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New methods for investigation of neuronal migration in embryonic brain explants

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Abstract

Background—Proper migration of neurons is essential for the formation and normal functioning of the nervous system. Defects in neuronal migration underlie a number of neurologic diseases in humans. Although cell migration is crucial for neural development, molecular mechanisms guiding neuronal migration remain to be elucidated fully. Newborn neurons from the embryonic medial ganglionic eminence (MGE) migrate a long distance dorsally in the developing brain, giving rise to several types of interneurons in the neocortex.

New Method—In this study, we developed an immunocytochemistry (ICC) protocol to stain neurons migrating out of the MGE explant embedded in Matrigel. We also established a protocol to efficiently transfect cells in MGE explants, achieving a transduction efficiency of more than 30%.

Comparison with Existing Method—In addition, we developed microfluidic chambers for explants that allow visualization of the vectorial migration of individual neurons from mouse embryonic MGE explants. Our microfluidic system allows monitoring of the distribution of cellular organelles (e.g. Golgi) within migrating neurons which have been stained with commercial molecular dyes or transfected with adeno-associated virus (AAV) expressing reporter proteins.

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Conflict of interest

Authors have no conflict of interest.

Conclusion—These methods provide new paradigms to study neuronal migration in real-time.

Keywords

neuron migration; MGE explant; microfluidic device; transduction

1. Introduction

Neurological syndromes caused by disruption of neuronal migration can be devastating human disorders, which are mostly due to genetic causes (for an extensive review see reviews Reiner et al., 2013; Valiente and Marin, 2010). Studies have shown that disruption of cytoskeleton underlies a large spectrum of neuronal migration disorders (Métin et al., 2008). Migration defects of cortical interneurons can cause abnormal inhibitory responses in the developing cortex (Valiente and Marin, 2010). Ganglionic eminence (GE) neurons move tangentially and across the plane of the glial fiber system during brain development (Marín and Rubenstein, 2001). These GE-derived neurons contribute significantly to the GABAergic cortical cell population (Hernández-Miranda et al., 2010). The gamma amino butyric acid (GABA) interneurons must be produced and deployed to the cortex during fetal life in order for the neural circuits to function properly in the mature brain. Many GABA neurons migrate tangentially to reach their final destination. Tangential migration of GABA neurons is widespread in the embryonic rodent brain (Parnavelas et al., 2000), and three major routes of such migration have been recently characterized in detail: (1) from the medial GE (MGE) to the cortex; (2) from anterior entopeduncular area (AEP) to the neocortex and hippocampus; and (3) from the lateral GE (LGE) to the olfactory bulb. This latter migratory route persists into adulthood as the rostral migratory stream. In this study we have focused on MGE neurons because they have a unique migratory potential, and may have applications in cell replacement therapies in certain neurodegenerative diseases. MGE neurons are the only primary neuronal precursors known to be able to disperse when grafted into the adult brain (Wichterle et al., 1999).

Here, we describe novel methods to study MGE migration, including a microfluidic chamber to culture MGE explants. Our methods may open new avenues for informative neurobiological investigations. An advantage of the neurotechnology system we have developed is that, once validated, it could be used for screening of drugs, which can affect neuronal migration. In addition, immunocytochemical (ICC) staining methods can be used to detect changes in the distribution of proteins thought to contribute to migration defects, such as DISC1, Lis1, and Ndel1 (Richter et al., 1999), and be used to express mutant proteins involved in neurological diseases instead of using transgenic animal models.

2. Materials and methods

2.1. Fabrication of the microfluidic microchambers

To probe the migration of neurons from a MGE explant towards a naturally chemotrophic cortical explant, we designed a microfluidic device with two compartments connected by an array of small channels. The two compartments, in the form of circular wells of 1.5 mm diameter and 3 mm depth were filled with gel matrix. Also filled with gel was the parallel

array of microchannels of 25 μm width, 10 μm height, and average 150 μm length, connecting the two compartments. The cross section of the channels was chosen such that the migration and mechanical orientation of individual neurons could be recorded by time lapse microscopy. The device was fabricated by directly bonding polydimethylsiloxane (PDMS - Dow Corning Midland, MI) on glass coverslips, inside glass-bottom Petri-dishes (MatTek, Ashland, MA) after exposing the surfaces to be bonded to oxygen plasma for 30 seconds. The PDMS piece was cast on a microfabricated silicon wafer, to produce a replica of the photolithographic features on the wafer, baked for 12 hours at 65°C and punched using a 1.5mm puncher (Harris Uni-Core, Ted Pella, Reading, CA) to define the two tissue compartments. The geometry of the array of microchannels and their connection to the two compartments was precisely defined using photolithography techniques, in a 10 μm thick layer of SU-8 photoresist (Microchem, Newton, MA). Immediately after fabrication, devices are kept on ice and filled with the liquid Matrigel (BD Biosciences, Franklin Lakes, NJ), which is then allowed to gel at room temperature, and covered with Neurobasal medium (BD Biosciences).

2.2. Animals

C57BL/6 breeding pairs were obtained from Charles River Laboratories (Wilmington, MA). C57BL/6 matings were set up and the females monitored for vaginal plugs. The day vaginal plug was observed was considered embryonic day 0 (E0), and the male was separated from the female. The pregnant dams were housed in a temperature- and humidity-controlled environment on a 12-hour light/dark cycle with food and water available *ad libitum*.

2.3. Embryo collection and processing

For collection of embryos, the dams were anesthetized by intraperitoneal (i.p.) injections of a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) and the embryos were removed by hysterotomy. The embryos were collected at E15 and decapitated immediately upon removal from the dam, as described previously (McCarthy et al., 2012).

2.4. Analysis of neuronal migration in MGE explants

Slice preparations were prepared from brains of E15 embryos as described with modifications (Crandall et al., 2007; McCarthy et al., 2011; McCarthy et al., 2012). Briefly, embryonic brains were removed and placed in 8% agarose (type VII; Sigma-Aldrich, St. Louis, MO). Coronal sections were cut at a thickness of 250 μm using a Vibratome. A sterile biopsy punch (Miltex Inc., York, PA) was used to collect disks of MGE from the vibratome sections (McCarthy et al., 2011). The majority of the neurons produced in the MGE are migratory neurons destined to become cortical GABA neurons (Butt et al., 2005; Rallu et al., 2002; Anderson et al., 2001). The disk explants from MGE additional punch-out from the cortical region were collected from the same embryonic slice. For staining experiments, MGE explants were cultured in 50 μl Matrigel covered with Neurobasal medium containing 2% B27 supplement, 0.25% glutamine, 0.0125% glutamate, 1 $\times$ penicillin/streptomycin in glass bottom culture dishes (BD Biosciences). Following 48 hours in culture, the explants were fixed with 4% paraformaldehyde (PFA) for 1 hour, and stored in 1 $\times$ Phosphate Buffered Saline (PBS) buffer at 4°C until ICC was performed. For microfluidic devices

experiments, both wells were filled with Matrigel and covered with Neurobasal medium, described above. MGE explant was placed in one of the wells and a cortical explant placed in the other well.

2.5. Immunocytochemistry (ICC) of MGE explants

PFA fixed explants were treated with warm (37°C) 1× PBS for 90 sec (three 30 second incubations with warmed PBS), followed by incubation with 0.1% Triton X-100, 10% goat serum (Jackson Immuno Research) in 1× PBS for 1 hour. After washing 3 times for 10 min with 1× PBS, the samples were incubated overnight at 4°C with primary antibodies to β III-tubulin and pericentrin (β III-tubulin mouse monoclonal antibody, MAB55441, 1:1,500; EMD Millipore, Billerica, MA; pericentrin rabbit polyclonal antibody, PRB-432C, 1:800; Covance, Dedham, MA) to reveal the processes and of the centrosome respectively in migrating cells. The next day, explants were washed three times with 1× PBS and incubated at room temperature for 1 hour with secondary antibodies - anti-mouse Alexa 488 (1:1,000) or anti-rabbit Alexa 555 (1:800) (Invitrogen/Life Technologies, Grand Island, NY). Nuclei were labeled with ToPro3 (1:1,000; Invitrogen/Life Technologies, Grand Island, NY) by incubation for 15 min.

2.6. Lentiviral (LV) and adeno-associated virus (AAV) vectors and MGE transfection

Lentiviral vectors [LV-CMV-GFP (green fluorescent protein); Sena-Esteves et al., 2004] and AAV vectors (AAV-CBA-GFP; Broekman et al., 2006) packaged with serotype 8, 9, or rh10 capsids were kindly provided by Dr. Sena-Esteves (UMass Medical School, Worcester, MA). Viral titers of 5×10^7 transducing units (t.u.)/ml for lentiviral vectors and 5×10^8 genome copies (g.c.)/ml for AAV vectors were used to infect explants. For transduction with viral vectors, MGE explants were placed in 96-well plates and treated with 50 μ l lentivirus or 5 μ l AAV in 200 μ l Neurobasal media containing 10 mM HEPES buffer, pH 7 for 1 hour. To improve the infection efficiency, samples were centrifuged at $1000 \times g$ for 30 minutes in the 96-well plates. After centrifugation, the explants were placed in Matrigel and cultured, as described above.

2.7. Cell organelles labeling

Viable MGE explants were stained with Hoechst 33258 and BODIPY FL C5-ceramide dyes (both from Molecular Probe Inc., Eugene, OR) according to the manufacturer's instructions. BODIPY FL C5-ceramide localizes to the Golgi apparatus, and Hoechst 33258 dye stains the nuclei.

2.8. Microscopy

Confocal images were acquired using an Olympus FluoView 1000 confocal microscope with independent photostimulation and imaging channels. This microscope has a variety of lasers covering the visible and near infrared spectrum. It also has a tunable two-photon source for reduced photodynamic damage, as well as DIC optics and a photomultiplier collector for transmitted light imaging. There are two sets of objectives, both water immersions: one set corrected for a coverslip and one set for direct immersion. There is a culture chamber attachment that surrounds the stage, which maintains temperature and CO₂

levels and the acquisition software can be programmed for time-lapse acquisition. The current confocal laser lines available are 405, 488, 514, 561, and 635 nm. The 2 photon is a chameleon hidden photons (HP), with a range of 690-1020 nm and peak power of 3 Watts.

3. Results and discussion

3.1. Immunocytochemistry (ICC) of non-dissociated MGE explants embedded in Matrigel

MGE explants were cultured in Matrigel and placed in: 1) glass bottom culture dishes, or 2) microfluidic chambers (described below). Neurons migrated out from MGE explants in a symmetrical fashion over 48 hours (Fig. 1 C–E). By fixing and co-staining the cells with the neuronal microtubular marker, β III-tubulin, the centromere marker, pericentrin, and the nuclear dye, ToPro3, we were able to visualize the morphology of individual neurons (Fig. 1E). This ICC method of analyzing neurons, which have migrated out of the MGE explants can be used to monitor alterations in neuronal morphology and expression of endogenous proteins/or receptors in neurons. Also, this assay can be used as a tool to compare the distribution of the neurons as a function of distance from the edge of the explant, as related to different genotypes (McCarthy et al., 2012), or to record epileptiform discharges in organotypic cultures of cortex and hippocampus (Berdichevsky et al., 2010).

3.2. Loading and culturing MGE explants in the microfluidic chambers

MGE explants were isolated from brains of E15 mice embryos as previously described. MGE explants previously infected with AAV or lentivirus, or labeled with specific dyes, were placed in the micro-wells (Fig. 1F). Explants were allowed to attach to the Matrigel for 10 min at 37°C, after which Neurobasal medium containing 2% B-27 supplement, penicillin, streptomycin and glutamine, was added to the microfluidic system. Following 48 hours in culture, the neurons migrating out of the explant into the microchambers (Fig. 1 G–I) were examined under a microscope using phase-contrast optics and by time-lapse image.

3.3. Adeno-associated virus (AAV) transduce MGE explants more efficient than lentivirus

In this study, we developed a protocol to transduce MGE explants with high efficiency. We compared transduction efficacy of AAV serotypes (AAVrh10, AAV 8, and AAV 9) and LV. We packaged AAV-CBA-GFP vector in cells transfected with expression plasmids for the following capsids: AAVrh10 (Fig. 2 A–B), AAV 8 (Fig. 2C), or AAV 9 (Fig. 2D), and lentiviral vector (LV-CMV-GFP) with an expression construct for vesicular stomatitis virus glycoprotein (VSV-G) (Fig. 2C). All AAV and LV vectors demonstrated GFP expression by transduction of MGE explant within 72 hours, but with different expression levels. Highest expression was detected with AAVrh10 while lowest was with LV.

Further, MGE explants were stained with the Golgi marker BODIPY FL C5-ceramide (B-22650; Molecular Probes; Fig. 2 F–G). The BODIPY FL C5-ceramide dye was resuspended in sterile deionized water and diluted to 5 μ M in 1 $\times$ Hank's buffered salt solution (HBSS) immediately prior to use. This working dilution was added directly to the explants at a final concentration of 5 μ M and incubated for 30 min at 4°C followed by several cold washes with 1 $\times$ HBSS and 30 min room temperature incubation with explants in the microfluidic system filled with Matrigel. Live-cells microscopy showed neurons migrating out of

explants inside of microchambers and labeled with the Golgi marker (Fig. 2G). All the neurons showed a similar staining pattern with red- emitting punctae for BODIPY FL C5-ceramide along the processes. In MGE cells, the centrosome and Golgi moved within the neurons a remarkably long distance away from the nucleus (up to 30 μ m), and at the end of its forward migration, the centrosome and the Golgi apparatus folded into a single complex localized to a swelling distinct from the nuclear compartment, as previously observed (Métin et al., 2008)

4. Conclusions

Here we describe methods for culturing and staining neurons migrating out of MGE explants. Our ICC method allows co-staining of these with different intracellular probes. In addition, we also labeled neurons with live cell Golgi and nuclear trackers and imaged neurons in real time. Further, MGE explants showed differences in transduction efficiencies using AAV and lentiviral vectors. Most cells in the MGE explants were preferentially transduced with AAV vectors, serotype rh10 being the best and all better than with a lentiviral vector. Transduction efficiency could presumably be enhanced by increasing the titer of AAV vectors. In addition, our microfluidic devices can be used to culture MGE explants in different compartments and permits the visualization of the vectorial migration of individual neurons in culture chambers. The microfluidic platforms are amenable to high-resolution microscopy because they are constructed on glass coverslips. These attractive features of our staining techniques and microfluidic platforms make them ideal for studying neuronal migration at both a cellular and a molecular level.

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Abbreviations

AAV	adeno-associated virus
AEP	anterior entopeduncular area
GABA	gamma amino butyric acid
GE	ganglionic eminence
g.c.	genome copy
GFP	green fluorescent protein
HP	hidden photon
ICC	immunocytochemistry
i.p.	intraperitoneal

LGE	lateral GE
LV	lentivirus
MGE	medial ganglionic eminence
PFA	paraformaldehyde
PBS	Phosphate Buffered Saline
t.u.	transducing units
VSV-G	vesicular stomatitis virus glycoprotein

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Highlights

- ◆ We established an immunocytochemistry (ICC) protocol to stain neurons migrating out of the MGE explant embedded in Matrigel.
- ◆ We also established a protocol to efficiently transfect cells in MGE explants
- ◆ We developed microfluidic chambers for explants that allow visualization of the vectorial migration of individual neurons from mouse embryonic MGE explants.
- ◆ Our microfluidic system allows monitoring of the distribution of cellular organelles (e.g. Golgi) within migrating neurons which have been stained with commercial molecular dyes or transfected with adeno-associated virus (AAV) expressing reporter proteins.
- ◆ Our methods provide new paradigms to study neuronal migration in real-time.

Fig. 1. Experimental models to study MGE neurons migration from the explants of mouse embryos

A timed-pregnant female mice was sacrificed and brains of the E15 embryos isolated, as described in the methods (1 A). The MGE explants were dissected out from 250 µm coronal Vibratome slices of brains (1 B). MGE explants were placed in a glass bottom culture dish containing 10 µl of Matrigel (1 C). Neurons migrated out of the MGE explant in a symmetrical and radial way, shown here after 48 hours (1 D). The explants were fixed and neurons in Matrigel were co-stained with the neuronal marker β III-tubulin (red), the centromere marker pericentrin (green) and the nuclear marker ToPro3 (blue), shown at low magnification 20 $\times$ and high magnification 100 $\times$ (1 E). Based on the experimental model schematized on the right (2), an MGE explant was placed in one of the wells of a microfluidic device and a cortical explant placed in the other well (1 F). Both wells were filled with Matrigel and covered with Neurobasal medium. Neurons migrated away from MGE explant within microchannels filled with Matrigel towards the cortical explant in the opposite well (1 G–I). Images of a neuron that migrated out of the explant inside of the microfluidic chambers are shown (1 I). The direction of migration is indicated by the white arrow.

Fig. 2. Transduction and labeling of MGE explants

MGE explants from the brains of E15 mouse embryos were transfected with AAV or LV vectors expressing GFP and placed in microchambers filled with Matrigel and covered with Neurobasal medium. After 72 hours neurons expressing GFP migrated out of the explants. (A–E) MGE explants infected with either AAV serotyped with one of the following capsids: AAVrh10 (A–B), AAV 8 (C), and AAV 9 (D), or with lentivirus (E), showing infected neurons expressing GFP after 72 hours. In B, an individual neuron infected with AAVrh10 is shown inside of the microchannel (magnification bar = 20 μ m). In addition, we monitored the migration in real-time of live neurons moving out of the explants (F), and labeled with BODIPY TR C5 (Golgi-tracker red, Molecular Probe; F–G). Forty-eight hours after placing MGE explant labeled with Golgi-tracker in a microfluidic system filled with Matrigel, individual neurons were observed inside of microchannels expressing the Golgi-tracker (G).