Video Article

In ovo Electroporation in Chick Midbrain for Studying Gene Function in Dopaminergic Neuron Development

Ben Yang*[1], Lauren B. Geary*[1], Yong-Chao Ma[1,2]

[1]Northwestern University Feinberg School of Medicine, Children's Hospital of Chicago Research Center

[2]Departments of Pediatrics, Neurology and Physiology, Northwestern University Feinberg School of Medicine

*These authors contributed equally

Correspondence to: Yong-Chao Ma at ma@northwestern.edu

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Abstract

Dopaminergic neurons located in the ventral midbrain control movement, emotional behavior, and reward mechanisms[1-3]. The dysfunction of ventral midbrain dopaminergic neurons is implicated in Parkinson's disease, Schizophrenia, depression, and dementia[1-5]. Thus, studying the regulation of midbrain dopaminergic neuron differentiation could not only provide important insight into mechanisms regulating midbrain development and neural progenitor fate specification, but also help develop new therapeutic strategies for treating a variety of human neurological disorders.

Dopaminergic neurons differentiate from neural progenitors lining the ventricular zone of embryonic ventral midbrain. The development of neural progenitors is controlled by gene expression programs[6,7]. Here we report techniques utilizing electroporation to express genes specifically in the midbrain of Hamburger Hamilton (HH) stage 11 (thirteen somites, 42 hours) chick embryos[8,9]. The external development of chick embryos allows for convenient experimental manipulations at specific embryonic stages, with the effects determined at later developmental time points[10-13]. Chick embryonic neural tubes earlier than HH stage 13 (nineteen somites, 48 hours) consist of multipotent neural progenitors that are capable of differentiating into distinct cell types of the nervous system. The pCAG vector, which contains both a CMV promoter and a chick β-actin enhancer, allows for robust expression of Flag or other epitope-tagged constructs in embryonic chick neural tubes[14]. In this report, we emphasize special measures to achieve regionally restricted gene expression in embryonic midbrain dopaminergic neuron progenitors, including how to inject DNA constructs specifically into the embryonic midbrain region and how to pinpoint electroporation with small custom-made electrodes. Analyzing chick midbrain at later stages provides an excellent *in vivo* system for plasmid vector-mediated gain-of-function and loss-of-function studies of midbrain development. Modification of the experimental system may extend the assay to other parts of the nervous system for performing fate mapping analysis and for investigating the regulation of gene expression.

Video Link

The video component of this article can be found at http://www.jove.com/video/4017/

Protocol

1. Supply Preparation (not in video)

1. Prepare a fresh 1X dilution of Penicillin/Streptomycin (Pen/Strep) in 1X PBS. No more than 50 mL is necessary. Filter with a 0.22 µm syringe filter.
2. Prepare injection constructs by combining desired plasmid constructs. We put all constructs in the pCAG-Flag vector with appropriate stoichiometry to a final volume of ~10 µL. Also, pCAG-GFP[14] or pCAG-mCherry should be added for tracking electroporation efficiency and electroporated cells. We use pCAG-mCherry for this experiment. Lastly, add 0.22 µm-filtered Fast Green dye into the plasmid DNA mixture at a final concentration of 0.05% to visualize the injection efficiency into embryonic chick midbrains.

2. Egg Preparation (in video)

1. In order to acquire HH stage 11 embryos, eggs need to be incubated at 100 °F (37.8 °C) for about 41 hours after fertilization with 25-40% humidity. Place eggs on their side and mark the top of each with a pencil dash. Place trays in a pre-warmed, humidified incubator. Slowly rock eggs by setting the turning function of incubator platforms to "Automatic." Check the humidity and water level every 24 hours.
2. Right before starting the experiment, remove eggs from the incubator and leave at room temperature. This will help to slow down the development and prevent over development.

3. Clean the surface of each egg with 70% ethanol and wipe down with a Kimwipe.
4. Take two 4 cm pieces of scotch tape and stick them side-by-side on the egg to make a large rectangle over the pencil dash. Smooth the tape against the egg. This is where the window will be made. The tape will prevent small pieces of eggshell from falling off when the window is cut open.
5. Use a 10 mL syringe and 18½ gauge needle to draw 5 mL of albumin out of the egg. After piercing the shell, angle the needle away from the yolk as to not disturb or damage the embryo while removing albumin. Clean the needle with 70% ethanol between eggs, but be sure the needle is dry before piercing the next egg.

3. Glass Injection Needle Preparation (in video)

1. Injection needles are made using the following settings on a Flaming/Brown Micropipette Puller (Sutter Instrument Model P-97): Heat 670, Pull 120, Vel 40, Time 165. World Precision Instruments (API) 4 inch 1.0 mm thin wall capillary glass (API TW100F-4) is used to make needles.
2. Load a pulled glass needle using a P10 pipette and the long-tip capillary loading pipette tip (Eppendorf 5242956.003). Load 5-10 µL, depending on how many eggs are to be injected. Fill the glass needle slowly, creating a continuous column of plasmid solution absent of air bubbles.
3. Place the loaded needle in the needle holder attached to the micromanipulator (Narishige MM3) and the Picospritzer III microinjector. View the needle under a dissection microscope and trim the needle at the point where the green fluid stops. It helps to use a dark background to better visualize the needle. Proper needle trimming comes with practice.

4. Midbrain/Neural Tube Injection (in video)

1. Use spring scissors to cut a 2-2.5 cm diameter window in the egg. Start the window from the previous needle hole made when drawing albumin, and rotate the egg in your hand as you cut. Be sure to clean off the scissors with a Kimwipe saturated in 70% ethanol between eggs.
2. View chick embryos under a dissection microscope. It may be necessary to train the eyes. It may help to visualize the embryo by injecting 0.22 µm-filtered 20% India ink in PBS under the chick embryo. However, we have found that this step should be avoided if possible, as it tends to decrease survival.
3. Injection works best if the embryo is positioned in parallel to the path of the needle, with the head away from the needle. Slowly pierce the neural tube at a 45 degree angle to the midbrain-hindbrain junction. It is very critical to only fill the neural vesicle representing the midbrain with plasmid DNA and Fast Green dye, without significant diffusion into the forebrain and hindbrain regions. Start injecting with the Picospritzer microinjector's duration set at 25 ms; this may need to be changed depending on needle trimming. In general, use between 10-50 ms.

5. Electroporation (in video)

1. Retrieve the injection needle. Place 3 drops of 1X Pen/Strep in PBS solution in the egg on the embryo.
2. Check the settings of the BTX EMC830 electroporator:

VOLTAGE:	**20 V**
PULSE LENGTH:	25 ms
#PULSES:	3
INTERVAL:	500 ms
POLARITY:	UNIPOLAR

3. Place the 2 mm long L-shaped platinum electrodes at the same horizontal level as the bottom of the neural tube, with the embryonic midbrain sitting in the middle between two 3 mm apart electrodes. Aligning the electrodes with the bottom of the neural tube is critical for efficient electroporation of the ventral midbrain. To achieve midbrain-specific electroporation, we replaced the original BTX electrodes with two custom-made platinum L-shaped electrodes with short 2 mm long tips. These platinum electrodes are made of 0.5mm diameter platinum wires (Alfa Aesar). These platinum electrodes are just long enough to cover the midbrain of a stage 11 chick embryo, which is about 0.9 mm long, without targeting much of the forebrain or the hindbrain regions. Press the BTX footswitch once. Air bubbles should be generated around the electrodes with each successful electroporation. Carefully remove the electrodes from the egg and wipe the electrodes off with a 70% ethanol-covered Kimwipe. Place another 3 drops of 1X Pen/Strep solution in the egg, on the embryo.
4. Cover the window with a 5 cm x 5 cm piece of clear packaging tape. Be sure to seal the egg well and that the egg contents do not come in contact with the tape.

6. Incubate and Harvest

1. Place the eggs back into the incubator. Make sure the turning of incubator platforms is OFF. Incubate eggs until the desired HH stage 23 is achieved.
2. Harvest living embryos and place them into common PBS-filled Petri dishes for each condition. Screen harvested embryos under a fluorescent dissection microscope, keeping only embryos with mCherry expression.
3. Transfer embryos into individual wells of a 24-well dish. Fill each well with 1 mL of freshly made 2% paraformaldehyde (PFA) and allow to fix 2-3 hours at 4 °C.
4. Wash fixed embryos three times for 10 minutes each in PBS. Place embryos sequentially in 15% and 30% sucrose until equilibrated. Embryos will initially float in sucrose, but will sink upon equilibration.

5. Check embryos under a fluorescent dissection microscope, taking notes on the mCherry expression pattern and level for each embryo. Embed each embryo in OCT. It is very critical to orient embryos properly to generate optimal midbrain cross sections for subsequent analyses (**Figure 1**).
6. Prepare 18 µm thick midbrain sections using a Leica CM3050S cryostat. Analyze midbrain development and dopaminergic neuron differentiation by immunostaining with appropriate cell type markers.

7. Representative Results

A diagrammatic representation of electroporating and analyzing embryonic chick midbrain is shown in **Figure 1**. In successfully injected and electroporated chick embryos, mCherry expression should be visible in the midbrain region after further development (**Figure 2A, B, C**). Assuming that mCherry correlates with the expression of other co-electroporated genes, embryos exhibiting proper mCherry expression can then be fixed and embedded to prepare midbrain cryosections for further study (**Figure 2D**). Generally, about 50% of ventral midbrain neural progenitors can be efficiently transfected with the above protocol. The specification of dopaminergic fate in electroporated neural progenitors can be examined by the co-localization of mCherry, Flag-tagged gene, with dopaminergic neuron markers such as Tyrosine Hydroxylase (TH) (**Figure 3**). The patterning of progenitor domains in ventral midbrain can be investigated by co-immnostaining cryosections with different progenitor domain markers.

Insufficient mCherry expression suggests that ectopic genes are likely not efficiently expressed. Embryos under this condition should not be used for further studies. Similarly, embryos that did not survive to the end of the experimental procedure can't be used for data collection and analysis.

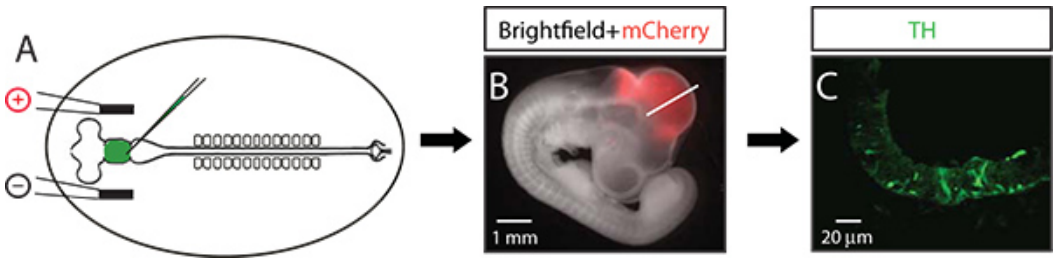

Figure 1. Illustration of the embryonic chick midbrain *in ovo* electroporation assay. HH stage 11 embryonic chick embryos are injected with pCAG-mCherry together with genes of interest mixed with fast green to visualize the injection process (A). After filled with DNA constructs, midbrains are electroporated with a square wave electroporator. Embryos are further incubated until desired HH stage 23 is reached. Then mCherry-positive embryos are harvested, fixed, and embedded (B). Midbrain cryosections are prepared with cutting planes indicated by the white line in Figure 1B, and analyzed by immunostaining with midbrain markers such as Tyrosine Hydroxylase (TH) (C). Click here to view larger figure.

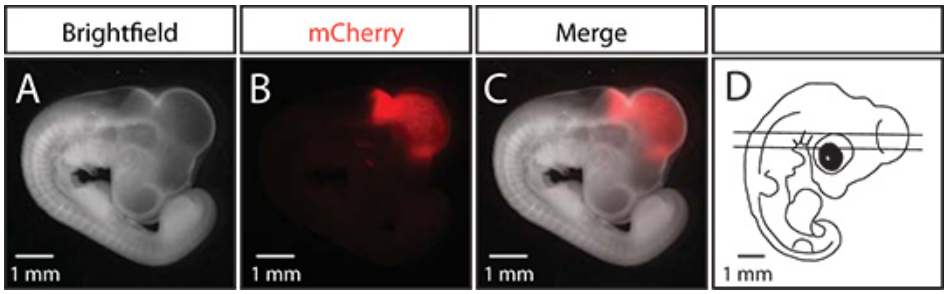

Figure 2. Preparation of cryosections from embryonic chick midbrain. After 41 hours incubation, HH stage 23 chick embryos expressing mCherry and genes of interest are harvested (A, B and C), fixed with PFA, treated with sucrose, and embedded in OCT for cryosectioning. Chick embryos are carefully oriented to ensure the preparation of midbrain sections. A typical embryo morphology and the cutting plane for obtaining midbrain sections are shown (D). Click here to view larger figure.

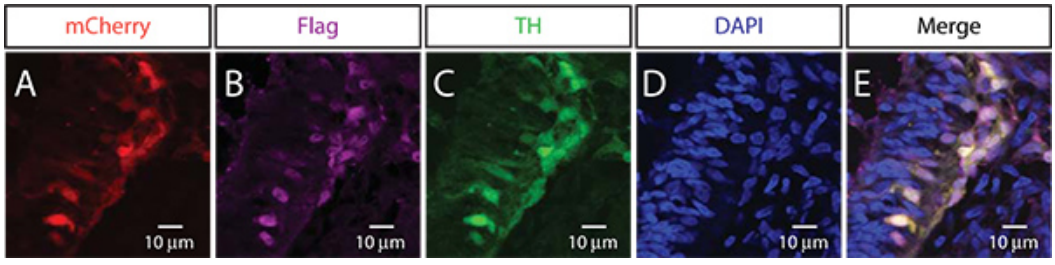

Figure 3. Analysis of electroporated midbrain sections. Cryosections from embryos with high levels of mCherry expression (A) are prepared and stained with antibodies that recognize the Flag-tagged gene of interest (B) and midbrain dopaminergic neuron marker TH (C). Embryonic midbrains electroporated with pCAG-mCherry and pCAG-Flag empty vector serve as control. Click here to view larger figure.

Discussion

The *in ovo* electroporation of embryonic chick midbrain offers a low cost and rapid alternative to the generation of transgenic or knockout animals to perform *in vivo* study of gene functions in midbrain dopaminergic neuron development. Using short 2 mm long L-shaped platinum electrodes together with embryonic midbrain-specific DNA injection is the key for achieving efficient expression of the gene of interest in midbrain dopaminergic neuron progenitors. In addition, using correct HH stage 11 chick embryos is critical. This is because first, at HH stage 11, the development of chick midbrain is advanced enough such that the forebrain, the midbrain, and the hindbrain are morphologically distinguishable. This allows for the accurate positioning of the injection needle and electrodes at the midbrain region, and thus efficient DNA injection and transfection. Second, at HH stage 11, the anterior neuropore of the chick embryo is not yet completely closed, allowing the injected plasmid DNA to easily enter specifically into the midbrain region. The narrow junctions between embryonic forebrain, midbrain and hindbrain also help to prevent fast diffusion of DNA constructs injected specifically into the midbrain. Lastly, embryos at later stages tend to curl their heads to the side of the neural axis, making it very difficult to target the electrode specifically at the midbrain. The incubation time necessary to obtain HH stage 11 embryos may vary slightly in response to deviations in incubation temperature, as well as natural differences between eggs. Under our experimental conditions, it takes about 41 hours incubation at 100 °F.

To obtain embryonic chick midbrain sections for analysis, embedding and cutting midbrains at the correct orientation is very critical. Forty-one hours after electroporation, chick embryos often display a morphology similar to **Figure 2D**. Preparing cryosections with a cutting plane parallel to the ones shown in **Figure 2D** maximizes the chance of getting correct midbrain sections for further immunostaining and quantification.

The fact that midbrain-specific ectopic gene expression can be achieved by electroporation opens up the possibility that this approach can be applied to other regions of the central nervous system. The location of the injection, as well as positioning of the electrodes, could significantly affect the brain regions that are efficiently transfected with DNA constructs[7,15]. This technique may also prove useful in reducing specific gene expression in midbrain by RNAi-mediated knockdown[16]. Similarly, slightly modified techniques may be implemented to study gene regulation by expressing enhancers linked to reporter genes to identify, map, and characterize new regulatory regions from genes in the chick. When applied to mouse embryos, this technique would be much quicker and easier to use than classic transgenic approaches.

Disclosures

The authors have no potential conflicting interests to disclose.

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References

1. Wightman, R.M. & Robinson, D.L. Transient changes in mesolimbic dopamine and their association with 'reward'. *J. Neurochem.* **82**, 721-735 (2002).
2. Kelley, A.E. & Berridge, K.C. The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.* **22**, 3306-3311 (2002).
3. Moore, D.J., West, A.B., Dawson, V.L., & Dawson, T.M. Molecular pathophysiology of Parkinson's disease. *Annu. Rev. Neurosci.* **28**, 57-87 doi:10.1146/annurev.neuro.28.061604.135718 (2005).
4. Dailly, E., Chenu, F., Renard, C.E., & Bourin, M. Dopamine, depression and antidepressants. *Fundam. Clin. Pharmacol.* **18**, 601-607 (2004).
5. Sesack, S.R. & Carr, D.B. Selective prefrontal cortex inputs to dopamine cells: implications for schizophrenia. *Physiol. Behav.* **77**, 513-517 (2002).
6. Ang, S.L. Transcriptional control of midbrain dopaminergic neuron development. *Development.* **133**, 3499-3506 (2006).
7. Andersson, E., *et al.* Identification of intrinsic determinants of midbrain dopamine neurons. *Cell.* **124**, 393-405 (2006).
8. Hamburger, V. & Hamilton, H.L.A Series of Normal Stages in the Development of the Chick Embryo. *J. Morphol.* **88**, 49 (1951).
9. Bellairs, R. & Osmond, M. *The atlas of chick development*. 2nd edn, Elsevier, (2005).
10. Itasaki, N., Bel-Vialar, S., & Krumlauf, R. 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat. Cell Biol.* **1**, E203-207, doi:10.1038/70231 (1999).
11. Momose, T. *,et al.* Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev. Growth Differ.* **41**, 335-344 (1999).
12. Muramatsu, T., Mizutani, Y., Ohmori, Y., & Okumura, J. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem. Biophys. Res. Commun.* **230**, 376-380, [pii] S0006291X96958829 (1997).
13. Nishi, T., *et al.* High-efficiency *in vivo* gene transfer using intraarterial plasmid DNA injection following *in vivo* electroporation. *Cancer Res.* **56**, 1050-1055 (1996).
14. .Matsuda, T. & Cepko, C.L. Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America.* **101**, 16-22, doi:10.1073/pnas.2235688100 (2004).
15. Blank, M.C., Chizhikov, V., & Millen, K.J. In Ovo Electroporations of HH Stage 10 Chicken Embryos. *J. Vis. Exp.* (9), e408, DOI: 10.3791/408 (2007).
16. Chesnutt, C. & Niswander, L. Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis.* **39**, 73-78, doi:10.1002/gene.20028 (2004).

