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Multiple events of gene manipulation via in pouch electroporation in a marsupial model of mammalian forebrain development

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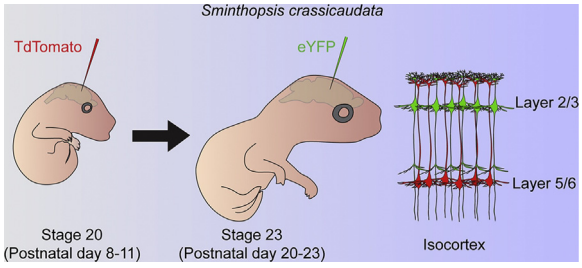
HIGHLIGHTS

- In pouch electroporation allows several transfection events in the same animal.
- Minimally-invasive procedure with high success and spatiotemporal accuracy.
- Enhanced access to manipulate distinct brain circuits independently.
- Marsupial neuron transgenesis *in vivo* enables molecular studies of forebrain evolution.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: The technique of *in utero* electroporation has been widely used in eutherians, such as mice and rats, to investigate brain development by selectively manipulating gene expression in specific neuronal populations. A major challenge, however, is that surgery is required to access the embryos, affecting animal survival and limiting the number of times it can be performed within the same litter.
New method: Marsupials are born at an early stage of brain development as compared to eutherians. Forebrain neurogenesis occurs mostly postnatally, allowing electroporation to be performed while joeys develop attached to the teat. Here we describe the method of in pouch electroporation using the Australian marsupial fat-tailed dunnart (*Sminthopsis crassicaudata*, Dasyuridae).
Results: In pouch electroporation is minimally invasive, quick, successful and anatomically precise. Moreover, as no surgery is required, it can be performed several times in the same individual, and littermates can undergo independent treatments.
Comparison with existing method: As compared to *in utero* electroporation in rodents, in pouch electroporation in marsupials offers unprecedented opportunities to study brain development in a minimally invasive manner. Continuous access to developing joeys during a protracted period of cortical development allows multiple and independent genetic manipulations to study the interaction of different systems during brain development.
Conclusions: In pouch electroporation in marsupials offers an excellent *in vivo* assay to study forebrain development and evolution. By combining developmental, functional and comparative approaches, this

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system offers new avenues to investigate questions of biological and medical relevance, such as the precise mechanisms of brain wiring and the organismic and environmental influences on neural circuit formation.

1. Introduction

The ability to characterise and manipulate specific neuronal populations independently has been critical for our understanding of the fundamental processes that underlie correct brain development, as well as the mechanisms involved in neurodevelopmental disorders. In the past few decades, an increasingly popular technique that allows gene manipulation of selected cellular populations in a spatially confined way has been *in utero* electroporation (Tabata and Nakajima, 2008; Matsui et al., 2011)

Electroporation was introduced in 1982, when Neumann et al. reported a method to transport DNA into cells *in vitro* by applying short electric pulses (Neumann et al., 1982). This procedure has the dual effect of increasing the membrane permeability and mobilising the negatively charged DNA molecules towards the positive electrode (Neumann et al., 1982). This technique has since been adapted to be performed *in vivo* to drive gene expression in selected populations of newborn neurons (Saito and Nakatsuji, 2001; Takahashi et al., 2002; Miyasaka et al., 1999), and has been so far successfully carried out in several vertebrates, such as zebrafish, xenopus, chicken, mouse, rats and ferrets (Saito and Nakatsuji, 2001; Takahashi et al., 2002; Hendricks and Jesuthasan, 2007; Haas et al., 2002; Nakamura and Funahashi, 2001; Borrell, 2010). In eutherian mammals, such as rats and mice, *in utero* electroporation consists of injecting plasmid DNA into the lateral ventricle of embryonic brains, typically using a pulled glass pipette, and then applying electric pulses using forceps-like electrodes (Tabata and Nakajima, 2008). Cell specificity can be achieved by controlling: 1) the developmental stage when selected populations are born, 2) the orientation and position of the electrodes, and 3) the DNA vector used to drive cell-specific gene manipulation (Dean, 2013). However, a major challange of *in utero* electroporation is that surgery is required to expose the uterine horns and access the embryos. Therefore, this technique can only be performed a limited number of times in the same pregnant female to minimize mortality risks for the mother and/or the embryos (Kozulin et al., 2016). In order to overcome this issue, and to provide an experimental model of mammalian brain development and evolution, we adapted this technique in an australidelphian marsupial, the fat-tailed dunnart (*Sminthopsis crassicaudata*), a mouse-sized member of the Dasyuridae family of carnivorous marsupials (Suárez et al., 2017). Marsupials are promising animal models to study forebrain development, as the overall pattern of neurogenesis, layer cytoarchitecture and molecular profiles of cortical neurons is widely shared with eutherian mammals (Cheung et al., 2010; Suárez et al., 2017; Wang et al., 2011; Puzzolo and Mallamaci, 2010). An important developmental difference, however, is that marsupials are born with a very immature forebrain and most of cortical neurogenesis occurs postnatally (Suárez et al., 2017; Puzzolo and Mallamaci, 2010; Smith, 2001). Therefore, electroporation can be performed without surgery inside the pouch, allowing multiple independent events of gene manipulation within and between littermates.

Here we present the method, applications and advantages of in pouch electroporation in postnatal fat-tailed dunnarts and the potential of this experimental model to study mammalian brain development. Moreover, this technique performed in marsupials also represents a versatile tool to test hypotheses about the evolution of brain circuits.

2. Materials and methods

2.1. Animals

Animal breeding and all experimental procedures were approved by The University of Queensland Animal Ethics Committee and the Queensland Government Department of Environment and Heritage Protection, and were performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013), as well as international guidelines on animal welfare (e.g. EU Directive 2010/63/EU for animal experiments). The male:female ratio in the breeding boxes was of 1:1 (virgin males) to 1:3 (experienced males). The pouches were inspected regularly to check for the presence of joeys. The inspection involved gently retrieving the females in the breeding cage from their bottomless hiding box with one hand and gently opening the pouch with the other hand. The pouch of pregnant or oestrous females is usually hairless and easier to open (Morton, 1978). Females with joeys have a moist pouch and their nipples are prominent and highly vascularised. Staging of postnatal dunnarts was performed as described before (Suárez et al., 2017), and the equivalent stages in terms of isocortical development are indicated in Table 1.

2.2. Intralitter identification

From developmental stage 18 at postnatal day (P)0 (day of birth), until the end of stage 26 (P35), dunnart joeys usually remain attached to the same teat, which simplifies the identification of littermates that received different treatments, such as electroporation at diverse developmental stages. For joeys collected after stage 26, when swapping between teats is more common, a tattooing system was used for identification. Briefly, one of the paws and/or the base of the tail of each joey was immobilised with forceps and a small scratch was made on the skin with a fine hypodermic needle (30G) embedded with a green tattoo paste (Ketchum Mfg. Co., NY).

2.3. Adult anaesthesia

For temporary sedation, adult female dunnarts with pouch young were transferred into a gas anaesthesia induction chamber with 5% isoflurane in medical oxygen, delivered at a flow rate of 200 mL/Kg/min. The anaesthesia was then maintained by supplying 2–5% isoflurane through a silicone mask (Zero Dead Space MINI

Table 1
Developmental stages of dunnart and equivalent mouse isocortical development. E, embryonic day; P, postnatal day.

Stage	Fat-tailed dunnarts	Mouse
18	P0-3	E10.5
19	P4-7	E11.5
20	P8-11	E12.5
21	P12-15	E13.5
22	P16-19	E14.5
23	P20-23	E15.5
24	P24-26	E16.5
25	P27-30	E17.5
26	P31-35	E18.5
27	P36-40	P0-P4
28	P41-50	P4-P10