Video Article

Preparation and Culture of Chicken Auditory Brainstem Slices

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Abstract

The chicken auditory brainstem is a well-established model system that has been widely used to study the anatomy and physiology of auditory processing at discreet periods of development [1-4] as well as mechanisms for temporal coding in the central nervous system [5-7].

Here we present a method to prepare chicken auditory brainstem slices that can be used for acute experimental procedures or to culture organotypic slices for long-term experimental manipulations.

The chicken auditory brainstem is composed of nucleus angularis, magnocellularis, laminaris and superior olive. These nuclei are responsible for binaural sound processing and single coronal slice preparations preserve the entire circuitry. Ultimately, organotypic slice cultures can provide the opportunity to manipulate several developmental parameters such as synaptic activity, expression of pre and postsynaptic components, expression of aspects controlling excitability and differential gene expression

This approach can be used to broaden general knowledge about neural circuit development, refinement and maturation.

Video Link

The video component of this article can be found at https://www.jove.com/video/2527/

Protocol

1. Preparation of Dissecting Area

1. Continuously bubble artificial cerebral spinal fluid (ACSF) with a mixture of 95% O_2 / 5% CO_2 (pH between 7.2-7.4, osmolarity 295-310 mOsm/l).
2. While ACSF is bubbling, clean working area with 70% EtOH. Also clean the vibratome and slicing blade. Rinse blade with distilled water (dH_2O).
3. Place clean fluid absorption pad on dissecting area with appropriate dissecting tools.
4. Glue agar block (40 mg agarose / mL, i.e. 4%, in dH_2O) to the stage of a vibratome-slicing chamber*.
   *Tip: prepare agar prior to dissection. Store pre-made agar inside a covered petri dish in the refrigerator. Agarose purchased from EMD Chemicals or Invitrogen.

2. Preparation of 6-well Plates with Culture Medium

1. Under a sterile hood, fill 1 well per animal of a 6-well plate with 1 mL of culture medium and incubate at 35°C and 5% CO_2.
2. Prepare as many culture membrane inserts as needed and place them in hood for later use.

3. Isolation of Chicken Auditory Brainstem

1. Place egg under a bright light source to determine the air filled space void of the embryo (typically the large side of the egg).
2. Break open large side of egg exposing membrane sac. Make slice in membrane sac with scalpel and gently remove the head of the chicken embryo.
3. Rapidly decapitate head with scissors.
4. Place tip of razor blade slightly posterior to and between the eyes. Make a rostral-to-caudal midline incision through the skull applying only slight pressure*.

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*Tip: pressure applied is dependent on the age of the embryo (i.e., younger = less, older = more)
5. Gently push aside skin and feathers to expose skull and verify midline cut.
6. Block rostral portion of skull by slicing with a razor blade. Position blade posterior to eyes and rapidly cut through entire skull and brain tissue*.
*Tip: strong pressure is required to completely remove rostral section.
7. Make midline-to-lateral incisions with small scissors in the caudal region of the skull, slightly anterior to visible neck muscles on both sides of the head. Pull away skull and tissue to expose cerebellum and brain.
8. Gently remove brainstem from skull base by cutting attached tissue with small scissors. Once all tissue is cut, brainstem should move freely away from the skull*.
*Tip: you may need to flip the skull (brain down) to cut attached tissue.

4. Preparation of Brainstem for Slicing

1. Once removed from the skull, pin brainstem down through the optic tecta.
2. Completely remove the cerebellum by gently cutting the peduncles with small scissors, exposing the floor of the fourth ventricle.
3. Remove any membranous tissue and blood vessel from the surface of the brainstem with tweezers and make lateral cuts through the optic tecta to isolate the brainstem.
4. Place a small amount of super glue on the vibratome stage in front of the agar block (towards the vibratome blade).
5. Using tweezers, gently and swiftly lift the brainstem at the spinal cord and place tissue onto the super glue with the rostral side down and dorsal side towards to vibratome blade*.
*Tip: excess glue should be absorbed with a kim-wipe or filter paper before next step.
6. Gently pour oxygenated ACSF into the vibratome stage*.
*Note: this ACSF can be continuously bubbled with O_2/CO_2. However, bubbling of ACSF in such a small volume of solution results in unwanted movement (and possible damage) to brainstem slices. If performed rapidly, already oxygenated ACSF from the dissection is sufficient.
7. Vibratome blade should be at a 20-22° angle. Start vibratome (oscillation should be set at maximum amplitude) and move stage up towards the blade so that the top of the tissue is parallel with the blade before slicing.
8. Quickly move blade towards brainstem tissue. Slow down considerably just prior to blade contact with tissue.
9. Begin slicing tissue with slowest possible forward motion. Once through the entire coronal section of tissue, gently move slice away from the blade with a brush or a broken glass, fired polished pipette, fit at one end with a rubber bulb.
10. Lower stage in 300-500 µm steps and slice again. Repeat until anatomical signatures are visible in brainstem tissue. At this time, slice brainstem tissue containing auditory structures at 200-1000 µm thicknesses, depending on the age of tissue and experimental needs.
11. Carefully maintain usable slices in the order of slicing, i.e., the 1st slice represents the most caudal region of the circuit (low-frequency sound processing) and the last slice represents the most rostral section (high-frequency processing)*.
*Note: for placement of slices for culture use, go to section 6. For *in-vitro* physiology storage, see next section.

5. Slice Storage for *in-vitro* Physiology

1. Depending on the age of the chicken embryo and the thickness of the slice, each animal should provide 1 to 6 slices.
2. Gently place individual slices in chamber using fire polished glass pipette fitted at one end with a rubber bulb. Chamber should be numbered. Place only one slice per well, in order to maintain some tonotopic specificity (e.g., most caudal slice in chamber 1 and the most rostral slice in the last chamber). Chamber should be filled with ACSF and continuously bubbled with a mixture of 95% O_2 / 5% CO_2 (pH 7.4, osmolarity 295-310 mOsm/l).
3. Allow slices to equilibrate by placing the chamber into a warm bath (36° C) for approximately 1 hour.
4. Remove chamber from warm bath and allow to rest at room temperature for approximately ½ hour.
5. After ½ hours at room temperature, slices can be transferred from the holding chamber to a ~0.5 mL recording chamber mounted on a microscope for electrophysiological experiments.
6. Slices can be used for up to ~6 hours after removal from the warm bath.

6. Preparation of Organotypic Slice Cultures [8]

1. Immediately following the slicing procedure (see Section 6) transfer slices with ~500 µL ACSF using a fire polished glass pipette fitted at one end with a rubber bulb to a 48 well plate on ice. One slice per well*.
*Tip: Slices need to be at least 300 µm and up to 1000 µm thick.
2. After slices have been collected, transfer the 48-well plate to the hood.
3. Remove a 6-well plate containing culture medium out of the incubator and transfer it to the hood.
4. Just prior to placing slices from a single brain onto membranes, place one of the prepared membrane inserts (see Section 2.2) into a well with culture medium. Make sure no air bubbles are present under membrane.
5. Using a glass pipette, transfer a slice in a drop of ACSF onto the culture membrane and remove excess ACSF with a micropipette. Repeat for remaining slices (max 4 slices per membrane)*.
*Tip: Make sure slices do not touch each other and place slices centrally on the membrane so that they don't touch the membrane rim.
6. Repeat for as many brain slices as needed*.
*Tip: If cultures are manipulated at a later time point, it might be advantageous to limit the amount of membranes to 3 per 6-well plate as to reduce the time the cultures are removed from the incubator.
7. Change culture medium 3 times a week under a hood: Remove old medium with a vacuum tube with a sterile tip while membrane insert is lifted out of the well with a pair of forceps. Add 1 mL of fresh culture medium in well while membrane insert is lifted out of the well. When all medium is changed, put plate back into the incubator.

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7. Representative Results:

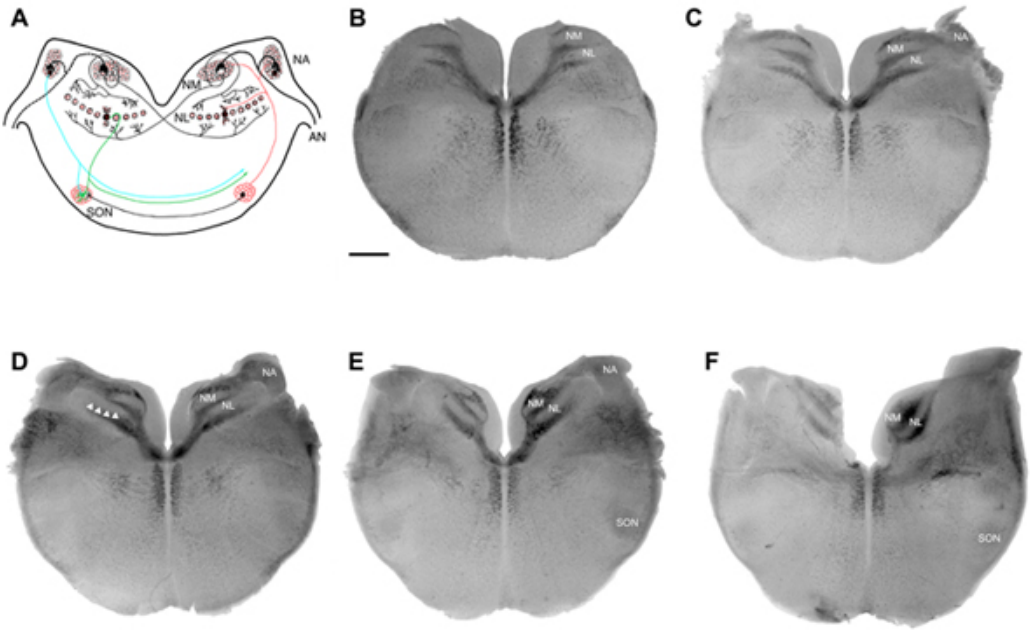

Figure 1. Binaural circuitry of the chicken auditory brainstem. (A) Schematic of a coronal section and (B-F) *in-vitro* slice images of the chicken auditory brainstem. The circuit in (A) shows afferent excitatory inputs from the auditory nerve (AN) to nucleus magnocellularis (NM) and nucleus angularis (NA). NM projects bilateral excitatory inputs to nucleus laminaris (NL) on both sides of the brainstem. An inhibitory input from the superior olivary nucleus (SON) projects to NA, NM and NL. The *in-vitro* slices in (B-F) are sequential images (200 µm each) going from caudal (B) to rostral (F) of the chicken auditory brainstem, representing the low- to high-frequency regions, respectively. Circuitry is responsible for coding temporal properties of sound used primarily for sound localization. Scale bar in (B) = 500 µm and applies for images in (C-F). Arrows in (D) point to the single cell body layer of NL.

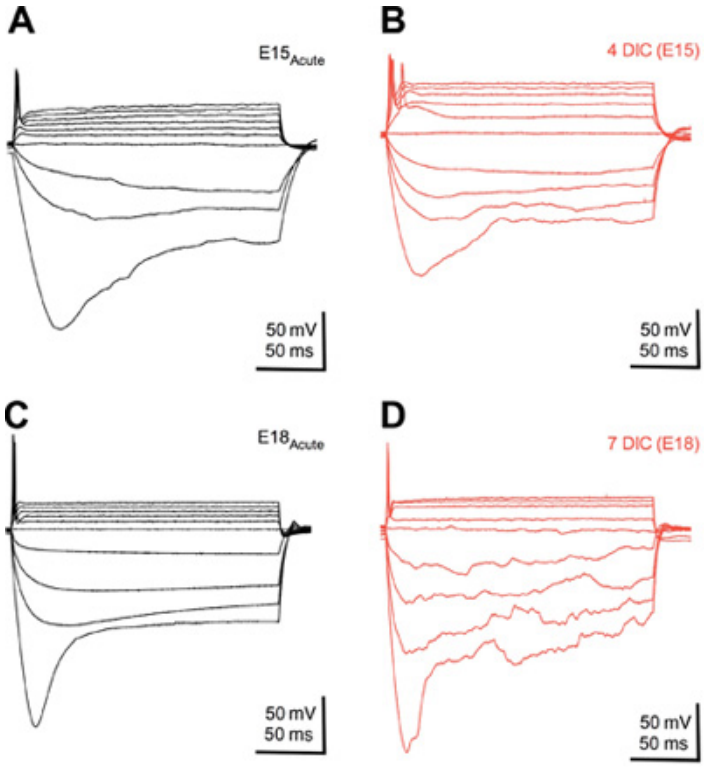

Figure 2. Cultured neurons develop normal physiologic response properties. Superimposed membrane voltage changes in response to hyperpolarizing and depolarizing current steps from acute (A&C) and cultured tissue at 4 (B) and 7 (D) days in culture (DIC). Note changes

in hyperpolarizing voltage "sag", strong reduction in outward current, and single AP firing that develop similarly in the slice culture approach compared to age equivalent acute tissue. Current injections were 200 ms, steps of 50-100 pA. RMPs were between -55 and -60 mV.

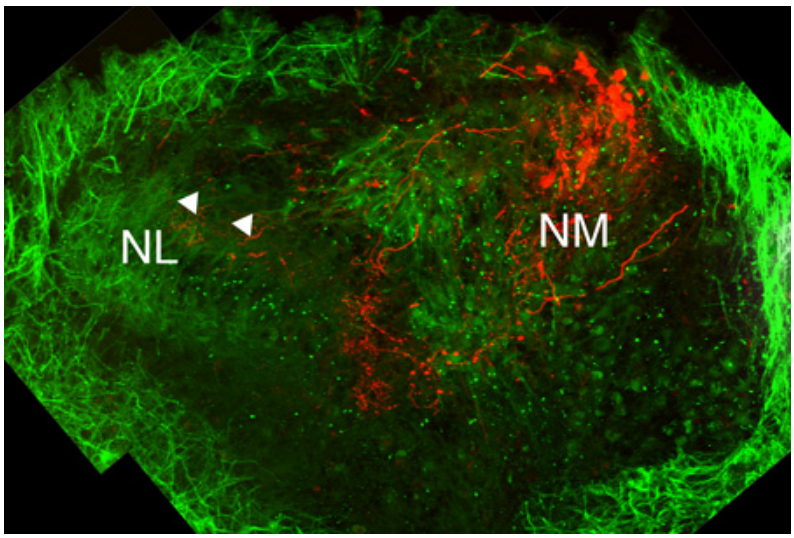

Figure 3. Anatomical structure in culture slices. Left half of the auditory brainstem of an E11 chicken after 7 days in culture (DIC). An antibody against the microtubule-associated protein 2 (MAP2), which occurs in the soma and dendrites, is labeled in green. A cluster of nucleus magnocellularis (NM) neurons and its axons are labeled in red via electroporation of an Alexa dextran dye. Both NM and the NL cell line are visible. Ipsilateral NM axon terminals can be seen projecting to the dorsal NL dendrites (white arrowheads).

Discussion

For several decades, the acute slice preparation of the chicken brainstem has been used to study auditory processing [9, 10]. This approach has provided a tremendous amount of *in-vitro* physiological data on binaural processing from both developmental and mature states [4, 11, 12]. Much is known about this highly specialized circuit and the role each nucleus plays in the temporal processing of sound [13, 14]. In fact, short-term experimental manipulations and their effects on the structure and function of this well-characterized circuit have proven advantageous [15]. From a long-term developmental perspective however, such manipulations are not possible and techniques to address this issue are warranted. Here we propose a novel technique that provides the opportunity to investigate such questions. Ultimately, organotypic slice cultures provide the prospect to manipulate synaptic activity, intrinsic channel regulations, postsynaptic receptor responses, and differential gene expression at specific and over long developmental periods. This approach will further build on the extensive knowledge of the chicken auditory circuit and better assist scientists in understanding basic questions about neurobiological development.

Disclosures

No conflicts of interest declared.

Acknowledgements

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