Short communication

An inhalation anaesthesia approach for neonatal mice allowing streamlined stereotactic injection in the brain

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ABSTRACT

Background: Investigating brain function requires tools and techniques to visualise, modify and manipulate neuronal tissue. One powerful and popular method is intracerebral injection of customised viruses, allowing expression of exogenous transgenes. This technique is a standard procedure for adult mice, and is used by laboratories worldwide. Use of neonatal animals in scientific research allows investigation of developing tissues and enables long-term study of cell populations. However, procedures on neonatal mice are more challenging, due to the lack of reliable methods and apparatus for anaesthesia of these animals.

New method: Here, we report an inhalation-based protocol for anaesthesia of neonatal (P0 − 2) mice and present a custom 3D-printed apparatus for maintenance of anaesthesia during surgical procedures. Our optimised method of anaesthesia enables a rapid method of stereotactic injection in neonatal mice for transduction of brain tissue.

Results and comparison with existing methods: This approach significantly enhances animal welfare and facilitates wider and simpler use of neonatal rodents in scientific research. We demonstrate this procedure for targeted labelling of specific brain regions, and in vivo modification of tissue prior to organotypic culture.

Conclusions: Our protocol for reliable delivery of inhalational anaesthetics can be readily adopted by any laboratory and will enable safer use of neonatal rodents across a diverse spectrum of scientific disciplines. Application to stereotactic injections allows a rapid and efficient method for modification of brain tissue.

1. Introduction

Understanding the mechanisms of brain function requires approaches that reveal the structure, connectivity, and activity of neuronal networks. Recombinant DNA technology has enabled development of powerful genetically encoded tools for visualisation of cell populations, editing of genomic DNA, and tracing of neuronal circuits. These tools can be delivered in repurposed viruses into specific brain areas of adult rodents using intracerebral stereotactic injections (Cetin et al., 2006). This widely performed technique involves surgically opening the skull before injection, requiring approximately 40 min. per animal (Cetin et al., 2006), but is highly efficient and provides reliable neuronal transduction in adult animals.

Research using neonatal rodents is less prevalent, but no less valuable, allowing analysis of developmental processes, long-term transgene expression, and use of young tissue for sensitive electrophysiology experiments. However, the use of neonates is complicated by a lack of experimental equipment for animals of this size, and critically, the difficulty of anaesthesia in these animals. Neonatal anaesthesia is notoriously unreliable and uncontrollable, posing a significant welfare challenge to their use. Historically, anaesthesia has been performed using hypothermia, where pups are incubated on ice for around 15 min, but the depth of hypothermic anaesthesia is unknown and difficult to control. It is unclear whether hypothermia produces anaesthesia or simply immobilisation (Flecknell, 2009), and the recovery from which has been suggested to be painful and damaging. Therefore, in accordance with 3Rs principles, this approach is now being restricted (Herrmann and Flecknell, 2019).

Here, we present a procedure for inhalation anaesthesia of neonatal mice at P0 − 2 (Fig. 1A). Using a 3D-printed apparatus for anaesthetic delivery (see Materials & Methods), we detail a protocol for the controlled maintenance and reliable recovery from inhalation anaesthesia using isoflurane. This method can be readily applied by any laboratory and provides important improvements in neonatal rodent welfare, facilitating their use in a range of scientific disciplines. Using this approach, we perform intracerebral injection of adeno-associated viruses.
(AAV) into discrete brain areas of P0−2 mice. A complete litter of postnatal animals can be safely and reliably injected in less than 2 h, offering a rapid and efficient means for brain region specific transduction.

2. Materials and methods

2.1. Materials

| Syringe Preparation:          | WPI, 18100–3          |
|-------------------------------|-----------------------|
| Borosilicate glass capillary  | WPI, 18100–3          |
| Pipette puller                | Narishige, PC-10      |
| Microforge                    | Narishige, MF-830     |
| Mineral oil                   | Sigma Aldrich, 330,779|
| Hamilton syringe, 5 μL        | Model 75, 7634–01 Hamilton Company |
| Dual Ferrule Adaptors         | Hamilton, 55751–01    |
| 1 mL Syringe and 30G Needle   | Becton Dickinson; 303172, 304000 |
| Parafilm®M                    | Bema, PM-996          |
| Surgery Cover                 | Buster, 141765        |
| Surgical setup:              | Kopf, Model 940      |

2.2. Animal use

Procedures were performed in accordance with UK Home Office regulations. Experiments conducted in the UK are licensed under the UK Animals (Scientific Procedures) Act of 1986 following local ethical approval (Animal Welfare Ethical Review Body). P0−2 C57BL/6J0a
2.6. Immuno

cell

2.4. Imaging labelled tissue

2.5. Organotypic slice preparation

2.7. Electrophysiology

2.3. Viruses

pAAV.CAG.LSL.tdTomato (Addgene #100,048) was produced by Addgene, and AAV9.hSyn.HLEGFPCre.WPRE.SV40 (Addgene #105,540) was produced by U-Penn Vector Core.

2.1. Setup & preparation

2.8. Custom mould production

2.3. Induction of anaesthesia

The entire nest, including pups and nesting material is moved from the parent cage to an empty cage containing an absorbent pad immediately prior to starting the procedure. Induction of anaesthesia is conducted in a small induction chamber with 2L/min flow of 4 % isoflurane in O2 for 3–4.5 min. After 3 min., anaesthesia is tested at 30-second intervals by performing a gentle foot pinch using forceps. Loss of motor response denotes sufficient induction. The neonate is then transferred to the anaesthetisation mould with inflow of 4 % isoflurane at 2L/min, secured in place using a tissue covering and micropore tape (Fig. 1C1). To minimise exposure of the experimenter to isoflurane delivery system, and the outflow to a scavenging unit using flexible tubing, and sealed with Parafilm (Fig. 1C2).

2.2. Syringe preparation

Borosilicate glass capillaries are pulled into tapered glass needles using a single-step heating protocol on a pipette puller (Fig. 1D). Bevelled tips are created by breaking the glass using forceps at a diameter of ~50 to 80 μm (Fig. 1D2). Tip dimensions of every pipette are confirmed under magnification (e.g. using a microscope).

Excluding air from the syringe ensures accurate volume dispensing; therefore the syringe is entirely filled with mineral oil. The needle is backfilled using a Luer syringe and 30G needle, secured in the Hamilton syringe using glass pipette adaptors, and positioned on the frame.

AAV is loaded into the glass needle through the tip to minimise the wasted viral volume. A drop of AAV-containing PBS is placed on Parafilm beneath the tip and the Hamilton syringe plunger is retracted using the stereotactic frame to draw the required volume into the needle (Fig. 1D1). The AAV volume should not exceed that of the glass pipette to prevent cross-contamination between experiments. Glass needles are replaced after injecting 3 animals to prevent clogging or blunting. Replacing needles after every animal requires significant preparation time, prolonging the procedure, and risking maternal re

2.9. Intracerebral injection

Injection positions for specific brain regions are measured relative to lambda (Fig. 1C2 insert). Under LED illumination, the pipette is positioned above the injection site, and depth axis (z) is zeroed at the height of the skin. Injection is performed by rapidly and firmly lowering the glass needle to penetrate both the skin and skull. After penetration,
the injection depth is adjusted to the desired location, and virus is slowly dispensed under hand control. We use 0.5 μL injection of virus at around 1 × 10^{13} GC ml^{-1} per hemisphere. The glass pipette is retracted gently from the skull, and injection is repeated on the second hemisphere if required.

3.5. Recovery

The total duration of anaesthesia is approximately 6–10 min s. After injection, the animal’s tail is marked with pen to discriminate from uninjected animals, and returned to the nest with the remainder of the litter for recovery from anaesthesia. Subsequent animals are injected following the same procedure. Once all injections have been completed, pups are checked for successful recovery (usually 10–20 min), as determined by spontaneous limb mobility. The nest is returned to the parent cage after successful recovery of all animals. Injection of a litter of 6–8 pups usually takes around 1.5–2 hours from setup to recovery.

4. Results

4.1. Induction of neonatal inhalation anaesthesia

We aimed to develop a robust and reliable technique for anaesthetising neonatal mice, as an alternative to hypothermia. We monitored the induction of anaesthesia of P0–2 mice using 4% isoflurane in an induction chamber, as is typically used for anaesthesia of adult mice. At least 3 min of isoflurane exposure was required for reliable and sufficient anaesthetic depth, which is significantly longer than for adult mice. However, the duration must be confirmed on every animal using a gentle foot pinch, as movement levels of neonatal mice can be minimal even without complete anaesthetic induction.

We have noticed that increased duration of anaesthesia can correlate with non-recovery, however this has not been experimentally examined for ethical reasons. Due to this observation, we induce pups for no more than 4.5 min., and if response to foot pinch is still evident, the animal will not undergo the procedure. Using induction of between 3 min. to 4.5 min., a trained user can expect minimal non-recovery occurrences (30/30 mice recovered across 7 injected litters).

4.2. Maintenance of anaesthesia using a custom frame

Maintenance of anaesthesia requires apparatus for circulation of anaesthetic at the animal’s snout, while allowing access to the cranium. We designed and produced a 3D-printed gas flow apparatus consisting of a neonate-shaped mould with a nose-cone formed of concentric rings while at P0, as infant mortality occurring in the first 24 h post birth is avoided.

For adult animals, access to the brain requires drilling through the skull, while at P0–2, the soft skull can be penetrated by a glass injection needle. This greatly simplifies the procedure, dramatically decreasing the protocol duration, and allows injection of a complete litter (6–8 pups) in around 1.5–2 hours from setup to recovery. Due to the developmental thickening of the skull, this approach can only be used for animals up to P3.

4.3. Consideration of neonatal mouse age

To limit the loss of injected pups, in particular from maternal cannibalism, we and others (Li and Daly, 2002) perform injection at P1 rather than P0, as infant mortality occurring in the first 24 h post birth is avoided.

For adult animals, access to the brain requires drilling through the skull, while at P0–2, the soft skull can be penetrated by a glass injection needle. This greatly simplifies the procedure, dramatically decreasing the protocol duration, and allows injection of a complete litter (6–8 pups) in around 1.5–2 hours from setup to recovery. Due to the developmental thickening of the skull, this approach can only be used for animals up to P3.

4.4. Intracerebral injection for neuronal labelling

We demonstrate this procedure for two experimental approaches. Firstly, we injected two discrete brain areas for sparse fluorescent...
labelling of neuronal populations by coinjection of AAVs expressing Cre-dependent tdTomato (lox-Stop-locx cassette) and Cre-EGFP at a ratio of 10,000:1 (Fig. 2A1, see Weiler et al., 2018). 0.5 μl of viral mixture was injected per hemisphere at a total concentration of 1.5 × 10^{12} GCMl^{-1} into either the hippocampus (From lambda, in mm: Rostral (+) / caudal (−): 0.0, Lateral: ± 1.3, Ventral: 1.4) or frontal cortex (Rostral (+) / caudal (−): +1.5, Lateral: ± 1.0, Ventral: 1.0). On brain dissection at P8, robust expression was evident in the corresponding brain areas (Fig. 2A2-3).

4.5. Intracerebral injection for organotypic tissue preparation

Hippocampal organotypic slice culture is a powerful method allowing ex vivo analysis of neuronal physiology (Gähwiler et al., 1997), but requires tissue harvesting at around P7. Neonatal injection allows in vivo transduction of tissue prior to harvesting, increasing the flexibility of experimental timings. This is particularly important for genomic manipulations such as Cre-dependent gene excision, which can require weeks for removal of highly abundant or stable target proteins. For instance, the AMPA receptor (AMPA), a glutamate-gated synaptic receptor, requires at least 14 days from Cre-DNA transduction before complete protein loss is achieved in conditional knockout mice (Li et al., 2009). Neonatal injection of AAV-Cre before tissue harvesting at P7 allows recording of AMPAR knockout tissue from as early as 8 days in vitro.

To optimise organotypic study of AMPAR knockout tissue, we performed injection of AAV-Cre-EGFP (0.5 μl at 3 × 10^{12} GCMl^{-1}) into the hippocampus of P1 Gria1−/− mice (Conditional GRIA1−/− knockout). Cre transduction will prevent expression of the three AMPAR subunits present in hippocampal pyramidal cells. Robust EGFP expression was seen in organotypic slices on tissue harvesting at P7 (Fig. 2B1), and slices were cultured until DIV8 for analysis. At this time-point, immunofluorescent detection of AMPARs (antibody to GluA2 protein) in fixed slices showed a mosaic pattern, with AMPAR expression specifically absent from Cre-expressing CA3 cells (Fig. 2B2). Similarly, dual patch-clamp recording of synaptic currents at CA1 pyramidal cells showed an absence of AMPAR currents in Cre-transduced neurons, confirming successful modification of organotypic tissue in vivo (Fig. 2B3). These examples demonstrate the utility of neonatal injection for modification of developing brain tissue, and add a further tool to the neurophysiologist’s toolkit.

5. Discussion

Intracerebral injection of neonatal rodents is a powerful approach for neuroscience research, however reliable anaesthesia of early postnatal animals has been difficult to achieve. Previous reports have been reliant on either hypothermic anaesthesia (Cheetham et al., 2015; Kim et al., 2014), or even a lack of anaesthesia (Li and Daly, 2002), raising major welfare issues (Herrmann and Flecknell, 2019).

Our inhalation-based technique offers a refinement of neonatal rodent anaesthesia that can be employed for recovery procedures on early postnatal animals. Induction of anaesthesia is performed in an induction chamber, however, due to the rapid elimination of isoflurane from the body, maintenance of anaesthesia requires constant delivery throughout the procedure. Apparatus for neonatal stereotactic injection has been reported, but is designed for hypothermic anaesthesia (Cunningham and McKay, 1993). We therefore designed a 3D printable mould for delivery of inhalational anaesthetics. 3D printing allows simple size-scaling of the apparatus for application to various ages or rodent species, and offers affordable production requirements. This anaesthetic procedure can be used not only for intracerebral injections, but any other cranial procedures, and simple redesign of the printed apparatus would allow access to other regions of the animal, for surgical procedures across a range of scientific disciplines. We report the use of isoflurane, however the procedure can be applied to other inhalational anaesthetics. Brief isoflurane exposure has no negative effects on cognition in adulthood (Rosenholm et al., 2017), however transient synaptic changes as possible and should be considered in experimental design (Qi et al., 2016).

We have demonstrated the value of neonatal intracerebral injection for in vivo tissue transduction before organotypic culture, and have previously employed this approach in synaptic receptor research (Watson et al., 2017). Our technique offers a rapid and efficient protocol for neuronal transduction, and published neonatal brain maps (Pilpel et al., 2009) will aid stereotactic targeting of any brain area. This technique may present an alternative to established methods for developmental neuroscience research such as in utero electroporation, with substantial benefits in mouse welfare. The protocol and apparatus that we report provides a significant improvement in rodent welfare, and can be easily adapted to facilitate greater use of neonatal animals in both neuroscience and wider scientific research.

CRediT authorship contribution statement

Hinze Ho: Investigation, Methodology, Writing - review & editing.
Adam Fowler: Methodology, Resources.
Marisa Coetzee: Methodology, Writing - review & editing.
Ingo H. Greger: Funding acquisition, Writing - review & editing.
Jake F. Watson: Conceptualization, Investigation, Methodology, Data curation, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no competing interests. The 3D-printed mould presented in this report is a registered design with the EUIPO (Design #: 007571286 – 0001 to 0004).

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