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Real-time selective detection of dopamine and serotonin at nanomolar concentration from complex in vitro systems

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ABSTRACT

Electrochemical sensors provide means for real-time monitoring of neurotransmitter release events, which is a relatively easy process in simple electrolytes. However, this does not apply to in vitro environments. In cell culture media, competitively adsorbing molecules are present at concentrations up to 350 000-fold excess compared to the neurotransmitter-of-interest. Because detection of dopamine and serotonin requires direct adsorption of the analyte to electrode surface, a significant loss of sensitivity occurs when recording is performed in the in vitro environment. Despite these challenges, our single-walled carbon nanotube (SWCNT) sensor was capable of selectively measuring dopamine and serotonin from cell culture medium at nanomolar concentration in real-time. A primary midbrain culture was used to prove excellent biocompatibility of our SWCNT electrodes, which is a necessity for brain-on-a-chip models. Most importantly, our sensor was able to electrochemically record spontaneous transient activity from dopaminergic cell culture without altering the culture conditions, which has not been possible earlier. Drug discovery and development requires high-throughput screening of in vitro models, being hindered by the challenges in non-invasive characterization of complex neuronal models such as organoids. Our neurotransmitter sensors could be used for real-time monitoring of complex neuronal models, providing an alternative tool for their characterization non-invasively.

1. Introduction

Dopamine and serotonin are neurotransmitters, which play an important role in various functions including cognition, memory, movement, motivation, and mood Schultz (2007). Because of significant differences in the genomics but also nervous system Herculano-Houzel (2009, 2014) between humans and other animals, both academia and pharmaceutical industry are rapidly adopting the use of human brain organoids for research and development applications Vaez Ghaemi et al. (2021), especially if one is at large excess compared to another. Chemical oxidation occurs at similar potentials and distinguishing them can be difficult with conventional electrode materials Li et al. (2017); Di Santo et al. (2017). Due to chemical similarity of the two molecules, their electrochemical oxidation occurs at similar potentials and distinguishing them may be possible with conventional electrode materials. Li et al. (2021); Abbaspour and Noori (2011); Jin et al. (2004); Jiang et al. (2005); Kim et al. (2021), especially if one is at large excess compared to another.

Neuronal signal transmission occurs mainly at synapses, containing both a presynaptic terminal of signaling cell and postsynaptic terminal of the signal-receiving cell. Before neurotransmitter is released at the presynaptic terminal, membrane potential of the neuron is depolarized.

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Membrane depolarization initiates at the cell soma region and spreads along the axon to presynaptic terminal. Laughlin and Sejnowski (2003) Because membrane potential change is an electrical event, neuronal activity can also be analyzed with electrophysiological recording. However, the main drawback in performing electrophysiological recording with microelectrode array (MEA) is the lack of selectivity between different neuronal subtypes. Electrophysiological MEA recording can provide valuable information from simple cell cultures but also about general activity in organoids Tasnim and Liu (2022), however distinguishing activity in more complex brain models becomes impossible due to lack in selectivity. While it is possible to fluorescently label cells and patch clamp the desired cell-type, the measurement setup is often limited to one or two cells at maximum Wood et al. (2004); Suk et al. (2019).

In contrast to electrophysiological recordings, electrochemical recordings can be used to achieve desired selectivity between neuron populations. In electrochemical recording, a potential is applied to working electrode and current is measured as a function of time (or vice versa). If different molecules are chemically distinct, they often undergo redox reactions at different potentials and thus selectivity between molecules can be obtained. Glutamate, gamma-aminobutyric acid (GABA), and acetylcholine are not directly electrochemically active molecules on most electrode materials and thus require enzymatic reagent actions for their electrochemical detection Shadilagni et al. (2019); Chu et al. (2023), thus signals arising from neurons that release these non-electroactive molecules are automatically eliminated. In contrast, amine neurotransmitters such as dopamine and serotonin have molecular similarity and are both directly electrochemically active, causing possible interference between one another.

In brain-on-a-chip applications, cells are cultured directly on the electrodes. Material biocompatibility thus plays an essential role in dictating design rules for the device, as its materials are not allowed to alterate the survival, development, or behavior of the culture. Special care for biocompatibility must be considered when dopaminergic neurons Gaven et al. (2014); Er et al. (2020); Airavaara et al. (2020) or stem-cell derived brain organoids Poli et al. (2019) are used, as both of these are extremely sensitive to culturing conditions and substrate effects. Biocompatibility of the electrodes should thus be evaluated with the corresponding cellular model, e.g. dopaminergic neurons when evaluating dopamine sensors, instead of using highly-robust immortalized cell lines.

Although dopamine and serotonin have been earlier measured selectively from simple electrolytes such as phosphate buffered saline (PBS) and in vivo at nanomolar sensitivity Swamy and Venton (2007); Cernat et al. (2010); Castagnola et al. (2023), it has not been possible to simultaneously measure them under in vitro conditions at physiologically relevant concentrations in real-time. The cell culture medium is a much more fouling environment compared to in vivo, as the cerebrospinal fluid (CSF) is mainly composed of salts and non-aromatic molecules Spector et al. (2015). Few aromatic compounds such as folate Jimenez-Jimenez et al. (1999), thiamine Ormazabal et al. (2005), and the metabolites of amine neurotransmitters Ormazabal et al. (2005), are however present in the CSF at a total concentration of $\approx 1 \mu$M. In this work, we investigated the detection of dopamine and serotonin at nanomolar concentrations under the highly fouling in vitro cell culture systems, where the competitively adsorbing molecules are present at a concentration up to $\approx 900 \mu$M.

2. Materials and methods

2.1. Electrochemistry

All electrochemical procedures were carried out with Reference 620 potentiostat (Gamry Instruments). A platinum wire was used as counter electrode (99.95% purity, Alfa Aesar), while an Ag/AgCl pseudoreference electrode was prepared by treating a silver wire (99.9% purity, Alfa Aesar) in 10% sodium hypochlorite solution (FF-Chemicals) for 45 min. Electrolyte solutions were not deaerated and all solutions were freshly prepared prior to being used, unless otherwise stated. Recordings were performed from sterile 12-well plates (VWR), which were maintained at 37 °C temperature during all experiments.

An illustration of our electrochemical experimental protocol alongside with a picture of the electrochemical setup can be found at the Supplementary Information.

2.1.1. Preparation of electrodes

Electrodes were prepared from single-walled carbon nanotube (SWCNT) network sheet, which was provided by Canatu Oy (Finland) and has been thoroughly characterized earlier by Leppanien et al. Leppanien et al. (2021). Glass coverslips (12-mm diameter, Fisher Scientific) were first washed sequentially in acetone, isopropanol, and deionized water. Next, the coverslips were dried by nitrogen gun and were then silanized by hexamethyldisilazane (HMDS) inside a priming oven at 148 °C temperature. SWCNT sheets were press- transferred onto HMDS-primed coverslips and the network was densified by 99.5% ethanol (Anora, Finland). Conductive silver paste (Electrolube) was added to provide a mechanically robust electrical contact to the SWCNT network and the samples were dried overnight in air. To provide additional mechanical support, a thin polyethylene terephthalate piece was placed beneath the coverslip. Conductive copper tape (Ted Pella) was used to contact the silver paste to copper strip, after which the electrode was insulated with 50-µm thick PTFE tape (Irpola) everywhere except a pre-cut 2-mm diameter hole that was positioned above the SWCNT sheet.

2.1.2. Electrolytes and solutions

Electrochemical experiments were performed either in Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco) or organoid medium that can be used to culture human midbrain and hindbrain organoids Jurazo et al. (2021) that was prepared as instructed under sterile conditions. In short, the base medium N2B27 was first prepared by mixing DMEM/F-12 (Gibco) and Neurobasal (Gibco) media at 1:1 ratio, after which it was supplemented with N-2 supplement (Gibco) at 1:200 dilution, B-27 supplement (Gibco) at 1:100 dilution, GlutaMAX supplement (Gibco) at 1:100 ratio, and Penicillin-Streptomycin (Gibco) at 1:100 dilution. This N2B27 medium can be stored in fridge for a few weeks before completing the medium with following supplements. In order to make a complete organoid culturing medium, the N2B27 medium was further supplemented with 10 ng/mL human glial cell line-derived neurotrophic factor (bDNF, Peprotech), 10 ng/mL human brain derived neurotrophic factor (hBDNF, Peprotech), 1 ng/mL transforming growth factor beta 3 (TGF-/β3, Peprotech), 500 µM dibutyryl cyclic adenosine monophosphate (dbcAMP, Sigma), and 200 µM ascorbic acid (Sigma). Reagents for the complete organoid medium were a kind gift by PhD Gemza Gomez-Giro from Prof. Schwamborn’s laboratory.

Complete organoid medium was placed into humidified incubator (37 °C, 5% CO$_2$) and was incubated overnight for minimum of 20 h to let ascorbic acid become oxidized away Rantatauro et al. (2022). Because of alkalinity shift that occurs in culture media under ambient conditions without 5% CO$_2$ Michel et al. (2019), all recordings were performed within 30 min to avoid pH shifting above pH 8.

Both dopamine hydrochloride (Sigma) and serotonin hydrochloride (Sigma) were first dissolved into DPBS to create concentrated stock solutions, which were then further diluted into DPBS to create 50 µM stock solution of each analyte. These stock solutions were used to create concentration series measurements with cyclic voltammetry, but they were also used to create the injection solutions for chronoamperometric measurements.

Chronoamperometric concentration series experiment had injection solutions made of the same electrolyte solution that was present inside the well-plate. If the electrode was placed inside DPBS in the well-plate,
then the injection solution was made of DPBS. Similarly, organoid medium recording had injection solution made of organoid medium. Injection solutions were prepared into Eppendorf vials with varying concentration (0, 2.5, 5, 10, 25, 50, 100, 250, 500 nM) of either dopamine or serotonin alone, or alternatively the same concentration series was prepared of dopamine but each of the solutions had also 1 μM of serotonin in the injection solution (dopamine + 1 μM of serotonin).

2.1.3. Electrochemical treatment
Prior to using the SWCNT electrodes, electrochemical oxidation treatment was done at +1.4 V (vs. Ag/AgCl) potential for 30 s in Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco) to overcome super-hydrophobicity of the surface, followed by a stabilization treatment at +0.4 V (vs. Ag/AgCl) for 60 s.

2.1.4. Cyclic voltammetry
Cyclic voltammetry was used to study redox characteristics of dopamine and serotonin but also to evaluate sensitivity of our SWCNT electrodes, the electrolyte being either DPBS or organoid medium. Cyclic voltammograms were recorded at four different scan rates (200, 400, 600, 2000 mV/s), the scan window being between −0.2V and +0.5 V (vs. Ag/AgCl).

2.1.5. Chronoamperometric injection series
Chronoamperometry was used to study real-time detection of dopamine and serotonin at physiological concentration range (2.5–500 nM). Based on the oxidation potential data obtained in cyclic voltammetry, we selected holding potentials accordingly. In DPBS, holding potential for dopamine detection was +0.15 V and for serotonin detection +0.30 V (vs. Ag/AgCl), whereas the holding potential was 0.15 V (vs. Ag/AgCl) for dopamine detection when there was 1 μM serotonin present in all of the injection series solutions. In organoid medium, holding potential for dopamine detection was +0.215 V and for serotonin detection +0.30 V (vs. Ag/AgCl), whereas the holding potential was +0.200 V (vs. Ag/AgCl) for dopamine detection when there was 1 μM serotonin present in all of the injection series solutions.

To let the electrode stabilize and reach equilibrium state with electrolyte, the first injection was done 30 s after starting the measurement. Injection volume was selected to be 25 μL, being sufficiently large to avoid excess dilution before reaching the electrode but small enough to enable rapid wash-out from the surface. This volume was injected by manual pipetting onto the electrode that was kept submerged in the electrolyte. Injection series was performed from smaller concentration to larger concentration (0 nM → 500 nM), and we waited 15 s between each injection to let previous solution diffuse away from electrode surface. Current was sampled at 1.0 s time-point after injection, after which the non-Faradic component had essentially dissipated.

2.2. Cell culture
All cell culturing procedures were conducted under sterile conditions.

2.2.1. Preparation of culture substrates
Electrode samples were prepared with similar protocol as introduced for the electrochemical experiments. After preparation, the electrodes were sterilized by 70% ethanol immersion for 30 min, after which they were left to dry in air. Samples were stored under sterile conditions, inside a sterile 12-well plate until being used for cell culturing. Immediately prior to being used, they were sterilized from both sides for 15 min under UV illumination, after which they were moved into a new 12 well-plate and were coated overnight with Poly-L-ornithine (0.5 mg/mL, washed thrice with DPBS, and air-dried before seeding cells).

2.2.2. Primary cell culture preparation
NMRI mice were housed at a 12-h light–dark cycle, with water and food ad libitum. All animal experiments were approved by the Finnish National Board of Animal Experiments (license number: E93/13959/2019/KEK21-01) and performed according to the European legislation on the protection of animals used for scientific purposes.

2.2.3. Postnatal midbrain culture
Animal care and license are presented in Section 2.2.2. Ventral midbrains of 1-2-day-old mice were obtained by the following protocol. The midbrain was acquired by first coronally slicing out the hindbrain and forebrain areas to collect the midbrain flexure, then sagittally cutting out the dorsal (cortex, hippocampus) and ventral (thalamus) parts to account only for the ventral midbrain, i.e., ventral tegmental area and substantia nigra. After this, the final piece was cut into approximately 1 mm³ pieces, which were then treated with papain for neurons solution (David Sulzer laboratory ventral midbrain culture protocol Sulzer and Kanter (2011)) for 10 min. The neuron-containing pieces were washed twice with Neurobasal-A medium (NB-A) (Sigma-Aldrich, Saint Louis, USA), triturated three times with a siliconized glass Pasteur pipette in NB-A, and centrifuged for 5 min at 1000 RPM. Cells were plated on top of the electrode samples in a 50 μL microisland (1 × 10⁵ cells/sample). Poly-L-ornithine (0.5 mg/mL overnight, washed thrice with PBS, and air-dried before seeding cells) coated glass coverslips were used as control samples. Cultures were maintained in a humidified incubator (37 °C, 5% CO₂, saturated humidity, RH 80–100%). After 1h, NB-A medium (Gibco, Waltham, USA) supplemented with 2% B27 (Gibco, Waltham, USA), 40 μM L-glutamine (Sigma-Aldrich, Saint Louis, USA), 25 ng/mL GDNF (Cyt-243, ProSpec, Rehovot, Israel) and 1% penicillin-streptomycin (Gibco, Waltham, USA) was added. On the following day, FDU-solution was added to the cultures for mitotic inhibition of the glia. Half of the culture media supplemented with FDU-solution was changed 4–5 days after seeding.

2.3. Immunofluorescence of midbrain cultures
After culturing for 13 days in vitro (DIV), ventral midbrain neuronal cultures were washed thrice with DPBS and were then fixed with 4% paraformaldehyde (PFA) solution for 20 min in room temperature, followed by washing thrice with DPBS. Electrodes were then disassembled, recovering the coverslip that only had the 2-mm open region remaining intact. Midbrain neuron cultures were stained with following protocol. Cells were permeabilized with 0.2% Triton X-100 in DPBS (TX-DPBS) for 15 min, after which they were blocked with 5% normal horse serum (NHS, S-2000, Vector Laboratories, Newark, USA) in 0.2% TX-DPBS for 1 h in RT. After this, cells were incubated with primary antibodies; mouse anti-PSD95 PDZ domain (124 011, Synaptic Systems, Göttin gen, GER), rabbit anti-Synaptophysin (101 002, Synaptic Systems, Göttin gen, GER) and sheep anti-TH (AB1542, MilliporeSigma, Burling ton, USA) in 5% NHS and 0.2% TX-DPBS for 1h in RT. Next, the cells were washed thrice with DPBS and then cells were incubated with secondary antibodies; anti-mouse AF488 (A21202, Thermo Fisher Scientific, Waltham, USA), anti-sheep AF568 (A11015, Thermo Fisher Scientific, Waltham, USA, ab175477, Abcam Cambridge, UK) and anti-rabbit AF647 (A32795, Thermo Fisher Scientific, Waltham, USA) in 5% NHS and 0.2% TX-DPBS for 1h in RT. All antibodies (both primary and secondary) were used with a 1:500 dilution. Finally, cells were washed thrice with DPBS and mounted on glass slides with Fluoromount-G (SouthernBiotech, Birmingham, USA) mounting medium. Glass coverslip control samples were also incubated with 200 ng/mL DAPI in DPBS for 10 min at RT before the final wash, whereas DAPI staining was omitted for the electrode samples because they were disassembled by tweezers before staining.

Cells were imaged with ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices LLC, San Jose, USA) using a 10x objective and pseudocolored using ImageJ/FIJI.
2.4. Detection of dopamine from cell culture

Electrode samples were prepared using a similar protocol as illustrated earlier, with the exception of having a polymer substrate instead of glass coverslip and without Poly-L-Lysine coating, after preparation, the electrodes were sterilized by 70% ethanol immersion for 30 min, after which they were left to dry in air. Electrodes were then sterilized from both sides for 15 min under UV illumination, after which they were moved into 12 well-plate.

A culturing medium was prepared by supplementing Ham’s F-12K (Kaihn’s) medium (Gibco) with 15% horse serum (Gibco), 2.5% fetal bovine serum (Gibco), and 1.0% penicillin-streptomycin, from now on referred as PC12 medium. Wells that contained electrodes received 1.5 mL of the PC12 medium, after which the plate was placed into humidified incubator (37 °C, 5% CO2) for 30 min before seeding cells.

PC12 Adh (ATCC, CRL-1721.1) cells (Passage 4) were thawed rapidly at 37 °C water bath, after which the vial contents were moved into pre-incubated PC12 medium. Cells were centrifugated at 1000 RPM for 5 min, after which the supernatant was aspirated away and cell pellet was resuspended into pre-incubated PC12 medium. The 30-min incubated medium was removed from wells, after which cells were seeded at 138 000 cells/cm² density and were placed into the incubator. After 2 h, wells were washed twice with DPBS (Corning) and then 1.5 mL of pre-incubated medium was added into the wells. After culturing for three days, wells were gently washed with DPBS and medium was changed.

Electrochemical recordings were performed at 5 DIV under ambient air conditions but maintaining the cultures (N = 3) at 37 °C, the electrolyte being PC12 medium. Potassium chloride (KCl, Sigma) was dissolved into deionized water to create a 100 mM KCl solution, which was pre-heated to 37 °C. Chronoamperometric recording was performed at holding potential of +0.250 V (vs. Ag/AgCl), recording first spontaneous events and then stimulating the cells with 50 μL injection of the 100 mM KCl solution sequentially three times. After this recording, the samples were washed once in deionized water and were then submerged into deionized water for 45 min to destroy the cell culture without denaturing proteins onto electrode surface. Next, the same chronoamperometric recording with KCl injection was performed without having cells on the electrode.

For staining the PC12 Adh cells, electrode preparation was similar except a 12-mm diameter HMDS primed glass coverslip was used as the substrate beneath SWCNT sheet. Otherwise, electrode preparation and cell culturing protocol were similar as earlier described. At 5 DIV, the electrode preparation and chronoamperometry. Two different electrolytes were used: (1) a simple salt solution, PBS, that is often selected for benchmarking purposes due to the absence of surface fouling components. (2) A complete organoid medium with highly complex formulation that has been optimized for the survival and development of cell cultures, causing severe fouling of electrode surfaces and often a complete loss of sensitivity in the nanomolar concentration range.

3. Results and discussion

3.1. Nanomolar detection of dopamine and serotonin

Dopamine and serotonin were electrochemically detected at nanomolar concentration with SWCNT electrodes by using cyclic voltammetry and chronoamperometry. Two different electrolytes were used: (1) a simple salt solution, PBS, that is often selected for benchmarking purposes due to the absence of surface fouling components. (2) A complete organoid medium with highly complex composition that has been optimized for the survival and development of cell cultures, causing severe fouling of electrode surfaces and often a complete loss of sensitivity in the nanomolar concentration range.

3.1.1. Cyclic voltammetry

Cyclic voltammograms were averaged with Descriptive statistics tool in Origin software (version 2023, OriginLab). Measurement data was analyzed in Echem Analyst software (Gamry). After obtaining oxidation currents for dopamine and serotonin, data values were transferred to Origin software. Descriptive statistics tool was used to obtain mean average and standard deviation (SD) values from the data sets. We plotted the mean values with SD into graphs, after which fitting was done with linearized fit equation (\( y = a + bx \)) without weighing.

Chronoamperometric traces shown in Fig. 2A, Fig. 3A and Fig. 4A were also smoothed by performing Fast Fourier Transformation (FFT) to the raw data. Smoothing was done based on low-pass parabolic filter with Cutoff frequency at 10 Hz and selecting Points of Window to value 5. Chronoamperometric trace in Fig. 4A inlet was smoothed by Savitzky-Golay filter selecting Points of Window to value 50.

Limit of detection (LOD) was calculated using the following equation:

\[
X_{LOD} = \frac{3.3 \sigma_{blank}}{S}
\]

, where \( \sigma_{blank} \) is the standard deviation of blank measurements and \( S \) is the sensitivity that was calculated based on calibration curve slopes obtained nearby the detection limit region, and knowing the electrode diameter.

3.2. Data processing and statistical methods

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This reduction peak was located at –58 mV (vs. Ag/AgCl) potential, giving rise to ΔE_p of 310 mV that indicates severely decreased reaction kinetics for dopamine in organoid medium. On the contrast, serotonin reduction peak remained to be visible in the organoid medium and appeared at +258 mV (vs. Ag/AgCl) potential (Supplementary Fig. S7). Accordingly, the ΔE_p of serotonin increased to 61 mV that indicates only slightly slower reaction kinetics in the organoid medium.

Dopamine and serotonin were detected at 25 nM concentration in PBS, both individually in solution but also together (Fig. 1A and Supplementary Fig. S9). In organoid medium, dopamine could not be directly detected with cyclic voltammetry at concentrations below 500 nM. However, dopamine oxidation peak characteristics became visible after performing background-subtraction (Supplementary Figs. S10–S11) and remained to be detectable down to 25 nM concentration in organoid medium. On the contrast, serotonin was observable at 25 nM...
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Fig. 2. Nanomolar detection of dopamine (DA) and serotonin (5-HT) in PBS, both analytes detected separately but dopamine also in the presence of 1 μM serotonin in the injection solutions. (A) Chronoamperometric traces recorded during injection series (0–500 nM) of dopamine and serotonin in PBS. Thick traces correspond to denoised data after FFT-filtering. Black lines drawn from the traces at concentration range 0–10 nM depict the background current, onto which the analyte oxidation current was compared to. Individual traces have been manually offset. (B) Calibration curves for dopamine and serotonin detection in PBS, error bars representing standard deviations of the measurements (N = 3–4). Clear outliers were omitted from calibration curves.

Organoid medium contains several molecules with aromatic nature at exceedingly high concentration (Supplementary Tables S3–S4), which compete against the adsorption of dopamine and serotonin onto electrochemically active surface sites of the electrode surface. The total concentration of these aromatic molecules was ≈900 μM, of which vast majority (≈800 μM) contain only one aromatic ring, whereas 68.5 μM contain two aromatic rings and 21.5 μM contain three aromatic rings (Supplementary Tables S3–S4). Strong adsorption can occur through π-electron coupling interaction between aromatic rings of the fouling molecules and the aromatic hexagonal arrangement of carbon atoms in CNTs Chen et al. (2007), with the adsorption strength increasing as the number of aromatic rings (aromaticity) increases Lin and Xing (2008).

Dopamine has highly similar molecular structure with most of the competitive adsorbing species that have a single aromatic ring and thus, low probability for dopamine adsorption is expected when dopamine is present at nanomolar concentration. Furthermore, it is energetically unfavorable for dopamine to competitively adsorb over molecules with higher aromatic nature. Due to this, there are only a small number of available electrochemically active surface sites for dopamine adsorption and thus, also for the occurrence of oxidation reaction because dopamine acts an inner-sphere molecule on carbonaceous electrodes Peltola et al. (2020). Accordingly, we observed a substantial shift (+131 mV) in the dopamine oxidation peak once measuring from organoid medium (Supplementary Table S1) but also significant decrease in sensitivity that is illustrated by slopes in calibration plots (Fig. 1B). Lastly, the reduction current arising from dopamine-quinone was barely detectable, also at higher scan rates (Supplementary Figs. S5–S8), indicating rapid replacement of the oxidation product by competitively adsorbing species.

On the contrast, serotonin has two aromatic rings and thus its adsorption onto the SWCNT electrode is expected to be stronger compared to dopamine and most of the competitive adsorbing molecules. Adsorption of serotonin and thus also its electrochemical
oxidation current are not expected to be affected as much by the introduction of organoid medium as the electrolyte. Serotonin peak position did not substantially shift (+23 mV) once changing the electrolyte from PBS to organoid medium (Supplementary Table S1), suggesting its continued ability to adsorb to some extent also in the highly fouling environment. In addition, the proportion between reduction and oxidation currents remained largely unchanged (Supplementary Table S2) that indicates stronger adsorption of serotonin oxidation products compared to dopamine. Significantly decreased slope in the calibration plots (Fig. 1B) however indicates a great reduction in the number of available adsorption sites in organoid medium, while the effect of fouling environment decreased at higher serotonin concentrations, as expected.

Analysis of the log_{10}(I_{ox}) vs. log_{10}(Scan Rate) graphs supports this notion (Supplementary Figs. S12–S13), as serotonin showed slope values closer to 1 that indicates larger proportion of adsorption component in the oxidative reaction, compared to dopamine. Once shifting from PBS to organoid medium, both analytes show a significant decrease in the adsorption component, serotonin from ≈0.7 to ≈0.22 and dopamine from ≈0.43 to ≈0.11. Analyte diffusion to surface sites thus has much more importance in organoid medium, indicating that only a small percentage of the initially electrochemically active sites are present on the electrode surfaces.

Dopamine and serotonin both show two linear regimes in the calibration plots of cyclic voltammetry (Fig. 1B and Table 1) as measured in both electrolytes. Detection of serotonin at nanomolar concentration was not largely affected by the presence of excess dopamine, indicated by close resemblance in the slopes with and without dopamine (Fig. 1B and Table 1). Thus, a large excess of dopamine did not interfere with the detection of serotonin.

3.1.2. Chronoamperometry

After analyzing the onset and peak oxidation potentials for dopamine and serotonin, the holding potential for chronoamperometry measurement could be selected to be slightly above the peak oxidation potential of analyte-of-interest to ensure diffusion-limited conditions. In organoid medium however, the onset potential to both serotonin oxidation but also medium components occurred before dopamine peak oxidation. When detecting dopamine in the presence of excess serotonin, we accordingly set the holding potential to +200 mV (vs. Ag/AgCl) to be sufficiently large to oxidize dopamine but remain below the onset oxidation potential of serotonin.

Fig. 3. Nanomolar detection of dopamine (DA) and serotonin (5-HT) in Organoid Medium, both analytes detected separately but dopamine also in the presence of 1 μM serotonin in the injection solutions. (A) Chronoamperometric traces recorded during injection series (0–500 nM) of dopamine and serotonin in Organoid Medium. Thick traces correspond to denoised data after FFT-filtering. Black lines drawn from the traces at concentration range 0–10 nM depict the background current, onto which the analyte oxidation current was compared to. Individual traces have been manually offset. (B) Calibration curves for dopamine and serotonin detection in Organoid Medium, error bars representing standard deviations of the measurements (N = 3–4). Clear outliers were omitted from calibration curves.
Table 1
Analytical parameters for the detection of dopamine (DA) and serotonin (5-HT) with SWCNT electrode, obtained by cyclic voltammetry and chronoamperometry, electrolyte being PBS or organoid medium.

<table>
<thead>
<tr>
<th>Sensor material</th>
<th>Detection technique</th>
<th>Electrolyte</th>
<th>Analyte</th>
<th>LOD (nM)</th>
<th>Smallest shown concentration (nM)</th>
<th>Selectivity at nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWCNT</td>
<td>CA</td>
<td>PBS</td>
<td>Dopamine</td>
<td>0.025</td>
<td>2.5</td>
<td>Yes</td>
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<tr>
<td>Au-MPy</td>
<td>CA</td>
<td>PBS</td>
<td>Dopamine</td>
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<td>10</td>
<td>No</td>
</tr>
<tr>
<td>ERGO-P/GCE</td>
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<td>PBS</td>
<td>Dopamine</td>
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<td>–</td>
<td>Yes</td>
</tr>
<tr>
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<td>TRIS</td>
<td>Dopamine</td>
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<td>Yes</td>
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<tr>
<td>SWCNT</td>
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<td>Dopamine</td>
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<td>Yes</td>
</tr>
<tr>
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<td>aCSF</td>
<td>Dopamine</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>SWV</td>
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<td>FSCV</td>
<td>Tris</td>
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<td>Dopamine</td>
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<td>100</td>
<td>Yes</td>
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<td>CA</td>
<td>Culture medium</td>
<td>Serotonin</td>
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<td>2.5</td>
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<td>ERGO-P/GCE</td>
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<td>60</td>
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<td>Serotonin</td>
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<tr>
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<td>DPV</td>
<td>Serotonin</td>
<td>–</td>
<td>10</td>
<td>Yes</td>
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<tr>
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<td>PBS</td>
<td>Serotonin</td>
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</tbody>
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Table 2
Summary of electrochemical detection for dopamine and serotonin and comparison to state-of-the-art electrodes. Only electrodes capable for sub-second temporal resolution were selected for comparison. Because LOD is simply a calculative value and often extrapolated from calibration curve obtained with excessively large concentration, we also note the smallest shown concentration.

<table>
<thead>
<tr>
<th>Sensor material</th>
<th>Detection technique</th>
<th>Electrolyte</th>
<th>Analyte</th>
<th>LOD (nM)</th>
<th>Smallest shown concentration (nM)</th>
<th>Selectivity at nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWCNT</td>
<td>CA</td>
<td>PBS</td>
<td>Dopamine</td>
<td>0.025</td>
<td>2.5</td>
<td>Yes</td>
</tr>
<tr>
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<td>CA</td>
<td>Culture medium</td>
<td>Dopamine</td>
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<td>500</td>
<td>No</td>
</tr>
<tr>
<td>ITO/CNP</td>
<td>SWV</td>
<td>Culture medium</td>
<td>Serotonin</td>
<td>0.33</td>
<td>5</td>
<td>No</td>
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</tbody>
</table>

In PBS, signals arising from 2.5 nM injection solutions of dopamine and serotonin were detected (Fig. 2), the calculative LOD being 0.025 nM for dopamine and 0.017 nM for serotonin (Table 1). Because of the highly fouling nature of organoid medium, we expected a significant decrease in sensitivity upon the change of electrolyte. Despite that, we were able to detect signals arising from dopamine at 10 nM and serotonin at 5 nM concentration in organoid medium (Fig. 3), the LOD being 1.28 nM for dopamine and 0.33 nM for serotonin (Table 1). Based on this, the decrease in sensitivity was ≈98% for dopamine and ≈81% for serotonin upon shifting from the highly simple PBS
electrode into complete culture medium. We must note that all electrode types are expected to undergo a substantial loss in sensitivity due to the presence of fouling molecules at high concentration, some of them having highly similar molecular structure than dopamine and serotonin itself. This has been observed earlier with various different electrode types Kouzar et al. (2021); Rantataro et al. (2022); Nekoueian et al. (2023). In spite of a significant decrease in sensitivity, the performance of our SWCNT electrodes in organoid medium was comparable to those earlier state-of-the-art electrodes that were measured in PBS (Table 2).

Once measuring dopamine from organoid medium in the presence of serotonin, the holding potential was slightly smaller (+200 mV vs. Ag/AgCl) compared to when dopamine was measured alone (+215 mV vs. Ag/AgCl). Because both potentials were slightly below dopamine oxidation peak, even minor increases in the holding potential will significantly affect dopamine oxidation current. As expected, dopamine oxidation current was notably smaller in the recording where serotonin was present (Fig. 3B). In addition to the effect of holding potential, we expect competitive adsorption of serotonin to also participate in the decreased dopamine oxidation current. Nevertheless, our SWCNT sensor was capable of detecting 25 nM of dopamine with the presence of 1 μM serotonin in the injection solution, having LOD of 2.74 nM (Table 1).

Compared to earlier state-of-the-art electrodes (Table 2), our SWCNT electrode shows superior analytical performance towards the detection dopamine and serotonin from the viewpoint of both sensitivity and selectivity. We must note here that research and results obtained with inherently slow electrochemical detection methods, such as differential pulse voltammetry (DPV) or the use of aptamer sensors Nakatsuka et al. (2018) whose temporal resolution is limited to ≈ 1 s Farjami et al. (2013); Álvarez-Martos and Ferapontova (2016); Shahdost-Fard and Ghazizadeh (2023), were omitted from comparison because our interest lies in the detection of transient neurotransmitter release events in real-time. While dopamine has been earlier detected at 5 nM concentration in PBS Ma et al. (2020) and 50 nM in culture medium Nekoueian et al. (2023) by using differential pulse voltammetry (DPV), obtaining sub-second temporal resolution in the analytical window is not possible with this technique.

The injection series experiment consisted manual pipetting and thus, systematic error can arise from slightly varying distance between the pipette tip and electrode. As this distance increases, the initially injected neurotransmitter-containing solution undergoes more dilution by mixing with the electrolyte before reaching electrode surface. However, we observed only relatively minimal variation in the measured oxidation currents between individual recordings (Figs. 2 and 3) and thus, we can conclude that the experimental setting is sufficiently well controlled.

3.2. Real-time detection of dopamine from cell culture

Dopamine was detected from in vitro cell culture with chronoamperometry, using electrode holding potential above dopamine oxidation potential. We first recorded spontaneous activity of the cultures (N = 3) for ≈ 10 s, during which a positive current fluctuation was observed that disappeared after destroying the culture with deionized water (Fig. 4A inlet and Supplementary Figs. S14–S15). We attribute these current signals to arise from the oxidation of spontaneously released dopamine from the culture, as PC12 cells are known to spontaneously release neurotransmitters already at 3–5 DIV Brauchi et al. (2008). The detected spontaneous dopamine release events typically lasted ≈1 s and on average had peak current of 95.4 ± 47.7 pA, which corresponds to 43.1 ± 21.6 nM of released dopamine. The measured dopamine oxidation current however ranged between 27.4 and 212.3 pA, corresponding to dopamine at 12.4–95.9 nM concentration.

Because our electrode was a macroelectrode with 2-mm diameter, the background current was exceedingly large compared to dopamine released by a single cell. Only synchronous activity of multiple cells could thus be detected, causing individual signals to become superimposed and broadening the electrochemical oxidation signal. Supporting our reasoning of detecting synchronized dopamine release events, PC12 cultures have been reported to be capable of spontaneously synchronizing their action potential firing up to the distance of 500 μm Cui et al. (2020).

After recording spontaneous activity, we stimulated the culture with 50 μL of 100 mM KCl that is known to depolarize cell membrane and evoke the release of dopamine Westerink and Ewing (2008). A sharp oxidation peak occurred after each stimulation event and the oxidation current gradually decreased towards baseline (Fig. 4A), as both the stimulant diffused away from cells and thus dopamine concentration at the electrode surface decreased. The first stimulated dopamine release peak had amplitude of 14.3 ± 6.0 nA, second peak 8.6 ± 3.2 nA, and third peak 6.68 ± 1.1 nA. Based on the calibration curve for dopamine in culture medium (Fig. 3B), oxidation current of dopamine released by PC12 Adh cells after KCl stimulation corresponds to 2.6 ± 1.1 μM dopamine at the first peak, whereas 1.5 ± 0.6 μM at the second and 1.2 ± 0.2 μM at the third peaks. These concentration values are slightly larger than dopamine released in vivo in the absence of stimulation Robinson et al. (2003), which was expected because we stimulated the cells with high-concentration KCl. Secondly, the culture was highly confluent at 5 DIV (Fig. 4B) and the cells were mostly present at spheroid-like morphology at close proximity to one another, which is a hallmark of high-density culture of PC12 Adh cells. This ensured near-complete coverage of the entire electrode.

When dopamine is measured from in vitro cultures, the electrolyte is often changed to PBS or other highly simple buffer solution for the measurement duration Senel et al. (2020); Li et al. (2021); Mir et al. (2015); Perebikovsky et al. (2020); Vasudevan et al. (2019). In addition, cells are often incubated in dopamine precursor (L-DOPA) containing medium prior to performing the stimulatory recording Poolakkandy et al. (2022); Perebikovsky et al. (2020), which increases the concentration of released dopamine by up to threefold in PC12 cells Westerink and Ewing (2008); Abe et al. (2015). Instead of that, we performed our recording in real-time, directly from the highly fouling culture medium and still observed a significant increase in oxidation current upon stimulating the culture. The comparison between our SWCNT electrodes and earlier state-of-the-art sensors for dopamine detection after KCl-stimulation can be found in the Supplementary Information Table S5. More importantly, our electrodes were capable of recording spontaneous dopamine release events from the culture in the absence of stimulation. This indicates outstanding performance of our SWCNT electrode, as it has not been earlier possible to detect neurotransmitters released from spontaneous cellular activity in the in vitro setting.

Immortalized cell lines are much more robust than primary neurons and thus, their use might not provide accurate information about biocompatibility of electrode materials. Because of that, we evaluated biocompatibility of our SWCNT electrodes by culturing a highly sensitive primary midbrain mouse culture. Dopaminergic neurons can be identified by tyrosine hydroxylase (TH) immunostaining Nickels et al. (2020), whereas the presence of synaptophysin (presynaptic) and PSD95 (postsynaptic) indicate mature synapses Southam et al. (2019). Despite observing slightly lower density of cells on the SWCNT electrodes compared to glass coverslips, we observed that dopaminergic neurons were healthy and appeared similar to those cultured on positive control glass coverslips at 13-DIV, illustrated by the presence of highly mature neurites on TH-positive cells and the formation of synapses (Fig. 4C). Although we cannot provide a conclusive explanation for the observed slightly lower cell density, we expect it to arise from sample manufacturing because the electrode was disassembled before staining. This could result into detachment of cells and thus apparently lower cell density. If the sample was not highly biocompatible, healthy dopaminergic neurons could not be observed on the sample.

Based on the immunological staining of TH and Synaptophysin, dopaminergic neurons on the SWCNT electrode can be assumed to be capable of producing and releasing dopamine. However, we cannot ascertain whether this would result in transmitting the dopamine-
related signal to postsynaptic targets because the exact colocalization of synaptic markers in three-dimensional space could not be thoroughly assessed. To exhaustively confirm the functionality of the neuronal networks on SWCNTs, this should be further studied with additional markers of dopaminergic signaling.

4. Conclusion

Our SWCNT sensors exhibit outstanding analytical performance, showing selective detection of both dopamine and serotonin at nanomolar concentration also in the highly fouling organoid medium. A drop of sensitivity as large as 98% was observed when changing from a simple electrolyte to culture medium, which undoubtedly concerns also other state-of-the-art electrodes. Despite that, our electrodes showed sensitivities in the culture medium comparable to those earlier state-of-the-art electrodes that were benchmarked in PBS. Our electrodes also have excellent biocompatibility, as they were able to sustain healthy development of primary midbrain dopamine neurons on them. Lastly, we measured spontaneous dopamine released from an in vitro cell culture, as well as KCl-stimulated neurotransmitter release. To the best of our knowledge, this is the first time that transient electrochemical signals have been recorded inside culture medium in real-time without stimulating the culture.

Considering these results together, our SWCNT sensor paves way for electrochemical monitoring of in vitro cultures in real-time. Because cultures can be grown directly on our sensor material and recording does not require changing of electrolyte, our neurotransmitter sensor system may become an invaluable non-invasive tool for evaluating the state of complex neuronal models and their response to given treatments.

Lastly, our SWCNT sensor material has been proven to be manufactured at industrial scale and reproducibility. Because of inherently simple electrode manufacturing process, exhaustive protocols containing exotic composite materials can be avoided, further decreasing sensor-to-sensor variation.

CRediT authorship contribution statement

Samuel Rantataro: Conceptualization of this study, Methodology,
Investigation, Data Analysis, Validation, Writing - Original Draft, Visu-
alization. **Ilmari Parkkinnen**: Methodology, Investigation, Data Anal-
ysis, Validation, Writing - Original Draft. Mikko Airavaara: Resources, Writing - Review and Editing, Supervision, Funding. Tomi Laurila: Resources, Writing - Review and Editing, Supervision, Funding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw data required to reproduce these findings is available to download from [Rantataro, Samuel (2023), “Real-time Selective Detection of Dopamine and Serotonin at Nanomolar Concentration from Complex In Vitro Systems”, Mendeley Data, V1, DOI:10.17632/zty2y4nt3b.1].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

References


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