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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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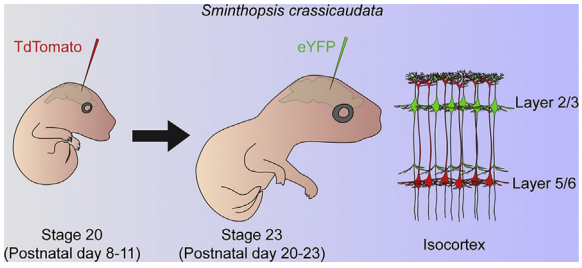
HIGHLIGHTS

- 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

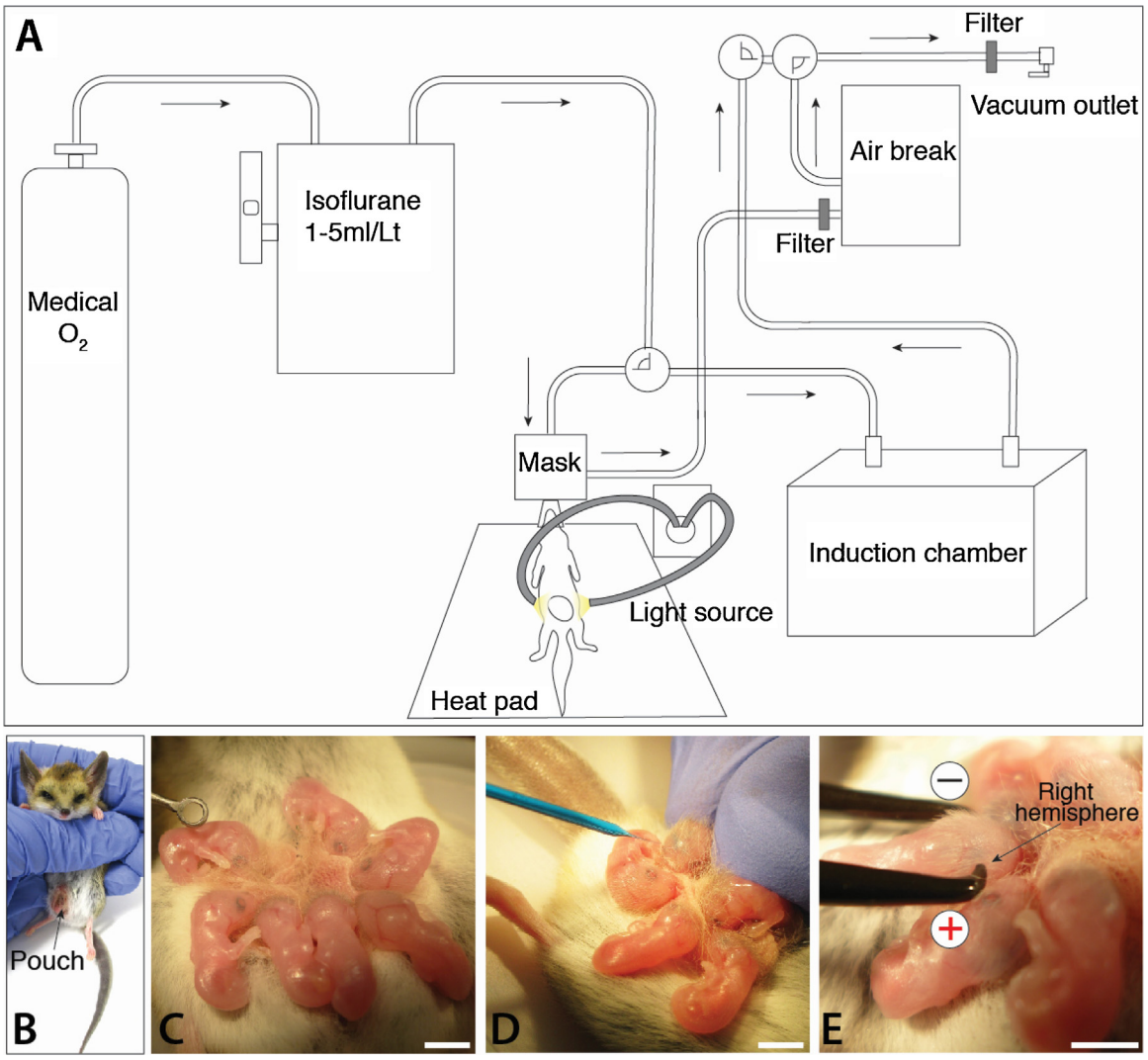

Fig. 1. Set-up and procedure of in pouch electroporation. A: Schematic of the set-up required for the anesthesia of female fat-tailed dunnarts and in pouch electroporation of pouch young. **B**: Female dunnart in supine position with joeys inside the pouch. **C**: Exposure of teat-attached joeys by eversion of the pouch. **D**: The plasmid solution is injected in the lateral ventricle and the dye allows detection of ventricle filling through the skull and skin. **E**: Forceps-like electrodes are positioned across the head of the joeys. The position of the forceps and orientation of the positive electrode allow controlled targeting of selected neuronal population. Scale bars: 4 mm.

Qube Anaesthetic System, AAS, AZ) throughout the procedure. The system connects directly to the vacuum outlet and incorporates an air brake to avoid the risk of lung collapse (Fig. 1A). This allows careful examination and manipulation of joeys in a minimally invasive manner.

2.4. In pouch electroporation

2.4.1. Plasmid injection

Once the female dunnart was anaesthetised and placed on a heatpad in supine position, the pouch was carefully everted, and the joeys were exposed gently, without detaching them from the teat, from stage 19 (P4-P7) to stage 23 (P20-P23; Figs. 1B, 1C). The joeys were injected with 0.5–1 μL of a 1 μL/μg solution of plasmid DNA, in 1 M sterile phosphate-buffered saline (PBS; Lonza, Basel), into the lateral ventricle using a pulled glass pipette (Thin Wall Glass Capillaries 1.2 mm OD/0.90 mm ID, WPI, FL) and air pulses delivered via a picospritzer (Parker Hannifin, NH; Fig. 1D). The tip of the glass pipette, previously prepared using a Flaming/Brown micropipette puller (heat 495, pull 100, vel 100, Sutter Instrument Co., CA), was trimmed obliquely using forceps as previously described (Tabata and Nakajima, 2008; Matsui et al., 2011). The plasmids used in this study were the fluorophores tdTomato and eYFP (red and green, respectively) (Niwa et al., 1991; Matsuda and Cepko, 2004) cloned into a pCAG expression vector (Suárez et al., 2014). To visualise the location of the plasmid solution into the lateral ventricle, 0.0025% v/v of the dye Fast Green (Sigma-Aldrich Co., MO) was added (Tabata and Nakajima, 2008; Matsui et al., 2011).

2.4.2. Position of the electrodes

The 1 mm forcep-type electrodes (Nepa Gene Co., Ichikawa) were positioned on the head of each joey immediately following plasmid injection (Fig. 1E). The spatial orientation was defined depending on the targeted neuronal population. Five 100 ms square pulses of 30–35 V were delivered to specific brain regions via an electroporator system (ECM 830, BTX, Harvard Bioscience, MA). The same electrode size and pulse parameters were used throughout stages and brain regions, resulting in consistent and well-defined electroporated patches. After the completion of the procedure, the joeys were replaced inside the pouch and the mother was allowed to recover.

2.5. Euthanasia and tissue collection

Once the mother with pouch young was anaesthetised as described above, joeys were removed from the teat by gently pulling with forceps, while holding the base of the teat with another forceps. Joeys younger than stage 28 (P41-50) were put in a petri dish and anaesthetised on ice for 5–15 min, while older joeys received an intraperitoneal injection of 0.05-0.5 mL solution of sodium pentobarbitone (1/50 v/v Lethabarb, Virbac, corresponding to 190 mg Lethabarb per kg body weight). Following deep anaesthesia, joeys younger than stage 21 (P12-15) were decapitated and drop fixed in 4% paraformaldehyde (PFA; ProSciTech, QLD) in saline (0.9% NaCl), while older joeys were transcardially perfused with saline followed by 4% PFA. Brains were post-fixed in 4% PFA for at least four days before further processing.

2.6. Immunohistochemistry

Collected brains were embedded in 3.4% agarose (Difco, Thomas Scientific, NJ) and sectioned coronally using a vibratome (VT1000S, Leica Biosystems, Nussloch). Brain sections of 50 μm were mounted on microscopy slides (Superfrost Plus, Thermo Fisher Scientific, MA), dried and covered with 4% PFA for 10 min for post-fixation. The slides were then incubated for 2 h in 10% (v/v) normal donkey serum (NDS; Jackson Immunoresearch Inc., PA) and 0.2% Triton X-100 (TX100, Sigma-Aldrich Co., MO) in PBS (pH 7.4), followed by overnight incubation in 10% NDS and 0.2% TX-100 in PBS with primary antibodies: chicken anti-Gfp (1:750; Abcam, Cambridge) and/or goat anti-tdTomato (1:1000; SICGEN, Coimbra). After PBS washes, slides were incubated with the appropriate fluorescent secondary antibodies Alexa Fluor 568 donkey anti-goat and/or Alexa Fluor 488 donkey anti-chicken (1:500; Invitrogen, Thermo Fisher Scientific, MA). The slides were then stained for 10 min with 0.1% 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI; Invitrogen, Thermo Fisher Scientific, MA), washed and coverslipped with antifade mounting media (ProLong Gold; Invitrogen, Thermo Fisher Scientific, MA). DAPI staining was used to outline isocortical layers based on cytoarchitecture, as previously done in mice Suárez et al. (2014) and opossum Puzzolo and Mallamaci (2010).

2.7. Image acquisition

Wide-field fluorescence imaging was performed with a Zeiss upright Axio-Imager Z1 microscope fitted with Axio- Cam HRc and HRm cameras and images were acquired with Zen software (Carl Zeiss AG, Oberkochen). High resolution images were acquired using a Diskovery spinning disk confocal microscope (Spectral Applied Research Inc, Ontario) built around a Nikon TiE body and equipped with two sCMOS cameras (Andor Zyla 4.2, 2048 × 2048 pixels) and controlled by Nikon NIS software (Nikon, Tokyo). All imaging was performed at the Queensland Brain Institute's Advance Microscopy Facility. Images were cropped, sized, and enhanced for contrast/brightness with Photoshop, and the figures assembled in Illustrator (Adobe Creative Suite 6, CA).

2.8. Statistical analysis

D'Agostino & Pearson tests were used to assess the normality of the datasets, which found them all to have non-normal distributions. Data analysis was therefore performed using Mann–Whitney or Kruskal–Wallis non-parametric tests (Prism 7, GraphPad Software Inc., CA). Probability values of $p < 0.05$ were considered significant. Data is presented as mean ± standard error of the mean (SEM).

3. Results

3.1. In pouch electroporation can be successfully performed in fat-tailed dunnarts

Considering that in pouch electroporation has never been performed before in a marsupial model, we first set out to establish a protocol and determine its feasibility as an experimental model. Much of the equipment and protocols for standard mouse *in utero* electroporation can be adapted to dunnarts, as their brain is slightly smaller than stage-matched mice (cortical thickness of stage 24 dunnarts is 64.6% that of E16.5 mice; in adult dunnarts it is 65.8% that of adult mice). The schematic in Fig. 1A illustrates the experimental set-up to examine female dunnarts with pouch young (see Methods for further details). Once the female was anaesthetised, the joeys were exposed by everting the pouch (Fig. 1B, C). The plasmid solution was then injected into the lateral ventricle (Fig. 1D), using a pulled glass pipette attached to a picospritzer with pedal control of air pulses, and the electrodes were positioned over the presumptive somatosensory cortex by clamping the head (Fig. 1E). We performed this technique at stage 23, collected the animals at stage 27, and found that it successfully induced expression of eYFP into the dunnart brain (Fig. 2A), allowing labelling of all the major components of layer (L) 2/3 neurons: dendrites, cell bodies and axons (Fig. 2B–D).

3.2. In pouch electroporation does not significantly affect the mortality rate of fat-tailed dunnarts

Given the *ex utero* development of the dunnart forebrain, no surgery of the mother is required to access the joeys at these stages, which makes in pouch electroporation a minimally invasive technique. This resulted in 100% survival rate of the mothers of electroporated joeys across different developmental stages of in pouch electroporation (mortality rate 0%, n = 42, Fig. 2E). We then calculated the mortality rate of joeys per litter after they received the first electroporation (1.2%, n = 42 litters, 273 total joeys, Fig. 2E) and after the same joeys were electroporated for the second time (7.4%, n = 42 litters, 269 total joeys, Fig. 2E). The baseline mortality rate for non-electroporated joeys per litter during the developmental stage in which the first electroporation was performed, i.e. between stage 19 and 21 (P7-P16), was 0% (n = 17 litters, 69 joeys), and during the developmental stage between the second electroporation and collection, i.e. between stage 22 and 28 (P17-P50), was 4.3% (n = 11 litters, 68 joeys). We then compared the mortality rate of joeys after one or two electroporations with the baseline of mortality rate of non-electroporated controls of the same age, and found that the difference was not statistically significant (Fig. 2E). A Mann-Whitney test comparing the joeys that died after the first electroporation and the baseline mortality rate between stage 19 and 21 resulted in $p = 0.5827$, and a Mann-Whitney test comparing the joeys that died after the second electroporation and the baseline mortality rate between stage 22 and 28 resulted in $p = 0.3921$, highlighting the feasibility of performing multiple electroporation events within single individuals.

3.3. The protracted neocortical neurogenesis in fat-tailed dunnarts facilitates the independent transfection of distinct neuronal layers with in pouch electroporation

Development of the marsupial neocortex is 2–3 times more protracted as compared to mice (Puzzolo and Mallamaci, 2010; Cooper, 2008; Smart and Smart, 1982), offering an extended period for developmental manipulations, including the differential transfection of progenitors that give rise to neurons of distinct neuronal layers. We electroporated the somatosensory cortex across devel-

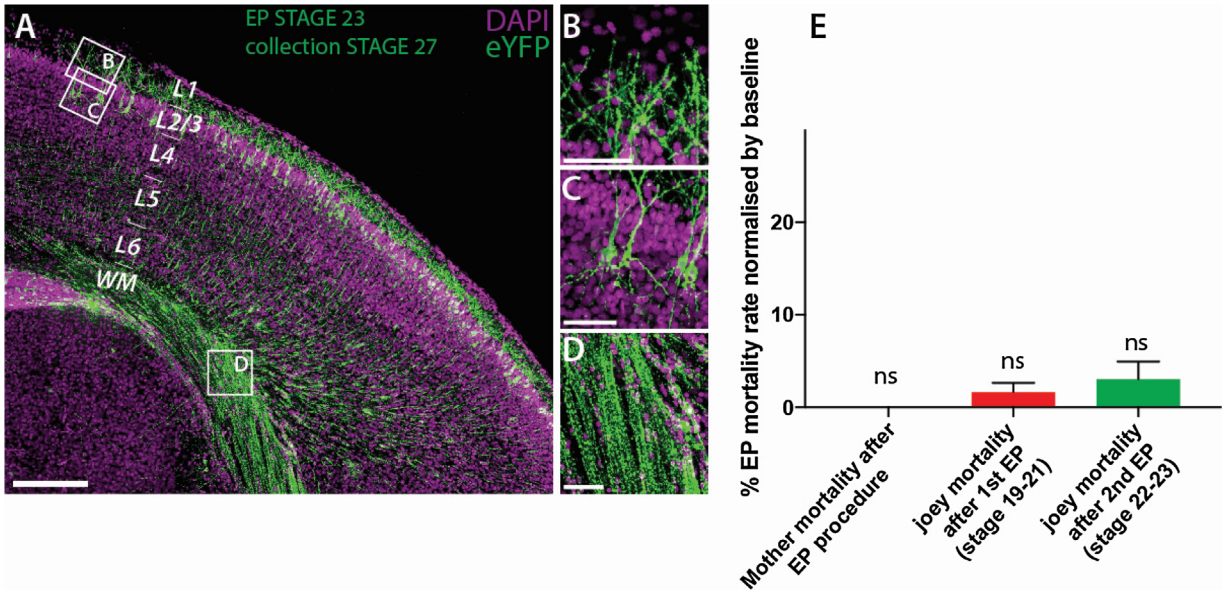

Fig. 2. In pouch electroporation successfully transfects neocortical neurons and does not affect dunnart mortality rate. A: Example of a successful electroporation with eYFP at stage 23. The electroporation area corresponds to the primary somatosensory cortex and the fluorophore labels all major components of upper layer (L) 2/3 neurons: dendrites in L1 (**B**), cell bodies in L2/3 (**C**), and axons along the white matter (WM; **D**). Scale bars: 200 μm in **A**, 100 μm in **B** and **D**, and 50 μm in **C**. **E**: The mortality of the mothers and joeys that received either one or two electroporations was compared to non-electroporated controls (mortality baseline). Normalised mortality rate of mothers (0%; n = 42; left), joeys after 1st electroporation (1.2%; control, n = 69; electrporated, n = 273; middle), and joeys after 2nd electroporation (3.1%; control, n = 68; electroporated, n = 269; right. Differences of mortality rates between non-electroporated controls and electroporated joeys was not statistically significant after either the first or second electroporations (p = 0.5827 and p = 0.3921, respectively; Mann-Whitney tests). The results are shown as mean ± SEM.

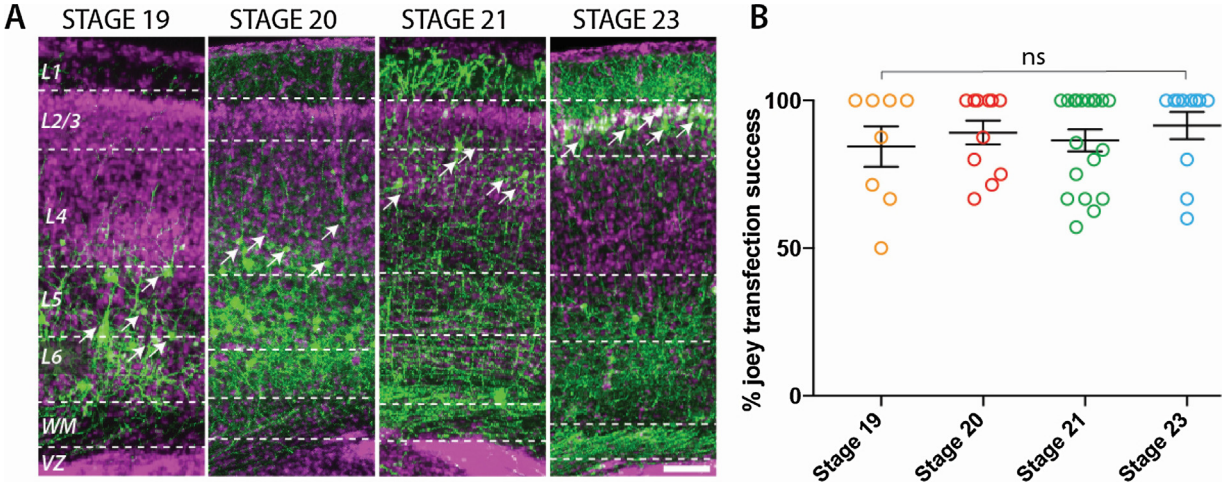

Fig. 3. In pouch electroporation performed at different developmental stages allows a high transfection efficiency in specific layers of the dunnart neocortex. A: Dunnart joeys were electroporated with eYFP at stages 19, 20, 21 and 23 and their brains examined at stage 27. The developmental stage on the day of the electroporation determines the layered position of transfected cells (arrows) within the neocortex, in a protracted inside-out neurogenic fashion. Scale bar: 100 μm. **B**: Scatter plot of transfection success across stages, as indicated by the presence of fluorescent neurons in the brain of electroporated joeys. The results, shown as mean ± SEM, indicate high electroporation efficiency across the developmental stages.

opment and examined the brains at stage 27, when the neurons have reached their final location in the different layers of the neocortex (Suárez et al., 2017). When the electroporation was performed at stages 19, 20, 21 or 23 (see Table 1 for respective postnatal brains), transfected neurons predominantly localised in layers (L) 5-6, L5-4, L4-3 and L2/3, respectively, corresponding to an inside-out neurogenic labelling pattern (Fig. 3A).

3.4. In pouch electroporation results in high transfection rates across development

We obtained a high success rate of transfection across developmental stages in which in pouch electroporation was performed (stage 19 = 84.4%, n = 8 litters, 41 total joeys; stage 20 = 89.1% n = 11 litters, 41 total joeys; stage 21 = 86.5%, n = 19 litters, 98 total joeys; stage 22 = 91.5%, n = 11 litters, 50 total joeys, Fig. 3B). Moreover, transfection success showed no statistically significant difference across stages (Fig. 3B, Kruskal-Wallis test, p > 0.99 for all comparisons), further demonstrating its potential to manipulate gene expression across multiple points of development.

3.5. Different genes can be successfully expressed in different neurons by electroporating at multiple time points

Given that in pouch electroporation does not significantly increase the mortality of the joeys or their mothers, even when

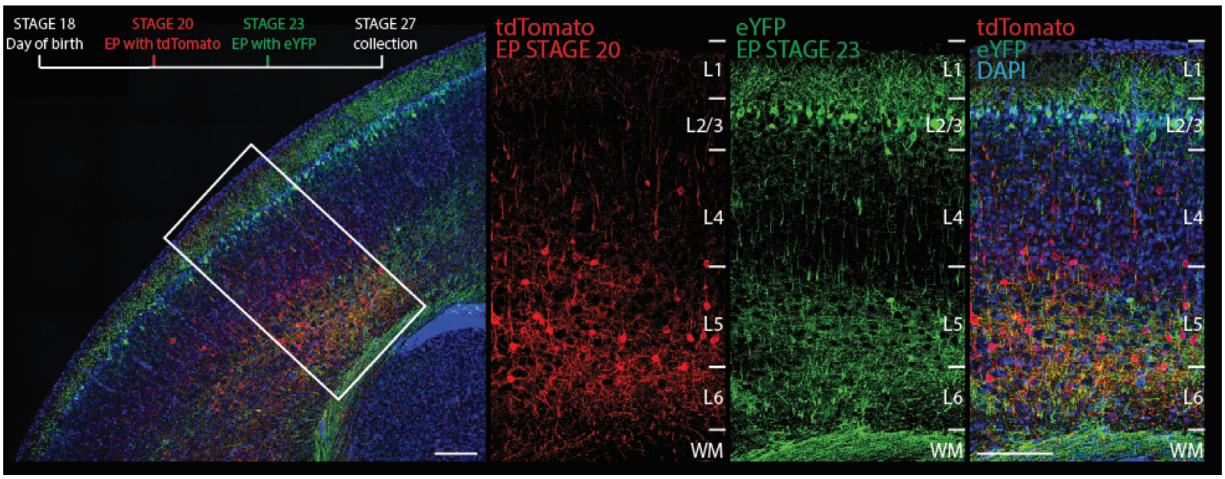

Fig. 4. In pouch electroporation can be performed multiple times in the same individuals to label distinct neuronal populations. The experimental procedure of the double electroporation is indicated on the top of left panel. The square in the left panel indicates the area of the right insets. Electroporation of tdTomato at stage 20 resulted in labeling neurons located in the deeper layers (red), while electroporation of eYFP in the same region at stage 23 labelled the upper layer neurons (green). The last panel on the right shows the distribution of cell layers by including both channels and DAPI staining (blue). Scale bars: 200 μm.

repeated in the same animals (Fig. 2E), this technique can be performed multiple times to differentially manipulate distinct neuronal populations within and between littermates. We electroporated the same neocortical hemisphere sequentially using two different constructs: tdTomato at stage 20 and eYFP at stage 23. This selectively labeled the deeper and upper layer neurons of the neocortex, respectively (Fig. 4). Considering that the main output neurons of the neocortex are located both in L2/3 and L5/6, the ability to differentially manipulate gene expression in these neuronal populations provides new experimental opportunities to investigate their respective roles in circuit formation.

3.6. *Different brain areas can be precisely targeted with in pouch electroporation*

In mice, *in utero* electroporation has been used to transfect numerous brain structures (Baumgart and Grebe, 2015; Taniguchi et al., 2012). However, positioning the electrodes across the uterine wall and amniotic sac restricts the accuracy and replicability of the electroporated site location. On the other hand, the head of marsupial joeys can be directly held with the electrodes without these barriers, facilitating the precise targeting of the electroporated region. To establish a set of examples of regions that can be reliably transfected in dunnarts, we controlled the position of the electrodes aiming at different forebrain locations. We generated numerous cases of well-confined sites of electroporated neurons. Examples of forebrain regions that can be successfully transfected include the olfactory bulb, the anterior olfactory nucleus, prefrontal cortex, hippocampus, neocortex, piriform cortex, striatum, thalamus, and entorhinal cortex (Fig. 5).

4. Discussion

In vivo electroporation is a technique widely used across species to study many aspects of brain development, from the generation of specific cellular populations to the formation of functional brain circuits (Takahashi et al., 2002; Hendricks and Jesuthasan, 2007; Haas et al., 2002; Nakamura and Funahashi, 2001; Borrell, 2010; Saito, 2006).

Here, we successfully adapted this technique to be performed in pouch-young of the Australian marsupial fat-tailed dunnart, as it presents several advantages. First, as opposed to eutherian mammals, the forebrain of marsupials develops predominantly postnatally, while in the pouch, so it is possible to study brain development from stages equivalent to human mid-embryogenesis in a minimally invasive manner (Suárez et al., 2017). Second, while in eutherians a surgery is required to access the embryos, exposing the dunnart joeys in anaesthetised females has a lower mortality rate as compared to mice (Kozulin et al., 2016). As a result, this technique has a high success rate, does not significantly increase the mortality of the joeys nor their mothers, even when carried out twice within the same individuals or littermates (Figs. 2–4), and can be successfully used to specifically transfect different neuronal populations within and between brain regions (Figs. 4 and 5). The low mortality rate of joeys, combined with the possibility of independently manipulating and collecting littermates, offers a substantial reduction of animals required for each experiment as compared to mice. In addition, in pouch electroporation can be performed relatively quickly, taking about 5–10 min for a litter of 6–10 joeys. In mice, *in utero* electroporation success depends upon the embryonic day (E) on which the procedure takes place, with 50% of embryos with effective transfection at E11.5 (Shimogori and Ogawa, 2008) and more than 90% at later stages (Saito, 2006; Shimogori and Ogawa, 2008; Szczurkowska et al., 2013). The mortality rate of electroporated mouse embryos also depends on the strain and the age in which the technique is performed, and can reach up to 40% when the embryos are electroporated before E12.5 (Baumgart and Grebe, 2015; Shimogori and Ogawa, 2008; Szczurkowska et al., 2013) down to less than 10% after E15.5 (Saito, 2006; Borrell et al., 2005). Similarly, the mortality rate of mouse mothers is dependent on the strain, the electroporation parameters, and the duration of the procedure, and it can reach up to 8–10% when performed at very early stages (E9.5-E10.5) (Punzo and Cepko, 2008), while in contrast in dunnarts it is negligible throughout developmental stages.

Another important advantage of using postnatal marsupials as models of mammalian forebrain development is the unprecedented opportunity for continuous access, observation, manipulation and collection of individual littermates across development, without the need to sacrifice the mother or littermates. Moreover, the ability to transfect multiple brain regions across developmental stages can be used to elucidate the specific contribution of different neuronal populations in the formation of brain circuits via selective expression of genetic constructs.

In pouch electroporation, using a mouse-sized marsupial species that breeds well in captivity (Suárez et al., 2017), will also provide useful tools to investigate comparative and evolu-

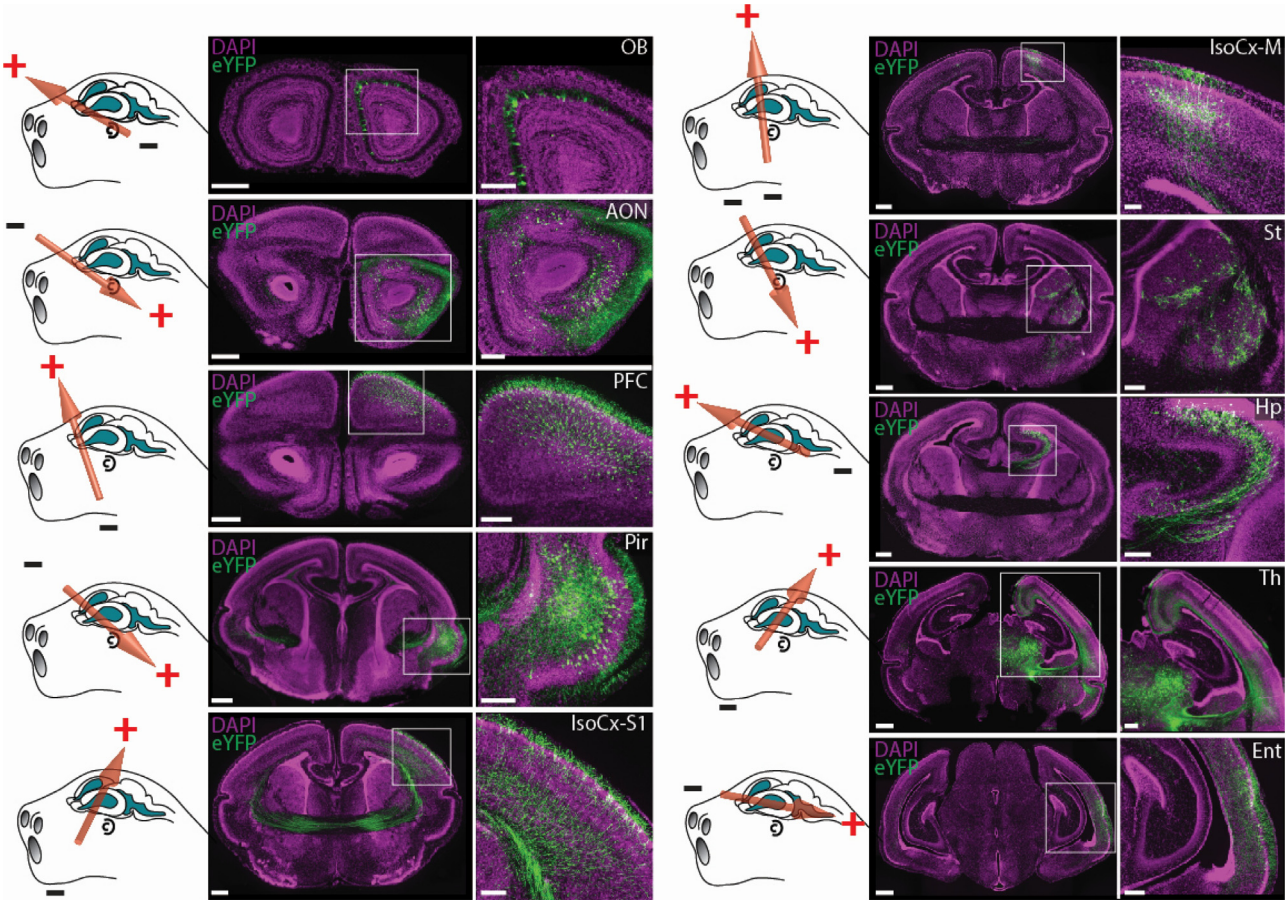

Fig. 5. High-precision labelling of different forebrain areas with in pouch electroporation in dunnarts. By combining the timing of electroporation and the position of the electrodes, it is possible to label specific neuronal populations in the dunnart brain. Left schematics indicate the approximate position and direction of current delivered via forceps-type electrodes. We labelled the olfactory bulb (OB), the anterior olfactory nucleus (AON), prefrontal cortex (PFC), piriform cortex (Pir), primary somatosensory cortex (IsoCx-S1), motor cortex (IsoCx-M), striatum (St), hippocampus (Hp), thalamus (Th), and entorhinal cortex (Ent). Insets of each case are indicated on right panels. Electroporations were performed at stage 18 to label olfactory bulb and anterior olfactory nucleus; stage 19 to label thalamus; stage 20 to label piriform cortex and striatum; stage 21 to label entorhinal cortex; stage 23 to label prefrontal cortex, primary somatosensory cortex, motor cortex and hippocampus. All the joeys were collected at stage 27. Scale bars: 200 μm for whole brain sections, and 100 μm for the insets.

tionary questions. To our knowledge, this is the first description of marsupial electroporation, and its feasibility opens numerous experimental possibilities. For example, considering that this technique labels the cells that are born on the day of the procedure, it allows comparative studies about the relative timing of neurogenesis in marsupials and eutherians. Questions of brain evolution might also be resolved by comparing the developmental mechanisms, sequence of events, and functional features of various brain components between dunnarts and mice. Furthermore, dunnarts can serve as useful animal models to investigate different stages of circuit development, such as neuronal differentiation, migration and maturation, dendrite and axon formation, and the establishment of functional connections. This technique may, therefore, help to elucidate not only crucial events during the evolution of the mammalian brain, but also important basic mechanisms of cortical wiring of biomedical relevance.

The establishment of a method for multiple and selective gene manipulation in a mammalian model of postnatal brain development also opens exciting possibilities to study how early environmental factors might affect this process. For example, specific populations of neurons could be genetically manipulated and combined with paradigms of sensory stimulation and/or disruption to investigate their individual contribution to brain formation. Exploiting this model may provide crucial insights into the early mechanisms of forebrain formation, including what aspects of eutherian and marsupial biology are related to their differences in brain organisation.

5. Conclusion

In this study, we show the potential uses of in pouch electroporation in marsupials to perform multiple transfection events in the developing forebrain, with high success and low mortality. In pouch electroporation could be used to obtain crucial insights into the early mechanisms of mammalian brain formation, by combining experimental techniques that would not be feasible in rodents or other eutherian animal models. In addition, this approach also offers opportunities to address comparative and evolutionary questions, as well as to explore the roles of genetic and environmental influences on circuit formation in health and disease.

Declaration of interests

The authors declare no conflict of interests

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