whole-cell recordings overcome these limitations but are challenging to perform, even in brain regions which are easily accessed. Technical challenges of single-cell resolution experiments are further amplified in certain neuron populations that are located deep in the brain, or in spatially diffuse populations that require either genetic tools to locate cells in vivo (e.g., genetic expression of channelrhodopsin paired with optrode recording) or post-hoc histochemical identification after recording site labeling (e.g. with neurotransmission-specific markers). Being located diffusely near the ventral surface of the brainstem, medial olivocochlear (MOC) suffer from the above limitations[1], making them extremely difficult to access for in vivo experimentation.

Brain slices (~100-500 µm thickness) have long been used to study brain circuitry, including auditory brainstem circuitry, because of the physical segregation of connected neurons that are contained within the same slice[2–9]. Experiments using much thicker slices (>1 mm) have been employed in other labs to understand how bilateral inputs integrate in areas of the superior olivary complex (SOC) including the medial superior olive[10,11]. These slices were prepared such that axons of the auditory nerve (AN) remained intact within the slice and were electrically stimulated to initiate synaptic neurotransmitter release in the CN, mimicking activity of first order auditory neurons as they would respond to sound. One major disadvantage of these thick slices is visibility of neurons for patch-clamp electrophysiological recordings ("patching"). Patching becomes increasingly difficult as the numerous axons in this area become myelinated with age[12–15], making the tissue optically dense and obscuring neurons even in a typical, thin brain slice. Our goal is to create in vitro preparations that more closely resemble the circuit connectivity of in vivo recordings, but with the high-throughput and high-resolution recording abilities of visually guided patch-clamp electrophysiology in brain slices.

Our lab investigates the physiology of neurons of the auditory efferent system, including MOC neurons. These cholinergic neurons provide efferent feedback to the cochlea by modulating the activity of outer hair cells (OHCs)[16–20]. Previous studies have shown that this modulation plays a role in gain control in the cochlea[21–26] and protection from acoustic trauma[27–33]. In mice, MOC neurons are diffusely located in the ventral nucleus of the trapezoid body (VNTB) in the auditory brainstem[1]. Our group has utilized the ChAT-IRES-Cre mouse line crossed with the tdTomato reporter mouse line to target MOC neurons in brainstem slices under epifluorescent illumination.

We showed that MOC neurons receive afferent inhibitory input from the ipsilateral medial nucleus of the trapezoid body (MNTB), which is excited, in turn, by axons from globular bushy cells (GBC) in the contralateral cochlear nucleus (CN)[34–38]. Additionally, MOC neurons likely receive their excitatory input from T-stellate cells in the contralateral (CN)[39–41]. Taken together, these studies show MOC neurons receive both excitatory and inhibitory inputs derived from the same (contralateral) ear. However, the presynaptic neurons, and their axons converging on MOC neurons, are not quite close enough to each other to be fully intact in a typical coronal slice preparation. To investigate how integration of synaptic inputs to MOC neurons affects their action potential firing patterns, with a focus on newly described inhibition, we developed a preparation in which we could stimulate the diverse afferents to MOC neurons from one ear in the most physiologically realistic way possible, but with the technical benefits of in vitro brain slice experiments.

The wedge slice is a modified thick slice preparation designed for investigation of circuit integration in MOC neurons (schematized in **Figure 1A**). On the thick side of the slice, the wedge contains the severed axons of the auditory nerve (termed "auditory nerve root" hereafter) as they enter the brainstem from the periphery and synapse in the CN. The auditory nerve root can be electrically stimulated to evoke neurotransmitter release and synaptic activation of cells of the fully intact CN[42–46]. This stimulation format has several benefits for circuit analysis. First, instead of directly stimulating the T-stellate and GBC axons that provide afferent input to the MOC neurons, we stimulate the AN to allow activation of intrinsic circuits abundant in the CN. These circuits modulate the output of CN neurons to their targets throughout the brain, including MOC neurons[46–51]. Second, the polysynaptic activation of afferent circuits from the AN through the CN upstream of MOC neurons allows for more natural activation timing and for plasticity to occur at these synapses as they would in vivo during auditory stimulation. Third, we can vary our stimulation patterns to mimic AN activity. Finally, both excitatory and inhibitory monaural projections to MOC neurons are intact in the wedge slice, and their integration can be measured at an MOC neuron with the precision of patch-clamp electrophysiology. As a whole, this activation scheme provides a more intact circuit to the MOC neurons compared to a typical brain slice preparation. This brainstem wedge slice can also be used to investigate other auditory areas which receive inhibitory input from ipsilateral MNTB including the lateral superior olive, superior olivary nucleus and medial superior olive[10,11,52–56]. Beyond our specific preparation, this slicing method can be used or modified to evaluate other systems with the benefits of maintaining connectivity of long-range inputs and improving visualization of neurons for a variety of single-cell resolution electrophysiology or imaging techniques.

This protocol requires the use of a vibratome stage or platform which can be tilted approximately 15°. Here we use a commercially available 2-piece magnetic stage where the "stage" is a metal disc with a curved bottom placed in a concave magnetic "stage base." The stage can then be shifted to adjust the slice angle. Concentric circles on the stage base are used to estimate the angle reproducibly. The stage and stage base are placed in the slicing chamber, where the magnetic stage base can also be rotated.

**PROTOCOL:**

All experimental procedures were approved by the National Institute of Neurological Disorders and Stroke/National Institute on Deafness and Other Communication Disorders Animal Care and Use Committee.

**1. Experimental preparations**

NOTE: Details regarding slice preparation including slicing solution, slicing temperature, slice incubation temperature and apparatus (etc.) are specific for brainstem preparation performed in this experiment. Slice incubation details can be altered per laboratory experience.

1.1. Prepare internal solutions for patch-clamping.

1.1.1. Prepare voltage clamp containing (in mM) 76 Cs-methanesulfonate, 56 CsCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 Na-GTP, 2 Mg-ATP, 5 $Na_2$-phosphocreatine, 5 QX-314, and 0.01 Alexa Fluor-488 hydrazide. Adjust the pH to 7.2 with CsOH.

1.1.2. Prepare current clamp solution containing (in mM) 125 K-gluconate, 5 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 1 EGTA, 0.3 Na-GTP, 2 Mg-ATP, 1 $Na_2$-phosphocreatine, and 0.01 Alexa Fluor-488 hydrazide. Adjust the pH to 7.2 with KOH.

1.2. Prepare 100 mL of 4% agar by adding 4 g of agar to 100 mL hot (near boiling) water. Place on heated stir plate to maintain temperature and stir until completely dissolved. Pour into 100 mm plastic Petri dishes to approximately 1 cm depth and let cool. Refrigerate until needed.

1.3. Prepare 1 L artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 1.2 $CaCl_2$, 1.3 $MgSO_4$, 5 KCl, 26 $NaHCO_3$, 1.25 $KH_2PO_4$, and 10 dextrose. Bubble with carbogen (5% $CO_2$ / 95% $O_2$) for at least 10 min, then adjust final pH to 7.4 with 1 M NaOH if needed. Maintain oxygenation and pH of solution by bubbling continuously with carbogen throughout experiment.

1.4. Prepare 200 mL slicing solution by adding 1 mM kynurenic acid to ACSF. Sonicate solution in a sonicating water bath for 10 min until kynurenic acid is dissolved. Continuously bubble with carbogen and place on ice.

CAUTION: Use appropriate personal protective equipment when handling kynurenic acid.

1.5. Mount an appropriate blade in the vibratome following the manufacturer's instructions. Chill vibratome slicing chamber by surrounding it with ice.

**2. Brain removal with intact auditory nerve root for stimulation**

NOTE: Mice for these experiments were obtained by crossing ChAT-IRES-Cre transgenic mice on a C57BL/6J background with tdTomato reporter mice (Ai14). Mice used for histology and electrophysiology were post-hearing onset (P14-P23), which is around P12 in mice. Neurons expressing tdTomato in the ventral nucleus of the trapezoid body (VNTB) have been previously

characterized as MOC neurons in this mouse line[57].

2.1. Euthanize (e.g., $CO_2$ asphyxiation) and decapitate the animal using approved institutional procedures.

2.2. Using a razor blade, cut the skin at the midline of the skull from the nose to the back of the neck. Peel back skin to expose the skull.

2.3. Using small scissors, make an incision in the skull through the midline starting at the base (caudal end near spinal cord) of the skull and continuing towards the nose.

2.4. At the lambda suture, make cuts in the skull from the midline, lateral toward the ear on both sides. Peel back the skull to expose the brain.

2.5. Starting at the rostral end, gently lift the brain away from the skull with a small lab spatula or blunt forceps. Cut the optic nerve and continue to gently work the brain backwards, exposing the ventral surface.

2.6. Cut the trigeminal nerves by pinching them with fine forceps near the ventral surface of the brainstem.

NOTE: Do this carefully as the vestibulocochlear nerve lies just below this and needs to be intact for eventual stimulation.

2.7. Place the preparation in a glass Petri dish filled with cold slicing solution. Place the dish under a dissecting microscope. Gently bubble with carbogen.

2.8. Trim the facial nerve close to the brainstem and expose the vestibulocochlear nerve.

2.9. Using fine forceps, push the tips into the foramina where the vestibulocochlear nerve exits the skull as far as possible and pinch the nerve to sever it, leaving the nerve root attached to the brainstem. Repeat this on the other side.

2.10. Once both nerve roots are free, remove the meninges and vasculature from the ventral surface of the brainstem near the trapezoid body.

2.11. Free the brain completely from the skull by pinching the remaining cranial nerves and connective tissue taking care to preserve the remaining spinal cord if possible.

**3. Block and mount brain on stage (magnetic disc)**

3.1. Prepare the surface of the brain to fix to the stage by blocking the brain at the level of the optic chiasm.

3.1.1. With the ventral surface up, stabilize the brain using a blunt tool to gently immobilize the spinal cord so that the brain does not tilt during the following step.

3.1.2. At the level of the optic chiasm, use open forceps to create the plane for blocking the brain by inserting through the brain down to the bottom of the dish. Insert the forceps at an angle of approximately 20˚ from vertical so that the tips exit the dorsal surface of the brain caudal to the optic chiasm.

3.1.3. Cut along the forceps using the razor blade.

3.2. Glue the brain to the surface of the stage.

3.2.1. Prepare a small block (~1 $cm^3$) of 4% agar for supporting the brain.

3.2.2. Place a small drop of glue on the stage and spread it into a rectangle so both the brain and agar block can be glued down.

3.2.3. Using forceps, carefully lift the brain and gently dab the excess liquid using the edge of a paper towel. Place the blocked surface onto the glue, ventral surface will be towards the blade during slicing.

3.2.4. Push the agar block gently against the dorsal surface of the brain to support it during slicing and to ensure proper brain positioning (i.e., angle).

**4. Slice brain to create wedge slice**

NOTE: Prepare a brain slice using vibratome that has the cochlear nerve root on the thick side and medial olivocochlear (MOC) neurons and the medial nucleus of the trapezoid body (MNTB) on the thin side.

4.1. Place the magnetic disc with attached brain onto the stage holder and place it in the slicing chamber with the ventral surface of the brain oriented towards the blade.

4.2. Fill the chamber with ice cold slicing solution and bubble with carbogen.

4.3. Lower the blade into the solution and cut slices caudal to the region of interest to make sure the slices are symmetrical. If the slices appear asymmetrical, tilt the stage slightly to obtain symmetry.

NOTE: Blade speeds between 0.05-0.10 mm/s were effective for cutting healthy slices and may vary depending on animal age and brain region.

4.4. Once the slices are symmetrical, shift the stage ~15˚ (corresponding to approximately 3 concentric rings on the stage base) to one side.

NOTE: Shift the stage away from the auditory nerve root that you want to preserve in the slice.

4.5. Continue slicing carefully until the auditory nerve root is close to the surface on one side, and the facial nerve can be seen at the surface of the other side.

4.6. Shift the stage back 15˚ to the original position.

4.7. Move the blade away from the tissue and spin the stage base 90° so that the lateral edge of the thin side is facing the blade. Lower the blade several hundred microns and then slowly bring the blade close to the edge of the tissue. Repeat this until the blade touches the lateral edge. Lower the blade to the desired thickness of the thin edge of the slice, here an additional two hundred microns.

NOTE: The resulting slice is ideally ~300 μm thick at the level of the ventral nucleus of the trapezoid body (VNTB) on the side where patch clamping will take place.

4.8. Move the blade back away from the tissue and spin the stage base back so that the ventral surface is facing it.

4.9. Make the cut that designates the rostral surface of the wedge slice. Transfer the slice to a piece of interface paper (1 $cm^2$) caudal surface down. Move the slice to the incubation chamber or other suitable incubation apparatus for recovery (30 min at 35 °C).

NOTE: The facial nerve should be visible on both hemispheres of the slice on the rostral surface (see **Figure 1B**).

**5. Electrophysiology set-up and recording**

5.1. Place the wedge slice in the recording chamber and secure slice with a harp or stabilizing system. Perfuse the tissue continuously at a rate of 7-10 mL/min with warm (35 °C) ACSF bubbled with carbogen.

5.2. Identify genetically labeled MOC neurons in the VNTB using epifluorescence with 561 nm emission filters for patch-clamp recordings. Flip slice if there are no potentially patchable cells.

5.3. Using DIC optics, focus on the auditory nerve root on the thick side of the slice and use a micromanipulator to move the bipolar tungsten stimulating electrode down to the auditory nerve root and gently into the surface of the tissue.

NOTE: Suction electrodes have been used in auditory nerve stimulation experiments in other labs. Theta glass electrodes, or optical stimulation methods can be employed if applicable to other specific preparations.

5.4. Move the field of view back to the VNTB to choose an MOC neuron to target for patch clamp electrophysiology.

5.5. Fill a recording pipette with appropriate internal solution for the proposed experiment.

5.6. Patch and record from the MOC neuron in the whole-cell configuration. Compensate membrane capacitance and series resistance if required.

5.7. Adjust electrical stimulation amplitude of the auditory nerve root to obtain consistent postsynaptic events in the MOC neuron.

NOTE: It may be necessary to move the stimulation electrode.

5.8. Run appropriate stimulation protocols to observe evoked synaptic currents in MOC (voltage clamp) or action potential patterns (current clamp).

NOTE: The wedge slice preparation can be used with any typical patch-clamp tools such as loose patch recordings, pharmacology, optogenetics, calcium imaging, neurotransmitter uncaging, etc.

**6. Histological confirmation of brainstem nuclei**

NOTE: This is done with cresyl violet staining, in fixed, re-sectioned wedge slice. This method allows for visualization of nuclei which are contained in the slice.

6.1. After preparing a wedge slice, submerge slice in fixative (4% PFA in PBS) overnight. Rinse the slice 3x for 10 min in PBS (room temperature on a shaker), then place in 30% sucrose in PBS overnight at 4 °C to cryoprotect.

6.2. Re-section the slice on a freezing microtome (40-70 μm) and collect serial sections in a 24 well plate in PBS.

6.3. Mount sections on gelatin coated slides and let dry completely. Place slides in slide carriage.

6.4. Prepare cresyl violet solutions

6.4.1. Prepare 1% cresyl violet acetate by mixing 5 g cresyl violet acetate in 500 mL $dH_2O$

6.4.2. Prepare acetate buffer by first preparing 90 mL solution A (540 μL glacial acetic acid + 89.46 mL $dH_2O$) and 10 mL solution B (136 mg sodium acetate in 10 mL $dH_2O$). Combine solution A and solution B yielding the acetate buffer.

6.4.3. Combine 1% cresyl violet acetate with the acetate buffer 1:1 for 0.5% cresyl violet in acetate buffer. Filter before use.

6.4.4. Prepare 95% and 70% ethanol by diluting 100% ethanol with appropriate volumes of $dH_2O$

6.5. Perform cresyl violet staining protocol. Move the slide carriage through solution trays, blotting excess solution on a paper towel between trays: xylene – 5 min; 95% ethanol – 3 min; 70% ethanol – 3 min; $dH_2O$ – 3 min; 0.5% cresyl violet solution – 8-14 min monitoring frequently until nuclear staining becomes dark purple; $dH_2O$ – 3 min; 70% ethanol – 3 min; 95% ethanol – 1-2 min; 100% ethanol – dip slides twice; xylene – 5 min; xylene: 25 min until mounting is performed.

CAUTION: Use xylenes only under a fume hood.

6.6. Remove slides from xylene one at a time and immediately place cover slips on slides using mounting medium. Allow mounting medium to dry (overnight).

6.7. Image sections.

**7. Biocytin labeling for anterograde tracing of axons in live, unfixed tissue**

7.1. Prepare a wedge slice as above (Steps 2-4)

7.2. Transfer the slice to interface paper (~1 $cm^2$). Under a dissecting microscope, locate the CN on the thick side of the slice.

7.3. Carefully remove excess ACSF from the area surrounding the slice by twisting up a corner of a tissue paper to draw the ACSF away from the tissue. This prevents the biocytin from spreading to surrounding areas of the slice which could lead to uptake into cells outside the CN.

7.4. With fine forceps, select a small crystal of biocytin and place it on the surface of the CN. Gently press the crystal into the tissue to promote contact with neurons and subsequent uptake into somata. Repeat this step to cover the desired region of interest, in this case CN regions containing T-stellate and GBC neurons.

7.5. Place the slice in an incubation chamber. Allow the slice to incubate for 2-4 h at 35 °C to allow for the uptake and transport of the biocytin. After incubation, rinse the slice in ACSF to remove any biocytin particles.

7.6. Place slice in fixative (4% PFA in PBS) overnight. Rinse 3x for 10 min in PBS.

7.7. Cryoprotect slice in 30% sucrose in PBS overnight at 4 °C or until the slice sinks.

7.8. Resect the tissue to produce transverse sections on a freezing microtome at 70-100 μm.

7.9. Process tissue using standard immunohistochemical methods with a fluorescently conjugated streptavidin.

NOTE: Additional immunohistochemistry can be performed on the sections if helpful for labeling presynaptic cell bodies, axons, receptors, or other synaptic molecules important for circuit visualization (i.e., primary antibody steps should not adversely affect biocytin secondary visualization).

7.10. Image the tissue.

**REPRESENTATIVE RESULTS:**

**Histological examination of wedge slice**

For our investigation of auditory brainstem neuron function, the wedge slice preparation was designed to contain the auditory nerve root and CN contralateral to the MOC neurons targeted for recordings (example slice shown in **Figure 1B**). Initial histological examination of the preparation is important to confirm that the slice contains the nuclei necessary for circuit activation and that axonal projections are intact. Two cell types within the CN provide sound information to MOC neurons. T-stellate cells are hypothesized to provide the excitatory input to MOC neurons[39–41,58]. Globular bushy cells (GBC) excite MNTB neurons in the contralateral hemisphere (via the specialized calyx of Held synapse)[34,36–38,59,60] which, in turn, provide inhibitory input to MOC neurons[57] (schematic **Figure 1A**). To confirm the presence of both T-stellate cells and GBCs, we re-sectioned (to 50 μm) a wedge slice that was fixed by submersion in 4% PFA and performed cresyl violet staining to label somata. In the thick side of the wedge slice (**Figure 2**, left hemisphere), the CN was present in nearly its full rostro-caudal extent. Additionally, the dorsal and ventral subdivisions of the CN were intact (**Figure 2**; arrow and arrowhead in S19). T-stellate neurons and GBCs cluster in the ventral cochlear nucleus near where the auditory nerve (**Figure 2**; arrow in S17) enters the CN[61–65]. The wedge slice also contains neurons of the MNTB ipsilateral to MOC neurons from which recordings are performed (thin hemisphere of the original wedge slice, right side in **Figure 2**). This confirms that at least part of the inhibitory input to MOC neurons is intact (**Figure 2**, slices 1-15, highlighted by dashed ovals in S11).

In separate experiments, we confirmed that the axons and presynaptic terminals of CN neurons were intact in the wedge slice using anterograde labeling with biocytin. First, the live wedge slice was prepared and placed on interface paper. Immediately after preparing the wedge slice, biocytin crystals were placed in the CN which allowed uptake and anterograde transport along axons during an incubation period. Then fixing and re-sectioning of the tissue (70 μm sections) was performed. Staining of sections with fluorescently labeled streptavidin was performed to visualize axons labeled with the biocytin. Confocal images of these sections show bright labeling in the CN where the crystals were placed and taken up into cell bodies (**Figure 3A**, left hemisphere, dashed area). Axons exiting the CN along the ventral acoustic stria (**Figure 3A**, white arrowheads) were clearly labeled and could be followed to their termination points. Biocytin-positive puncta surrounding contralateral MOC neurons suggest our preparation preserves synaptic contacts originating from the CN (**Figure 3B**). Likewise, labeled calyces of Held in the

contralateral MNTB indicate axons projecting from GBCs to MNTB neurons are preserved in the wedge slice (**Figure 3C**). These histological examinations confirm our wedge slice contains both the cell bodies and axonal projections of the afferent input circuitry to MOC neurons, which, therefore, allows us to measure postsynaptic responses evoked by stimulation of the auditory nerve and subsequent propagation of activity through ascending circuitry.

**Synaptic physiology in wedge slice**

Integration of excitatory and inhibitory synaptic inputs critically shapes neuronal activity. We recently described inhibitory inputs to MOC neurons from neurons of the MNTB[57], but the effect of integration of these inputs with excitatory inputs on MOC neuron activity is unknown. In a wedge slice from a ChAT-IRES-Cre x tdTomato mouse, voltage-clamp recordings were performed from an MOC neuron. Current was applied via a bipolar tungsten stimulating electrode driven by a stimulus isolation unit to evoke neurotransmitter release from presynaptic axons. First the ventral acoustic stria (VAS) at the midline was electrically stimulated to activate T-stellate axons directly and MNTB neurons via GBC axon stimulation, to measure the latency to post-synaptic responses in a recording configuration that mimics typical thin-slice experiments (**Figure 4B**, example traces, grey, holding potential -60 mV). In separate experiments, the auditory nerve root was stimulated to activate monaural ascending circuitry and measured post-synaptic responses at MOC neurons as described above. Electrical stimulation in either location evoked a fast-electrical artifact followed by multipeaked current responses (example responses from AN stimulation in **Figure 4C**, black traces, holding potential -60 mV). We compared onset latency measures of the first postsynaptic current (PSC) evoked with direct stimulation of the VAS with those evoked with auditory nerve stimulation and found a significantly longer latency in AN stimulation event. This was attributed to the synaptic delay incurred at the AN/CN synapse (AN stimulation: 5.27 ± 0.43 ms, median ± median absolute deviation (MAD), range 4.26-5.93 ms, n = 8; VAS stimulation: 1.98 ± 0.28 ms, median ± MAD, range 0.75-3.46 ms, n = 17; Wilcoxon Signed Ranks Test, $p$ = 0.014, **Figure 4D**). These results confirm that stimulation of the auditory nerve root results in synaptic activation of CN neurons and subsequent circuit activity, more closely representing in vivo – like timing than direct stimulation of T-stellate or GBC/MNTB axons.

With our cesium based, high [$Cl^-$] internal solution used in voltage clamp, excitatory (glutamatergic) and inhibitory (GABA and glycinergic) PSCs are both inward at resting membrane potential (-60 mV) and therefore indistinguishable. While evoking circuit activity in the AN-stimulating configuration, we electrically isolated the presumed inhibitory input by shifting the holding potential to 0 mV, the approximate reversal potential for AMPA mediated glutamatergic currents. In our example neuron, outward current responses were observed at 0 mV (**Figure 4Ci**, red traces) indicative of chloride conductances. These are likely to be GABA- or glycinergic synaptic responses. These data demonstrate the utility of the wedge slice to activate both excitatory and inhibitory inputs to MOC neurons by stimulating the auditory nerve root, with activation of subsequent afferent circuitry. Further, diverse patterns of post-synaptic responses were evoked by AN stimulation, suggesting that even under conditions of identical stimulation of AN axons, activity of the entire circuit is dynamic and complex. This experimental paradigm allows for a detailed analysis of how complex auditory stimuli propagate through the brainstem and integrate at MOC neurons, determining the MOC efferent system's output and eventual

impact on the cochlea.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Wedge slice schematic and example image.** (**A**) Schematic of the medial olivocochlear feedback circuit. Blue arrows indicate the afferent ascending pathway to MOC neurons and black arrows indicate the descending feedback pathway from MOC neurons to the base of outer hair cells (OHC). (**B**) Brightfield image of a wedge slice with labels of the auditory nerve root (ANR) and cochlear nucleus (dashed outline) on the thick side. Asterisk indicates the approximate location of the ventral nucleus of the trapezoid body where MOC neurons are targeted for patch-clamping on the thin side of the wedge slice. Dashed black lines indicate the facial nerves which can be seen in both hemispheres of the slice on the rostral surface. IHC - inner hair cell, GBC - globular bush cell, SPN - superior paraolivary nucleus, MNTB - medial nucleus of the trapezoid body, VNTB - ventral nucleus of the trapezoid body, LSO - lateral superior olive.

**Figure 2: Cresyl violet stained sections from a wedge slice that was re-sectioned at 50 µm.** Every other section was imaged. Sections are numbered rostral --> caudal. Wedge slices tended to contain the entirety of the cochlear nucleus (CN) including both the dorsal CN (arrow in S19) and ventral CN (arrowhead in S19), auditory nerve root (open arrowhead in S17) and much of the MNTB (dark area near ventral surface in S3-S15, highlighted with dashed ovals in S11). D and V on scale bar represent dorsal and ventral in slice orientation.

**Figure 3: Axons of ascending input to MOC neurons from the contralateral cochlear nucleus remain intact in the wedge slice.** (**A**) The rostral-most section from a wedge slice taken from a P23 ChAT-IRES-Cre x tdTomato (red fluorescence) mouse that was re-sectioned (70 µm) and processed for biocytin visualization. Confocal image is a tiled, maximum intensity projection z-stack. Axons in the ventral acoustic stria are highlighted by white arrowheads. Dashed outline indicates the small portion of the cochlear nucleus remaining in this rostral-most slice. Scale bar 500 µm. (**B**) Confocal image of a ChAT-IRES-Cre x tdTomato positive neuron in the VNTB with biocytin positive puncta in the surrounding neuropil. Scale bar 50 µm. (**C**) Biocytin labeled axons shown crossing the midline and terminating in the contralateral MNTB as calyces of Held. Vertical dashed line represents the midline of the slice. Scale bar 100 µm.

**Figure 4: Electrical stimulation of afferent inputs in voltage clamp yields multipeak postsynaptic currents in MOC neurons.** (**A**) Schematic of wedge slice with recording set-up for both ventral acoustic stria (VAS) stimulation (grey stimulating electrode) and auditory nerve (AN) stimulation (black stimulating electrode) of afferent inputs to MOC. (**B**) Examples of postsynaptic currents (PSCs) from an individual P17 neuron evoked with a single electrical stimulus near the midline at -60 mV. (**C**) PSCs evoked during AN stimulation at -60 mV in a P15 neuron. (**Ci**) Example PSCs in the same cell as C evoked at 0 mV holding potential (the approximate reversal potential for AMPA mediated currents in our recording setup, red). (**D**) Population data for quantification of latency to first PSC for VAS and AN stimulation. Boxes: quartiles, line inset: median, square inset: mean, whiskers: mean absolute deviation. * $p < 0.05$.

**DISCUSSION:**

The slicing procedure described here termed a wedge slice is powerful for maintaining intact presynaptic neuronal circuitry, but with the accessibility of brain slice experimentation for analysis of neuronal function. Great care must be taken in several initial steps in order to maximize utility of the preparation for circuit analysis. The dimensions of the wedge should be confirmed using histological examination, which is integral for confirmation that both presynaptic nuclei and their axonal projections are contained within the prepared wedge slice. Slice geometry may require modification if projections are severed or few axons reach the target nuclei. More generally, the finishing cut on the vibratome for the wedge slice is critically important. Optimal wedge slice preparation will require a combination of consistent use of vibratome configurations including use of concentric circle markings on the stage base, along with adjustment of settings based on known brain landmarks. After optimization of slice geometry, we recorded consistent PSCs in MOC neurons evoked by electrically stimulating the auditory nerve root in 8 of 18 wedge slices. In our previous work we were able to evoke inhibitory PSCs via direct stimulation of MNTB axons in approximately 60% of MOC neurons[57], suggesting that our success rate here is only a modest reduction given the long range of the inputs and necessity for polysynaptic circuit activation. When preparing the slice, it is advisable to err on the thicker side as a decrease in visibility due to a thicker slice is favorable over an unusable section which is incomplete or lacks circuit connectivity. Any slice configuration or dimensions can be used, as long as the slice fits under the recording microscope objective, is accessible by patch and stimulating electrodes (or other probes or equipment), and is thin enough for optically-based patch-clamping at the postsynaptic cell of interest. Rapid, gentle dissection and proper incubation and recovery conditions are also important to maintain viability of the circuit for patch-clamp experiments. Specific to our auditory brainstem preparation, the brain must be removed very carefully from the skull in order to preserve intact and functional auditory nerve roots. Stretching or tearing the nerve will impact the ability to stimulate the fibers and elicit activity in auditory neurons. Due to the larger volume of tissue in the slice, modification of traditional slicing solutions, temperatures, incubation details, and perfusion systems may improve the health of the slice. Here we employ slight modifications to our normal slice preparation. These include shorter recovery incubation times (30 minutes vs. 60 minutes) and faster flow rates in the slice chamber perfusion system.

Once the slice dimensions and incubation details have been determined, the function and connectivity of different components of circuitry within the slice should be demonstrated. In our preparation, we ensure that both excitatory and inhibitory inputs, hypothesized to originate in the cochlear nucleus with T-stellate and GBC (via the MNTB), are present as expected. Alternative stimulation methods such as suction electrodes for the auditory nerve, or optical stimulation methods such as optogenetics or focal neurotransmitter uncaging may also increase circuit activation or allow for cell-type specific activation when paired with genetic targeting of cell subtypes.

While this slicing method will hopefully be useful for many systems and circuits, some of the limitations of standard thin-slice sections are also relevant to this preparation. Generally, it may be difficult to preserve circuits with less planar projection patterns, as axons would likely be severed. Activating the circuit at the cranial nerves, as done here to mimic auditory inputs, may

not be feasible in many circuits. As with other slice preparations, the network effects of any pharmacology must be considered. For example, bath application of glutamate receptor blockers to isolate inhibitory (GABA- or glycinergic) or other modes of transmission cannot be used with polysynaptic circuit activation when glutamate is necessary for activation of neurons upstream of the patched target neuron. This is true in our case as both AN/CN and GBC/MNTB synapses are glutamatergic, therefore, all transmission would be eliminated at MOC neurons with bath application of glutamate receptor blockers. Additionally, application of GABA or glycine receptor blockers to eliminate MNTB-MOC synaptic responses would have the unintended consequence of eliminating intrinsic inhibitory connectivity within the CN that may shape the patterns of afferent inputs to MOC neurons. Focal application of receptor blockers, with pressure ejection or iontophoresis, could be used to restrict pharmacological function.

Finally, the main limitation of this, and any, in vitro technique is that although this preparation maximizes activation of monaural ascending auditory circuitry, the rest of the nervous system, including peripheral receptors that encode stimuli, is absent. This includes the cochlea itself, excitatory inputs from the other ear[66], commissural CN connections[67–70], and descending cortical[71–74] and collicular[75, 76] projections and modulatory inputs[77–80] known to influence activity of CN and SOC nuclei. While it is possible that a portion of descending IC projections are maintained it would be impossible to include both cortical projections and commissural CN projections due to slice geometry. Hence, we focus on the ascending auditory circuitry from the cochlea with the current experiments. The minimal thickness of the slice on the thin side also reduces the ability to perform binaural polysynaptic circuit analyses, which is an advantage of symmetrical thick slice preparations[10,11]. Additionally, we are unable to stimulate the auditory nerve with sound to evoke natural patterns of circuit activity. Auditory nerve responses are tonotopically varied, jittery, and plastic[81–84], making it difficult to perfectly simulate with our electrical stimulation method. This is a major drawback of in vitro experimentation in the auditory system. Tonotopic restriction of our stimulation is not possible since stimulating the entire AN root will elicit spiking in AN fibers across the tonotopic gradient. Accurately mimicking the diversity of AN fiber responses (i.e., low vs. high spontaneous rate fibers) to an electrical stimulus pattern is also not possible. It is also difficult to precisely match the dynamic intensity coding of multiple AN fibers at the CN. However, we are able to use our electrophysiology software to produce a variety of stimulation patterns aimed to mimic appropriate auditory nerve output during different acoustic stimuli (e.g. short, loud sounds, quiet, prolonged sounds or sounds in background noise) by varying the stimulus frequency both between electrical stimulus protocols and also within an individual protocol to approximate how combined AN inputs would look (modeled in ref.[85]). Monitoring MOC output during these experiments will test our hypotheses regarding what stimulus patterns may favor inhibition or excitation at MOC neurons.

Despite the limitations described above, a wedge slice preparation method has benefits compared to in vivo and typical in vitro slice physiology methods and can be used to approach in vivo circuit activation as closely as possible in the slice for cells that are difficult to access. In vivo whole-cell recordings in the auditory brainstem have been rare due to difficulties accessing this area surgically[86]. Instead the slice was prepared to include ascending inputs to MOC neurons beginning with the auditory nerve, which is stimulated directly to activate the entire monaural

ascending circuit. We demonstrate activation of both excitatory and inhibitory synaptic inputs, and responses to these inputs provide valuable information about timing of synaptic inputs as they reach the MOC neurons. This provides a platform for high throughput experimentation where we can employ a large repertoire of in vitro electrophysiology tools such as calcium or voltage imaging, neurotransmitter uncaging, and both intracellular (via the patch pipette) and extracellular (via bath application or iontophoresis) pharmacology. The preparation should also offer an increase in throughput over thick slice preparations due to better visibility of target neurons using DIC optics, which blur with increased tissue thickness, especially in the ventral brainstem. Overall, this technique provides improvements in targeting and throughput over in vivo methods, and better opportunities for circuit analysis than traditional slice physiology methods.

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The authors have nothing to disclose.

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Figure 1

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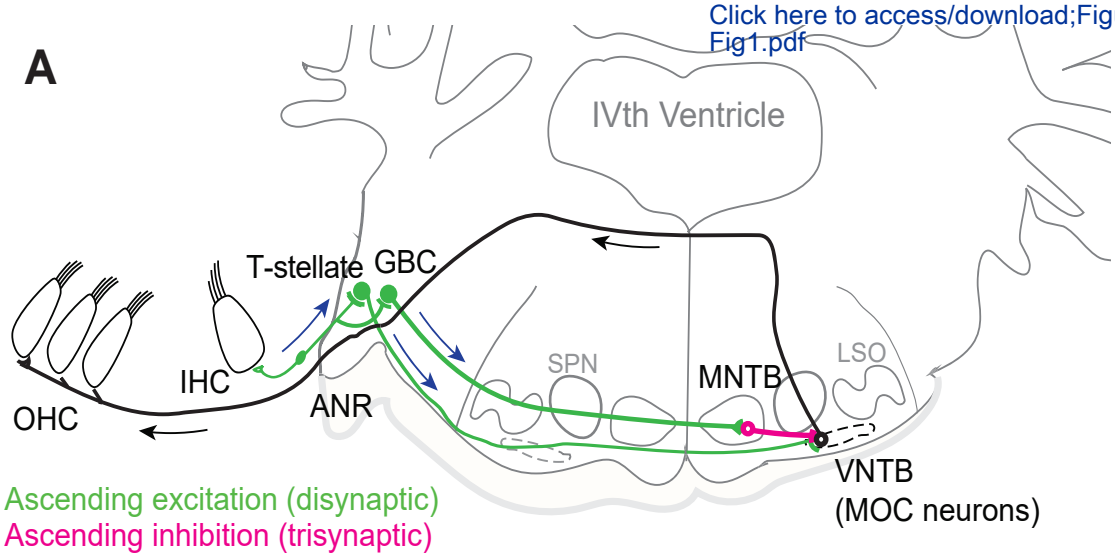


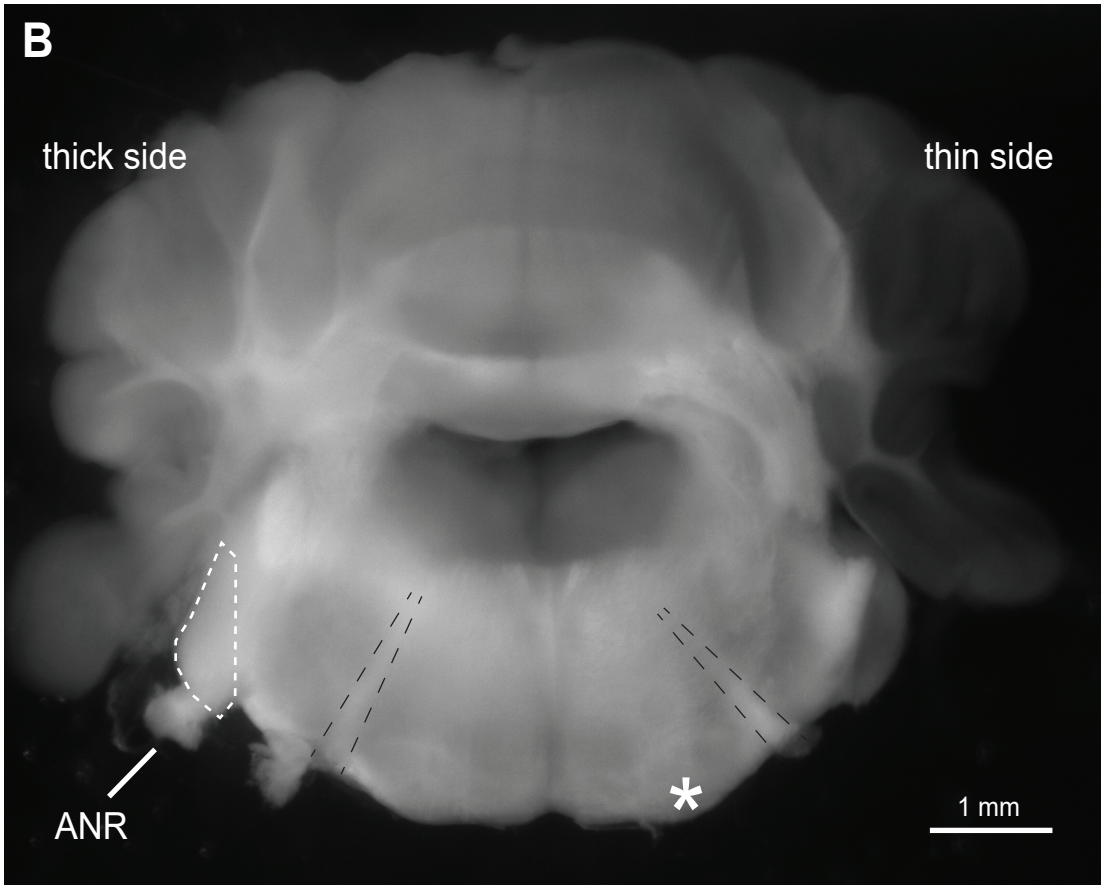



Figure 2



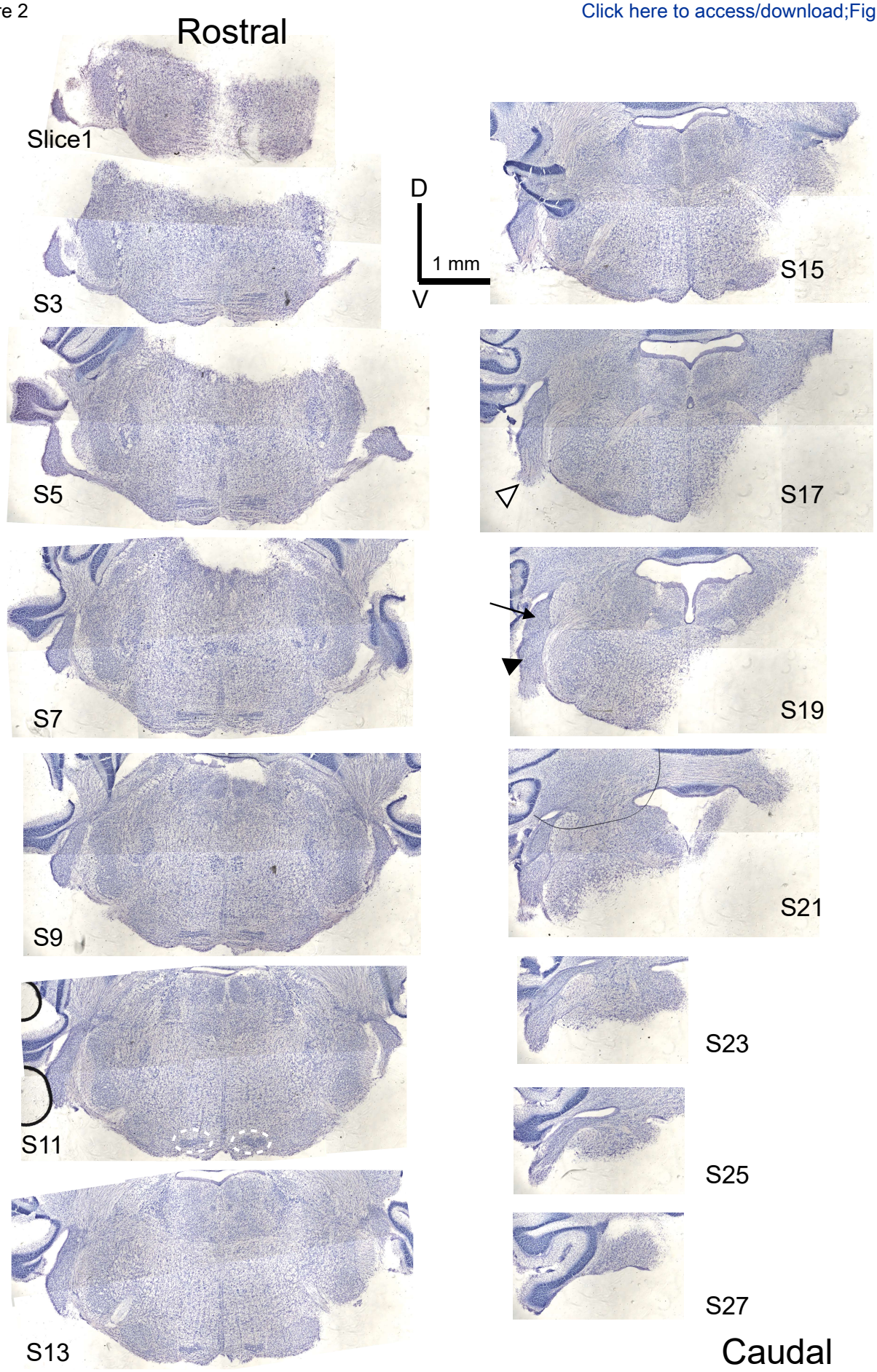


Figure 3



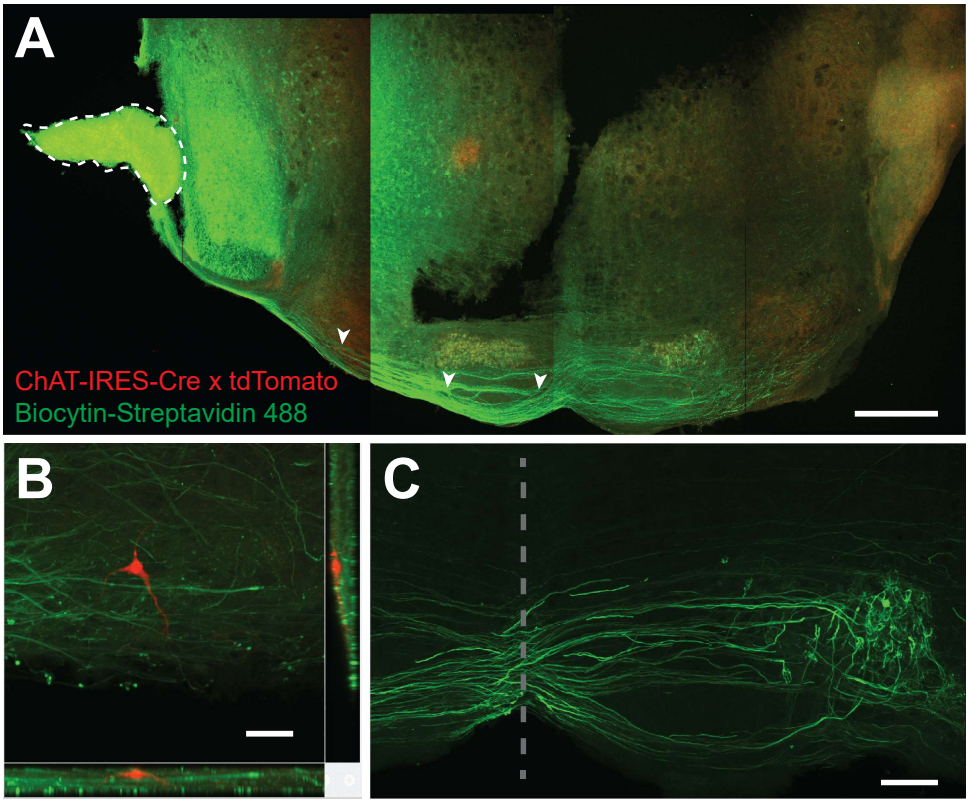
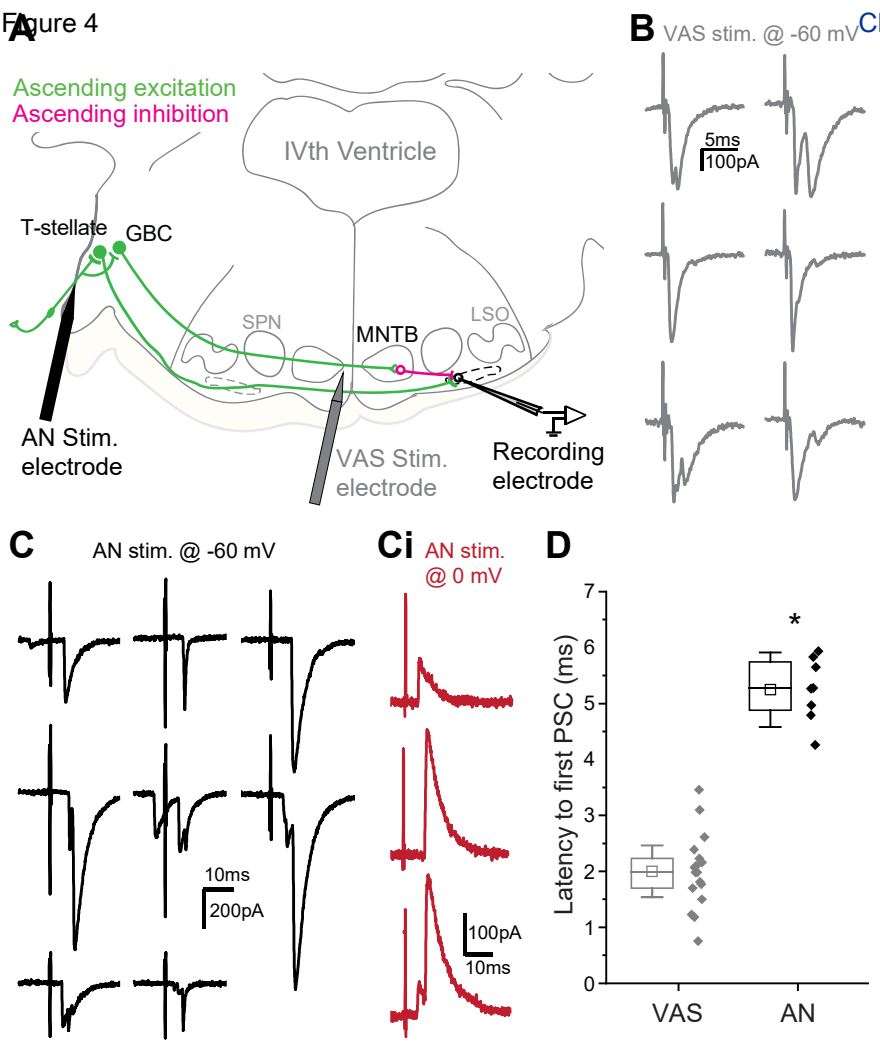


Figure 4





| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| **Experimental Preparations** | | |
| Agar, powder | Fisher Scientific | BP1423500 |
| AlexaFluor Hydrazide 488 | Invitrogen | A10436 |
| Analytical Balance | Geneses Scientific (Intramalls) | AV114 |
| Double edged razor blade | Ted Pella | 121-6 |
| Kynurenic acid (5g) | Sigma Aldrich | K3375-5G |
| pH Meter | Fisher Scientific (Intramalls) | 13-620-451 |
| Plastic petri dishes 100mm dia X 20mm | Fisher Scientific (Intramalls) | 12-556-002 |
| Stirring Hotplate | Fisher Scientific (Intramalls) | 11-500-150 |
| | | |
| **Dissection and Slicing** | | |
| Biocytin | Sigma Aldrich | B4261-250MG |
| Dissecting Microscope | Amscope | SM-1BN |
| Dumont #5 Forceps | Fine Science Tools | 11252-20 |
| Economy tweezers #3 | WPI | 501976 |
| Glass Petri Dish 150mm dia x 15mm H | Fisher Scientific (Intramalls) | 08-747E |
| Interface paper (203 X 254mm PCTE Membrane 10um) | Thomas Scientific | 1220823 |
| Leica VT1200S Vibratome | Leica | 1491200S001 |
| Mayo scissors | Roboz | RS-6872 |
| Single-edged carbon steel blades | Fisher Scientific (Intramalls) | 12-640 |
| Specimen disc, orienting | Leica | 14048142068 |
| Spoonula | FisherSci | 14-375-10 |
| Super Glue | Newegg | 15187 |
| Vannas Spring Scissors | Fine Science Tools | 91500-09 |
| | | |
| **Electrophysiology** | | |
| A1R Upright Confocal Microscope | Nikon Instruments | |
| Electrode Borosilicate glass w/ Filament OD 1.5mm, ID 1.1 | Sutter Instrument | BF150-110-10 |
| Electrode Filler MicroFil | WPI | CMF20G |
| In-line solution heater | Warner Instruments (GSAdvantage) | SH-27B |
| Multi-Micromanipulator Systems | Sutter Intruments | MPC-200 with MP285 |
| P-1000 horizontal pipette puller for glass micropipettes | Sutter instruments | FG-P1000 |
| Patch-clamp amplifier and Software | HEKA | EPC-10 / Patchmaster Next |

| | | |
|---|---|---|
| Recording Chamber | Warner Instruments | RC26G |
| Recording Chamber Harp | Warner Instruments | 640253 |
| Slice Incubation Chamber | Custom Build | |
| Stimulus isolation unit | A.M.P.I. | Iso-Flex |
| Syringe 60CC | Fischer Scientific (Intramalls) | 14-820-11 |
| Temperature controller | Warner Instruments (GSAdvantage) | TC-324C |
| Tubing 1/8 OD 1/16 ID | Fischer Scientific (Intramalls) | 14-171-129 |
| Tugsten concentric bipolar microelectrode | WPI | TM33CCINS |

**Histology**

| | | |
|---|---|---|
| 24 well Plate | Fisher Scientific (Intramalls) | 12-556006 |
| Alexa Fluor 488 Streptavidin | Jackson Immuno labs | 016-540-084 |
| Corning Orbital Shaker | Sigma | CLS6780FP |
| Cresyl Violet Acetate | Sigma Aldrich (Intramalls) | C5042-10G |
| Disposable Microtome Blades | Fisher Scientific | 22-210-052 |
| Filter-syringe Nalgene 4mm Cellulose Acetate 0.2um | Fisher Scientific (Intramalls) | 09-740-34A |
| Fluoromount-G Slide Mounting Medium | Fisher Scientific | OB100-01 |
| glass slide staining dish with rack | Fisher Scientific (Intramalls) | 08-812 |
| Microm HM450 Sliding Microtome | ThermoFisher | 910020 |
| Microscope Cover Glasses: Rectangles 50mm X 24mm | Fisher Scientific (Intramalls) | 12-543D |
| Permount mounting medium | Fisher Scientific | SP15-100 |
| Superfrost Slides | Fisher Scientific | 22-034980 |

**Comments/Description**

4% agar block used to stabilize brain tissue during vibratome sectioning
Fluorophore used in internal solution to confirm successful MOC neuron patch
Weighing chemicals
Vibratome cutting blade
Slicing ACSF additive used to reduce neuron activity during dissection and slicing in order to improve tissue health for patch clamping
Solution pH tester
4% Agar Prep
Heating for 4% Agar preparation

Chemical used for axonal tracing (conjugated to Streptavidin 488)
For precision dissection during brain removal
Fine forceps dissection tool
Forceps dissection tool
Dissection dish
Slice incubation/biocytin application
Vibratome for wedge slice sectioning
Dissection tool
Razor blade for dissection
Specialized vibratome stage for reproducible tilting
Dissection tool
Used for glueing tissue to vibratome stage
Dissection tool

Electrophysiology and imaging microscope, can be any microscope compatible with electrophysiology
Patch clamping pipette glass
Patch electrode pipette filler
Slice perfusion system heater
Micromanipulators for patch clamp and stimulation electrode placement
Patch clamp pipetter puller
Can be any amplifier/software

Slice "bath" during recording
Stablizes slice during electrophysiology recording
Heated, oxygenated holding chamber for slices during recovery after slicing
Stimulus isolation unit for electrophysiology
Electrophysiology perfusion fluid handling
Slice perfusion system temperature controller
Electrophysiology perfusion fluid handling
Stimulating electrode for electrophysiology

Histology slice collection and immunostaining
Secondary antibody for biocytin visualization
Shaker for immunohistochemistry agitation
Cellular stain for histology
Sliding microtome blade
Syringe filter for filling recording pipettes with internal solution
Immunohistochemistry fluorescence mounting medium
Cresyl Violet staining chamber
Freezing microtome for histology

Histochemistry slide cover glass
Cresyl violet section mounting medium
Histology slides

**Authors' responses are in bold**

**Comments from Peer-Reviewers:**

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.

NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

**Editorial Comments:**

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

**Thank you, we have done this.**

• **Abstracts:**

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write the text indicated in red in the attached document to avoid this overlap.

**After communicating with the editor, it was determined that this section does not apply to our manuscript, and we should disregard the comment**

• **Introduction:** Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

**Reviewers have indicated that our introduction and consideration of other techniques and literature is sufficient.**

• **Protocol Language:** Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

**Thank you, we have done this.**

• Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.

**Text added: line 125 "All experimental procedures were approved by the National Institute of Neurological Disorders and Stroke/National Institute on Deafness and Other Communication Disorders Animal Care and Use Committee."**

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

**Complete**

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

**Spaces have been added, and numbering adjusted where necessary**

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

**The portions of the protocol to be filmed have been highlighted.**

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

**We have ensured that the discussion is methods-focused**

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**Figures and tables have not been previously published**

**Reviewer #1:**

Manuscript Summary:

In this work Catherine Weisz and Matthew Fischl have developed a new slicing procedure termed "Wedge Slice". This new technique is a modified thick slice preparation designed for maintaining intact presynaptic neuronal circuitry with the accessibility of brain slice investigation for analysis of neuronal function. The technique consists in generating in vitro preparations that more closely resemble the circuit connectivity of in vivo recordings, but with the high performance and resolution recording abilities of patch-clamp electrophysiology in brain slices. It comprises an asymmetrical brain slice which is thickest in the rostro-caudal domain at the lateral edge of one hemisphere (containing the axons of the auditory nerve as they enter the brainstem and synapse in the cochlear nucleus) and then thins towards the lateral edge of the opposite side for optically-based patch-clamping at the postsynaptic cell of interest.

This new technique is very well designed and as a whole provides a more intact circuit compared to a typical brain slice. Results obtained are straightforward in showing the utility of the wedge slice to activate both excitatory and inhibitory inputs to medial olivocochlear neurons by stimulating the auditory nerve root, with activation of subsequent afferent circuitry.

Overall, the results obtained with this new technique are compelling and demonstrate that with the wedge slice authors can mimic in vivo circuit activation as closely as possible within the slice. Moreover, this technique can be applicable to other brain circuits.

Major Concerns:

I don't have any concerns.

**Thank you for your support of this technique and manuscript.**

**Reviewer #2:**

Manuscript Summary:

This is an interesting and mainly well-documented study that describes a seemingly minor, but potentially powerful variation of the brain slice method. By using a wedge rather than a uniformly thick coronal slice, the authors demonstrate (both anatomically and physiologically) that it is possible to preserve vital long range circuit elements and so study the brainstem inputs to neurons on the VNTB. Unusual slice configurations have been employed before (for example the Oertel parasagittal slice for maintaining cochlear nucleus circuitry) but this paper is a significant advance in allowing long range connections crossing the midline to be investigated. Though focused on the medial olivocochlear system neurons (the use of a transgenic animal with fluorescent marker is clever!), the technique could be applied to other parts of the brain where planar elements of circuitry are present. The authors provide a useful discussion of the limitations of the method (but see below).

**Thank you for your support of this technique, and for constructive comments to improve the manuscript.**

Major Concerns:

The authors provide a large amount of some rather trivial aspects of the method and these could be trimmed substantially.

**We understand that some details may seem trivial, but to appeal to a broader audience, we provide many specifics which may be necessary for readers with less experience in anatomy/histology or electrophysiology experimental methods. We try to attend to the specific omissions below.**

However there are some rather important elements left out.

1. What is the nature of the slice chamber itself, including flow rates and temperature?

**Per journal style, we have included the slice chamber manufacturer and part # in the materials list.**

My own experience of brainstem slices is that especially with thicker slices, flow rate and chamber design can be critical in maintaining good physiology.

**Thank you, we also use a faster flow rate compared to typical slice thicknesses thanks to advice from developers of thick slice preparations. We have not tested a different chamber design but will look into it. Thank you for the comment! We have amended the text to now read: line 529 "Due to the larger volume of tissue in the slice, modification of traditional slicing solutions, temperatures, incubation details, and perfusion systems may improve the health of the slice. Here we employ slight modifications to our normal slice preparation. These include shorter recovery incubation times (30 minutes vs. 60 minutes) and faster flow rates in the slice chamber perfusion system."**

2. The authors rightly mention in the introduction, that in more mature brains, myelination and reduced optical properties make patching very difficult even in thin slices. It seems that the thin edge of their preparation is 200-500um thick and so this limitation presumably still exists unless they are using neonatal brains. They should state what ages of animal they have used and whether this preparation only works well in immature brains.

**The data shown in figure 4 is from a P15 (AN stim) and a P17 (VAS stim) neuron. While the reviewer is correct that visualization and patching is easier in younger animals, our recordings come from post-hearing mice (>P14) ranging up to P21 in the wedge slice experiments performed so far. Given that the wedge slice is the approximate thickness of a normal slice (300 μm) at the level of the MOC, recording in adult animals should be feasible (as in Torres Cadenas et al 2020; recordings up to P36) albeit with a lower yield. The details regarding the age range of animals used in these experiments has been added to the protocol. Text reads: line 129 "Mice for these experiments were obtained by crossing ChAT-IRES-Cre transgenic mice on C57BL/6J background (The Jackson Laboratory, 028861) with tdTomato reporter mice (Ai14, The Jackson Laboratory, 007914). Mice used for histology and electrophysiology were post-hearing onset (P14-P21). Neurons expressing tdTomato in the ventral nucleus of the trapezoid body (VNTB) have been previously characterized as MOC neurons in this mouse line [57]. "**

3. In the discussion they provide advice on how to proceed if circuitry between the cochlear nucleus and contralateral VNTB is found not to be intact in a particular slice. Fair enough, but it would be useful to have some information on how often this happens i.e. what is the success rate?

**We have added text to address the success rate: line 514 "After optimization of slice geometry, we recorded consistent PSCs in MOC neurons evoked by electrically stimulating the auditory nerve root in**

**8 of 18 wedge slices. In our previous work we were able to evoke inhibitory PSCs via direct stimulation of MNTB axons in approximately 60% of MOC neurons [57], suggesting that our success rate here is only a modest reduction given the long range of the inputs and necessity for polysynaptic circuit activation."**

Minor Concerns:
line 60-suggest they replace "fulfill both criteria" with "suffer from the above limitations"

**The text has been amended as suggested.**

Suggest add references by Wang and Robertson who recorded from identified MOC neurons in VNTB
Two types of actions of norepinephrine on identified auditory efferent neurons in rat brain stem slices. Wang X, et al. J Neurophysiol. 1997. PMID: 9325349 Substance P-induced inward current in identified auditory efferent neurons in rat brain stem slices. Wang X, et al. J Neurophysiol. 1998. PMID: 9658043

**Thank you, we have added these references in the discussion in line 564, where we discuss pre-synaptic circuitry of MOC neurons that is likely absent in the wedge slice.**

Line 150-mention that carbogen is 95%O2/5%CO2

**We have ensured that carbogen is defined at its first use in the manuscript.**

The assumption is that all the cholngergic neurons they see in VNTB are MOC neurons, but Godfrey (Immunohistochemical evaluation of cholinergic neurons in the rat superior olivary complex.
Yao W, Godfrey DA. Microsc Res Tech. 1998 May 1;41(3):270-83) showed 2 distinct populations of Chat-positive neurons in VNTB. Can they be sure of this?

**Thank you, we are aware of this previous work and understand the concern that non-MOC neurons may be labeled in the ChAT-IRES-Cre mouse line used here. We shared the same concern when beginning research using this mouse line. In our recent publication (Torres Cadenas et al 2020), we characterized the fluorescently labeled cholinergic neurons in the VNTB of the ChAT-IRES-Cre x tdTomato mouse line and confirmed that they are MOC neurons using several lines of evidence. First, we co-labeled the genetically identified neurons with an anti-ChAT antibody to confirm that they were cholinergic. Next, we characterized the morphology of somata and dendrites of individual neurons from which recordings were performed using a diffusible biocytin tracer to confirm that they were larger, multipolar cells, with dorsally-projecting axons. In biocytin fills from two neurons, we traced the axon to the contralateral cochlear nucleus, the characteristic morphology of "ipsilateral" MOC neurons. Finally, we used a retrograde tracer applied to the cochlea to co-label the genetically-labeled neurons, confirming that they have axon projections to the cochlea and are therefore cochlear efferents. These mice appeared to lack genetic labeling of the more numerous group of round cholinergic neurons found in the rat in the above reference. We are not sure why there is a discrepancy between patterns of anti-ChAT antibody labeling in the above references and the genetic labeling in the ChAT-IRES-Cre mouse line that we use, but we are confident that, at least in this mouse line, the genetically labeled neurons in the VNTB are MOC neurons.**

Line 515-replace "leveraged" with plain English "used"

**The text has been amended as suggested.**

**Reviewer #3:**
Fischl and Weisz detail a novel brain slice preparation, "the wedge slice," that keeps the polysynaptic circuitry from the auditory nerve to the medial olivocochlear (MOC) nucleus intact while also optimizing conditions for targeting patch clamp recordings to MOC neurons. This novel approach will be useful for studying the input pathways to several auditory brainstem nuclei in addition to the MOC, and the approach can be readily adapted for studies in other brain regions. The protocol is elegantly written and easy to follow, and the results demonstrate successful application of the wedge slice to studying MOC inputs. I have only a few very minor suggestions for edits.

**Thank you for your support of this technique, and for constructive comments to improve the manuscript.**

Minor concerns:
1. Line 66: I believe SOC was not defined previously, so please spell it out here.

**Thank you, the text has been amended**

2. Lines 140, 143: Verb tense - Suggest changing "The pH was adjusted" to "Adjust the pH."

**Text amended**

3. Line 144: Approximately what temperature should the water be for dissolving agar? Near boiling?

**Text amended to include (near boiling)**

4. Line 240: More details are provided later, but it might be helpful to provide some info here about the types of stimulating electrodes that can be used for the auditory nerve root.

**We now define the type of stimulating electrode used in our experiments in the protocol.**

5. Line 304: Does anything need to be done to make the biocytin crystal small or are the biocytin crystals present in a standard commercial vial already small enough?

**Indeed, the crystals are of varying sizes. Many of the small crystals are small enough, but larger crystals can be pinched with forceps to make smaller if necessary. Text amended to read "select a small crystal of biocytin." (line 361)**

6. Line 333: I don't believe Figure 1B is ever referenced in the main text.

**Thank you for pointing out this omission, Figure 1B is now referenced at the beginning of the representative results in line 384.**

7. Line 394: I think the reference to Fig 4B should be changed to Fig 4Ci.

**Thank you, text amended**

8. Figure 2: This might be a journal website issue, but the resolution of this figure was quite low, making it difficult to make out much detail. When I downloaded what I thought might be a higher resolution version of the figure, using the link at the top of the Fig 2 page in the PDF, the downloaded figure had a resolution of only 150 dpi. It would be great if this could be increased to 300 dpi for the final version.

**This is a massive file and had to be reduced quite a bit for upload to the submission site. We apologize for the poor quality of the submitted figure. For the final version, we will use the journal's ftp server.**

9. Figure 4: Minor point, totally up to the authors' preference, but it might be helpful to include small subheadings over the traces in panels B, C, and Ci. For example, "VAS stim -60 mV," "AN stim -60 mV," and "AN stim 0 mV."

**Thank you, the figure has been amended to include the panel subheadings for clarity as suggested.**

**Reviewer #4:**
In this manuscript Weisz and Fischl describe the method of a specialized slice preparation called the wedge slice. This preparation is basically an oblique, tapering brain slice that contains the complete cochlear nucleus on one half of the slice and a gradually thinning (to 200-500µm) slice of the contralateral side. The thin side allows visually guided whole-cell recording of genetically identified neurons. In the case presented here, medial olivo-cochlear neurons (MOC) in the superior olivary complex are investigated. The wedge slice combines the advantages of regular acute brain slices and of the thick slice technique. It contains intact excitatory and inhibitory input stages to the MOC neurons in the thick part of the section and thus allows for natural timing and dynamics of inputs when stimulated from the auditory nerve root. The authors present microscopic and electrophysiological data that convincingly shows the feasibility of the approach.

This is a very interesting und useful method that is clearly and convincingly presented in this paper. The authors not only did a good job in explaining the procedure but also in carefully evaluating the pros and cons (pharmacology, cell survival etc) of this specific method. Although the slice preparation is designed for the study of MOC neurons, it can be easily adapted to study other binaural neurons in the superior olivary complex that receive both excitatory and inhibitory inputs from the contralateral side. Thus overall the technique is of great methodological interest for cellular neuroscience in the auditory system. Furthermore I think that the wedge slice technique described here again shows the great potential of slice preparations specifically designed for a certain experiment. Naturally, most of these "specialized slice approaches" are of limited scope and thus interest for a broader community. However, in this case I think the versatility of the approach in terms of target neurons and also the growing interest in descending projection like the olivocochlear system together should be highlighted.
All in all I am quite happy with this method paper (especially when supported by the visual documentation of the procedure) and think this should be published as it is.

**Thank you for your support of this technique, and for constructive comments to improve the manuscript.**

I have only a few minor comments:
1) The method heavily relies on the free movement of the magnetic specimen disc. As an owner of the same vibratome I do know that it is quite hard to position in any reproducible and exact manner. How

do the authors control the angles? Or is this not critical for the success? I am a bit worried about the repeatability of this approach, as the authors themselves write "This step requires knowledge of the circuit anatomy, careful examination of the tissue and experience estimating and making the cut." (ll. 454-455). Can the authors give some hints what to look for and how to estimate the cutting angle? Maybe a range of angles or some landmarks or so?

**The reviewer is correct that this is a difficult task. We take advantage of the concentric circles on the stage base and use them as reference points with the edge of the specimen disc, but also need to combine this with knowledge of slice surface landmarks. We have added more detail to the manuscript to better document our procedures. The text now reads: line 138, "The concentric circles on the stage base are used to estimate the angle reproducibly." Line 254, "4.4. Once the slices are symmetrical, shift the stage ~15° (corresponding to approximately 3 concentric rings on the stage base) to one side." We also added additional details regarding landmarks on the rostral cut surface of the slice and reference these landmarks in Figure 1B. Text reads: line 275 "NOTE: The facial nerve should be visible on both hemispheres of the slice on the rostral surface (see Fig 1B)."**

2) In order to increase the versatility of the approach: can the authors comment on connections from the side ipsilateral to the MOC neurons under consideration, are they intact? Is the CN on the thin side largely intact or does it taper out to "extremely thin" on this side? This could be relevant for future applications of this technique to for example binaural nuclei in the SOC.

**This is a good point that we failed to highlight in the original manuscript; the wedge slice does not include much if any of the CN ipsilateral to the patched MOC neuron. This prevents dual AN stimulation protocols from being employed as in thick slices. Presynaptic axons from the ipsilateral side could still be stimulated monosynaptically while recording from MOC neurons, but without the upstream plasticity and timing that occurs in the contralateral circuitry. The text has been amended to include: line 568 "The minimal thickness of the slice on the thin side also reduces the ability to perform binaural polysynaptic circuit analyses, which are an advantage of symmetrical thick slice preparations[10, 11]"**

3) The descriptions of Step 2 and 2.2 slightly confuse me: now, does the glue go on the brain or to the stage? Under Step 2 it says "only to the brain", but later glue is applied to the stage surface.

**We have removed text "taking care to only apply glue to the cut surface of the brain" for clarity.**

4) Step 7-9 seem critical to for determining the thickness of the thin part of the slice. Are the "additional few hundred micrometers" (l.223) the thickness of the thin part of the slice? Can the authors make the description of this critical step a bit more concrete?

**We agree that these are key parts of the protocol, and have added more detail. The section now reads: line 266 "Lower the blade to the desired thickness of the thin edge of the slice, here an additional two hundred microns. NOTE: The resulting slice is ideally ~300 μm thick at the level of the ventral nucleus of the trapezoid body (VNTB) on the side where patch clamping will take place."**

5) l. 349: is it a fluorescently labelled strepavidin or is it a secondary antibody (IgG)? I am confused about the nomenclature. Do people call streptavidin-molecules "antibodies" as well?

**We have amended text, deleting "anti-biotin secondary antibody" for clarity.**

6) l. 466: can the authors share some of the modifications of traditional slice solution they had to make in order to increase survival of the thick section?

**We do not use alternative slicing solutions. For typical slice preparations especially those using older animals, other researchers have suggested solutions based on NMDG or sucrose to reduce swelling, some with additives such as thiourea. However, we are not currently using those. We slice using ice cold solution and include 1 mM kynurenic acid in the slicing solution which we find promotes tissue health for patching. We have now noted the alterations we made specifically for the wedge slice preparation in the discussion. Text added: line 531 "Here we employ slight modifications to our normal slice preparation. These include shorter recovery incubation times (30 minutes vs. 60 minutes) and faster flow rates in the slice chamber perfusion system."**

7) ll.508-512: as electrical stimulation of the nerve root will always simultaneously activate a (potentially large) number of axons I doubt that the authors will be able to mimic natural auditory nerve output very well. I think in this section it should either be explained more specifically what can be done or it should be toned down. This is really one of the major drawbacks of the in vitro approach.

**You are absolutely right that we cannot mimic true auditory nerve activity, and have modified our discussion of the benefits and limitations of our preparation. We have added text and and additional reference, which now reads: line 570 "Additionally, we are unable to stimulate the auditory nerve with sound to evoke natural patterns of circuit activity. Auditory nerve responses are tonotopically varied, jittery, and plastic [77–80], making it difficult to perfectly simulate with our electrical stimulation method. This is a major drawback of *in vitro* experimentation in the auditory system. We are unable to tonotopically restrict our stimulation since stimulating the entire AN root will elicit spiking in AN fibers across the tonotopic gradient. We also cannot accurately mimic the diversity of AN fiber responses (i.e. low vs. high spontaneous rate fibers to an electrical stimulus pattern. It is also difficult to precisely match the dynamic intensity coding of multiple AN fibers at the CN. However, we are able to use our electrophysiology software to produce a variety of stimulation patterns aimed to mimic appropriate auditory nerve output during different acoustic stimuli (e.g. short, loud sounds, quiet, prolonged sounds or sounds in background noise) by varying the stimulus frequency both between electrical stimulus protocols and also within an individual protocol to approximate how combined AN inputs would look (modeled in [81]).**"