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µNeurocircuitry: Establishing *in vitro* models of neurocircuits with human neurons

Joseph A. Fantuzzo^{1,2}, Lidia De Filippis^{1,3,4}, Heather McGowan^{1,3}, Nan Yang⁵, Yi-Han Ng⁵, Apoorva Halikere^{1,3}, Jing-Jing Liu^{1,3}, Ronald P. Hart⁶, Marius Wernig⁵, Jeffrey D. Zahn², and Zhiping P. Pang^{1,3,7}

¹Child Health Institute of New Jersey, 89 French Street, New Brunswick, NJ 08901, USA

²Department of Biomedical Engineering, Rutgers University, 599 Taylor Road, Piscataway, NJ 08854, USA

³Department of Neuroscience and Cell Biology, Rutgers University, 675 Hoes Lane West, Piscataway, NJ 08854, USA

⁴Casa Sollievo della Sofferenza, Viale Cappuccini 1, 71013 San Giovanni Rotondo (FG), Italy

⁵Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, 265 Campus Drive, Stanford, CA 94305, USA

⁶Department of Cell Biology and Neuroscience, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA

⁷Department of Pediatrics, Robert Wood Johnson Medical School, Rutgers University, 1 Robert Wood Johnson Place, New Brunswick, NJ 08903, USA

Abstract

Neurocircuits in the human brain govern complex behavior and involve connections from many different neuronal subtypes from different brain regions. Recent advances in stem cell biology have enabled the derivation of patient-specific human neuronal cells of various subtypes for the study of neuronal function and disease pathology. Nevertheless, one persistent challenge using these human-derived neurons is the ability to reconstruct models of human brain circuitry. To overcome this obstacle, we have developed a compartmentalized microfluidic device, which allows for spatial separation of cell bodies of different human-derived neuronal subtypes (excitatory, inhibitory and dopaminergic) but is permissive to the spreading of projecting processes. Induced neurons (iNs) cultured in the device expressed pan-neuronal markers and subtype specific markers. Morphologically, we demonstrate defined synaptic contacts between selected neuronal subtypes by synapsin staining. Functionally, we show that excitatory neuronal stimulation evoked excitatory postsynaptic current responses in the neurons cultured in a separate chamber.

Keywords

Microfluidics; Soft Lithography; Induced Neurons; Neurocircuitry; Microchannels; Optogenetics; Compartmentalized Culture; Polydimethylsiloxane; Lentiviral Vectors

Innovation

While human-induced pluripotent stem cells (iPSCs) have been used to study different psychiatric disorders, *in vitro* models are often limited to single-cell type analysis and mechanistic studies in a manner that does not account for circuit-level connections. Microfluidics have been used to create compartmentalized devices for neuronal cell culture for a variety of applications, including circuit studies, but have either been limited to primary cell sources or single-subtype human neurons. We demonstrate that we can generate excitatory, inhibitory and dopaminergic induced neurons (iNs) from human iPSC sources and these can recapitulate neuronal connections to form a multi-subtype μ Neurocircuitry when cultured in our custom-designed culture devices. These human iNs possess neuronal morphology and display interchamber complex connectivity, as demonstrated here through morphological characterization and optogenetic manipulation. This novel μ Neurocircuit can be used as a tool for studying human neuropsychiatric disorders, with a goal of providing a platform for developing novel therapies.

Introduction

Brain connections are complex, involving afferent and efferent processes from neurons residing in distinct brain areas forming an intricate neurocircuitry that governs behavior. For example, neurons located in the nucleus accumbens receive dopaminergic (DA) neuronal control from the midbrain and excitatory inputs from the prefrontal cortex, forming a circuitry that regulates motivation and reward^{1,2}. Dysfunction of neural circuitry may result in neuropsychiatric disorders, including autism spectrum disorders (ASDs), schizophrenia and addiction. There are often no effective therapies for these disorders, largely due to the lack of a mechanistic understanding of the pathophysiology. While animal models have provided significant insight into mechanisms underlying behavioral disorders, they may not capture the entire complexity of the human brain³. Furthermore, studies focused on the role of genetics and protein function may rely upon species differences that cannot be modelled in animals, unless the gene of interest is ectopically expressed^{4,5}. Studies using human tissue have been limited, due both to scarce availability of suitable post-mortem or surgical tissues and the highly invasive tissue acquisition procedures. Ever since the discovery of the Yamanaka factors, iPSCs have served as a transformational tool to produce differentiated human cells for a variety of purposes^{6,7}. This novel technology has enabled researchers to consider different strategies for modelling human neuropsychiatric disorders in a culture dish⁸. However, studies performed on cell populations that are often composed of mixed neuronal subtypes in a conventional two-dimensional (2D) format cannot recapitulate the complexity of the physiological circuitry *in vivo*. Three-dimensional (3D) models provide a greater degree of cyto-architecture^{9,10}, but still have not yet provided a compartmentalized context for neurocircuit formation. When the goal is to reproduce a cyto-architecture

involving multiple brain regions, *in vitro* models must compartmentalize different neuronal subtypes, while allowing them to establish functional neuronal connections.

Several approaches have taken advantage of neuronal morphology as a means of modelling synaptic connections. The Campenot chamber, formed with a plastic divider affixed to a glass slide by silicone grease, sections of areas of culture while allowing axons to project through a series of channels created in a collagen layer^{11,12}. More recently, approaches using soft lithography have produced more precisely defined channels and compartment structures^{13,14}. These microfluidic devices have enabled studies of axonal injury^{15–17}, axon pathfinding¹⁸ and cellular migration¹⁹. They have also been utilized to investigate synaptogenesis²⁰ and circuit connectivity^{21–24}. A compartmentalized model of synaptic competition has been devised allowing the study of differences in individual neurons by chemically treating one chamber and performing synapse counting in another²². Axon diodes have also been created as a means of providing directionality in circuit models²³. Renault *et al.* have demonstrated the use of optogenetics and calcium imaging as tools to evaluate neuronal communication in a compartmentalized device²¹. These approaches have provided a proof of concept for microfluidics as a strategy to compartmentalize culture in brain circuit studies, but thus far they have only used primary neuronal cultures or are limited to human induced neurons of a single subtype²⁴. Furthermore, in most cases, they are not permissive to high-definition functional studies (e.g. patch clamp electrophysiology).

Building on these previous approaches, we have developed a μ Neurocircuitry model of neurons capable of receiving multiple inputs, which allows the study of a simplified version of human brain circuitry, with a goal of elucidating neuropsychiatric disorder pathology. We have developed a five-compartment neurocircuit model fulfilling the following requirements: 1) chemically distinct compartmentalization with microchannels, enabling defined synaptic connections, 2) a large, open central chamber, allowing spatial access for patch clamp apparatus and 3) feasibility for the culture of human-derived neurons to model the human-specific aspects of these disorders. We have used iN cell technology to generate excitatory (glutamatergic), DA and inhibitory (GABAergic) subtype human neurons from patient-specific iPSC sources. We demonstrate that these neurons express subtype-specific markers and develop functional synaptic connections between the outer and central culture chambers. We anticipate that this device will be useful for the study of brain circuits between various neuronal subtypes as they relate to neuropsychiatric disorders.

Materials and Methods

We designed the microdevice to mimic the architecture of a brain nucleus receiving 2–4 different inputs from other brain regions (Fig. 1a). The device has five chambers — four outer chambers and one large inner chamber (Figs. 1b,c). Outer and inner chambers are connected through a series of microchannels.

Device fabrication

A master template was prepared using soft lithography (Fig. 1d) on a four-inch silicon wafer (University Wafer, Boston, MA) in a manner similar to Taylor *et al.*^{15, 25} A first layer of photoresist defined 10 μ m wide and 100, 200 or 500 μ m long axonal extension

microchannels (Fig. 1e,f). SU-8 2002 (Microchem, Woburn, MA) was spun onto the wafer at 1,000 rpm to give a 3 μm layer. The layer was exposed to UV using a photomask on an EVG620 (EV Group, Tempe, AZ) mask aligner. The wafer was then developed for approximately 60 seconds in SU-8 developer (Microchem, Woburn, MA) and subsequently hard-baked at 150°C for 30 minutes.

A second layer of photoresist (SU-8 2075), which defined the overall culture chamber structure and walls between each compartment, was next spun onto the wafer. This layer was then soft baked for 10 minutes at 65°C and 50 minutes at 95°C, and then allowed to cool to room temperature. On top of this SU-8 2075 layer, another layer of SU-8 2025 was spun at 1,000 rpm for additional chamber height. Together, the chamber height was estimated to be 320 μm . The wafer was then aligned to the first layer, such that microchannels spanned the gap between side chambers and central chamber. Next, the wafer was exposed to UV and developed in SU-8 developer. A slow hard-baking protocol was followed by ramping the temperature from room temperature (20°C) to 105°C, which was held for 20 minutes, and then ramped down back to room temperature. This step is important to reduce peeling of SU-8 layers from the wafer due to residual stress.

The completed master was treated with approximately 60 μl trichloro (1H,1H,2H,2H perfluoro-octyl) silane vaporized in a vacuum chamber for 1 hour. Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI) was prepared at a 10:1 ratio with its curing agent and poured onto the master, degassed and baked at 65°C overnight to cure. Holes were punched into the PDMS to provide pipettor access. The central hole was punched to approximately 10 mm in diameter. Three side access holes per side chamber were punched at 3 mm in diameter. Devices were then sonicated in acetone for 1 hour followed by sonication in isopropanol for 40 minutes. The devices were bonded to a glass coverslip (22 mm $\times$ 22 mm) using an ETP high frequency generator (Electro-technic Products, Inc., model BD-10A, Chicago, IL), creating ozone to facilitate surface oxidation. The device was then placed on a hotplate at 95°C for 30 minutes under light weight. Once bonded, the devices were re-exposed to the ozone to create an oxidized surface and placed in distilled water for storage. To prepare the devices for culture, the water was removed and the devices were placed under UV light for 1 hour. Matrigel™ (Corning) was then added to each of the chambers of the device. It was then placed in an incubator for 1 hour prior to seeding.

Lentivirus production

To generate lentiviral vectors, we used calcium phosphate transfection of human embryonic kidney (HEK293) cells, as described by Tiscornia *et al.*²⁶ Briefly, lentivirus packaging vectors RRE, REV2, VsVG and DNA of interest were added to a glass vial followed by 60 μl of 2.5 M calcium chloride. The solution was then totaled to 500 μl using deionized water. This mixture was then added dropwise to 500 μl 2 $\times$ HBS solution while vortexing. The final solution was incubated at room temperature for 30 minutes and then added to a HEK293 culture plate at Day 0. DMEM from HEK293 culture on Day 1 was discarded. Media from Days 2 and 3 were collected and ultracentrifuged at 106,490 rcf (25,000 rpm) for 2 hours at 4°C. Concentrated virus was then resuspended in 100 μl MEM and aliquoted for later use. All viruses were generated for expression under the TetOn promotor (Tet-On).

iN cell derivation from iPSCs

Preparation of iPSC from human primary lymphocytes using Sendai viral vectors (CytoTune™, Life Technologies, Grand Island, NY) has been described^{27,28}. All source biomaterials were de-identified repository specimens and therefore are exempt from human subjects regulations. Feeder-free mTeSR™1 medium (Stem Cell Technologies) was used.

Human iNs were generated according to protocols described elsewhere^{27,29} with slight modifications. Briefly, iPSCs were seeded onto a Matrigel™-coated six-well plate at a density of 300,000 cells per well (Day -1) in mTeSR. Lentivirus and Y-compound (Y-27632; 5 µM) were added the following day (Day 0). To generate excitatory neurons, lentivirus expressing the transcription factor Ngn2 (with puromycin resistance) was added. For inhibitory neurons, viruses expressing Dlx2 (with hygromycin resistance) and Ascl1 (with puromycin resistance) were added. For dopaminergic neurons: Ascl1, Nurr1 and Lmx1a viruses were added. EN1, Foxa2 and Pitx3 were also added on Day 3. All of the factors utilized for the iN cell protocol were expressed under the TetO promoter (Tet-On), and thus, all infections included a virus expressing the reverse tetracycline-controlled transactivator (rtTA) protein. The following day (Day 1), the cultures were switched to Neurobasal media supplemented with B27 and L-glutamine. Doxycycline (2 ng/µl) was added on Day 1 to induce expression of subtype-specific transcription factors, along with Y-compound (5 µM) to limit apoptosis. On Day 2, iPSCs underwent a puromycin (1 µg/ml) selection. Day 3 cultures underwent additional puromycin selection with addition of secondary viral vectors as needed. Channelrhodopsin 2 (ChR2)-tdTomato lentivirus was added to excitatory neurons on Day 3 as necessary. Inhibitory neurons underwent an additional hygromycin (25 ng/ml or higher as needed) selection on Days 3 and 4 prior to seeding into the device. iNs were kept in Neurobasal media with L-glutamine, B27, doxycycline and puromycin (hygromycin also for inhibitory iNs) until plated on Day 6.

Glial and human iN cell culture

Mouse glial cells, obtained from postnatal day 0 (P0) pups, were seeded into the devices on Day 5 (one day prior to neuron seeding). In the central chamber, 20,000 glial cells were seeded (~300 glia/mm²). In the side chambers, 12,000 glial cells were seeded (~210 glia/mm²). For neurons, 150,000 neurons were seeded in the central chamber (~1,580 neurons/mm²) and 75,000 neurons were seeded in each side chamber (~1,875 neurons/mm²). iNs were removed from the well plate surface using Accutase (Innovative Cell Technologies, San Diego, CA) and plated with Neurobasal media containing L-glutamine, B27, NT3 (10 ng/ml), GDNF (10 ng/ml), BDNF (10 ng/ml), 5% FBS, 1% penicillin/streptomycin and 2 µg/ml doxycycline. After cell attachment, and as needed during the course of the culture, 2 µM cytosine arabinoside (AraC) was added to the media to control glial cell division. Devices were fed every 2–3 days. Devices were housed individually in 35 mm culture dishes. Two 35 mm dishes were placed in a 10 cm culture dish and approximately 1 ml of sterile water or PBS was added to the 10 cm culture dish to limit evaporation of media.

Immunocytochemistry

Human iNs were fixed for 15 minutes in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS at room temperature. Cells were permeabilized in 0.2% Triton X-100 in PBS for 10 minutes and then incubated in blocking buffer (1× PBS, 20% Goat serum) for 1 hour 30 minutes. Cells were then incubated overnight with primary antibodies. The primary antibodies used were: anti-MAP2 (Sigma, M3696, 1:750; Sigma, M1406 1:1,000 and 1:500), anti-vGlut2 (NeuroMab clone N29/29, 1:1,000), anti-tyrosine hydroxylase (TH, Millipore AB152, 1:500), anti-GAD6 (Sigma, G5038, 1:1,000), anti- β -III-tubulin (Tuj1, Covance, 1:500), anti-synapsin (rabbit anti-synapsin, E028, a gift from the Südhof lab, 1:3,000) and Hoechst (1×). Excitatory (glutamatergic) neurons were stained for MAP2, vGlut2 and β -III-Tubulin. Dopaminergic neurons were stained for MAP2 and TH. Inhibitory (GABAergic) neurons were stained for GAD6. Confocal images were collected using a Zeiss LSM700 laser scanning microscope (Carl Zeiss, Dublin, CA). Three-way circuit images were acquired by taking Z-stacks and processing the stacks as maximum intensity projections.

Microchannel diffusion study

For diffusion studies, we used a device previously seeded with neurons and glia and cultured for 6 weeks. Devices were placed on a heated stage (kept at 37°C). About 150 μ l of fresh media was added to the central chamber, and 21.8 μ l of media was added to one of the side chambers. Fluorescein was added to the side chamber (13.2 μ l) to generate a final concentration of 1 mg/ml fluorescein. Time-lapse images were collected every 15 seconds on a Zeiss confocal microscope (Zeiss LSM 700, Carl Zeiss, Dublin, CA) at excitation wavelengths of 488 (for fluorescein) and 563 (for tdTomato). Images were processed in the Fiji package of ImageJ³⁰. Diffusion distance was measured from the channel entrance to the diffusion front for every other channel in each sequential frame up to the 10th frame. The breakthrough time was determined by averaging all microchannels in which breakthrough (diffusion distance equaling 200 μ m) was seen. A least-squares fit of the model ($l = \sqrt{4Dt}$) was performed to determine the diffusion coefficient of fluorescein. The diffusion was repeated in multiple side chambers and the data were pooled.

Electrophysiology

Standard electrophysiology was performed as described by Vierbuchen *et al.*³¹ and Pang *et al.*²⁹ Whole-cell patch clamp recordings were made in the neurons located in the central chamber. The bath solution contained (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES and 10 glucose (pH 7.4, adjusted with NaOH). The whole-cell pipette solution contained (in mM) 126 K-Gluconate, 4 KCl, 10 HEPES, 0.3 Na-GTP, 4 Mg-ATP and 10 Phosphocreatine (pH 7.2, adjusted with KOH). Both current and voltage clamp experiments were performed. For the current clamp experiment, spontaneous action potentials were recorded at resting membrane potential; for evoked stem current clamp recordings (steps of 5 pA from −20 pA to 35 pA), negative currents were injected to keep the membrane potential at around −60 mV. For voltage clamp experiments, spontaneous postsynaptic currents (PSCs) were recorded at a holding potential of −70 mV; whole-cell current

responses were collected by a step depolarization protocol (step voltage injections were given from -100 mV to 0 mV with a step size of 10 mV).

Optogenetic experiments were performed by stimulating ChR2-infected excitatory neurons with 470 nm LED light (Thorlabs). Light evoked synaptic responses were recorded from central chamber neurons at a holding potential of -70 mV. All electrophysiological experiments were performed using a rig containing a Molecular Devices 700B amplifier (Molecular Devices), a Digidata 1400 A/D converter, a Sutter 385 manipulator and an Olympus IX50 with an infrared video camera. Data were filtered at 2 kHz and digitized at 5 kHz. Data were collected using pClamp10 software (Molecular Devices).

Results

Construction of a μ Neurocircuit of human neuronal cells with multiple inputs

To mimic a simplified neurocircuitry in the brain where a group of neurons receives two defined inputs (Fig. 1a), we designed a novel multi-compartmental device where the side chambers are linked with the central chamber through microchannels (Fig. 1b). We microfabricated the device using the procedure described in Methods (Fig. 1). Human iN cells^{29,31} derived from iPSCs were seeded into both outer and central chambers, producing cultures with normal neuronal morphology with extensive and complex branches (Fig. 2). We first evaluated different center wall widths (microchannel lengths) (100 μ m, 200 μ m [Fig. 2a] and 500 μ m [Fig. 2b]). While devices with either 200 μ m or 500 μ m walls were easily fabricated, those with 100 μ m walls were more challenging, due to difficulty in removing un-crosslinked photoresist from the master wafer during SU-8 development. We continued experimentation with the 200 μ m wall device, as it balanced successful fabrication with a shorter path for process growth into the central chamber, as compared with the 500 μ m wall.

To validate the formation of synaptic circuits in this system by different subtypes of iNs, we generated excitatory, inhibitory and DA neurons using different sets of transcription factors. Since glial cells are important for synapse formation^{32,33}, we cultured mouse glial cells in the different compartments of the chamber system. We then initially seeded excitatory (glutamatergic) neurons into the outer chamber and DA neurons into the central chamber (Fig. 2c). The cultures were maintained in a humidified environment and the medium was changed every 2–3 days. The outer chamber solution was originally kept at a slightly higher fluid level compared with the central chamber to create hydrostatic pressure to direct the extension of axons from the side chambers through the microchannels. In subsequent experiments, we found that excitatory projections from the side chambers dominated axonal progression through the channels in spite of equal fluid levels, and so the medium height was thereafter maintained equally between chambers. After 4–7 days of culture, massive extended neuronal processes were observed to pass through the microchannels connecting the outer and the central chamber (Fig. 2c) which formed synapselike boutons with neurons in the central chamber at 4–6 weeks post seeding (Fig. 2d,e). These results indicate that human neurons survive in the device for 4–6 weeks and sense diffusible, trophic signals from communicating chambers to drive process extension and potential synaptic interaction between chambers.

iN expression of neuronal markers in different compartments within the μ Neurocircuit

Given the survival of neurons grown in the microdevice, we sought to determine if their subtype identity was preserved by a morphological analysis. We seeded inhibitory neurons in the center and excitatory and DA neurons in the outer chambers. At 4 weeks post plating, we fixed the cultures and stained for pan-neuronal and subtype-specific markers. Excitatory neurons expressed MAP2 and Tuj1, along with vGlut2, indicating an excitatory neuronal phenotype (Fig. 3a,d). DA neurons showed expression of MAP2 and TH, consistent with a dopaminergic phenotype (Fig. 3b). Inhibitory neurons expressed GAD6, the rate-limiting enzyme for the production of GABA (Fig. 3c). Human iNs also expressed synapsin (Fig. 3e,f), suggesting synapse formation within the device. While generally neurons of a specific subtype remained within their distinctive chamber, there was a small percentage ($\sim 0.001\%$) of cells that apparently migrated through the microchannels. In spite of these few cells, these images demonstrate that iPSCs were converted into mature neurons, and that these three subtypes can be maintained in distinct compartments within the microdevice.

Additionally, since the device was designed to enable central chamber neurons to receive inputs from neurons in two distinct side chambers, we wanted to demonstrate the capacity of the device to facilitate formation of a three-way circuit. We infected excitatory neurons with either GFP or ChR2-tdTomato lentivirus prior to seeding, and placed them in adjacent side chambers. Non-labeled excitatory neurons were seeded in the central chamber. After 4 weeks, both GFP and tdTomato-labeled projections were observed to fill the microchannels (Fig. 4a). Interestingly, axons projected outward and turned to follow the circular edge of the device. Central chamber neurons located near the intersection between side chambers were found to have nearby projections from both GFP and tdTomato iNs. These neurons stained positive for synapsin, suggesting synapse formation between side chamber projections and central chamber iNs (Figs. 4b–d). These data suggest that axonal projections originating from neuronal bodies located in different outer compartments could form mature synapses onto the same neurons in the central chamber.

We also demonstrated that the number of projecting processes from the side chambers can be substantially increased by seeding 45,000 iNs per side chamber (instead of 25,000) and by seeding side chambers with iNs 3 days prior to the central chamber (Supplementary Fig. 1). This device was seeded with a different cell line, which shows reproducibility of the approach across different iPSC lines for circuit modeling.

Functional characterization of human μ Neurocircuit activity

We next asked whether human neurons would establish functional connections. Our system is designed to contain a large, open center well with room for a microscope objective and patch clamp apparatus (see Fig. 1,5a) or suitable to be used for functional analysis using an inverted microscope. Previous studies have shown the ability of compartmentalized systems to record inter-chamber communication using calcium imaging^{12,21}. While our system has the capacity for calcium imaging, we designed the system to have a large open well to allow for electrophysiology, which measures high temporal resolution (in millisecond scale) neuronal firing or synaptic transmission. This aspect strengthens the relevance of our approach, as it enables more complex functional analyses of neurocircuitries. Whole-cell

patch clamping experiments were performed on neurons cultured in the central chamber. These neurons are capable of firing spontaneous repetitive action potentials (Fig. 5b) as well as induced action potentials (Fig. 5c). Whole-cell current recordings under voltage clamp revealed both fast activating and inactivating sodium current responses as well as outward currents mediated by potassium channels (Fig. 5d). Finally, post synaptic currents could be recorded in these neurons, which are blocked by application of CNQX, a specific AMPA receptor blocker (Fig. 5e,f), suggesting they receive glutamatergic neuronal inputs from the excitatory neurons in the outer chamber.

Pathway-specific activation of neuronal inputs

One rationale for designing this strategy of culturing different neuronal subtypes in the microdevice is to have the ability to specifically manipulate individual subtypes. As a proof of principle, we expressed ChR2²¹ in the excitatory neurons before seeding them into the outer chambers of the microdevices (Fig. 6a,b). We then performed whole-cell recordings combined with optogenetic stimulation. Photostimulation of the presynaptic neuronal inputs expressing ChR2 to the neurons located in the central chamber can reliably evoke synaptic responses (Fig. 6c) as well as short-term synaptic plasticity induced by a train of photostimulation (Fig. 6d). Interestingly, sometimes the train stimulation can induce some asynchronous synaptic activity between and shortly after the train stimulations (Fig. 6e). These evoked responses were abolished by the addition of CNQX, indicating excitatory synaptic transmission in a network (Fig. 6f). This experiment demonstrates that the device promotes formation of an active circuit of iNs, and that we can accurately measure physiological properties of trans-synaptic signaling and synaptic plasticity.

Discussion

To harness the power of patient-specific neuronal cells for studying neuropsychiatric disorders, we created a simple and scalable system that can mimic the intricately connected neuronal circuitry in the brain. In this study, we have developed a five-compartment μ Neurocircuit model, where a population of neurons analogous to the mid-brain region receives input from at least two different populations. The system allows the use of functional assays such as synaptic physiology combined with optogenetic activation of a specific neuronal pathway. Our results validate this device as a unique tool for dissecting the complex mechanisms underlying neuropsychiatric disorders. Through morphological as well as functional analysis, we have the capacity to investigate various functional/morphological disease phenotypes found in psychiatric disorders (e.g. altered dendritic branching, synapse number and altered synaptic potentiation), while accounting for the role of upstream neurons in circuit with their synaptic targets.

Compartmentalized cultures as models for neurocircuitry

In neuropsychiatric disorder research, animal models have provided much insight into affected pathways; however there are certain aspects of human specific pathophysiology that cannot be recapitulated by animal models^{4,5}. Recent developments in stem cell biology enable the study of the pathophysiology of neuropsychiatric disorders using human neurons; however, the human neurons are often cultured in a 2D, single-chamber configuration.

Synaptic connections between neurons in these cultures are apparently random. In pursuit of *in vitro* circuit-level models, researchers have taken advantage of soft lithography techniques to generate microdevices for synaptic communication. Previous studies have successfully demonstrated compartmentalized primary neuronal cultures for a variety of applications^{22,23}. They have also demonstrated the use of calcium imaging and optogenetics as tools for investigating communication between chambers²¹. Here, we have extended this work by seeding human neuronal cells into a custom-designed compartmentalized device which can mimic one group of neurons receiving 2–4 different types of inputs. Different subtypes of human iNs survive in co-culture conditions within the device through 4 weeks and are primarily maintained within their respective compartments, while communicating to the central chamber via microchannels. The neurons display the morphology of mature neurons by expressing both pan-neuronal markers and subtype-specific markers, and demonstrate single-cell functionality and functional connectivity between chambers. Furthermore, near the walls separating side chambers, axonal projections extended through the microchannels toward the same population of central chamber neurons, suggesting the formation of a three-way circuit. This is significant for circuit modeling as many brain nuclei receive inputs from different regions of the brain.

Electrophysiological functional analysis has high-time resolution and thus is important to reveal the pathology associated with synaptic dysfunction. Ability to investigate these changes offers an additional level of information over most current models³⁴. By using an optogenetic approach, we can record from one neuronal subtype after stimulating another subtype (in this case, an excitatory to inhibitory circuit connection). We have therefore created a subtype-specific functional neurocircuit using human-induced neurons.

There are some limitations with the current approach. We initially planned to use chemical compounds to activate or inhibit neurons cultured in different compartments, which requires a slow diffusion rate between the outer and the central chamber; however the small molecule diffusion time scale determined using fluorescein predicts a 45 second period before a diffusing molecule from one chamber reaches another (Supplementary Fig. 2). This limits the utility of studying specific molecules within the device. However, even though the diffusion window is short, electrophysiological recordings can still be obtained before the window closes, enabling the completion of an experiment within the short time frame of isolated small molecule activity prior to diffusion into adjacent chambers. We may also be able to alter the height of the media in the central chamber to provide a counter force to diffusion as described in Taylor *et al.*²⁵ Nevertheless, small molecules that react with receptors specific to one neuronal subtype may still be investigated effectively. We may be able to lengthen diffusion time by tagging a molecule with a dextran (thereby increasing the diffusion coefficient), or by doubling the length of the channels, which quadruples the time for diffusion according to the equation for diffusivity ($l = \sqrt{4Dt}$).

Furthermore, as an *in vitro* model of a brain circuit, our device cannot fully model the complexity of the *in vivo* physiology, due at least to several challenges: the axonal projections that make up the circuit are largely unmyelinated; the devices do not contain all types of non-neuronal cells (e.g. endothelial cells and microglia); and we are not able to model all possible connections of the pathway. We can choose, however, to highlight

different regions of a given brain circuit by seeding other subtypes into the central chamber. Lastly, in cases where the focus is on a three-way circuit, the regions of the device of greatest interest are the locations where all three neuron types are closely apposed. The current four-side chamber design may limit the number of central-chamber neurons available for patch clamp analysis for a three-way circuit. Additional side compartments (e.g. eight instead of four), with neurons plated in an alternating fashion will greatly increase the area of interaction between three distinct subtypes.

The establishment of a μ Neurocircuitry model using human neurons derived from patients with known genetic background provides the opportunity to ultimately pursue personalized studies of neuropsychiatric disorders, such as schizophrenia, drug addiction or Parkinson's. Genome wide association studies have identified numerous polymorphisms that render carriers at greater risk for such diseases^{35,36}. By using human neurons with known risk alleles of a specific disease, we can study the role of a selected allele in mediating disease in a given neuronal subtype with its circuit connections.

Conclusions

In this paper, we have demonstrated the proof-of-principle use of soft lithography to generate a compartmentalized system for modeling human brain neurocircuits. Subtype specific human iNs are cultured within individual, interconnected chambers and recapitulate defined synaptic connections. This system not only can specify neuronal dysfunction at a circuitry level but has the capability to capture human-specific elements of disorders (e.g. single-nucleotide polymorphisms only present in human neurons). Using this system, we can monitor circuit-level changes unique to the patient's genetic background with a goal of drug screening for personalized medicine which operates at the neurocircuitry level. We expect that this approach will enable more accurate *in vitro* models of neuropsychiatric disorders and that it will facilitate translation of drug candidates into clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Diagram of μ Neurocircuitry device designed for interconnecting three different subtype neurons

(a) Diagram of a brain nucleus receiving two inputs. (b) Device schematic. Four outer chambers surround one large central chamber. Axons project through 10 μ m wide microchannels to communicate from the outer to central chamber. (c) Completed device bonded to glass cover slip. Colored dye indicates different chambers. (d) Fabrication protocol. Soft lithography was used to create a 3 μ m layer followed by a 320 μ m layer. PDMS is poured onto the master and cured. Holes are punched in the PDMS and the device is bonded to glass to yield the final device. (e) 3D representation of device with insets showing 3 μ m channel height. (f) Bright field image of microchannels and corresponding dimensions.

Figure 2. Human neuronal culture within the microdevice

(a) Induced neurons (iNs) cultured within a 200 µm walled device. (b) GFP-positive neurons cultured in a 500 µm walled device across from non-fluorescent iNs in the central chamber. (c–e) GFP-positive excitatory neurons exhibit extensive axonal projections to the central chamber and interact with TH-positive (red) DA-ergic neurons. Dashed area in (c) is showing in (d). Arrowhead showing a possible synaptic contact.

Figure 3. Morphological analysis

(a) Excitatory neurons express pan-neuronal marker MAP2 and vGlut2, an excitatory marker. (b) Dopaminergic (DA) neurons express MAP2 and synapsin, and tyrosine hydroxylase (TH), indicative of a DA phenotype. (c) GABAergic neurons express GAD6, indicative of an inhibitory phenotype. (d) Excitatory neurons infected with GFP express pan-neuronal marker β -III-tubulin (Tuj1). (e) DAergic neurons co-localizing with synapsin-positive boutons. (f) Interaction of inhibitory (green) and excitatory (red) neurons with synapsin-positive boutons.

Figure 4. Three-way circuit connectivity among neurons cultured in different compartments
(a) Two side chambers project axons into the central chamber. GFP-labeled induced neurons (iNs) were seeded on the right side and tdTomato-labeled iNs were seeded on the left. **(b–c)** Central chamber neurons interact with processes from both GFP and tdTomato-labeled side chamber iNs. These iNs stain positive for synapsin (blue) and MAP2 (white). **(d)** A MAP2-positive neuron receiving projections from both tdTomato-positive and GFP-positive axons. White arrowheads indicate synapsin-positive boutons.

Figure 5. Basal functional characterization of human neurons in the microdevice

(a) Microdevice under electrophysiology patch clamp recording setup. (b) Spontaneous action potentials were recorded showing functional maturation of iNs. (c) Step current experiment performed on GABAergic neurons. They fire repetitive action potentials upon depolarization. Step size is 5 pA. (d) Whole-cell currents were recorded in these cells showing a fast activation and inactivation sodium currents and outward potassium currents. Step size is 10 mV. (e) Spontaneous post-synaptic currents recorded from central chamber (GABAergic neurons). (f) Spontaneous post-synaptic currents (PSCs) recorded from central chamber could be blocked by 20 μM CNQX indicating glutamatergic transmission.

Figure 6. Optogenetics-aided synaptic functional analysis for outer-to-center neurocircuitry (a) Device wall with microchannels. ChR2-tdTomato (red)-infected excitatory neurons project to β -III-tubulin-labeled inhibitory neurons (green). (b) Extensive excitatory projections inside the central chamber co-localize with MAP2-labeled neurons (green). Nuclei labeled with Hoechst (blue). Scale bar 50 μ m. (c) Response to single pulse of blue light (470 nm). (d) Response to pulse train stimulation. (e) Post-synaptic currents (PSCs) recorded from central chamber neurons after excitation of terminals from projecting side chamber excitatory neurons with blue light. Arrow indicates asynchronous synaptic activity. (f) Addition of 20 μ M CNQX eliminates AMPAR-mediated response to light pulse. Red traces show a single trace for visualization.