Video Article

Electroporation of the Hindbrain to Trace Axonal Trajectories and Synaptic Targets in the Chick Embryo

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Abstract

Electroporation of the chick embryonic neural tube has many advantages such as being quick and efficient for the expression of foreign genes into neuronal cells. In this manuscript we provide a method that demonstrates uniquely how to electroporate DNA into the avian hindbrain at E2.75 in order to specifically label a subset of neuronal progenitors, and how to follow their axonal projections and synaptic targets at much advanced stages of development, up to E14.5. We have utilized novel genetic tools including specific enhancer elements, Cre/Lox - based plasmids and the PiggyBac-mediated DNA transposition system to drive GFP expression in a subtype of hindbrain cells (the dorsal most subgroup of interneurons, dA1). Axonal trajectories and targets of dA1 axons are followed at early and late embryonic stages at various brainstem regions. This strategy contributes advanced techniques for targeting cells of interest in the embryonic hindbrain and for tracing circuit formation at multiple stages of development.

Video Link

The video component of this article can be found at http://www.jove.com/video/50136/

Introduction

The hindbrain represents a key relay hub of the nervous system by communicating between the central and peripheral nervous systems via ascending and descending neuronal networks. It regulates basic functions including respiration, consciousness, hearing, and motor coordination [1-3]. During early embryonic development, the vertebrate hindbrain is transiently subdivided along its anterior-posterior (AP) axis into repetitive rhombomeres, in which distinct neuronal cell types are formed and generate multiple brainstem nuclei centers [4]. The hindbrain is also divided along its dorsal-ventral (DV) axis into a basal and alar plate, at which discrete neuronal progenitors become specified and differentiate in distinct DV locations [3,5,6]. How the early AP and DV-specific neuronal patterns are governing the establishment of functional brainstem circuitries is largely unknown.

To gain knowledge on this fundamental question, tools are required in order to label specific subsets of neurons in the early hindbrain and to trace their axonal trajectories and connectivity at more advanced stages. We have previously utilized specific enhancer elements, and a Cre/LoxP-based conditional expression system for tracking axonal trajectory of dorsal spinal interneurons in the early chick embryo [7-9]. In the current manuscript we have targeted the hindbrain and upgraded the experimental paradigm for labeling late embryonic hindbrain interneurons, axons and their synaptic targets, using a modified electroporation strategy and the PiggyBac - mediated DNA transposition. Our new strategy allows the tagging of distinct neuronal subtypes in one side of the hindbrain and the tracking of their axonal projections and synaptic sites at various embryonic stages, from 2 up to 12 days following electroporation. Based on this method, we labeled the dorsal-most subgroup of hindbrain interneurons (dA1/Atoh1$^+$ cells) and revealed two contralateral ascending axonal projection patterns, each derives from a different AP location and elongates in a distinct funiculus. dA1 axons were found to project and form synapses in the auditory nuclei, midbrain and in multiple layers of the cerebellum [10].

The combination of chick electroporation, genetic tracing of neurons and analysis of projection sites at much advanced stages of development provides a unique platform to study the formation of neuronal networks in the brain and to elucidate molecular mechanisms that govern circuit formation.

Protocol

1. Hindbrain Electroporation

1.1 Egg handling

1. Place the eggs horizontally in a humidified incubator (37-38.5 °C). Embryos are electroporated after 65-70 hr of incubation, when they reach 16-17 (HH) stage (25-30 somites).
2. Remove the eggs from the incubator; they remain in the horizontal position.

1.2 Preparations

1. Pull glass capillaries (0.5 mm diameter).
2. Connect bent L-shaped gold Genetrodes electrodes (3 mm diameter) with an adaptor holder to the pulse generator. Electroporation parameters include 25 volts, 5 pulse numbers, 45 msec pulse length, 300 msec pulse intervals.
3. Prepare a mouth pipette, 10 ml syringe needle, parafilm strips, a soft brush, sharp dissecting scissors and 25 ml sterile PBS solution. This amount is usually sufficient to handle an egg tray (18 eggs).
4. Prepare an adequate mixture of DNA plasmids (5 µg/µl each) and add about 0.025% Fast Green dye to visualize DNA injection. Usually a volume of 4 µl is sufficient for the injection of a tray.

1.3 Windowing, Injection and Electroporation

1. Clean egg shells with 70% ethanol.
2. Handle one egg at a time to minimize embryonic exposure time to air.
3. Make a hole at the egg pole with the scissors ends and remove 3-4 ml of albumin with the syringe. Do not remove a larger amount of albumin as it reduces viability.
4. Open a small oval window (2.5 x 2 cm size) at the upper center of the egg shell using the dissecting scissors.
5. Drip few drops of PBS on top of the embryo to prevent dryness during the whole process.
6. Load a small amount of the DNA mixture into the glass capillary using a mouth pipette.
7. Place the embryo with its posterior end towards you. No ink injection is required at this stage as the embryo is clearly visible. Avoiding ink injection increases embryonic survival. Penetrate the caudal hindbrain by holding the capillary at ~45°. Do not remove any membrane from around the embryo. Inject DNA solution carefully into the hindbrain lumen and exhale without forming air bubbles. DNA solution spreads anteriorly.
8. Immediately after DNA injection, place the parallel electrodes at the precise AP and DV hindbrain position you aim to target. Push the electrodes slightly ventrally without touching the heart, as it may reduce viability. Pulse the electroporator. Electrodes should be covered with bubbles to indicate electrical conductivity (**Figure 1A**).
9. Carefully remove the electrodes and drip few drops of PBS to cool the embryo and to remove bubbles. Seal the egg opening properly with a parafilm strip to prevent drying during incubation. Wash electrodes in PBS with the soft brush.
10. Place egg in the incubator for the desired length of time. Survival of embryos is approximately 90% 2-3 days after electroporation, and reduces up to 50% 12 days following electroporation.

2. Analysis of Embryos

2.1 Flat-mount preparation and immunofluorescence

1. Flat-mount preparations of the hindbrain can be performed up to E6.5 to easily visualize axons under a microscope or a stereo-microscope.
2. Dissect the embryo carefully by separating it from surrounding membranes and blood vessels. Place the embryo in a small *silicone-coated* Petri dish containing PBS. Attach the embryo to the dish with tungsten pins facing dorsally. Perform a dorsal slit in the hindbrain roof-plate using sharp glass capillaries.
3. Using sharp tweezers and micro-scissors hold the upper part of the head and pull the hindbrain tissue very carefully from rostral to caudal. After separation of the hindbrain remove remaining ventral tissues to reach a clean preparation.
4. Incubate the hindbrain with 4% paraformaldehyde (PFA) solution for 1-2 hr at room-temperature (RT). Wash the hindbrain with PBT (PBS/0.1% Triton X-100) to remove PFA.
5. For Immunofluorescence in flat-mounted hindbrains add 500 µl of blocking solution (5% of goat serum in PBT and incubate for 2 hr at RT Incubate with 1st antibody ON at 4 °C. Wash with PBT (15 min x 3). Incubate with 2nd antibody for 2 hr at RT. Wash with PBT (15 min x 3).
6. Place the hindbrain facing dorsally on a glass slide. Add fluorescent mounting media onto the hindbrain. Use glass capillaries or tungsten needles to flatten the hindbrain on the slide.
7. Add small amount of grease at each corner to prevent squeezing of the tissue by the cover slip. Carefully place a cover slip on top of the hindbrain and avoid air bubbles. For adherence add nail polish to cover glass edges.

2.2 Cryo-sections and Immunofluorescence

1. Cryo-sections of the brainstem can be performed at any stage to visualize axonal trajectories. Yet, after E6.5 this should be the preferred method due to the difficulty to visualize axons in the flat-mounted embryo, which becomes too thick. Moreover, demonstration of synapses requires sectioning of the tissue and a high-magnification view under a microscope.
2. Dissect the embryo and remove all surrounding tissues. Rinse with PBS. Cut the embryo at the caudal part of the medulla using micro-scissors and sharp tweezers. Make a wide incision between the cerebellum and the midbrain and remove the entire brainstem. The tissue should contain the medulla, pons and cerebellum.

3. Fix the brainstem in 4% PFA for overnight (ON) at 4 °C, rinse with PBS (10 min x 3), and incubate with 30% sucrose for ON at 4 °C until sinking.
4. Embed the hindbrain in a cryo-mold with OCT (Optimal Cutting Temperature) compound, and place it at -20 °C until freezing, as described elsewhere [11].
5. Place the OCT block in the cryostat at the desired sectional axis. Section width is 12 µm.
6. Dry the slides. Add 100 µl blocking solution on each slide for 2 hr at RT. Add 100 µl of antibodies (diluted in the blocking solution to the desired concentration). Incubation period for 1st and 2nd antibodies is ON at 4 °C or 2 hr at RT, respectively. For each incubation time, gently add parafilm strip on top of the slide to prevent dryness. Wash with PBS (10 min x 3) between antibodies. If required, add 1:1,000 Dapi (4'-6-Diamidino-2-phenylindole), diluted in PBS for 20 min at RT. Rinse with PBS.
7. Mount the slide with fluorescent mounting media. Let dry for 1 hr before analysis.

Representative Results

This protocol was recently used to uncover the axonal patterns and projection sites of dA1 subgroup of interneurons in the chick hindbrain [10]. To specifically label these axons, an enhancer element (Atoh1), that has previously been characterized as specific for spinal dI1 neurons [8,12,13], was confirmed to be expressed in hindbrain dA1 cells [10]. The element was cloned upstream to Cre recombinase and co-electroporated at E2.75 along with a Cre dependent cytoplasmic GFP reporter plasmid (pCAGG-LoxP-Stop-LoxP-cGFP; **Figure 1BI**). Hindbrains were analyzed 48 hr following electroporation by flat-mount preparation and revealed two contra-lateral ascending axonal projection patterns (**Figure 2A**); one tract was exclusively derived from dA1 neurons located at rhombomeres 6-7 that elongated in a dorsal funiculus, while the other originated from rhombomeres 2-5 and extended in a lateral funiculus.

To map the axonal projections of dA1 interneurons at later stages of development, the PiggyBac transposition method was applied [14,15]. A reporter Cre-conditional-myristoylated-GFP (mGFP) cassette, cloned between the two PB arms (PB-CAG-LoxP-STOP-LoxP-mGFP-PB), was electroporated at E2.75 along with the Atoh1::Cre enhancer and the transposase vector (CAG::PBase). In this strategy, the electroporated Cre-conditional mGFP is integrated into the chick chromosomes and consequently the STOP cassette is removed only in dA1 neurons, enabling prolonged expression of GFP in dA1 cells (**Figure 1BII**; [10]). The myristoylated form of the GFP tethers it to the membrane such that it is localized along the axonal membrane rather than being diluted in the cytoplasm. Brainstems were collected at E13.5 and analyzed in sagittal sections (**Figure 2B**). dA1 axons were found to accumulate in the cerebellum and to extend towards the external granular layer (EGL), which is marked by $Zic1^+$ cells.

Synaptic targets of dA1 axons in the cerebellum were also analyzed. E2.75 embryos were electroporated with synaptic vesicle protein 2 (SV2)-GFP reporter plasmid [16,17], along with Atoh1::Cre enhancer and the PB transposase (**Figure 1BIII**). This method enables the expression of the GFP reporter in the presynaptic vesicles of dA1 axonal termini. The cerebellum was sectioned at E13.5 and stained with the general pre-synaptic marker synaptotagmin [18,19], as well as with calbindin that labels the Purkinje layer (**Figures 3A, 3B**). $SV2\text{-}GFP^+$ synaptic vesicles were detected in multiple cerebellar regions, including the Purkinje layer, indicating the synaptic connections of dA1 hindbrain interneurons in the cerebellum.

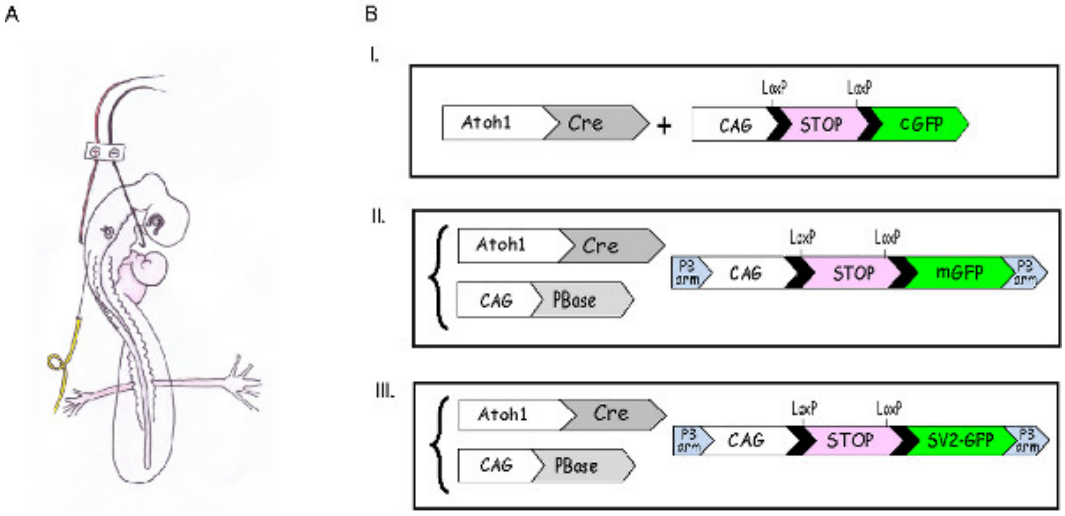

Figure 1. (**A**) **Illustration of the electroporation technique in E2.75 chick hindbrain.** (**B**) **Scheme of the plasmids used to conditionally label hindbrain dA1 interneurons**; *(I):* Constructs include the Atoh1 enhancer element cloned upstream to Cre-recombinase cDNA and a conditional reporter plasmid, in which a transcriptional STOP cassette is inserted between the CAGG enhancer/promoter module and the cytoplasmic GFP gene (cGFP). The conditional expression of GFP in dA1 neurons is driven by Atoh1::Cre. *(II):* The PiggyBac transposition method to constitutively label hindbrain cells. Constructs include the Atoh1::Cre enhancer plasmid, a Cre-conditional reporter cassette, in which myristoylated GFP (mGFP) is cloned between two PB arms (PB-CAG-LoxP-STOPLoxP-mGFP-PB), and a Pbase transposase plasmid. The integration of the reporter cassette into the genome of dA1 neurons is driven by the CAG::PBase and Atoh1::Cre vectors. (*III):* A similar PiggyBac transposition method to label synaptic targets of hindbrain interneurons. The Cre-conditional reporter plasmid includes a synaptic SV2-GFP flanked between two PB arms (PB-LoxP-STOP-LoxP-SV2-GFP-PB). Click here to view larger figure.

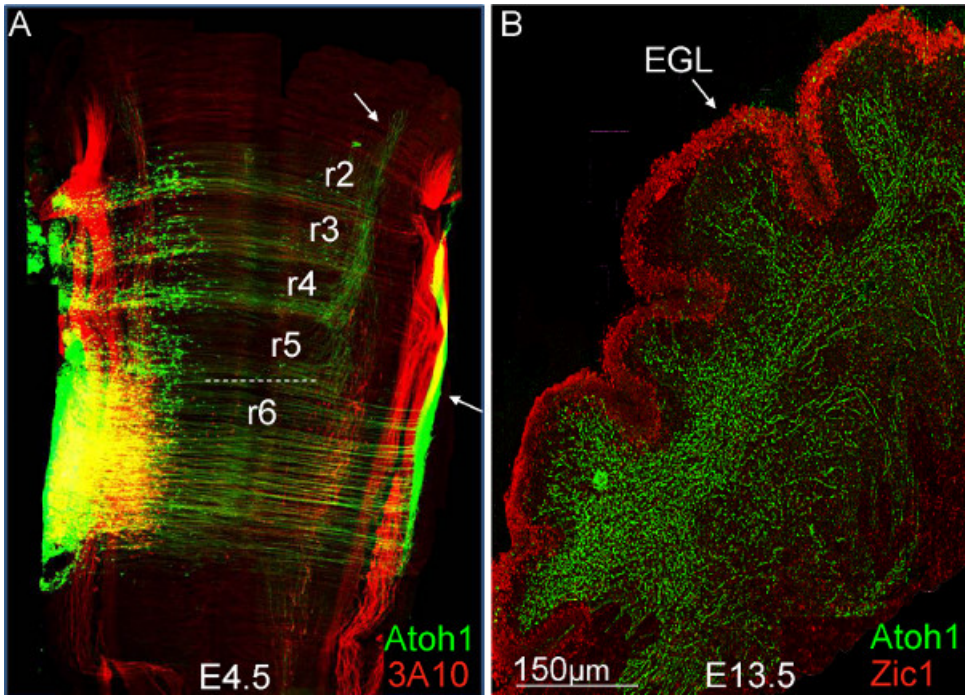

Figure 2. Axonal trajectories of dA1 interneurons. (**A**): A flat-mounted preparation of E4.5 hindbrain demonstrates two ascending dA1 axonal tracts (green, arrows). The neurofillament marker 3A10 (red) is used to mark rhombomere borders. (**B**) A sagittal section of the cerebellum of E13.5 embryo demonstrates dA1 axons (green) accumulating in the cerebellum. The EGL cells are labeled with Zic1 (red). EGL, External Granular Layer, Scale bar is marked.

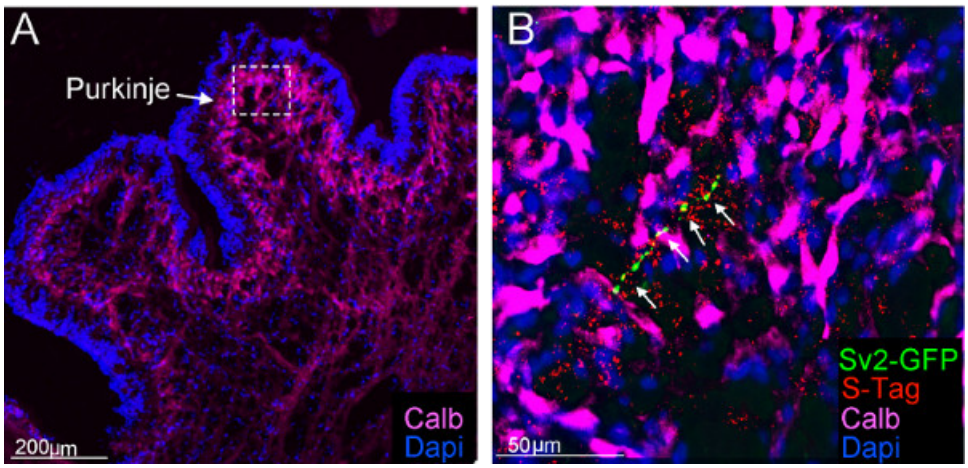

Figure 3. Synaptic targets of dA1 interneurons. (**A**): A sagittal section of E13.5 cerebellum stained with Calbindin (pink) to mark Purkinje layer and DAPI (blue) to label nuclei. (**B**). A higher magnification view of the marked area in A. SV2-GFP labeled dA1 synapses (green) are shown in the Purkinje layer of the cerebellum (pink). The general pre-synaptic marker synaptotagmin (red) is co-expressed with SV2-GFP^{+} synapses (yellow, arrows). Calb, Calbindin; S-Tag, synaptotagmin. Scale bars are marked.

Discussion

In ovo electroporation is a feasible, reliable, and effective tool to examine cell specification and axonal guidance during chick nervous system development [20]. In this protocol we describe a mode of electroporation in the chick hindbrain at E2.75 using enhancer elements which enable the conditional labeling of specific interneurons. This strategy is combined with the PiggyBac-mediated transposition system to insert foreign genes into the chick genome, which enables tracking of axonal routes, projections and synaptic sites at advanced stages of embryogenesis.

Previous methods to label axons/synapses in the chick hindbrain included classical retrograde or anterograde dye labeling or grafting experiments [21-23]. These studies contributed significant findings on hindbrain ascending and descending axonal tracts. However, some trajectories and projections were overlooked by these cellular approaches. In addition, molecular identities of subgroups of neurons that projected into the reported funiculi could not be revealed. Our combined method of electroporation and sustained genetic tracing provides the

unique ability to follow the development of selected hindbrain neurons from their specification stages to axonal navigation and projection targets [10]. Notably, our approach was previously applied to trace the axonal trajectories of spinal cord dorsal interneurons for shorter periods of time [8,9].

The described tools can also serve to uncover molecular mechanisms that govern axonal projections; Genetic codes (such as the Lim homeo-domain code) were selectively modified in a certain neuronal subgroup using the enhancer-Cre/Lox-based approach and impact of axonal growth and connectivity was followed [8,10]. Hence, this protocol may be useful not only for cell labeling but also for genetic manipulations on desired cells.

One of the benefits of electroporation as an *in ovo* gene delivery method is the rapidity of expression, which is observed already within 3 hr following electroporation [24]. However, the transient expression of the introduced gene fades away with cell divisions at later developmental stages. To override this limitation we made use of the PiggyBac transposition method for *in ovo* gene insertion. This tool enables the tracking of axons and synapses in a robust manner for much advanced stages after electroporation, compared to previous methods. As we could easily detect labeled neurons up to 12 days following electroporation (E14.5), it is highly plausible to use this approach even for longer periods of time, including after hatching.

Another benefit of the chick neural tube electroporation relays on the unilateral targeting of cells in a spatial and temporal restricted manners. This allows tracing axonal trajectories on both sides of the hindbrain in a stage-by-stage basis, which is harder to follow in mammals. This is mostly because germ-line transgenesis results in labeling of desired cells in both neural tube sides, complicating the ability to trace unilateral projections and to separate between the origin of ipsi and contra-lateral axonal tracts. Ultimately, based on the high degree of homology between mice and chick brainstem neurons [3,5], our technique provides a powerful tool to gain new knowledge on the assembly of wiring and neurons in the developing vertebrate hindbrain.

Albeit the advantages of our technique, the usage of enhancer elements to drive conditional expression of reporter genes in the chick neural tube requires careful evaluation for cell-specificity and timing of expression of the enhancer-GFP constructs; introduction of some enhancer elements were found in our system to drive a non-restricted expression of GFP in the hindbrain, whereas few others yielded a non-specific expression at early, but not late, neural tube stages (data not shown). Furthermore, our strategy does not currently support a temporal-specific expression of the reporter gene. Hence, we cannot distinguish between early or later-born subgroups of dA1 interneurons, which may possibly project different axonal tracts to target distinct cerebellar areas. Genetic construction of additional tools to temporally switch on/off the expression of GFP in hindbrain neurons will be beneficial to follow more precisely the development of axonal trajectories and synapses in the embryonic/adult brainstem. Finally, a technical drawback of our electroporation strategy is the inaccessibility of the embryo to this manipulation at stages beyond E2.75-3, due to its position in the egg, blood vessels and enveloping membranes. This limitation prevents the targeting of our plasmids into additional subtypes of hindbrain neurons which are born later in development.

Disclosures

No conflicts of interest declared.

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