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A microinjection technique for targeting regions of embryonic and neonatal mouse brain *in vivo*

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Abstract

A simple pressure injection technique was developed to deliver substances into specific regions of the embryonic and neonatal mouse brain *in vivo*. The retrograde tracers Fluorogold and cholera toxin B subunit were used to test the validity of the technique. Injected animals survived the duration of transport (24–48 hrs) and then were sacrificed and perfused with fixative. Small injections (≤ 50 nL) were contained within targeted structures of the perinatal brain and labeled distant cells of origin in several model neural pathways. Traced neural pathways in the perinatal mouse were further examined with immunohistochemical methods to test the feasibility of double labeling experiments during development. Several experimental situations in which this technique would be useful are discussed, for example, to label projection neurons in slice or culture preparations of mouse embryos and neonates. The administration of pharmacological or genetic vectors directly into specific neural targets during development should also be feasible. An examination of the form of neural pathways during early stages of life may lead to insights regarding the functional changes that occur during critical periods of development and provide an anatomic basis for some neurodevelopmental disorders.

Keywords

fetus; embryo; retrograde; mouse; development; tracer

1. INTRODUCTION

Many contributions have been made to improve our understanding of the nervous system by targeting specific regions of the brain. As information about the localization of function has increased, the ability to precisely inject neural tracers, pharmacological agents or gene vectors has become increasingly important. Tools to manipulate the genome of mice have made this species the principal model for the study of mammalian neural development. However, technical challenges such as small size and anesthetic requirements have prevented investigators from targeting specific neural structures in the embryonic and neonatal mouse

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brain. Recently, injection of genetic material for electroporation into the ventricles of embryonic mice has been described (Saito 2006; Shimogori and Ogawa, 2008). However, a simple method for the injection of neural tracers, pharmacological agents or gene vectors into targeted regions of the grey matter has not been communicated. Here we used neural tracers to demonstrate the targeting of specific regions of the perinatal mouse brain. The technique is fast and simple and requires minimal equipment.

In vivo microinjection provides opportunities to examine neuroanatomy during development and also to conduct subsequent experiments with identified projection neuron populations. For example, in vivo microinjection of retrograde tracers permits the examination of functional characteristics of identified projection neurons in slice or culture preparations. Such studies have been previously performed, but only in older rats which prevent the option to exploit transgenic strategies that are available in the mouse (Dahlhaus et al., 2005; Sooksawate et al., 2005; Evans et al., 2003). Targeting of specific brain regions with tracers during developmental periods can be combined with immunohistochemical methods and the administration of pharmacological treatments. This type of study could lead to a more complete understanding of the cellular and molecular mechanisms that regulate perinatal pharmacology. For example, little is known about the neural mechanisms that regulate the often altered effects of medications and other drugs when administered during the perinatal period, including during pregnancy (Thompson et al., 2009; Fitzgerald and Walker 2009).

Despite technical difficulties, some progress has been made in microinjecting substances into perinatal organisms. Stereotaxic placement of electrodes or pipettes has been described for neonatal rats (Hoorneman, 1985; Joosten and van Eden, 1989), and a stereotaxic apparatus for the neonatal rat or adult mouse is now commercially available. The direct injection of a neural tracer into the cortex of embryonic rats has been demonstrated in vivo (Sbriccoli et al., 1999). However, mice are now the model organism of choice for many fields including developmental neurobiology because of the availability of strains with targeted genetic alterations. Despite the rapid growth in use of the mouse, knowledge of mouse neuroanatomy during early development is lacking and therefore complete descriptions of the neurons that express identified genes are missing. This report introduces a basic in vivo technique for microinjection of small volumes of biologically interesting substances into targeted brain regions of perinatal mice. Neuroanatomical tract tracing combined with immunohistochemistry is used to demonstrate the targeting of regions within the deep forebrain and cerebellum with the widely adopted retrograde tracers Fluorogold and cholera toxin B subunit (CTB).

2. RESULTS

Neonatal Injections

Under hypothermia-induced anesthesia, neonatal mice (postnatal day 0 – 5) were positioned upon a stage in either a forebrain or hindbrain configuration and injected through the skull using a pulled glass pipette containing the retrograde tracer Fluorogold or CTB. All animals recovered quickly after the injection and their behaviors, including nursing, were indistinguishable from non-injected littermates. Two days after the injection neonates were perfused with 4% paraformaldehyde and dissected. Whole brains injected with the tracer Fluorogold were inspected under a handheld UV light to determine whether the injection was successful (Figure 1A). In some cases the dye failed to flow from a clogged pipette tip and these brains were discarded before sectioning. All CTB injected brains were sectioned. After a couple of litters for learning trials, the success rate for hitting selected neural targets in neonates reached a plateau of about 70%.

An example of a mouse cerebellum injected at P0 with 50 nL of 4% Fluorogold is shown in Figure 1. A coronal section through the cerebellum shows the injection site and extent of tracer

spread (Fig. 1B). Retrogradely labeled neurons were found in the inferior olive of the contralateral medulla (Fig. 1C) and in Clarke's column of the ipsilateral thoracic spinal cord (Fig. 1D). To show that alternatives to Fluorogold can be injected using this technique, CTB conjugated with Alexa Fluor 594 was injected into P0 neonates. CTB can work as a retrograde tracer and has the advantage of not inducing necroses at the injection site. The results of a successful injection of 50 nL of CTB show the tracer contained completely within the thalamus (Fig 2A). Another injection of CTB is shown into a well circumscribed region within the striatum at the level of the anterior commissure (Fig 2B).

Embryonic Injection

Injections into 4–7 embryos typically took 30 minutes. Twenty-four hours after tracer injections the dam was re-anesthetized and embryos were removed from the uterus for perfusion. In successful experiments, over 90% of embryos within a litter survived. Sometimes (~20%) an entire litter was lost due to early parturition, or less frequently the entire litter did not survive but remained within the uterus until the second laparotomy. Surviving whole embryos were placed under a UV light to determine whether Fluorogold was injected successfully and embryos containing tracer were perfused. All CTB injected embryos were perfused and sectioned. Of the injected embryos approximately 25% contained dye in the targeted region that did not spread appreciably into adjacent grey matter. Therefore a single pregnant dam would typically yield 1–2 successfully injected embryos. A typical example of a successful injection into the thalamus of an E17 mouse is shown in Figure 3A. Labeled cells of origin were observed in the spinal cord (Fig 3B) and the morphology of individual cells including their processes could be observed (Fig 3C). Another embryo received an injection into the basal ganglia at the level of the anterior commissure (Fig 4A). Labeled cells of origin were observed within the substantia nigra pars compacta (SNC) and the ventral tegmental area (VTA; Fig 4B).

Combined retrograde labeling and immunohistochemistry

Retrograde labeling was combined with immunohistochemistry to determine the effectiveness a double labeling strategy in perinatal mice. Fluorogold (50 nL) was injected into the striatum of a P0 neonatal mouse and the retrograde labeling was observed in the ipsilateral SNC and VTA (Fig 5A). Morphological features of individual projection neurons are shown at higher magnification in Figure 5B. An antibody against tyrosine hydroxylase (TH) was used to determine whether retrogradely labeled neurons could be characterized as dopaminergic. Tissue processed for immunoreactivity to TH displayed bilateral labeling for cells within both the VTA and SNC (Fig 5C). Numerous double labeled cells located only on the side ipsilateral to the injection site are shown in Figure 5D, indicating that dopaminergic neurons project to the striatum by P2 (arrows). Additionally, some retrogradely labeled neurons were observed without immunoreactivity for TH, suggesting a population of neurons projecting to the striatum at P2 that do not produce dopamine (arrowheads).

3. DISCUSSION

The direct injection of substances into the grey matter of adult organisms has lead to more precise localization of function and a better understanding of anatomic connectivity. However, the difficulty of targeting substances into specific brain areas of living embryos and neonates has retarded the analyses of neural development. The focus of this report was to demonstrate that precise microinjections of biologically active substances can be made into regions of the embryonic and neonatal mouse brain. We used the neural tracers Fluorogold and CTB to show that neural structures can be specifically targeted with small, well circumscribed injections in the perinatal mouse. Retrogradely labeled cells of origin were identified in several neural pathways, demonstrating that tracing studies using direct microinjection techniques is a

feasible strategy for the study of perinatal mouse neuroanatomy. Additionally, we showed that immunohistochemistry can be used in combination with tracing studies.

Comparison of the pattern of retrogradely labeled cells from injections into the striatum, thalamus and cerebellum were consistent with previously described patterns of labeling in rats and adult mice. Each of the projections systems that were targeted: the spinothalamic tract, the climbing fibers of the inferior olive, and the substantia nigra-ventral tegmental area dopaminergic pathway, appeared homologous to previously reported patterns in adult rats and mice (Sotelo et al., 1984; Burstein et al., 1990; Anderson et al., 2001; Hu et al. 2004). This suggests that many long neural pathways are formed early in mouse development and are amenable to further study. Immunohistochemistry methods and Fluorogold were demonstrated here to be combined successfully in perinatal mice. In prior studies, in situ for RNA detection and immunohistochemistry have been successfully combined with Fluorogold labeling in adult rodents (Burgunder and Young, 1988; Fried et al., 1989; Ju et al., 1989) suggesting possible future strategies for experiments with perinatal mice.

The study of perinatal organisms has shown that sensory and motor function depends on critical periods of plasticity that occur during development in which proper growth and refinement of neural circuitry is established (Hensch, 2004; Innocenti, 2007; Trachtenberg and Stryker, 2001). Aberrant development of neural pathways from environmental stress or genetic mutation is thought to contribute to the establishment of neurodevelopmental disorders (Fumagalli et al., 2007). An understanding of the molecular mechanisms involved in plasticity during critical periods has advanced quickly with the use of mutant mouse models (Taha and Stryker, 2005). However, neuroanatomical observations have been missing from studies of neurons at the origin of specific pathways in wild type and transgenic mouse models. In addition, transcription factors and other molecular markers that are expressed transiently in the developing embryo and neonate are used now as a principal means of identification of populations of neurons in the mouse spinal cord (Lee and Jessell, 1999; Helms and Johnson, 2003), cortex (Molyneaux et al., 2007; Leone et al., 2008), cerebellum (Wang and Zoghbi, 2001) and in the thalamus (Vue et al., 2007). However, these studies have lacked the capability to determine whether molecularly identified neural populations are interneurons or part of a projection system. The microinjection of neural tracers into embryonic and neonatal mouse brain provides a means to identify and quantify projection neurons that are also molecularly identified. Furthermore, transgenic mice may be examined for altered pathway formation, which may explain certain developmental phenotypes and catalyze the determination of gene function.

Several methods have been previously described to circumvent direct injection into embryos and neonates in vivo. For example, crystallized lipophilic dyes (e.g. DiI) or horseradish peroxidase (HRP) have been placed directly into fixed neural tissue and allowed to passively diffuse along axonal pathways (Godement et al., 1987; van der Togt and Feirabend, 1990; Hu et al., 2004). Unfortunately, these dyes travel relatively short distances, can require long transport times and may produce spurious or unwanted transcellular labeling (Godement et al., 1987; Kageyama and Robertson, 1993). Other studies have taken advantage of explanted tissue preparations to trace longer pathways using dextran-amines or HRP (Smalheiser et al., 1981; Glover et al., 1986). The value of these methods is limited by the dyes available for transport in fixed tissue or the selection of model organisms and tissues for viable explants.

More importantly, the use of fixed or ex vivo tissue prevents the subsequent examination of physiological characteristics. For example, retrogradely labeled neurons have been used in conjunction with in slice electrophysiology or primary cell culture preparations; but such studies have previously used only older rats (Dahlhaus et al., 2005; Sooksawate et al., 2005; Evans et al., 2003) which are, unlike mice, impractical for the examination of the functional

roles of specific genes. Combining electrophysiology or calcium imaging with identified projection neurons in transgenic and wild type mice may yield important and novel contributions to our understanding of the functional significance of gene expression during development.

The flexibility of the injection apparatus permits the introduction of other fluid or suspended substances. Vectors for genetic materials such as adeno- and lentiviruses and pharmacological agents can be injected into targeted embryonic and neonatal brain sites. For example, Welch et al. (2007) injected into the striatum of young null mutant mice a lentivirus containing a copy of the deleted gene. The reintroduction of the gene into a targeted neural region produced a relatively quick and inexpensive tissue specific knock-in model which allowed the study of the expression of the gene on behavior during adulthood. The reintroduction of a deleted gene or over-expression of a gene into a targeted neural structure can provide information on the role of that gene in specific regions of the brain.

The retrograde tracers Fluorogold and CTB were used in these experiments because each provides a means to clearly identify the site of the injection. Fluorogold is thought to pass through the lipid membrane of axons and terminals and is then driven into vesicles by a pH gradient and carried to the soma (Dado et al., 1990; Wessendorf 1991). Although easy to use, Fluorogold produces necrosis at the injection site prohibiting its use in behavioral experiments. CTB on the other hand is non-toxic and can be conjugated to various fluorescent probes to label multiple pathways simultaneously (Conte et al., 2009). The technique introduced here was designed to allow classical microinjection techniques to be applied to embryonic and neonatal mice. The advantage of a pressure injection system is that pipettes can be loaded with the substance of the investigators choice. The retrograde labeling provides evidence that neural pathways in embryonic and neonatal mouse are extant and appropriate for further study. The incorporation of microinjection into experiments on the developing mouse should help to reconcile classical neuroanatomy with studies in perinatal mice and may be an important tool for increasing our understanding of the neural mechanisms that produce developmental disorders and for determining the success of therapeutic interventions during early life.

4. EXPERIMENTAL PROCEDURE

Animals

Timed pregnant female mice (CD1, Charles River) and neonatal mice, born on postnatal day 0 (P0), were used in this study with the approval of the University of Minnesota Institutional Care Facility. Neonates were housed with their mother before and after the injection.

Stereotaxic Apparatus

Most manipulators from stereotaxic systems can be adapted for these experiments. We used an electrode manipulator stand with a 3-axis manipulator and an attached microinjection unit (Model 1449 Electrode Manipulator Stand, Model 1460-61 Electrode Manipulator, and Model 5000 Microinjection Unit; David Kopf Instruments, Teajunga, CA). A 5 μ L manual syringe (Model 95, Hamilton, Reno, NV) was fitted to the Microinjection Unit. Pipette tips were pulled from thin walled borosilicate glass capillary tubes (with filament) with an outer diameter of 1.0 mm and inner diameter of 0.76 mm. The pipette was attached to the needle of the syringe with cyanoacrylate adhesive. Pipettes were backfilled with light mineral oil and then forward filled with 4% Fluorogold (Fluorochrome, LLC, Denver, CO) or CTB conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA) dissolved at 5.0mg/ml in PBS. For injections into embryos, a short length of PE-10 plastic tubing was glued surrounding the pipette shaft leaving only a portion of the tip exposed. The tubing worked as a stopper so that the depth of the injection would not exceed that which was desired. All injections were done with the aid of a

dissecting microscope. Anatomical borders in the perinatal mouse brain were determined with the assistance of the Atlas of the Developing Mouse Brain (Paxinos et al., 2007).

Neonatal Injections

For injections into neonatal mice, a stage was cut from a small block of expanded polystyrene foam and fixed to the center of the base plate with adhesive. This stage was used for pups from P0 to P5 to position the body and help stabilize the head (Figure 6).

A neonate was placed in a container of crushed ice for about two minutes until the onset of hypothermia induced anesthesia. In albino mice the transverse and superior sagittal sinuses can be easily seen through the skin and skull. In pigmented mice the sinuses become difficult to discern after about P3. The sinuses were used as landmarks to identify the desired pipette insertion point. A dot was drawn on the skin surface at the insertion point with a fine felt-tipped marker (Figure 7A). For injections into the thalamus of P0–P5 neonates, the pipette insertion point was 0.7 mm lateral of the superior sagittal sinus and 1.2 mm rostral of the transverse sinus. The pipette was inserted to a depth of 2.2 mm from the surface of the skin. For injections into the caudate/putamen the insertion point was 1.3 mm lateral of the superior sagittal sinus, 2.2 mm rostral of the transverse sinus and the depth from the skin was 2.0 mm. The cerebellum could be directly observed through the bone and skin and a dot was placed on the skin at the injection site. The depth for injections into the cerebellum was 1.8 mm from the surface of the skin. For neonates at P5 (and older), the parietal bone is thick enough to prevent easy penetration by the pipette. In these animals a 30 gauge needle was first used to make a small puncture at the pipette insertion point.

Once anesthetized, the neonate was removed from the ice and placed on the stage in the appropriate orientation for forebrain (Figure 6B) or hindbrain/cerebellar (Figure 6C) injections. The head was held in position by the finger tips of a second investigator. Importantly, the skin around the pipette insertion point was gently stretched taut with the fingertips while the head was held in place (Figure 7B). This greatly improved the ability of the pipette to penetrate the skin and bone at the designated location by preventing the skin from slipping over the bone. The pipette was lowered to the skin surface under a dissecting microscope, and then lowered to the appropriate depth. Thirty to 50 nL of 4% Fluorogold or CTB was delivered over 15 seconds and the pipette was removed after 60 seconds. The neonate was immediately placed under a warming lamp and on a warming pad. After approximately one minute color, movement, and reaction to touch appeared normal and the neonate was returned to the home cage. No animal failed to recover after this procedure. Neonates could be injected one after another as long as care was taken to ensure the tip of the pipette did not clog with tissue or crystallized dye. A typical litter of 10 pups could be injected in about an hour.

Embryonic surgery and injections

All instruments were sterilized and sterile saline was used to keep the exposed organs moist during the surgery. Timed pregnant females carrying embryonic day 17 (E17) embryos were anesthetized with sodium pentobarbital (60 mg/kg ip) and placed on a heating pad that was placed over the base plate of the stereotaxic apparatus. The hair covering the abdomen was clipped and the skin swabbed with an antiseptic. A 2.5 cm longitudinal incision was made with scissors along the midline of the abdomen to gain entry to the abdominal cavity. A portion of the uterus containing 2 or 3 embryos was gently extracted at one time. Ritodrine hydrochloride (100 µL at 14 mg/ml in saline) was applied directly to the surface of the exposed uterus to relax the smooth muscle. An individual embryo was manipulated in utero under a dissecting microscope using hand-held ring forceps (Figure 8). Care was taken to avoid contact of the forceps with the placenta. A second forceps with flat, blunted edges was used to assist in positioning the embryo within the uterine sac. The head of the embryo was gently positioned

so as to be upright with the crown facing the pipette tip. For injections into the striatum the head was rotated slightly along the longitudinal axis so that the pipette would penetrate more laterally. The head of the embryo was held in direct contact with the uterine wall. The transverse and superior sagittal sinuses can be seen through the uterine wall and through the skin and bone of the embryo and were used as landmarks. Once the embryo was held stationary a second investigator lowered the pipette through the uterine wall, to the depth of the stopper (Figure 8, inset) and 30 nL of Fluorogold was ejected. The pipette remained in place for 10 seconds after completion of the injection and then was withdrawn. A few easily accessible embryos on each side were injected and then placed back into the body cavity. The mother was sutured and recovered on a warming pad for 1 hour and then moved back to her home cage. The mother was re-anesthetized 24 hours later with pentobarbital and the abdominal incision reopened to access the embryos for hypothermia induced anesthesia and fixation by perfusion.

Fixation and Histology

Fluorogold transports quickly after an injection and animals sacrificed after two hours for transport displayed bright labeling of fiber bundles up to 2 mm away from the injection site (unpublished observations). For robust filling of cells we allowed a transport time of 24 hours for embryonic injections and 48 hours for neonatal injections. After this time the embryo or neonate was put on ice for hypothermia induced anesthesia. The mouse was then pinned ventral side up to a polystyrene block. Under a dissection microscope the ribcage was cut out with fine scissors to provide access to the heart. The right atrium was lanced and a blunted 27 gauge needle inserted into the apex of the heart. Five milliliters of 0.9% saline and then 5 mL of 4% paraformaldehyde was perfused to fix the tissue. Solutions were delivered by pump at 1 mL/minute. Animals were then dissected so that only the skull and vertebral column remained intact, i.e. organs, limbs, skin were all removed. The combined skull and vertebral column containing the brain and spinal cord were placed in a container of 4% PFA at 4 degrees overnight. The next day the nervous system was carefully removed from skull and vertebrae under a dissecting scope. The brain and spinal cord were separated at the level of the tectum and embedded in cutting medium. Both were then sectioned in 50 micron sections on a cryostat, mounted onto a gel coated slide, dried and coverslips applied.

For immunohistochemistry after tracer injection, brains were fixed by intracardiac perfusion of 4% paraformaldehyde/0.1M phosphate buffer. Brains were submerged overnight in the same fixative. After washing with phosphate buffered saline (PBS) and cryoprotection in 30% sucrose, brains were frozen in OCT compound (Sakura). Twenty micron thick sections were collected on Superfrost Plus slides (Fisher, Pittsburgh, PA) and dried on a slide warmer overnight. Sections were then rinsed in PBS and fixed 4% paraformaldehyde/0.1M phosphate buffer. After three washes in PBS, the slides were immersed in boiled 10 mM citrate buffer (pH 6) for 5 minutes prior to blocking with 3% donkey serum / 0.3% Triton X-100/PBS for 1 hour. Anti-tyrosine hydroxylase mouse monoclonal antibody (Millipore, MAB318) was used at 1:200 dilution in the blocking solution and incubation was performed overnight at 4°C. On the second day, after washing in PBS, sections were incubated for 1 hour with Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA). After dehydration in ascending concentrations of ethanol and clearing in xylene, slides were mounted in DPX mounting medium (EM Sciences, Hatfield, PA).

Fluorescence was visualized with an Olympus BX50 microscope under custom filter sets (for Alexa Fluor 594 and Cy3: exciter 541–551; emitter 572–608; for Fluorogold: exciter 351–371; emitter 420, longpass). Micrographs were captured digitally with a Scion 1.4 megapixel grayscale scope-mounted camera and Scion Capture v2.0 software. Brightness and contrast of whole images was uniformly adjusted using Adobe Photoshop CS3.

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Figure 1.

Example of an injection of Fluorogold into the cerebellum of a P0 mouse fixed 48 hours later with 4% PFA. A) Whole brain after an injection of Fluorogold into the lateral cerebellum. The skull is removed and the injection confirmed under UV light. Arrow shows the injection point. Scale bar: 1.0 mm. B) Coronal section through the plane of maximum tracer spread. Inset: plane of section is illustrated in sagittal (upper) and coronal (lower) schematic. Fluorogold can be seen outside the brain, within the choroid plexus and meninges covering the cerebellum, presumably from the insertion of pipette or an artifact of sectioning. C) Labeled neurons with projection to the cerebellum located in the contralateral inferior olive (climbing fibers). Arrow indicates the dorsal part of the inferior olivary nucleus and arrowhead show the medial part of

the inferior olivary nucleus. D) Clarke's column (arrow) of the ipsilateral thoracic spinal cord. Abbreviations: 4V, fourth ventricle; Cb, cerebellum; SC, superior colliculus; Sp5, spinal trigeminal nucleus.

Figure 2.

Injections of cholera toxin subunit b conjugated to Alexa Fluor 594 into neonatal mouse brains. A) Injection of 50 nL into the thalamus. Inset: anterior-posterior level of injection (top) and coronal illustration of the plane of injection (bottom). B) Injection of 50 nL into the striatum. Abbreviations: 3V, third ventricle; ac, anterior commissure; cc, corpus callosum; CPu, caudate/putamen; Ctx, cortex; Hb, habenula; Hipp, hippocampus; VB, ventrobasal complex.

Figure 3.

Injection of Fluorogold into the thalamus of an E17 mouse. A) After 24 hours the tissue was sectioned. The injection site was localized to the lateral thalamus and did not spread to the contralateral side. Scale bar: 500 μ m. B) Labeled cells of the spinothalamic tract observed in the dorsal horn of the lower cervical spinal cord. A large number of neurons are labeled in the superficial dorsal horn with single neuron visible in lamina V. Scale bar: 100 μ m. C) Processes of individual neurons in the dorsal horn are visible (arrowheads). Scale bar: 50 μ m. Abbreviation: ic, internal capsule.

Figure 4.

Injection of Fluorogold into the striatum of an E17 mouse. A) After 24 hours for transport of a 50 nL injection of Fluorogold in the caudate-putamen the tissue was sectioned and photographed under a UV light source. ac, anterior commissure; LS, lateral septal nucleus; LV, lateral ventricle. Scale bar: 500 μ m. B) Labeled cells in the substantia nigra pars compacta (SNC) and the ventral tegmental area (VTA) that project to the striatum. Abbreviations: LV, lateral ventricle; LS, lateral septal nucleus; SNR, substantia nigra pars reticulata; fr, fasciculus retroflexus. Scale bar: 100 μ m.

Figure 5. Retrograde tracer and immunohistochemistry double labeling A) Fluorogold labeled projection neurons in the substantia nigra and ventral tegmental area. B) High magnification image of Fluorogold labeled cells from box in (A). C) Immunoreactivity for tyrosine hydroxylase identifies dopaminergic neurons bilaterally. D) High magnification image of immunoreactive neurons. Arrows indicate neurons double labeled with Fluorogold (shown in B). Arrowheads indicate projection neurons with no tyrosine hydroxylase activity. Abbreviation: Aq, cerebral aqueduct.

Figure 6.

Stage dimensions and positioning of neonates. A) The stage was cut from expanded polystyrene foam (often used as packing material) into the dimensions shown. B) Position of the neonate on the stage for injections into forebrain structures. The head of the neonate is held to the stage by hand using the fingers to stretch the skin at the injection point. C) Position of the neonate used for injections into hindbrain/cerebellum. The head is bent down with the jaw in contact with the side of the stage to provide access to the hindbrain and cerebellum.

Figure 7.

Injection of tracer into neonatal mouse in vivo. A) A dot of ink marks the injection target for a thalamic injection. The transverse and mid-sagittal sinuses can be seen through the skin (arrowheads) and were used as landmarks. B) The pipette is lowered through the skull and into the brain at the target location. The fingertips of the second investigator apply gentle pressure to keep the head in position and to stretch and make taut the skin at the insertion point.

Figure 8.

Injection of tracer into embryonic mouse in utero, in vivo. Under a dissecting microscope, the embryo is manipulated within the uterine sac with ring tipped forceps to position the head below the tip of the pipette. Once positioned and stable, the pipette is lowered the distance permitted by the “stopper” and the tracer is delivered. Inset: the “stopper” is made of PE-10 tubing fitted over the tip and glued to the glass so that only the desired length of the tip is exposed. In this case, for thalamic injections the exposed tip length was 2.0 mm.