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Subcellular Probes for Neurochemical Recording from Multiple Brain Sites

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Abstract

Dysregulation of neurochemicals, in particular, dopamine, is epitomized in numerous debilitating disorders that impair normal movement and mood aspects of our everyday behavior.

Neurochemical transmission is a neuron-specific process, and further exhibits region-specific signaling in the brain. Tools are needed to monitor the heterogeneous spatiotemporal dynamics of dopamine neurotransmission without compromising the physiological processes of the neuronal environment. We developed neurochemical probes that are ten times smaller than any existing dopamine sensor, based on the size of the entire implanted shaft and its sensing tip. The microfabricated probe occupies a spatial footprint (9 μm) coordinate with the average size of individual neuronal cells (~ 10 μm). These cellular-scale probes were shown to reduce inflammatory response of the implanted brain tissue environment. The probes are further configured in the form of a microarray to permit electrochemical sampling of dopamine and other neurotransmitters at unprecedented spatial densities and distributions. Dopamine recording was performed concurrently from up to 16 sites in the striatum of rats, revealing a remarkable spatiotemporal contrast in dopamine transmission as well as site-specific pharmacological modulation. Collectively, the reported platform endeavors to enable high density mapping of the

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Author contributions

H.N.S., M.K., and D.H. initiated the project. H.S., D.H., M.J.C., and A.M.G. guided the studies. H.N.S. and M.K. designed the experiments and wrote the paper with comments from all other authors. H.N.S., M.K., E.K., and H.Y. performed rat surgeries and experiments. H.N.S. developed instrumentation, software, and probes, and analyzed data. S.A. performed perfusion surgeries. S.A. and T.Y. performed histology and immunohistochemical analyses. H.N.S. and E.K. built instrumentation. M.J.C., A.M.G., and R.L. supervised all aspects of this work.

Competing financial interests

The authors declare no competing financial interests.

chemical messengers fundamentally involved in neuronal communication through the use of minimally invasive probes that help preserve neuronal viability of the implant environment.

Graphical Abstract

Cellular-scale ($< 10\ \mu\text{m}$) probes developed for minimally invasive, subsecond, and multi-site monitoring of neurochemicals in rat brain.

Keywords

striatum; voltammetry; implantable sensors; microelectrodes; dopamine

Introduction

The brain is made up of billions of neurons that chemically communicate with each other through various neurotransmitters. Dopamine is a type of neurotransmitter involved in motivation, learning, and movement. Its dysregulation and deficiency is implicated in a number of pathological conditions, including, Parkinson's disease, other movement-related diseases, and mood disorders^{1,2}. Dopamine signals display extensive variability across the striatum and other sites of the basal ganglia, having widespread connections to many other brain regions^{3–7}. When released through exocytosis, neurotransmitters flow directly across the synapse of two neurons and/or spillover to the extrasynaptic space to influence surrounding neuronal activity. The neurons carrying these chemicals are usually tens of microns in size, while the space in which neurotransmitters flow between the cells is at least a hundred times smaller—tens of nanometers⁸. Thus, sensors introduced into the brain should occupy minimal volumes to precisely probe the highly localized changes in neurotransmitter concentrations without perturbing the delicate surrounding cellular environment⁹. Furthermore, smaller sizes help reduce brain damage, which will help increase opportunities to safely employ these sensors in therapeutic applications, for example, in improving deep brain stimulation treatment in Parkinson's disease patients. Integrating chemical sensors may help normalize the remedial effects of the stimulator if the stimulation schedules can be tuned adaptively to the level of dysregulated neurochemical activity, as monitored by the sensors, in these patients¹⁰. In addition to miniaturization, the sensors should be widely distributed in order to examine dopamine's neuron-specific and heterogeneous spatiotemporal dynamics. Despite the enormous spatial scales attained with electrophysiological monitoring of neuronal electrical activity^{11–14}, similar progress has not been seen with recording of chemical activity. Neurochemical transmission largely governs

the electrical output of these neurons and for that reason should prescribe a major component of mapping brain activity and informing functional connectivity.

Of all *in vivo* methods used to probe dynamically these neurotransmitters, fast scan cyclic voltammetry (FSCV) provides the optimal performance in terms of aggregate temporal resolution (100 ms or less), sensitivity, and selectivity^{9,15}. The sensors for measuring dopamine are usually in the form of a carbon fiber (CF) microelectrode (CFM) comprising a 7 μm diameter CF protruding out of a fused silica or glass shaft, in which it is threaded for insulation and structural support. CF has been shown to provide an optimal electrochemical sensing interface for measuring dopamine due to its biocompatibility, highly adsorptive surface, electrocatalytic properties, and small size^{9,16}. CFM sensors have realized prevalent implementation in analyzing the mechanisms of dopamine signaling and its function in our everyday behavior and in pathology^{10,17–19}. Considerable studies have further attempted to investigate the spatial diversity of dopamine neurotransmission in the striatum using the CFM sensor^{3,4,17,20,21}. These single channel investigations, however, are limited by the inability to reproduce precisely dopamine measurements at specific brain sites, as they require serial repositioning of the sensor and/or impractical numbers of specimens to assess dopamine heterogeneity. Very few have employed concurrent recording from more than a single channel: 2 channel monitoring from two individually-implanted CFM sensors to assess cross-hemispheric dopamine projections²², or 4 channel monitoring with a microfabricated axial array¹⁵ or threaded multi-barrel glass-pulled CFM electrodes²³.

Size reduction of the implanted probe, especially around the CF sensing interface, is critical to ensure measurement accuracy²⁴. The conventional CFM shaft, which makes up the bulk of the implanted device has a nominal diameter of around 100 μm (**ESI**[†], Fig. S1), is large enough to compromise healthy vasculature, mechanically compress adjacent neurons, and activate a cohort of inflammatory processes in the surrounding brain environment^{13,25–27}. In addition to perturbing brain circuits along the implant path, this multifarious tissue response can deter measurement accuracy made at the CF neurochemical interface²⁴. For these larger ($\sim 100\ \mu\text{m}$) implant profiles, activated glia and neuronal cell death can extend up to 150 μm or further from the implant interface^{26,28–30}. Even probes of subcellular geometry (8–10 μm), such as those presented here, are capable of producing astrocytic response, which has been shown to correlate to their proximity to surrounding vasculature¹³. Thus, the (7 μm) CF neurochemical sensing interface, is also susceptible to tissue response, which may be heightened relative to the size of the adjoining shaft. As the estimated distance between striatal vasculature is around 60–70 μm ²⁵, reducing the size of the implant can improve the probability of avoiding these susceptible microstructures. Smaller footprints could further enhance the reproducibility of ascertaining viable recording volumes in the brain during the implantation procedure.

Here, we report the development and use of subcellular (< 10 μm) sensors for measuring changes in dopamine from multiple sites in the brain (Fig. 1). Key advancements are reducing implant footprint (one order of magnitude smaller than state of art¹⁵ and conventional CFM) (**ESI**[†], Fig. S1) and stiffness, ability to record concurrently from 16

[†]Additional methods are provided in Electronic Supplementary Information ESI.

channels, and high manufacturing reproducibility and yield. Our ability to construct these small and flexible sensors was leveraged by the inherent nature of the two materials that form their backbone: (1) the 7 μm CF electrochemical conduit and (2) the conformal parylene-C insulation. Cellular-scale devices have previously been constructed for electrical recording of cortical unit activity^{13,14}, but these present more straightforward construction—the insulated CF is bluntly cut to expose a radial surface area ($\sim 40 \mu\text{m}^2$) whereby extracellular potentials are conducted. FSCV requires a larger surface area ($> 500 \mu\text{m}^2$) for sampling nanomolar dopamine concentrations³¹. Simple lift-off or etching processes were used to bare a discrete length of CF from its parylene encapsulation for a larger electrochemical sensing surface area. As the bared CF sensing elements are of the same geometries and materials to those used in conventional CFM sensors, the sensitivities attained are equivalent to the well-established sensors^{3,4,10,17–21}. Batch fabrication methods enable high-yield (8 probes per array and 10 arrays per batch) and reproducible construction of arrays with well-defined pitch (250 μm), lengths (up to 6 mm), and exposed CF sensor length (50–200 μm). However, the resulting, smaller, softer probes cannot penetrate the brain unaided. Therefore, the probes are temporarily stiffened by biocompatible polyethylene glycol (PEG), which dissolves readily during the insertion process and allows the small probes to be lowered to their deep brain targets¹⁴ with limited brain damage. The resulting arrays permit multiple channels to be recorded concurrently, and therefore we pursued the design of 16 channel FSCV instrumentation to examine synchronous dopamine measurement from numerous distributed sensors in the brain. Probes were implanted in the striatum of anesthetized rats to demonstrate function in multichannel dopamine recording as well as make preliminary assessments of their induced inflammatory response.

Materials and methods

Device design and fabrication

Neurochemical probe arrays are fabricated as follows (Fig. 2). (1) A printed circuit board (PCB) with conductive traces interfacing a connector socket (Mill-Max, 853-93-100-10-001000) is aligned to a micromold: a glass substrate with trenches (250 μm pitch) (P-M Optics, PGVG-2082205). The glass micromold is used to temporarily align and hold individual CF's (7 μm diameter and ~ 10 mm long) (Goodfellow, C 005722). Silver epoxy (Epo-tek, H20S) is applied to the individual PCB traces to structurally and conductively connect each CF to the PCB. Ten such arrays can be aligned on a standard 100 mm glass wafer (University Wafer, 1631). The epoxy is cured by placing the wafer on a hot plate for 2 hours at 120°C. (2) The PCB-CF assembly is extended 5–6 mm so that only the CF sensing tips (50–200 μm long) are anchored to the glass substrate, which are then masked by applying a small amount of photoresist (PR) (AZ Electronics, AZ P4330). PR is then cured by placing the wafer on a hot plate at 120°C for 15 min. The wafer is immersed in isopropanol for 15–30 min, followed by an adhesion promoter consisting of A174 (Sigma-Aldrich, 440159), isopropanol, and distilled water at a volumetric ratio of 1:100:100 for 15–30 min. The wafer is air dried for 30 min and then soaked again in isopropanol for 5 min, followed by air drying for > 30 min. Parylene-C (0.5–1.5 μm) is uniformly deposited (Specialty Coating Systems, PDS 2010 Labcoater). (3) The release process to expose CF sensing tips is preceded by gently scoring the surface of the parylene atop photoresist layers

to allow diffusion of subsequent acetone. CF sensing tips are exposed by exposing and dissolving PR masks in acetone (5–15 min dissolution time depending on amount applied), followed by rinsing in isopropanol. As the array assembly is pulled away from the micromolds after the PR dissolved, residual parylene (few microns in length) may be observed hanging to the CF tips. Because of the highly conformal nature of parylene, these hanging films of parylene are unavoidable during the lift-off process (**ESI**[†], Fig. S1E and F). Another method explored to expose CF tips was flame-etching (Fig. 1C and D and **ESI**[†], Fig. S1C), which obviates the need for PR and lift-off procedures. Instead, a butane torch is used to heat the probe ends protruding ~1 mm above water to remove the parylene. The CF's are further trimmed with a razor-blade so that the exposed length is within 50–200 μm . In both cases, sensitivity of the probes was similar and functional yield of the sensors fabricated with either method was ~90%, but the flame-etching technique usually expended increased time. Obviously, these fabrication techniques will need to be optimized in future work to provide greater accuracy and reproducibility. Reactive ion etching (RIE) or oxygen plasma could be used to more uniformly pattern the CF sensing surfaces if etch rates can be properly controlled around CF tips. Difficulties in controlling temperature distribution at the suspended CF tips, which led to highly variable etch rates, impeded our abilities to employ plasma etching techniques in the current work. The fabricated CF arrays have individual electrode lengths of 5–6 mm with a pitch of 250 μm and diameters of 8–10 μm . Fig. 1A displays the completed array. Polyethylene glycol (PEG) (4000 to 8000 g/mol) (Sigma-Aldrich) is used to temporarily stiffen probes and provide a dissolvable shuttle for brain insertion (**ESI**[†], Fig. S2). The dissolvable shuttle is created by aligning arrays to micromolds (brushed with sterile mineral oil) and applying molten PEG (melted on a hot plate set at 50–80°C). Once the PEG has hardened completely, the arrays can be detached from the micromolds. Because this coating is manually applied, the geometry varies, with an overall thickness of 0.1–1 mm, length of 4–5 mm, and width of 3–5 mm. Larger volumes will increase dissolution times in saline from few seconds to ~1 min per penetration depth (0.5–1 mm) during implantation.

Experimental design

A total of 10 rats were used for implantation of the neurochemical probes. Among them, 9 rats were also implanted with medial forebrain bundle (MFB) stimulation electrodes to controllably evoke and measure dopamine release, and the remaining rat was used for histological assessment of inflammatory response. In 7 of the 9 rats implanted with the MFB electrodes, evoked dopamine was discerned as conforming to aforementioned standards. To ensure reproducibility of the measurements, a minimum of 4 stimulation-evoked dopamine signals were collected for each stimulation paradigm and typically > 40 stimulation-evoked dopamine signals were acquired in each rat.

FSCV instrumentation

Two systems were used for FSCV recording of dopamine: a 4 channel system obtained from Dr. Scott B. Ng-Evans at the lab of Dr. Paul E.M. Phillips at University of Washington (UW) that has been used for numerous reported studies, and a custom built 16 channel system (**see ESI**[†]). The UW system was used in initial studies to confirm dopamine recording functionality of the implanted sensors with well-validated hardware and software. To

practically make use of the channel capacity of the arrayed implants, hardware and software were built in-house for recording up to 16 channels concurrently. Recording performance is comparable for both systems: noise of ~0.1 nA (measured with a dummy load circuit mimicking tissue impedance consisting of a series capacitor of 3.3 nF and resistor of 10 k Ω , with digital filtering turned off and standard recording parameters), dynamic range of ± 2000 nA, and samples per scan of 175 (UW-4 channel) or 214 (custom built). The scan refers to the period in which a ramping voltage is applied to the working electrode (i.e., sensor) to detect electrochemical current. The scans are applied at a cycle frequency of 10 Hz for 100 ms temporal resolution.

FSCV dopamine recording parameters

During recording measurements, the implanted CF probes were applied with a triangular waveform ramping from -0.3 to 1.4 to -0.3 V at 400 V/s for each scan at a cycle frequency of 10 Hz and a hold potential of -0.3 V between scans (*in vitro*, the ramp was from -0.4 to 1.3 V with a hold potential of -0.4 V). Prior to recording, the implanted probes were conditioned by applying the same ramp at a cycle frequency of 60 Hz for a period of 5 – 15 min. Ear-bars were connected to system ground to suppress coupling of external high frequency noise.

Chemometric analysis

Current recorded at each scan were concatenated to generate background-subtracted plots of the change in current along the applied voltages versus time. These plots represent current as a nonlinear color scale, the y-axis as the applied voltage, and the x-axis as time. At each time point (i.e., at each scan), the current change versus voltage plot (cyclic voltammogram, CV) is evaluated to distinguish the voltages at which current change occurs, which usually confer oxidation (positive change) or reduction (negative change) of an electroactive substance. For dopamine, the voltages at which peak current deflections occur, or the redox potentials, are -0.2 and 0.6 V. Dopamine concentration change is linearly proportional to the current at the oxidation potential (0.6 V). However, current deflections also occur at potentials outside of the targeted dopamine redox potentials, and these interfering signals may be attributed to other molecules or ionic changes at the CF sensor. To verify that the measured current corresponds to dopamine redox without significant interference, correlation to *in vitro* measurements of dopamine standards as well as principle component regression are required. Finally, based on these corroborated measurements, apparent change in dopamine concentration can be computed. In some measurements, a digital Gaussian filter was used with a standard deviation of 1 for scan variable and 0.1 for the voltage variable (i.e., sample point) and filtering is indicated in these plots. Otherwise, background-subtracted measurements were displayed without any digital filtering. All of these analyses were performed in Matlab (Mathworks, Matlab 2016a).

In vitro measurements

CF arrays were tested *in vitro* using a flow cell apparatus to extract dopamine sensitivity measurements and CV standards (ESI[†], Fig. S2). Solutions used in the flow cell were phosphate buffered saline (PBS) or artificial cerebrospinal fluid (aCSF) buffer and dopamine

hydrochloride (Sigma-Aldrich, H8502) as dissolved to a concentration of 0.25, 0.5, and 1 μM in the buffer. A syringe pump (KD Scientific, KDS 200) was used to generate a fixed flow rate ($\sim 1\text{--}3\text{ ml/min}$) to the outlet of a flexible tube (1/8 inch inner diameter) where the sensors were positioned. A sample injection valve (Valco, Model 22Z) was used to switch the sample flowing across the sensors between PBS and dopamine solutions with an electronic actuator. As the CF sensing interfaces comprise identical surfaces to traditional CFM sensors (same CF material), observed dopamine sensitivity ($2\text{--}50\text{ nA}/\mu\text{M}$) ($\text{ESI}^\dagger$, Fig. S2) is within range of prior work^{3,4,9,15,17,18,32,19–21,31}.

Identifying operational probes in vivo

Working channels were identified based on the recorded current before and after implantation. Only arrays having at least 6 working channels were used for implantation. Probes were defined as nonfunctional if they met any of the following 2 criteria: (1) a mechanical break of the probe along its shaft or sensing tip resulting in a measured maximum background current of less than 100 nA, or (2) a perforated insulation resulting in current noise (defined as the root mean squared of 10 scans along the oxidation potential) greater than 1 nA or resulting in current saturation (i.e., magnitude of background current at any potential $> 2000\text{ nA}$). Characterization of all successfully implanted probes based on the aforementioned operating criteria are shown in $\text{ESI}^\dagger$, Fig. S4.

Surgical procedures

All animal procedures were approved by the Committee on Animal Care at the Massachusetts Institute of Technology and were conducted in accordance with the U.S. National Research Council Guide for the Care and Use of Laboratory Animals. A total of 10 Long-Evans male rats (Taconic, 350–450 g) were used for implantation of the neurochemical probes and were individually or pair caged with ad lib food and water. Rats were anesthetized with isoflurane (vaporizer set at 1.5–2.0%) supplied with oxygen at a flow rate of 1 l/min on a stereotaxic apparatus (Stoelting, 51600) fitted with a rebreathing circuit and mask (VetEquip, 921434). An analgesic, meloxicam (2 mg/kg) was administered subcutaneously. After incision and retraction of the tissue atop of the skull, holes were drilled (Stoelting, 51449) and craniotomies performed for the probes targeting striatum (anteroposterior [AP] +1.5 mm, mediolateral [ML] +2.1 mm), MFB stimulation electrodes (AP -4.1 mm , ML +1.7 mm), and reference electrode (AP -2.3 mm , ML -3.5 mm). In some procedures 8–10 bone screws (Stoelting, 51457) were installed on the circumference of the exposed skull to robustly secure implanted devices. After carefully removing the dura mater above the striatal target, the non-PEG-coated probe tips were lowered into the brain, as driven manually at a rate of $\sim 0.1\text{ mm/s}$ with a micromanipulator. While retaining view of probes through a stereo microscope, if more than 2 probes deflected (this usually occurred if too long of the probe was unanchored from the rigid PEG shuttle or the dura was not completely removed), the array was immediately replaced with another prepared device or the dura was more carefully removed. After $\sim 1\text{--}2\text{ mm}$ of the array was lowered, a small amount of PBS was introduced to the exposed brain surface to dissolve $\sim 0.5\text{--}1\text{ mm}$ lengths of the basal portions of the PEG shuttle. The array was lowered again with effort to drive only the non-PEG-coated portions of probes into brain. These dissolve and lower steps were repeated until the array was lowered to a final depth of dorsoventral (DV) $4.5\text{--}5.5\text{ mm}$.

relative to cortical surface. The array was then secured to the skull with acrylic cement (Ortho-Jet, 0206). In some procedures, a second array was implanted in the same manner immediately posterior to the first array. A Ag/AgCl electrode comprising a perfluoroalkoxy alkanes (PFA)-insulated and chloridized silver wire with an exposed tip of 0.5–1 mm (A-M Systems, 787000) was placed on the supradural surface of the contralateral hemisphere and secured to the bone with cement. MFB stimulation electrodes consisted of a pair of Pt/Ir electrodes, either parylene-C insulated 81 μm diameter (Microprobes, PI20030.1A3) or teflon insulated 125 μm diameter (Plastics One, MS333/18-A/SPC), with a tip-spacing of 200–500 μm . These electrodes were lowered to an initial depth of 6.0 mm towards the MFB targets and then slowly driven further downwards at increments of 0.1–0.2 mm while stimulating at each depth to determine optimal depth (DV 6.0–8.7 mm) for evoked dopamine release, as recorded by the sensors in the striatum. Upon locating optimal depth, the electrodes were secured by filling the hole with silicone sealant followed by encapsulating the electrode and remaining exposed skull with cement. The implantation procedures are displayed in **ESI[†]**, Fig. S3.

MFB stimulation parameters

Current-controlled stimulation consisted of 24–48 biphasic pulses (2 ms per pulse) with amplitude of 100–300 μA and frequency of 60 Hz, as supplied by a stimulus isolator (WPI, A365) that was triggered by the software of the recording instrumentation. The MFB stimulation was usually delivered in intervals of 3–5 min to avoid depletion of the dopamine terminals. It is speculated that in the 2 rats that did not produce discernable dopamine response as recorded by the implanted arrays, stimulation electrode positioning along AP-ML coordinates was not optimal. In 2 of the 7 rats producing successful recording of dopamine, the MFB stimulation electrode had to be removed and re-implanted twice to optimize its positioning for evoking measurable dopamine release. Imprecise positioning of the MFB electrode seemed to be the largest failure mechanism in the recording experiments.

Conferring dopamine chemical identity of in vivo measurements

Dopamine chemical identity of the measured electrochemical signals was conferred by analysis of the CVs of the background-subtracted data at maximum evoked oxidation current (i.e., current at the oxidation potential for dopamine, $E_{ox} = 0.6 \pm 0.05$ V, determined as the voltage at which peak current is measured), following MFB stimulation for each channel that showed change in current with a signal-to-noise ratio of > 5 . These measured CVs were then analyzed against *in vitro* dopamine and pH standards (14 *in vitro* CVs) generated in the flow cell to compute Pearson's correlation coefficient, R . Measurements were only identified as dopamine when $R \geq 0.8$ as correlated to a dopamine standard. Furthermore, principle component regression (PCR) was used to assess the degree of residual or remaining current in the CVs that cannot be accounted as either dopamine or pH³³. This added regulation ensures that measured CVs highly correlated ($R \geq 0.8$) to the dopamine standards maintain extraneous variance (Q , or residual sum of squares) below the calculated tolerance level ($Q_\alpha = 585$) for the principle components provided (i.e., 7 dopamine standards and 7 pH standards) that collectively estimate expected primary contributions to recorded electrochemical current with a confidence interval of 95%. An example of this analysis is shown in the **ESI[†]**, Fig. S5.

Computation of dopamine concentration changes from in vivo measurements

Changes in apparent dopamine concentrations were calculated based on the current measured at the oxidation potential of dopamine (0.6 V). This dopamine oxidation current is linearly proportional to dopamine concentration, which can be shown in theory ²¹ as well as *in vitro* measurements ($ESI^{\dagger}$, Fig. S2). Dopamine concentration change is estimated by dividing the measured (and conferred) dopamine oxidation current by the measured *in vitro* dopamine sensitivity of the probes (nA/nM). To account for variation in sensitivity amongst probes, we show that the sensitivity is directly proportional to the nonfaradaic background current of the individual probe, as this background current is proportional to the surface area of the exposed CF sensor ³⁴ and the resulting sensitivity ^{35,36}. The background current is mostly equivalent to the displacement current generated through the double layer capacitor formed by the CF-brain electrolyte interface and the applied voltage ramp across this capacitor. By normalizing the *in vitro* sensitivity to background current, the calibration factor can be applied to all measurements regardless of the individual background current characteristics.

Histology

Electrolytic micro-lesions were made at the tip of some of the sensors to mark their locations in the striatum (cathodal DC current with amplitude of 20–30 μ A applied for 20–30 s) ($ESI^{\dagger}$, Fig. S6). Brains were fixed for immunohistochemical analysis after probes had been implanted immediately after testing (in 2 rats with 8 channel arrays) or for 1–3 months (in 2 rats with 8 channel arrays and 1 rat with 4 conventional CFM sensors) to assess inflammatory tissue response induced by probes. A ketamine/xylazine mixture (80 mg/kg ketamine and 10 mg/kg xylazine) was administered intraperitoneally (I.P.) to the rats. Upon confirming sufficient depth of anesthesia, the rats were perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M PBS through the ascending aorta after incising the right atrium to create an outlet for the infused solutions. The brains were removed and were stored in 4% paraformaldehyde overnight, followed by 25% glycerol in 0.1% sodium azide (Sigma, 438456) in 0.1M PBS at 4°C. Brains were blocked and frozen in dry ice. The block was cut at 30 μ m intervals in the coronal (3 rats) or horizontal plane (2 rats) on a sliding microtome. Sections are stored in 0.1% sodium azide in 0.1M PBS. Some sections were used for immunofluorescence staining, and the other sections were used for Nissl staining. Sections were imaged with an automatized slide scanner (TissueGnostics, TissueFAXS Whole Slide Scanner) with 10 $\times$ objectives.

Immunohistochemistry

For immunofluorescent staining, sections were rinsed 3 times for 2 min in 0.01M PBS containing 0.2% Triton X-100 (Tx) (Sigma-Aldrich, T8787) and then pre-treated with 3% H₂O₂ in PBS-Tx for 10 min. Sections were rinsed 3 times for 2 min in PBS-Tx, and incubated in tyramide signal amplification (TSA) blocking reagent (PerkinElmer, FP1012) for 1 hr. Then the sections were incubated with primary antibody solutions containing rabbit anti-GFAP (z0334; DAKO) and mouse anti CD11b/c (Abcam, ab1211) in TSA blocking reagent in PBS-Tx for 24 hr at 4 °C. After primary incubation, the sections were rinsed 3 times for 2 min in PBS-Tx, and then incubated them for 2 hr in the secondary antibody

solution containing goat anti-rabbit Alexa Fluor 488 [1:300] (Life Technologies, A11034) and goat anti mouse Alexa Fluor 647 [1:300] (Life Technologies, A21236) in TSA blocking reagent in PBS-Tx. The sections were rinsed 3 times for 2 min in 0.1M PBS, then were mounted onto glass slides and coverslipped with ProLong Antifade Reagent with DAPI nuclear stain (Life Technologies, P36931).

Results and discussion

Recording MFB stimulation evoked dopamine in the striatum with pharmacological modulation

Two arrays (16 probes) were implanted in the striatum of 3 rats to assess heterogeneity of dopamine signaling. A simplified setup of the apparatus is shown in Fig. 3A and the projected configuration of the implanted probes is shown in Fig. 3B and C. The multichannel measurements reveal striking differences in dopamine release dynamics across the striatum, as can be visualized by the background-subtracted current measured from all 16 channels from a representative MFB stimulation trial (Fig. 4A). In the representative experiment shown in Fig. 4, 2 channels were identified as non-operational based (see “Identifying operational probes *in vivo*” in **Materials and methods**) demonstrating noise > 1 nA on channel 13 and background current < 100 nA on channel 15. The change in dopamine oxidation current (color intensity at ~ 0.6 V) and the timespan of these signals, in response to stimulation (at ~ 2 s) differ largely across channels. CV plots extrapolated from these measurements at ~ 1 s post-stimulation reveal 7 (out of 16) channels displaying discernable dopamine (Fig. 4B), which were further conferred for chemical selectivity by dopamine standards (**ESI[†]**, Fig. S5) for the selected stimulation trials. Measurements were further normalized to individual probe sensitivities to quantify, more precisely, actual dopamine concentration changes, which revealed contrasting differences in the temporal characteristics of the dopamine signal (Fig. 4C and D) and peak stimulation evoked dopamine (Fig. 4E) across sites. Spatially-dependent variability in the evoked amplitude of dopamine release are expected based on the differences in density of the dopamine releasing terminals in the striatum^{15,21}. The decay and rise time of the evoked dopamine signal also varied across measurement sites (Fig. 4F and G). The temporal characteristics of dopamine release and clearance are regulated by the overflow of these molecules following exocytosis from a nearby neuron and their uptake, primarily, via the dopamine transporter (DAT), respectively³⁷. Kinetic diversity of these dopamine signals has been speculatively attributed to the differences in number of uptake sites and/or affinity of the transporter at different locations^{3,21,38}.

Following intrastriatal measurements of stimulated dopamine, we investigated the effect of raclopride, a dopamine D2-receptor antagonist, on the spatially distributed changes in evoked striatal dopamine. Pharmacologic agents are often used as a means to corroborate selectivity of the electrochemical measurement^{39,40}. Raclopride, a D2-receptor antagonist, was administered to modulate the evoked dopamine response. Before administration, stimulation parameters were optimized to evoke consistent significant dopamine release in the channels demonstrating a dopamine response. Once the stimulation parameters were optimized (60 Hz, 48 pulses, 250 μ A), 4 trials were performed with these stimulation

parameters every 3–5 min. After collecting these pre-drug measurements, raclopride was administered (2.5 mg/kg I.P.), and 1 min later, measurements of stimulated dopamine release were resumed for at least 4 trials with the same MFB stimulation parameters. Raclopride administration amplified stimulated dopamine amplitudes on all channels that displayed evoked dopamine prior to treatment (7 channels) (Fig. 4E). Such amplification is thought to reflect inhibition of autoreceptors on dopamine axon terminals, which leads to increased dopamine efflux when these neurons are innervated. Furthermore, this inhibition may also diminish DAT uptake^{15,40,41}. Decay time increased at 5 of the 7 sites following raclopride (Fig. 4F), according well with evidence that raclopride has mixed effects on the decay time of evoked dopamine, either maintaining⁴¹ or increasing its value⁴⁰.

Recording stimulation evoked dopamine in relation to MFB stimulation electrode depth

The concentration of evoked dopamine at any given striatal measurement site depends on the placement of the stimulation electrode in the MFB as the dopamine releasing terminals branch out topographically in the striatum^{7,38}. The multichannel probes were used to explore this spatial variability in 5 rats. In a representative set of trials, increased depth of the stimulation electrode was shown to reverse the distribution of peak evoked dopamine release along the mediolateral axis of the striatum (Fig. 5). Because of the heterogeneity of evoked signals observed across striatal sites and subjects, inter-subject correlation is not useful without a better understanding of the spatial distribution of these neurochemical dynamics. Nevertheless, this knowledge may be accessible in future studies through the use of a higher density of probes. These multichannel methods would also allow reproducible mapping of dopamine neuron projections to striatum by targeting stimulation to the cell bodies in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). In this study, only the axon bundle (i.e., MFB), which contains fibers from many brain regions including those outside of SNc and VTA, was targeted to induce dopamine release in the striatum, making it difficult to map specific connections.

Functional reproducibility of probes

The distribution of measured dopamine across all implanted and recorded operational probes (51 out of 58 successfully implanted probes were operational—see **ESI[†]**, Fig. S6C for distribution) was assessed across the 8 rats with successfully-implanted MFB stimulating electrodes to evaluate functional reproducibility of the microfabricated probes based on the tested MFB stimulation parameters for each rat and set of probes (Fig. 6). The ratio of sites demonstrating discernable dopamine release versus those that did not detect dopamine is in line with our observed yield (~50%) for the ability to discern evoked dopamine from individually acutely-implanted CFM sensors, as well as the evidence for distinctive striatal microenvironments that do not present measurable dopamine release^{3,4,15,21,23}. This observation is also not surprising as most single-channel experiments usually require precise optimization of the location of the sensor in the striatum to find a region with measurable dopamine release. Collectively, the arrayed platform reported here affords increased yield of measurable dopamine sites in addition to its greater throughput.

Initial evaluation of brain tissue response to implanted probes

Histological examinations were performed to verify location of implanted sensors in the caudoputamen ($ESI^{\dagger}$, Fig. S6D) as well as to qualitatively evaluate the extent of their immunoreactive response (Fig. 7 and $ESI^{\dagger}$, Fig. S6). Except for sites marked with applied electrolytic lesions, no visible difference in expression for astrocytes (GFAP) or microglia (CD11) was observed along the estimated tracks of the sensing arrays (Fig. 7A and B and $ESI^{\dagger}$, Fig. S6B), relative to the unperturbed contralateral hemisphere ($ESI^{\dagger}$, Fig. S6A), as evaluated in 4 rats. The small sensor-incurred tissue damage contrasts with that of standard CFM sensors (Fig. 7C and D and $ESI^{\dagger}$, Fig. S6C). These findings are consistent with the inflammatory responses reported for similar cellular-scale probes used in electrophysiology^{13,25}.

Conclusion

Multichannel and minimally invasive probes were shown to measure dopamine concurrently from multiple spatially distributed sites in the striatum of rats and also induce minimal immunoreactive response in the brain. The reported arrays should open up new possibilities in probing the heterogeneous mechanisms of neurochemical transmission. The reported methods do not address the challenge of co-localizing functionally specific measurement sites across specimens, but they do offer the practical potential to classify dopamine transmission dynamics in terms of their spatial domains in the striatum in a high throughput manner. Synchronous measurements of dopamine from spatially distributed brain sites will be important to reveal the manner in which dopamine encodes, for example, motivational and/or cognitive states, in normal and disease conditions. Furthermore, the reduced inflammatory response associated with cellular-scale probes may help promote functional longevity of the dopamine measurements for chronic implant applications. These size-reduction strategies also clearly reduce brain damage, which is essential for translating use towards clinical applications, such as for closed-loop treatment in Parkinson's disease. Future work will involve improving batch fabrication techniques for greater uniformity across probes, increasing the channel capacity and density of the array, and implementation in freely moving animals. Although not explored here, the platform could readily be implemented for detection of other important neurotransmitters, such as serotonin, norepinephrine, and adenosine. Furthermore, these systems could be used with electrophysiological techniques, which would permit multimodal measurement of the fast changes in both electrical and chemical activity in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Minimally invasive probes for neurochemical recording. **(A)** A fully assembled array comprising 8 neurochemical probes (outlined box) integrated on a circuit board that directly connects to an external pre-amplifier and current-voltage converter to monitor neurochemical signals on a computer. **(B)** Close-up of outlined area in **A** showing 8 parylene-coated CF probes with individual lengths of 5.5 mm capable of reaching deep brain targets in small animals. **(C)** Scanning electron microscope image of outlined area in **B**, showing exposed 200 μm long CF sensing tip and the parylene insulated CF below this exposed tip. **(D)** Close-up of the outlined area in **C**, showing the delineation (arrowhead) between exposed sensing CF (fibrous structure) and parylene insulation (smooth structure). The diameter of the exposed CF is 7 μm, and the parylene insulated CF is 8.5 μm, which represents the maximal diameter of the probe.

Fig. 2. Fabrication process. (A) 7 μm diameter CF's are aligned and bonded to conductive pads on a printed circuit board (PCB) using a micro-groove array mold. (B) CF tips masked with photoresist (PR) for lift-off patterning of parylene chemical vapor deposited encapsulation and insulation. (C) Parylene deposition and lift-off of PR masks with acetone to expose CF dopamine-sensing tips. (D) Bulk fabrication of 10 arrays on a standard 100 mm diameter pyrex wafer.

Fig. 3.

Experimental setup for 16 channel neurochemical recording. (A) Simplified setup showing the sagittal view of brain targets (at ML 2.9 mm) and implanted devices—2 sensor arrays in green in the striatum (red outline) and the stimulation electrode targeting the MFB (blue) for controlling striatal dopamine release. The FSCV recording system is connected to the arrays, and also sends a trigger to the stimulus isolator (stim) to control MFB stimulation. (B) Array with 8 probes in the striatum along coronal axis (AP 1.5 mm). (C) Horizontal view showing projected locations of the 16 neurochemical sensing tips at DV 4.5 mm and channel colors associated with results in Fig. 4.

Fig. 4.

Synchronous measurements of stimulation evoked dopamine from 16 channels (each color plot represents a single channel) using 2 implanted arrays (Fig. 2C indicates color-channel mapping scheme) in the striatum of a rat. (A) Recorded background-subtracted current from all 16 channels. Color represents the background-subtracted current (proportional to dopamine concentration) with scale shown at right. MFB stimulation is applied at 2 s (artifact visualized as a randomly colored bar in all plots). Stimulation parameters are 60 Hz, 48 pulses, and 300 μ A. Dopamine release is seen immediately following stimulation as discerned by the current change at the dopamine redox potentials (-0.2 and 0.6 V), at channels 3, 4, 7, 8, 11, 12, and 14. Channels 13 and 15 are non-operational as noise > 1 nA, and background current is < 100 nA, respectively. (B) Overlaid CV plots for all channel data in A immediately following stimulation, confirming dopamine chemical identity based on peak currents at redox potentials of -0.2 and 0.6 V. (C) Computed dopamine (DA) concentration change vs. time for the data in A. (D–G) Pharmacologically modulated dopamine release measurements with MFB stimulation parameters uniformly set at 60 Hz, 48 pulses, and 250 μ A for all trials before (3 trials) and after (4 trials) raclopride administration. (D) Overlaid average stimulation evoked dopamine vs. time traces, before (solid) and after (dashed) administration of raclopride. Scale bars represent 50 nM (vertical) and 1 s (horizontal). (E) Peak evoked dopamine across all 7 channels displaying measurable dopamine, all of which display significant increase following raclopride. (F) Bar graph of time from peak evoked dopamine oxidation current to time to half its peak (i.e. decay time) for data in D. Channels 4, 7, 11, 12, and 14 display significant increase in decay time and channel 3 shows significant decrease in decay time following raclopride. (G) Bar graph of time to peak evoked dopamine oxidation current (i.e. rise time) shown in a corresponding manner to F. Channels 4, 7, 8, 11, and 14 display significant increase in rise time following raclopride. Error bars and gray shading represent $\pm$ standard error of mean (SEM).

Fig. 5.

Peak stimulation evoked dopamine recorded in the striatum at two different MFB stimulation electrode depths (DV) of 6.7 and 7.7 mm in a rat (representative measurements averaging 3 stimulation-evoked trials for each MFB depth). Left-to-right (channel numbers in **B**) direction represents increasing laterality along the mediolateral axis of the striatum as represented in Fig. 3A. (**A**) Background-subtracted current converted to projected dopamine concentration changes (scale bar on right) at DV 6.7 (top panel) and DV 7.7 (bottom panel) mm as taken from recorded data averaged over 3 stimulation-evoked trials for each depth. MFB stimulation occurs at 5 s (no marking artifacts shown in these measurements) and with fixed parameters across trials (60 Hz, 48 pulses, and 200 μ A). (**B**) Computed dopamine concentration change vs. time for the same measurements as **A**. Dashed and solid curves indicate measurements from DV 6.7 and DV 7.7 mm, respectively. All 3 channels show statistically significant change in evoked dopamine between the two MFB depths. Scale bars represent 50 nM (vertical) and 1 s (horizontal). Gray shading represents $\pm$ SEM.

Fig. 6.

Characterization of all implanted and operational probes (51 operational probes out of 58 successfully implanted probes in 8 rats—see **ESI[†]**, Fig. S4 for histograms of operational and non-operational channels from all experiments with MFB stimulation). **(A)** Histogram showing distribution of working channels that either detected dopamine (+) or did not detect dopamine (nd), as evoked by MFB stimulation, showing that most operational sensors implanted in the gross striatum region do not detect dopamine release, concurring with previously reported observations^{3,4,14,21} and our measurements with traditional single-channel sensors that require optimization of their intrastriatal placement to find regions with measurable dopamine. Data are from operational channels measured across 8 rats (2 rats recorded with 16 channel system and 6 rats recorded with 4 channel system) for different MFB stimulation parameters. Colors represent the stimulation amplitude (indicated in legend) applied at 60 Hz, 48 biphasic pulses, 2 ms per pulse and the total # of probes employed for each set of stimulation parameters is indicated in the legend. **(B)** Histogram of measured evoked dopamine concentrations (extrapolated from + column in **A**) from all experiments for operational channels.

Fig. 7.

Immunohistochemical examination of implanted sites for the subcellular probes (**A** and **B**) and conventional CFM sensors (**C** and **D**). DAPI primarily stains cell bodies. (**A**) Horizontal brain slice implanted with an array of 8 probes, ~0.7 mm dorsal to their sensing tips in striatum (where electrolytic lesions were made). (**B**) Close-up of outlined area in **A**, revealing small hole putatively induced by probe shaft. (**C**) Same as **A** for an implantation of 4 standard CFM sensors. (**D**) Close-up of outlined area in **C** revealing CFM track and the heightened intensity of astrocyte (GFAP) and microglia (CD11) expression around it.