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Short communication

Optimisation of *in ovo* electroporation of the chick neural tube

Louis-Philippe Croteau[a,b], Artur Kania[a,b,c,*]

[a] Institut de recherches cliniques de Montréal (IRCM), Unité de developpement des circuits neuronaux, Montréal, QC, Canada
[b] Faculté de Médecine, Université de Montréal, Montréal, QC, Canada
[c] Departments of Biology, Anatomy and Cell Biology, Division of Experimental Medicine, McGill University Montréal, QC H3A 2B2, Canada

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ABSTRACT

Chick *in ovo* neural tube electroporation has become a widely used method for assaying gene function during embryonic development. Since its first description, many variants of this technique have been described, with varying values for specific parameters such as electrode type and spacing, voltage, pulse duration and plasmid DNA concentration. Here we examine the influence of some of these variables and derive a detailed and optimal protocol for electroporating the caudal neural tube during the third day of embryonic development. Our findings highlight the importance of electrode placement and DNA dilution buffer for optimal expression and absence of electroporation artifacts.

1. Introduction

Chick *in ovo* neural tube electroporation of DNA or siRNA has several advantages over mouse transgenesis. These include low cost of assaying gene function, possibility of generating data relatively quickly, refined targeting of specific neuronal populations through the use of specific promoters or electrode placement, and easier in vivo accessibility. Existing *in ovo* electroporation protocols suggest that efficiency of targeting and expression depends on a variety of specific parameters such as voltage, electrode placement, spacing and nucleic acid concentration. However, some of these parameters have only been developed for specific embryonic ages or tissues. For example, some protocols optimise these parameters for early embryos of Hamburger–Hamilton (HH) st. 7–12 (Hamburger and Hamilton, 1951) (embryonic day (E) 2 equivalent) neural tube and optic placode (Nakamura et al., 2004; Sauka-Spengler and Barembaum, 2008; Momose et al., 1999) while Rao and Sockanathan (2005) focus on the cervical spinal cord. Others (Krull, 2004) suggest determining empirically the parameters, within a suggested range, for different developmental stages in the neural tube or limb. Since optimal conditions of electroporation will differ depending on the thickness and density of the targeted and surrounding tissues, we varied voltage and electrode spacing parameters as well as DNA dilution buffer to derive an optimal protocol for E3 prospective lumbar spinal cord. We find an optimal voltage–electrode distance combination allowing efficient transgene expression and low incidence of electroporation artifacts, and that an appropriate nucleic acid dilution buffer is critical for maintaining neural tube integrity.

2. Materials and methods

2.1. Chick in ovo electroporations

Fertilized eggs (Couvoir Simetin, Mirabel, QC) were incubated (Lyon Technologies, model PRFWD) at 39 °C according to standard protocols (Hamburger and Hamilton, 1951). Chick spinal cords were electroporated with *pN2-eGFP* expression plasmids at Hamburger–Hamilton stage (HH st.) 18–19, generally as previously described (Kao et al., 2009; Momose et al., 1999). In brief, the vitelline membrane covering the embryo was torn from thoracic to sacral level of the spinal cord. 1.5–3 μg/μl of plasmid DNA (electroporation efficiency did not differ significantly within this range) in water or TE buffer pH 7.5 (10 mM Tris–Cl (Fisher Scientific) and 1 mM EDTA (Invitrogen)) and 0.05% Fast Green FCF (Sigma), was injected into the neural tube at thoracic level, in a caudal direction, using a glass needle inserted into a mouth suction pipette (Sigma–Aldrich). Glass needles were made from borosilicate glass capillaries (Harvard apparatus) pulled with a micropipette puller (Sutter instruments, model: P1000, settings: 2.5 mm box filament, heat: 550, pull: 29, velocity: 150, time: 49, pressure: 200), tips were broken to obtain a needle point diameter of approximately 25 μm, with a tapering length of 9 ± 1 mm. Following injection of plasmid DNA, platinum/iridium electrodes (FHC, cat. # UEPMGBVNNNND, mounted on plastic forceps, bent at a 90° angle to obtain a tip length

* Corresponding author at: Neural Circuit Development Laboratory, Institut de recherches cliniques de Montréal (IRCM), 110 avenue des Pins Ouest, Montréal, QC, Canada H2 W 1R7.
E-mail address: artur.kania@ircm.qc.ca (A. Kania).

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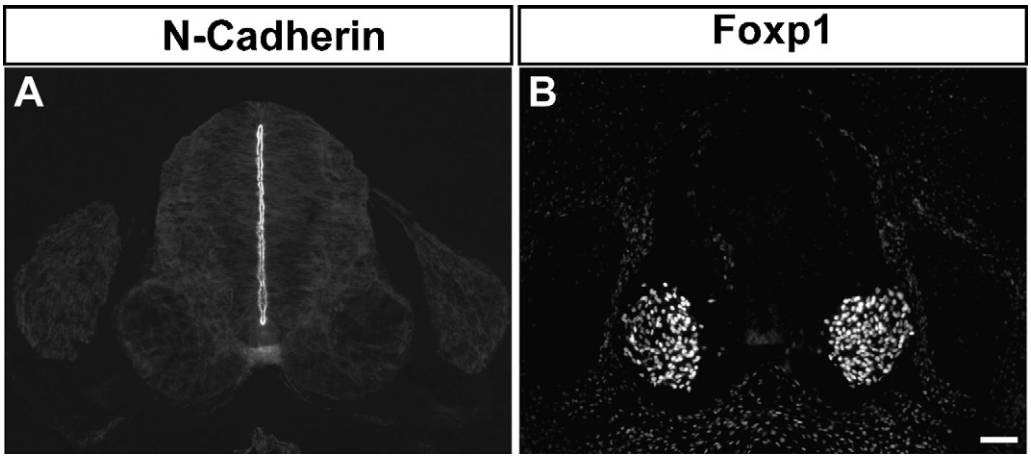

Fig. 1. Chick HH st. 26 lumbar spinal cord. N-cadherin (A), Foxp1, (B) expression in cryosections of the lumbar spinal cord, detected using specific antisera. Scale bar: 50 μm.

of approximately 1.4 mm), were placed at the lumbar spinal cord level, parallel to the spinal cord and aligned with each other and placed just beneath the surface, submerged in albumen. Experiments were carried out by varying the distance between the anode and cathode at an accuracy of ±0.1 mm, as well as varying the position of the electrodes relative to the embryo as described in Fig. 2. Current was applied via a TSS20 Ovodyne electroporator (Intracel; settings: 30 V or 15 V, 5 pulses 50 ms wide in a 1 s interval) (the voltage values indicated by the electroporator were confirmed by measuring voltage values by multimeter (Fluke model 87)). Following electroporation, 100 μl of a mixture of antibiotics (10% Penicillin Streptomycin (Invitrogen cat. # 15140-148), 1% Fungizone (Gibco cat. # 15290-018) in Tyrode's Saline solution (0.8% NaCl, 0.02% KCl, 0.005% $NaPO_4{\cdot}2H_2O$, 0.1% glucose, in H_2O) was applied on top of the embryos, the shell was sealed with a double layer of Parafilm (Pechiney Plastic Packaging Company) and incubated at 39 °C until harvesting at HH st. 26–27, approximately 48 h following electroporation.

2.2. Immunohistochemistry

Embryos were cut transversely at approximately 3 segments anterior to the hindlimbs, and fixed in a 4% solution of cold paraformaldehyde (Sigma) in PB (1.0 M phosphate buffer: 20.66% (w/v) $Na_2HPO_4{\cdot}7H_2O$, 3.2% (w/v), $NaH_2PO_4{\cdot}H_2O$, in H_2O) for 75 min at 4 °C. Embryos were then washed 3 consecutive times in PBS followed by 3 washes of 5 min at 4 °C on a slowly shaking mixer (VMR International), and cryoprotected with 30% sucrose in PB overnight. The next day, embryos were embedded in O.C.T. (Sakura Finetek), and stored at −80 °C. 12 μm sections were collected using a Leica cryostat microtome (model CM3050S). Sectioned tissue was allowed to air dry, and then washed with PBS, incubated in blocking solution (1% heat inactivated horse serum in 0.1% Triton-X/PBS (Sigma)) for 5 min, followed by incubation overnight at 4 °C with primary antibodies diluted in blocking solution. The following primary antibodies and dilutions were used: guinea-pig anti-Foxp1 (1:16,000) (Dasen et al., 2008), sheep anti-GFP (1:1000) (Molecular Probes), mouse anti-N-cadherin (6B3) (1:100) (Developmental Studies Hybridoma Bank). Samples were incubated overnight with primary antibodies followed by three 5 min washes in PBS and 1 h incubation with the appropriate secondary antibody. Slides were then rinsed several times with PBS and mounted with Mowiol mounting medium (9.6%, w/v Mowiol (Calbiochem), 9.6% (v/v) 1 M Tris–HCl (Fisher Scientific), 19.2% (v/v) Glycerol (Fisher Scientific), in H_2O). The following secondary antibodies were used: Cy3-conjugated AffiniPure donkey anti-mouse IgG (1:1000), Cy5-conjugated AffiniPure donkey anti-guinea-pig IgG (1:500) (Jackson ImmunoResearch Laboratory), Alexa Fluo 488-conjugated donkey anti-sheep IgG (1:1000, Molecular Probes). Immunofluorescence images were acquired with a Leica (DM 6000) epifluorescence microscope.

2.3. Molecular biology

pN2-eGFP expression plasmid (Invitrogen) was amplified in bacteria using standard protocols (Joseph Sambrook, 2001) and purified using a Qiagen plasmid maxiprep kit. Plasmid concentration was quantified using a Thermo Scientific Nanodrop 1000 spectrophotometer, diluted in TE, concentrated by ethanol precipitation followed by 70% ethanol washes, air dried and resuspended in TE or water to a final concentration of 1.5–3 μg/μl.

3. Results and discussion

In order to optimise chick *in ovo* spinal cord electroporations, we tested different voltage and electrode placement parameters in the context of five 50 ms pulses in 1 s intervals using the Ovodyne TSS20 square pulse generator. Hamburger–Hamilton stage (HH st.) 18–19 chick neural tubes were injected at lumbar level with the eGFP expression plasmid *pN2-eGFP* diluted in TE buffer. Following current application, embryos were incubated until HH st. 26–27 and harvested. Electroporation efficiency was determined by eGFP expression in whole-mount preparations, embryos were fixed, embedded, frozen at −80 °C and cryosectioned. To evaluate the effects of electroporation on spinal cord development and the presence of electroporation artifacts, we analyzed the expression of specific neuronal markers by immunohistochemistry. To determine spinal cord integrity we assessed N-Cadherin expression, which is normally confined to a characteristic dorsoventral line on the apical surface of ventricular zone (Shiga and Oppenheim, 1991) (Fig. 1A). To assess the effects of electroporation on cell fate specification, we used the transcription factor Foxp1, which is normally expressed by differentiated motor neurons in the ventral horn of the spinal cord (Dasen et al., 2008; Rousso et al., 2008) (Fig. 1B). We first tested the consequence of applying five 50 ms 15 V pulses with the electrodes 5 mm apart (Krull, 2004). Under these conditions N-Cadherin and Foxp1 expression was normal, but the electroporation efficiency, as assed by eGFP expression, was extremely low (Fig. 2G). Higher current values and thus greater electroporation efficiency would possibly be obtained by applying a buffered saline solution, such as Ringer's solution, prior to electroporation, thereby