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Sensing serotonin secreted from human serotonergic neurons using aptamer-modified nanopipettes

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Abstract

The serotonergic system in the human brain modulates several physiological processes and altered serotonergic neurotransmission has been implicated in the neuropathology of several psychiatric disorders. The study of serotonergic neurotransmission in psychiatry has long been restricted to animal models but advances in cell reprogramming technology have enabled the generation of serotonergic neurons from patient-induced pluripotent stem cells (iPSCs). While iPSC-derived human serotonergic neurons offer the possibility to study serotonin (5-HT) release and uptake, particularly by 5-HT-modulating drugs such as selective serotonin reuptake inhibitors (SSRIs), a major limitation is the inability to reliably quantify 5-HT secreted from neurons in vitro. Herein, we address this technical gap via a novel sensing technology that couples 5-HT-specific DNA aptamers into nanopores (glass nanopipettes) with orifices of ~10 nm to detect 5-HT in complex neuronal culture medium with higher selectivity, sensitivity, and stability than existing methods. The 5-HT aptamers undergo conformational rearrangement upon target capture and serve as gatekeepers of ionic flux through the nanopipette opening. We generated human serotonergic neurons in vitro and detected secreted 5-HT using aptamer-coated nanopipettes in a low nanomolar range, with the possibility of detecting significantly lower (picomolar) concentrations. Further, as a proof-of-concept, we treated human serotonergic neurons in vitro with the SSRI citalopram and detected a significant increase in extracellular 5-HT using the aptamer-modified nanopipettes. We demonstrate the utility of such methods for 5-HT detection, raising the possibility of fast quantification of neurotransmitters secreted from patient-derived live neuronal cells.

Introduction

Serotonin (5-HT) is a phylogenetically ancient molecule involved in biological systems that are central to mammalian physiology as well as to higher-order brain functions.¹ The serotonergic system has been implicated in the neuropathology of psychiatric disorders including major depressive and autism spectrum disorders, as well as schizophrenia.¹ Further, several psychotropic drugs including antidepressants and neuroleptics target serotonergic neurotransmission.² Interestingly, only a small fraction of the total 5-HT content in the body is found within the brain (<5%); most 5-HT is located peripherally, in the blood and gut.² Early experiments demonstrated that the 5-HT does not cross the blood brain barrier,³ and that the 5-HT found within the brain is generated exclusively by a small fraction of serotonergic neurons residing in the raphe nuclei of the hindbrain.⁴ Thus, while human serotonergic neurons are relevant systems to study, they have presented the combined challenge of low percentage and relative inaccessibility in the human brain.

The last decade has seen great advances in cellular reprogramming technologies that enabled the generation of induced pluripotent stem cells (iPSCs) from adult somatic cells.⁵⁻⁷ A challenge to harnessing the power of iPSC-technology is the optimization of protocols for generating each neural subtype of the brain.⁸ We and others have established methods to generate human serotonergic neurons from adults and demonstrated their utility for neurological disease modeling in vitro.⁹⁻¹¹ Collectively, these data indicate that human serotonergic neurons in vitro exhibit spontaneous electrical activity, express 5-HT transporter, and release 5-HT, and that these processes can be targeted pharmacologically.^{4,12}

The advantage of using iPSC-derived serotonergic neurons is the ability to investigate uniquely human cellular processes. Furthermore, with an iPSC-based model system, serotonergic neurons can be derived from patients with known genetics, family history, diagnoses, and response to treatment. Moreover, iPSC-derived cells enable the manipulation of patient-

derived cells genetically and pharmacologically *in vitro*, which is a substantial advantage for studying psychiatric disorders that are challenging to recapitulate with animal models alone.⁸ However, to utilize the full potential of the *in vitro* neural system to model disease and discover new drugs, it is necessary to have a method for reliably measuring 5-HT concentration that is both accurate and sensitive to small changes.

Conventional methodologies for 5-HT analysis in biological samples include separative (high performance liquid chromatography; HPLC coupled to mass spectrometry; MS),^{10,13–15} electrochemical (fast scan cyclic voltammetry; FSCV),^{16–19} antibody-based (enzyme-linked immunosorbent assay; ELISA),^{20–22} and optical (genetically modified 5-HT receptors)^{23–26} platforms. While each technique has advantages, the limitations of large sample volumes, extensive sample treatment, or complex and expensive instrumentation (e.g. HPLC-MS),²⁷ as well as issues of selectivity due to cross-reactivity (ELISA, FSCV, optical biosensors),^{18,28} have hindered progress in detecting 5-HT from human serotonergic neurons. The lack of an accurate and sensitive quantification of secreted 5-HT directly in the culture medium manifests as a major gap in the field. Overcoming this hurdle is key for studying patient-derived serotonergic neurons and how pharmacological agents modulate 5-HT levels.

To address these limitations, we have developed a novel technology for 5-HT detection *in vitro*, that couples quartz nanopipettes that are low-cost, fabricated in a high-throughput manner, and necessitates low volumes, with DNA aptamers that endow these sensors with the necessary specificity and selectivity in complex media.²⁹ Aptamers are short single-stranded oligonucleotides that have been designed to serve as artificial receptors for targets of interest.^{30,31} Recently, a 5-HT-specific aptamer that undergoes significant conformational rearrangement upon 5-HT recognition demonstrated high specificity and selectivity in brain tissue.³² When functionalized to the inside surface of quartz nanopipettes with ~10 nm orifices, DNA aptamer rearrangement upon 5-HT binding gates ionic flux through the pore opening resulting in measurable changes in current. Importantly, the nanoscale pore opening is resistant to

nonspecific binding to the sensor surface compared to conventional biosensors, which improves the sensor's lifetime. Further, the nanoconfinement of receptor-target binding events leads to high sensitivity (low picomolar capabilities) that exceeds existing methods by at least two to three orders of magnitude.²⁹

Herein, building on our previous work, we generated human serotonergic neurons from iPSCs and deployed novel DNA-aptamer-coated nanopipettes to detect 5-HT in culture medium.^{4,22,33,34} We validated the specificity, selectivity, and stability of 5-HT-specific aptamer-modified nanopipettes in complex matrices in the presence of chemically similar interferents. As a proof-of-concept, we treated human serotonergic neurons with the most commonly prescribed antidepressant, the selective serotonin reuptake inhibitor (SSRI) citalopram, and observed a significant two-fold increase in extracellular 5-HT. These results raise the possibility of detecting physiological differences in 5-HT flux from patient-derived serotonergic neurons and to correlate these differences with pharmacological agents that modulate these processes.

Results

Generating iPSC-derived human serotonergic neurons in vitro

To examine 5-HT concentration in complex culture medium, we generated serotonergic neurons from iPSCs of three healthy individuals (Lines 1-3). Using a previously described method, we neuralized iPSCs to generate serotonergic neural precursor cells (NPCs), which were then further differentiated to serotonergic neurons (Fig. 1a).^{10,11,22} To determine the serotonergic identity of differentiated serotonergic neurons, we confirmed expression of serotonergic markers. Initially, at the NPC stage, nestin-immunopositive serotonergic NPCs coexpressed Nkx2.2 (Fig. 1b). Following three-four weeks of differentiation, NPCs acquired typical neuronal morphology and MAP2ab-immunopositive neurons expressed tryptophan hydroxylase (TPH) as well as 5-HT (Fig. 1b).

Next, we observed that differentiated 5-HT neurons were also immunopositive for the 5-HT transporter (SERT). We observed typical SERT-immunopositive punctae colocalizing with counterstained neurites (Fig. 1b), highlighting that the derived serotonergic neurons express the transporter that is also the direct binding target of SSRIs. Quantification of the number of TPH+ and 5-HT+ neurons of all MAP2ab+ neurons showed a high efficiency of differentiation with >90% of differentiated neurons adopting a serotonergic fate (Fig. 1c). To examine the functionality of serotonergic neurons, we used ELISA to detect 5-HT secreted into the culture medium. Confirming previous findings, we detected 5-HT concentrations up to 25 nM, which is substantially lower than serum or plasma 5-HT levels that ELISA assays are optimized to process, and close to the lower bound of the detection limit with ELISA (Fig. 1d).^{10,11}

Sensing 5-HT in complex media using DNA-aptamer modified nanopipettes

To detect 5-HT secreted by serotonergic neurons, we employed a novel biosensor that combines the high selectivity of 5-HT-specific DNA aptamers as bioreceptors with solid-state conical glass nanopores (nanopipettes) that confine molecular recognition events within an orifice of ~10 nm for enhanced sensor sensitivity (Fig. 2a). The 5-HT aptamers covalently functionalized on the inside surface of the quartz nanopipette undergo a significant conformational change upon analyte recognition,³² which leads to alterations in the permeability of the aptamer layer near the opening. We previously validated the covalent functionalization of 5-HT aptamers to quartz surfaces, characterized aptamer-modified sensors, and investigated the sensing mechanisms.²⁹

Aptamer gating of ionic flux through the nanopipette is transduced as an increase in current response upon 5-HT recognition in complex cell culture media (Fig. 2b). To interrogate 5-HT aptamer sequence specificity, we conducted a negative control by combining nanopipettes with a scrambled DNA sequence, where the same number of nucleobases were conserved, but their order was scrambled to alter molecular recognition to the target. Scrambled sequence-

modified nanopipettes exposed to pure 5-HT showed negligible changes in current response, demonstrating the specificity of the 5-HT aptamer for robust target detection.

In addition to validating the 5-HT aptamer specificity in complex cell culture media that contains high micromolar concentrations of nonspecific molecules including *L*-tryptophan, a 5-HT precursor, we tested selectivity towards other similarly structured neurochemicals. We exposed the 5-HT aptamer-modified nanopipettes to another 5-HT precursor, *L*-5-hydroxytryptophan (*L*-5-HTP) and a 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Fig. 2c). The sensors were selective towards 5-HT with a significantly larger response compared to the nonspecific molecules. The selectivity ratios for 5-HT relative to *L*-5-HTP and 5-HIAA were 12:1 and 7:1 respectively. Traditional microelectrodes used for 5-HT detection via FSCV have selectivity ratios of 2.5:1 for 5-HT relative to 5-HIAA, the major 5-HT metabolite for electrode fouling.³⁵ While this high cross-reactivity of 5-HT:5-HIAA has been minimized via Nafion coatings³⁶ (17:1) or by altering the stiffness of carbon fibers (7:1),³⁵ 5-HT aptamer-modified nanopipettes require no further chemical or physical modifications due to the inherent selectivity of the aptamers.

Upon validating the specificity and selectivity of the 5-HT aptamer-modified nanopipettes, we interrogated the stability of these sensors in complex cell culture medium. The sensors remained stable with minimal change in current response in neural differentiation medium (NDM) for at least 4 h without clogging, indicative of minimal nonspecific binding (Fig. 2d). This stability in complex media that contains many interferent molecules is likely due to the highly negatively charged DNA aptamers occluding nonspecific molecules from entering the ~10 nm nanopore. To this end, we demonstrate that unfunctionalized quartz nanopipettes with the same nanopore size, clog within minutes in NDM.

Functional modulation of 5-HT levels by citalopram and KCl

Upon corroborating the specificity, selectivity, and stability of the 5-HT aptamer-functionalized nanopipettes, we deployed these sensors to quantify the 5-HT content in media collected from

iPSC-derived 5-HT neurons versus iPSC-derived astrocytes that served as a negative control, as they do not produce 5-HT (Fig. 3a). Further, to examine functional changes in extracellular 5-HT levels upon increased activity of serotonergic neurons, human serotonergic neurons were treated with potassium chloride (KCl), which causes membrane depolarization leading to an influx of calcium, resulting in synaptic secretion of 5-HT into the extracellular space (Fig. 3b).

For current-voltage measurements with serotonergic neurons treated with citalopram or KCl, NDM with equal concentration of the treatment chemical was used to obtain the initial baseline. Using the specific baseline that correlates with the experimental condition is critical as the nanopipettes are sensitive to changes in ionic content, *e.g.* addition of 30 mM KCl alters the ion current rectification of the nanopipette.²⁹ Upon obtaining a stable baseline, 5-HT aptamer-modified nanopipettes were immersed in a new bath containing NDM in which serotonergic neurons were cultured (Fig. 3c). We expected to detect an unknown 5-HT concentration in the nanomolar regime based on previous reports^{9,22} and ELISA results,¹¹ which was within the detection range of the 5-HT aptamer-modified nanopipettes.²⁹

Subsequently, samples with higher expected concentrations (*i.e.* post-KCl or citalopram treatment) were tested in the same manner and a measurable increase in current response was observed from the baseline (Figure 3c,d). Finally, sensors were immersed in NDM solutions with high concentration of pure 5-HT (1 μ M), orders of magnitude above the 5-HT aptamer binding affinity ($K_d \sim 30$ nM),³² to demonstrate concentration-dependent target detection and to validate that the 5-HT content in clinical samples were within the detection regime of the sensors. Media in which iPSC-derived astrocytes were cultured as a negative control, did not elicit a sensor response (Fig. S1). While negligible changes in current response were observed upon sensor exposure to the astrocyte media, the visible current increase upon addition of 1 μ M pure 5-HT, demonstrated the functionality of the tested sensors.

The method of standard addition was used to quantify unknown 5-HT concentrations in the cell culture medium to minimize matrix effects that interfere with measurement signals (Fig. S2).³⁷ While 5-HT sensors were unable to detect any 5-HT in serum-free astrocyte culture medium, measurements in samples where serotonergic neurons were cultured led to quantification of ~1 nM concentration of 5-HT (Fig. 3e). After KCl treatment, 5-HT levels increased three-fold to ~3 nM compared to the 5-HT concentration before treatment, demonstrating modulation of 5-HT levels by inducing neuronal activity.

Human serotonergic neurons take up synaptic 5-HT via the serotonin transporter and this process constitutes an important mechanism for maintaining extracellular 5-HT content. The SSRI and clinically approved antidepressant citalopram directly binds SERT, blocking 5-HT uptake in serotonergic neurons.^{2,38} We hypothesized that preventing 5-HT uptake via SERT would lead to increased 5-HT levels in the culture medium. Therefore, we next examined whether treatment with citalopram altered extracellular 5-HT levels. Serotonergic neurons were treated with 5 μ M citalopram and culture medium was collected at 2 h and 24 h after treatment (Fig. 3f). Citalopram treatment significantly increased the amount of secreted 5-HT (~2 nM), demonstrating the ability to detect physiological changes in 5-HT concentration upon drug treatment.

Interestingly, we did not observe a significant difference between the 2 h and 24 h citalopram treatment groups (Fig. 3f). This finding suggests that 5-HT does not detectably accumulate over 24 h potentially due to steady-state degradation of 5-HT in the culture medium and/or increased feedback inhibition of 5-HT release from serotonergic neurons. Taken together, the 5-HT aptamer-modified nanopipettes differentiated physiological differences in 5-HT concentrations in real samples, with statistical relevance between serotonergic neurons that were untreated versus treated with citalopram or with KCl.

Discussion

We have developed novel technology for detecting and quantifying 5-HT with higher selectivity and sensitivity than previously possible. By virtue of size exclusion of nonspecific proteins in complex culture medium, nanopores are ideal for detecting small molecules including neurotransmitters. The combination of nanopipettes with conformation-changing DNA aptamers confers high specificity and selectivity for 5-HT detection.²⁹ Limitations of this approach include the challenge of multiplexing, as detection of target analytes relies on the specific conformational rearrangement of the aptamer inside the nanoscale orifice and thus detection of different molecules simultaneously with one biosensor is not feasible.

Further, the mechanism relies on aptamers undergoing significant conformational change,²⁹ which necessitates the isolation of sequences that adopt an aptamer target-bound structure that substantially differs from the free aptamer. Thus, while this methodology is generalizable for small-molecule sensing, target-induced aptamer conformational rearrangements must be validated by complementary techniques based on fluorescence (e.g. Förster resonance energy transfer) or other principles (e.g. circular dichroism or surface-enhanced Raman spectroscopy).^{32,39-41} However, there are strategies to incorporate the structure-switching functionality directly into the aptamer selection process for optimized design of novel sequences for specific targets of interest; these methodologies hold great promise for targeting diverse neurochemicals.²⁹⁻³³

Using chemically modified nanopipettes, we detected 1-3 nM 5-HT in the culture medium, which is within a comparable range to that from ELISA measurements (0-25 nM) but at lower concentrations. Slightly higher quantities of 5-HT detected via ELISA could appear due to antibody cross-reactivity resulting in binding to other molecules present in the complex sample that often results in false positives.²⁸ Nanopipette biosensors have recently been shown to have the potential to detect analytes over lower concentration ranges compared to equilibrium sensors

with conventional receptor-ligand interactions (e.g. antibody-based detection in ELISA), which are typically limited to detection within an order of magnitude in either direction of the recognition element dissociation constant (K_D).²⁹ This phenomenon may be due to the capacity of nanopipettes to detect a small number of 5-HT molecules interacting with aptamers confined within the 10 nm orifice^{47–49} or due to the macromolecular crowding effect in complex media that has been previously reported to increase detection sensitivity significantly.^{50,51}

What is more, the sensitivity of these 5-HT aptamer-modified nanopipettes that can detect concentrations of 5-HT in the low picomolar regime does not come at the cost of chemical selectivity. While other voltammetric techniques such as FSCV have existed for over five decades and have seen progress to advance rapid, sub-second neurotransmitter monitoring,⁵² to date, the sensing surface (i.e. microelectrodes) still suffers from a high degree of fouling due to protein adsorption.⁵³ In particular, 5-HT and its metabolite, 5-HIAA produce highly reactive radicals during oxidation that polymerize to form films on the electrode surfaces, which decreases the sensitivity and limits the accuracy of these measurements.^{54,55}

To address the issue of fouling for better sensitivity, various waveforms have been implemented for 5-HT detection.^{18,56} However, often an improvement in the detection limit (low nanomolar range) comes at the cost of reduced chemical selectivity, due to overlapping reduction potentials of the analytes or matrix components.¹⁸ While the whole sensing surface (tens of micrometers) is exposed for electron transfer reactions in FSCV, the sensing area for aptamer-modified nanopipettes is confined inside of the ~10 nm orifice, which significantly reduces nonspecific binding of larger proteins and improves sensor lifetime in complex media. Further, repulsive forces between the highly negatively charged aptamers and other negatively charged molecules that compose the neural culture matrix may further reduce nonspecific binding at the nanopipette tip.⁵⁷

While many approaches are hindered by high salt content, which is inherently present in biological samples, the sensing mechanism of chemically modified nanopipettes exploits high

ionic flux to maximize the signal change upon aptamers gating the current through the nanopore upon target recognition.⁵⁸⁻⁶¹ Techniques such as HPLC-MS, while capable of detecting 5-HT at high picomolar concentrations when thoroughly optimized, suffer from contamination and interference from sample matrices that lower the ionization efficiency of the analyte and leads to incomplete separation of the compounds of interest.⁶² In general, separation-based techniques followed by quantification via MS or electrochemical detectors require time-consuming sample pre-treatment and refinement of instrument parameters, rendering the technology incompatible with in situ measurements.⁶³

Alternatively, aptamer nanopipette-based measurements are suitable to track in situ neurochemical flux in the complex cell media. These properties make 5-HT aptamer-modified nanopipettes an ideal method for examining secreted 5-HT from iPSC-derived serotonergic neurons from multiple individuals, including psychiatric patients and for further study with serotonergic drugs that induce 5-HT depletion. Though not investigated here, this novel technology could likely be applied to detect 5-HT in other systems including cultured brain slices, enterochromaffin cells, or human platelets to study other aspects of 5-HT dynamics. Moreover, the generalizability of the reported technology will facilitate the development of this sensing strategy for quantification of diverse small molecules and neurotransmitters secreted by human neurons in vitro, serving as model systems to elucidate how pharmacological agents modulate neurochemical flux.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) unless otherwise noted. For DNA-modified nanopipette measurements, undiluted phosphate buffered saline at pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, ThermoFisher

Scientific AG, Reinach, Switzerland) was used as received. All DNA sequences were purchased from Microsynth AG (Balgach, Switzerland). The single-stranded 5-HT aptamer is thiolated on the 5' end with sequence: (5'/Thiol/-CGA CTG GTA GGC AGA TAG GGG AAG CTG ATT CGA TGC GTG GGT CG-3'). The 5-HT aptamer molecular weight is 13,969.8 g/mol with a melting point of 74.0 °C. The scrambled sequence with the same number and ratio of nucleotides as the 5-HT aptamer but with the order jumbled and modeled via Mfold⁶⁴ to confirm a significant difference in the secondary structure versus the 5-HT aptamer has the following thiolated sequence: (5'/Thiol/-CCC GGG AAT TCC GGA ATT GGG GCA AATT GAT GAG GGG GTC ATG GG-3'). The scrambled sequence has a molecular weight of 13,969.8 g/mol with a melting point of 71.4 °C. All DNA solutions (stock concentration 100 µM) were HPLC purified, aliquoted, and stored at -20 °C until use.

DNA-modified nanopipette fabrication and functionalization

Nanopipettes were fabricated from quartz capillaries with outer diameters of 1 mm and inner diameters of 0.5 mm purchased from Friedrich & Dimmock Inc. (NJ, U.S.A.) using a P-2000 laser-based pipette puller from Sutter Instrument (CA, U.S.A.). The pulling parameters for nanopipettes with apertures of ~10 nm were: (line 1) Heat 750, Filament 4, Velocity 40, Delay 150, Pull 80; (line 2) Heat 700, Filament 3, Velocity 60, Delay 135, Pull 180. The DNA sequences (5-HT aptamer or scrambled sequence) were immobilized on the inside of the nanopipette using a previously validated three-step surface chemistry protocol.²⁹

First, an (3-aminopropyl)trimethoxysilane (APTMS) monolayer was vapor-deposited at 40 °C for 1 h on the quartz nanopipette surfaces. Second, MicroFil syringe tips purchased from World Precision Instruments (FL, U.S.A.) were used to incubate APTMS-functionalized nanopipettes with 1 mM 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) dissolved in a 1:9 (v/v) mixture of dimethyl sulfoxide and PBS for 1 h. The MBS couples the amine-terminated silanes to

thiolated DNA. In the final step, thiolated DNA sequences were prepared for functionalization by reducing the disulfide bonds by co-incubation with 50-fold excess tris(2-carboxyethyl) phosphine (TCEP) relative to DNA concentration (100 μ M) for 1 h. The DNA solutions were then diluted to 5 μ M in PBS and desalted to remove unreacted TCEP, which has been shown to be reactive towards maleimides,⁶⁵ using 7K MWCO Zeba™ spin desalting columns from ThermoFisher Scientific AG (Reinach, Switzerland). Prior to surface attachment, DNA sequences were denatured at 95 °C for 5 min and then renatured by cooling to room temperature. Nanopipettes were rinsed twice with PBS using MicroFil tips and then incubated in the DNA solution for 2 h. Any remaining aptamer solution was rinsed out by PBS three times prior to experimental use.

Sensing 5-HT via aptamer-modified nanopipettes

The electrical current was measured with a custom-built high gain current amplifier between two Ag/AgCl quasi-reference counter electrodes, one inside the nanopipette and the other in the bulk solution (Fig. S2). Data recording was performed using a 2017 LabVIEW interface and data were collected using an FPGA card PCIe-7852R from National Instruments (TX, U.S.A.) based on WEC-SPM package provided by the Warwick Electrochemistry and Interfaces Group. The current responses and potentials reported in the manuscript are denoted with respect to the grounded electrode in the solution bulk. Cyclic voltammograms were acquired by sweeping voltage from -0.5 V to +0.5 V with a 0.05 V s⁻¹ scan rate.

iPSC-derived serotonergic neuron cultures

Methods for generating iPSCs from human fibroblasts and deriving serotonergic neurons and astrocytes have been previously described.^{22,34} Briefly, adult neurotypical donor-derived iPSCs ($N=3$ individuals) were used to form embryoid bodies that were patterned with hindbrain- and raphe-specifying growth factors and morphogens in the culture medium for three weeks to generate serotonergic neural progenitor cells (NPCs). Serotonergic NPCs were seeded and

further differentiated in neural differentiation medium (NDM) for four weeks before use in experiments. The NDM contains basal components neurobasal medium (Thermo Fisher Scientific; Waltham, U.S.A.), N2- and B27-supplements (Life Technologies; Carlsbad, U.S.A.), BDNF (10 ng/mL; Peprtech, Rocky Hill, U.S.A.), GDNF (10ng/mL; Peprtech, Rocky Hill, U.S.A.), ascorbic acid (200 nM; Stemcell Technologies, Vancouver, CA), cAMP (250 µg/mL; Tocris Bioscience, Bristol, U.K.), and laminin (1 µg/mL; Peprtech, Rocky Hill, U.S.A.). As a negative control for cells that do not secrete 5-HT, astrocytes, were derived from the same three iPSC lines as previously described.³⁴ For this study, astrocytes were differentiated from glial precursor cells in a defined medium of DMEM F12 + GlutaMAX with 10% Knockout Serum Replacement (Thermo Fisher Scientific; Waltham, U.S.A.), N2-supplement (Life Technologies; Carlsbad, U.S.A.), CNTF (20 ng/mL; Proteintech, Rosemont, U.S.A.), BMP4 (20 ng/mL; Peprtech, Rocky Hill, U.S.A.), FGF2 (20 ng/mL; HumanZyme, Chicago, IL), and EGF (20 ng/mL; HumanZyme, Chicago, IL).

Human serotonergic neuron media preparation

For measuring 5-HT concentration in the culture media of human serotonergic neurons and the negative control astrocytes, media were collected 24 h after a media change. Serotonergic neurons were treated with media containing either 5 µM citalopram (Sigma-Aldrich; St Louis, U.S.A.) for 2 h and 24 h or 30 mM KCl (Sigma-Aldrich; St Louis, U.S.A.) for 30 min prior to media collection. Human serotonergic neuron media were shipped from the Salk Institute for Biological Studies (San Diego, U.S.A.) to ETH Zurich (Zurich, Switzerland) on ice and were stored immediately at -80 °C. To conduct measurements, samples were thawed and kept on ice during the duration of the experiment and freeze-thaw cycles were limited to twice per sample to avoid 5-HT degradation. For accurate calculations of 5-HT concentrations, respective data were normalized to either base media (NDM), media + 5 µM citalopram, or media + 30 mM KCl that had no contact with cells.

Immunocytochemistry and Imaging

Immunocytochemistry was performed as previously described.^{11,22} Briefly, cells were fixed with 4% paraformaldehyde (EMS Chemie; Domat/Ems, Switzerland) for 15 min at 4 °C followed by antigen-blocking for 30 min at 4°C in blocking solution (0.5% Triton-X100 and 5% Normal Horse Serum in Tris-buffered saline; Sigma-Aldrich, St Louis, U.S.A), followed by overnight incubation with primary antibodies at 4 °C. Cells were washed and incubated with secondary antibodies for 1 h at room temperature. To visualize nuclei, DAPI (1 µg/mL, 10 min) was used. Primary antibodies used were Nestin (Mouse/Rabbit 1:500; EMD Millipore, Burlington, U.S.A.); Nkx2.2 (Mouse 1:100; Hybridoma Bank, Iowa City, U.S.A.); 5-HT (Rabbit 1:1000; Protos Biotech, New York, U.S.A.); TPH (Sheep 1:500; Millipore, Burlington, U.S.A.); MAP2ab (Chicken 1:1000; Abcam, Cambridge, U.K.); SERT (Rabbit 1:1000; Abcam, Cambridge, U.K.). Brightfield and fluorescent images were taken as previously described.¹¹ Briefly, images were taken in phase contrast during different states of differentiation using the Olympus IX51 inverted microscope at 10x/20x magnification; fluorescent images were taken using the Zeiss LSM 880 laser scanning confocal microscope at 20x magnification.

5-HT ELISA

Culture medium from wells of six- to eight-week-old serotonergic neurons was collected and frozen with dry ice and stored at -20°C. The concentration of 5-HT was measured using a competitive ELISA kit (Enzo Life Sciences; Farmingdale, U.S.A.) according to the manufacturer's instructions, as previously described.¹¹ Briefly, 5-HT standards were serially diluted and the percentage of displaced 5-HT was measured. The percentage of still bound 5-HT was inversely proportional to the concentration of 5-HT in the samples. A standard curve and linear regression analysis were used to determine the concentration of 5-HT within our samples.

Statistics

Statistical analyses were carried out using GraphPad Prism from GraphPad Software Inc. (CA, U.S.A.). Standard deviations were calculated from means for data from cells derived from three individuals. Data are reported as means \pm standard errors of the means (SEM) with probabilities $P < 0.05$ considered statistically significant. Comparative data were evaluated by one-way analysis of variance followed by Tukey's multiple group comparisons.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure. 1

(a) Bright field representative microscopy images of serotonergic neuron derivation from human induced pluripotent stem cells (iPSCs). Three individual lines of iPSCs were neuralized into neural progenitor cells (NPCs) that were differentiated for three to four weeks with neural differentiation media (NDM) for all experiments. Scale bar 200 μm . (b) Representative confocal microscopy images of serotonergic neuron markers detected via immunofluorescence. (Top panel) Serotonergic NPCs are immunopositive for Nestin (red) and Nkx2.2 (green) (scale bar 25 μm), while differentiated serotonergic neurons (bottom panel) are immunopositive for serotonin (5-HT, green), tryptophan hydroxylase (TPH, red) (scale bar 25 μm ; arrow heads show double positive cells); and (b, right panel) MAP2ab (red) and serotonin transporter (SERT, green) immunopositive (scale bar 5 μm). (c) Quantification of serotonergic differentiation efficiency for each line showing percentage of cells immunopositive for TPH or 5-HT out of MAP2ab positive neurons. Bars show means \pm SEM. (d) Scatter-plot showing quantification of 5-HT concentration using inverse competitive ELISA. Black dots are known concentrations on a standard curve; green dots are 5-HT concentrations from serotonergic neuron media samples measured at the lower end of the detection range (0-25 nM of calculated 5-HT).

Figure. 2

(a) Schematic of serotonin (5-HT) aptamer-modified quartz nanopores for sensing secreted 5-HT from neuronal cultures. Aptamer structural rearrangement upon 5-HT recognition alters the nanopore permeability to ions and leads to an increase in measured current. Nanoscale confinement of molecular recognition elements within a pore size of \sim 10 nm enables highly sensitive, selective, and specific sensing of 5-HT molecules while minimizing interactions with nonspecific interferents. (b) Representative voltammograms of 5-HT aptamer-modified

nanopipettes tested in complex neural differentiation medium (NDM) ($N = 3$). Exposure to 5-HT (100 μM) resulted in a measurable increase at +0.5 V. Sequence specificity is demonstrated by testing scrambled sequence-modified nanopipettes as a negative control, which shows negligible change upon addition of the same concentration of 5-HT ($N = 2$). (c) To validate selectivity of 5-HT aptamer-modified nanopipettes to similarly structured molecules, sensors were exposed to *L*-5-hydroxytryptophan (*L*-5-HTP) and 5-hydroxyindoleacetic acid (5-HIAA) in 100 μM concentrations in phosphate buffered saline. Negligible responses were observed for nonspecific molecules at +0.5 V compared to the specific target, 5-HT [$F(2,12)=175.6$, $***P<0.001$]. Error bars are standard errors of the means for $N = 3$ nanopipette sensors. (d) The 5-HT aptamer-functionalized nanopipettes exhibit high stability in NDM for up to 4 h, indicative of minimal non-specific binding. In comparison, for bare unfunctionalized quartz nanopipettes, clogging is observed with a significant reduction in the current response within 10 min.

Figure. 3

(a) Schematic of cell culture protocol: media collected from serotonin (5-HT) neurons and astrocytes derived from induced pluripotent stem cells (iPSCs). (b) Schematic of treatment paradigm: 5-HT neurons incubated with either control neural differentiation media (NDM) for 24 h, 5 μM citalopram for 2 h, or 30 mM KCl for 30 min before media collection and subsequent 5-HT detection with aptamer-modified nanopipettes. (c) Representative current-voltage curves demonstrating the increase in current from the stabilized baseline in NDM when testing media in which untreated serotonergic neurons were cultured. The NDM containing serotonergic neurons treated with KCl (30 min) further increased the current response. A high concentration of 5-HT (1 μM) was added to show maximal sensor response (d) Similar trends were observed for serotonergic neurons treated with citalopram (2 h) with a measurable increase in current

response. The current magnitude for KCl-treated samples were higher due to the increased ionic content (+ 30 mM of KCl) compared to the citalopram samples. The same concentration of KCl was added to the NDM baseline and untreated serotonergic neuron samples to obtain the same ionic concentration for comparison. (e) The astrocyte culture media did not elicit a response (*ND* = not detectable) from the 5-HT aptamer-modified nanopipettes whereas ~1 nM 5-HT was detected in the 5-HT neuron culture media. Treatment of the culture with KCl, induced a three-fold increase in 5-HT content ($***P < 0.001$). Group means were significantly different [$F(2,6) = 75.96$; $P < 0.001$]. (f) Treatment of the 5-HT neuronal culture with citalopram generated a significant increase in 5-HT ($**P < 0.01$) while treatment duration of 2 versus 24 h did not result in significant changes (*ns* = not significant; $P > 0.05$). Measurements from untreated 5-HT neurons are the same as in Fig. 3e, shown for statistical comparisons. Group means were significantly different [$F(2,6) = 19.27$; $P < 0.01$]. Error bars are standard errors of the means for $N = 6$ nanopipette sensors.