A MICROCHIP FOR STUDYING THE EFFECTS OF DOPAMINE AND ITS PRECURSOR ON NEUROSPHEROIDS

Subin Mao[1], Catherine Fonder[2,3,6], Fazlay Rubby[1], Renyuan Yang[1], Gregory Phillips[5,6], Donald Sakaguchi[2,3,4,6,], and Long Que[1,*]*
[1]Department of Electrical and Computer Engineering
[2] Molecular, Cellular, and Developmental Biology Program
[3]Department of Genetics, Development and Cell Biology, [4]Neuroscience Program
[5]Department of Veterinary Microbiology, [6]Nanovaccine Institute
Iowa State University, Ames IA 50011, USA

ABSTRACT

This paper reports a MEMS chip for studying the effects of the neurotransmitter dopamine (DA) and its precursor L-DOPA on adult rat hippocampal progenitor cell (AHPC) neurospheroids for the first time. This chip allows DA or L-DOPA in one chamber to diffuse to AHPC neurospheroids, a promising *in vitro* brain model, cultured in an adjacent chamber through an integrated diffusion barrier, used to mimic the blood-brain barrier (BBB) by an array of intentionally misaligned micropillars. After cell fixation and immunostaining were conducted, the fluorescence images of AHPC neurospheroids were analyzed. AHPC neurospheroids cultured in these devices remained highly viable following DA or L-DOPA treatment. Cell proliferation and neuronal differentiation have also been shown following DA or L-DOPA treatment, indicating the AHPC neurospheroids as a valuable *in vitro* brain model for neurogenesis research.

KEYWORDS

Neurotransmitter, dopamine, neurospheroids, blood-brain barrier (BBB), microchip, neurogenesis

INTRODUCTION

It was discovered that the adult mammalian brain can generate new neurons and integrate them into existing circuits [1], which enables new perspectives in understanding how the central nervous system (CNS) functions in health and disease. Research has found that mature cells in all neural lineages, including neurons, can be generated throughout adulthood in two distinct areas of the forebrain [2]. It has been reported that neurotransmitters are associated with chemical communication between differentiated neurons and can also contribute to neurogenesis [3-6]. For instance, some research groups reported that dopamine (DA), a type of neurotransmitter, plays a role in regulating endogenous neurogenesis in the adult mammalian brain [3-4]. However, it remains controversial if neurogenesis exists *in vivo* in the adult mammalian substantia nigra, a midbrain dopaminergic nucleus [4]. Most research efforts utilized *in vivo* approaches to study possible neurogenesis due to neurotransmitter involvement.

While the *in vivo* studies are critical for reliable evaluation of the effects of neurotransmitters on neurogenesis, *in vitro* studies can provide some clear advantages, given their cost-effectiveness, quick turn-around time, and no requirement of ethical approval. However, so far, very few efforts have been reported to study the neurotransmitter effects on an *in vitro* brain model on-chip.

Herein, a MEMS chip is reported for studying the effects of one type of neurotransmitter (i.e., DA) and its precursor (i.e., L-DOPA) on an *in vitro* brain model (i.e., AHPC neurospheroids) [7-8]. On this device the AHPCs are cultured in one chamber separated by a diffusion barrier, mimicking the BBB from a second chamber containing DA or L-DOPA.

MATERAILS AND METHODS

Materials

Adult rat hippocampal progenitor cells (AHPCs) were generously gifted by Dr. F.H. Gage, Salk Institute, La Jolla, CA. Poly-L-ornithine was purchased from Sigma-Aldrich, St. Louis, MO. Laminin was purchased from Cultrex by Trevigen, Gaithersburg, MD. Maintenance media (M) composed of Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1) was purchased from Omega Scientific, Tarzana, CA. GlutaMAX was purchased from Thermo Fisher Scientific, Waltham, MA. N2 supplement was purchased from Gibco by Thermo Fisher Scientific, Waltham, MA. Basic fibroblast growth factor (bFGF) was purchased from Promega Corporation, Madison, WI. Uncoated tissue-culture polystyrene (TCPS) cultureware was purchased from Thermo Fisher Scientific, Waltham, MA. Propidium Iodide (PI) was purchased from Thermo Fisher Scientific, Waltham, MA. DA and L-DOPA were purchased from Sigma-Aldrich, St. Louis, MO.

Chip description and fabrication

(i) Illustration and operational principle

The proposed chip is schematically shown in **Fig. 1**. It consists of two adjacent chambers separated by systematically misaligned micropillars as a diffusion barrier for DA or L-DOPA. One chamber is used for culturing neurospheroids, and the other acts as a reservoir for DA or L-DOPA solution. The diffusion barrier between the two chambers serves as a mimic of the blood-brain barrier (BBB), allowing DA or L-DOPA solution to diffuse into an adjacent chamber and thus react with the neurospheroids, which are used as an *in vitro* brain model. The topside view of the diffusion barrier (i.e., arrayed micropillars) is illustrated in **Fig. 1b**. As shown, the adjacent rows of the micropillars are systematically misaligned, reducing the effective gaps between them, thereby slowing down the fluidic diffusion.

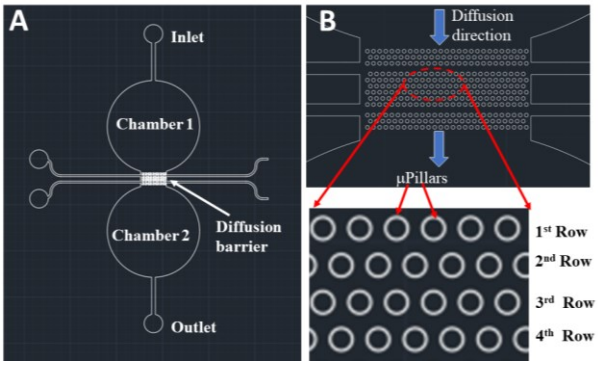

Figure 1: (A) Schematic illustration of the chip with two chambers: Dopamine or L-DOPA in Chamber 1, AHPC neurospheroids cultured in Chamber 2; (b) Close-up of the diffusion barrier: It consists of 12 rows of micropillars. The micropillars in adjacent rows are intentionally misaligned to reduce the gaps among them, resulting in a diffusion barrier to mimic the BBB.

The diffusion barrier is currently under further improvement and modifications to mimic the BBB by (1) injecting curable PEG prepolymer into the spacing/gaps among the micropillars to form a permeable hydrogel barrier with micro/nanoscale pores, or (2) culturing cortical astrocytes and endothelial cells inside spacing/gaps among the micropillars.

(ii) Fabrication of the chip

The chip was fabricated using a soft lithography process [7-8]. Briefly, a mold was first fabricated from resist AZ40XT. Then polydimethylsiloxane (PDMS) was poured on the mold followed by 2-hour degassing and 2-hour curing at 75 °C. Thereafter, the PDMS microfluidic layer was peeled off from the mold, followed by the formation of the chambers by punching through the PDMS layer using a biopsy puncher. Finally, the microfluidic layer was bonded with a glass substrate after plasma treatment to complete the fabrication of the chip.

Neurospheroid cultures

AHPC neurosphereoids were generated as previously described [9]. Briefly, AHPCs were cultured in uncoated T25 culture flasks under proliferation conditions with DMEM/F-12, supplemented with 2.5 mM GlutaMAX, 1 × N2 supplement, and 20 ng/mL bFGF. This resulted in AHPCs spontaneously aggregating and generating neurospheroids that continued to proliferate.

Cell Staining and Immunocytochemistry

All cell staining and immunocytochemical procedures were conducted on chip. Briefly, the chamber with neurospheroids was rinsed with 0.1 M PO_4 buffer, and then fixed with 4% paraformaldehyde (PFA, Thermo Fisher Scientific) in 0.1 M PO_4 buffer for 20 minutes followed by phosphate buffered saline (PBS) wash. The cells were incubated in blocking solution: 0.2% Triton X-100 (Thermo Fisher Scientific), 5% normal donkey serum (Jackson ImmunoResearch), and 0.4% bovine serum albumin (Sigma-Aldrich) in PBS at room temperature for 1.5 h. Primary antibodies Rabbit α Ki-67 (1:100, IgG; Abcam), and Mouse α TuJ1 (1:100, IgG; R&D Systems) were diluted in the blocking solution. The cells were incubated with the primary antibody solution overnight at 4 °C. After rinsing with PBS, the microchamber was loaded with secondary antibody solution. To prepare the secondary antibody solution, Donkey α Rabbit Cy3 (1:100, IgG; Jackson Immunoresearch), and Donkey α Mouse AF488 (1:100, IgG; Jackson ImmunoResearch) were diluted with the blocking solution containing 4',6-diamidino-2-phenylindole (DAPI; diluted 1:1,000; Invitrogen). Again, the cells were incubated with the secondary antibody solution under room temperature in the dark for 1.5 h. After rinsing with PBS, the cells were ready for fluorescence imaging.

Propidium iodide (PI) staining and cell viability test

We used PI to stain dead cells. A 1.5 µM PI solution was prepared by diluting the stock solution in culture medium, which was then loaded into the culture chamber, followed by incubation for 20 min at 37˚C in 5% CO_2. As a control, another chamber containing AHPC neurospheroids was loaded with 70% ethanol for 5 min to intentionally kill all the cells prior to adding the PI solution. After PI incubation, the chamber was rinsed with 0.1 M PO_4 buffer and then fixed with 4% PFA. After being rinsing with PBS, the cells were incubated with DAPI (1:1,000) diluted in PBS for 60 min at room temperature in the dark, followed by a final rinse with PBS.

Imaging

Phase contrast images of AHPC neurospheroids were taken with a Nikon Diaphot inverted phase contrast microscope (Nikon Corp.) equipped with a Q Imaging Retiga 2000R (Q Imaging) digital camera daily so that the cell growth and neurospheroid development could be monitored. Corresponding fluorescence images were taken using a Leica DMI4000 B (Leica Microsystems) inverted microscope equipped with standard epifluorescence illumination and a Leica DFC310 FX digital camera. A 20× objective was used to obtain images for quantitative data analysis.

Data acquisition and statistical analysis

ImageJ software (http://imagej.nih.gov/ij) was used to analyze and quantify the images of neurospheroids. Ki-67 and TuJ1 positive cells were counted using the Cell Counter tool in ImageJ and the % of Ki-67 or TuJ1 immunoreactive cells was determined as the number of Ki-67 labeled nuclei or TuJ1 labeled cytoskeleton over the total DAPI-labeled nuclei. All means are reported with standard error of the mean (mean ± SEM). Graph Pad Prism 9 (Graph Pad Software, Inc. San Diego, CA) was used for statistical analysis and graph-making. Means were compared using ordinary one-way ANOVA. Statistical significance determined using Tukey's multiple comparison test, with alpha = 0.05.

RESULTS AND DISCUSSION

Fabricated chip

A photo of a fabricated chip is shown in **Fig. 2a**. An optical micrograph of the cross-sectional view of the micropillars in the diffusion barrier region is shown in **Fig. 2b**. The top-side view of the micropillars in the diffusion barrier region

is shown in **Fig. 2c**. In this case (**Fig. 2c**), the cured PEG polymer is inside the gaps among the micropillars. Typical width and height of the micropillars are 45 µm and 95 µm, respectively. The gap between adjacent micropillars in the same row is 50 µm.

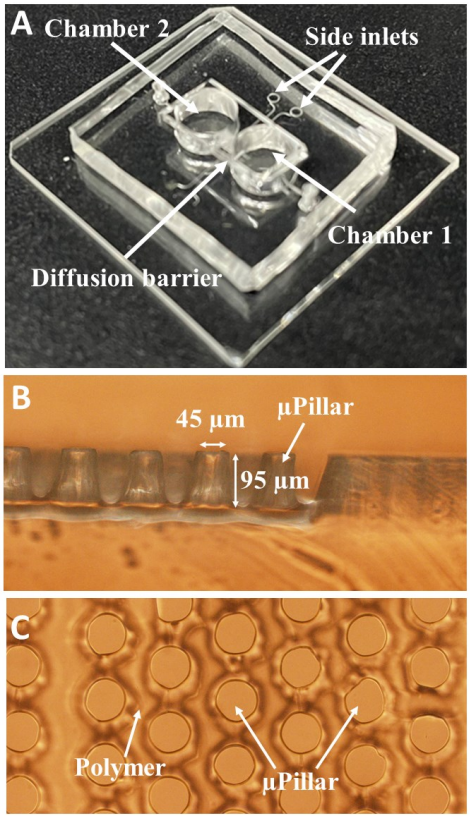

Figure 2: (A) Photo of a fabricated chip, (B) the close-up showing the micropillars as the diffusion barrier. The gap between adjacent micropillar is ~50 µm, (c) the close-up showing the topside view of the micropillars, the cured PEG polymer is distributed among the micropillars.

Diffusion demonstrations using food dye

Two types of chips have been tested. One type of chip (***Chip1***) has misaligned micropillars as the diffusion barrier. In the second chip (***Chip2***), the gaps among micropillars are filled with cured PEG polymers through the side inlets (**Fig. 2a**). Diffusion of a solution (green food dye for visibility) through the barrier has been demonstrated from *Chamber 1* to its adjacent *Chamber 2*.

Experiments have found that it took several minutes for the dye to diffuse from *Chamber 1* to *Chamber 2* for *Chip1*. In contrast, it took several days or longer than ten days for the dye to diffuse from *Chamber 1* to *Chamber 2* for *Chip2*, depending on the molecular weight of PEG polymer. Representative photos before and after food dye diffusion from *Chamber1* to *Chamber2* are given in **Fig. 3**. For Chip2, the concentrations of food dye essentially cannot be the same in both chambers because of the PEG polymer-based diffusion barrier. The diffusion barrier is under further modifications to mimic the BBB by culturing cortical astrocytes and endothelial cells inside spacing/gaps among the micropillars.

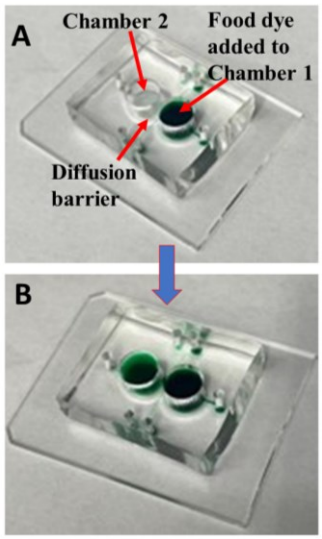

Figure 3: Dopamine diffused from Chamber 1 to Chamber 2 through the barrier layer between them. For visibility, green food dye was used to mimic the DA or L-DOPA sample.

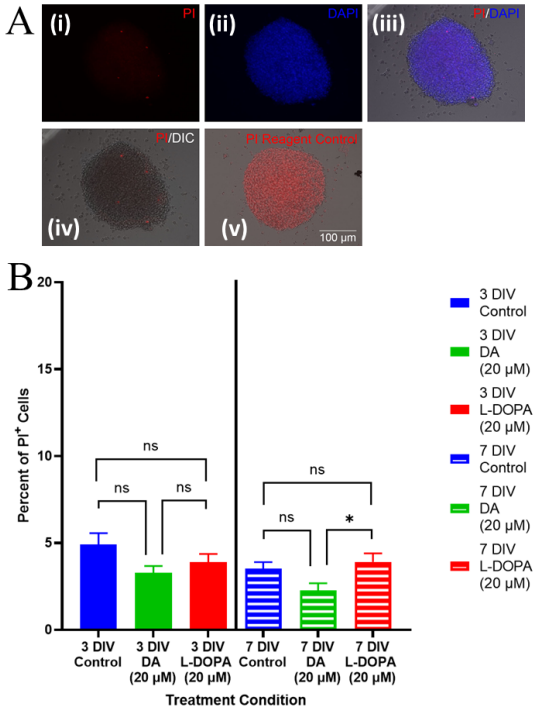

Figure 4: Cell viability under DA and L-DOPA treatment: (A) Representative fluorescence images of one neurospheroid labeled with PI and DAPI on 7 DIV; (B) 95-98% viability of cells inside neurospheroids cultured from 3 DIV to 7 DIV. Error bars represent standard error of the mean.

Cell viability analysis

After cell fixation and immunostaining, the fluorescence images were analyzed. It has been found that cells inside neurospheroids remained highly viable, showing 95-98% viability of cells inside neurospheroids after either DOPA or L-DOPA treatment (**Fig. 4**), similar to control.

Some representative fluorescence images for analyzing cell proliferation and differentiation of the neurospheroids are shown in **Fig. 5**.

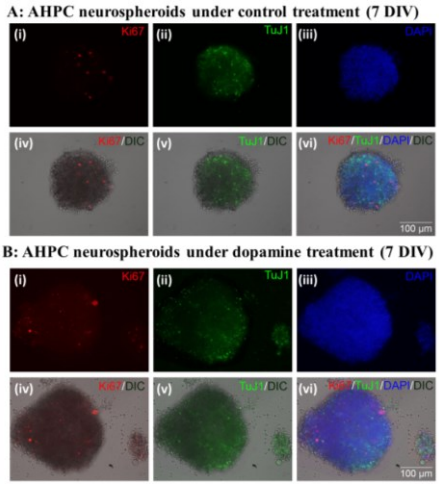

Figure 5: Representative fluorescence/light images of one neurospheroid stained with different markers on 7 DIV (A) under control and (B) under DA treatment.: (i) Ki67, (ii) TuJ1, (iii) DAPI, (iv) merged images of (i) to light image, (v) merged image of (ii) and light image, (vi) merged images of (iii) to (v).

Proliferation analysis

Ki-67 immunolabeling was used to analyze cell proliferation within NSs-AHPC growing inside the culture-chambers. The quantitative analysis of cell proliferation is summarized in **Fig. 6**. The percentage of Ki-67 positive cells over total nuclei count on 7 DIV is 2.3-5.7% for control, DA and L-DOPA treatment.

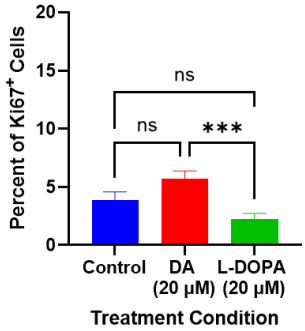

Figure 6: Proliferation: quantitative analysis of AHPC neurospheroids on 7 DIV with and without DA and L-DOPA treatment. Error bars represent standard error of the mean.

Neurogenesis analysis

TuJ1 immunolabeling was used to analyze neuronal differentiation. The quantitative analysis of cell neurogenesis is summarized in **Fig. 7**. The quantitative analysis for TuJ1 labeling indicates 11.4-15.8% immunoreactivity on 7 DIV for control, DA and L-DOPA treatment.

SUMMARY

A microchip with a diffusion barrier, mimicking the blood brain barrier, has been developed to evaluate the effects of DA and L-DOPA on APHC neurospheroids. It has been found the AHPCs remained highly viable and capable of proliferation and neuronal differentiation. All these results indicate that the AHPC neurospheroids can be used as an *in vitro* brain model for neurogenesis research.

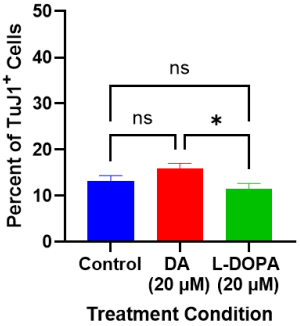

Figure 7: Neurogenesis: quantitative analysis of AHPC neurospheroids on 7 DIV with and without DA and L-DOPA treatment. Error bars represent standard error of the mean.

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CONTACT

*L. Que, tel: +1-515-294-6951; lque@iastate.edu
*D. Sakaguchi, +1-515-294-3112; dssakagu@iastate.edu