Journal of Visualized Experiments

A Pipeline using Bilateral In Utero Electroporation to Interrogate Genetic Influences on Rodent Behavior

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61350R2
Full Title:	A Pipeline using Bilateral In Utero Electroporation to Interrogate Genetic Influences on Rodent Behavior
Keywords:	in utero electroporation, prefrontal cortex, gene transfer, development, social behavior, bilateral transfection, neurodevelopmental diseases, pyramidal neurons, neuropsychiatric disorders
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Boston, MA, USA

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TITLE:

A Pipeline using Bilateral In Utero Electroporation to Interrogate Genetic Influences on Rodent Behavior

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SUMMARY:

The role of recently discovered disease-associated genes in the pathogenesis of neuropsychiatric disorders remains obscure. A modified bilateral in utero electroporation technique allows for the gene transfer in large populations of neurons and examination of the causative effects of gene expression changes on social behavior.

ABSTRACT:

As genome-wide association studies shed light on the heterogeneous genetic underpinnings of many neurological diseases, the need to study the contribution of specific genes to brain development and function increases. Relying on mouse models to study the role of specific genetic manipulations is not always feasible since transgenic mouse lines are quite costly and many novel disease-associated genes do not yet have commercially available genetic lines. Additionally, it can take years of development and validation to create a mouse line. In utero electroporation offers a relatively quick and easy method to manipulate gene expression in a cell-type specific manner in vivo that only requires developing a DNA plasmid to achieve a particular

genetic manipulation. Bilateral in utero electroporation can be used to target large populations of frontal cortex pyramidal neurons. Combining this gene transfer method with behavioral approaches allows one to study the effects of genetic manipulations on the function of prefrontal cortex networks and the social behavior of juvenile and adult mice.

INTRODUCTION:

Genome-wide association studies (GWAS) have driven the discovery of novel candidate genes that are associated with brain pathologies[1-4]. These studies have been particularly beneficial in understanding devastating neuropsychiatric disorders such as schizophrenia (SCZ), where the investigation of novel genes has served as a launching point for new lines of research and therapeutic intervention[5,6]. Genes harboring risk for SCZ show biased expression in the prefrontal cortex (PFC) during prenatal and early postnatal development, a region implicated in the pathology of several neuropsychiatric disorders[7]. Additionally, mouse models of psychiatric disorders exhibit abnormal activity in PFC networks[6,8,9]. These results suggest that SZC-associated genes might play a role in the developmental wiring of this region. Further investigation using animal models is required to understand the contribution of these candidate genes to the establishment of connections in the PFC and to determine whether these genes have a causative role in the pathogenesis of neuropsychiatric disorders. Genetic manipulation techniques in mice that allow for the study of gene expression changes on specific neuronal circuits during prenatal and early postnatal development area promising method to understand the molecular mechanisms that link gene expression changes to PFC dysfunction.

Genetic mouse lines offer a method to study the impact of particular genes on brain development and function. However, relying on transgenic mice can be limiting since there are not always commercially available lines to examine the effects of specific genes on developing neural circuits. Moreover, it can be extremely costly and time consuming to develop custom mouse lines. The use of intersectional genetic manipulation strategies that combine transgenic mice with viral approaches has revolutionized the understanding of the brain[10-12]. Despite much progress, viral strategies come with certain limitations depending on the viral vector type, including limits in packaging capacity that can restrict viral expression[13] and cell toxicity associated with viral expression[14]. Furthermore, in most experimental conditions, robust gene expression using adeno-associated virus (AAVs) requires approximately 2 to 4 weeks[15], making routine viral strategies unfeasible to manipulate genes during early postnatal development.

In utero electroporation (IUE) is an alternative approach that allows for a rapid and inexpensive gene transfer[16,17] that, when coupled with fluorescent labeling and pharmacogenetic or optogenetic approaches, provides a powerful platform to dissect the function of neuronal circuits. Additionally, with the development of CRISPR-Cas9 genome editing genes can be overexpressed or precisely altered through cell-type specific knock-down or knock-out of specific genes or through the modulation of promoters[18,19]. Gene manipulation approaches using IUE are especially advantageous when the effect of genes on neuronal circuits need to be tested during narrow developmental windows[20]. IUE is a versatile technique and overexpression can be easily accomplished by inserting a gene into an expression vector under a specific promoter. Additional control of gene expression can be achieved by driving expression using promoters of different

strengths or using inducible promoters capable of temporally controlling gene expression[21,22]. Additionally, IUE allows for the targeting of cells within specific cortical layers, cell types and brain regions, which isn't always feasible using other approaches[5,17]. Recent advances in the IUE configuration based on the use of three electrodes, which generates a more efficient electric-field distribution, have expanded the functional repertoire of this method and allowed scientists to target new cell types and increase the efficiency, accuracy, and number of cells that can be targeted[23,24]. This technique was recently used to determine the causative role of *complement component 4A* (*C4A*), a gene linked to SCZ, in PFC function and early cognition[5].

Presented here is an experimental pipeline that combines gene transfer approaches to target large populations of excitatory neurons in the frontal cortex, including the PFC, with behavioral paradigms that not only enables the study of cell and circuit-level changes, but also allows behavior to be monitored throughout early postnatal development and adulthood. First described is a method to bilaterally transfect large populations of layer (L) 2/3 pyramidal neurons in frontal cortical regions. Next, tasks to assay social behavior in juvenile and adult mice are outlined. Cell counts can be obtained upon the completion of behavioral tasks to quantify the extent and location of cell transfection. Furthermore, the number of cells transfected can be correlated with behavioral data to determine if a greater number of transfected cells leads to greater perturbations in behavior.

PROTOCOL:

All experimental protocols were conducted according to the National Institutes of Health (NIH) guidelines for animal research and were approved by the Boston University Institutional Animal Care and Use Committee (IACUC).

1. DNA solution preparation

1.1. Purchase a commercial plasmid or subclone a gene of interest into plasmid with desired promoter. Here, a plasmid containing EGFP under the CAG promoter (pCAG-EGFP) was used.

NOTE: Determine the desired promoter based off the level of expression needed. In general, plasmids under the CAG promoter can be used to achieve high levels of the transgene whereas cell-type-specific promoters (e.g., synapsin for neurons) tend to be less active. The experimenter should empirically determine the appropriate expression levels for each plasmid.

1.2. Transform bacteria and grow stocks in bacterial media with the appropriate antibiotic.

1.2.1. Remove competent cells (DH5α cells) from -80 °C and thaw on ice for 20 min.

1.2.2. Mix 100 pg -100 ng of the plasmid DNA with 30 µL of competent cells. Incubate the mixture on ice for 20 min.

1.2.3. Perform heat shock by incubating in a 42 °C water bath for 45 s and then returning the tube back to ice for 2 min.

1.2.4. Add 200 - 1,000 µL of LB media to the transformed competent cells and grow for 45 min at 37 °C on a shaking incubator.

1.2.5. Plate 200 µL of the transformed cells into an LB agar plate containing the appropriate antibiotic and incubate the plate overnight at 37 °C.

1.2.6. The next day, incubate one bacterial colony in 200 mL of LB broth with the appropriate antibiotic at 37 °C on a shaking incubator overnight.

1.3. Purify the plasmid DNA using a maxiprep kit.

1.3.1. Follow instructions provided in the obtained maxiprep kit. For elution step, do not elute DNA into the elution buffer. Instead, elute DNA using either 200 µL of sterile 1x PBS or molecular grade water.

1.3.2. Ensure that the final concentration of the DNA is greater than 1 µg/µL. If the plasmid containing the gene of interest does not contain a reporter gene, then also prepare a plasmid to co-transfect with a reporter molecule, such as green fluorescent protein (GFP), to allow for the visualization of transfected cells.

1.4. Prepare DNA solution for the surgery by diluting the plasmid DNA into 1x PBS to 1 µg/µL final concentration of each plasmid. Add fast green dye to the DNA solution to a final concentration of 0.1%. For bilateral injections, prepare 60 µL of solution per dam (for approximately 10 pups).

NOTE: If plasmid does not contain a reporter gene, co-electroporate with GFP. Co-electroporation rates are typically 95% or higher. In our hands, transfection efficiency was not affected by co-transfection. All electroporated plasmids should be diluted to 1 µg/µL.

2. Ordering or breeding timed-pregnant mice

2.1. If ordering timed-pregnant mice, order mice to arrive on embryonic day (E) 13 or earlier to allow the dams adequate time to acclimate to animal housing. In this protocol, CD-1 outbred mice are used for all experiments.

NOTE: Ordering a few days in advance will reduce animal stress and lead to a higher survival rate of the pups.

2.2. If breeding timed-pregnant mice, pair female mice with a male overnight, once a week. Check for the presence of a vaginal plug on the following morning (E0.5). Determine pregnancy by monitoring the weight of the female mice.

NOTE: Different mouse strains have different weight increases through pregnancy, so determine typical weight gain for the mouse strain used.

2.3. Whether ordering or breeding the mice, to reduce stress of the dams, place a nesting pad and mouse house in the cage. Reducing stress can help increase the survival rate of the pups.

3. Design and assembly of three prong electrode

3.1. Use grade 2 titanium sheets with a thickness of 0.063 in as a stock material for electrode contacts.

3.2. Using standard machining techniques or precision hand tools, make electrodes with the following dimensions: 20 mm x 5 mm with a rounded tip and grooved back. Remove any rough edges or burrs using fine grit sandpaper.

3.3. To wire the electrode contacts, wrap 22 G stranded copper wire around the grooves of the electrode and secure by soldering. Protect this joint using heat shrink tubing.

3.4. Then, attach the connected electrode to autoclavable, non-conductive forceps using an additional heat shrink tubing to make the two negative electrodes. Attach the single positive electrode to an autoclavable, non-conductive material (such as a toothbrush handle with a sawed off head). Fit the open end of the wire with a standard banana plug.

4. Preparation for surgery

4.1. Bring pregnant dams to the surgery area at least 30 min prior to the surgery to allow for the reduction in stress levels after transport from the animal facility.

4.2. Sterilize the entire surgery site using sterilizing germicidal wipes and then 70% ethanol.

4.3. Sterilize autoclaved tools in a glass bead sterilizer.

4.4. Transfer sterile 1x PBS (about 50 mL per dam) to 50 mL conical tubes and place in a tube rack in the water bath heated to 38-40 °C. Check the sterile saline temperature with a thermometer.

4.5. Turn on the water heating circulation pump so that it is warmed to 37 °C prior to the start of surgery. This will maintain the mouse's body temperature for the duration of the surgery.

4.6. Turn on the pressure-injector and electroporator and ensure proper function prior to the surgery.

4.7. Briefly spin the plasmid DNA solution (obtained in step 1.4) on a tabletop centrifuge and place it on ice.

4.8. Pull glass pipettes on a pipette puller so that the tip of the pulled-glass pipette is about 50 µm in diameter.

4.9. Fill pipette with 20-40 µL of DNA solution (obtained in step 1.4).

4.10. Set up all necessary items for surgery including hair removing lotion, iodine, 70% ethanol, cotton swaps, eye drops, sutures, gauze, etc.

4.11. Prepare a surgery sheet and fill out the necessary information, such as mouse ID and weight, date of surgery, surgeon name, etc.

5. In utero electroporation surgery

5.1. Weigh the mouse prior to the surgery and note this on the surgery sheet.

5.2. Anesthetize a pregnant mouse (E16) by inhalation in an induction chamber with 4% (v/v) oxygen-isoflurane mixture. Once the mouse has been induced, move to a mask inhalation and maintain isoflurane at 1-1.5% (v/v) and monitor breathing throughout the surgery. Check that the mouse is fully anesthetized, the breath rate should be ~55-65 breaths per min.

5.3. Administer preoperative analgesics: buprenorphine (3.25 mg/kg; SC) and meloxicam (1–5 mg/kg; SC) at a max volume of 10-30 ml/kg.

5.4. Use hair removal cream or carefully use a razor to remove the fur from the abdomen. Sterilize the abdomen by swabbing with povidone-iodine and 70% ethanol and repeat this at least 3 times. Create a sterile field around the abdomen using a sterile gauze, sterile draping can also be used.

5.5. Make a midline incision (3-4 cm) in the abdominal skin, being sure to lift the skin up with forceps to avoid cutting through the muscle. Then cut through the muscle, again taking care to lift the muscle up to avoid cutting vital organs.

5.6. Carefully pull the uterine horns out of the dam using ring forceps and place them gently onto the sterile field, making sure that the uterine horn is supported with padding and isn't tugging too far away from the dam. From this point on, keep the uterine horn moistened throughout the rest of the surgery with the pre-warmed sterile 1x PBS.

5.7. Position an embryo using either forceps or fingers. Carefully insert the pulled glass pipette into the lateral vertical, which can be visually identified between the midline of the brain and the eye. Inject about 2-3 µL into each lateral ventricle by either inserting the pipette into one and then the other ventricle (recommended) or by injecting the DNA solution into one ventricle until it passes into both lateral ventricles. The ventricle has been successfully targeted if a crescent shape is present after injection.

NOTE: The tip of the glass pipette could break during surgery. If this happens, replace the glass pipette, ensuring that the uterine horns are kept moistened while a new pipette is prepared and

filled with the DNA solution.

5.8. To transfect cells bilaterally in the frontal cortex, position the two negative electrodes on the sides of the embryos head just lateral and slightly caudal to the lateral ventricles and position the positive electrode between the eyes, just in front of the developing snout.

5.9. Ensure the embryo is generously moistened. Apply four square pulses (pulse duration = 50 ms duration, pulse amplitude = 36 V, interpulse interval = 500 ms).

5.10. Inject and electroporate all embryos, going one-by-one so that each embryo is electroporated immediately after the DNA solution injection. Once all embryos have been electroporated, carefully insert the uterine horns back into the abdominal cavity. During this step, coat the abdominal cavity in sterile PBS (1x) to aid uterine horn placement.

5.11. Fill the abdominal cavity with sterile 1x PBS so that no air pockets remain after suturing is complete. Suture the muscle with absorbable sutures and the skin with silk non-absorbable sutures.

5.12. Allow dam to fully recover in a heated chamber for at least 1 h. In the next 48 h, check on the dams regularly. As the dam recovers from the anesthesia and regains consciousness, it will start moving and whisking.

5.13. Administer post-operative analgesics if dams are showing signs of pain, such as balling their bodies up and breathing rapidly. Only administer if there are signs of pain since SC injections could stress out the dams, but if administered to the experimental group also administer to the control group, or vice versa.

6. Assaying early social behavior in a maternal interaction task

NOTE: This protocol is adapted from previous publications[5,25]. Perform this task after mice have been born from postnatal day (P) 18-21.

6.1. Maternal homing behavior in the maternal interaction I (MI1) task.

6.1.1. Ensure that cage bedding is not changed in the week before the task will be performed.

6.1.2. Obtain or build an open field (OF) arena that can be easily cleaned (acrylic is recommended) with the following dimensions: 50 x 50 x 30 cm (length-width-height). Lighting conditions during performance of behaviors can vary depending on experimental question and can influence levels of arousal and anxiety-like behavior. Record behavioral experiments under a dim light (approximately 20 lux) positioned over the center of the arena.

6.1.3. For two days prior to the behavior testing, acclimate the dam to the arena by placing it beneath a mesh wire cup (such as a pencil cup) in a corner of the arena for five min per day.

6.1.4. On the testing day, which can be from P18-21, before mice have been weaned, clean the arena thoroughly with sanitizing wipes and 70% ethanol.

6.1.5. Set up the arena with two opposing corners both containing clean bedding and one corner containing soiled nest bedding from the pup's home cage.

6.1.6. Allow each pup to explore the arena for 3 min, placing each pup in the neutral, empty corner at the start.

NOTE: Record the behavior with a video camera at 30 fps. Thoroughly clean the arena between every pup and replace the fresh bedding. Alternate which corner is the fresh versus nest bedding as a control to avoid corner preference due to other reasons (i.e., ambient noise or light). If running multiple litters across the P18-21 developmental window, run behavioral experiments at the same time between days. Also ensure that during a given day, control and experimental groups are run in parallel.

6.2. Maternal social interaction in the maternal interaction II (MI2) task

6.2.1. Perform this task immediately after the MI1 task.

6.2.2. Set up the arena so that one corner contains an empty wire mesh cup and the opposing corner contains the dam under a mesh wire cup. If mice are able to move the cup, weigh the cup down with a weight that can be taped to the top of the cup to prevent movement. Put soiled nest bedding from home cage in another corner.

6.2.3. Run the MI2 task. Place the pup in the empty corner and record behavior for five min at 30 fps, allowing the pup to explore. Run each pup separately and thoroughly clean the entire arena and wire mesh cups between every pup.

7. Assaying adult social behavior task

7.1. Run adult social behavior in the same mice that were run in the MI1 and MI2 task once they are adults (P60-P70 or older). Data collected here was done in a separate cohort.

7.2. Handle the adult mice for 3 consecutive days to allow habituation to the experimenter. Ensure that only experimenters that have been familiarized to the mice run behavior experiments, ideally have the same person run all tasks.

7.3. Habituate mice to the OF arena for 3 days for 5 min each day.

7.4. Assay behavior in a novel object recognition task to measure general locomotion and interest in a novel object. This will allow more meaningful interpretation of social behavior if mice have a specific deficit in social interactions.

7.4.1. Place mice in the arena for 5 min with a novel object (small plastic toy with smooth, cleanable surfaces) in one corner of the arena. Clean the arena thoroughly between mice with 70% ethanol.

7.4.2. For novel object recognition, place the previously exposed ‘novel object’, which is now familiar, in one corner and place a new novel object in the opposing corner. For all tasks, switch the corners between trials as a control.

7.4.3. For novel social interaction, ensure that the novel mice are age, strain and sex-matched and are acclimated to the mesh wire cup for 2 consecutive days for 5 min each day. For each trial, place a novel mouse under a mesh wire cup and in the opposing corner place an empty mesh wire cup. Let the mice explore the arena for 5 min while recording behavior. Clean the arena and mesh wire cups thoroughly between each trial.

8. Analyzing behavioral data

8.1. Use DeepLabCut (https://github.com/AlexEMG/DeepLabCut) to perform basic body part tracking). Detailed notes on how to install and use DeepLabCut can be found on its GitHub page. Also available is a custom python-based library 'dlc_utils' (https://github.com/balajisriram/dlc_utils) for further analysis of the data after basic body parts tracking is completed. More details about how to use this library can be found in the GitHub page.

8.1.1. Install DeepLabCut using the anaconda installation process. Install a GUI capable CPU-only version of DeepLabCut as well as the GPU-enabled version for training the network.

8.1.2. Follow the instructions available in the link below to create a project for tracking body parts. Breifly, choose a sample of frames from your data set and manually mark the relevant body parts in these sampled frames. Train the DeepLabCut network to predict the body parts and verify that the trained network performs adequately.
https://github.com/AlexEMG/DeepLabCut/blob/master/examples/Demo_yourowndata.ipynb

8.1.3. For the purposes of tracking body position and identifying simple interactions in an open field, identify the centroid of the animal, the head (operationally defined as the midpoint between the ears), the left and right ears as well as the snout and the base of the tail. Having multiple body parts tracked allows for the appropriate substitution when some body parts are missing in the frame due to occlusion.

8.1.4. Apart from animal body parts, track a variety of points related to the environment: such as the edges of behavior boxes. These allow for repeatable estimation of such points across multiple sessions - even if the position of the behavioral setup slightly changes relative to the camera between sessions.

3.1.5. After tracking the body parts from the behavior data, take care to filter the predicted body part locations based on the confidence associated with the prediction on each frame of the video. Low confidence predictions are usually associated with occluded body parts. For such predictions, substitute a given body part with another (if such substitution is appropriate) or use the locations of other body parts to predict where the relevant body part is likely to be. For most open field applications, the centroid of the rodents' body is rarely occluded and can be predicted with high accuracy and precision.

3.1.6. Use the predicted location of the centroid as well as the location of the tracked points in the environment to estimate a number of features of the animal's behavior. For example, in the open field data, the time derivative of the position can be used to calculate the speed of the animal.

3.2. To avoid bias, perform all experiments “blind” with respect to the experimental group when possible, particularly when there is any subjective element in assessing the results. Test for the effect of sex differences on the main experimental outcomes by the pooling of data into male and female groups. All statistical tests are designed to test an equal number of animals between groups.

9. Post hoc cell counting to characterize extent of cell transfection

9.1. Determine the number of cells transfected per mouse since not all mice will have successful transfection and there will be variation in the number of transfected cells. One method to achieve this involves counting the number of transfected neurons in alternating coronal sections followed by an interpolation to estimate the total number of transfected cells. For this, image and count every other coronal section (50 µm).

9.1.1. Use transcardial perfusions with 4% PFA to fix tissue and then dissect the brain. After cryoprotection, section the brain in coronal sections at 50 µm.

9.1.2. For the frontal cortex, count cells within +2.75 and + 1.35 mm from Bregma. These coordinates contain frontal cortical areas and includes part of somatosensory cortex (S1). Using this method, there were no observed transfected cells in more caudal cortical regions or subcortical areas.

9.1.3. Be sure to denote the left from right hemisphere, such as marking one hemisphere with a needle hole during sectioning, and count cells bilaterally. Use an automated cell counting software or count cells manually, confirming the presence of a cell body using DAPI.

9.2. Once cell counts are obtained, set a threshold for inclusion. For example, only include mice that are bilaterally electroporated in analysis. For further analysis, correlate number of cells transfected with behavioral responses to see if there is an association. This technique will target multiple brain areas, so it is necessary to provide information on which brain regions have been genetically manipulated.

NOTE: It is possible that manipulating certain genes could alter neuronal migration, specification and/or death. Ensure during cell counting that brain anatomy is examined and each transfected neuron is within the layer that was supposedly transfected (i.e. L2/3). Gross anatomical measurements such as cortical thickness can also be quantified by measuring the distance from the pia to cortical L6.

REPRESENTATIVE RESULTS:

Successful development and implementation of a custom-built electroporator and three prong-electrode.

For IUEs, an inexpensive custom-built electroporator was built based on a previously described design[27] (**Figure 1A** and **Figure 2**). A three prong electrode was made[23,24] using plastic forceps with 2 negative electrodes attached to the tips of the prongs and the positive electrode was attached to the end of a toothbrush handle (**Figure 1B**). The electroporator and three prong electrode were tested to ensure proper function. IUE was performed by exposing the uterine horns, injecting plasmid DNA and electroporating each embryo (**Figure 1C**). The three prong electrode can be held fairly easily in two hands as shown (**Figure 1B**, right), using the prongs to stabilize the embryo's head. L2/3 PFC pyramidal somas and their processes were labeled with GFP via IUE, thus confirming the success of the gene transfer experiment.

Targeting a large population of neurons bilaterally in the frontal cortex of mice.

The total number of transfected cells and the distribution of transfected neurons can be quantified for both juvenile and adult mice[5]. Using this bilateral IUE method, about 4000-6000 L2/3 pyramidal neurons were transfected with the pCAG-GFP plasmid (**Figure 3A**). Additionally, most of these cells were localized to frontal cortical regions including the frontal association cortex, motor association areas, prelimbic and infralimbic cortex and the orbital and anterior cingulate cortex (**Figure 3B**). A representative example shows the rostral-caudal distribution of transfected neurons in an adult control mouse (P60, **Figure 3C**). This confirms the ability of bilateral IUEs to target and genetically label large populations of L2/3 pyramidal neurons in the frontal cortex.

Social behavior in juvenile and adult mice.

The first part of the maternal interaction task tested the ability of control P18 transfected mice (IUE with pCAG-GFP plasmid) to find nest bedding (maternal interaction 1 [MI1]). This tests the sensorimotor abilities of the juvenile mice. Control mice spent more time exploring their nest bedding than exploring fresh bedding. Thus, suggesting that, as expected, these mice have intact sensorimotor abilities and exploratory behavior (**Figure 4A** and **Figure 3B**). The second part of the task (MI2) takes advantage of the tendency of mice to be motivated to interact with and be near their mother. In this task, pups spent most of their time near their mother while spending significantly less time exploring the empty cup or nest bedding (**Figure 4C**,**D**). These results suggest that IUE control mice exhibit normal homing behavior.

Adult control mice (P60) spent approximately 35% of the time exploring a novel object (total time

spent in the arena = 5 min, **Figure 5A**,**B**). When presented with a novel and familiar object, adult mice spent more time exploring the novel object, suggesting intact interest in novelty (**Figure 5C,D**). In the sociability task, control adult mice spent similar amounts of time exploring a novel mouse and empty cup (**Figure 5E,F**). This behavior was automatically tracked using the freely available DeepLabCut software[26]. Example videos show successful labeling of various points on a mouse, including the limbs, centroid, head and ears (**Video 1**). DeepLabCut was used to determine when mice were rearing by examining the length of the mouse's body, since this distance becomes shorter when the mouse rears (**Video 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: In utero electroporation using a custom-built electroporator and a three prong electrode. (**A**) Image of the custom-built electroporator (left) and its internal circuit (right). 1: Power Indicator. 2: Power Switch. 3: Pulse Indicator. 4: Test Mode Switch. 5: Voltage Selector. 6: Pulse Width Control. 7: Electrode (+). 8: External Trigger (TTL). 9: Electrode (-). (**B**) Image of the custom-built three prong electrode (left) and the recommended method to hold the three prong electrode during the IUE (right). (**C**) Left: Diagram depicting IUE surgery performed in E16 dams. Right: representative 20X confocal image of IUE with GFP targeted to L2/3 mPFC. Yellow asterisk: L2/3 GFP+ neurons. Left panel scale bar = 250 µm. Right panel scale bar = 75 µm. Figures and data adapted from Comer et al., 2020[5].

Figure 2: Circuit diagram of the custom-made electroporator. A diagram depicting the custom-made electroporator circuit based off previously described examples[27].

Figure 3: Targeting large populations of L2/3 frontal cortex neurons using IUE. (**A**) Total number of GFP+ cells in juvenile control mice. N = 15 mice. (**B**) Percentage of GFP+ cells per area in adult control mice. N = 22 control mice. (**C**) Representative sections showing rostro-caudal extent of transfected cells in the frontal cortex. Images in left panels are zoomed areas from the right panels (red square). Frontal association cortex: FrA. Supplementary motor cortex: M2. Prelimbic cortex: PL. Infralimbic cortex: IL. Anterior cingulate cortex: ACC. Medial orbitofrontal cortex: MO. Ventral orbitofrontal cortex: VO. Lateral orbitofrontal cortex: LO. Anterior insular cortex: AI. Frontal cortex area 3: Fr3. Primary motor cortex: M1. Primary somatosensory cortex: S1. Piriform cortex: Pir. Anterior olfactory nucleus: AO. Caudate-putamen: Cpu. Black numbers: Bregma coordinates. Left panel scale bar = 0.5 mm. Right panel scale bar = 1 mm. Mean $\pm$ SEM. (**D**) Data showing the number of cells electroporated on the right versus left hemisphere within each mouse. Showing uninterpolated cell counts from 14 adult mice electroporated with pCAG-GFP plasmid. Paired t-test. $p = 0.4757$. Data adapted from Comer et al., 2020[5].

Figure 4: Assessing sensorimotor abilities, exploratory behavior and early social interactions in juvenile transfected mice. (**A**) Representative example of path traveled (black trace) by a P18 control pup in MI1 task. Fresh bedding corners (fresh 1 and 2, pink) and nest bedding corner (green). (**B**) Control pups spent more time exploring the nest bedding than the fresh bedding in the MI1 task. ***$p < 0.001$, ****$p < 0.0001$. Two-way ANOVA and Sidak's post test. (**C**) Representative example of path traveled (black trace) by a P18 control pup in MI2 task. Dam's

cup (dam: blue), Empty cup (empty cup: yellow), Nest bedding corner (nest: green). (**D**) Control pups spent more time interacting with their dam than with the empty cup or nest bedding. Two-way ANOVA and Sidak's post test. ****$p < 0.0001$. N = 15 control mice. Figures and data adapted from Comer et al., 2020[5].

Figure 5: Assessing social interactions in adult transfected mice. (**A**) Representative example of path traveled (black trace) by P60 control adult mouse in novel object interaction task. pink corner = location of novel object. (**B**) Control mice spent approximately 35% of the time exploring the novel object (5 min total time). Percent time spent in corner with novel object shown. (**C**) Representative examples of path traveled (black trace) by a P60 control adult mouse in novel object recognition task. pink corner: location of novel object. green corner: location of familiar object. (**D**) Control mice spent more time exploring the novel object than the familiar object. Discrimination index (DI) shown; DI = ((time with novel object – time with familiar object) / (time with novel object + time with familiar object)). (**E**) Representative examples of path traveled (black trace) by P60 control adult mouse in sociability task. pink corner: location of novel mouse under mesh wire cup. green corner: location of empty mesh wire cup. (**F**) Control mice spent on average equal time exploring an empty cup and a cup containing a novel mouse. Graph shows DI ((time with novel mouse – time with empty cup) / (time with novel mouse + time with empty cup)). Since mice were not socially isolated prior to the task, the drive to interact with a novel mouse might have been diminished. However, there was still exploration of the novel mouse. N = 22 control mice. Figures and data adapted from Comer et al., 2020[5].

Video 1: Using DeepLabCut to automatically track animal position in behavioral tasks. A representative video of an adult mouse in the social interaction task that has been labeled using DeepLabCut. Various parts of the mouse can be labeled such as the limbs and head. Using the centroid is appropriate to track the position of the animal but other points can be used to identify more complex behaviors, such as grooming or rearing.

Video 2: Using DeepLabCut to automatically track rearing in behavioral tasks. A representative video of an adult mouse in the social interaction task that has been labeled using DeepLabCut. The red arrow shows the length of the mouse with the arrow pointing towards the head of the mouse. The length of the arrow can be used to determine with the mouse is rearing, since the length of the mouse, and the arrow, becomes smaller when the mouse rears.

DISCUSSION:

Herein, a pipeline is described that combines the manipulation of novel genes of interest in large populations of frontal cortical neurons with behavioral assays in mice. Moreover, this pipeline allows for the longitudinal study of behavior in the same mice both during early postnatal development and in adulthood. This technique bypasses the need to rely on genetic animal models that can be costly in terms of time and expenses. The strength of this protocol is that it can be used to study neurodevelopmental and neuropsychiatric disorders for which recent GWAS have discovered novel genetic associations[28,29]. Although this method provides cell-type specific transfection of excitatory neurons, one limitation is that it is less feasible to target other brain cell types such as interneurons or glial cells. However, multiple studies suggest a modified

approach to target other brain cell-types[30,31]. Additionally, by modifying the position of the electrodes relative to the embryo's head and changing the timing of the IUE, other brain regions can be bilaterally transfected including the hippocampus, amygdala, cerebellum, and the visual, somatosensory and motor cortices[24,32]. Additionally, different cortical layers can be targeted by performing IUE at different developmental stages.

Although IUE can have a high success rate, there are critical steps and troubleshooting of the method that is required at times. It is necessary that plasmids are carefully designed and validated in cell lines. As with all cloning, care must be taken to ensure proper gene expression such as confirming the sequence is in frame. Additionally, the effect of gene manipulation (e.g., overexpression or silencing) should be confirmed taking into consideration that expression levels could vary across the developmental time course of the mouse and developmental date of IUE. Western blot and qPCR can be used to determine the extent of genetic overexpression[5]. It is recommended to co-electroporate a reporter gene, such as GFP, in a separate plasmid since proteins tagged with GFP can be mis-folded or lose their function. Alternatively, if a reporter is not used the experimenter can use in situ hybridization, qPCR or western blot to determine expression levels of the gene of interest[5].

If plasmids have been verified but there are no animals that appear to be positive for transfection, thoroughly check all equipment, especially the electroporator, to ensure proper function. When delivering voltage pulses to embryos, the uterine horns should be moistened well with warmed saline and the electrodes should produce bubbles upon generation of the voltage pulse. If no bubbles are present when the voltage is delivered, there is likely a problem with the electroporator. Alternatively, the cDNA might not have been injected properly into the ventricle. When cDNA is injected properly into the lateral ventricle, the fast green dye will be visible in the shape of a crescent. Lastly, the position of the electrodes is important. If the electrodes are positioned slightly incorrectly, cells might not be transfected in the region of interest. Therefore, when checking for a successful transfection, save some more caudal brain sections to see, if perhaps, the wrong brain region was transfected. Once this method has been practiced, an experienced surgeon can expect to achieve a success rate of nearly 90%. This protocol can be modified to target other brain regions of interest. For example, it is possible to target most cortical regions bilaterally and even certain subcortical regions, including the hippocampus[23] . It is also possible to further cut down costs by building a custom electroporator, which was used in the data presented here[5,27].

Future studies could make use of this method to understand the role of newly discovered gene candidates in various neurological diseases. The presented pipeline offers a relatively quick assay to test the effects of specific genetic manipulations on early postnatal development and behavior into adulthood. Future efforts using this method have the potential to discover which genes play a causative role in certain brain disorders, including SCZ and autism spectrum disorder.

ACKNOWLEDGMENTS:
We thank Lisa Kretsge for critical feedback and editing to the manuscript. We thank all research assistants in the Cruz-Martín lab who were invaluable in helping with perfusions and cell counting

of behavior brains. We thank Andrzej Cwetsch for input on the design of the tripolar electrode, and Todd Blute and the Boston University Biology Imaging Core for use of the confocal microscope. This work was supported by a NARSAD Young Investigator Grant (AC-M, #27202), the Brenton R. Lutz Award (ALC), the I. Alden Macchi Award (ALC), the NSF NRT UtB: Neurophotonics National Research Fellowship (ALC, #DGE1633516), and the Boston University Undergraduate Research Opportunities Program (WWY). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES:

The authors declare no competing interests.

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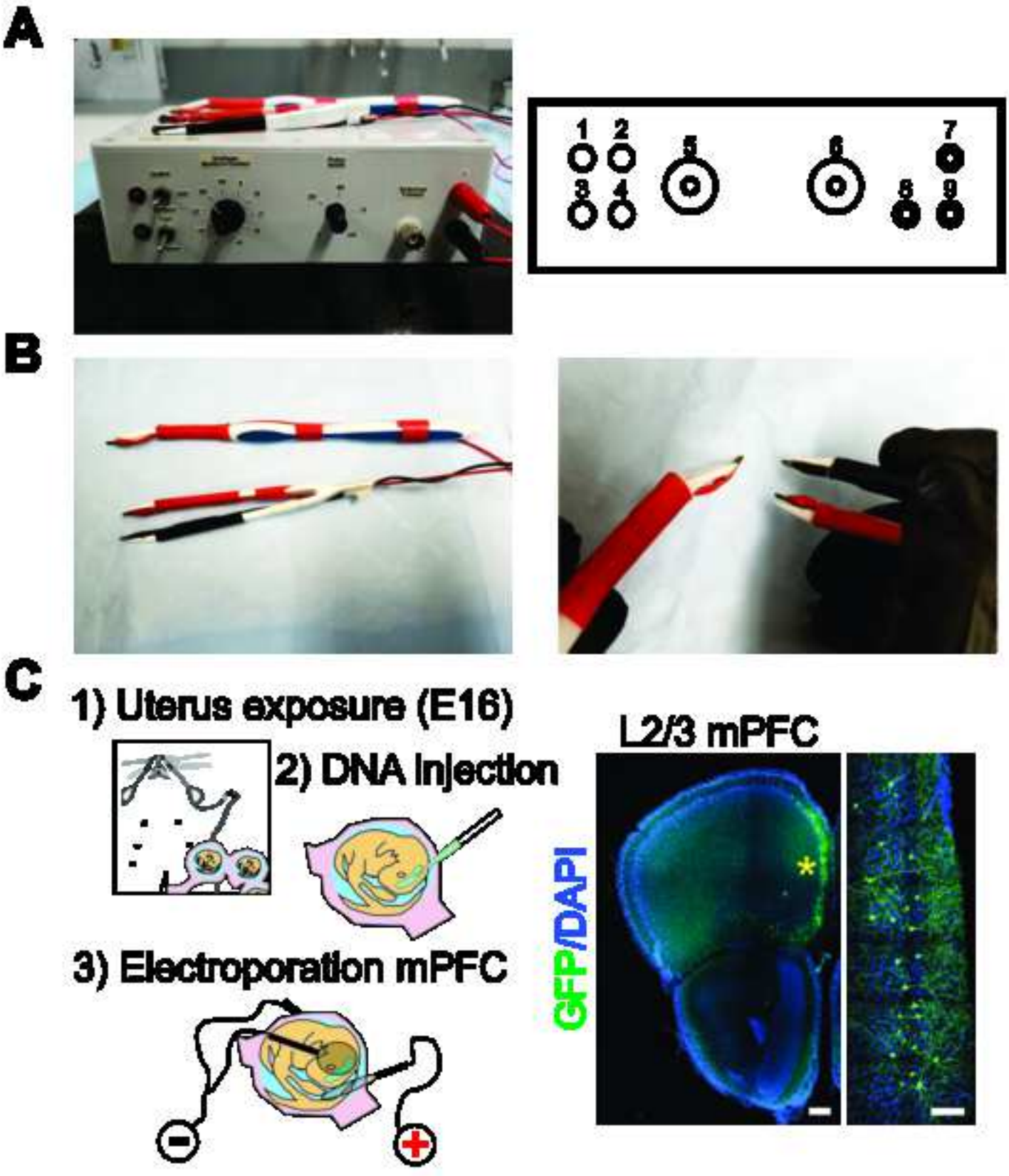

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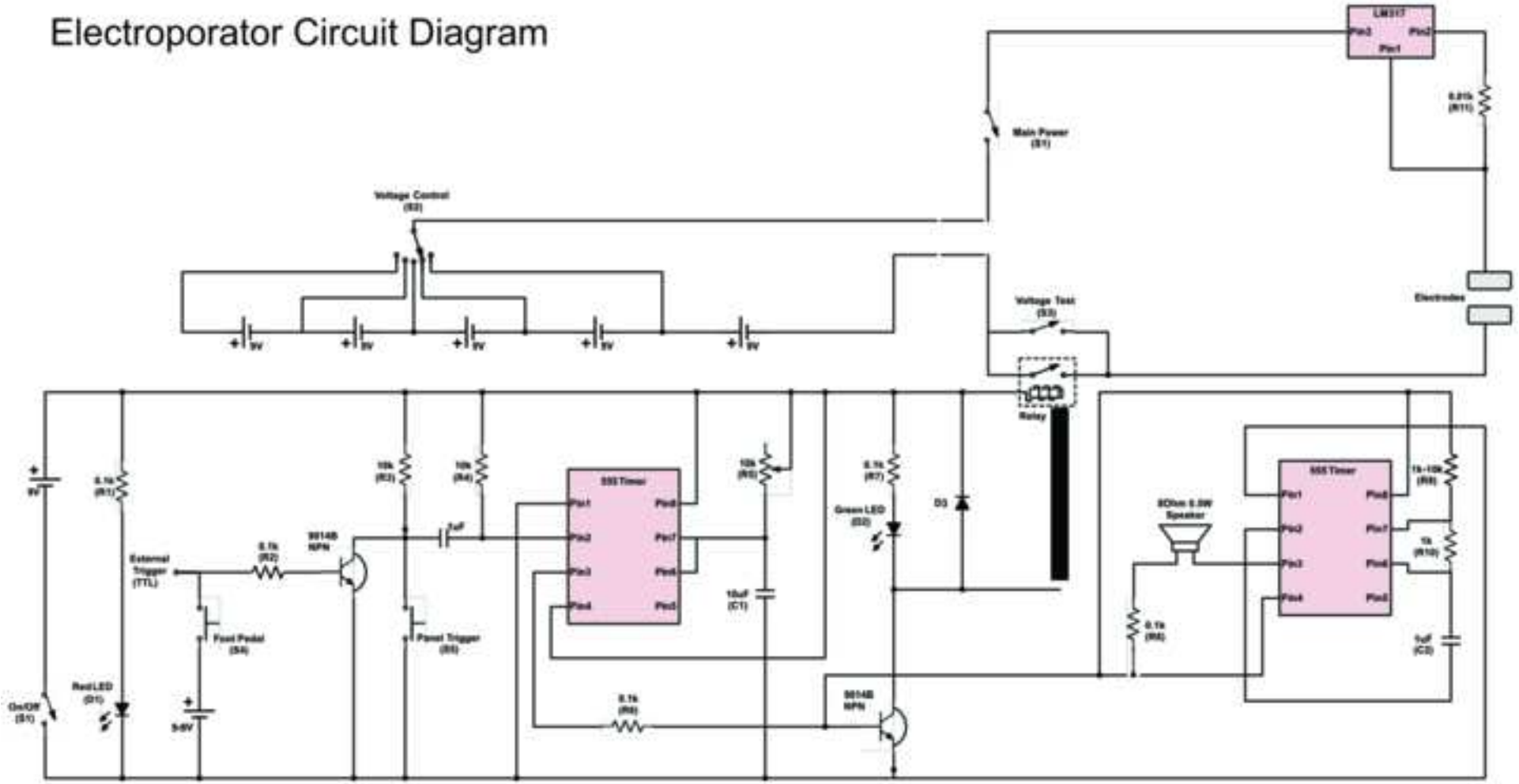

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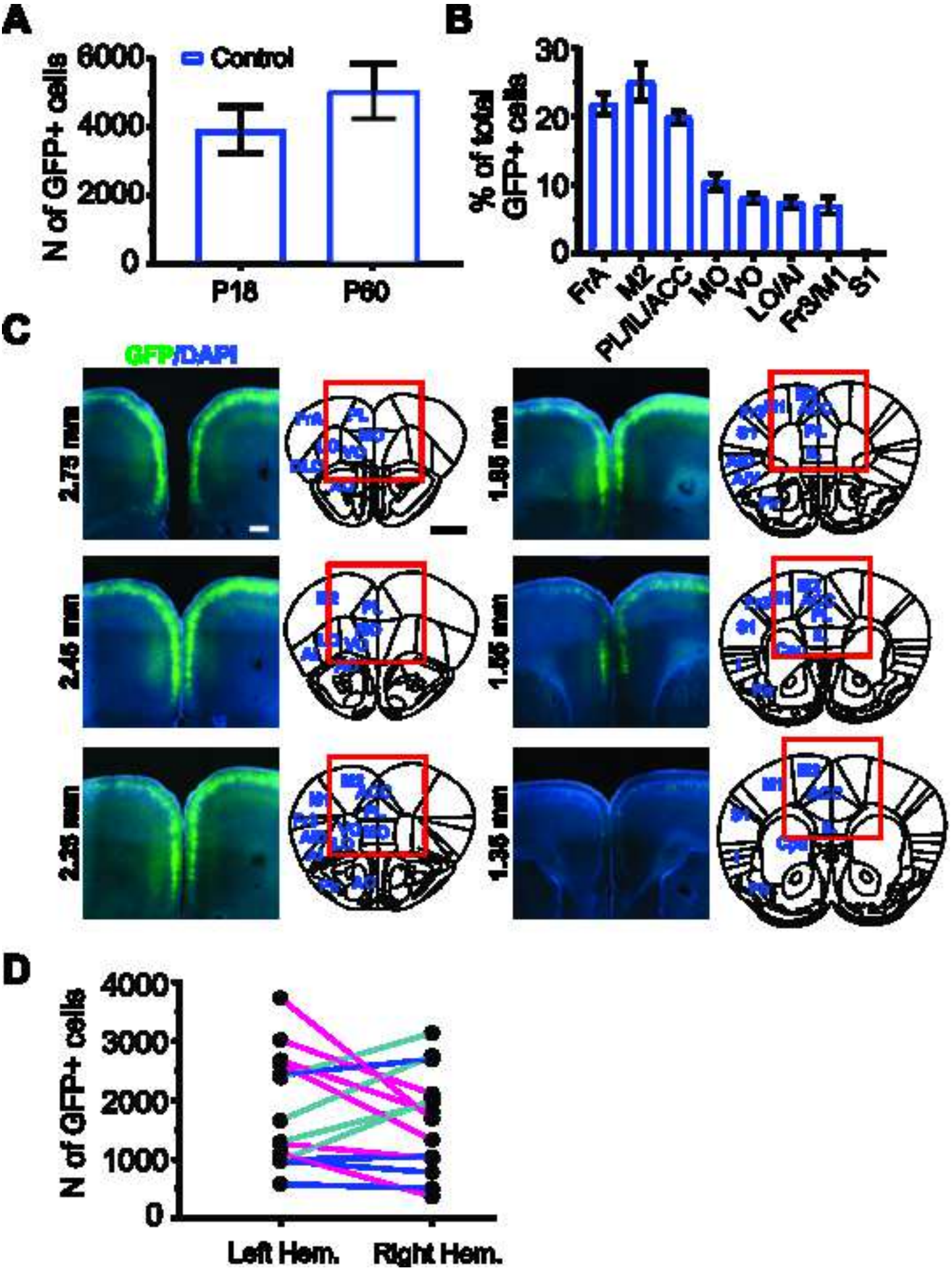

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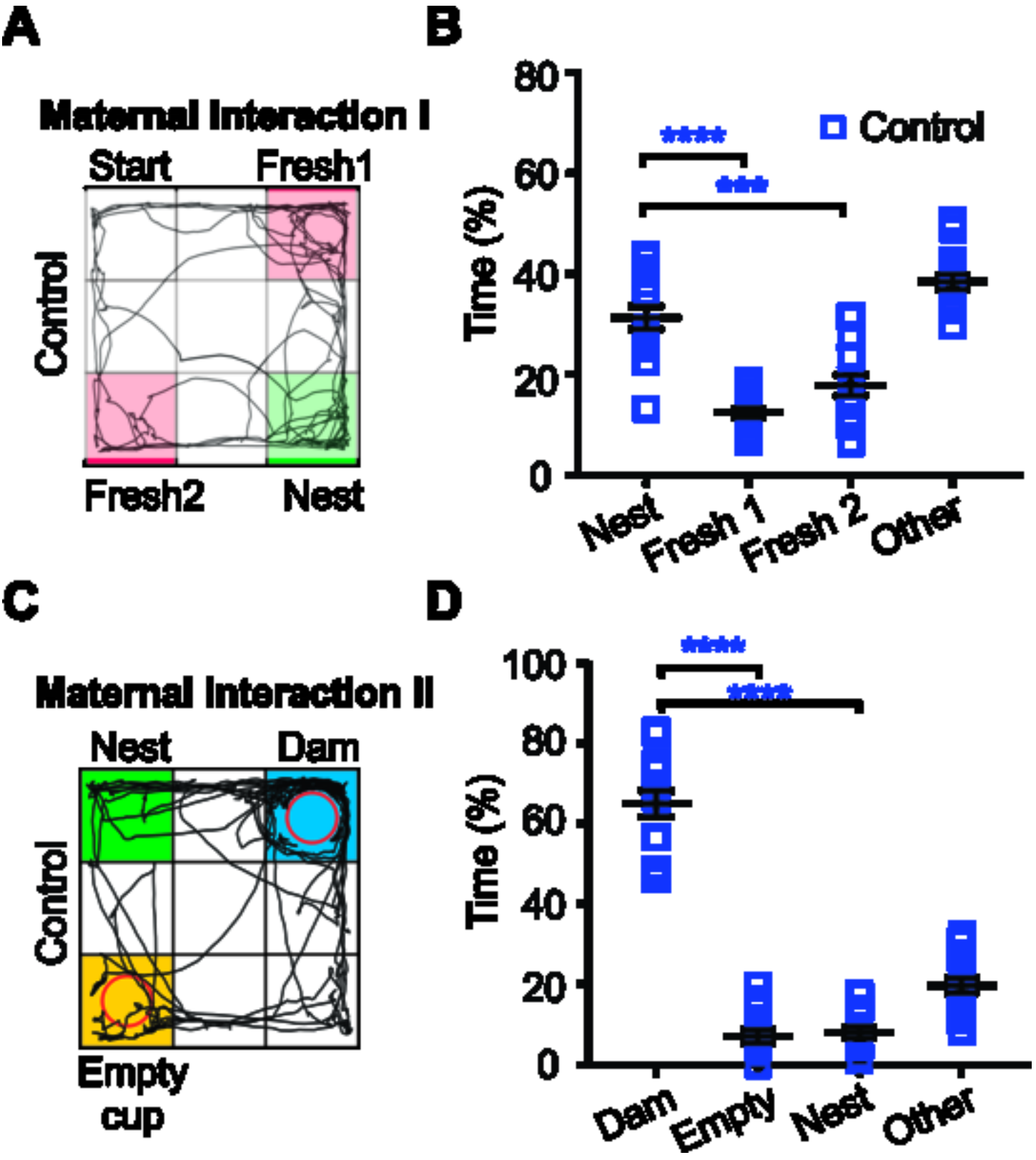

Figure 5

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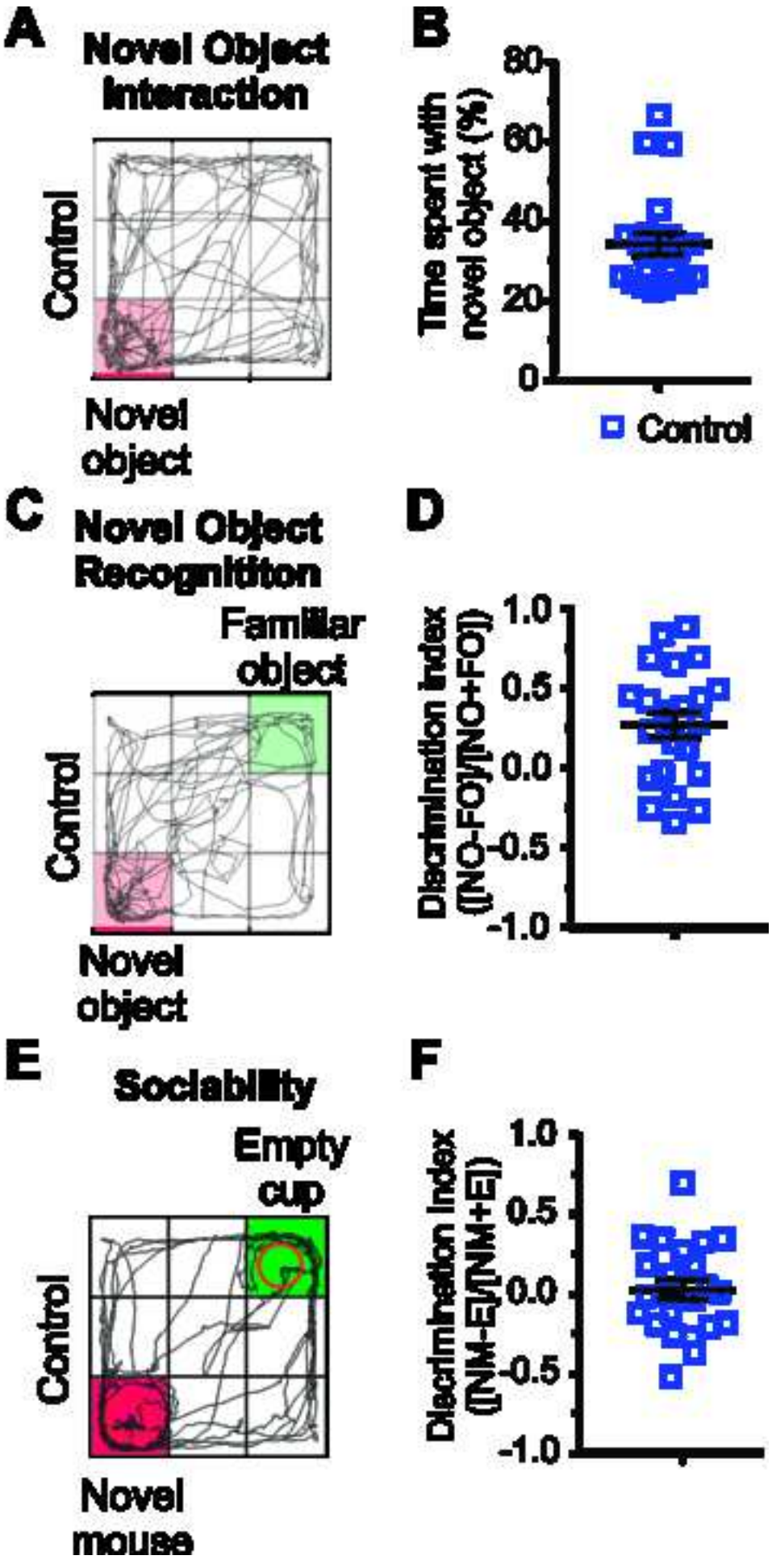

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Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
13mm Silk Black Braided Suture	Havel's	SB77D	Suture skin
Adson Forceps	F.S.T.	11006-12	IUE
C270 Webcam	Logitech	N/A	Record behavior
Electroporator	Custom-built	N/A	See Figure 1 and 2 and Bullmann et al, 2015
EZ-500 Spin Column Plasmid DNA M	Bio Basic Inc.	BS466	Pladmid preparation
Fast Green FCF	Sigma	F7252-5G	Dye for DNA solution
Fine scissors- sharp	F.S.T.	14060-09	IUE
Fisherbrand Gauze Sponges	Fisher Scientific	1376152	IUE
Gaymar Heating/Cooling	Braintree	TP-700	Heating Pad
Glass pipette puller	Sutter Instrument,	P-97	IUE
Glass pipettes	Sutter Instrument,	BF150-117-10	IUE
Hair Removal Lotion	Nair	N/A	Hair removal
Hartman Hemostats	F.S.T.	13002-10	IUE
Open field maze- homemade acrylic arena	Custom-built	N/A	50 × 50 × 30 cm length-width-height
pCAG-GFP	Addgene	11150	Mammalian expression vector for expression of GFP
Picospritzer III	Parker Hannifin	N/A	pressure injector
Retractor - 2 Pronged Blunt	F.S.T.	17023-13	IUE
Ring forceps	F.S.T.	11103-09	IUE
Sterilizer, dry bead	Sigma	Z378569	sterelize surgical tools
SUTURE, 3/0 PGA, FS-2, VIOLET FOI	Havel's	HJ398	Suture muscle
Water bath	Cole-Parmer	EW-12105-84	warming sterile saline

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