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In vitro biocytin injection into perinatal mouse brain: a method for tract tracing in developing tissue

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Abstract

Injection of biocytin provides an effective method for labeling axonal projections. Several difficulties arise when this technique is employed in fetal or early postnatal animals in vivo, including limited access to injection sites and extended post-injection survival periods. To circumvent these problems, we adapted the technique of extracellular biocytin injection for use in explanted brain hemispheres of developing mice. Briefly, entire brain hemispheres from perinatal mice (E16–P9) were removed and placed in oxygenated aCSF in a brain slice recording chamber. Following visually guided injection of biocytin (2%) into the prelimbic cortex, the brains were then incubated in oxygenated artificial cerebrospinal fluid (aCSF) for varying periods of time and then immersion-fixed in 4% paraformaldehyde and 0.5% glutaraldehyde. The next day, the brains were sectioned and processed for biocytin histochemistry using the avidin–biotin-complex method. We examined the method of injection, electrode type, time of injection, and post-injection incubation period. We found that in E16–P9 animals iontophoresis of biocytin using 8- to 12-megaohm patch clamp electrodes for a duration of 10 min provides optimal axonal labeling. Post-injection incubation times of four or more hours are sufficient for labeling fine caliber collaterals as well as axon bundles that reach distances over 3 mm. In vitro injection of biocytin into explanted brain hemispheres provides a quick and easy method for tract tracing in developing brains.

Keywords: Anterograde tracer; Biocytin; Development; Quantification; Tract-tracing

1. Introduction

Biocytin, a low molecular weight complex of biotin and the amino acid lysine, was first introduced by Horikawa and Armstrong (1988) as an intracellularly injected label for electrophysiologically-identified neurons. In addition to intracellular injections, King et al. (1989) demonstrated that extracellular application of biocytin can be used for anterograde tract-tracing. Since the original description of biocytin as a neuronal tract-tracer, it has been extensively and successfully utilized to visualize neuronal morphologies and axonal projections at both the light and electron microscopic level (Izzo, 1991; Helm et al., 1993; Pinault, 1996; Khalilov et al., 1997).

The utility of this molecule for tracing studies is based both on its high degree of solubility as well as the high affinity of biotin for avidin, which allows for sensitive detection with avidin-labeled fluorochromes or chromogenic enzymes.

Whereas the majority of investigations utilizing extracellular biocytin for tracing axonal projections have been conducted in adult tissue (Kenan-Vaknin et al., 1992; de la Cruz et al., 1994; Stezhka and Lovick, 1994), or in slice cultures (Mouginot and Gahwiler, 1995) relatively few, if any, developmental studies have employed biocytin as an extracellular tracer in the intact brain. Factors contributing to this lack of use include the long post-injection survival periods (24–96 h) traditionally associated with in vivo biocytin injections, as well as the difficulty associated with targeting in vivo injections in small perinatal animals. Developmental studies have, therefore, primarily used in vitro application of fluorescent molecules such as the carbocyanine dye, DiI, on fixed brain tissue (Godement et al.,

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1987; Santacana et al., 1990; Nisenbaum et al., 1998). Although the carbocyanine dyes have proven extremely useful, several disadvantages exist. For example, DiI labeling is incompatible with many other histochemical techniques, such as immunocytochemistry and in situ hybridization. Second, it is sometimes difficult to obtain quantitative data with DiI due to the transient nature of the fluorescent image. Although it is possible to photoconvert the fluorescent signal (Linke and Frotscher, 1993; Papadopoulos and Dori, 1993), labeling can be inconsistent, particularly for fine collaterals. A third disadvantage inherent to carbocyanine dyes is the long incubation times necessary for transport of the tracers in fixed tissue. To avoid these problems and maximize the ability to quantify the development of axonal projections into immunohistochemically defined targets, we have adapted the biocytin technique for investigations in prenatal and early postnatal mouse brain. The present report characterizes a method for the injection of biocytin into explanted whole brain hemispheres that allows for rapid and sensitive detection of intact neuronal pathways in developing tissue. To illustrate the utility of this technique, we have applied this procedure to the study of prelimbic corticofugal pathways in perinatal mouse brain. Recently, a similar technique using BDA injections instead of biocytin in isolated spinal cord preparations has been reported (de Boer-van Huizen and ten Donkelaar, 1999).

2. Methods

2.1. Experimental animals

CD1 mice were used in all experiments. Embryonic age was determined by the presence of a vaginal plug (this was designated as E0). The age was confirmed by measuring the crown-rump length of the embryo (Schambra et al., 1992). In the case of postnatal ages, the day of birth was designated as P0. Mice were maintained on a 12-h light, 12-h dark cycle with food and water ad libitum. All of the procedures employed in this study were approved by the University of Connecticut Animal Care and Use Committee and are in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.2. In vitro hemisphere preparation

Mice ranging in age from E16 to P9 were killed by decapitation. The brains were then removed and hemisected. Each brain hemisphere was placed immediately into room temperature, oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM D-glucose, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 26 mM $NaHCO_3$) and allowed to equilibrate at room temperature for at least 30 min. Brain hemispheres were then submerged with their medial sides up in a tissue slice recording chamber (Fine Science Tools, Foster City, CA) and stabilized with nylon netting. Hemispheres were superfused with room temperature aCSF (bubbled with 95% O_2/5% CO_2) at a rate of 3.3 ml/min.

2.3. Biocytin injections

Glass microelectrodes containing a 2% (weight to volume) biocytin (Ne-biotinyl-L-lysine; Sigma, St Louis, MO) solution dissolved in 0.5 M potassium acetate were lowered into the prelimbic cortex. Injection electrodes were placed with a micromanipulator into hemispheres that were obliquely illuminated with a fiber optic illuminator (Lumina, Chiu Technical Corporation, Kings Park, NY) and viewed with a Nikon dissecting microscope. Biocytin was injected either by iontophoresis (200 ms, 2.5 Hz, 8–20 V) or by pressure ejection. For iontophoretic injections, a Grass SD9 stimulator was used. The bath and electrode leads were silver chloride pellets (Warner Instruments). After injections were completed, the brains were placed in 60×15 mm Petri dishes containing approximately 12 ml bubbled aCSF. These dishes were stored in an oxygenated (95% O_2/5% CO_2). Tupperware chamber and brains were allowed to incubate at room temperature for either 15, 30 min, 1, 2, 4 or 8 h. They were then fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight at 4°C. The next morning, brain hemispheres were embedded in 1.5% agar in deionized distilled water and sectioned in the parasagittal plane at 80 μm on a vibratome (Pelco, St Louis, MO).

2.4. Detection of biocytin

Free-floating sections were processed for biocytin histochemistry in well-plates (12 Well Culture Cluster, Corning Inc., Corning, NY) using the avidin–biotin-peroxidase complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Approximately 15 sections were placed into each well. Sections were rinsed for 10 min in phosphate-buffered saline (PBS), followed by a 5-min rinse in 0.1 M glycine in PBS. After a final 10 min rinse in PBS, sections were incubated for 30 min in 0.5% hydrogen peroxide in PBS to saturate endogenous peroxidase activity. Sections were then rinsed $3\times$ in PBS (1–5 min) and blocked in 1% normal goat serum (NGS) containing 0.2% Triton X-100 in PBS for at least 60 min. Following this, they were rinsed $3\times$ in PBS (1–5 min) and incubated in a mixture of avidin and biotinylated horseradish peroxidase as specified in the Vectastain ABC Kit, Vector

Laboratories, containing 0.1% Triton X-100 in PBS for 60 min. Sections were then rinsed 3 × 10 min in PBS. They were reacted with 0.1% diaminobenzidine (DAB Substrate kit, Vector Laboratories) in the presence of 0.03% hydrogen peroxide with 0.04% nickel chloride for 25 min. Tissue was then rinsed five times in PBS to stop the peroxidase reaction. Sections were mounted onto Fisherbrand frosted, precleaned microscope slides (Fisher, Pittsburgh, PA), allowed to dry overnight at room temperature, and coverslipped with Accu-mount 60 (Baxter, Bedford, MA).

2.5. Double-labeling with tyrosine hydroxylase immunocytochemistry

To achieve double-labeling with biocytin and immunocytochemistry (ICC) for tyrosine hydroxylase (TH), sections were first processed as above for biocytin. Sections were then placed in PBS containing 5% NGS to block non-specific antibody binding and incubated for 40 h at 4°C in PBS/2.5% NGS containing rabbit anti-TH (Chemicon, Temecula, CA) diluted 1:1000. After rinsing 4 × 15 min in 2.5% NGS in PBS, sections were incubated for 2 h in biotinylated secondary antibody (goat-anti rabbit, Vector Laboratories) diluted 1:200, and processed using the ABC method. To enable differentiation between biocytin-filled axons and TH labeling, the DAB reaction following TH ICC did not include nickel chloride.

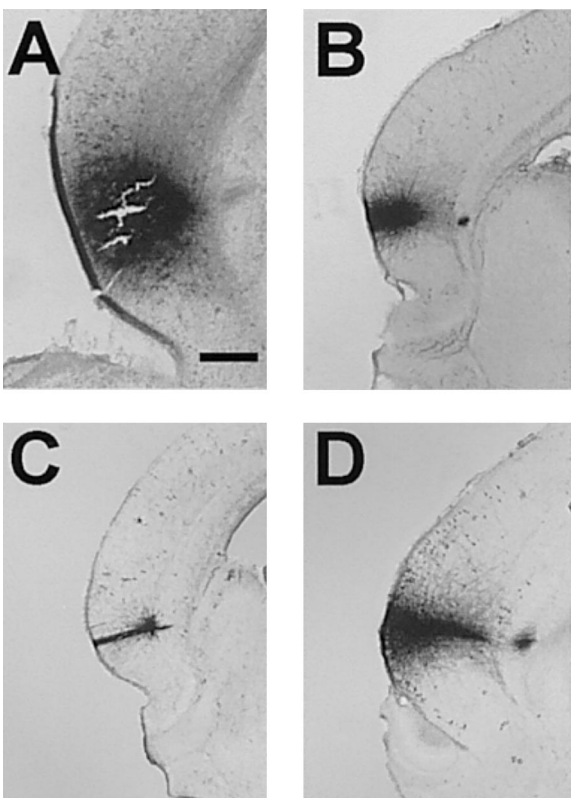

Fig. 1. Method of injection, time of injection, and type of electrode were varied to achieve optimal labeling. (A) Pressure ejection of biocytin into the prelimbic cortex was achieved by expelling biocytin through a patch pipette over a period of 5 s with a 10-ml syringe compressed approximately 1 cc. This often created a hole at the site of injection. (B) Iontophoresis into the prelimbic cortex (200-ms positive voltage pulses at a frequency of 2.5 Hz at 8 V) left virtually no tissue damage and size of injection was easily controlled by varying electrode type and time of injection. We found that patch-type electrodes were better for labeling groups of cells than were sharp microelectrodes. (C) A 5-min injection labeled fewer cells than a 10-min injection (D). Scale bar, 250 μm.

2.6. Tissue analysis

Sections were examined and photographed on a Nikon Eclipse microscope. Light microscopic images of injection sites and all resulting labeling were acquired using a Spot Camera (Diagnostic Instruments, Sterling Heights, MI). Images were analyzed and processed with a Macintosh G3 computer using Adobe Photoshop 4.0.1. Camera lucida was used to reconstruct injection sites.

3. Results

3.1. Injections

We first examined the effect of varying several different injection parameters on biocytin labeling of corticostriatal neurons. Initially, we compared biocytin labeling resulting from pressure and iontophoresis injections into the prelimbic cortex of perinatal mice. Pressure ejection was achieved by expelling biocytin through a patch pipette over a period of 5 s with a 10-ml syringe compressed approximately 1 cc. This produced a significant amount of tissue damage and did not yield a consistent amount of labeling (Fig. 1A; $n = 15$). Injection sizes ranged from small (0.02 mm^2) to fairly large (0.4 mm^2), the latter being more common. Iontophoresis of biocytin, on the other hand, left virtually no damage to the tissue under investigation (Fig. 1B), and injection size was easily controlled by varying the type of electrode and the time of injection. Injection with sharp microelectrodes (resistance = 30 megaohms) resulted in the labeling of single neurons or small groups of cells. In contrast, patch electrodes (resistance = 8–12 megaohms) yielded labeling of groups of cell bodies and their axonal projections. Using patch electrodes, the size of injection varied systematically with the time of injection: with 2–5 min, injection sites ranged in size from 0.01 to 0.075 mm^2 (Fig. 1C), while at 10 min, injection sites ranged in size from 0.17 to 0.32 mm^2 (Fig. 1D). While we have primarily concentrated on anterograde labeling, we also observed apparent retrograde labeling. Retrogradely-labeled neurons in both distal neocortical regions and in thalamic nuclei

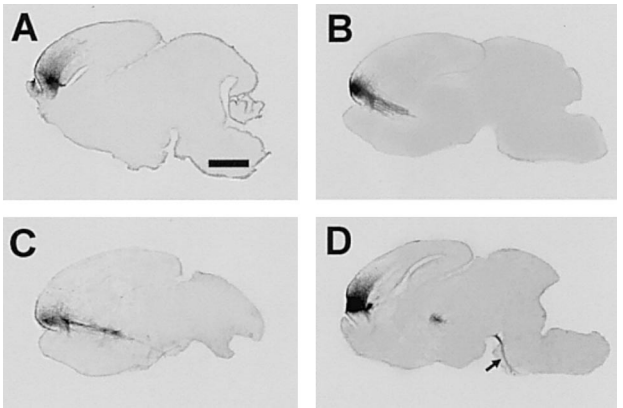

Fig. 2. Examples of labeling after different post-injection incubation periods. (A) A 15-min incubation resulted in relatively little transport of biocytin. Labeling just began to reach the striatum and rarely traveled more than 1.5 mm. (B) A 2-h incubation resulted in transport through the striatum and biocytin traveled distances over 2 mm. (C) After 4 h, more distant targets were reached, such as the reticular nuclei. (D) With a post-injection incubation period of 8 h, biocytin labeling reached distances of approximately 3.5 mm and was found as far as the pons (arrow). These injections were performed at 8 V for 10 min with a patch electrode in P1 animals. Scale bar, 1000 μm.

were observed following both large pressure and smaller iontophoretic injections.

3.2. Post-injection incubation

The relationship between post-injection incubation and distance of axonal labeling in the striatum was determined for post-natal hemispheres. Extent of labeling increased with the length of post-injection incubation. Brief incubations (15–30 min) resulted in shorter distances of axonal labeling (1–1.9 mm), whereas longer incubations (4–8 h) produced labeling ranging from 2.2 to 3.4 mm. The apparent rate of biocytin transport, however, decreased with increased incubation time. The largest drop-off in rate occurred at approximately 2 h of incubation. Brief incubation periods (15 min–2 h) resulted in transport through the striatum (Fig. 2B), whereas longer incubations (>4 h) produced labeling in distant targets, such as the pons and reticular nuclei (Fig. 2C,D) in addition to the striatum. Also, fine caliber collaterals (<1 μm) in the striatum were labeled with longer incubations (>4 h), but not after briefer (15 min–2 h) incubation times (Fig. 3).

3.3. Biocytin and immunocytochemistry

We performed double-labeling experiments with tyrosine hydroxylase (TH) immunocytochemistry (ICC) and biocytin labeling to demonstrate compatibility with ICC. After injection, tissue was first processed for biocytin histochemistry using nickel intensification and next for TH ICC without nickel intensification. With this dual-color labeling, biocytin appears dark purple and is easily distinguishable against the light-brown staining of TH-positive regions (Fig. 4A). At high magnification, collaterals can be visualized against striatal patch areas which stain positive for TH (Fig. 4B). While the results shown here use two chromagen indicators which does not allow for double-labeling the same axon fiber, we have also successfully used the method with double fluorescent probes in which we used avidin rhodamine to label biocytin-labeled fibers. With this method, it is therefore possible to determine the developmental course of axonal projections into immunologically defined compartments.

4. Discussion

The present study describes a method for in vitro extracellular biocytin injection into explanted brain

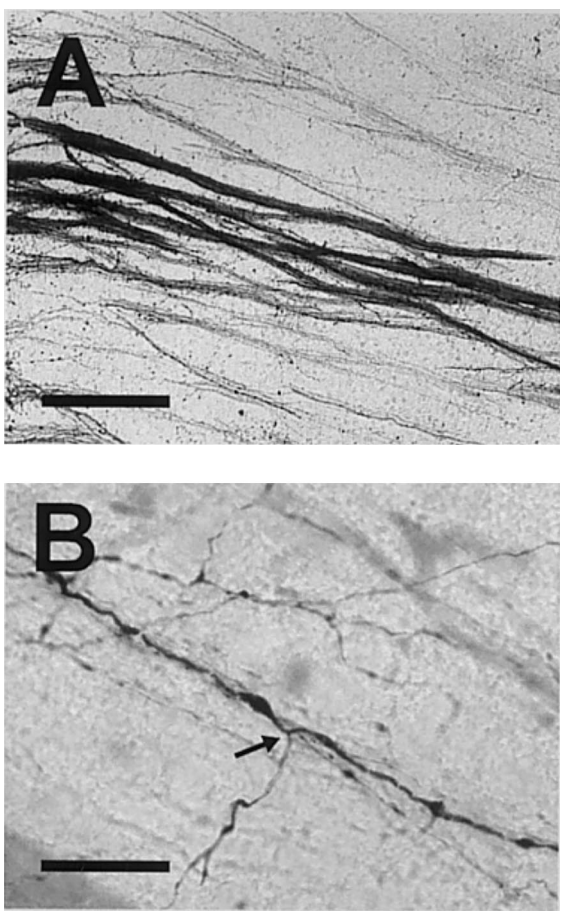

Fig. 3. Primary axons in the striatum were labeled at all post-injection incubations studied; however, it was only at the longer incubations that collaterals in the striatum were labeled. (A) Large striatal bundles of primary axons without collaterals labeled after brief incubations (15 min–2 h). Scale bar, 100 μm. (B) Fine caliber collaterals off of primary axons (arrow) in the striatum labeled after 4 h of incubation. Scale bar, 20 μm.

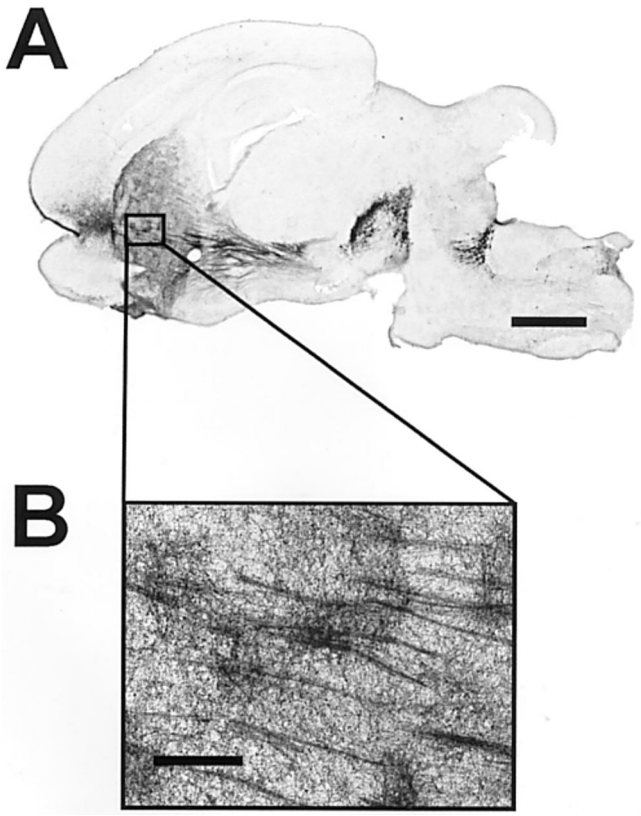

Fig. 4. Biocytin and tyrosine hydroxylase (TH) immunocytochemistry are compatible in double-labeling experiments. (A) Biocytin-labeling appears black against the gray of TH-positive regions in striatum. Scale bar, 1000 μm. (B) At high magnification, collaterals can be seen within striatal patch areas, which label intensely for TH. Scale bar, 100 μm.

hemispheres of perinatal mice. This method provides easy access to developmental tissue to allow quantification of axonal projections into immunohistochemically defined targets. Method of injection, electrode type, time of injection, and post-injection incubation period were varied in this study. We found that iontophoresis through a patch electrode for 10 min with a post-injection incubation period of at least 4 h is optimal for labeling the corticofugal pathways and fine caliber collaterals within the striatum.

There are several clear advantages to using biocytin for tracing neuronal projections. These include its relatively low cost, speed of processing, and versatility. Although the present study examined only the developing corticofugal pathway in mice between the ages of E16 and P9, biocytin has been used in previous developmental studies to examine projections of the auditory cortex in neonatal rabbit (de Venecia and McMullen, 1994), thalamocortical projections in neonatal cats (Erisir and Aoki, 1998), and pyramidal dendritic and axonal bundle development in the visual cortex of rat (Lohmann and Koppen, 1995). Thus, the use of biocytin for developmental studies is not limited to mouse.

Biocytin is generally regarded as an anterograde tracer; however, previous studies have also detected retrograde labeling resulting from biocytin injections (King et al., 1989; Izzo, 1991; Picano-diniz et al., 1992). King et al. (1989) found that biocytin is transported retrogradely in adult animals, but only with very large injections. We also observed retrograde labeling. However, in the present study the retrogradely-labeled neurons were observed following both large and small injections. Thus, retrograde labeling does not appear to result solely following tissue damage.

Varying post-injection incubation period produces different extents and types of axonal labeling (Figs. 2 and 3). Brief incubations (30 min–2 h) provide transport through the large bundles of axons in the striatum while longer incubation times (>4 h) result in labeling of distant targets, such as the pons, and labeling of finer caliber collaterals. In vivo transport rates of biocytin have been calculated at approximately 1.5–3 mm/h (Izzo, 1991; Pinault, 1996). Biocytin, a modified amino acid, likely travels by fast axonal transport which has been estimated at rates of approximately 20–400 mm/day (Vallee and Bloom, 1991). Previous studies employing biocytin as the tracer have also found rates which indicate that it travels by these means (King et al., 1989). Our data show that in vitro, at room temperature, biocytin is transported at approximately 1 mm/h within the first 2 h of post-injection incubation. After 2 h, the apparent transport rate drops to approximately 0.15 mm/h. Slower apparent rates were found at longer incubations periods and faster rates were calculated at the shorter incubations. The reason for this inverse relationship could be due to the drop-off in available tracer as incubation time increases. In a few instances, the electrode tip broke and excessive amounts of biocytin leaked into the brain. In these cases, we noted farther transport than those injections made with intact electrode tips. Nevertheless, in small, immature mouse brain, this transport rate is sufficient to label most axonal pathways within 4 h.

In conclusion, extracellular injection of biocytin into explanted brain hemispheres provides increased accessibility for mapping axonal projection patterns in embryonic and postnatal animals. The characterization of this technique allows for future studies of developing pathways and for quantifying alterations in axon collateral branching in immunologically defined compartments.

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