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Microfluidic Device for the Selective Chemical Stimulation of Neurons and Characterization of Peptide Release with Mass Spectrometry

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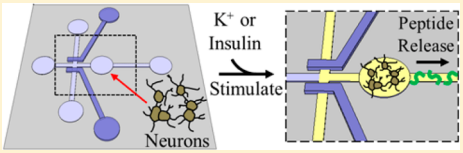
ABSTRACT: Neuropeptides are synthesized in and released from neurons and are involved in a wide range of physiological processes, including temperature homeostasis, learning, memory, and disease. When working with sparse neuronal networks, the ability to collect and characterize small sample volumes is important as neurons often release only a small proportion of their mass-limited content. Microfluidic systems are well suited for the study of neuropeptides. They offer the ability to control and manipulate the extracellular environment and small sample volumes, thereby reducing the dilution of peptides following release. We present an approach for the culture and stimulation of a neuronal network within a microfluidic device, subsequent collection of the released peptides, and their detection via mass spectrometry. The system employs microvalve-controlled stimulation channels to selectively stimulate a low-density neuronal culture, allowing us to determine the temporal onset of peptide release. Released peptides from the well-characterized, peptidergic bag cell neurons of *Aplysia californica* were collected and their temporal pattern of release was characterized with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. We show a robust difference in the timing of release for chemical solutions containing elevated K^+ (7 ± 3 min), when compared to insulin (19 ± 7 min) ($p < 0.000\,01$).



Neuropeptides are an important class of signaling molecules that are involved in cell-to-cell communication in both the central and peripheral nervous systems. They are synthesized and stored in neurons and, upon stimulation, are exocytotically released from the cells to function as neurotransmitters, neuromodulators, trophic factors, and neurohormones. Known to influence a wide range of physiological processes, neuropeptides influence learning and memory, food intake, pain, and disease.[1−3] Identifying neuropeptides that both establish and enrich neuron−neuron interactions and understanding the physical and chemical conditions necessary for their release will aid in uncovering the key effectors of formation and repair in neural networks. Characterizing neuropeptide release *in vitro* from low-density culturing environments allows cells of interest to be isolated from their potentially heterogeneous neighbors, which oftentimes contain a different chemical complement from nearby cells.[4]

The study of neuropeptide signaling can be challenging; because only a fraction of the mass-limited neuropeptide content of a cell is released after stimulation, sample amounts can be low. Furthermore, in the commonly employed dish-based culturing systems, released peptides are diluted by orders of magnitude before collection, rendering their characterization difficult. There is a need for neuronal culture, collection, and detection systems that both identify released neuropeptides and provide information regarding release conditions. Here we describe an approach to maintain a neuronal network in a controlled environment, selectively apply chemical stimulations, collect peptide releasate with minimal dilution, and couple the releasate collection to off-line mass spectrometry (MS) for peptide characterization.

Microfluidic devices are well suited for examining neuropeptide release. With the evolution of microvalves, fluid control, surface patterning, and chemical gradients, these devices provide the ability to precisely control the microenvironments around neurons while significantly reducing sample volumes.[5−8] By enabling detection of these mass-limited samples, we gain a broader understanding of peptide release conditions. Additionally, these devices offer a number of advantages that improve cell culture: biocompatibility, gas permeability, and optical transparency.

In recent years, progress has been made toward integrating microfluidics and neuroscience with cell culture, manipulation, and electrophysiology studies.[9−13] Compartmentalized devices fluidically isolate cell bodies from processes, allowing these structures to be examined separately.[14] Whole *Caenorhabditis*

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elegans, the well-studied neural development model, have been placed in microfluidic chambers for the interrogation of a number of factors, including odor recognition and Ca^{2+} imaging,[15,16] dynamic cellular processes,[17] and high throughput genes and drug screening.[18] Finally, several devices have been employed to study the injury and regeneration of neuron processes as well as to model disease within a device.[19−21] While microfluidic technologies have been used to address a wide range of topics in neuroscience, little of this research has involved the investigation of neuropeptides.

A number of detection platforms have been used to investigate neuropeptide release, including enzyme immunoassays, radioimmunoassays, and capillary electrophoresis coupled to laser-induced fluorescence.[22,23] While highly sensitive and quantitative, these techniques require analyte preselection prior to analysis, followed by either a derivatization procedure to render the peptide fluorescent or the creation of an antibody. In contrast, MS provides high sensitivity and information content for the entire peptide complement, without analyte preselection. Matrix-assisted laser/desorption ionization (MALDI) MS offers high salt tolerances and a relative ease of coupling to microfluidics. For all of these reasons, MS has proven to be a great detection platform for peptide release studies.[24−27]

We present a microfluidic design that allows the user to selectively apply chemical stimulations to neurons maintained in the device. The resulting neuropeptide releasates are collected off-line and detected with MALDI- time-of-flight (TOF)-MS. This approach provides information regarding peptide release content and the physiological conditions necessary for release. Previously, we reported several devices that employed MALDI-MS imaging to collect and quantify neuropeptides on chip.[28,29] In this study, we achieve further control over the extracellular microenvironment by creating a microvalve-controlled device that exposes a low-density neural network to measured time periods of exposure to a defined chemical stimulation. With the added temporal control over stimulation and collection, the onset of peptide release can be determined and compared. By collecting peptide releasates and characterizing them via MS, off-chip, multiple-day stimulations can be performed on the same neural network and the specific released peptides can be characterized. Bag cell neurons from the marine mollusk *Aplysia californica* are widely used in neuropeptide studies. We exposed these neurons to both general and bag cell-specific chemical stimulations, followed by the subsequent collection and detection of peptide releasates. We observed both the onset and refractory period of bag cell peptide release for elevated K^+ and insulin exposures; our results show a robust difference in the time frame required to detect peptides after exposure to distinct chemicals.

EXPERIMENTAL SECTION

Reagents. Chemicals were purchased from Sigma Aldrich (St. Louis, MO), and organic solvents were purchased from Fisher Scientific (Fairlawn, NJ) unless otherwise noted. The polydimethylsiloxane (PDMS) prepolymer kit, Sylgard 184, was purchased from Dow Corning (Midland, MI). 2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane (OEG, 90%) was purchased from Gelest, Inc. (Morrisville, PA). Filtered artificial seawater (ASW, pH 7.8) contained 460 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 22 mM $MgCl_2$, 26 mM $MgSO_4$, 2.5 mM $NaHCO_3$, and 15 mM HEPES. Negative photoresist SU-8 2075 and developer were obtained from MicroChem Corp. (Newtown, MA). Positive photoresist AZ 4620 and developer were from AZ Electronic Materials Corp. (Somerville, NJ).

Device Fabrication. The device consists of two separate layers, a flow channel and a pressure channel layer. Silicon masters were prepared using a traditional photolithographic process as reported previously.[30,31] The flow channel master was made with AZ4620 photoresist to a height of 25 μm. Wafers were heated to 200 °C for 20 min to create rounded corners in the channels. Octadecyltrichlorosilane was evaporated onto the wafer for 4 h or longer to allow for the removal of PDMS from the wafer. For the pressure channel master, SU-8 2075 photoresist was spun and patterned to make channels 100 μm in height. Small pieces of silicone tubing (Helix Medical, Carpinteria, CA) were glued onto the SU-8 features on the master. Uncured 20:1 PDMS was spun onto the flow channel master at 2050 rpm for 60 s; 5:1 PDMS was poured onto the pressure channel master. Both masters were allowed to partially cure at 70 °C until just set. Then both PDMS blocks were aligned and adhered together and cured at 90 °C overnight. The main reservoir and outlet were made with an AcuPunch biopsy punch (Acuderm, Inc., Ft. Lauderdale, FL) of 1 mm. The PDMS block was then adhered to a 30 mm cell culture dish (Greiner Bio-One, Monroe, NC), and uncured PDMS was poured around the device to prevent leakage. The device was cured again at 70 °C for 1 h and both the flow and pressure channels were filled with ASW. Pressure channels were connected to an in-house built pneumatic solenoid valve system that is controlled by LabView software (National Instruments, Austin, TX). The program controls the application or removal of pressure to the pressure channels for closing or opening the channels, respectively.

To reduce analyte losses into the PDMS material,[29,32] channels were treated with 5:1:1 $H_2O/HCl/H_2O_2$ for 5 min, rinsed with deionized water (DI; Milli-Q Biocel, Millipore Corporation, Billerica, MA) for 5 min, OEG for 30 min, and finally flushed with DI water for a minimum of 4 h to yield OEGylated PDMS, as described previously.[33] Prior to use, PDMS devices were washed with methanol and dried with N_2.

Cell Culture Experiments. *Aplysia californica* (100−150 g; National Resource for *Aplysia*, Miami, FL) were anesthetized by injecting 390 mM of $MgCl_2$ equal to 1/2 the animal's body weight. The abdominal ganglia was dissected and incubated at 34 °C in ASW containing 10 mg/mL protease from *Streptomyces griseus*, 60 mg/L penicillin G, 100 mg/L streptomycin, and 100 mg/L gentamicin for 90 min. This treatment of the ganglia allows for removal of the connective tissue surrounding the bag cell neurons. Individual bag cell neurons were then isolated and loaded into the main reservoir of the microfluidic device. A small, thin piece of PDMS was placed over the main reservoir to direct flow through the flow channels. Cells were cultured in ASW for 24 h prior to stimulation experiments.

Stimulation Experiments. Two syringes, containing either a solution of 60 mM of KCl in ASW or 5 μM of insulin in ASW, were attached to a syringe pump (Harvard Apparatus, Holliston, MA) and connected to the stimulation channels on the device. A syringe filled with ASW connected another syringe pump to the inlet reservoir. Pre- and postcontrols were performed with ASW as cell culture media. Each series of chemical stimulation measurements was performed on a network of cultured cells in the microfluidic device. Both the ASW and chemical stimulation solution were flowed through

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the device at a flow rate of 0.5−1 μL/min. When ASW was flowed, the microvalves were closed to prohibit chemical stimulation solution from entering the channels. When chemical stimulations flowed through the device, ASW flow was stopped and the microvalves opened. Small pieces of silicone tubing connected the pressure channels to an in-house built solenoid valve system. Prior to cell loading, each pressure channel was filled with water to prevent bubble formation in the channels. If bubbles entered the channels, a small amount of negative pressure was applied to the channel to remove them. During each stimulation experiment, cells were visualized to monitor cell viability with an inverted fluorescence microscope (Carl Zeiss, Inc. Peabody, MA).

Sample Preparation and Mass Spectrometric Detection. Sample solutions exited the device through polytetrafluoroethylene tubing (Cole Palmer Instrument Company, Vernon Hills, IL) and between 10 and 20 μL of sample were collected and then desalted and concentrated using ZipTip C_{18} pipet tips (Millipore, Billerica, MA) according to manufacturer instructions. Briefly, the ZipTip was wetted with 100% acetonitrile (ACN), equilibrated with 0.1% trifluoroacetic acid (TFA) in deionized water, loaded with sample containing 0.1% TFA, washed with 5% methanol/0.1% TFA in deionized water, and eluted onto a ground steel MALDI target (Bruker Daltonics Inc., Billerica, MA) first with 50% ACN and then with 75% ACN solutions. Samples were then combined with 50 mg/mL 2,5-dihydroxybenzoic acid matrix in 1:1 acetone/water for analysis. An ultrafleXtreme TOF mass spectrometer (Bruker Daltonics) in the reflectron mode was used to analyze peptide releasates. Mass calibration was performed with peptide calibration standard II (Bruker Daltonics). Between 500 and 1500 shots were collected per spot. Individual mass spectra were analyzed with flexAnalysis (version 3.3, Bruker Daltonics).

RESULTS AND DISCUSSION

Device Design and Fabrication. The goal of this work was to create a small-volume cell culturing region that allows neuronal network formation and access to cells. Fluidics were added to enable the extracellular media to be exchanged with various solutions (media or stimulation solutions) and a collection port/channel to enable off-line sample characterization, in this case via MS. In order to enable temporal control of the media surrounding the cells, we used microvalve-controlled stimulation channels (Figure 1A).

The main and outlet reservoirs are 1 mm in diameter; this reduces dilution within the device while allowing for effective fluid flow. The main reservoir serves as the cell culture chamber and is open for cell loading (and physiological recordings, if necessary). Flow channels are 200 μm wide by 50 μm high and rounded; pressure channels are 200 μm wide by 100 μm high. Pressure channels can be selectively opened (or closed) to allow for chemical stimulation additions to the cultured neurons within the device. Studies were performed with colored ink solutions (as shown in Figure 1B) to verify that this arrangement allows fluidic isolation between the stimulation channels and the main channel. PDMS channels are OEGylated to reduce peptide losses into the PDMS.[29] The culture chamber is where the neurons are cultured and form the network.

Cell Culture. A significant amount of chemical heterogeneity exists among different cell types and, perhaps surprisingly, even among cell populations that are considered to be homogeneous. One of the challenges in studying high-density or larger cultures is due to the small chemical differences that can be lost when measurement approaches report average values from within a high-density cell population. There are a number of reports documenting high-density cultures in microfluidic devices,[34−37] but significantly fewer protocols exist for low-density cultures. While individual cells can be assayed for their peptide contents using MS,[24,25,38,39] chemical signaling requires multiple cells and so sparse neuronal networks appear to be of the appropriate scale for investigation of signaling networks using microfluidics. For these reasons, we fabricated a device that is capable of maintaining low-density cultures. Each device reported here contained 10−20 bag cell neurons that were removed from the animal and cultured for 24 h prior to stimulation experiments. A representative image of a bag cell culture is shown in Figure 1C; this image shows the cells forming a network with putative chemical or electrical connections between the neurons. Even after 1 day *in vitro* (DIV), neurons appeared healthy and viable with long process growth and network formation. Cells can be maintained for at least 12 DIV (data not shown) with media changes, allowing for multiple-day stimulation experiments on the same cell cluster in the device.

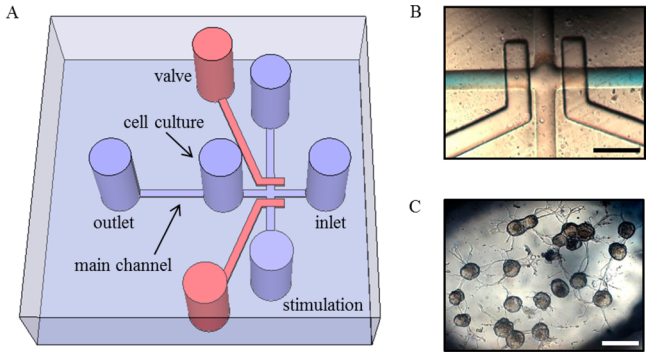

Figure 1. (A) Schematic of the device, 8 mm wide, 8 mm long, and 2 mm high. Bottom layer (blue) denotes the flow channels whereas the top layer (red) denotes the pressure channels. (B) Image of an ink solution in the stimulation channels with both valves closed. Ink is not observed in the main channel, indicating that valves fluidically isolate stimulation solutions from the main channel. Scale bar = 500 μm. (C) Representative bag cell culture after 1 DIV. Neurons cultured in the device appear viable and form networks. Scale bar = 100 μm.

Elevated K^+ Stimulation. Following cell culture, neurons within a device were exposed to media that was known or expected to cause the release of peptides. Elevated K^+ acts as a secretagogue, which causes the exocytosis of neurotransmitters from a neuron through the depolarization of the cell membrane.[40] The addition of K^+ to bag cell neurons and the detection of peptide release have been extensively studied, and their subsequent peptide profiles have been widely documented.[29,41−44] Therefore, *A. californica* provides an excellent model for validating this system; by observing known released peptides, we validate device function. To that end, nine different devices, with each containing a different network of bag cell neurons, were subjected to increasing time periods of K^+ exposure (Figure 2). A pre- and postcontrol was performed on each group of neurons with ASW before and after chemical stimulation, respectively. ASW is a minimal culture media (as it contains the required inorganic ions) and should not elicit peptide release. We did not observe peptide release with the addition of ASW, which suggests that we did not induce release

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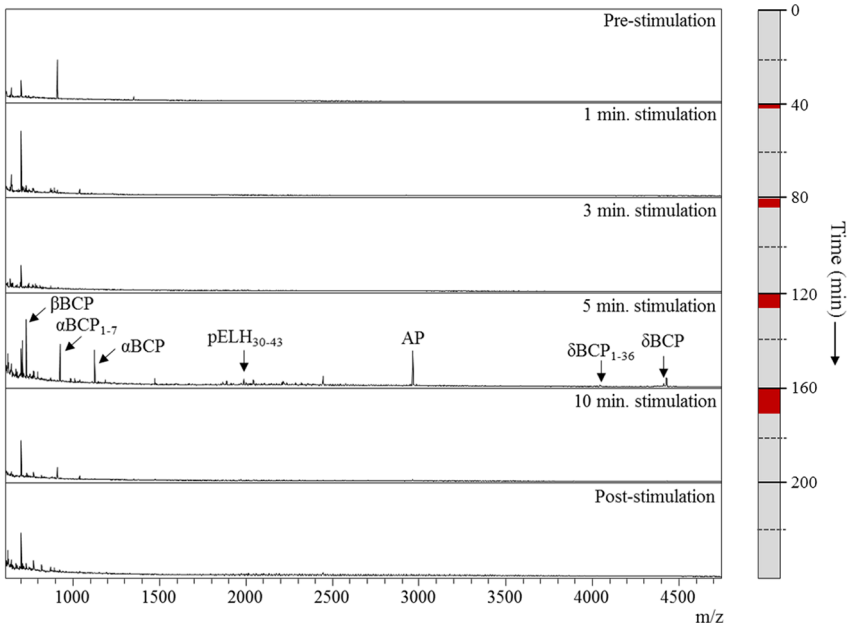

Figure 2. Series of representative mass spectra of bag cell neuron peptide release following KCl stimulation at increasing stimulation exposure times. A number of previously identified peptides from the bag cell neurons were observed following 5 min of exposure to KCl (βBCP, m/z 728.4; αBCP_{1-7}, m/z 922.5; αBCP, m/z 1122.6; AP, m/z 2959.5; $pELH_{30-43}$, m/z 1471.8; δBCP_{1-36}, m/z 4022.7; δBCP, m/z 4406.9). On the right of the mass spectra are an example of a time course for the chemical stimulation protocol series. Red bars represent chemical stimulation addition; gray bars indicate ASW addition. Each exposure was 40 min in duration with stimulation time and ASW addition varying. Two collections were done during each exposure time at t = 20 min and t = 40 min.

via flow-induced stress and motions across the culture chamber or by damaging the cells upon chemical stimulation.

Each neural network was exposed to elevated K^+ for a combination of exposure times. For example, a group of neurons may have been exposed to K^+ for 1 min, 3 min, 5 min, 10 min, and 20 min or a subset of these exposure times, sequentially, with ASW as the pre- and postcontrol. Following each stimulation exposure, the microvalves were closed and ASW was flowed through the device until the total time reached 40 min, allowing any released peptides to be washed out of the device for collection. Solutions were collected twice during the 40 min period to gain additional temporal release information. After collection, samples were desalted and concentrated with C_{18} pipet tips and eluted onto a MALDI target for analysis.

In this study, nine different groups of bag cell neurons, each in separate devices, received increasing periods of K^+ stimulations and peptide release was detected. The resulting mass spectra were collected twice during each exposure time. Figure 2 shows a representative series of intensity-normalized mass spectra following 1, 3, 5, and 10 min exposures to K^+. A number of well-studied peptides were detected following 5 min of K^+ exposure, demonstrating that a 5 min exposure is sufficient to cause release. In general, peptide release was observed in both 20 min collections after the 5 min stimulation period (n = 4; out of seven devices where peptide release was observed). Peptides were not observed in the pre- and postconditions, indicating that the shear stress caused by flow does not stimulate or lyse the cells. Because of its relatively good detection limits compared with the other egg-laying hormone-related peptides, the detection of acidic peptide (m/z 2959.5) at S/N $\geq$ 5 was used to indicate when peptide release had occurred. Figure 3 shows the percentage of the time that acidic peptide was detected versus the elevated K^+ exposure time.

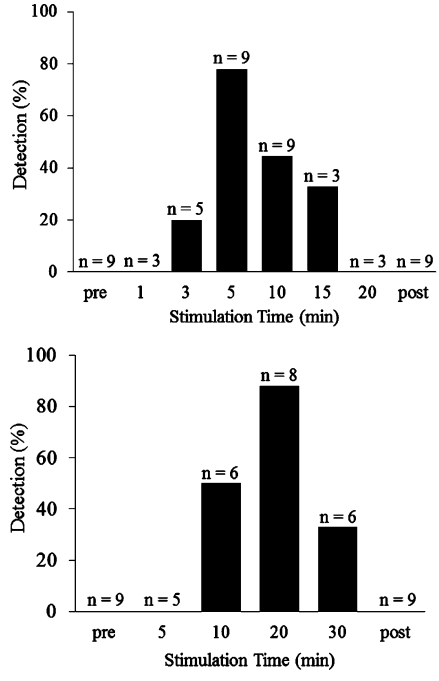

Figure 3. Percentage of the time that acidic peptide (m/z 2959.5), a known peptide of the bag cell neurons from *Aplysia californica*, is detected versus stimulation exposure time for KCl and insulin. The n represents the number of distinct repeats (individual devices containing a distinct network of neurons) used that were stimulated for the indicated time. Stimulation time courses were varied to demonstrate that peptide release is independent of previously applied stimulations to the cells. (Top) 5 min of KCl exposure time was necessary to elicit peptide release 79% of the time. (Bottom) 20 min of insulin exposure was necessary to elicit peptide release 88% of the time.

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Regardless of previously applied K^+, exposure to 5 min of K^+ resulted in the observance of release 80% of the time, whereas, when the same neurons were exposed to K^+ for a shorter period (<5 min), release was detected less than 50% of the time. This demonstrates that exposure times of less than 5 min usually did not elicit release. For subsequent exposure periods (>5 min), acidic peptide detection decreased. In part, this decrease is due to the refractory period that bag cells enter following release.[44] During this period, regardless of the length of stimulation applied, the cells are not able to sustain peptide release. Since bag cell neurons are involved in reproduction, this refractory period is consistent with the fact that the animals can only lay eggs periodically. Furthermore, this shows that the temporal resolution of our platform is able to capture both the onset of peptide release and the refractory period after release. We did observe differences in the temporal aspects of release between experiments, which may represent differences in the release dynamics between individual animals.

Insulin Stimulation. Elevated K^+ is a general secretagogue that causes release of vesicles (including dense core vesicles containing peptides) in a neuron through the depolarization of the cell membrane. Therefore, it should elicit release from most, if not all, neuronal subtypes. In contrast with the depolarization caused by K^+, which directly affects potassium channels, insulin is a cell-specific chemical stimulation that is internalized through the phosphorylation of receptor tyrosine kinases.[45,46] Therefore, cells without this receptor will not respond to insulin application. Previous studies have shown that bag cell neurons contain insulin receptors.[47] Unlike the widely accepted model of neurotransmitter exocytosis, which involves the influx of external Ca^{2+}, bag cell neurons are unusual because the release of intracellular Ca^{2+} (such as caused by insulin exposure) is enough to result in peptide secretion.[48] The application of 5 μM of mammalian insulin causes peptide release through such an extracellular Ca^{2+}-independent process.[48] While the structure of mammalian insulin is distinct from *Aplysia* insulin peptides, it is similar enough to still function in *Aplysia*.[39,47,48] To demonstrate that the networks maintained in our device exhibit a similar physiology to semi-intact animals in previous reports[39,47,48] and to document that small numbers of bag cell neurons in our reduced network respond to insulin (something not shown before), nine additional devices, each containing a distinct network of bag cell neurons, were exposed to increasing periods of insulin to determine the temporal dynamics of peptide release. In contrast to K^+, 20 min of insulin exposure was necessary to detect peptide release. The slower response is not surprising given that insulin-evoked release may require peptide internalization, a process that is expected to take longer than the release induced via potassium ions.

In addition to acidic peptide, a number of egg laying hormone-related peptides were detected (Figure 4). It was previously reported that internal Ca^{2+} increases within 5−20 min of exposure to insulin.[48] Our data agrees with this; at least 20 min was necessary for peptide release to occur. Furthermore, in the majority of devices where release occurred following 20 min of insulin exposure, peptides were only observed in the first collection (n = 4; out of 7 devices where peptide release was observed). Devices where peptides were collected in both 20 min collections, signal intensities were at least 2.5 times greater in the first collection (n = 3; out of 7 devices where peptide release was observed).

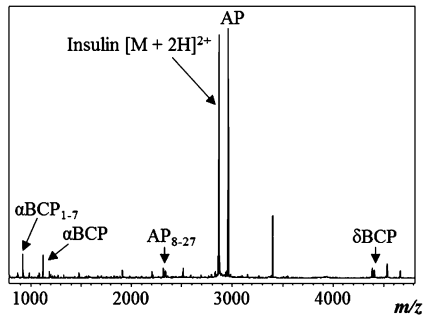

Figure 4. Representative mass spectrum of peptide release following 20 min of insulin exposure (αBCP_{1-7}, m/z = 922.5; αBCP, m/z = 1122.6; AP_{8-27}, m/z = 2316.2; AP, m/z = 2959.5; δBCP_{1-36}, m/z = 4022.7; δBCP, m/z = 4406.9).

Jonas et al.[48] showed that the egg-laying hormone (which is stored in the same peptidergic vesicle that contains acidic peptide) was released following insulin application. However, their use of radioimmunoassay required the preselection of one particular peptide of interest. MS allows us to view an entire complement of released peptides. The egg laying hormone prohormone, the source of these peptides, has a tetrabasic site that is cleaved prior to vesicle packaging, resulting in the N- and C-terminal ends of the prohormone being packaged into distinct vesicles.[49]

The α, β, and γ-bag cell peptides are located near the N-terminus of the egg laying prohormone, and acidic peptide and egg laying hormone are located near the C terminus and each are packaged into these two peptide-containing vesicle populations. We observed both N- and C-terminal peptides, indicating that both types of vesicles are released upon insulin stimulation, at least under our culturing conditions. Furthermore, insulin $[M + 2H]^{2+}$ was observed for all exposure periods. This demonstrates that the stimulation solution fills the device in the time allotted for exposure.

Acidic peptide detection versus exposure time was again plotted to show the amount of insulin exposure time necessary for peptide release. Our observations were robust, with acidic peptide being detected 88% of the time following 20 min of exposure, whereas release was detected less than 50% of the time following a shorter insulin exposure. Similar to the refractory period seen with K^+ stimulations, we observed fewer peptides in the 30 min exposure period.

In the study done by Jonas et al.,[48] entire bag cell clusters, each containing hundreds of cells, were perfused with insulin for 70 min. They found that the amount of insulin-induced peptide release was similar to that of an afterdischarge. Their application of electrical stimulation would resemble our application of K^+, in that both trigger action potentials and an afterdischarge. Here, we show our ability to see peptide release following both K^+ and insulin exposure. However, our microfluidic valve system enables release studies from significantly fewer cells with higher temporal resolution. This increase in temporal resolution shows that two similar peptide releases (in terms of amount and peptide content) have different kinetics and, presumably, biochemical pathways.

CONCLUSIONS

Overall, this approach can maintain a neuronal culture and detect peptide release following appropriate physiological stimulations. The difference in exposure time necessary to elicit peptide release between K^+ and insulin demonstrates that

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by further controlling the microenvironment, we can gain a greater understanding of the conditions necessary for peptide release to occur. This knowledge increases our ability to apply essential molecules of repair to restore function to a damaged neural network. The ease of fabrication and cell culture, as well as the coupling to MS, make this device readily amenable to many neuronal types.

The system demonstrated here can be extended to work with other cell types and culturing platforms to enable the interface of culturing, stimulation, and release characterization. Potential enhancements include the ability to quantify the amount of peptides released such as via the approach for Zhong et al.[29] or other direct MS approaches such as the method described by Rubakhin and Sweedler.[50] In addition, via appropriate interfacing to CE−laser induced fluorescence (LIF)[51−53] and CE−MS,[54,55] more complete characterization of the extracellular media can be performed. Finally, as this system is transparent and contains an accessible culturing platform, future efforts can include Ca^{2+} imaging or electrophysiology to enable a new range of studies on neuronal release dynamics.

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Notes

The authors declare no competing financial interest.

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