

Electroanalytical Engineering Technologies for Monitoring Molecules in Neuroscience

Brenna Parke

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Department of Bioengineering

Imperial College London

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Dedication

To my parents.

Declaration of Originality

I, Brenna Parke, declare that the work presented in this thesis is original and all else is appropriately referenced. All experimental data and analyses performed in collaboration are described in the text and figure captions. The work in this dissertation was performed at or in affiliation with Imperial College London.

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Abstract

Depression is a complex neuropsychiatric disease whose exact aetiology has eluded scientists for decades. Theories such as the monoamine hypothesis and the newer inflammation hypothesis attribute the physiological underpinnings of depression to be within the brain or a brain-immune axis. Validating these theories has been difficult to execute because, until recently, few tools were available to measure neurotransmitters in the brain. This technology gap has left diagnosis and treatment of depressed patients up to the opinion of physicians (i.e. no chemical basis). Over the last few decades reliable tools to probe the brain in real-time have been developed and used in animals. While many useful studies have resulted using these technologies, conclusions from animal experiments are not easily translated to the human condition. Therefore, the focus of this thesis is to probe peripheral organs that share an inflammation axis with the brain. This thesis begins by introducing the topics of neurochemical signalling, immune signalling and neurotransmitter measurement techniques. Chapter 2 describes the general methods used for the work with specific methods mentioned in each chapter. To bridge the gap between *in vivo* and *in vitro* measurements, chapter 3 optimizes histamine measurements in tissue slice preparations to prepare for the cell work. Chapter 4 describes neurotransmitter release from hair cells, which share an immune axis with the brain. Chapter 5 extends this work to skin cells, where we show ambient neurochemical release from dermal fibroblasts is mediated by histaminergic receptors. In chapter 6 we pivot to endocrine signalling in the pancreas, where we develop novel methodology to co-detect serotonin and insulin and apply our new technology to insulin-secreting cells for the first real-time measurements of insulin. Finally, chapter 7 summarizes the entirety of the work and I offer my own perspectives on where this work should be taken in the future.

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine, serotonin
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotrophic hormone
AI	analog input
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AO	analog output
ATP	adenosine triphosphate
AUC	area under the curve
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BNST	bed nucleus of stria terminalis
BPD	bipolar disorder
CFM	carbon fiber microelectrode
CPu	caudate putamen
CRH	corticotropin-releasing hormone
CV	cyclic voltammetry
DA	dopamine
DAQ	data acquisition
DAT	dopamine transporter
DMEM	Dulbecco's modified eagle's medium
DPH	diphenhydramine
DRG	dorsal root ganglia
ECM	extracellular matrix
FBS	fetal bovine serum
FIA	flow injection analysis
FSCAV	fast scan controlled-adsorption voltammetry

FSCV	fast scan cyclic voltammetry
GABA	gamma alpha butyric acid
H ₃ R	histamine receptor 3
HBSS	Hank's balanced salt solution
HPA axis	hypothalamic pituitary adrenal axis
HPLC	high-performance liquid chromatography
HTMR	high-threshold mechanoreceptor
IL	interleukin
IMDM	Iscoe's modified Dulbecco's medium
IT	current vs. time
K15	keratin 15
LPS	lipopolysaccharide
LTMR	low-threshold mechanoreceptor
MDD	major depressive disorder
mTOR	mammalian target of rapamycin
NE	norepinephrine
NET	norepinephrine transporter
NFH	neurofilament heavy chain
NMDA	N-methyl-D-aspartic acid
OCD	obsessive compulsive disorder
OCT	organic cation transporter
ORS cells	outer root sheath cells
ovBNST	oval nucleus of the bed nucleus of stria terminalis
PAN	polyacrylonitrile
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PGA	polyglutamic acid
PH	posterior hypothalamus
PKC	protein kinase C

PLSR.....	partial least squares regression
p/s	penicillin/streptomycin
ROS	reactive oxygen species
SEM	standard error of the mean
SERT.....	serotonin transporter
SSRI.....	selective serotonin reuptake inhibitor
STIM	stimulation electrode
SYN1	synapsin 1
TGF	transforming growth factor
TMN.....	tuberomammillary nucleus
TNF	tumor necrosis factor
TRD.....	treatment-resistant depression
VMAT	vesicular monoamine transporter

1. Chapter 1 Introduction

Abstract

Depression is a complex neuropsychiatric disease that affects a large portion of the global population. Despite its prevalence, the underlying physiology of depression is not well-understood. Furthermore, the majority of the depressed population do not respond to pharmacological treatments. The heterogeneous nature of depression has puzzled researchers for decades, and, as a result, several theories outlining the physiology of depression have been posited. The monoamine hypothesis was the first to show that a chemical imbalance in the brain due to low levels of monoamines (serotonin, dopamine, norepinephrine) associated with depression. This theory was refined to isolate low brain serotonin in the pathophysiology of depression, aided by the development of serotonin targeting drugs (selective serotonin reuptake inhibitors, SSRIs) that had improved efficacies. However, as the population of depressed individuals has rapidly increased since the creation of SSRIs, their efficacy has significantly decreased. Alternative theories delineating the physiology of depression have described low interconnectivity between serotonin neurons (neuroplasticity) and increased levels of cytokines and histamine due to chronic inflammation in its aetiology. While these theories describe different physiological systems involved in depression, there is much biochemical overlap between them, including the immune system and hypothalamic-pituitary-adrenal (HPA) axis. Therefore, the work in this thesis is performed under the central hypothesis that the separate theories of depression work best to describe the pathophysiology of depression when synergized under the HPA axis and/or immune system. As the chemistry of depression is still largely attributed to the brain, niche techniques have been used to characterize neurochemical signalling of associated messengers (serotonin and histamine). Here, the intricacies of microdialysis (a sampling technique) and electrochemical techniques for monoamine detection, with special focus on fast scan cyclic voltammetry (FSCV), are described in detail. Within this thesis, we utilize FSCV to characterize novel signalling pathways

in the developing brain (*ex vivo* slices) and in peripheral systems that share an immune/HPA axis with the brain (hair, skin and pancreas). With the collective work, we offer new perspectives into serotonin signalling throughout the body and how its involvement throughout the body could be leveraged for future diagnostics in the context of depression.

Neurochemical Signaling

Probing neurological processes in the brain poses a difficult measurement challenge, as the peripheral body (outside of the brain) does not reflect the central nervous system (CNS). Separated by a tightly controlled blood-brain barrier (BBB), the brain is separated chemically from the rest of the body. Communication within the brain is *via* chemical messengers (neurotransmitters) between neurons and relay signals. These signals are tightly controlled by biological feedback mechanisms. For example, monoamine neurotransmitters are taken back up by protein transporters, which are usually considered neurotransmitter-specific, but have been found to be 'promiscuous' in that they take up several monoamines¹. An example of this can be found in serotonin uptake, where, at high concentrations dopaminergic transporters (DATs), norepinephrine transporters (NETs) and organic cation transporters (OCTs) will take up serotonin (Uptake 2) in tandem with their designated serotonin transporters (SERTs) (Uptake 1)^{1, 2}. Some transmitters like glutamate and γ -aminobutyric acid (GABA) are so tightly regulated that they are largely confined to the synaptic cleft. However, less-tightly regulated messengers such as dopamine and serotonin will spill out into the extracellular space, taking part in volume transmission. In addition to uptake, another important control mechanism for neurotransmitters are autoreceptors. Located in and outside of the synaptic cleft, autoreceptors control how the pre-synaptic neuron signals based on the concentration of the messenger in the extracellular space. These control mechanisms, among others, are imperative for regulating neurochemical signalling within the brain.

Depression

Neuropsychiatric disorders are medical conditions that involve both neurological and psychiatric issues. Disorders such as schizophrenia, bipolar disorder (BPD), major depressive disorder (MDD), anxiety, attention deficit disorder, obsessive compulsive disorder (OCD) and Tourette have specific phenotypes³, but the underlying physiologies are not well-defined. Some genetic markers have been described for schizophrenia⁴, BPD⁵ and Tourette's⁶, but no accessible chemical biomarkers have been identified. This has left all metrics for diagnosis to be based on observable behaviors identified by the patient and the physician. Additionally, many of these disorders are co-morbid, interchangeably diagnosed or misdiagnosed. Ayano *et al.* reported that approximately 40% of patients presenting with neuropsychiatric disorders are misdiagnosed, with a disordinate number of schizophrenic patients being misdiagnosed 75% of the time and BPD 18% of the time⁷. This rate of misdiagnosis between schizophrenia and BPD is because they share many behavioral characteristics. Unfortunately, no chemical biomarkers in the peripheral body have been found for any of these disorders. Studies have tried to identify genetic unifying genetic markers and/or chemicals in bodily fluids (blood, urine, saliva, sweat) but revealed inconsistent results⁸. Most of the data that describes contributing physiological mechanisms of some neuropsychiatric disorders are based on invasive animal studies or non-invasive imaging techniques, as we are limited by safe technologies to probe the human brain. Of the neuropsychiatric disorders, depression is one of the most pervasive, affecting over 270 million people and is cited as the leading cause of global disability⁹. Symptomatically, depression is characterized by feelings of sadness, hopelessness, disinterest in things once enjoyed, poor sleep, lack of appetite, low sex drive and miscellaneous aches and pains, which results in poor quality of life for long periods of time¹⁰. Being one of the most discussed diseases of the mind, there are several theories for depression's aetiology. The next section describes the current understanding of the physiological underpinnings of depression and how the data in this thesis was performed based around a synergy of these theories.

Serotonin in Depression

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter known colloquially as the 'happy molecule'. Within the brain, serotonin mediates a variety of biochemical processes: mood, sleep, appetite, learning, memory and sexual behavior¹¹. Serotonin levels are tightly controlled compared to other monoamines such as dopamine¹², as too high levels in the brain result in a fatal condition called serotonin syndrome. While this transmitter is imperative for neurological function, it is found in high concentrations throughout the body. Within the gut, serotonin is released from enterochromaffin cells and neurons in the lamina propria to control appetite and digestion¹¹. Moreover, serotonin has been found to crucially mediate endocrine function within the pancreas¹³. However, studying these peripheral serotonergic functions is a relatively new field, and new mechanisms are still being discovered. The ever-evolving understanding of serotonin in bodily processes is fascinating, but the most extensive research of serotonin signalling lies within the brain and its involvement in neuropsychiatric disorders.

Current antidepressant pharmacotherapies overwhelmingly target serotonin, based on the monoamine hypothesis of depression, implicating a dysregulation of the monoamines (dopamine (DA), norepinephrine (NE) and serotonin) in the brain. This hypothesis is based on low levels of these molecules found in post-mortem brain tissue from depressed patients¹⁴. Aided by some clinical efficacy of serotonin-targeting drugs (selective serotonin reuptake inhibitors, SSRIs), this theory has been refined to isolate low levels of brain serotonin in the physiology of depression¹⁰. SSRIs mechanistically work by blocking SERTs in the brain, prolonging the presence of serotonin in the extracellular space/synapse which propagates the chemical signal for longer. While SSRIs are the frontline treatment for depression, they only work for ~40% of patients¹⁵, leaving a majority left to seek alternative therapies (this condition is classified as treatment-resistant depression, TRD). Additionally, the efficacy of SSRIs is not drastically different from non-pharmaceutical therapies such as exercise, psychotherapy and placebo^{16,17}. This phenomenon has brought into question the validity of serotonin as a biomarker for depression, sparking much debate within the

community⁸. Additionally, with little new information from researchers to guide them, drug discovery efforts for depression have largely halted within the past 20 years¹⁸. The heterogeneity of depression has led to the development of alternative mechanisms outlining the underlying pathophysiology.

There is evidence based on the efficacy of alternative pharmaceutical therapies for depression that suggests that decreased neuroplasticity is involved in the aetiology of depression. Neuroplasticity is the morphological and functional adaptation to stressors in the environment. Its implication in depression originates from decreased neuroplasticity found in the hippocampus and prefrontal cortex of depressed patients¹⁹. Supporting this idea, is an alternative therapy for patients with TRD, ketamine, used primarily as an animal tranquilizer. It has been found that sub-anesthetic doses of ketamine given once every few months helps alleviate depressive symptoms in TRD²⁰. Ketamine's primary mechanism of action is to non-competitively antagonize N-methyl-D-aspartate (NMDA) receptors²¹. Although this agent shows some efficacy, how it works in the context of depression is not well-defined. It is theorized that ketamine is an effective antidepressant because its action on the α -3-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (co-localized with NMDA receptors) which upregulates brain-derived neurotrophic factor (BDNF) and mammalian target of rapamycin (mTOR) to promote synaptic plasticity²¹. A growing body of evidence has suggested that ketamine indirectly affects the serotonin system, with a recent publication by Witt *et al.* showing that an acute administration of ketamine raises basal serotonin levels in the CA2 region of the hippocampus in a similar way to SSRIs (escitalopram and fluoxetine)²², potentially linking the serotonin and synaptic plasticity hypotheses of depression. Another atypical therapy for depression is psilocybin. This drug is a psychedelic that is known to agonize serotonin receptors²³ and has data to support the role of neuroplasticity in depression²⁴. A seminal study by Daws *et al.* showed that psilocybin induced more brain network integration within 5-HT_{2A} receptor-rich higher-order functional networks from clinical trial data found in humans²⁵. Research into using psilocybin to treat depression has grown in popularity, but there are conflicting sentiments as to whether its efficacy lies in its mechanism of

action or if its psychedelic effects make people happier²⁶. Both atypical therapies for depression are associated with neuroplasticity but are also highly linked to serotonin. It is therefore highly likely that serotonin plays a central role in the aetiology of depression as the link between various hypotheses.

Histamine in Depression

Histamine is another example of a monoamine signalling molecule. Within the brain histamine is thought to regulate sleep-wake cycles and play a role in neurodevelopment²⁷. As an immune messenger, histamine is released from mast cells in the peripheral body and microglia within the brain during neuroinflammation²⁸. The involvement of histamine in depression has been postulated in the cytokine hypothesis, outlined below.

Straying from the solely neurological postulation of depression, the cytokine hypothesis posits that increased levels of proinflammatory cytokines during an inflammatory response elicits depressive phenotypes. When an immune response occurs, T cells and B cells release proinflammatory cytokines into the bloodstream. This hypothesis implicating this proinflammatory cytokine release has been developed over the years by several studies finding increased levels of interleukins (IL)-6, IL-10, tumor necrosis factor (TNF)- α and decreased levels of tumor growth factor (TGF)- β in blood serum in depressed individuals²⁹⁻³¹. Cytokines can cross into the brain through the BBB and some cytokines are endogenously produced by neurons, astrocytes and microglia³². This connection between the immune system and depressive phenotypes has been supported by behavioral studies that employ an induced immune response in rats. In these studies, rats given an immune challenge (lipopolysaccharide, LPS) exhibited depressive-like behavioral phenotypes, which persist 24-48 hours after proinflammatory cytokine levels in the serum and liver return to baseline³³. Additionally, patients given cytokine immune therapies for cancers and viral diseases frequently exhibit depressive symptoms³⁴. When standing alone, this hypothesis does not explain depression in its entirety but shares several links to the catecholamine hypothesis. First, proinflammatory

cytokines directly influence monoaminergic systems in the brain by disrupting synthesis, reuptake and release of monoamine neurotransmitters³⁵. Second, SSRIs such as fluoxetine are known to be excellent reactive oxygen species (ROS) scavengers³⁶. During an immune response there is an increased generation of ROS species that affect cellular oxidation processes, and the efficacy of these SSRIs may be attributed to this anti-inflammatory effect in addition to their effects on serotonin. Finally, high levels of stress have been linked to both worsening psychiatric phenotypes and high levels of proinflammatory cytokines through the hypothalamic-pituitary-adrenal (HPA) axis^{37, 38}. When a stressor is perceived, the HPA axis is activated³⁹. First, the hypothalamus releases corticotropin-releasing hormone (CRH), causing the pituitary gland to release adrenocorticotrophic hormone (ACTH) into the bloodstream. The ACTH signals to the adrenal glands on the kidneys to release cortisol into the bloodstream. These high levels of cortisol negatively affect memory and learning by blocking memory retrieval and consolidation, phenotypes strongly associated with depression⁴⁰. Cytokines can modulate the secretion of both ACTH and cortisol⁴¹. With a large body of evidence connecting the monoaminergic and immune systems, it can be inferred that their respective hypotheses are linked to the HPA axis.

In addition to proinflammatory cytokines, histamine levels are significantly increased during an immune response. In response to stress or injury, mast cells release histamine in large quantities, a process known as mast cell degranulation⁴². This histamine release induces vascular and tissue changes in the peripheral body. The stress response also induces neuroinflammation in the brain (the HPA axis) where histamine and cytokines are released from microglia and histamine from histaminergic neurons²⁸. Histamine neurons and their associated receptors have been shown by our group and others to modulate the serotonin system. Samaranyake *et al.* showed that a stimulated release of histamine in the PH induced a subsequent inhibition of serotonin⁴³. This phenomenon was found to be mediated by the H₃ receptor (H₃R). Building on this work, Hersey *et al.*, showed that an acute challenge of LPS increased histamine levels in the PH, further inhibiting serotonin release⁴⁴. Additionally, they found that the same acute LPS challenge

decreased basal levels of extracellular serotonin, and when given an SSRI to raise serotonin levels, the drug was most efficacious when paired with a histamine synthesis inhibitor (alpha-fluoromethyl histidine, α -FMH). This study showed that inducing an immune response in the peripheral body directly affects brain serotonin. Therefore, it can be posited that if a patient with depression is in a state of inflammation and does not respond to SSRIs, the high histamine levels in the brain are most likely working against the action of the antidepressant. Furthermore, it is feasible that the serotonin and inflammation hypotheses together more accurately outline the physiological underpinnings of depression when synergized.

Brain-Body Axis

While there is much evidence interconnecting the cytokine and monoamine hypotheses of depression, the defined physiology is confined within the brain. Efforts to identify peripheral biomarkers for depression have been measuring blood serotonin levels in MDD patients⁴⁵, screening for genetic variants⁴⁶, and identifying immune markers (IL-6) in the blood³¹. These studies have yielded heterogeneous results with no unifying conclusions. As the poor process for diagnosis and treatment of depression leaves a large global population of people to suffer, it is of high merit to explore different physiological models that might give some more chemical insight into the physiology of depression. For this task we hypothesize that the skin and endocrine systems are worthy avenues of exploration.

Brain-Skin Axis

There is a strong connection between neuropsychiatric disorders and the skin. Within the field of psychodermatology, it is well-accepted that skin conditions (many with underlying immunological aetiologies) are connected with psychiatric symptoms. Psychodermatological experts deem that flare ups of visible skin conditions (acne, eczema, psoriasis) can cause psychological reactions⁴⁷. This psychological component is reported in many cases of psoriasis. Psoriasis is a long-lasting autoimmune condition in which skin cells multiply 10 times faster than normal, and instead of shedding off the dead skin cells, they

build up on the surface⁴⁸. This cellular build-up results in scaly, itchy patches of skin. Many patients report psoriasis flare-ups during periods where their psychiatric symptoms worsen⁴⁹. Similar anecdotes have been described in eczema^{50, 51}. Psychiatric disorders can also manifest themselves in the skin, a condition called delusional parasitosis⁵². While the evidence is correlative, the underlying immune aspect of these skin diseases in conjunction with the known monoaminergic modulation of immune cells the HPA axis brings into question the connectivity between the brain and skin (Figure 1.1).

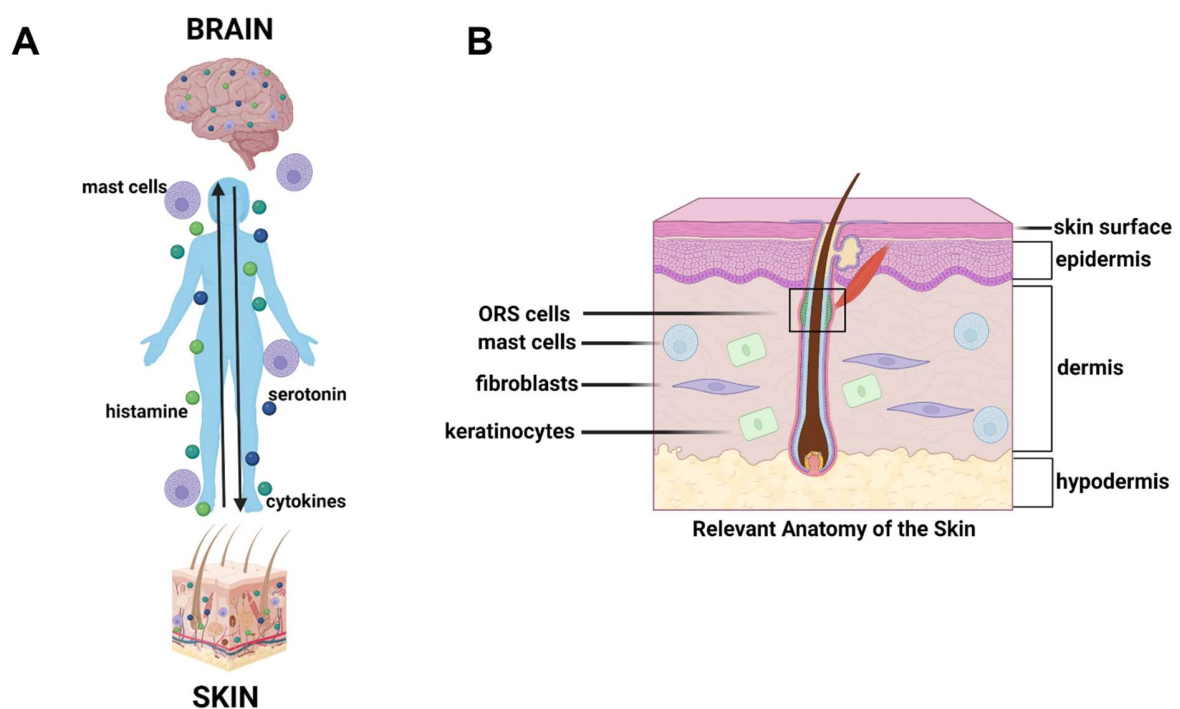


Figure 1.1. Illustration of the newly hypothesized skin-brain connection. (A) In this work it is theorized that the brain and skin communicate an immuno-sensory axis where afferent neurons receive serotonergic and histaminergic signals in response to tactile stimuli and transmit the information to the brain. Based on the concomitant worsening of skin and psychiatric symptoms, we believe this axis is bi-directional. (B) The skin and hair follicle cells we hypothesize or have been shown to release neurochemicals, in which some will be probed in later chapters. Made using Biorender.com.

Within the skin there are many cell types and layers that work in harmony to mediate the sense of touch and our interactions with the outside world. Chemical messengers associated with neurological and immune function have been found or postulated to be in the skin. Dermal histamine in the skin is known to come from mast cells⁴² and keratinocytes⁵³. Additionally, dermal fibroblasts exhibit histamine receptors on their surface, having been shown to secrete or degrade collagen in response to extracellular histamine levels⁵⁴. Excitingly, there is indirect evidence for serotonin signalling in the skin from biochemical analyses showing synthesis markers and the presence of serotonin receptors⁵⁵. Additionally, Merkel cells (epidermal sensory cells) have been shown to release serotonin⁵⁶ and norepinephrine⁵⁷ in response to mechanical stimuli. A hallmark study by Chang *et al.* found that low-threshold mechanoreceptors (LTMRs) on Merkel cells are activated, they transmit serotonin to A β -afferent neuron endings in the skin⁵⁶. This biological relationship, deemed a neuroepithelial unit, was the first to demonstrate how tactile information is chemically mediated. As afferent neurons are found throughout the skin and surrounding hair cells within the dermal papillary layer, it raises the question of how tactile information is chemically mediated from other cell types in the skin and the role it plays in depression.

The robust immuno-physiological connection in the skin is intriguing because, if found to be connected, the skin could serve as a chemical basis for depression. We explore these brain-skin connections in this work by characterizing the chemical signalling profiles of human-derived outer root sheath (ORS) cells that grow from the hair follicle (Chapter 4) and human dermal fibroblasts (Chapter 5).

Endocrine Loops

In addition to its role in the skin and brain, serotonin is known to mediate biochemical processes throughout the endocrine system. The endocrine system is a hormonal signalling network that uses hormones and peptides to maintain homeostasis within the brain and body. Endocrine glands consist of

the hypothalamus, pituitary gland, pineal gland, thyroid, parathyroid, pancreas, adrenal glands, testes (male) and ovaries (female). These glands secrete hormones into the blood stream to mediate almost every biological function in the body, notably: metabolism, development, mood, sleep, appetite, blood sugar and sexual function. The endocrine system is mediated by serotonin and the HPA axis in organs such as the hypothalamus (serotonin receptors), pancreas (beta/alpha cell regulation) and liver (tissue regeneration)¹¹. Induced stress in mice resulted in increased synthesis and metabolism of serotonin in the brain⁵⁸⁻⁶⁰. As all components of the HPA axis are endocrine organs, it is no surprise that this system is affected by stress responses. Prolonged stress, where cortisol levels are chronically high, can lead to endocrine dysfunction such as insulin resistance⁶¹. A serious endocrine disease is diabetes, a metabolic disease in which insulin signalling is disrupted. While its aetiology is still in question, consequences of leaving diabetes untreated are neuropathic pain, kidney failure, nerve damage, cognitive impairments and a weakened immune system (chronic infections). Recent work has shown that serotonin plays a critical role in insulin regulation. Serotonin has been shown to increase beta cell mass during metabolic challenges such as pregnancy and high-fat diets, where beta cells increase synthesis and release of serotonin to stimulate their own proliferation. Almaca *et al.* added to this pathway showing that serotonin from beta cells at high glucose stimulates the 5-HT_{1F} receptor on alpha cells to reduce glucagon secretion, showing serotonin's control over blood glucose levels. Clearly, serotonin plays a large role in endocrine functionality and has connections to the brain *via* the HPA. What remains unclear is how the pancreas is connected to psychiatric disease. connection between the endocrine system and brain.

To explore this avenue in the context of depression, we developed a new tool to directly probe insulin and serotonin quantitatively and qualitatively with one temporally resolved electrochemical measurement (Chapter 6)

Psychiatric disorders are a difficult public health issue to challenge, as they are not well-understood and efforts to find peripheral biomarkers have not yielded positive results. Depression is no exception and the

community's division in backing one physiological hypothesis for depression has made it difficult to define a unifying pathway. However, the serotonin, cytokine and neuroplasticity hypotheses have intertwined biochemical pathways. In this work, we hypothesized that the three hypotheses of depression are indeed synergized *via* immune and HPA axes extending to the skin and endocrine system (illustrated in Figure 1.2). Working under this hypothesis, we explored neurochemical signalling in peripheral systems that share an immune axis with the brain (skin and pancreas).

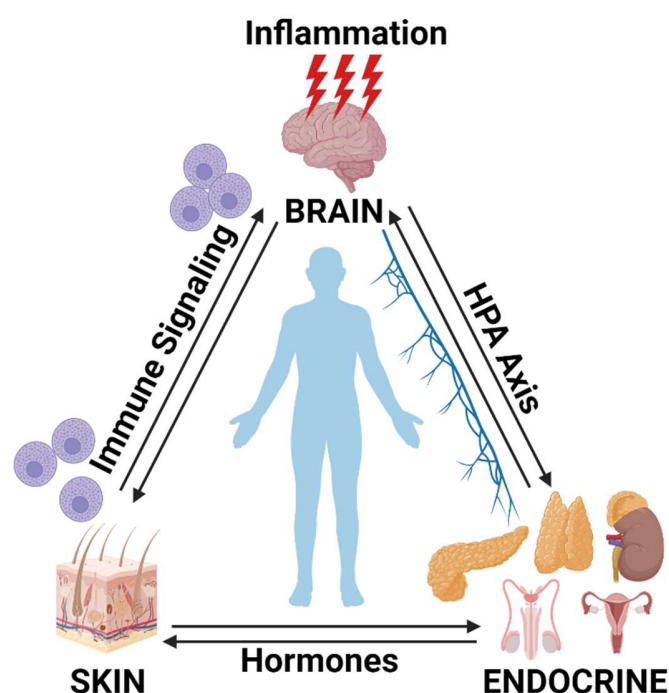


Figure 1.2. Hypothesized signalling axes between the skin, endocrine system and brain in depression. In this work we hypothesize that the immune system and HPA axis mediate the chemistry of depression between the skin, gut and brain. During a state of inflammation, the immune system releases proinflammatory cytokines that suppresses serotonin signalling in the brain. The endocrine system is affected by this stress response *via* the HPA axis, and serotonin in endocrine organs is then dysregulated. Together, this indicates that histamine and serotonin are promising peripheral biomarkers for depression. Made using Biorender.com.

Measurement Techniques to Probe Neurochemicals

Characterizing signalling mechanisms in the brain poses a difficult measurement challenge, as the brain is a complex matrix that is constantly in flux. Therefore, measuring neurochemicals requires a high degree of selectivity and sensitivity. Additionally, neurotransmission occurs on a millisecond timescale, a difficult temporal scale to achieve for many measurement techniques. Methods that meet some or all these criteria are: microdialysis and electrochemical methods. Microdialysis is a sampling technique that is considered the gold standard for neurotransmitter detection. Electrochemical techniques such as fast scan cyclic voltammetry (FSCV), fast scan-controlled adsorption voltammetry (FSCAV), high-speed chronoamperometry and amperometry with biosensors can be employed to monitor up to 2 redox-active molecules at a time. These electrochemical techniques are versatile in the sense that they are optimized for the brain but can easily be applied to other biological systems such as cultured cells. Compared to the brain, cells are more accessible biological models useful to probe complicated mechanisms because they are isolated from the body. Below I describe the specifics of microdialysis and other electrochemical techniques with a focus on FSCV, as the data in this thesis was collected in both the brain and in cells using FSCV.

Microdialysis

Microdialysis is a well-established sampling method for *in vivo* neurochemical detection (outlined in Figure 1.2). For this, a probe with micro-dimensions is inserted into the brain and infused with a perfusion solution that is similar to the osmotic makeup of the interstitial fluid. Then, the perfusate is flowed through the probe and molecules from the surrounding tissue are allowed to diffuse through the semipermeable membrane at the end of the probe, and flow out as the dialysate. The membrane allows for small molecules to pass through rather than larger bacteria and viruses. The dialysate is collected for separation and secondary analyses. Species collected in the dialysate are separated high performance liquid chromatography (HPLC)⁶² with secondary analysis using analytical techniques such as UV/Visible

spectroscopy⁶³, electrochemical detection⁶⁴ and mass spectrometry⁶⁵. For depression, microdialysis has been used to characterize serotonin dynamics through direct drug application⁶⁶. A study by Gardier *et al.* used a chronically implanted microdialysis probe to show that a chronic dose of an antidepressant, paroxetine, activated 5-HT_{1B} autoreceptors⁶⁷. While this important work has given us much insight into *in vivo* neurochemical signalling, there are some limitations to the technique. The probes for microdialysis are typically ~250 µm in diameter, and when inserted in the brain, damage the surrounding tissue and induce an immune response⁶⁸. Additionally, the dialysate is sampled at a frequency of ≥1 minute (typically ~10-20 minutes)⁶⁸. Therefore, microdialysis cannot capture neurotransmission in real-time (milliseconds). While microdialysis has shed light on important physiological mechanisms, alternative methods must be used to directly probe neurotransmission in real-time.

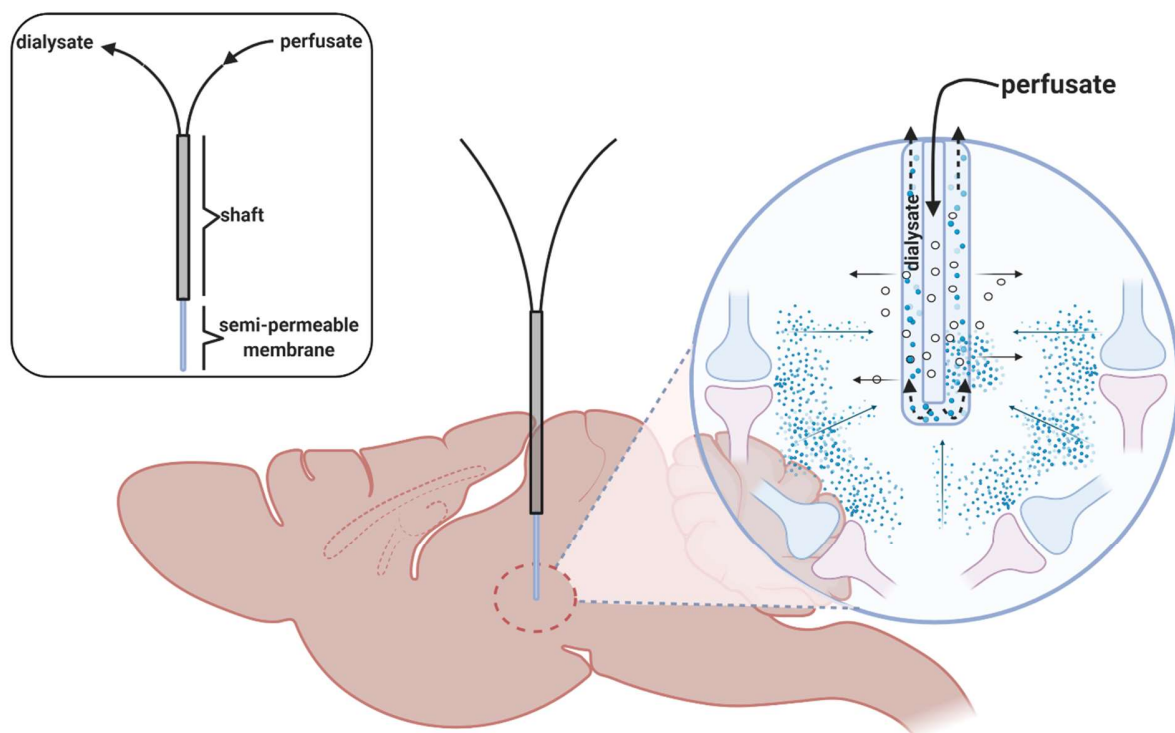


Figure 1.3. Illustration of an *in vivo* microdialysis sampling experiment in the brain. Microdialysis probes inserted in the brain are perfused with a solution similar to the osmotic makeup of the interstitial fluid, deemed the perfusate.

Neurochemicals in the surrounding extracellular space are allowed to diffuse through the semi-permeable membrane and flowed out as the dialysate. The dialysate is collected, and contents separated *via* HPLC for qualitative and quantitative analysis with an analytical method (UV/Vis, electrochemistry or mass spectrometry). This figure is used with permission².

Electrochemical Techniques

Electrochemical techniques for neurotransmitter detection offer high temporal resolution, high sensitivity and molecular specificity at (mostly) carbon-based electrodes. Carbon-based electrodes are often employed for electrochemical detection because of their biocompatibility, flexibility and customizable dimensions. In the last few decades, electrochemical technologies have been developed for *in vivo* neurotransmitter detection. As a result, researchers have been able to elucidate neurological phenomena and underlying disease circuitries. In this section we will discuss the nuances of amperometry and FSCV for probing cellular signalling in and out of the brain.

Amperometry is a technique in which a single potential is applied, and the resultant current is from any species that oxidizes at that potential. This technique does not have any temporal constraints since the potential is constantly applied. The oxidative currents collected are non-specific, therefore amperometric sensors aimed for detection in a complex matrix are usually modified with enzymes or other biospecific coatings for single-molecule specificity⁶⁹. Enzyme modifications are done by encapsulating an enzyme on the surface of the electrode. An example of an amperometric biosensor is for glutamate detection, where sensors are modified with glutamate oxidase to selectively capture glutamate signalling by measuring the peroxide oxidation generated by glutamate interacting with the enzyme⁷⁰. However, these sensors are limited in their sensitivity by how much enzyme can be loaded, and often requires large quantities of enzyme to be trapped onto the surface, resulting in large probes that induce an immune response. Alternatively, amperometry for biological detection can be useful for simplified models, like *in vitro* cell

cultures. The Kennedy lab has successfully used amperometry at planar carbon fiber microelectrodes to detect insulin pulses in isolated beta cell cultures⁷¹. Other groups have applied amperometry in mast cell cultures^{72, 73}, PC12 cells⁷⁴ and chromaffin cells⁷⁵. Due to the qualification limits of amperometry, this was not used in more complex matrices. An extension of amperometry, high-speed chronoamperometry, is indeed utilized in the brain extensively by the Daws group. They use this method to look at uptake dynamics for serotonin and dopamine by spritzing a bit of their analyte near their probe and observe its uptake⁷⁶. Again, the oxidative current is attributed to the analyte they've exogenously applied, but not qualified within the measurement.

Detection in more complex matrices requires the ability to identify and quantify molecules of interest, which can be done electrochemically using cyclic voltammetry (CV). CV is an electrochemical technique that applies sweeping voltages to an electrode to induce redox reactions at discrete potentials. Redox peak positions allow for molecular identification, and concurrently the peak heights correspond to concentration. Traditional CV employs scan rates between 0.01-1 V/s, meaning a single measurement could take up to several minutes depending on the applied potential window. Therefore, traditional CV does not have temporal resolution suitable to capture neurotransmission. Alternatively, FSCV (illustrated in Figure 1.4) is an electrochemical technique that employs scan rates ($1-10^5$ V/s), improving upon acquisition times compared to traditional CV. FSCV has been largely used for *in vivo* detection of electroactive neurotransmitters⁷⁷⁻⁷⁹. For this technique, voltage schemes are applied at fast scan rates to induce reduction/oxidation (redox) reactions captured, typically, at the carbon surface of a carbon fiber microelectrode. Within the bounds of the voltage schemes, molecules that are adsorbed to or within the vicinity of the carbon surface, will oxidize and reduce at discrete potentials, allowing molecular differentiation based on their peak potentials. Additionally, the peak currents produced by these redox reactions are directly proportional to the number of molecules exchanging electrons, making the singular

measurement both a qualification and quantification. As the waveforms are applied at fast rates (typically 400-1000 V/s), this allows for sub-second temporal resolution (typically 10 Hz) as some waveforms are scanned within 2-10 ms. This temporal resolution is within the time frame of neurotransmission, making FSCV suitable to detect fast redox reactions within the extracellular space of the brain. In the context of neuropsychiatric diseases, monoamines readily undergo redox reactions, and there are well-established FSCV protocols for their detection^{77,80,81}. However, each monoamine varies slightly in their redox reaction sites and electron transfer kinetics. Consequently, different voltage schemes have been optimized for each molecule within the FSCV repertoire for stable, sensitive and selective measurements in the brain. Hashemi *et al.* demonstrated the difference in FSCV detection methods for dopamine and serotonin⁷⁷. When detecting serotonin using the dopamine-optimized waveform, the serotonin signal was inconsistent over time, and vice versa, when detecting dopamine on the serotonin-optimized waveform, the dopamine peak position shifted to more positive potentials and broadened. The peak-broadening effect indicated that dopamine had slower redox kinetics than serotonin and was therefore not suitable to detect with the serotonin-optimized waveform.

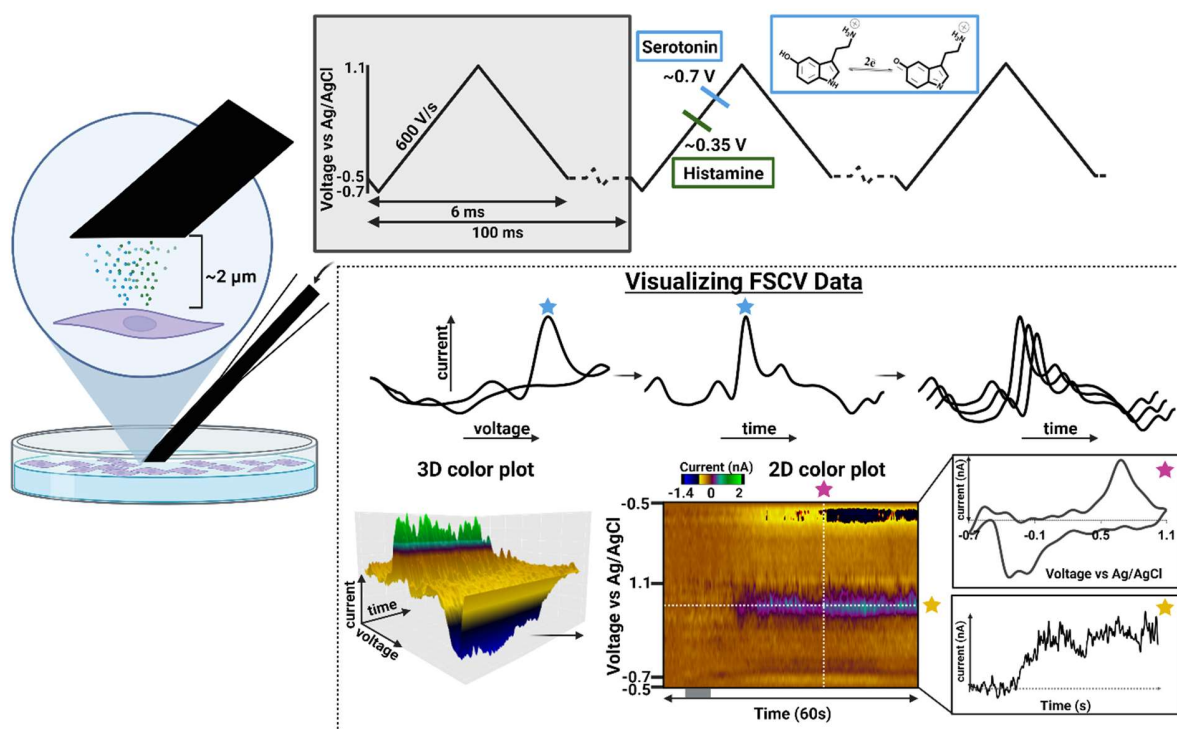


Figure 1.4. FSCV application of a waveform to co-detect serotonin and histamine at single-cell resolution. From a fibroblast cell, a bevelled CFM is placed $\sim 2 \mu\text{m}$ above the cell. A histamine-optimized waveform is applied to co-detect histamine ($\sim 0.35 \text{ V}$) and serotonin ($\sim 0.7 \text{ V}$) on the cyclic voltammogram. FSCV data is visualized by stacking CVs in their time-base in a 3D color plot. The 3D color plot is visualized in a 2D space (bird's eye view) with the current given in false color. Extrapolated data from the color plots are CVs (pink star) for analyte identification and current vs. time traces (yellow star) for biological information. Made with Biorender.com.

Another benefit of FSCV is that the CFM dimensions are tunable, with commercially available carbon fibers as low as $5 \mu\text{m}$ in diameter. As mentioned, large probes induce an immune reaction that results in a loss in sensor sensitivity and damage in the surrounding tissue, so the small dimensions of the CFMs significantly reduces the immune response. This is further evidenced by the difference in post-experimental histological analyses that show that microdialysis probes create a lesion at the implantation site, whereas CFMs do not leave this track mark. A normal protocol within FSCV labs is to apply a large voltage ($\sim 13 \text{ V}$) to their implanted CFM so that the large current creates a damage track mark in the tissue

at the implantation site for histological validation^{22, 82}. However, FSCV is indeed limited by the large capacitive background current that is subtracted out at the beginning of each measurement window, resulting in all measurements reflecting a change from baseline. Additionally, as certain molecules have similar or overlapping oxidation and/or reduction potentials, *in vivo* measurements often require pharmacological validation of the signal. Regardless, FSCV has been an essential tool used to uncover cellular signalling mechanisms in *in vivo* and *in vitro* systems.

Using FSCV, much important work has been done to elucidate circuitry involved in neurological diseases. Researchers have used FSCV to map cytoarchitecture⁸²⁻⁸⁴, determine the effects of monoamine targeting drugs^{85, 86}, and correlate dopamine dynamics to addictive behaviors^{87, 88}. The Wightman group performed many elegant behavioral studies with implanted electrodes in the nucleus accumbens. Notably, found that rats taught to self-administer cocaine had increased dopamine transients when they self-administered, that correlated with their learning they would get a cocaine reward⁸⁹. This study was the first to show that associated stimuli of an addictive reward would elicit dopamine transients, which modulate behavioral phenotypes in addiction. After pioneering the technique for serotonin detection, the Hashemi group elucidated the effects of antidepressant therapies on the serotonergic system^{85, 90}, found basal serotonin levels were inherently lower in a behavioral model of depression⁹¹ and a robust connection between serotonergic inhibition during an inflamed state⁴⁴. Their most recent work has shown that antidepressant therapies with different molecular targets all raise basal serotonin levels in a similar way providing a chemical basis for synergizing different theories for depression²².

While *in vivo* FSCV has been imperative for expanding our understanding of the brain, its application to cultured cells has teased out nuanced signalling mechanisms. Wightman notably showed through vesicular monoamine transporter 2 (VMAT2) knock outs in mast cells that the transporter is crucial for

serotonin and histamine accumulation in granules⁹². Isolated cellular models provide a simple system to understand otherwise complex signalling mechanisms. In this work we chose to simplify complicated physiological systems (the skin and pancreas) in cellular cultures to probe cellular signalling mechanisms with FSCV.

Scope of Dissertation

The aim of this dissertation is to explore synergistic biological models of serotonin release that are mediated by the HPA axis and/or the immune system in the context of depression. The work focuses on utilizing both existing and developing novel FSCV measurement techniques to characterize neurochemical and endocrine signalling molecules in real-time from a variety of tissue and cellular types: *ex vivo* brain tissue slices, hair cells, skin cells and mouse-derived pancreatic islets. This first chapter capitulated what is known about the physiology of depression with a special focus on serotonin and histamine. In Chapter 2, general methods common are described. In Chapter 3, we use *ex vivo* brain slices to evoke histamine release in the striatum from a bundle of nerves associated with psychiatric disorders, the bed nucleus of stria terminalis (BNST). Then, we pivot our animal practices to cell work. In Chapter 4 we adapt FSCV to cultured cells and, using human-derived hair cells, uncover that tactile stimuli are mediated by serotonin and histamine. Within this study we found that several skin and hair cell types all spontaneously release serotonin. We continue this work in Chapter 5 by characterizing the spontaneous activity of human dermal fibroblasts at a single-cell level. Finally, in Chapter 6, we offer a novel FSCV measurement technique for insulin and serotonin detection, validated in mouse-derived islets. The impact of this work is reflected upon in Chapter 7, where I offer my own perspectives and ideas for future directions.

Publications associated with each chapter:

Chapter 1: Buchanan, A. M.⁺; Parke, B.⁺; Hashemi, P., Experimental Methods for Investigating Uptake 2 Processes *In vivo*. *Handb Exp Pharmacol* **2021**, 266, 101-117.

Chapter 3: Marquez-Gomez, R.; **Parke B.**; Cras, Y.; Gullino, S. L.; Hashemi, P.; Ellender, T.; “Histamine originating from the BNST modulates corticostriatal synaptic transmission during early postnatal development”. *PNAS*. **2023** (*Under Review*)

Chapter 4: Agramunt, J.; **Parke, B.**; Mena, S.; Jimenez-Acosta, F.; Williams, G.; Limbu, S.; Hexter, M.; Knight, L.; Hashemi, P.; Kozlov, A. S.; Higgins, C. A.; “Mechanical Stimulation of human hair follicle outer root sheath cultures activates adjacent sensory neurons”. *Sci. Adv.* **2023**, 9 (43) eadh3273.

Chapter 5: **Parke, B.**[†]; Andriuskevicius, T.[†]; Rhodes, A.; Mena, S.; Higgins, C.; Hashemi, P.; “Monitoring Neurotransmitter Release from Dermal Fibroblasts in Real Time with Voltammetry”. *Anal. Chem.* **2023** (*In Prep*)

Chapter 6: **Parke, B.**; Suba, K.; Hansen, B.; Adan, C.; Lakhani, S.; Salem, V.; Hashemi, P.; “Real-time Insulin Measurements at Carbon-Based Microsensors with Fast Scan Cyclic Voltammetry”. *Anal. Chem.* **2023** (*In Prep*)

2. Chapter 2 General Methods

Abstract

Disrupted monoamine signalling is implicated in neuropsychiatric disorders such as depression, anxiety, obsessive compulsive disorder, Tourette's and addiction. A large body of work applying real-time electrochemical detection of monoamines in the brain with fast scan cyclic voltammetry (FSCV) has significantly improved the collective understanding of underlying pathophysiologies of psychiatric diseases. Measuring monoamine signalling in the brain using FSCV is typically performed using carbon fiber microelectrodes (CFMs), and therefore requires an in-depth understanding of electrochemical interactions at carbon surfaces. The work in this thesis was performed using several FSCV methods to probe neurochemical release in biological systems. While specific methods are described in each chapter, the commonalities are described here in detail including: FSCV hardware and software, CFM fabrication and surface modification and flow injection analysis. CFMs are fabricated to produce two types of electrodes: cylindrical and bevelled electrodes, which are typically made with different types of carbon fibers. Custom-built FSCV hardware and software are described with the inclusion of several hardware configurations with different capabilities. Finally, flow injection analysis using a custom-built flow cell is described for dynamic signal detection. The methods chronicled in this chapter are essential for FSCV experimentation and are used to probe monoaminergic signalling in *ex vivo* brain slices, hair cells, skin cells and pancreatic islets in subsequent chapters.

Introduction

The work presented in this thesis was made possible by utilizing several methodologies. While the specific methods will be described in each chapter, there are several methods consistent across the thesis. These methods (FSCV hardware, FSCV software, carbon fiber microelectrode (CFM) fabrication and surface

modification) are commonly used within the small community of FSCV researchers but are lesser known by the greater community and therefore, warrant a full description. There are many publications that have described the nuances of performing FSCV experiments^{80, 81, 93, 94}, but these details can sometimes be hard to source as they are often found within research articles. An additional hurdle is that there are certain, often crucial, experimental details that are performed but left out of the manuscripts. This process of tweaking experimental protocols without explicitly defining them in academic texts is common practice in many laboratories and the details are mostly passed down through generations of graduate students. FSCV research comes with a steep learning curve in terms of its theory but also the difficulty of working with custom-built hardware, software and delicate protocols. Therefore, it is my aim to not only describe FSCV methods used throughout this thesis but also disclose the important details and rationale behind each of them, citing their origin and/or giving some evidential basis. In this chapter, I delve into the theory behind carbon fiber microelectrode use and manufacturing, the hardware setup for an FSCV experiment and how to perform flow injection analysis (FIA) for controlled *in vitro* measurements. The references in this chapter have been carefully curated to aid future graduate researchers' understanding as to why they *must* perform certain steps for rigorous data turn around.

Carbon Fiber Microelectrode Fabrication

Carbon fibers are an excellent material for electrochemical detection in biological systems due to their biocompatibility, their dimensions are easily customizable and the striated surface full of negatively charged oxygen species readily promotes adsorption of electroactive species⁹⁵. Electrochemical reactions at CFMs predominantly take place at the carbon edge plane the fiber⁹⁶. This means that the carbon edge planes (lines of carbon atoms capped with different oxygenated moieties) must be exposed to the electroactive species rather than the basal plane (2D plane of sp^2 conjugated carbon atoms). Therefore, when choosing a carbon fiber material for electrochemical detection, it is imperative to know the orientation of the carbon sheets.

Carbon fibers generally come in two forms: pitch-based and polyacrylonitrile (PAN)-based. PAN-based carbon fiber is made with a synthetic fiber called polyacrylonitrile, which is first oxidized then carbonized at high temperatures in an inert atmosphere⁹⁷. These fibers have edge-plane sites containing the majority of functional oxide groups with greater electron density on the surface of the fiber⁹⁶. PAN-based fibers are best for making cylindrical CFMs as their carbon edge planes are exposed on the surface of the fiber and they are extremely flexible, a bonus for *in vivo* measurements. The other type of carbon fiber is pitch-based. These carbon fibers are made from a material called pitch, comprised of aromatic hydrocarbons produced by the distillation of carbon-based materials (plants, crude oil and coal)⁹⁸. The conjugated carbon rings align at their basal planes and wrap around each other vertically, like rose petals, so when pitch-based fibers are sliced horizontally, this exposes their carbon edge plane (which looks like the inside of an onion)⁹⁸. Bevelled CFMs are best made with pitch-based carbon fibers because the carbon edge plane is exposed when the carbon-epoxy-glass end is polished⁹³. Normally, cylindrical CFMs are made with fibers 5-7 μm in diameter (ideal for *in vivo* measurements) and bevelled CFMs with fibers 10 μm in diameter for single-cell resolution. Depending on the measurement, these dimensions can be altered as carbon fiber comes in various thicknesses. Recent work by the Sombers lab has characterized bevelled electrodes made with carbon fiber 34 μm in diameter, which they found minimized neurotransmitter escape from the electrode surface when measuring exocytotic events at single cells⁷⁵. While this much larger sensor was not employed in this thesis, it demonstrates the versatility in fabricating CFMs and is also worth considering in the design of future cell experiments.

The CFMs used in this work are cylindrical and/or bevelled (Figure 2.1). Cylindrical electrodes are used for insertion into *ex vivo* tissue slice preparations (Chapter 3), bulk neurotransmitter detection from hair and skin cells (chapter 4) and insulin detection (Chapter 6). Bevelled electrodes are used for neurotransmitter detection at single fibroblast cells (Chapter 5) as well as for insulin detection from INS-1 cells and islets (Chapter 6). The specific methods for making these CFMs are described below.

Cylindrical Carbon Fiber Microelectrode Fabrication

Cylindrical CFMs are made first by aspirating a carbon fiber 5-7 μm in diameter (T-650, Goodfellow, Pittsburg, PA, USA) into a single barrel borosilicate glass capillary (0.6 mm OD x 0.4 mm ID x 100 mm long (*in vivo* or *ex vivo* experiments), 1.0 mm OD x 0.58 mm ID x 100 mm long (cell experiments), Science-Products, Hofeim, Germany). Then, the filled capillary is pulled under heat and gravity using a micropipette puller (PE-22, Narishige, Tokyo, Japan) to forge a carbon-glass seal. The protruding fiber is cut to 50-150 μm (depending on the experiment) with a scalpel under an optical microscope (Olympus CX43, Olympus life science, Tokyo, Japan). An electrical connection is made by inserting a pinned stainless-steel wire coated with silver epoxy (Silver Print II, Farnell, Leeds, UK) into the opposite end of the protruding carbon fiber.

Carbon Fiber Microelectrodes

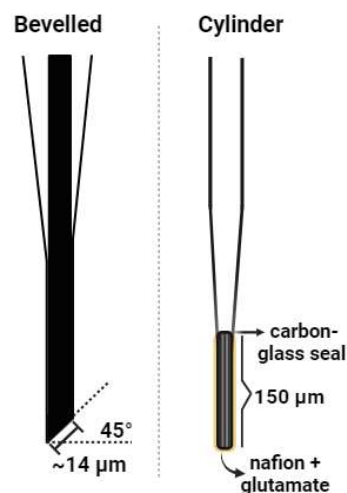


Figure 2.1. Carbon fiber microelectrode illustration. (A) depicts a bevelled CFM made with a pitch-based carbon fiber 10 μm in diameter sealed in a pulled glass pipette and epoxy. The exposed, elliptical carbon surface is polished at a 45° angle. (B) depicts a cylindrical CFM made with a PAN-based carbon fiber 5-7 μm in diameter. The exposed carbon fiber from the carbon-glass junction is cut to 150 μm and surface-modified with a thin layer of Nafion™ and polyglutamic acid. Made using Biorender.com.

Bevelled Carbon Fiber Microelectrode Fabrication

Bevelled CFMs, a planar form of CFM utilized for probing cellular signalling, are made in a similar manner as cylindrical CFMs. This method is described in detail elsewhere⁹³ but is recounted here for clarity. First, a carbon fiber ~10 µm in diameter (P-55, Cytec, Woodland Park, NJ, USA— the Ewing Lab) is aspirated into a single barrel borosilicate glass capillary (1.0 mm OD, 0.58 mm ID, 100 mm in length, Science-Products, Hofeim, Germany). Then, the filled capillary is pulled under heat and gravity using a micropipette puller (Narishige, Tokyo, Japan) to forge a carbon-glass seal. The protruding carbon fiber is cut as close to the carbon-glass seal as possible (within 10 µm). The electrode is dipped in a solution of 14% (w/w) m-phenylenediamine and Epon-828 (Sil-Mid, Birmingham, UK) heated to 80 °C for ~30s. The epoxied electrodes are left to gel overnight at 25 °C, then cured at 100 °C then 150 °C for 2 hours each. The cured electrodes are bevelled to a 45° angle on a bevelling wheel (BV-10, Sutter Instruments, Novato, CA, USA) covered with a solution of 1% Kodak Photo-Flo fluid (Parallax Photographic Coop, Brixton, London, UK) in 0.9% NaCl. An electrical connection is forged by inserting a pinned stainless-steel wire coated with silver epoxy into the end of the capillary opposite the bevelled fiber.

Carbon Fiber Microelectrode Surface Modifications

Carbon surface modifications can improve the detection limits and selectivity of sensors towards their target analyte and have been critical for optimal detection of serotonin, histamine and many other analytes with FSCV. There are several ways to modify the carbon surface to enhance sensitivity and selectivity: over-oxidation of the surface, electrochemical etching, attaching ionic polymers and trapping enzymes for biosensors. These modifications can be used alone or in combination for optimized measurements towards redox-active molecules. Several surface modifications were used in this thesis and are discussed below.

In its pure form, the carbon fiber surface is ideal for detecting adsorption-driven redox reactions. The combination of the rough surface covered in negatively charged oxygen groups readily promotes molecular adsorption. An additional benefit of carbon fiber is that when the applied potential is swept ≥ 1.0 V the outermost layer of the carbon surface will oxidize, exposing a clean, etched surface with more negatively charged oxygen groups⁹⁹. The potential limits of a waveform can influence this behavior by increasing (wide potential window) and decreasing (small potential window peaking at <1.0 V) the amount of negatively charged oxygen groups on the surface to tune the affinity of adsorption¹⁰⁰. A study by Holmes *et al.* demonstrated this principle by pre-treating the CFM with a wide potential window (-1.2 to 1.3 to -1.2 V at 400 V/s for 10 minutes at 60 Hz) to expose more oxygen groups by etching the carbon surface¹⁰¹. This pre-treatment was shown to significantly increase sensitivity towards serotonin and dopamine. As such, over-oxidizing the surface of CFMs is useful to improve detection limits for adsorption-driven redox reactions. In this work, I utilize this protocol to roughen the surface of bevelled electrodes for insulin detection as the process of bevelling makes the carbon surface smooth, almost eliminating adsorption completely (Chapter 6, Figure 6.5).

A more complicated, but common way to modify the CFM surface is by attaching polymers to exclude or promote adsorption of certain molecules. A hallmark study by Hashemi *et al.* showed that electrodepositing a thin layer of NafionTM significantly increases CFM sensitivity to serotonin and repels 5-hydroxyindolacetic acid (5-HIAA), a degradation product of serotonin that interferes with the measurement at CFMs⁷⁷. This surface modification was critical to selectively detect serotonin in the brain. As a result, over a decade of important *in vivo* serotonin work has come out of the Hashemi lab^{12, 44, 82, 91, 102, 103}. In addition to our success with NafionTM, other groups have used PEDOT-PSS¹⁰⁴⁻¹⁰⁶ and other polymers¹⁰⁷ to enhance their measurements. Holmes *et al.* also found that applying the wide potential window (mentioned above) induced the polymerization of polyglutamic acid (PGA) onto the carbon surface¹⁰¹. They found that coating the CFM with PGA alone significantly enhanced the sensitivity of the

CFM to serotonin, but a compounded coating of NafionTM and PGA was optimal for serotonin detection.

The specific method with materials is described below.

Electrodeposition of NafionTM and polyglutamic acid

The electrodeposition protocols for NafionTM and PGA are the same for both bevelled and cylindrical CFMs. NafionTM electrodeposition is described in detail elsewhere⁷⁷, but briefly: a constant potential of 1 V is applied to a CFM dipped that is into a solution of NafionTM (LQ-1105, Ion Power, New Castle, DE, USA) for 30 s vs a pseudo-Ag/AgCl reference electrode. The CFMs are baked at 70 °C for 10 minutes and left to rest overnight before use. The electropolymerization of PGA was performed by first placing the CFM in a solution of 1 μ M L-glutamic acid and then applying a waveform with a wide potential window (-1.2 to 1.3 to -1.2 V at 400 V/s) for 10 minutes at 60 Hz versus a pseudo-Ag/AgCl reference electrode¹⁰⁸. Both surface modifications can be performed alone or in combination. When both NafionTM and PGA are on the CFM, the NafionTM procedure must be performed before the PGA procedure, as the NafionTM needs to stabilize overnight.

FSCV Hardware and Software

Potentiostats are specialized instruments that apply voltage to an electrochemical system and detect the current response at the connected electrodes. These instruments can be purchased from many different suppliers to run a multitude of electrochemical methods. As FSCV runs voltage schemes at high scan rates (typically 400-1000 V/s), this exceeds the capabilities of most commercial potentiostats. As such, for many years the only potentiostat capable of running FSCV waveforms was made by the Dagan corporation (CHEM-CLAMP). The rest of the complementary hardware was forged and modified by chemists in academia. These setups are bulky, complicated and need significant troubleshooting. The general setup includes data acquisition (DAQ) cards from national instruments (PCI-6341 and PCI-6221, National Instruments, Houston, TX, USA) placed inside a large desktop PC (any that have multiple PCIe and PCI

slots), connected to a hand-made 'breakout box' that is the interface between the software and the Dagan potentiostat, which reads the current at the CFM and reference electrodes (figure 2). Within the breakout box are two pinned blocked (CB-68LPR, National Instruments, Houston, TX, USA) that are custom-wired to have several connections: analog to digital converters, digital to analog converters, digital connections and electrical stimulation inputs/outputs (Figure 2.4). Over the years, depending on experimental needs and budgetary constraints, this hardware has been added to and adjusted to suit experimental needs. To improve understanding of how these systems work I have included a detailed configuration of the hardware used in this thesis (Figures 2.2-2.6) and offer a new *slightly* modified configuration I developed for a more portable FSCV system (Chapter 3, Figure 3.1).

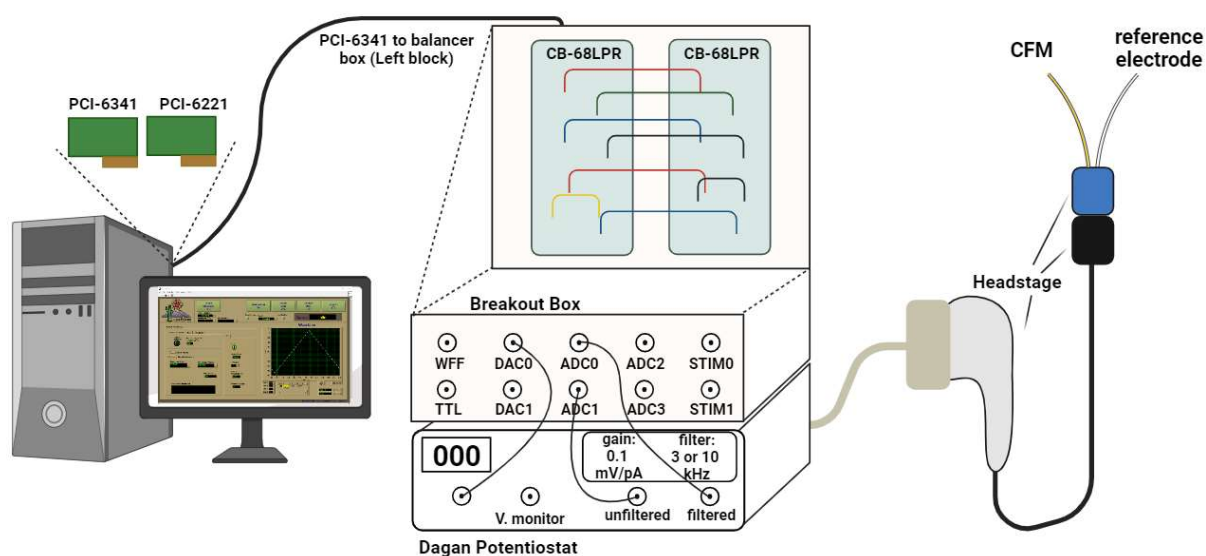


Figure 2.2. FSCV hardware for a desktop set-up. The waveform parameters are input into the WCCV 4.0 software. The software interfaces with the multifunctional I/O device (PCI-6341) in a PCI slot within the computer tower. The internal PCI-6341 is connected to a custom-wired breakout box with two CB-68LPR connector blocks. The internal wiring that interconnects the two blocks (specifically outlined in appendix A, figure A.2) has several types of outputs: digital to analog converters (DAC 0-1), analog to digital converters (ADC 0-3), digital connections (WFF and TTL) and electrical stimulation inputs/outputs (STIM 0-1). These are connected *via* BNC cables to a Dagan potentiostat. Finally,

the potentiostat is wired directly to a head stage fitted with connections to the CFM and reference electrode. The function of this set-up is to run an FSCV waveform and collect data (no stimulation). Made using Biorender.com.

There are three software programs written specifically for FSCV data acquisition and analysis: HDCV (Chapel Hill) (Wake Forest University) and Wildcat (shown in Figure 2.2, WCCV 4.0, Knowmad Technologies, LLC, Tuscon, AZ, USA). Both were developed by academics trained in FSCV to be functional for data collection and analysis. In the Hashemi lab, Wildcat is the software of choice for data collection. Recently, the Hashemi lab offered an improvement towards FSCV data analysis for all the 'Analysis Kid' (<https://analysis-kid.hashemilab.com/>)¹⁰⁹. The Analysis kid is an open access tool useful for any FSCV data that needs to be analyzed as it requires '.txt' files rather than the Wildcat-specific (undefined) file type and has tools to background-subtract, filter and model any uploaded data. The FSCV data in this thesis was analyzed with both Wildcat and Analysis Kid depending on the project and is explicitly stated in the following chapters.

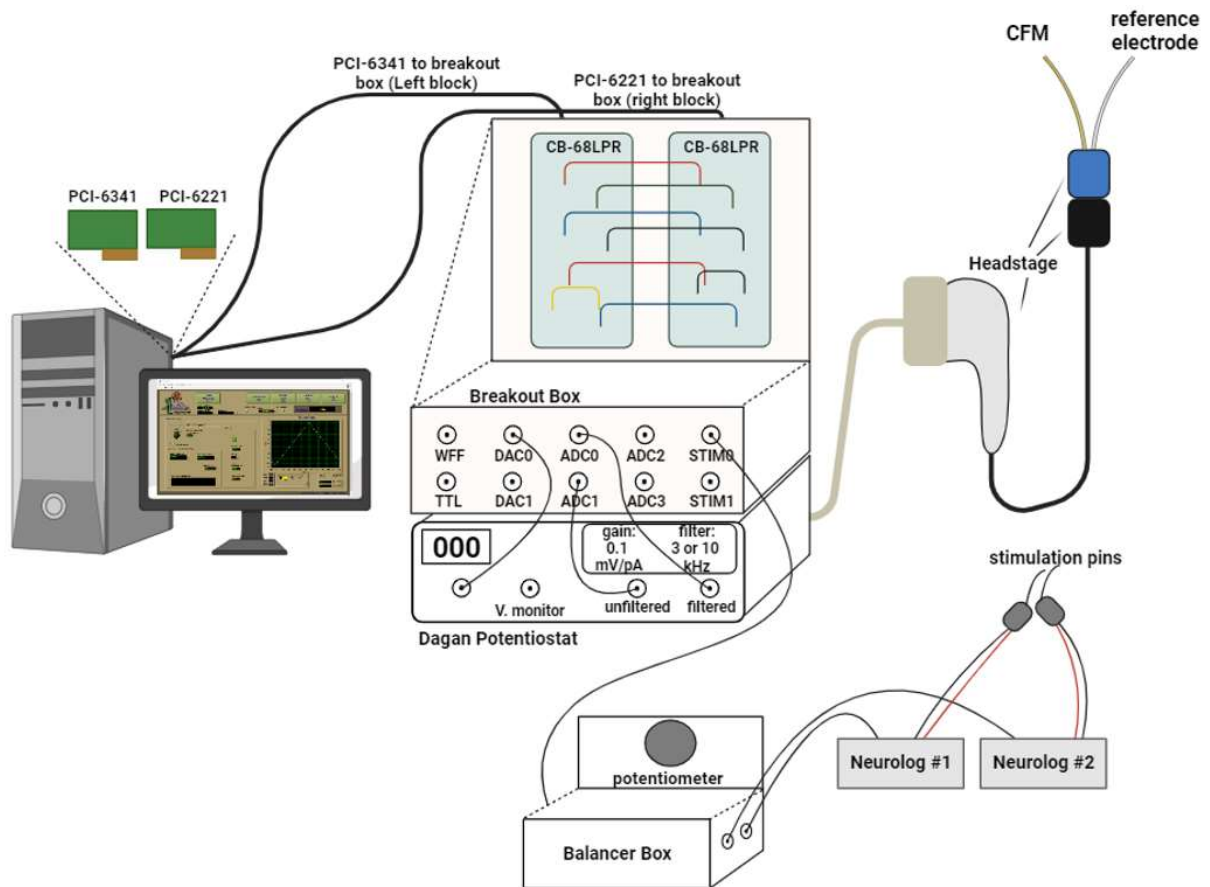
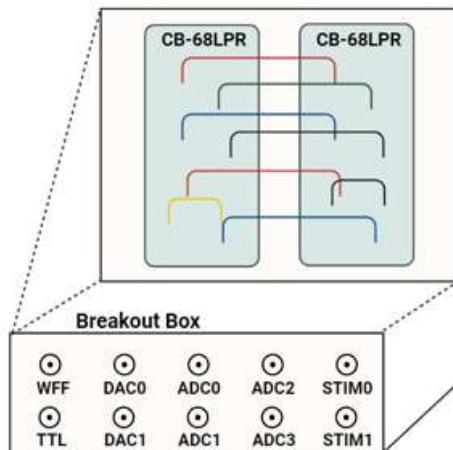


Figure 2.3. Electronic Hardware with Internal DAQ Cards to Run FSCV and Apply Electrical Stimulation. This system uses two multifunction I/O cards (PCIe-6341 and PCIe-6221) within the computer tower that interface between the WCCV software and the breakout box. The PCIe-6341 card runs the FSCV waveform for the potentiostat (connected to the left CB-68LPR block in the breakout box). This sends the waveform instructions to the potentiostat to apply to the CFM and reference electrode connected *via* the head stage. The PCIe-6221 card delivers the electrical stimulation paradigm the user puts in the software through the balancer box, which filters the analog data to the neurologs. The neurologs deliver the electrical pulse(s) to the stimulation pins. Made using Biorender.com.

Breakout Box Internal Connections

External BNC connection	Left Block		Right Block	
WFF	J2	J36		
TTL	J1	J35		
DAC 0	J22	J55		
DAC 1	J21	J54		
ADC 0	J68	J34		
ADC 1	J33	J66		
ADC 2	J65	J31		
ADC 3	J30	J63		
STIM 0			J22	J55
STIM 1			J21	J54
	J11		J1	
	J44		J35	
	J21	J25		
	J55	J23		
	J54	J58		
	J22	J57		



PCI-6341 pinout		breakout box connection
1	PFI14/P2.6	TTL + PFI0 (PCI-6221)
2	PFI12/P2.4	WFF
21	AO1	DAC 1
22	AO0	DAC 0
23	AI15 (AI7)	AO GND (6341)
25	AI6 (AI6+)	AO 1 (6341)
30	AI3 (AI3+)	ADC 3
31	AI10 (AI2-)	ADC 2
33	AI1 (AI1+)	ADC 1
34	AI8 (AI0-)	ADC 0
35	DGND	TTL + DGND (PCI-6221)
36	DGND	WFF
54	AO GND	DAC 1
55	AO GND	DAC 0
57	AI7 (AI7+)	AO 0 (J22)
58	AI14 (AI6-)	AO GND (J54)
63	AI11 (AI3-)	ADC 3
65	AI2 (AI2+)	ADC 2
66	AI9 (AI1-)	ADC 1
68	AI0 (AI0+)	ADC 0

PCI-6221 pinout		breakout box connection
11	PFI0/P1.1	PFI 14 (PCI-6341)
21	AO1	STIM 1
22	AO0	STIM 0
44	DGND	DGND (PCI-6341)
54	AO GND	STIM 1
55	AO GND	STIM 0

Figure 2.4. Breakout Box Configuration with Connections Referenced to the Internal DAQ Cards. The breakout box contains two CB-68LPR blocks wired between each other and to the male BNC outputs. The DAC ports are connected to analog outputs and the ADC ports are connected to analog inputs from the PCIe-6341 card *via* the left block. The TTL and WFF ports are connected to digital outputs between the PCIe-6341 and PCIe-6221 cards. The STIM ports are connected to the analog outputs from the PCIe-6221 card *via* the right block. Made using Biorender.com.

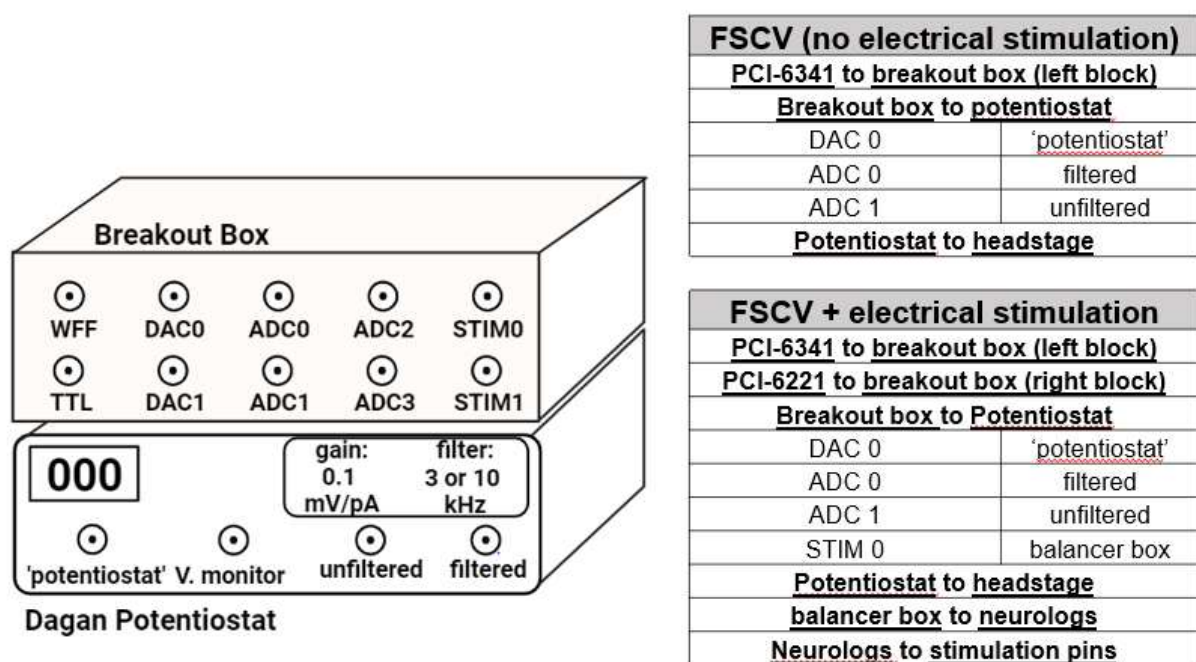


Figure 2.5. FSCV Hardware Connections With and Without Electrical Stimulation using Internal DAQ cards (PCI-6341 and PCIe-6221). Made using Biorender.com.

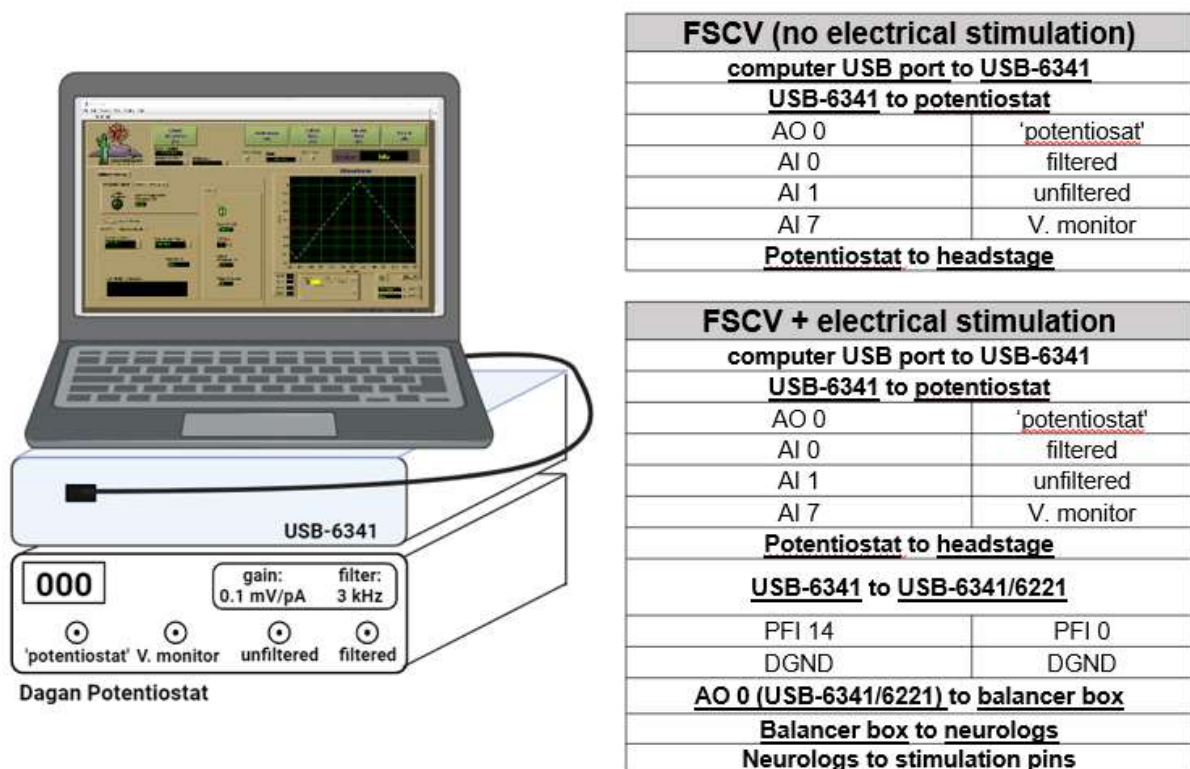


Figure 2.6. FSCV Hardware Connections With and Without Electrical Stimulation Using External DAQ (USB-6341 and USB-6221). Made using Biorender.com.

FSCV Data Acquisition and Analysis

All FSCV data was acquired using WCCV 4.0 software (Knowmad Technologies, LLC, Tuscon, AZ, USA), a PCIe- or USB-6341DAC/ADC (National Instruments, Austin, TX, USA) device, a Dagan potentiostat (Dagan Corporation, Minneapolis, MN, USA) and a Pine Research head stage (Pine Research Instrumentation, Durham, NC, USA). Histamine, serotonin and/or dopamine were co-monitored using a histamine-optimized waveform (-0.5 to -0.7 to 1.1 to -0.5 V at 600 V/s) at 10 Hz⁷⁸. Serotonin was monitored alone using the “Jackson waveform” (0.2 to 1.0 to -0.1 to 0.2 V at 1000 V/s) at 10 Hz¹¹⁰. Prior to each experiment, a pseudo-Ag/AgCl reference electrode was made) by electroplating AgCl onto a silver wire applying 5 V (A-M Systems, Sequim, WA, USA) in 1 M HCl for ~30 s to deposit silver chloride onto the surface. Additionally, prior to taking measurements, the waveform of choice was applied to the electrode at 60 Hz for 10

minutes, then at the acquisition frequency of 10 Hz for another 10 minutes. This paradigm is employed because this was found to efficiently equilibrate the electrode to the environment, stabilizing the background current⁸⁰.

Flow Injection Analysis

The time resolution of FSCV is one of the most advantageous that allows for real-time detection of a dynamic signalling system (the brain, cells, organoids, etc). As such, proper characterization of FSCV sensors requires a controlled setup that can mimic a fluctuating system. FIA is an experimental system that flows a solution (i.e. a salt buffer) at a fixed rate through a 2-position HPLC valve to a CFM in a flow cell. The 2-position valve (Figure 2.7) can flow the buffer directly to the electrode (position A) or push through the sample loop (position B), filled with an analyte of interest to the CFM to induce a change in signal. Although this sort of system has been used by the FSCV community for years, not much has been published on it¹¹¹, and until recently home-made flow cells have been the standard. While the idea and execution of FIA is one of the simpler facets of FSCV research, the home-made systems come with several problems that disrupt the flow and require electrodes to be creatively fashioned with a nut to screw into the top (increasing the level of difficulty when fabricating them). Recent work by the Hashemi lab has improved on the elbow joint by producing a troubleshoot-free flow cell for streamlined FIA measurements, where the CFM is placed within a small opening in the flow cell so that it makes contact with the flowing solution, depicted in detail by Hexter *et al.*¹¹². All FIA experiments in this work were done using this custom flow cell, a 2-position six-port HPLC valve (VICI Valco, Houston, TX, USA), and 1/16" PEEK tubing (VICI Valco, Houston, TX, USA).

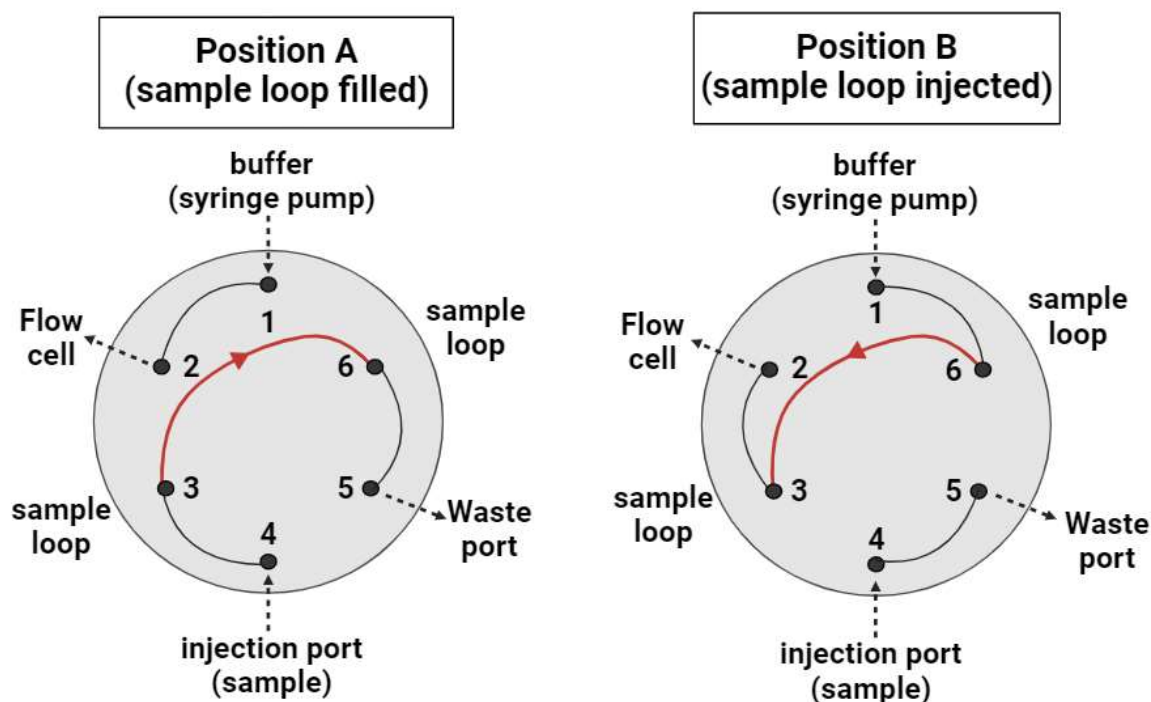


Figure 2.7. Flow Injection Analysis 6-Port Valve Diagram. Position A depicts the buffer (port 1) inlet to the CFM mounted in the flow cell (port 2) with the sample loop bypassed. In this position the sample is injected into the sample loop (ports 3 and 6) the injection port (port 4) and excess solution in the sample loop is directed to the waste port (port 5). When the valve is changed to Position B, the buffer (port 1) is pumped through the sample loop (ports 3 and 6), delivering the pre-loaded sample to the CFM mounted in the flow cell (port 2). Made using Biorender.com.

Each FIA experiment uses a syringe infusion pump (Harvard Apparatus, model 70-4500, Cambridge, UK) controlling the flow of a salt buffer at 1.7 mL/min. A freshly made pseudo-Ag/AgCl reference electrode is placed in a designated space just below the CFM and the hole is plugged with blue tack (warmed up by fingers) to seal the system (when dry). The CFM is mounted and lowered into the flow stream until the exposed fiber is fully submerged (visualized with the oscilloscope). An analyte-specific optimized waveform is applied at 60 Hz for 10 min and then at 10 Hz for 10 min prior to data collection. For each

injection, the analyte is introduced to the flow stream for 10 s *via* a six-port HPLC loop injector (Cheminert valve, VIVI, Houston, TX, USA) resulting in a rectangular plug.

In this work, the FIA set-up was used to assess sensor performance by metrics of sensitivity (calibrations), selectivity (multi-analyte injections), stability (repeated injections over several hours) and to optimize waveform parameters (Chapter 6). For calibrations, 5- or 7-point curves (check any analytical chemistry textbook) were generated by applying different concentrations of an analyte, with each point repeated in triplicate. Stability experiments were performed by injecting one concentration of an analyte every 2 minutes over several hours to replicate the time a sensor would be used to probe a biological system. Selectivity was characterized by injecting known concentrations of multiple analytes within the same sample. These methods are used throughout this thesis, and the specific methods for developing a waveform for insulin and serotonin co-detection will be described within chapter 6.

Conclusions

The methods outlined in this chapter are required for almost all FSCV experimentation. While they are innately difficult, a clear understanding of these concepts is imperative to successfully perform experiments with FSCV. CFM fabrication and surface modification are crucial for high quality, sensitive measurements, FIA is critical for sensor development and validation, and the hardware connections need to be understood in the case of any systemic malfunctions or modifications. The intention behind this chapter was to provide a concise resource to help future FSCV researchers understand why certain experimental details are crucial and where they come from. The remaining chapters in this thesis describe novel, highly collaborative research that uses diverse biological models and methods to characterize neurochemical signalling.

3. Chapter 3 Histamine from the ovBNST Crosses Anatomical Borders into the Striatum

The work presented in this chapter was done in collaboration with the Ellender laboratory at the University of Antwerp. All data presented here was performed by Brenna Parke with the assistance of Dr. Tommas Ellender for Figure 3.4.

Abstract

Histamine is a neuromodulator manufactured in the cell bodies of the tuberomammillary nucleus and is thought to be released all along the projecting axons. Histamine is implicated in the pathophysiology of neuropsychiatric diseases such as Tourette's syndrome, depression and anxiety. There is a need for robust biological models to ask physiological questions about histamine transmission within the context of disease states. Our group has optimized fast scan cyclic voltammetry (FSCV) at carbon fiber microelectrodes (CFMs) for *in vivo* histamine detection, however these studies are difficult to perform and are not high throughput. A higher throughput way to ask physiological questions is by using tissue slice preparations, *via* which a whole cohort of data can be obtained within a day or two. Therefore, in this work I develop the first *in vitro* system to measure histamine with FSCV in brain slices. First, I configure a portable FSCV hardware system to facilitate a niche collaboration. Then I determine that the oxygenated buffer used for tissue slice experiments causes a significant downshift in histamine's oxidation potential. Finally, I show the first histamine measurements in brain slices *via* a collaboration with the Ellender group at the University of Antwerp in Belgium. Together, this work offers new means for collaboration with FSCV and that histamine detection in brain slices is a feasible, high throughput way to understand the physiological mechanisms of histamine release.

Introduction

Histamine in the brain is synthesized in neurons found in the tuberomammillary nucleus (TMN) of the hypothalamus¹¹³⁻¹¹⁵. While it has been mostly associated with the wake-sleep cycle^{116, 117}, disruptions in histamine signalling have been implicated in the pathophysiology of neurodevelopmental processes¹¹⁸⁻¹²⁰ and psychiatric diseases¹²¹ such as Tourette's syndrome, anxiety and depression. Findings in mice have correlated lower histamine levels with the aetiology of Tourette's syndrome¹²²⁻¹²⁴, a neuropsychiatric illness characterized by involuntary behavioral ticks¹²⁵. Highly comorbid with Tourette's, depression and anxiety both have altered brain histamine in their pathophysiology. Disruptions in brain histamine levels have been correlated to anxiolytic behaviors in rodents¹²⁶, and high histamine levels resulting from immune activation is associated with depression²⁸. There are several research groups including our laboratory and newer collaborators who desire robust biological models to study histamine transmission to ask physiological questions. Our group has optimized *in vivo* histamine measurements using fast scan cyclic voltammetry (FSCV) at carbon fiber microelectrodes (CFMs). However, these experiments are difficult to perform and are not high throughput, evidenced by the best scenario being to perform one successful experiment per day which is largely due to the dorsal location of the PH. A higher throughput way to ask physiological questions is with tissue slice preparations, with which one can perform around five experiments per day. Many groups have applied FSCV to brain tissue slices to ask physiological questions about neurochemical signalling. Wightman notably mapped dopamine uptake across different regions in brain slices and found similarities to *in vivo* dopamine dynamics¹²⁷. West *et al.* have recently used brain tissue slices to elucidate novel signalling mechanisms mediating serotonin reuptake¹²⁸. However, histamine signalling in tissue slices has not yet been characterized with FSCV.

In this chapter, I develop the first *in vitro* system to measure histamine with FSCV in brain tissue slices to be used within our own laboratory and for niche collaborations *via* a portable system. First, I discuss the development of a portable FSCV hardware system to streamline collaboration. Second, I characterize

histamine signals in standard buffer used for tissue slice measurements. Then, I detail the experimental set-up and optimization of the tissue slice measurements, and finally present the first measurement of histamine release in acute striatal brain slices for a collaboration with the Ellender Lab at the University of Antwerp in Belgium. In sum, I have configured the first portable system for FSCV histamine measurements in tissue slice preparations.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. 'Cutting' aCSF (65 mM sucrose, 85 mM NaCl, 25 mM NaHCO₃, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 7 mM MgCl₂, and 10 mM glucose, pH 7.4) was prepared and chilled overnight at 4°C. Prior to slicing, 0.5 mM of CaCl₂ was added to ~300-400 mL of cutting aCSF, and this solution was kept on ice while bubbled with carbogen gas (95% O₂-5% CO₂) for ~30 min. 'Recording' aCSF (126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄·2H₂O, 10 mM glucose, 1.35 mM NaHCO₃, 2 mM CaCl₂, and 2 mM MgCl₂) was prepared and bubbled with carbogen gas for ~30 min prior to experimentation. For FSCV calibrations, histamine (5, 1, 0.5, 0.25, and 0.1 µM) and dopamine (1, 0.5, 0.25, 0.1, 0.05 µM) calibration solutions were prepared from stocks made immediately prior to experimentation (Figure 3.5). All FSCV calibrations were performed in recording aCSF bubbled with carbogen gas for at least 30 min prior to measurements.

Animals

All experiments were conducted on C57BL/6 mice of mixed sex with *ad libitum* access to food and water. Experiments were designed to use all littermates across developmental age ranges to control for effects of litter sizes and maternal care factors that could affect the degree of neuronal and circuit maturity. All mice were bred and housed in a temperature-controlled animal facility (12-hour light/dark cycles) and

used in accordance with the European Ethics Committee (decree 2010/63/EU) and were approved by the Ethische Commissie Dierproeven at the University of Antwerp, Belgium.

Brain slice preparation

For striatal slice preparation, mice aged postnatal day (P)9-15 were anesthetized with isoflurane then euthanized *via* decapitation. The brain was immediately removed post-mortem and put into ice-cold cutting aCSF (pH 7.2–7.4) bubbled with carbogen gas. The brain was sliced into 350-400 μ m thick coronal sections in the cold cutting aCSF using a vibratome (Microm HM650V). The slices were collected and allowed to rest in recording aCSF (pH 7.2–7.4, at 32°C) bubbled with carbogen gas for at least 60 minutes prior to experimentation.

Portable FSCV Hardware for Data Collection

The hardware for FSCV experiments was modified from the original configuration (chapter 2, figure 2.2) to be easily transported. In this work, FSCV data was acquired using WCCV 4.0 software (Knowmad Technologies, LLC, Tuscon, AZ, USA), a multifunction I/O device (USB-6341, National Instruments, Austin, TX, USA), a Dagan potentiostat (Dagan Corporation, Minneapolis, MN, USA) and a Pine Research head stage (Pine Research Instrumentation, Durham, NC, USA). The hardware connections and physical set-up is depicted in Figure 3.1.

FSCV Recordings in Striatal Slices

For FSCV experiments, brain slices containing the bed nucleus of stria terminalis (BNST) and striatum were placed into a bath of recording aCSF (32°C and perfusion speed of 2 mL/min) bubbled with carbogen. The slice was secured with a metal harp with nylon threads. The CFM was placed in the striatum and allowed to equilibrate with the histamine waveform applied at 60 Hz for 10 minutes followed by 10 Hz for 10 minutes. A bipolar stimulating electrode (FHC Inc., Bowdoin, ME, USA) was placed on top of the oval nucleus (ov) BNST after the CFM was secured. Electrical stimulation consisted of 30 pulses at 20 Hz (120-

200 μ A) to the ovBNST. FSCV data were acquired using the portable system described above. Histamine and dopamine were co-monitored using a histamine-optimized waveform (-0.5 to -0.7 to 1.1 to -0.5 V at 600 V/s) at 10 Hz⁷⁸ during and immediately after electrical stimulation. All FSCV measurements are reported against a pseudo-Ag/AgCl reference electrode made prior to experimentation by applying 5 V across two silver wires (A-M Systems, Sequim, WA, USA) in 1 M HCl for ~30 s to deposit silver chloride onto the surface.

All data collected at the CFM were filtered in WCCV using a 4th order Butterworth low-pass filter with a cut-off frequency of 3 kHz (scan rate*Nyquist frequency) and smoothed twice. To remove electrochemical drift, all data were filtered in MatLab (2nd order Butterworth bandpass filter with cutoff frequencies of 0.01 and 1.5 Hz).

FIA Experiments

CFMs were calibrated in a flow cell as described in chapter 2 with known concentrations of dopamine and histamine. Dopamine and Histamine 2HCl were dissolved in recording aCSF bubbled with carbogen for at least 30 min prior to experimentation. Serial dilutions were performed to create calibration solutions of dopamine (1, 0.5, 0.25, 0.1 and 0.05 μ M) and histamine (5, 1, 0.5, 0.25, 0.1 μ M). Once the CFM was placed in the flow cell, the histamine-optimized waveform was applied at 60 Hz for 10 min and then at 10 Hz for 10 min prior to data collection. For each calibration point, the analyte was introduced to the flow stream for 10 seconds resulting in a rectangular plug. Each point was repeated three times per electrode.

Statistics

All data is reported as mean $\pm$ standard error of the mean (SEM). Statistical tests are two-tailed and were performed using GraphPad (GraphPad software, RRID: SCR_002798). Continuously collected data were assessed for normality and parametric (ANOVA, paired t-test or unpaired t-test) tests were performed as appropriate. Statistical significance is denoted as * p <0.05, ** p <0.01 and *** p <0.001.

Results

Portable Hardware System for FSCV Data Collection

As shown in Chapter 2, FSCV hardware are typically run with PCI-type data acquisition (DAQ) cards that require a desktop tower to function. This bulky system limits portability. However, as interest in collaborative FSCV measurements has accrued, we have opted to alter the hardware configuration to be transportable, with the goal to fit within a backpack.

For a collaboration with the Ellender lab in Belgium, we developed a transportable FSCV system for data acquisition using a laptop, a USB-connected DAQ card and a potentiostat with associated cables. We approached this task by first understanding the inputs and outputs of the hardware and software: the software (WCCV 4.0) first sends a digital signal that is converted to analog through the DAQ card. The analog signal is then sent to the potentiostat, which applies the specified waveform to the CFM and reference electrode connected *via* the head stage. The current data is collected at the CFM and reference electrode, which is sent back as analog information through the potentiostat, then converted to a digital signal in the DAQ card and output into a color plot in the software. To facilitate this flow of information, the analog output (AO)0 connection on the DAQ card is connected to the main connection of the potentiostat (unmarked). The analog input (AI)0 and AI1 connections on the DAQ card are connected to the filtered and unfiltered positions on the potentiostat, respectively. The output voltage is monitored at channel 7 on the oscilloscope within the software, so the AI7 position on the DAQ card is connected to the voltage monitor on the potentiostat. The portable system is depicted in Figure 3.1. The connections for the portable system are similar to the configuration outlined in chapter 2 (Figure 2.2), but here we eliminated the need for a tower, monitor and breakout box by connecting an external USB-connected DAQ card to a laptop. With this configuration, we can easily minimize an FSCV system to fit within a backpack.

We also configured the portable system to deliver an electrical stimulation. To add the stimulation function, an additional USB-connected DAQ card (USB-6341 OR USB-6221) is connected from its PFI0 position to the PFI14 position on the main DAQ card. From the stimulation DAQ card, a balancer box is connected to the AO0 port and individual BNC connections are made from the balancer box to the neurologs (similar to Figure A.1), which output the stimulation.

Thus, I successfully configured a portable FSCV set-up to be used for niche collaborations around the world. To prepare for measurements in tissue, we next determined how a presumably low histamine signal would look at a CFM in a buffer bubbled with carbogen gas.

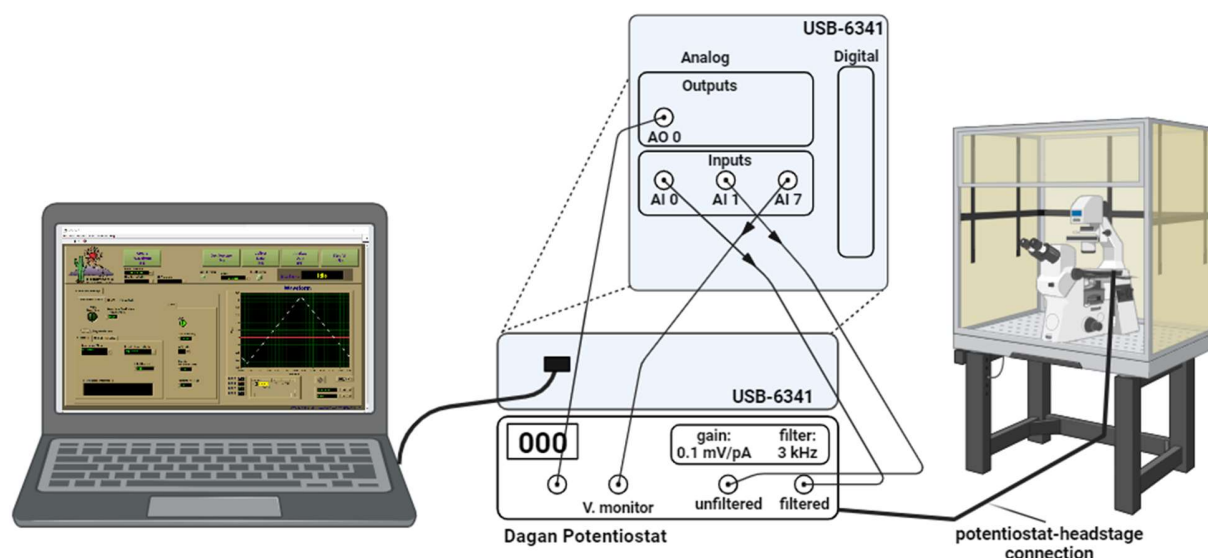


Figure 3.1. Hardware configuration used to collect FSCV data. The waveform parameters are user-defined in WCCV 4.0 software within the laptop (left). The laptop interfaces directly with the USB-6341 multifunction I/O device (blue) *via* a USB connection. The USB-6341 ports (AO 0, AI 0, AI 1, and AI 7) are connected to the Dagan potentiostat (white) ports (unlabeled port, unfiltered, filtered and v. monitor) respectively *via* BNC cables. The potentiostat is connected to the head stage where the current is read at the CFM and reference electrodes within the electrophysiology rig (right). Made using Biorender.com.

Histamine oxidation peak downshifts in a carbogen environment

Prior to our involvement, the Ellender lab identified a dense bundle of histamine axons in brain slices early in development (postnatal week 2) confined to the oval nucleus of the bed nucleus of stria terminalis (ovBNST). The planned collaborative experiment was to use FSCV to determine if evoked histamine in the ovBNST could cross over into the striatum. As the striatum is sparsely innervated with histaminergic axons, and the ovBNST is not within the anatomical borders of the striatum, we expected our histamine signal to be extremely low. Therefore, we replicated the experimental conditions of an acute brain slice experiment prior to traveling. For this we placed a CFM in a beaker of stirred aCSF bubbled with carbogen gas at 32°C, then spiked histamine into a bulk solution (Figure 3.2A-B).

We found a low concentration of histamine (100 nM) exhibited a downshift in peak potential (~ 0 V) compared to unbuffered aCSF at room temperature (~ 0.3 V) (Figure 3.2C). The buffering capacity of aCSF is due to the dissociation of bicarbonate (HCO_3^-) and this reaction is stabilized at pH 7.2-7.4 when bubbled with carbogen. Based on these results, the observed potential shift is likely due to the increased oxygenation and temperature of the aCSF. Regardless, with this expected potential shift, we next performed FSCV in acute striatal brain slices at the University of Antwerp.

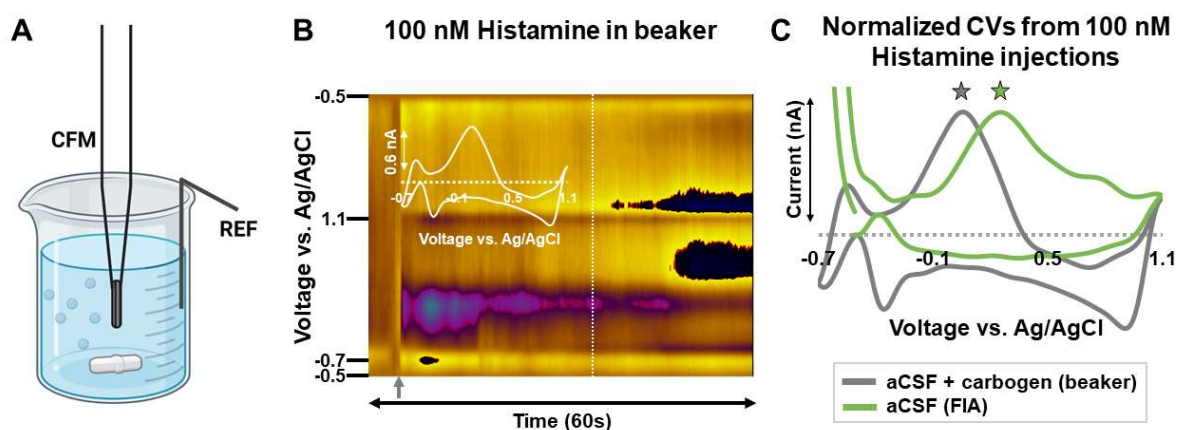


Figure 3.2 Carbogen induces a downshift in histamine oxidation peak potential. (A) Illustration of a CFM and reference (REF) electrode in a stirred beaker with aCSF bubbled with carbogen gas. (B) Representative color plot of

100 nM histamine injected into a beaker as described in panel A. The gray arrow denotes the injection time. (C) Normalized cyclic voltammograms (CVs) taken from 100 nM histamine in a CSF injected in a flow cell (FIA) (green) or a beaker bubbled with carbogen (gray). The oxidation peaks are denoted with color-coordinating stars. Made using Biorender.com.

Evoked histamine from the ovBNST is detectable in the striatum

To determine whether electrically evoked histamine from the ovBNST is detectable in the striatum, we set up the portable FSCV system in our collaborator's laboratory. We placed the system on a table near a Faraday cage on an anti-vibration table containing the aCSF bath and two quadrupole micromanipulators. After testing the system's functionality, we prepared acute brain slices from C57BL/6 mice aged 9-15 days postnatal. Brain slices containing the striatum and ovBNST were chosen for experimentation based on anatomical landmarks similar to the mouse brain atlas¹²⁹.

For each experiment, the brain slice was secured with a nylon-stringed harp in a flowing aCSF bath (2 mL/min, 32°C) bubbled with carbogen (Figure 3.3A). We used micromanipulators to place the CFM within the striatum close to the ovBNST then the twisted tungsten stimulating electrode so that it contacted the ovBNST (Figure 3.3B-C). The distance between the CFM and stimulating electrode was $497 \pm 51 \mu\text{m}$ for $n=6$ brain slices from 3 mice.

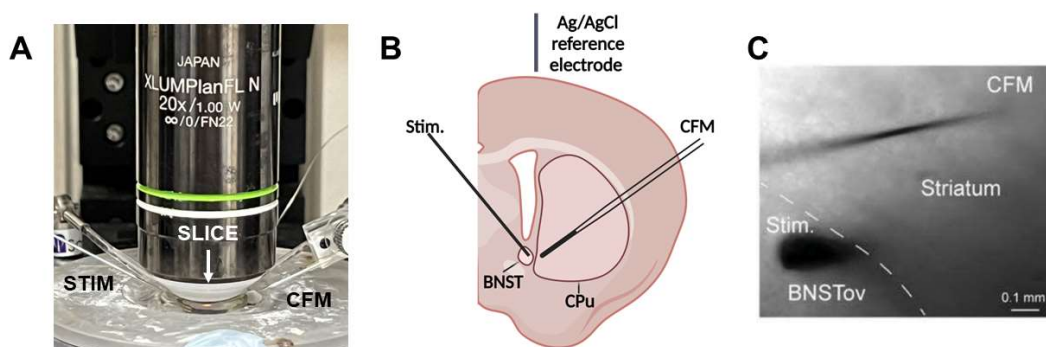


Figure 3.3. Experimental set-up for FSCV measurements in *ex vivo* striatal brain slices. (A) striatal brain slice containing the ovBNST is placed in a flowing recording aCSF bath with the stimulation electrode (STIM) and CFM

placed on and in the slice, respectively. (B) Anatomical illustration of the STIM and CFM placed on the ovBNST and caudate putamen (CPu) in the striatum. The reference electrode is placed within the aCSF bath. Made using Biorender.com. (C) Image of the stimulation (Stim.) electrode placed on the ovBNST while the CFM is placed near the stim within the striatum. Made using Biorender.com.

A histamine-optimized waveform was applied to the CFM to detect evoked histamine and dopamine at their distinct oxidation potentials (0.0 V and 0.6 V, respectively). Upon electrical stimulation of the ovBNST, a fast dopamine release was observed with a slightly delayed, slower histamine release (Figure 3.4A). The cyclic voltammograms (CVs) from brain slices were validated by comparing CVs from CFMs placed in a stirred beaker of oxygenated aCSF and spiking in 100 nM of both histamine and dopamine (Figure 3.4B). Oxidative current for dopamine and histamine signals were converted to concentration *via* calibration curves taken in a FIA system (Figure 3.5). Significant increases in dopamine (45.3 ± 5.6 nM, paired t-test, $p=0.0002$) and histamine (178.5 ± 57.7 nM, paired student's t-test, $p=0.009$) as a result of electrical stimulation were observed, with dopamine peaking earlier than histamine (5.6 ± 0.07 s, 7.0 ± 0.2 s respectively; paired t-test, $p=0.0016$) (Figure 3.4C). Additionally, half-life decay ($t_{1/2}$) analysis showed histamine lingered in the tissue significantly longer than dopamine (dopamine: 6.6 ± 0.04 s, histamine: 18.7 ± 3.7 s, paired t-test, $p=0.019$, $n=6$ brain sections from 3 mice) (Figure 3.4C). Together, these data show that histamine evoked from the ovBNST is detectable in the striatum.

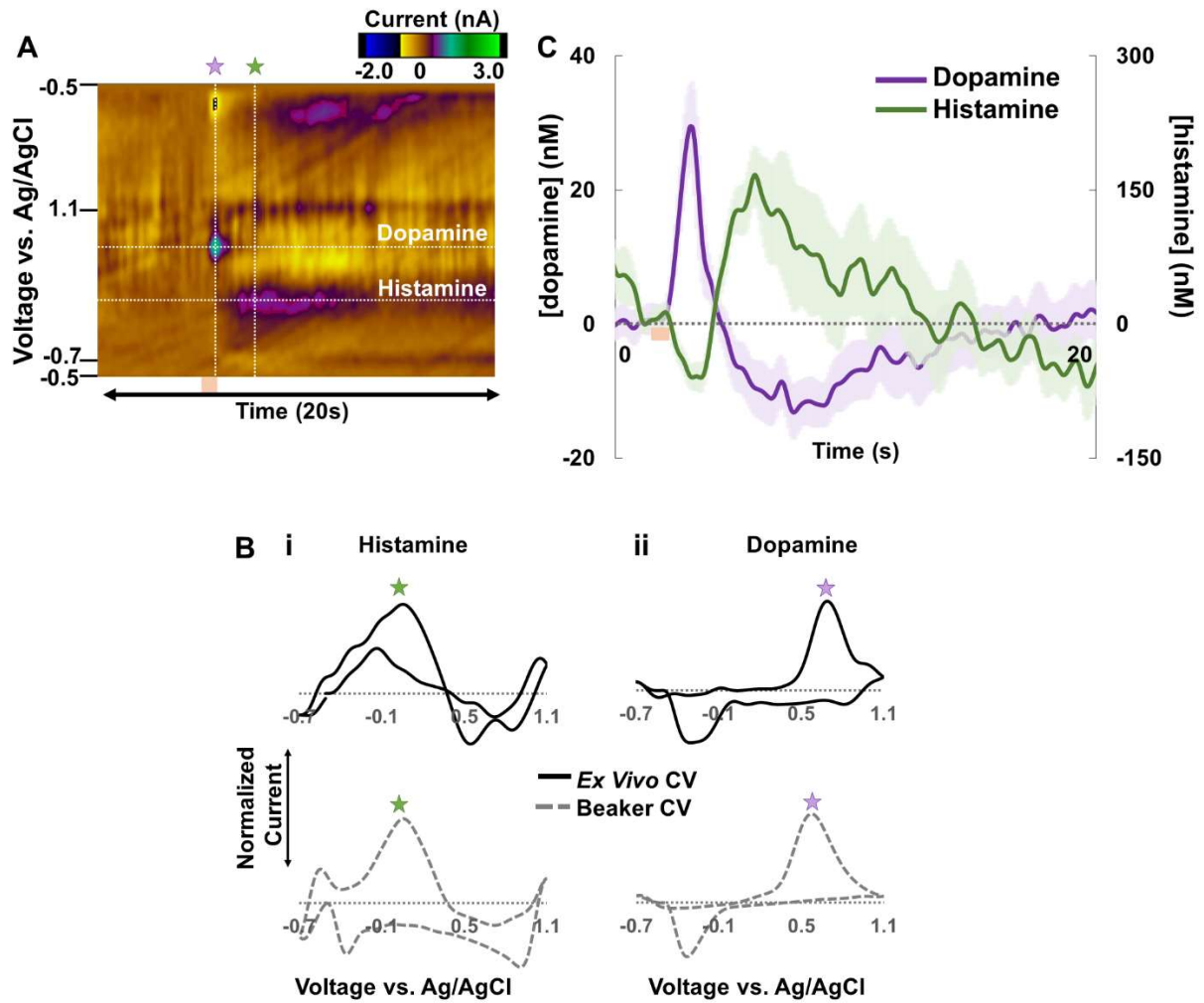


Figure 3.4. Electrically evoked histamine from the ovBNST is detected in the striatum. (A) Representative color plot of a stimulated response detected at CFM with FSCV. The first vertical line (purple star) is a dopamine release, and the second vertical line (green star) is a histamine release. (B) The cyclic voltammograms (CVs) from the brain (Bi and ii top) were verified by injecting low concentrations of histamine (Bi bottom) and dopamine (Bii bottom) in a stirred beaker of recording aCSF (30°C) bubbled with carbogen. (C) Bandpass filtered (2nd order Butterworth with 0.01 to 1.5 Hz cutoff frequencies) current vs. time (IT) traces for dopamine and histamine, averaged (n=6 stimulations from 3 mice) with error reported as standard error of the mean. (D) A representative image of the experimental set up where the CFM was placed in the caudate putamen (CPu) close to the stimulation site, the stimulation electrode

placed on the ovBNST and the Ag/AgCl reference electrode in the bath of recording aCSF (30°C) bubbled with carbogen.

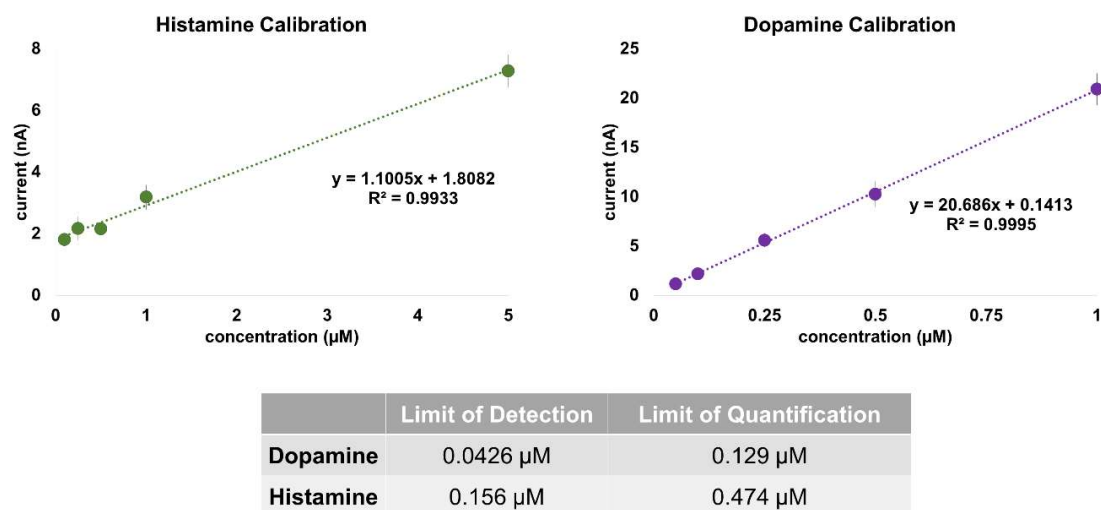


Figure 3.5. Calibration of CFMs for histamine and dopamine in aCSF. Histamine (green, left) and dopamine (purple, right) were calibrated in aCSF using FIA. Inset is the linear regression statistics for each molecule. The table below outlines the limits of detection and quantification for dopamine and histamine. (n=3 electrodes each)

Discussion

In this work we offer a novel, portable FSCV hardware configuration that is transportable. With this system we were able to travel to Belgium to take the first FSCV histamine measurement in acute brain slices. We observed a delayed histamine release compared to a fast dopamine release in the striatum and that the oxygenation of the buffer significantly downshifted histamine's peak potential. Physiologically, our measurements showed that histamine evoked from a nearby brain region can cross anatomical borders to modulate other areas.

Traditionally, the hardware needed to run FSCV systems use internal DAQ cards that require a computer tower, meaning the researcher is limited to experimentation within their institution. Technologies have been developed to miniaturize and make FSCV hardware more accessible. A miniaturized potentiostat,

“TinyFSCV” was specifically developed for dopamine detection at CFMs and can apply scan rates up to 400 V/s¹³⁰. Kimble *et al.* developed the Bluetooth-enabled WINCS system capable of running various FSCV waveforms for *in vivo* neurotransmitter detection of adenosine, dopamine and serotonin¹³¹. However, there are no miniaturized systems capable of applying the histamine-optimized waveform used in this work. For this, we replaced the PCI-type DAQ cards with a USB-connected DAQ card, eliminating several pieces of equipment resulting in a transportable FSCV system. This system has alleviated a limiting factor in many FSCV collaborations.

We observed a significant downshift in histamine’s peak potential when replicating the conditions for tissue measurements. Fundamentally, the histamine signal at the CFM has been known to shift its peak potential in different buffers and tissues, while generally keeping its broad shape. Conflicting reports have been made on the oxidation potential of histamine^{132, 133}, leaving researchers to debate about how best to capture histamine with FSCV. In cultured mast cells, Wightman identified histamine release by a peak that appears just after the scanning potential switches directions (~ 1.3 V)⁷². However, quantitating peaks at this position is controversial as they are more likely a result of capacitive current discharging when the scanning potential changes directions. The Venton group has shown evidence that histamine electropolymerizes onto CFMs at potentials above 1.1 V¹³⁴. However, the N-shaped histamine-optimized waveform developed by the Hashemi lab, used in this work, reports a histamine peak at ~ 0.3 V⁷⁸. While this signal is consistent, its contributing redox mechanism (Faradaic or not) is not known. In this work, the significant potential shift in an oxygenated salt buffer (carbogen-bubbled aCSF) has given us some clues as to what the source of the signal might be. We postulate that using this waveform, histamine is pre-concentrated at the carbon surface when the holding potential is applied. Then, when the potential is scanned this induces a charge transfer within the conjugated 5- and 6- member carbon rings of the histamine molecule that is detected by the CFM. As the histamine-optimized waveform peaks at 1.1 V, according to Venton’s work, this potential does not induce oxidative electropolymerization of histamine onto the CFM’s surface, evidenced

in our color plots by a small capacitive peak on the back-scan. While the underlying mechanism contributing to the histamine signal on this waveform has not yet been proven, the reason for its continued implementation is that the histamine signal comes in on the forward scan, ensuring it is not a capacitive artifact.

Histamine evoked from the ovBNST was detected in striatal brain slices. We based our experimental set-up on published protocols for FSCV measurements in brain slices^{135, 136}. For our work, the CFM was placed in the striatum and the stimulation electrode onto the surface of the ovBNST. Upon electrical stimulation of the ovBNST we found a detectable histamine signal in the striatum. It is well-documented that extracellular dopamine is quickly cleared from the extracellular space in striatal slices¹³⁷⁻¹³⁹, but histamine is detected for much longer (tens of seconds to minutes)¹⁴⁰⁻¹⁴². This indicates that histamine likely is transmitted *via* volume transmission, like adenosine and serotonin. Additionally, the slow clearance of histamine is likely due to the slower kinetics of organic cation transporter 3 (OCT3) and histamine N-methyl transferase (HNMT)^{143, 144}, aiding its ability to cross anatomical borders^{145, 146}. Most definitely, the long lifespan of evoked histamine in the extracellular space of the striatum suggests that the ovBNST is a significant source of histamine for the developing striatum and other nearby structures. As our work was done in early developing brains, it is unclear whether this phenomenon carries into adulthood where the striatum remains sparsely innervated with histamine neurons.

In vivo histamine measurements typically take weeks to obtain a full n-size, with the difficulty largely attributed to the hard-to-reach location of the PH within the brain (close to the base of the brain). With the ex vivo slice protocol described in this work, we were able to perform a full n-size within a few days. Thus, with our protocol, future measurements of neuronal histamine with FSCV can be streamlined and physiological phenomena can be discerned in a shorter time frame.

Conclusion

We have shown the first measurements of histamine in brain tissue slices for high throughput studies. These measurements have shown that histamine evoked from a nearby brain region can cross anatomical borders to modulate other regions. Additionally, the observed potential shift in an oxygenated environment helped shed light on the underlying mechanism of the signal. Finally, the development of a portable FSCV system that is not limited in its waveform applications have opened the door for more collaborations such as this one. We intend to expand upon this study to understand the role of histamine in psychiatric disorders and work with new collaborators to push the boundaries of FSCV measurements.

4. Chapter 4 Detection of Neurochemicals in Human Hair cells with Fast Voltammetry

The work presented in this chapter was done in collaboration with the Higgins laboratory at Imperial College London. The data presented here was performed by Brenna Parke unless otherwise denoted as performed by members of the group run by Dr. Claire Higgins.

Abstract

Tactile stimuli activate mechanoreceptors that transmit information from the skin to the brain. Recently, epidermal cells, Merkel cells and keratinocytes, have been classified as mechanoreceptors that facilitate communication of mechanical information to sensory neurons in the skin. The human hair follicle is surrounded by a complex network of sensory neurons, but mechanotransduction capacity has not been attributed to epithelial cells in the outer root sheath of the follicle. It is well-accepted that hair deflection activates sensory neurons surrounding the hair follicle to transmit tactile information. Here, we hypothesized that hair follicle outer root sheath (ORS) cells act as transducers of mechanical stimuli to sensory neurons around the hair follicle. After optimizing electrochemical measurements for cell culture preparations, electrochemical analysis on *in vitro* hair follicle preparations showed that ORS cells release ATP, serotonin and histamine in response to mechanical stimulation. We used calcium imaging and pharmacological interventions in a co-culture of ORS cells and dorsal root ganglia neurons to find that the release of these signalling molecules leads to sensory neuron activation. We conclude that hair follicle ORS cells are mechanotransductive and form a neuroepithelial unit with sensory neurons in human skin. We therefore uncover a circuit to show that cells in the human hair follicle have a role in transmission of mechanical information to sensory neurons.

Introduction

Touch is a sensation that mediates social behaviors, psychological health and developmental processes. Exemplified by the COVID-19 pandemic, prolonged social and physical isolation negatively affects peoples' mental health¹⁴⁷. Additionally, people that report touch 'starvation' have increased feelings of stress, depression and anxiety¹⁴⁸. This has been demonstrated in rodents where pups deprived of maternal touch had decreased expression of stress-resilient genes and enhanced stress response¹⁴⁹. Touch is largely mediated within the skin, where tactile stimuli are relayed to the brain through the activation of sensory neurons, which leads to molecular and behavioral responses¹⁵⁰.

Sensory neurons are the conduit between physical sensation and perception in the brain. Low-threshold mechanoreceptors (LTMRs) are sensory neurons that innervate the skin and transfer tactile information to the brain. There are two types of skin: non-hairy (glabrous) and hairy. Glabrous skin is found on the palms of hands and soles of feet, which facilitates tactile understanding¹⁵¹. In contrast, hairy skin with LTMR-associated hair follicles transfers tactile information to the brain that influences psychiatric behaviors.¹⁵² This is evidenced by the calming effect a gentle stroke can have on a newborn child. Unique to hairy skin, C-LTMRs are known to mediate social interactions. Mice that lack C-LTMRs exhibit social isolation behaviors, whereas activation of these receptors leads to touch-seeking and social behaviors¹⁵³. In humans, C-LTMRs are hypothesized to facilitate affective touch, exhibited by those with reduced C-LTMR densities who have an impaired perception of pleasant touch¹⁵⁴. Additionally, patients with genetic mutations for low C-LTMR densities have greater activation of primary sensorimotor cortices during acute pain tasks¹⁵⁵. Therefore, C-LTMRs have an integral role in social and affective touch, and variations in their densities affects neuronal processing of tactile stimuli.

Mechanical stimulation of the skin (stretching, poking, vibration, hair deflection) activates different classes of mechanoreceptors^{156, 157}. While these mechanoreceptors were previously thought to be expressed solely on sensory neurons, recent data shows that they are present on Merkel cells and keratinocytes (epidermal cell types). In response to tactile stimuli, Merkel cells release norepinephrine⁵⁷ and serotonin⁵⁶ and keratinocytes release ATP¹⁵⁸. The neurotransmitter-mediated interaction between Merkel cells to sensory neurons is known as a neuroepithelial unit in the skin. While much is known about these cells in mouse models, little is known in humans. Additionally, mechanotransduction capacity has not been attributed to cells within the hair follicle.

We hypothesized that when a hair fiber is deflected, hair follicle epithelial cells (outer root sheath (ORS) cells) act as a transducer to chemically activate the sensory neuron afferents around the follicle. For this study, in a highly collaborative effort with the Higgins lab, we employed a combination of immunolabeling, calcium imaging and a newly optimized fast voltammetry protocol to *in vitro* and *ex vivo* preparations of human hair follicle explants to evaluate their role in processing tactile information.

Materials and Methods

Subjects and Samples

Occipital scalp skin containing hair follicles was taken from male subjects aged 23-54 undergoing hair transplant surgery at the Mediteknia Clinic (Las Palmas de Gran Canaria) and Fario Hair Institute (London, UK). For these samples, consent forms approved by either the Universidad de las Palmas de Gran Canaria Research Ethics Committee or Imperial College Research Ethics Committee were used to obtain written consent. Keratinocyte cultures were established from skin left over abdominoplasties, with informed written consent obtained from patients using consent forms approved by the Imperial College Research Ethics Committee. (Higgins lab)

Expression Profiling

Whole-mount immunolabeling

Sample processing for volumetric imaging was performed using a modified version of iDISCO (immunolabeling enabled 3D imaging of solvent-cleared organs) technique¹⁵⁹. Freshly biopsied samples were fixed overnight at 4°C in 4% paraformaldehyde (PFA) (Agar Scientific, Stansted, UK) in phosphate buffered saline (PBS) (Gibco, Waltham, MA, USA) then washed in two 30-minute PBS washes. Fixed samples were next dehydrated using a series of 20%, 40%, 60%, 80% then 100% methanol/H₂O (1 hour per wash). After methanol dehydration, samples were incubated overnight at 4°C with 66% dichloromethane/33% methanol for 10 minutes then bleached with 5% H₂O₂ in methanol overnight at 4°C. After bleaching, samples were rehydrated in 20%, 40%, 60% and 80% H₂O/methanol series followed by a 1-hour PBS wash. Lastly, samples were washed twice with PBS in 0.2% Triton-X-100 (1 hour per wash).

After fixation and pre-treatment, follicles were incubated for 12 hours at 37°C in a permeabilization solution consisting of PBS with 0.2% Triton-X-100, 20% DMSO and 0.3 M glycine. Samples were then incubated for 24 hours at 37°C in a blocking solution of PBS with 0.2% Triton-X-100, 6% donkey serum, 6% goat serum and 10% DMSO. After blocking, samples were incubated with primary antibodies diluted in PBS with 0.2% of Tween-20, 0.1% of 10 mg/mL Heparin solution, 5% DMSO, 3% donkey serum and 3% goat serum (Vector Labs) for 24 hours at 37°C. Samples were then washed 4 times in PBS with 0.2% of Tween-20, 0.1% of 10 mg/mL Heparin solution (4 hours per wash) prior to incubation for 24 hours at RT with secondary antibodies diluted in PBS with 0.2% of Tween-20, 0.1% of 10 mg/mL Heparin solution, 3% donkey serum and 3% goat serum. After secondary antibody incubation, samples were washed 4 times (4 hours per wash) in PBS with 0.2% of Tween-20, 0.1% of 10 mg/mL Heparin solution.

After immunolabelling samples were dehydrated again with a series of 20%, 40%, 60%, 80%, 100% methanol/H₂O (1 hour per wash). Samples were then incubated for 3 hours at room temperature on a

rocking platform, in 66% dichloromethane/33% methanol. They were next incubated for two 10-minute washes in 100% dichloromethane. Finally, clearing was performed incubating the samples with 100% dibenzyl ether for 1 hour. Overall, the protocol takes 9 days to complete with the timeline optimized for whole human follicles.

Samples were mounted on a glass slide and covered with a coverslip raised 2mm above the sample to avoid sample compression. This allowed the sample to be in a contained 3D space for volumetric imaging. Samples were imaged with Leica SP8 confocal microscope with HyD detectors to depths between 200-500 μm . (Higgins lab)

Gene expression analysis in tissue and cells

RNA was extracted from plucked hair fiber or ORS cells grown in explant culture for 2 weeks, using a Qiagen miniRNA extraction kit (Qiagen, Hilden, Germany). Superscript IV (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate cDNA with OligoDT primers. A PCR reaction was performed using HotStart Taq (Thermo Fisher Scientific, Waltham, MA, USA) with primers designed against build Hg39 sequences. Products were run on a 2% Agarose Gel and product size was checked against a 1 Kb ladder. (Higgins lab)

Sc-RNA-seq data analysis

Seurat

From a publicly available dataset, anagen human hair follicle single-cell RNA expression data was obtained Gene Expression Omnibus (GEO) number GSE193269¹⁶⁰. Anagen-specific human hair follicle tissue samples were extracted for further downstream analysis. Inclusion criteria were cells that expressed more than 500 unique molecular identifier (UMI) counts, <6500 genes, >200 genes, and <30% mitochondrial gene expression in UMI counts. Any doublets were excluded by computationally comparing cell barcodes, pooling the raw count for each gene for those samples and dividing by the total number of barcodes per cell identifier so that the end number of raw counts accurately reflected the counts for that barcode.

Datasets from multiple donors were merged using derivation from the standard integration protocol described in Seurat version 4.2.0¹⁶¹ was used. Gene expression matrices were normalized using log normalization. Then, the 2000 most variably expressed genes were selected within each sample with local polynomial regression as a selection method prior to integration. Data was integrated using anchor-based canonical correlation analysis (CCA) integration with 30 CCA dimensions to obtain the integration anchors between sample origins. To conserve information of the gene expression outside of the highly variable genes, a binary reduce function was applied to combine the elements of the given gene list and initial values before integration, preserving gene count information throughout the integration process. For graph-based clustering and data visualization, the remaining 19,083 cells were used. First, the dataset was scaled using a linear regression model, then dimensions were reduced by clustering cells using principal component analysis (PCA) followed by uniform manifold approximation and projection (UMAP) using the Seurat R package with 50 dimensions based on the non-plateauing trend in an elbow plot where the standard deviation of the principal components is shown. Cells belonging to subpopulations were reanalyzed separately by subsetting the relevant cluster and rerunning the neighbor and cluster identification to build second-level clusters. Wilcoxon's rank sum testing was used with a return threshold of 0.01, log range of 0.25-fold difference and a minimum fraction detection range of 0.25, which showed balanced results between the number and size of expected clusters within the anagen human hair follicle, without redundancy following clustering. The cluster labelling was performed based on the observed protein marker expression within each cluster. (Higgins lab)

Cotan

For quality control, in addition to gene expression analysis in the cell clusters using Seurat, we also used COTAN¹⁶², an R package for gene pair analysis theory. Here, we analyzed both the anagen hair follicle dataset (GEO number GSE193269)¹⁶⁰ and a publicly available healthy human skin scRNA dataset (GEO number GSE147424)¹⁶³. For the skin dataset, matrices for the seven healthy control subjects were

extracted to be used for downstream analysis. This quality control protocol with COTAN uses a standardized method (as described above in the Seurat section) with the addition of cells with low information density are removed by setting a threshold for UMI detection efficacy cells depicted by the value obtained from PCA. After performing quality control with COTAN, we had 18,832 cells for the human anagen hair dataset (GSE193269) and 23,813 cells in the healthy skin dataset (GSE147424) for analysis. Rather than comparing cell identity across datasets, a comparison is drawn using generalized contingency tables, where these tables are computed for each dataset using a discrete UMI count variable R_{gc} . First, for each dataset a uniform population of cells is assumed to act according to a negative binomial distribution such that gene count is modelled as Poisson random variables with a rate constant following gamma distribution. Therefore, R_{gc} is considered to operate according to a discrete Poisson distribution and normalized so that the UMI counts may be collapsed, resulting in the binarization of expression values of each UMI into two categories ($R_{gc} = 0$) and ($R_{gc} \geq 1$), neglecting to consider values larger than 1 as different. Using this method, the co-expression of two genes, A and B, of interest is computed and can be categorized into co-expressed genes ($R_{Ac} + R_{Bc} \geq 1$). Using this approach, we directly compared co-expression between datasets without bias. (Higgins lab)

Primary Cell Cultures

Hair follicle epithelial cells

ORS explant cultures¹⁶⁴ were established from human hair follicles taken from the occipital scalp. First, follicles were incubated in dispase for 30 minutes at 37°C. Forceps were used to separate the hair follicle epithelium from the hair follicle mesenchyme. Hair fibers containing epithelial tissue were then placed on mitomycin treated NIH3T3 feeder layers in 35 mm culture dishes and left to adhere for 2-3 minutes. FAD medium (a 1:1 ratio of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA) and Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 ng/ml epidermal growth factor, 5 µg/mL insulin, 0.18 mM adenine, 0.4 µg/mL hydrocortisone, 0.1 nM cholera toxin, 2 nM

triiodothyronine and 1X penicillin-streptomycin (P/S)) was then added to cultures which were incubated at 37°C in 5% CO₂/95% humidity for 7-10 days when they were used in experimental procedures. (Higgins lab)

Keratinocytes

Keratinocyte explant cultures were isolated human skin discarded after abdominoplasty procedures. Briefly, excess fat and dermis was removed from samples, which were then incubated overnight in dispase at 4°C. The following morning, the epidermis and dermis were manually separated with forceps. The epidermis was then chopped roughly with scissors and placed onto mitomycin treated NIH3T3 feeders. The epidermis was left to dry for 2-3 minutes to adhere to the feeders before FAD media was added. Dishes were incubated at 37°C in 5% CO₂/95% humidity for 7 days, to facilitate outgrowths of keratinocytes from the explanted epidermis, prior to use in experimental procedures. (Higgins lab)

DRG neurons

Dorsal root ganglia (DRG) neurons were isolated from C57BL/6J mice, which were sacrificed for other research purposes. The spine was removed and opened transversally and the DRGs from C2-L5 removed using fine forceps. DRGs were incubated with Hank's Balanced Salt Solution (HBSS) (Gibco, Waltham, MA, USA) containing 2 mg/mL collagenase II at 37°C for 1 hour. The DRG neurons were dissociated with a fire-polished pasteur pipette with small tip diameters and plated onto petri dishes pre-coated with a 0.1% poly-L-lysine solution. DRG neurons were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% FBS and 1X P/S and incubated at 37°C in 5% CO₂/95% humidity. (Higgins lab)

Co-culture of epithelial cells and DRG neurons

For the co-culture system we combined mouse DRG neurons and human ORS cells. Briefly, mouse DRG neurons, isolated as described above, were seeded onto a mature ORS cell culture on NIH3T3 feeders. This co-culture was maintained in FAD media at 37°C in 5% CO₂/95% humidity. Co-cultures were used after

observing an axonal network developing towards the ORS cells, which usually occurred after approximately 10 days. (Higgins lab)

Calcium dynamics experiments

Calcium imaging and analysis

Calcium imaging was performed on ORS cultures, or ORS LTMR co-cultures grown in 35 mm dishes with glass bottoms (Ibidi, Gräfelfing, Germany), using the fluorescent calcium indicator Cal-520 (AAT Bioquest, Sunnyvale, CA, USA). Cultures were incubated with 10 μ M Cal-520 in HBSS with 0.04% Pluronic F-127 for 90 minutes at 37°C followed by 30 minutes at room temperature. Cells were then washed once with HBSS prior to incubation in fresh HBSS for experimentation. Dishes were placed onto an inverted Leica SP5 confocal microscope (Leica, Wetzlar, Germany) with the temperature maintained constant at 30°C. Calcium transients were imaged once per second for 60 seconds using a Sentech Stc-TC33USB-AS camera (Omron Sentech, Kanagawa, Japan). Prior to stimulation, a 60 second baseline recording was taken to ensure there were no fluctuations in the calcium-fluorescence within cells. An Injectman micromanipulator (Eppendorf, Hamburg, Germany) was used in combination with a glass pipette with a 1 μ m diameter tip was used to stimulate. The pipette was lowered slowly until it touched an ORS cell at which point-of-contact distance was recorded. The pipette was then raised, and the cell was left to rest for at least 2 minutes or until no activity was observed. To mechanically stimulate cells, the pipette was lowered until the point-of-contact + 5 μ m. Recordings were obtained for 120 seconds after mechanical stimulation. Cells (either LTMRs or ORS) were distinguished from one another by size and shape. ORS cells are flatter with well-defined borders, no axonal structures, and they grow in confluent monolayers and are surrounded by other ORS cells. In contrast, LTMRs have rounded and raised cell bodies, with axonal structures protruding from the cell body. We also performed post hoc staining to confirm our observations.

Calcium transients were quantified and analysed using Image J. After manually selecting the cells the Multi ROI intensity profile for a time series macro was used to quantify the calcium transients. We calculated the change in intracellular calcium concentration ($\Delta F/F$) relative to the baseline concentration prior to stimulation. This was plotted as a function of time, while peak $\Delta F/F$ values were used for statistical analysis. (Higgins lab)

Drug interventions

To evaluate if the observed increases in calcium were due to a calcium source in the extracellular space or the endoplasmic reticulum, cells pre-loaded with Cal-520 were incubated at 37°C in 3 different conditions for 8 hours prior to cell stimulation; HEPES-buffered HBSS (+Ca²⁺, -TPSG), HEPES-buffered HBSS with 3 μ M TPSG (+Ca²⁺, +TPSG), and modified HEPES-buffered HBSS without calcium (-Ca²⁺, -TPSG). Different cultures were used for each experiment.

To assess the role of mechanosensitive ion channels in the response of ORS cells to mechanical stimuli, GsMTx-4, a mechanosensitive and stretch-activated ion channel inhibitor was used. Cultures pre-loaded with Cal-520 were mechanically stimulated and calcium transients recorded. GsMTx-4, at a concentration of 8 μ M in HEPES-buffered HBSS was added to cultures for 20 minutes at 30°C, after which time the cells were mechanically stimulated and calcium transients recorded.

To deplete synaptic vesicles of their neurotransmitter in ORS cells, we incubated co-cultures with 10 μ M bafilomycin A1 in FAD media for 1 hour at 37°C. We then loaded co-cultures with Cal-520 as described previously, prior to mechanical stimulation and recording of calcium transients. For FSCV, we stimulated cells with the 1 stim protocol as described below.

To assess the role of ATP in cell response we used apyrase, an enzyme that converts ATP to ADP and ADP to AMP. Cultures pre-loaded with Cal-520 were mechanically stimulated and calcium transients recorded.

Apyrase, at a concentration of 200 units/mL diluted in HEPES-buffered HBSS was added to cultures for 20 minutes at 30°C, after which time the cells were mechanically stimulated and calcium transients recorded.

Lastly, mirtazapine, a serotonin (5-HT_{2A}, 5-HT_{2B}) and histamine receptor (H₁R) antagonist, was used to assess LTMR response to ORS stimulation. Cultures pre-loaded with Cal-520 were mechanically stimulated and calcium transients recorded. Cultures were then incubated with 200 µM mirtazapine diluted in HEPES buffered HBSS, for 30 minutes at 37°C. After 30 minutes, different cells within the same original culture dish were mechanically stimulated and calcium transients recorded. (Higgins lab)

Fast Scan Cyclic Voltammetry

Carbon Fiber Microelectrodes

This process is described in detail in Chapter 2. For this work, CFMs cut between 147-150 µm were coated with Nafion™. For the data shown in Figures 4.1 and 4.2, Nafioned CFMs were modified by applying the polymer waveform (-1.2 V to 1.3 V to -1.2 V at 400 V/s) at 60 Hz for 10 minutes in DMEM or IMDM (Thermo Fisher, Waltham, MA, USA). For Figures 4.3-4.5 the Nafioned CFMs had the polymer waveform applied at 60 Hz for 10 minutes in a 1 µM solution of L-glutamic acid (Sigma Aldrich, St. Louis, MO, USA)¹⁰¹.

FSCV Data Acquisition and analysis

FSCV data were acquired using the setup described in Figure 2.2. For Figures 4.1 and 4.2 a serotonin-optimized waveform was applied (0.2 V to 1.0 V to -0.1 V to 0.2 V at 1000 V/s) at 10 Hz¹¹⁰. Then, for data shown in Figures 4.3-4.5, a histamine-optimized waveform was applied (-0.5 V to -0.7 V to 1.1 V to -0.5 V at 600 V/s) at 10 Hz⁷⁸. Reference wires were made as described in Chapter 2 prior to each experiment. ORS cells (5 dishes from 4 patients) or keratinocytes (3 dishes from 1 patient) in 35 mm dishes were covered in a solution of HBSS + 10 mM HEPES at pH 7.4. All dishes were probed in the same manner. Each dish was mounted on an inverted microscope (Olympus, Tokyo, Japan), and the CFM was placed 5 µm above the cells with a micromanipulator (Sutter Instruments, CA, USA). For all experiments, the waveform was

applied at 60 Hz for 10 minutes, then at 10 Hz for 10 minutes to equilibrate prior to data collection. Prior to stimulation, 10 control files were collected. The stimulation was applied near the CFM by indenting the cells by 5 μm with a blunted pipette tip 1, 3 or 5 times, with indents one second apart. A stimulation file was collected once every 10 minutes until each stimulation protocol was applied to the area. The CFM was then moved to a different location in the dish, allowing for re-equilibration for 5 minutes prior to data collection. The process was repeated for a total of 3 locations in each dish, totaling 15 stimulation files for the ORS cultures and 9 stimulation files for the keratinocytes.

ORS cell dishes treated with mirtazapine were first probed with FSCV, as described above, then incubated with the drug (200 μM) for 30 minutes. The FSCV stimulation protocol was repeated for 3 locations over 5 ORS cell dishes, totaling 15 stimulations. ORS cell dishes treated acutely with bafilomycin first had the FSCV stimulation protocol applied to 3 locations, then 10 μM bafilomycin A1 was spiked in. The drug was allowed to sit for 60 minutes prior to repeating the FSCV stimulation protocol.

Flow injection analysis experiments

FIA was performed as described in Chapter 2. All chemicals were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA) unless otherwise indicated. For Figures 4.1 and 4.2, stock solutions were prepared fresh daily by dissolving serotonin hydrochloride in 1X TRIS buffer (15 mM TRIS, 126 mM NaCl, 2.5 mM KCl, 25 mM NaHCO_3 , 2.4 mM CaCl_2 , 1.2 mM NaH_2PO_4 , 1.2 mM MgCl_2 and 2.0 mM Na_2SO_4) at pH 7.4, DMEM (Thermo Fisher, Waltham, MA, USA) or IMDM (Thermo Fisher, Waltham, MA, USA). The stock was diluted in their respective buffer or media to create calibration solutions (10, 25, 50, 75, 100, 500 and 1000 nM) of serotonin, which were injected in triplicate for each CFM calibration. Stability experiments were performed by injecting boluses of 1 μM serotonin to the CFM in 1X TRIS buffer, DMEM or IMDM once every 2 minutes for 2 hours (60 injections total).

The CFMs used for cell experiments were post-calibrated in a flow cell with known concentrations of serotonin and histamine. Serotonin HCl and histamine dihydrochloride were dissolved in 1X HBSS (Thermo Fisher Scientific, Waltham, MA, USA) + 10 mM HEPES at pH 7.4 to create stock solutions. Serial dilutions were performed to create calibration solutions of serotonin (1000, 500, 100, 50, 25 and 10 nM), histamine (20, 10, 5, 2, 1 and 0.5 μ M) and a mixed solution (150 nM serotonin + 5 μ M histamine). Each calibration point was measured in triplicate for each electrode.

FSCV Data analysis

Computational models were developed and trained using Python 3.10 and the scikit-learn library. A partial least-squares regression (PLSR) model for each CFM was used to convert oxidative current to concentration for serotonin and histamine. The calibration model was designed to reduce the dimensionality of the CV from 3000 samples (inputs) to 15 features and to simultaneously predict the concentration of serotonin and histamine (2 outputs). The number of components of the regression were chosen to reduce the predictive error of FIA injections used to train the model and avoid overfitting. CVs were normalized by removing the mean and scaling to unit variance (standardization). Models were trained using k-fold cross-validation with 5 different train-test sets, a limit of 1000 iterations and $1e^{-6}$ tolerance. FIA injections of mixed serotonin and histamine in HBSS were used to train the model to predict concentrations of serotonin and histamine. The root mean square errors of test predictions were 14.63 nM for serotonin and 0.69 mM for histamine. Area under the curve (AUC) of concentration vs. time traces was calculated using the Simpson's rule between 5 seconds (start of stimulation) and 60 seconds (end of recording). The slope of the concentration release after stimulation was calculated *via* linear regression between 5 and 30 seconds.

Statistics

Statistical significance is defined at $p < 0.05$. Statistical analyses were performed using Python 3.10 SciPy library, Matlab2020b and Excel. Distribution of samples are mean $\pm$ standard error of the mean (SEM) or in box-whisker plots with a line at the mean value. Calcium transient data were tested for significance using a one-way analysis of variance (ANOVA) or unpaired t-tests depending on the experimental conditions. In these experiments, each cell was regarded as a separate data point. Differences in serotonin and histamine across the data set were tested for significance using a two-way ANOVA and Tukey-Kramer post-hoc multiple comparisons of the AUC and slope of release. In FSCV experiments, each location in the culture dish was regarded as a separate datapoint.

Results

Cell media impairs voltammetric measurements

As the aim of this study was to make robust measurements to characterize ORS cell signalling in cell preparations, we initially determined whether a neurotransmitter (serotonin) could be detected in cell media. We compared the CFM response to serotonin in a salt buffer (1X TRIS) and two common types of cell media (DMEM and IMDM). The response to a 1 μ M serotonin bolus in a flow cell was significantly diminished when taken in the two types of cell media compared to 1X TRIS buffer (TRIS: 28.36 ± 1.60 nA, DMEM: 2.84 ± 0.08 , $p < 0.001$, IMDM: 3.27 ± 0.33 nA, $p < 0.001$) (Figure 4.1A,C). Additionally, serotonin oxidation peak shifted towards more positive potentials in media (DMEM and IMDM) and exhibited peak broadening (IMDM) compared to 1X TRIS buffer (Figure 4.1B). Having found diminished capacity for serotonin detection in cell media, we next determined whether cell media would irreversibly foul the sensor.

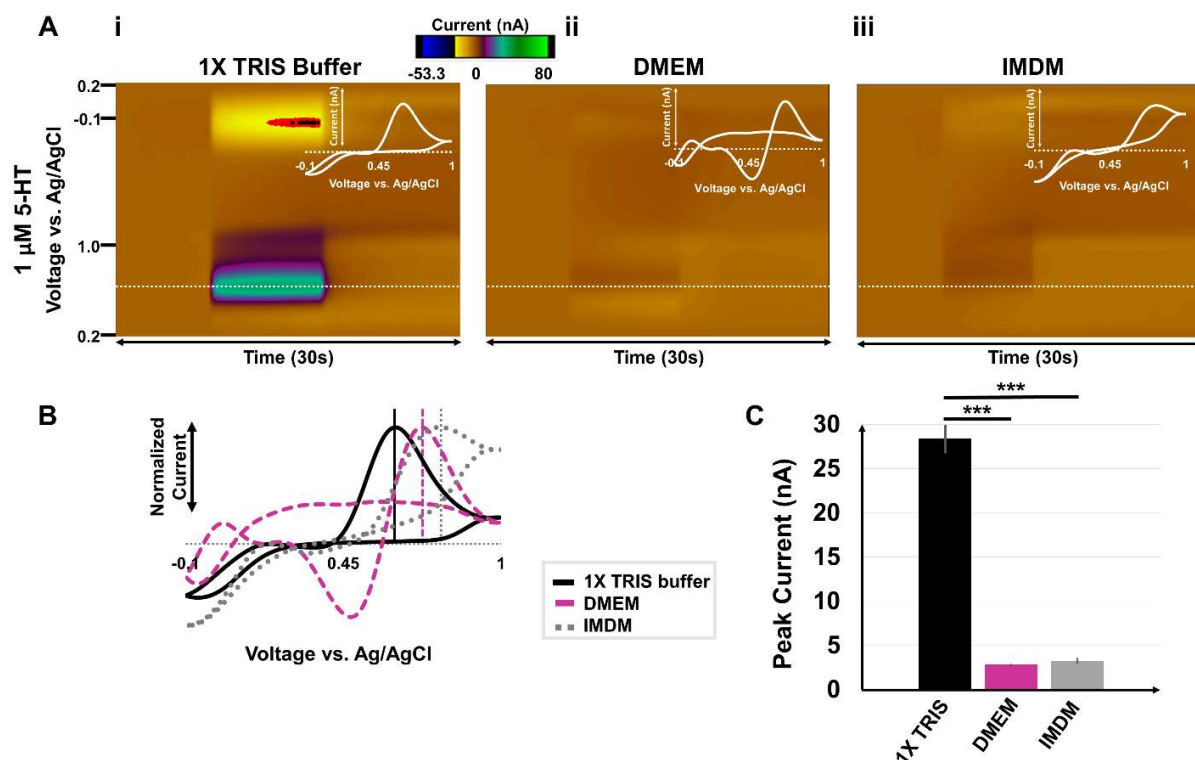


Figure 4.1. Serotonin measurements at CFMs are diminished in cell media. (A) Representative color plots of 10s bolus injections of serotonin onto a Nafioned CFM in 1X TRIS buffer (i), DMEM (ii) and IMDM (iii). (B) Normalized to maximum amplitude of the oxidation peak, serotonin CVs are shown taken in TRIS buffer (black), DMEM (pink dashes) and IMDM (gray dots). The vertical lines denote the position of the oxidative current. (C) Averaged peak current from 1 M serotonin boluses in 1X TRIS buffer (black), DMEM (pink) and IMDM (gray). Error bars represent the standard error of the mean (SEM) from $n=3$ electrodes. An unpaired t-test was used to determine significance (***) $p < 0.001$.

Cell media is composed of a specialized balance of salts, amino acids, vitamins and other nutrients formulated to keep the cells healthy and functioning in an external environment. Since the signal for serotonin was significantly diminished in media, we explored whether this effect was irreversible or could be mitigated. Based on previous publications, we know that overoxidation of the carbon surface enhances monoamine adsorption¹⁰⁰ and that amino acids such as glutamate readily polymerize onto the carbon surface¹⁰¹. As cell media is made up of many amino acids, we hypothesized that this could enhance serotonin measurements at the CFM surface. For this, we evaluated sensitivity and stability of the CFM

towards serotonin before and after overoxidizing the carbon surface in DMEM and IMDM (Figure 4.2). We first calibrated Nafioned CFMs to serotonin in 1X TRIS buffer (Figure 4.2Ai, Bi), then applied a wide potential window (-1.2 V to 1.3 V to -1.2 V at 60 Hz for 10 minutes, 'polymer waveform') to the CFM in cell media (DMEM or IMDM). Then, the CFM was calibrated to serotonin in the media it was cycled in (figure 4.2Aii, Bii), but we observed no improvement when detecting serotonin in cell media (Figure 4.2A,Bii), and the CV was, again, shifted towards more positive potentials (Figure 4.2Aiv, Biv). However, calibrating the overoxidized CFMs in 1X TRIS buffer resulted in a significant increase in sensitivity post-DMEM ($p<0.01$) and an insignificant increase post-IMDM ($p=0.31$). Decreases in the limits of detection (DMEM: $p<0.001$, IMDM: $p=0.81$) and quantification (DMEM: $p<0.001$, IMDM: $p=0.81$) were observed with DMEM being a significant increase (Figure 4.2C,D). This indicates that over-oxidizing the carbon surface in both media types improves serotonin detection. We next evaluated whether this effect would improve the stability of the response over time.

Overoxidizing the carbon surface in cell media improves sensitivity to serotonin

We investigated the stability of the current over 2 hours (one injection every two minutes for a total of 60 injections) by applying 1 μ M serotonin boluses in TRIS buffer at untreated CFMs (black dots), CFMs overoxidized in DMEM (dark red dots), CFMs overoxidized in IMDM (dark blue dots) and the current response to 1 μ M serotonin boluses in cell media at CFMs overoxidized in DMEM (light red dots) and IMDM (light blue dots) (Figure 4.2E). The response to serotonin in cell media post-overoxidation still resulted in significantly less current compared to untreated CFMs (DMEM: 1.26 ± 0.34 nA, $p<0.001$, IMDM: 4.27 ± 0.16 nA, $p<0.001$) (Figure 4.2E). However, the CFMs post-overoxidation in DMEM and IMDM had significantly higher responses to serotonin in TRIS buffer and significantly less signal loss compared to untreated CFMs (Control: 12.75 ± 0.47 nA; $74.5 \pm 3.2\%$ current loss, DMEM: 71.32 ± 0.87 nA, $p<0.001$; $36.1 \pm 3.7\%$ current loss, $p<0.001$, IMDM: 71.02 ± 0.65 nA, $p<0.001$; $36.1 \pm 3.7\%$ current loss, $p<0.001$). Compared to each other, the overoxidized CFMs in TRIS buffer were not significantly different from each

other in peak current ($p=0.897$) but pre-treating in IMDM improved stability significantly more than DMEM ($p=0.0492$) (Figure 4.2E). Together, these data show that while cell media is not conducive for serotonin measurements, the detrimental effects are not irreversible, and the chemical makeup of the matrix can significantly improve sensitivity, detection limits and stability in a salt buffer when paired with an overoxidation protocol.

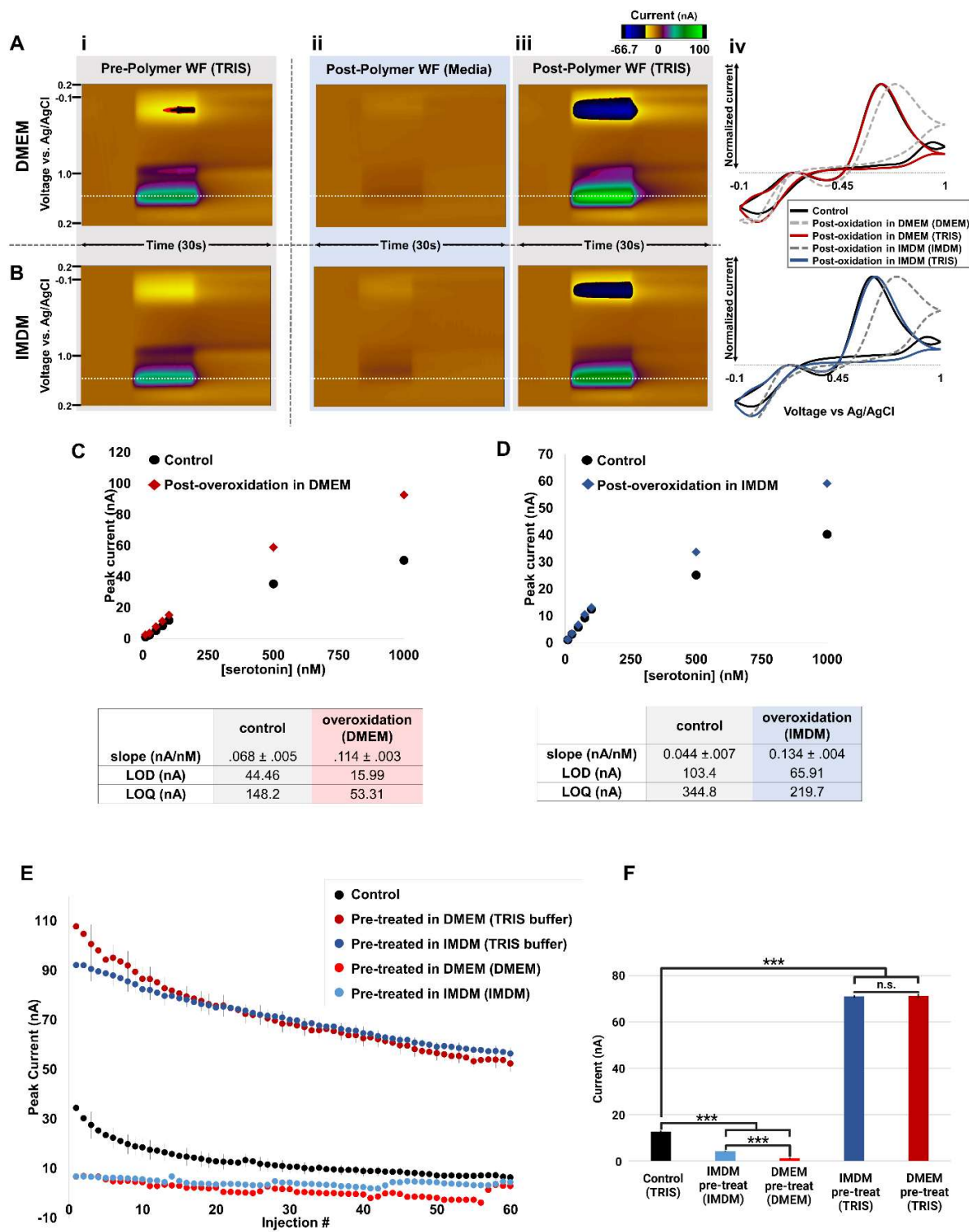


Figure 4.2. Overoxidation of the carbon surface in cell media enhances serotonin measurements. Color plots of 1 μ M serotonin boluses (10s) before (i) and after (ii, iii) cycling in DMEM (A) and IMDM (B) on the polymer waveform.

Panel (ii) shows serotonin in DMEM, and panels (I and iii) show serotonin in TRIS buffer. CVs from each condition are compared in (iv). (C) Nafioned CFMs were calibrated in TRIS buffer to serotonin before (black dots) and after (red diamonds) being cycled in DMEM (D) Nafioned CFMs were calibrated in TRIS buffer to serotonin before (black dots) and after being cycled in IMDM. Associated sensitivities (slope, nA/nM), limit of detection (LOD, nM) and limit of quantification (LOQ, nM) are reported in the tables below C and D. (E) Repeated injections of 1 μ M serotonin in TRIS buffer over 2 hours (1 injection every 2 minutes) at Nafioned CFMs before (black) and after cycling DMEM (dark red) or IMDM (dark blue) on the polymer waveform. Post-polymer waveform CFMs were given the same repeated 1 μ M serotonin boluses in DMEM (light red) or IMDM (light blue). (F) The signals in E were averaged for each condition and error bars are the standard error of the mean. Changes between conditions were compared *via* unpaired t-tests. n.s.=not significant, * $p<0.05$, ** $p<0.01$ and *** $p<0.005$

While a salt buffer is necessary to probe cellular cultures using FSCV, we found that TRIS can be cytotoxic to many cell types, making our TRIS buffer unsuitable for cell work. Another pH buffering molecule, HEPES, has been used for probing cells with FSCV^{72, 92} and has also been shown to facilitate ATP signalling in cultures¹⁶⁵. Subsequent figures in this work reveal that ORS cells release ATP, so for this we chose to use HEPES to buffer a salt solution for FSCV measurements in cells. A sterile, pre-mixed salt solution (Hank's balanced salt solution, HBSS) with 10 mM HEPES was chosen for FSCV measurements (Figure 4.3) as it has a similar osmolality to the body's interstitial fluid¹⁶⁶. Next, we began our collaborative efforts with the Higgins group to probe neurochemical release from human hair follicle explant cultures (ORS cells).

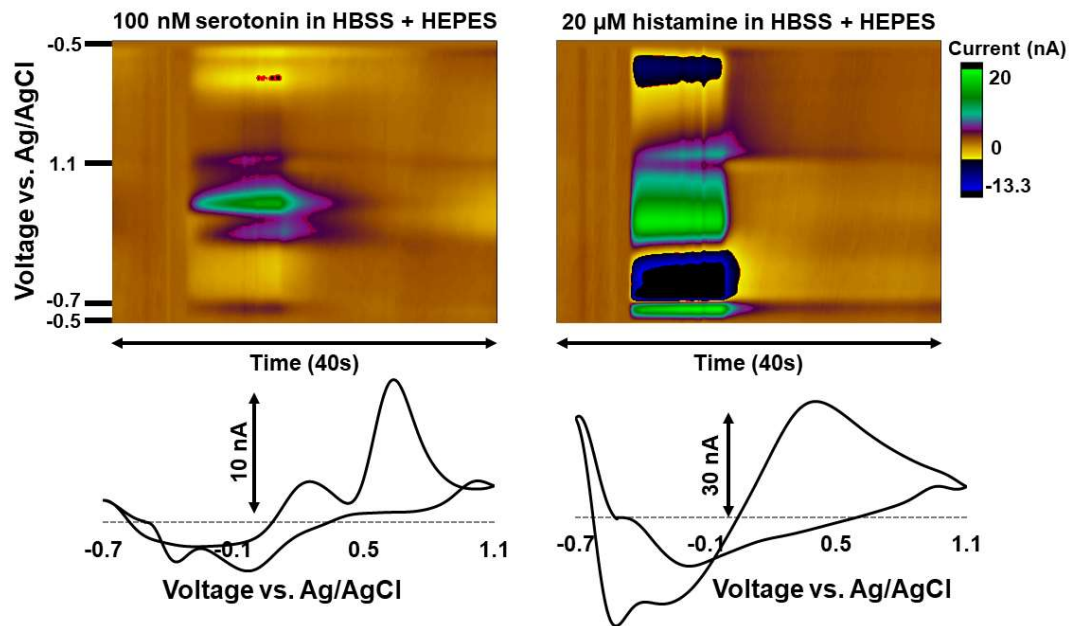


Figure 4.3. Neurochemical detection in HBSS + 10 mM HEPES. A 100 nM serotonin FIA injection (left) onto a CFM modified with Nafion and polyglutamic acid in HBSS + 10 mM HEPES with extracted CV below. A 20 M histamine FIA injection (right) onto the same CFM in HBSS + 10 mM HEPES with extracted CV below.

Mechanical stimulation of epithelial cells evokes neurochemical release

While we optimized our FSCV protocols for neurochemical detection in cell cultures, the Higgins lab identified sensory neurons that wrap around the human follicle. Using whole-mount immunolabeling they found several types of LTMRs, including C-LTMR, and an HTMR innervate human hair follicles taken from occipital scalp skin (Figure 4.4). Additionally, they found that outer root sheath cells cultured from hair follicle explants respond to mechanical stimuli *in vitro*. Using published datasets of single cell RNA from human hair follicle and skin preparations, the hair follicles had enhanced mechanosensitive machinery compared to the skin (Figure 4.5A,B). Then, using calcium imaging in a co-culture of ORS cells and neurons taken from the dorsal root ganglia of mice (LTMRs), a mechanical stimulus to the ORS cells elicited an immediate influx of calcium and delayed influx into the LTMRs 70-200 μm away from the stimulus point (Figure 4.5C,D). Separate experiments of calcium depletion of the co-cultures and inhibiting

mechanosensitive channels with GsMTx-4 decreased the response to mechanical stimulation (Figure 4.5E-I). Finally, mechanical stimulation of the co-cultures was found to activate LTMRs *via* ATP, which was partially inhibited after inhibiting ATP release *via* apyrase incubation (Figure 4.6). The partial activation of LTMRs after inhibiting ATP release indicated that the ORS cells must be communicating with other signalling molecules. This raised the question as to what molecules are mediating this ORS-LTMR communication.

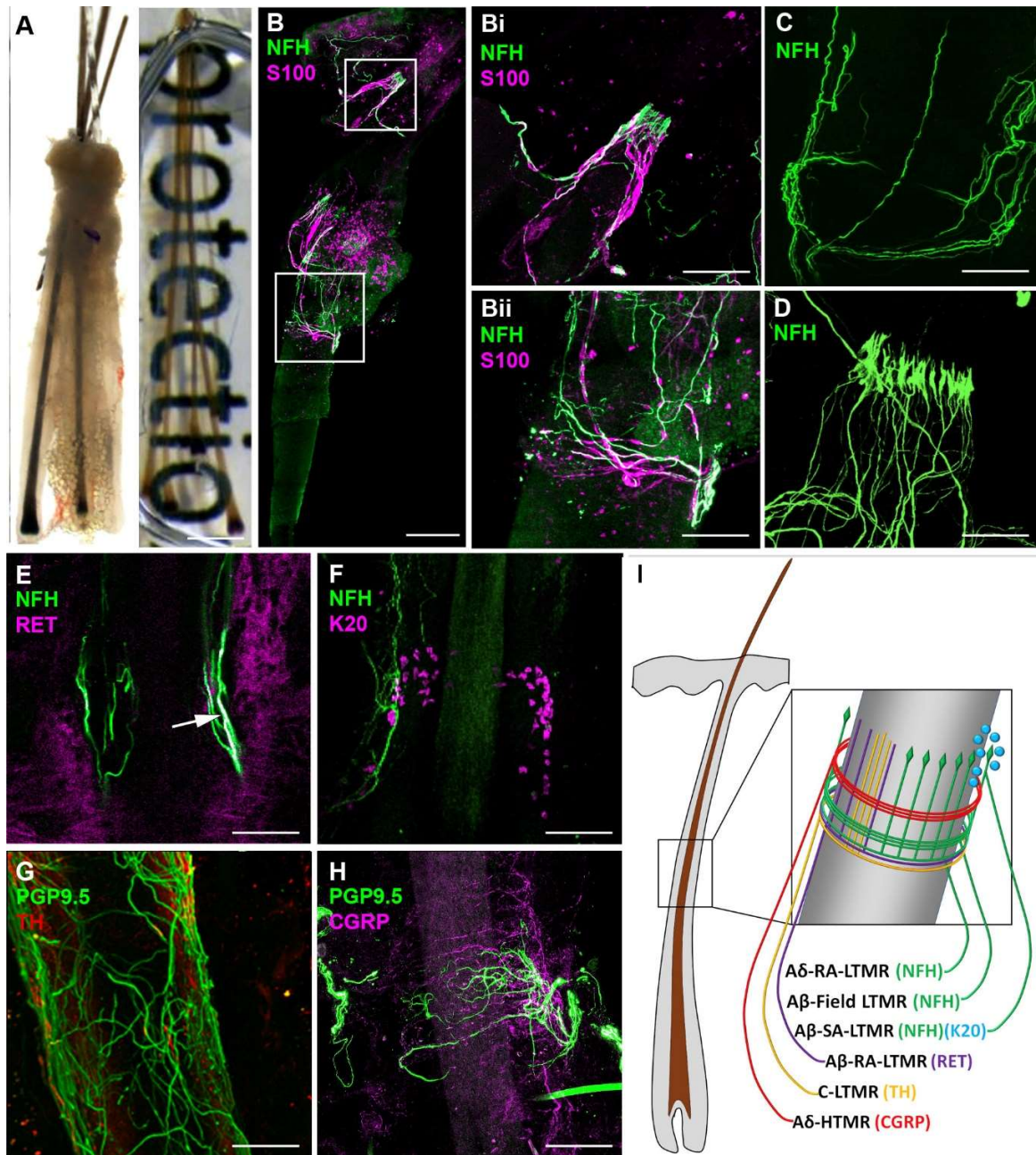


Figure 4.4. Human occipital scalp hair follicles are innervated by LTMRs and an HTMR (sensory neurons). (A) Representative human follicular unit from scalp skin composed of terminal hairs before (left) and after (right) clearing. (B) Follicular unit stained with NFH and S100 showing longitudinal (Bi) and circumferential (Bii) nerve endings from A δ -RA-LTMR, A β -RA-LTMR and A β -Field-LTMR. (C) Volumetric stacking of human follicular units showing LTMR circumferential endings. (D) Whole-mount images created using stacking of human follicular units and

3D rendering that show the lanceolate shape of LTMR longitudinal endings. (E) Overlap (arrow) of RET and NFH antibody staining reveals A β -RA-LTMR longitudinal nerve ending. (F) NFH antibody staining shows A β -SA-LTMR innervating K20(+) Merkel cells. (G) Antibody staining against TH identifies C-LTMR longitudinal endings. (H) CGRP antibody staining shows A δ -HTMR forming circumferential endings around human hair follicles. (I) Synoptic illustration of the different LTMRs and HTMR found to innervate human hair follicles based on staining in panels A-H. Depicted for visual clarity, we do not know whether these are present on every terminal hair follicle or if there is heterogeneity across the scalp. Scale bars: A) 500 μ m, B) 200 μ m, Bi) 100 μ m, C-H) 50 μ m Performed by Julia Agramunt (Higgins lab)

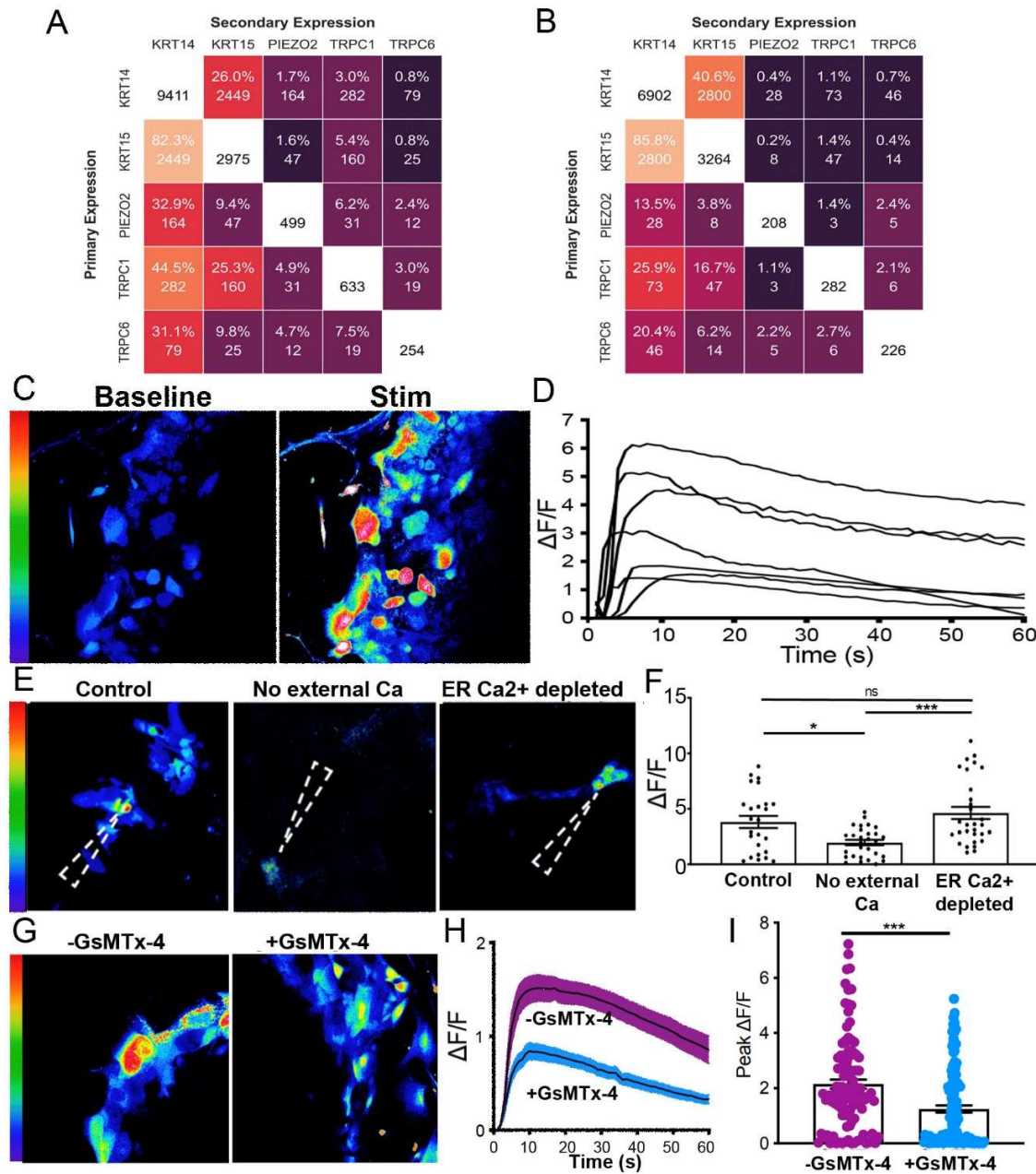


Figure 4.5. ORS cells respond to mechanical stimulation *in vitro*. (A) Heatmap depicting co-expression of scRNA-seq data of human scalp hair follicles showing 5.4% of K14 cells also express TRPC1. (B) Co-expression analysis of scRNA-seq data of human skin showing 1.1% of K14 cells also express TRPC1. (C) Representative images of ORS cells at baseline and after mechanical stimulation. (D) $\Delta F/F$ traces of mechanically stimulated calcium release in ORS cells from panel C. (E) Representative images of ORS cells after mechanical stimulation in (+)Ca²⁺ (-)TPSG (control), (-)Ca²⁺

(-) TPSG (no external Ca^{2+}) and (+) Ca^{2+} (+)TPSG (ER Ca^{2+} depleted) conditions. (F) Comparison of peak $\Delta F/F$ from $n=2$ ORS cultures from 2 patients (27-30 cells in each condition). A one-way ANOVA with Bonferroni post-hoc analysis was performed to determine significant changes between the three conditions. (G) Representative images of ORS cells after mechanical stimulation with and without GsMTx-4. (H) Mean $\Delta F/F$ vs time traces of mechanically stimulated calcium in ORS cells with and without GsMTx-4. (I) Peak $\Delta F/F$ values from $n=7$ ORS cultures from 4 patients (total of 106 cells in the (-)GsMTx-4 group and 110 cells in the (+) GsMTx-5 group). An unpaired t-test was used to determine compare $\Delta F/F$ values between conditions. Performed by Julia Agramunt (Higgins lab)

As ORS cells partially use ATP to transmit the mechanical stimuli to activate LTMRs, we sought to identify other molecular intermediates between mechanical stimulation of ORS cells and LTMR activation. Using an antibody for a synaptic vesicle protein, synapsin 1 (SYN1), we found SYN1(+) synaptic vesicles between nerve endings from neurofilament H (NFH)(+) LTMRs and keratin 15 (K15)(+) ORS cells (Figure 4.7A). The positive expression of SYN1 showed that the ORS cells can release neurotransmitters, and that they likely participate in the activation of LTMRs. We next employed an electrochemical technique, fast scan cyclic voltammetry (FSCV) at carbon fiber microelectrodes (CFMs) to identify and quantify mechanically evoked neurotransmitter release in ORS cell explant cultures (Figure 4.7B). An in-depth discussion of FSCV can be found in Chapters 1 and 2. For this work we chose to employ a histamine-optimized waveform because the voltage bounds allow for serotonin, dopamine and histamine to be co-detected. As we were initially unsure as to what neurochemicals would be released, we thought it best to cast the net wide and use an inclusive waveform. Experimentally, we placed the CFM $\sim 1-3 \mu\text{m}$ above the ORS cells and the stimulating electrode (blunted glass pipette), as shown in Figure 4.7B, with a pseudo-Ag/AgCl reference electrode on the outskirts of the dish. We used a stimulation paradigm with assigned intensities associated with the number of stims, where 1 stim was the lowest, 3 the intermediate and 5 the highest. Each stim was a $5 \mu\text{m}$ indent into the ORS cells and repeated indents for the different intensities were performed every other second. Upon mechanical stimulation, the ORS cells were found to release serotonin and histamine based

on the inset cyclic voltammogram (CV) of the release (Figure 4.7Ci). The release was validated to be serotonin and histamine by introducing a mixed solution of 150 nM serotonin and 5 μ M histamine to the CFM using FIA and normalizing the resultant CV to the one taken from the ORS cell response (Figure 4.7Di). The overlapping oxidative peaks for histamine (~ 0.3 V) and serotonin (~ 0.65 V) confirmed their presence in the ORS cultures. Using a partial least squares regression (PLSR) model, we mathematically separated the current vs time traces for histamine and serotonin, as their peak currents are not well-resolved in the raw data. FSCV currents were converted to concentration based on post-calibration curves of histamine and serotonin captured using an FIA system. The stimulations evoked current vs time (IT) traces proportional to their intensities for histamine and serotonin (Figure 4.7E,F) with AUC quantitation for histamine (1 stim: 0.65 ± 0.20 μ M*min, 3 stims: 1.47 ± 0.35 μ M*min, 5 stims: 2.96 ± 0.3 μ M*min) and serotonin (1 stim: 6.63 ± 1.20 nM*min, 3 stims: 14.47 ± 5.50 nM*min, 5 stims: 33.82 ± 11.32 nM*min). These data show that ORS cells co-release serotonin and histamine in response mechanical stimulation and that the magnitude is proportional to the number of applied stimuli.

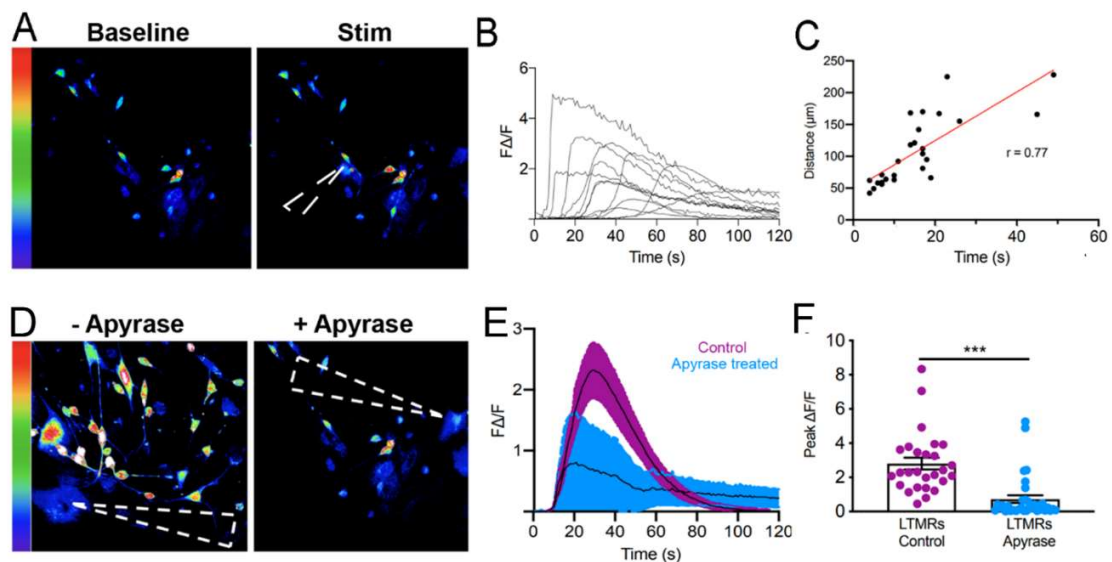


Figure 4.6. LTMRS are partially activated by mechanically stimulated ORS cells with ATP inhibition *via* apyrase in co-culture. (A) representative images of LTMRS (white arrow) at baseline and mechanically stimulated (B) $\Delta F/F$ vs

time traces of LTMR activation upon ORS cell stimulation. (C) Correlation of LTMR distance from stimulation and time of peak activation. (D) representative images of activated LTMRs (white arrow) *via* mechanical stimulation of ORS cells with (right) and without (right) apyrase incubation (E) average $\Delta F/F$ vs time response of LTMR activation after mechanical stimulation of ORS cells with (blue) and without (purple) apyrase treatment. (F) Comparison of peak $\Delta F/F$ responses from LTMRs treated with (blue) and without (purple) apyrase. (Higgins lab)

We next investigated whether the response of ORS cells to increasing stimulation number was specific to hair follicle epithelial cells or common to skin epithelia *in vitro* by repeating our experimental paradigm to explant cultures of interfollicular skin keratinocytes. Mechanical stimulation of keratinocytes elicited a release of histamine (Figure 4.7Cii). As with ORS cells, the inset CV from the keratinocyte response was normalized to a CV taken from a FIA-injected histamine bolus onto a CFM (Figure 4.7Dii). FSCV signals were converted to concentration based on post-calibrations of CFMs using FIA. Based on AUC analysis of the IT curves, the evoked histamine response was proportional to stimulation number (1 stim: $1.96 \pm 0.77 \mu\text{M} \cdot \text{min}$, 3 stims: $2.13 \pm 0.82 \mu\text{M} \cdot \text{min}$, 5 stims: $3.20 \pm 1.28 \mu\text{M} \cdot \text{min}$) (Figure 4.7G), whereas serotonin was not released (1 stim: $-13.75 \pm 2.97 \text{ nM} \cdot \text{min}$, 3 stims: $-33.55 \pm 12.25 \text{ nM} \cdot \text{min}$, 5 stims: $-26.50 \pm 11.37 \text{ nM} \cdot \text{min}$) (Figure 4.7H). The observed drop in signal post-stimulation is likely a stimulation artifact which can also be observed in the absence of release. Compared to the ORS cells, the amount of histamine released in keratinocytes was not significantly different (Figure 4.7J), but the ORS cells released a significantly greater amount of serotonin than the keratinocytes (1 stim: $p < 0.005$, 3 stims: $p < 0.01$, 5 stims: $p < 0.05$) (Figure 4.7I). These data support previous reports of stimulated histamine release in keratinocytes, but also show that hair follicle and skin epithelial cells proportionally respond to mechanical stimulation. Additionally, they show that mechanically evoked serotonin release is specific to hair follicle ORS cells. Based on these data, we next investigated the release mechanism of serotonin and histamine from ORS cells.

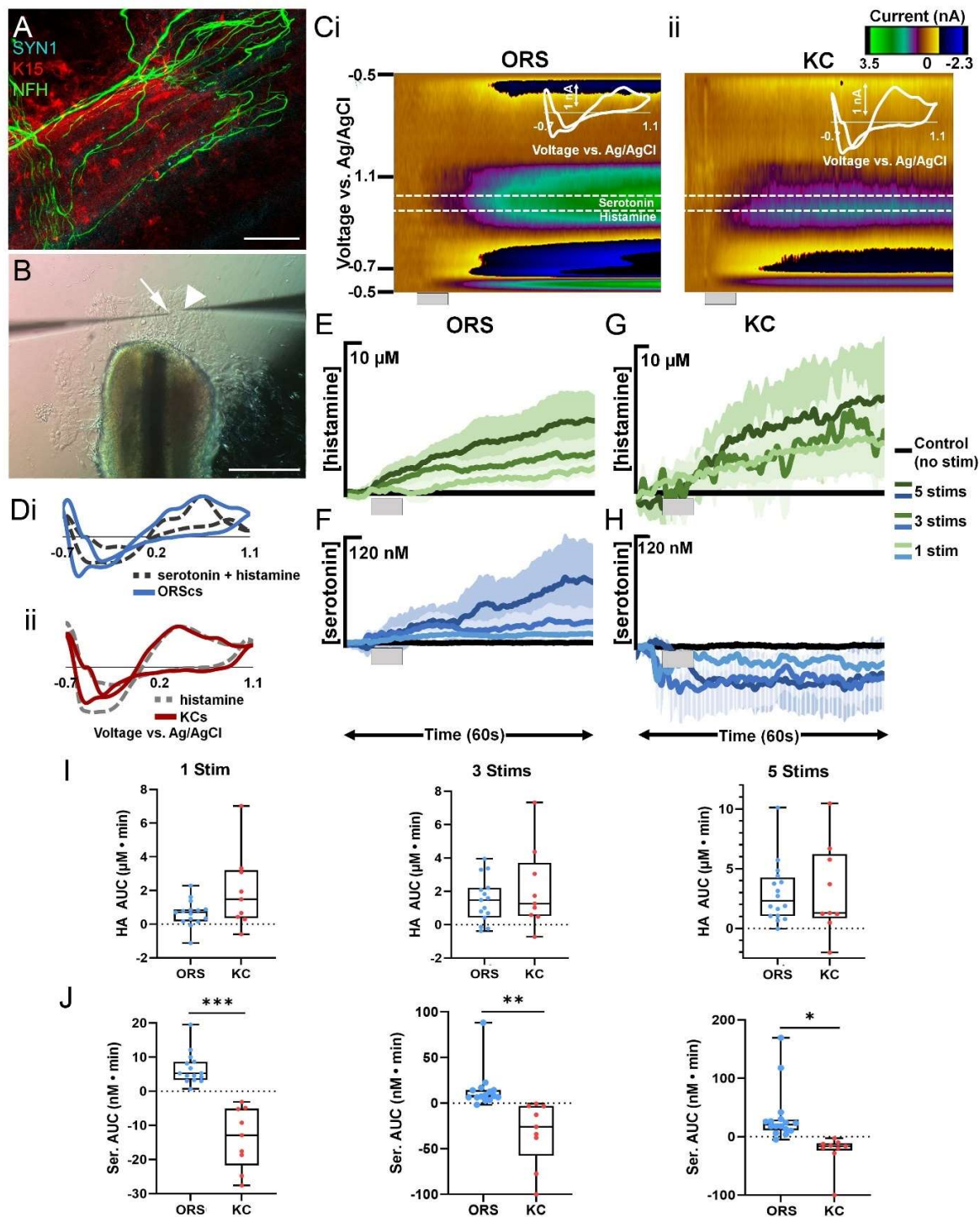


Figure 4.7. Epithelial cells release neurochemicals in response to mechanical stimulation. (A) Whole-mount immunostaining of cleared human follicular units using SYN1, NFH, and K15 antibodies. (B) Image of an ORS cell culture with the CFM (arrow, left) and stimulating pipette (triangle, right). (C) Representative color plots of

mechanically stimulated ORS cells (i) and keratinocytes (ii) with inset CVs taken from the signal. The gray bar denotes the time and duration of the stimulation. (D) normalized CVs from the (i) ORS cells (blue) and a mixed FIA injection of serotonin and histamine (dark gray dashes), and from the (ii) keratinocytes (red) and an FIA injection of histamine (light gray dashes). (E) Mechanically evoked histamine release from ORS cells from 1 to 5 stimulations. Gray bar denotes maximum stimulation time and duration. (F) Mechanically evoked serotonin release from ORS cells from 1 to 5 stimulations. Gray bar denotes maximum stimulation time and duration. (G) Mechanically evoked histamine release from keratinocytes from 1 to 5 stimulations. Gray bar denotes maximum stimulation time and duration. (H) Mechanically evoked serotonin release from keratinocytes from 1 to 5 stimulations. Gray bar denotes maximum stimulation time and duration. (I) Box and whisker plots comparing AUC analysis of mechanically evoked histamine release between ORS cells and keratinocytes from 1 to 5 stimulations (left to right). Statistical significance was determined using a two-way ANOVA with Tukey-Kramer post-hoc analysis. (J) Box and whisker plots comparing AUC analysis of mechanically evoked serotonin release between ORS cells and keratinocytes from 1 to 5 stimulations (left to right). Statistical significance was determined using a two-way ANOVA with Tukey-Kramer post-hoc analysis.

Neurochemicals in ORS cells are released from synaptic vesicles

After finding that ORS cells co-release serotonin and histamine in response to mechanical stimulation, we sought determine if the stimulated signal would activate the LTMRs. We co-cultured human ORS cells with LTMRs isolated from the dorsal root ganglia (DRG) of mice. In the cultures we observed the LTMRs and ORS cells resided in close proximity to one another, suggesting this *in vitro* model is suitable to investigate signalling between the two cell types.

Next, we used the ORS-LTMR co-cultures to evaluate whether there was an excitatory connection between the ORS cells and LTMRs. To assess LTMR activation, we used calcium imaging to visualize intracellular calcium levels (cultures were incubated with Cal-520). ORS cells were identified and stimulated as described for Figure 4.7. We found that neurons located 70-200 μm from the point of stimulation had increased intracellular calcium levels in response to the stim (Figure 4.8A). LTMR activation began ~10 seconds after ORS cell stimulation, with some responding just over 50 seconds after stimulation (Figure

4.8B). Comparing the time of LTMR activation with the distance from the stimulation point showed a strong correlation ($R=0.77$), suggesting that molecules released from the ORS cells diffuse through the media to activate the neurons (Figure 4.8C). While this is quite different to the *in vivo* human condition, this preparation gives us insight into the ability of ORS cells to activate sensory neurons.

Next, we explored whether the signalling molecules released from ORS cells (ATP, serotonin and histamine) would mediate LTMR signalling. We incubated the co-cultures with bafilomycin A1, a vacuolar-type H^+ -ATPase inhibitor to dissipate the vesicular proton gradient necessary for vesicular transporters to pump transmitters into the vesicles, therefore depleting the synaptic vesicles. We mechanically stimulated the co-cultures as described above and measured their intracellular calcium levels (pre-loaded with Cal-520) (Figure 4.8D). The LTMRs without bafilomycin exhibited a large calcium influx whereas the calcium signal from the culture incubated with bafilomycin was significantly dampened (Figure 4.8E,F).

We next applied an acute dose of bafilomycin to an isolated culture of ORS cells to determine whether the ORS cells activating the small population of LTMRs was due to vesicular neurotransmitter release. In response to mechanical stimulation, the untreated ORS culture elicited a release of serotonin and histamine, but upon acute bafilomycin application, the mechanical stimulation did not evoke a release (Figure 4.8G). The area under curve analysis of the IT curves showed a significant decrease in stimulated release of both serotonin and histamine (Figure 4.8H,I).

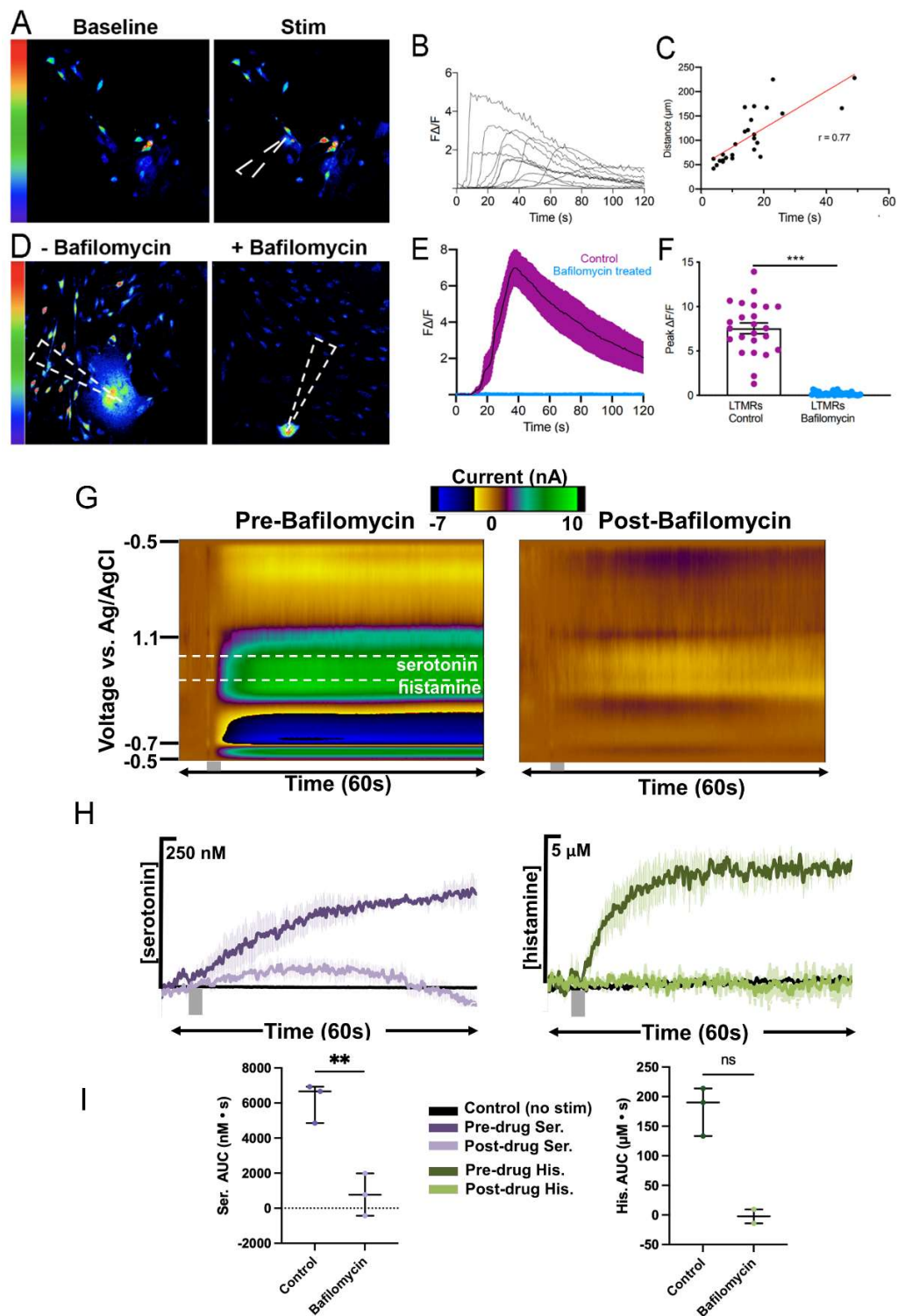


Figure 4.8. Neurochemicals are released from synaptic vesicles in ORS cells. (A) Representative images LTMR calcium responses pre (left) and post-mechanical stimulation (right) of ORS cells in co-culture with the LTMRs (white

arrow). (B) $\Delta F/F$ vs time traces of calcium release from LTMRs after ORS mechanical stimulation in ORS-LTMR co-culture. (C) LTMR distance from ORS stimulation vs activation time correlation. (D) Representative images of LTMRs after mechanical stimulation of ORS cells in co-culture with LTMRs (white arrow) with (right) and without (left) Bafilomycin A1. (E) Averaged $\Delta F/F$ traces from LTMRs after mechanical activation of ORS cells with (blue) and without (purple) bafilomycin. (F) Peak $\Delta F/F$ from activated LTMRs with and without bafilomycin (n=2 cultures from 1 patient, 26 different cells at each condition). (G) representative FSCV color plots of stimulated release of ORS cultures with (right) and without (left) bafilomycin. Gray bars denote the stimulation time. (H) Current vs time traces of stimulated serotonin (left, purple) and histamine (right, green) release. Control (no stimulation) is denoted in black, stimulation pre-bafilomycin is the darker color and the post-bafilomycin trace is the lighter color. (I) AUC analysis of mechanically stimulated serotonin (left, purple) and histamine (right, green) before (dark color) and after (light color) bafilomycin. Scale bars in A and D are both 50 μm . **p<0.01, ***p<0.001

Neurochemicals modulate LTMR response through serotonergic and histaminergic receptor activation

After determining that ORS cells co-release serotonin and histamine in response to mechanical stimulation, we next determined whether the neurotransmitter release from ORS cells can modulate LTMR response. For this used the ORS cell and DRG co-culture, as previously described in this work, and treated them with mirtazapine, an antidepressant that antagonizes 5-HT₂, 5-HT₃ and H₁ receptors.

After pre-incubation with the fluorescent calcium dye (Cal-520), calcium signals at baseline and evoked in the co-culture were recorded in both ORS cells and LTMRs (Figure 4.9A,B). The co-cultures were then incubated with mirtazapine for 30 minutes and calcium signals (baseline and evoked) were recorded (Figure 4.9C,D). Mirtazapine had no effect on the ORS response (p=0.967) (Figure 4.9E), but significantly dampened the LTMR response (p<0.005) by a factor of 2.42 (Figure 4.9F).

Finally, to confirm that the reduced LTMR response is due to the antagonistic effect of mirtazapine on serotonin and histamine receptors rather than a consequence of impaired neurotransmitter release from ORS cells, we monitored mechanically evoked release of neurotransmitters from ORS cells treated with

mirtazapine using FSCV (Figure 4.9G). We found that mirtazapine induced a 3.6-fold increase in serotonin release ($p=0.010$) and a 2.1-fold increase in histamine release ($p=0.17$) with 1 stim (Figure 4.9H,I). We also applied 3 and 5 stimulations which also resulted in increases in neurotransmitter release (Figure 4.10). This result highlights that in the presence of more serotonin and histamine as a result of mirtazapine in ORS cells, the LTMR response is still halved.

Overall, these data suggest that ORS cells in human hair follicles can release serotonin and histamine in response to mechanical stimulation, which can modulate LTMR intracellular calcium levels through 5-HT₂, 5-HT₃ and H₁ receptor activation.

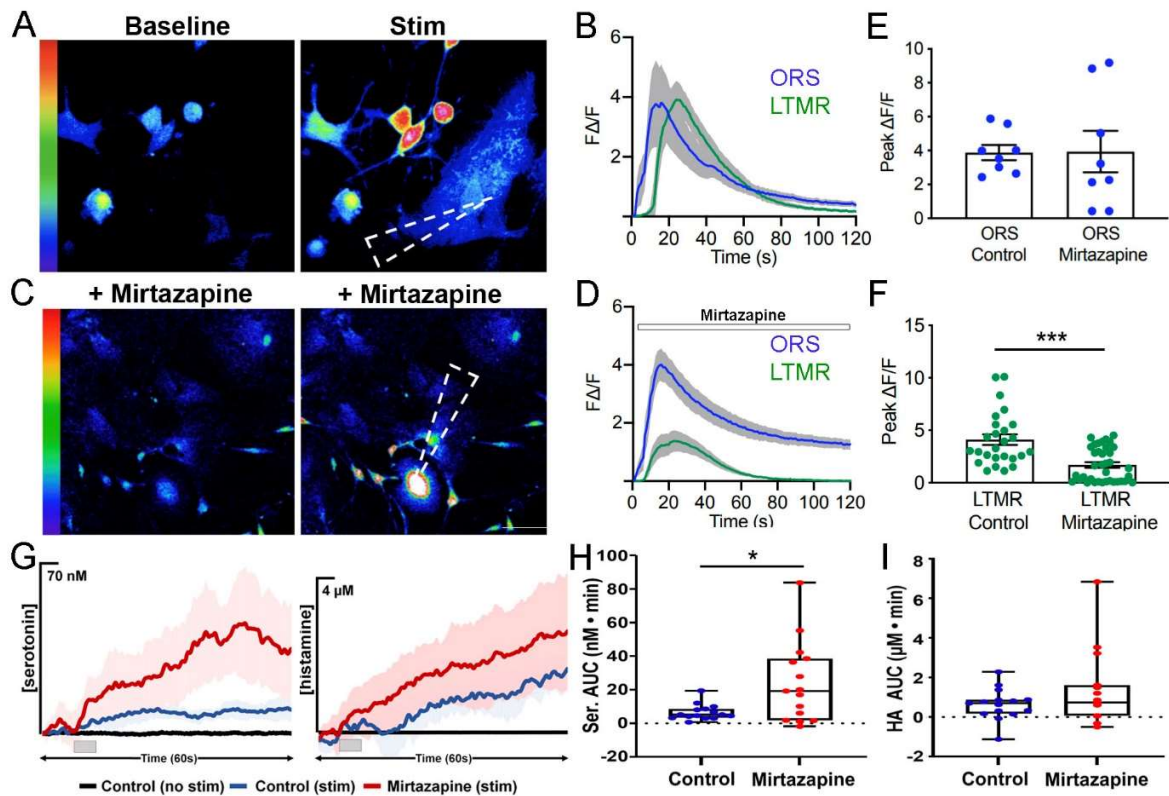


Figure 4.9. Serotonin and histamine release from ORS cells modulates LTMR response. (A) Images of baseline and stimulated calcium transients in ORS cells and associated LTMRs in co-culture. (B) Traces of $\Delta F/F$ in ORS cells and LTMRs after mechanical stimulation of ORS cells (C) Images of baseline and stimulated calcium transients in ORS cells and associated LTMRs in co-culture treated with mirtazapine. (D) Traces of $\Delta F/F$ in mechanically activated ORS cells

and LTMRs after mirtazapine treatment. (E) Peak $\Delta F/F$ in n=8 co-cultures from 3 patients (a different ORS cell was stimulated in each co-culture). An unpaired t-test was used to compare peak $\Delta F/F$ values in control cells and after mirtazapine treatment. (F) Peak $\Delta F/F$ in n=8 co-cultures from 3 patients (36 LTMRs total). An unpaired t-test was used to compare peak $\Delta F/F$ values in control LTMRs and after mirtazapine treatment (different cells within the same dish were used in the two conditions). (G) Average traces of stimulated release of serotonin (left) and histamine (right) from ORS cells using 1 stimulation. (H) Box and whisker plots comparing the AUC from the current vs time traces from stimulated (1 stim) serotonin release in ORS cells. A two-way ANOVA with Tukey-Kramer post-hoc analysis was used to determine significance. (I) Box and whisker plots comparing the AUC from the current vs time traces from stimulated (1 stim) histamine release in ORS cells. A two-way ANOVA with Tukey-Kramer post-hoc analysis was used to determine significance. (n=15 stimulations in 5 ORS cultures from 4 different patients)

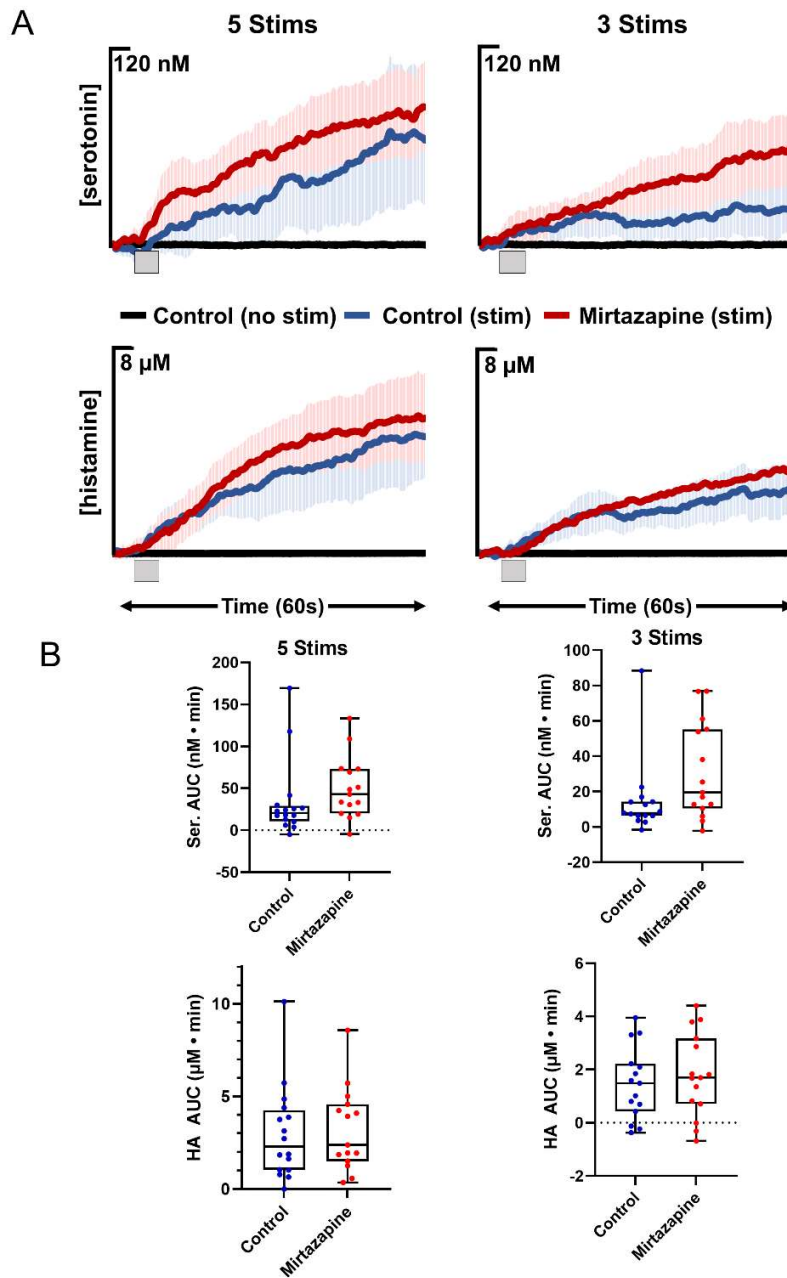


Figure 4.10. Mechanical stimulation of ORS cells induces more neurochemical release when pre-incubated with mirtazapine. (A) Current vs time traces of stimulated release of histamine and serotonin from ORS cells. Control files were taken prior to stimulation (black traces, 10 per location), then ORS cells were mechanically stimulated 3 or 5 times by a 5 μ m indent with and without mirtazapine treatment at 3 locations per dish (n=15 stimulations in 5 ORS

cultures from 4 patients). (B) AUC analysis for serotonin (top) and histamine (bottom) was compared between the two conditions. A 2-way ANOVA with Tukey-Kramer post hoc analysis was performed to determine statistical significance. * $p < 0.05$

Discussion

In this study we evaluated our hypothesis that hair follicle ORS cells can act as a signal transducer for mechanical stimuli, leading to the excitation of sensory neurons surrounding the hair follicle. For this we collaborated with the Higgins lab, who showed that 5 types of LTMRs and an HTMR innervate hair follicles from human occipital scalp samples, while we simultaneously optimized FSCV measurements for cellular cultures by exploiting the effects of cell media to improve serotonin detection at CFMs. Then, to understand the interaction between ORS cells and LTMRs we assessed the mechanosensitive properties of ORS cells and identified the mechanically evoked release of serotonin and histamine from ORS cells using FSCV. Based on the presence of a synaptic vesicle marker, SYN1, in ORS cells, it is likely that the neurochemical release is *via* synaptic vesicles. In contrast, interfollicular skin keratinocytes release comparable levels of histamine, but negligible serotonin in response to mechanical stimulation. Finally, we showed that antagonizing serotonin and histamine receptors on LTMRs dampened their response to excitatory stimuli from ORS cells. Together, these results show that hair follicle ORS and LTMRs form a neuroepithelial unit.

Initially, the optimization of serotonin measurements showed that cell media is not conducive for sensitively probing neurochemicals. However, applying an overoxidation protocol at CFMs in cell media significantly improved detection limits and sensitivity towards serotonin in a salt buffer. This finding coincides with Holmes *et al.*, where overoxidation of the CFM in the presence of glutamate induced the amino acid to polymerize onto the carbon surface, resulting in enhanced monoamine detection¹⁰¹. It can be postulated here that the amino acids in the cell media also polymerize onto the CFM. However, confirmation studies need to be performed to validate this notion. Importantly, for future FSCV work in

cells, the carbon surface is not irreversibly fouled when exposed to media and it can even be used to improve measurements.

ORS cells have the capacity to release serotonin, but this was not conserved in the keratinocytes, which only released histamine. Importantly, ORS cells are the equivalent to keratinocytes in the hair follicle, and within the skin, keratinocytes differentiate into hair follicle epithelial cells (ORS cells). Additionally, in response to injury, hair follicle ORS cells can de-differentiate into keratinocytes to aide in wound healing. However, we found that only ORS cells released serotonin¹⁶⁷. This finding suggests a novel signalling pathway for tactile stimulation that is mediated by cells within the hair follicle. The mechanical stimulation paradigm employed in this work resulted in a large, sustained release of serotonin and/or histamine from the ORS cells and keratinocytes. While this paradigm worked well for this study, it would be interesting to elucidate how different kinds of stimulation (light, vibration, indentation depth) effect neurochemical release from these cell types.

We found that ORS cells release ATP in response to mechanical stimulation and use this to communicate with LTMRs. This finding supports previous work demonstrating mechanically evoked ATP release from keratinocytes¹⁵⁸. Our finding builds on this to show that ATP release from hair follicle epithelial cells in response to mechanical stimuli facilitates their communication with LTMRs. However, serotonin and histamine play an important role in LTMR activation, as inhibiting ATP release resulted in a partial activation of the LTMRs. We found that a full LTMR inhibition occurred when disrupting vesicular release within the ORS cells, indicating the essential role of serotonin and histamine in the communication of tactile information from the follicle to sensory neurons.

Finally, both mouse and human keratinocytes from skin have histamine synthesis machinery. A mouse keratinocyte line (Pam 212) release histamine in response to alpha-melanocyte stimulating hormone⁵³.

Our work supports this and provides clues about skin conditions with poorly defined aetiologies, such as dermatographia.

Conclusion

Together, our findings strongly suggest that ORS cells from the human hair follicle with nearby LTMRs form a neuroepithelial unit. We found that ORS cells release ATP, serotonin and histamine, which modulate LTMR activation, and the differential release of these signalling molecules facilitate communication with sensory neurons within the follicle. This epithelial-neuronal communication leads to physical and psychological outcomes in the central nervous system and brain. Additionally, the makeup of cell media can enhance serotonin detection at carbon microelectrodes when paired with an overoxidation protocol. Our work in this study has laid an important foundation for future work in probing cells with FSCV and in how tactile evocation of neurochemicals from scalp hair follicles affects touch perception.

5. Chapter 5 Monitoring Neurotransmitter Release from Dermal Fibroblasts in Real Time with Voltammetry

The work presented in this chapter was done in collaboration with the Higgins laboratory at Imperial College London. The entirety of the data presented here was performed by Brenna Parke with assistance from Tomas Andriuskevicius or Anna Rhodes for Figures 5.2-5.5.

Abstract

The cellular makeup of the skin chemically mediates the body's interactions with the world, and visually reflects happenings within the body. A brain-skin connection *via* an immune axis has been described where skin conditions with immunological aetiologies are correlated with worsening psychiatric symptoms. Additionally, a collaboration between the Hashemi and Higgins labs has recently shown that epithelial cells associated with hair follicles within the skin mediate the transfer of tactile information to sensory neurons *via* serotonin and histamine release. This robust neuro-epithelial connection led us to explore whether other skin cells have the capacity for neurochemical release. Dermal fibroblasts have been shown to respond to changes in extracellular levels of histamine and serotonin in the context of wound healing. However, release of these neurochemicals from dermal fibroblasts has not been shown. Fast scan cyclic voltammetry (FSCV) is an electrochemical method used to detect release of redox-active molecules in real-time, typically at carbon fiber microelectrodes (CFMs). In preliminary work, we identified spontaneous serotonin release from epithelial cell types in the skin and hair follicle (outer root sheath cells, keratinocytes and fibroblasts). As neurochemical release from dermal fibroblasts has not yet been identified, in this work we employed FSCV at CFMs to characterize spontaneous human fibroblast activity with single-cell resolution. We first observed the spontaneous release of histamine and serotonin which increased in frequency in response to an H₁ receptor antagonist. An investigation into the mechanisms of release found no ATP-mediation, indicating a non-vesicular release process. Finally, we evaluated the

capacity for fibroblasts to uptake serotonin and histamine by incubating fibroblast cultures with an excess of both neurochemicals, resulting in decreased and increased signalling frequency, respectively. Within this exploratory study, the novel identification of serotonin and histamine release was found to be regulated by H₁ receptor activity. Overall, our data sheds important light on the release of neurochemicals within the skin and lays the foundation for future work to correlate dermal signalling dysfunction to neurological diseases.

Introduction

The skin mediates the body's interaction with the outside world. Conversely, it is also a window into dysregulation within the body, evidenced by physicians using the appearance of certain rashes and other skin abnormalities to diagnose and treat internal diseases. However, the interconnectivity between the skin and internal physiological processes is still being uncovered. A relationship between the skin and brain has been described, where chronic skin conditions with immunological underpinnings (eczema, psoriasis) are correlated worsening psychological symptoms.^{47, 49} From this, it is thought that the brain and skin are chemically connected *via* an immune axis, for example, mast cells (an immune cell type) are found within both the skin¹⁶⁸ and brain¹⁶⁹ and potentially participate in this immune axis. Mast cells mediate immune function in skin during an inflammatory reaction or when the skin is wounded, a process in which dermal fibroblasts are integral^{170, 171}. While dermal fibroblasts respond to histaminergic stimulation during an immune reaction, their capacity for neurochemical release has not been shown. Our recent work, described in Chapter 4, showed that tactile information is transmitted *via* neurochemicals (serotonin and histamine) between epithelial cells in the skin and hair to sensory neurons surrounding the hair follicles¹⁷². As dermal fibroblasts respond to extracellular histamine and in conjunction with our previous findings, we sought to explore whether they also had the capacity to release neurochemicals.

Fibroblasts are a versatile cell type found throughout the body, with their function varying based on their location (i.e. cardiac fibroblasts are different from dermal fibroblasts). Within the dermis of the skin, fibroblasts maintain tissue homeostasis by regulating extracellular matrix (ECM) turnover with collagen production and participate in immune reactions, including wound healing. In the initial stage of wound healing, mast cells at the site of damage release histamine to recruit nearby dermal fibroblasts to differentiate into myofibroblasts, their immune-active form¹⁷³. These myofibroblasts restore the ECM by depositing collagen to close the wound¹⁷⁰. This has been demonstrated by Lin *et al.* where the application of excess histamine or an H₁ receptor antagonist in human dermal fibroblasts resulted in elevated levels of collagen⁵⁴. However, to our knowledge, fibroblasts have not been shown to release histamine as a chemical messenger.

In this work, we explore the spontaneous activity of dermal fibroblasts with FSCV. We first identified and validated the spontaneous release of serotonin and histamine from dermal fibroblasts. Then, find signalling activity increased in response to an H₁ antagonist (diphenhydramine). Application of bafilomycin, as in chapter 4, signalling decreased but did not cease, therefore the release of histamine and serotonin was not solely mediated by ATP. Finally, incubation with excess histamine and serotonin induced a differential response to diphenhydramine, shedding light on the integral role of the H₁R in neurochemical release from dermal fibroblasts. The detection of neurochemical release from dermal fibroblasts is novel, however the exploratory nature of this study had led us to posit biological mechanisms governing their release. We posit these fibroblasts store and release the chemicals in granules much like mast cells. Future work to validate granular release of histamine and serotonin in dermal fibroblasts is underway, and we aim to fully elucidate the role of neurochemical signalling within the skin and probe its role in psychiatric disease.

Materials and Methods

Chemicals

Sterile HBSS (pH 7.4) with calcium and magnesium was purchased from Thermo Fisher (product number: 14025050; Waltham, MA, USA) and used for FSCV measurements in fibroblasts. Calibration solutions for flow injection analysis (FIA) were prepared fresh each day by diluting from stocks of serotonin HCl (Sigma Aldrich, St. Louis, MO, USA) and/or histamine 2HCl (Sigma Aldrich, St. Louis, MO, USA) dissolved in HBSS (pH 7.4) made with the same formulation published by Thermo Fisher (140 mg/L CaCl_2 , 100 mg/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 400 mg/L KCl, 60 mg/L KH_2PO_4 , 350 mg/L NaHCO_3 , 8000 mg/L NaCl, 48 mg/L Na_2HPO_4 , 1000 mg/L D-glucose). Diphenhydramine HCl (Sigma Aldrich, St. Louis, MO, USA) stock was prepared daily prior to application to fibroblast cultures. Aliquots of 1 mM Bafilomycin A1 (Tocris Bioscience, Bristol, UK) were prepared and kept frozen at -20°C until needed for addition to fibroblast cultures.

Cell Culture

Keratinocytes and ORS cells (Figure 5.1) were cultured and prepared for FSCV experiments as described in the Materials and Methods section in Chapter 4.

Dermal fibroblasts were isolated from human skin discarded after abdominoplasty procedures with informed written consent obtained from patients using consent forms approved by the Imperial College Research Ethics Committee. Briefly, excess fat and dermis was removed from samples, which were then incubated overnight in dispase at 4°C . The following morning, the epidermis and dermis were manually separated with forceps. The epidermis was then chopped roughly with scissors and placed onto mitomycin treated NIH3T3 feeders. The epidermis was left to dry for 2-3 minutes to adhere to the feeders before FAD media was added. In parallel, the dermis was then minced with scissors and digested with 1 mg/mL collagenase (Thermo Fisher, Waltham, MA, USA) for 40 minutes and quenched with Dulbecco's Modified

Eagle's Medium (DMEM) with Glutamax (Thermo Fisher, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco) and 1X ABAM. The solution was then filtered through a 40- μ m cell strainer (Starlab, Milton Keynes, UK) to obtain a suspension of single cells and were incubated at 37°C in 5% CO₂/95% humidity for 7 days in DMEM + 20% FBS + 1X AMAM prior to use in experimental procedures. After the initial 7 days, fibroblasts were passaged into T75 flasks and 35 mm cell dishes (as needed for FSCV experiments) with DMEM + 10% FBS + 1X penicillin/streptomycin (p/s) (Thermo Fisher, Waltham, MA, USA) and kept in incubators with media changed every 2-3 days. Fibroblasts were used between passages 0 and 5 (P0-5).

Carbon Microelectrodes

Bevelled and cylindrical carbon fiber microelectrodes (CFMs) were fabricated as described in Chapter 2. For histamine and serotonin detection from fibroblasts and FIA, bevelled CFMs were coated with Nafion™ and polyglutamic acid (PGA) (as described in chapter 2 for both polymers). For measurements in the ORS cells, fibroblasts and keratinocytes in Figure 5.1, cylindrical CFMs with Nafion™ and PGA were used.

Flow Injection Analysis

Electrodes were post-calibrated after fibroblast measurements for histamine (0.25, 0.5, 1, 2, 5, 10, 15, 20, 30 and 50 μ M) and serotonin (10, 25, 50, 75, 100, 500 and 1000 nM) in HBSS using FIA, as described in Chapter 2. For each FIA experiment, the histamine-optimized waveform (-0.5 V to -0.7 V to 1.1 V to -0.5 V at 600 V/s) was applied to the CFM at 60 Hz for 10 minutes then at 10 Hz for 10 minutes prior to acquiring data files. Bolus injections (10 seconds) were applied in triplicate for each calibration point. However, a large variability in the current response was observed between bevelled CFMs. As such, we are exploring alternative methods of quantitation to concentration and, in this work, have compared currents from serotonin and histamine separately.

FSCV Data Acquisition and Analysis

FSCV data were acquired with the hardware described for Figure 2.2. Color plots acquired with the histamine-optimized waveform were zero phase filtered and filtered with a low pass 4th order Butterworth filter at 3 kHz and smoothed twice.

Keratinocytes and ORS cells

FSCV experiments for data acquired in Figure 5.1 were performed with the same experimental set-up in Chapter 4. Briefly, cylindrical CFMs were lowered to ~3-5 μm above a confluent area of cells and spontaneous activity was monitored for 10 minutes at 3 locations per cell dish. Fibroblasts: n=1 dish from 1 patient, keratinocytes: n=3 dishes from 2 patients, and ORS cells: n=5 dishes from 4 patients.

Fibroblasts

Prior to FSCV measurements, 2 mL of HBSS (pH 7.4, 37°C) was added to fibroblast cultures (P0-5) plated in 35 mm dishes exhibiting 70-90% confluency. The dishes were placed on a light microscope within a Faraday cage. A pseudo-Ag/AgCl reference electrode (made daily as described in chapter 2), was placed in the buffer on the side of the dish. Then, a bevelled CFM was lowered to ~2 μm above a fibroblast with a quadrupole micromanipulator (QUAD, Sutter Instruments, Novato, CA, USA). The light microscope and micromanipulator were turned off to eliminate noise within the Faraday cage. The histamine-optimized waveform (-0.5 V to -0.7 V to 1.1 V to -0.5 V at 600 V/s) was then applied at 60 Hz for 10 minutes then at 10 Hz for 10 minutes to equilibrate the carbon surface chemistry to the cellular environment. Control data files (30 seconds each) were acquired back-to-back for 60 minutes (120 data files) using a custom Python script prior to pharmacological intervention. An aliquot from a freshly made stock of diphenhydramine (H1 receptor antagonist, DPH) in HBSS was spiked into the cell dish and allowed to diffuse for several minutes (~5 minutes) to bring the total concentration of the dish to 10 μM DPH. Then 60 more minutes of data files (120 files) were acquired. This procedure was repeated for the addition of a vacuolar-type ATPase

inhibitor, Bafilomycin A1 (5 and 10 μM), but 2 hours of data were acquired as the effects of the drug are known to take longer.

For serotonin and histamine pre-incubation, the media was replaced with HBSS for 30 minutes to equilibrate to the salt buffer then by serotonin (100 μM) or histamine (10 and 100 μM) in HBSS for 60 minutes to allow for uptake of the neurochemicals. Fresh HBSS was added, and the dish was mounted on the microscope stage for FSCV measurements. Data was acquired in the same manner as described above (60 minutes of post-incubation data files, then the addition of DPH followed by 60 more minutes of data files).

For data analysis, current vs. time (IT) curves were extracted at the oxidation potentials for histamine (~ 0.3 V) and serotonin (~ 0.65 V). These plots were analyzed using a custom-coded peak detection program using the SciPy library in Python for peak height (nA) and frequency (Hz). Each dish was analyzed for peak height and averaged together based on their experimental condition (control, drug, serotonin/histamine incubation, etc). Frequency was analyzed for all dishes sharing the same experimental conditions rather than individually then averaged.

Statistical Analysis

Statistical analyses for all data were performed using Python SciPy library¹⁷⁴. The frequency and peak height data (above) was analyzed for normality, but none fit a normal distribution (Shapiro-Wilk test) (rejecting the null hypothesis (H_0)). As such, the non-parametric Mann-Whitney test was applied to determine statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between median values. All error bars represent the median absolute deviation (MAD).

Results

During the acquisition of control (non-stimulated) measurements in ORS cells and keratinocytes (reported in Chapter 4) we observed spontaneous releases of serotonin (Figure 5.1). The experimental protocol was

repeated in dermal fibroblast cultures where similar spontaneous releases of serotonin were observed (Figure 5.1). The signals from each cell type were validated against serotonin CVs taken in a flow cell. Based on this work, we began characterizing spontaneous neurochemical release from fibroblasts.

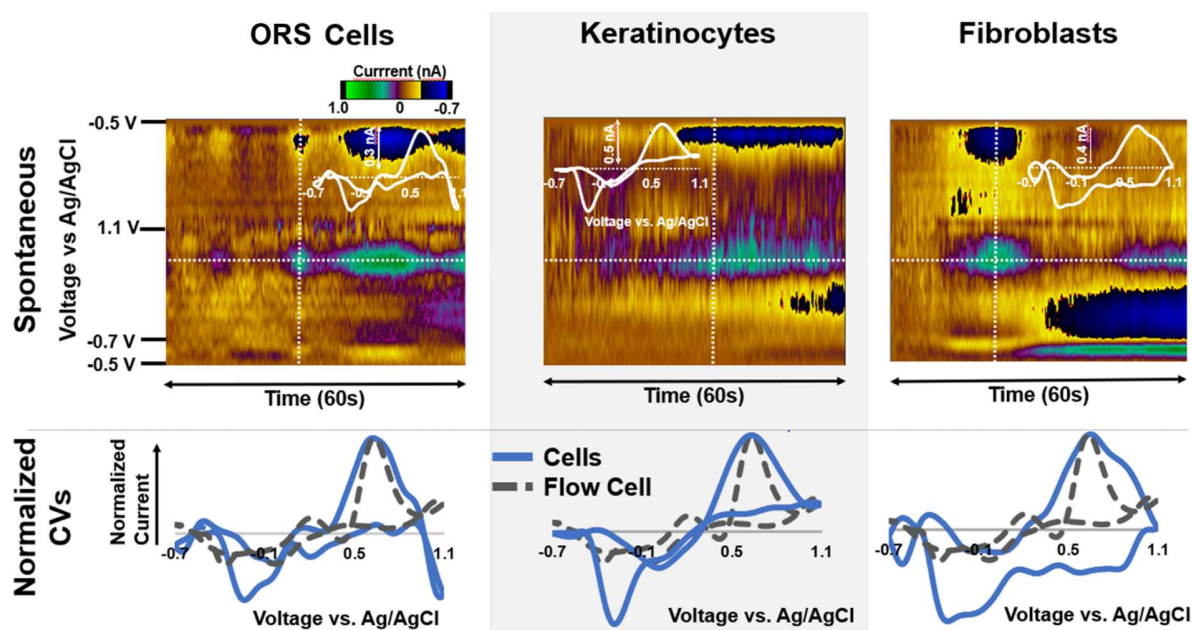


Figure 5.1. Skin and hair cells release serotonin. Representative color plots showing spontaneous release of serotonin from human hair cells (outer root sheath (ORS) cells), dermal keratinocytes, and dermal fibroblasts in HBSS (pH 7.4) detected at CFMs with FSCV. Inset cyclic voltammograms (CVs) are taken from the vertical line in each color plot, and the horizontal line denotes the serotonin oxidation peak. Below each color plot are their respective CVs plotted against one for serotonin taken from a flow cell. The oxidation peak currents are normalized.

We employed bevelled CFMs to probe the spontaneous activity of fibroblasts with single-cell resolution. For FSCV measurements, the bevelled CFM was placed $\sim 2 \mu\text{m}$ above a single fibroblast (Figure 5.2 A,B). After the histamine-optimized waveform was applied at 60 Hz for 10 minutes then at 10 Hz for another 10 minutes, 60 minutes of data was collected (120 x 30 second files). Spontaneous oxidative current events at $\sim 0.3 \text{ V}$ and $\sim 0.65 \text{ V}$ were observed (Figure 5.2Ci,Ei). To induce a signal change, based on reports of human dermal fibroblasts expressing H1 receptors⁵⁴, we applied a bolus of the H₁ receptor antagonist,

diphenhydramine (DPH), to the cell dish, bringing the total concentration to 10 μ M. The drug was allowed to diffuse for several minutes (~5 minutes) then data collection resumed for another 60 minutes (120 x 30 second files). Higher, more frequent oxidative currents at the same potentials were observed in response to the H₁ antagonist (Figure 5.2Cii,Eii). These signals were validated to be histamine (~0.3 V) and serotonin (~0.65 V) against cyclic voltammograms (CVs) from FIA injections of both analytes (Figure 5.2D,F). The current vs. time (IT) traces were extracted at the oxidative potentials for histamine and serotonin and analyzed with a peak detection program in Python. Identified peaks were averaged as current, as calibration curves from the CFMs used to acquire fibroblast signals had much variability, so we are exploring analysis methods to convert to concentration. From this analysis, the median peak amplitude and event frequency for pre- and post-diphenhydramine signals are plotted for serotonin and histamine in Figure 5.2E and F, respectively. Both histamine and serotonin significantly increased in peak amplitude (serotonin: 0.039 ± 0.007 nA pre-drug and 0.048 ± 0.013 nA post-DPH, $p < 0.001$ (Mann-Whitney test); histamine: 0.037 ± 0.005 nA pre-drug, 0.044 ± 0.010 nA post-DPH, $p < 0.001$ (Mann-Whitney test)) and frequency (serotonin: 0.060 Hz pre-drug and 0.28 Hz post-DPH; histamine: 0.037 Hz pre-drug, 0.24 Hz post-DPH) after the application of diphenhydramine. The novelty of spontaneous serotonin and histamine detection in dermal fibroblasts was compounded by an increase in signalling upon application to DPH. From these data, we next sought to determine the mechanism by which these neurochemicals are released from dermal fibroblasts.

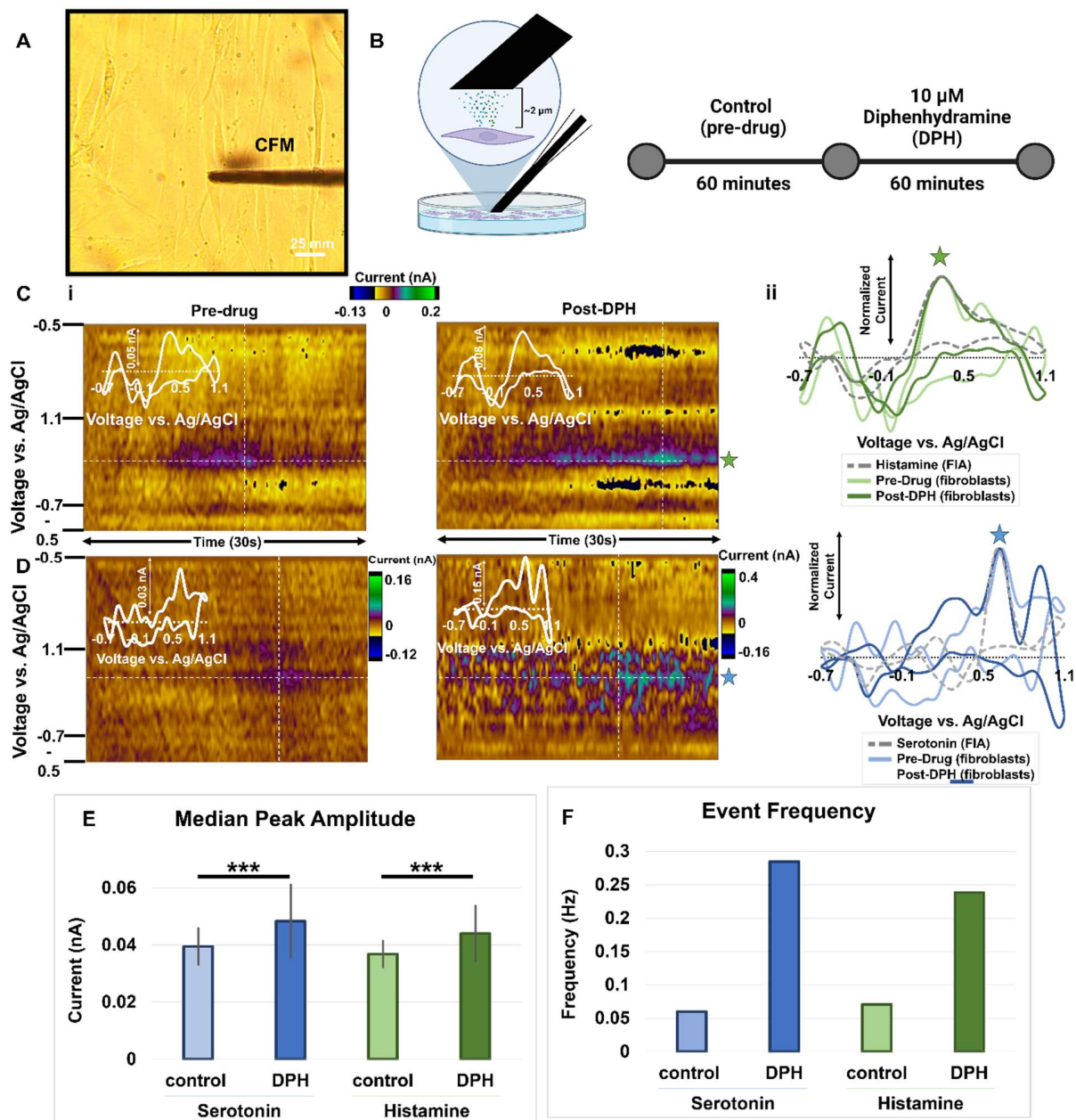


Figure 5.2. Human Dermal Sheath Fibroblasts respond to H1 antagonist (diphenhydramine). (A) Image of a CFM placed above a fibroblast. (B) Illustration of the experimental set-up and paradigm used in this work, where the CFM is placed ~2 μm above a fibroblast in culture then 60 minutes of control then another 60 minutes of drug (diphenhydramine) files were acquired. (C) (i) Representative color plots of histamine release from dermal fibroblasts in HBSS (pH 7.4) before (left) and after (right) spiking 10 μM diphenhydramine into the buffer. The inset CVs are taken

from the vertical line in each color plot. The horizontal line indicates the position of histamine oxidation (green star).

(ii) CVs from fibroblast release before (light green) and after (dark green) diphenhydramine overlaid with histamine from flow injection analysis (FIA) (dashed dark gray). The oxidative peaks (green star) are normalized to one. (D) Representative color plots of serotonin release from dermal fibroblasts in HBSS (pH 7.4) before (left) and after (right) spiking 10 μ M diphenhydramine into the buffer. The inset CVs are taken from the vertical line in each color plot. The horizontal line indicates the position of histamine oxidation (blue star). (ii) CVs from fibroblast release before (light blue) and after (dark blue) diphenhydramine overlaid with serotonin from flow injection analysis (FIA) (dashed light gray). The oxidative peaks (blue star) are normalized to one. (E) Median peak amplitudes of serotonin (blue) and histamine (green) release from fibroblasts before (light color) and after (dark color) the application of 10 μ M diphenhydramine (DPH). Data files were acquired for ~1 hour (120 data files) for each condition (n=8 dishes). The data did not fit the normal distribution (Shapiro-Wilk test) (reject null hypothesis, H_0), so a non-parametric (Mann-Whitney) test was used to determine statistical significance, *** $p < 0.001$. (F) The total frequency of serotonin (blue) and histamine (green) release from fibroblasts across 8 cell dishes before (light color) and after (dark color) the application of 10 μ M diphenhydramine (DPH) (960 data files for pre- and pos-drug conditions for serotonin and histamine). Data files were acquired for ~1 hour (120 data files) for each condition. As the data did not fit the normal distribution (reject null hypothesis, H_0), a non-parametric (Mann-Whitney) test was used to determine statistical significance, *** $p < 0.001$. Made using Biorender.com.

Based on our findings in Chapter 4, where serotonin and histamine were released from vesicles in ORS cells, we were curious whether this was also the case for fibroblasts. For this we applied a vacuolar-type ATPase inhibitor (Bafilomycin A1, BAF) to determine whether release is ATP-mediated (vesicular release). A bolus of BAF was acutely administered to fibroblast cultures after 60 minutes of baseline data files were collected. For these experiments, we applied 10 μ M and 5 μ M BAF and collected data for another 60 or 120 minutes, respectively (Figure 5.3). The higher concentration of BAF resulted in a decrease in histamine release amplitude, while the lower concentration did not induce any change (10 μ M BAF: control: 0.040 ± 0.007 nA, BAF: 0.038 ± 0.005 nA, $p < 0.001$; 5 μ M: control: 0.033 ± 0.002 nA, BAF: 0.033 ± 0.004 nA, $p = 0.56$)

(Figure 5.3C,E) and neither dose induced a significant change in histamine release frequency (10 μ M BAF: control: 0.187 Hz, BAF: 0.160 Hz; 5 μ M BAF: control: 0.010 Hz; BAF: 0.010 Hz) (Figure 5.3D,F). Serotonin peak amplitude did not significantly change response to either dose of BAF (10 μ M BAF: control: 0.035 ± 0.002 nA, BAF: 0.035 ± 0.004 nA, $p=0.278$; 5 μ M: control: 0.033 ± 0.002 nA, BAF: 0.033 ± 0.004 nA, $p=0.31$) (Figure 5.3C,E) nor in frequency (10 μ M BAF: control: 0.065 Hz, BAF: 0.057 Hz; 5 μ M BAF: control: 0.017 Hz; BAF: 0.018 Hz) (Figure 5.3D,F). The expectation with BAF is that the inhibition of ATPase would inhibit any vesicular packaging and therefore stifle release. However, this was not the case in fibroblasts, where signalling amplitude and frequency were largely unaffected by either concentration of BAF. This led us to postulate alternative mechanisms of neurochemical release, such as the secretory granules found in mast cells. As fibroblasts interact with mast cells in the skin, we thought their release mechanisms could be similar. This idea was determined toward the end of this study, so for the continuation of this study we designed an experiment to test this by first probing healthy fibroblasts then apply a protein kinase C (PKC) inhibitor (LY333531) to induce degranulation¹⁷⁵. The expected outcome is that the fibroblasts will release histamine and serotonin after the application of the drug then cease signalling afterward. We next tested the effect of exogenously applied histamine (10 and 100 μ M) and serotonin (100 μ M) on the spontaneous activity of fibroblasts.

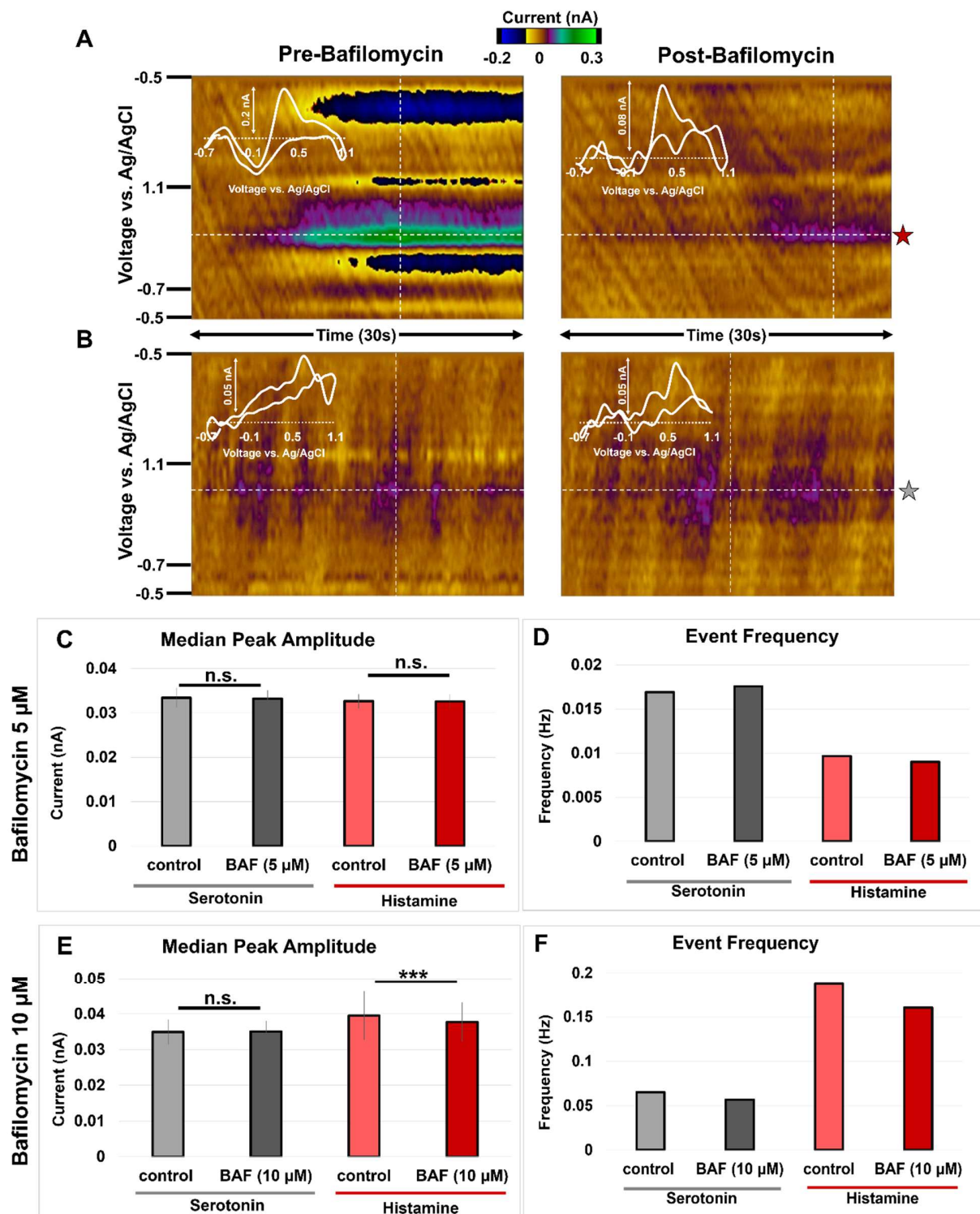


Figure 5.3. Human Dermal Sheath Fibroblasts respond to ATPase inhibitor (Bafilomycin A1). (A) Representative color plots of histamine release from fibroblasts in HBSS before (left) and after (right) an acute administration of 2.5

μM Bafilomycin. Inset CVs are taken at the vertical line in each color plot. (B) Representative color plots of serotonin release from fibroblasts in HBSS before (left) and after (right) an acute administration of 5 μM Bafilomycin. Inset CVs are taken at the vertical line in each color plot. Current vs. time traces extracted at the oxidation potentials for histamine (green star) and serotonin (blue star) were analyzed *via* peak detection program written in python and compared in panels C-F. (C) Median peak amplitude of serotonin (blue) and histamine (green) release before (light color) and after (dark color) 5 μM BAF. The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** $p < 0.001$. (D) Event frequency of serotonin (gray) and histamine (red) release before (light color) and after (dark color) 5 μM BAF. The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** $p < 0.001$. (E) Median peak amplitude of serotonin (blue) and histamine (green) release before (light color) and after (dark color) 10 μM BAF. The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** $p < 0.001$. (F) Event frequency of serotonin (gray) and histamine (red) release before (light color) and after (dark color) 10 μM BAF. The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** $p < 0.001$.

We pre-incubated fibroblast cultures with excess histamine (10 and 100 μM) (Figure 5.4) or serotonin (100 μM) (Figure 5.5) for 1 hour prior to FSCV experimentation, then after an hour of measurements in serotonin- and histamine-free HBSS, we applied diphenhydramine and monitored for another hour to further modify the signal to discern the regulatory role of the H_1R . We observed significant changes in peak currents from control with the combination of incubating with 100 μM histamine and DPH (serotonin: control: 0.039 ± 0.007 nA, post-100 μM incubation: 0.034 ± 0.003 nA, $p < 0.001$; post-100 μM incubation+DPH: 0.050 ± 0.016 nA, $p < 0.001$; post-incubation vs. post-DPH: $p < 0.001$) (histamine: control: 0.037 ± 0.005 nA, post-100 μM incubation: 0.034 ± 0.003 nA, $p < 0.001$; post-100 μM incubation+DPH: 0.046 ± 0.013 nA, $p < 0.001$; post-incubation vs. post-DPH: $p < 0.001$), and a notable increase in event frequency for both serotonin and histamine (serotonin: control: 0.060 Hz, post-100 μM incubation: 0.036 Hz, post-100 μM incubation+DPH: 0.182 Hz) (histamine: control: 0.071 Hz, post-100 μM incubation: 0.050

Hz, post-100 μ M incubation+DPH: 0.175 Hz) (Figure 5.4A,C). When repeating this paradigm for 10 μ M histamine (Figure 5.4B), we observed no significant change in release amplitude for both histamine (histamine: control: 0.037 ± 0.005 nA, post-10 μ M incubation: 0.035 ± 0.003 nA, $p < 0.001$; post-10 μ M incubation+DPH: 0.037 ± 0.004 nA, $p = 0.71$; post-incubation vs. post-DPH: $p < 0.001$) and serotonin (serotonin: control: 0.039 ± 0.007 nA, post-10 μ M incubation: 0.035 ± 0.004 nA, $p < 0.001$; post-10 μ M incubation+DPH: 0.037 ± 0.004 nA, $p < 0.001$; post-incubation vs. post-DPH: $p < 0.001$), and a similar increase in signal frequency for both histamine (histamine: control: 0.071 Hz, post-10 μ M incubation: 0.084 Hz, post-10 μ M incubation+DPH: 0.145 Hz) and serotonin (serotonin: control: 0.060 Hz, post-10 μ M incubation: 0.083 Hz, post-10 μ M incubation+DPH: 0.158 Hz) as for the larger concentration of histamine.

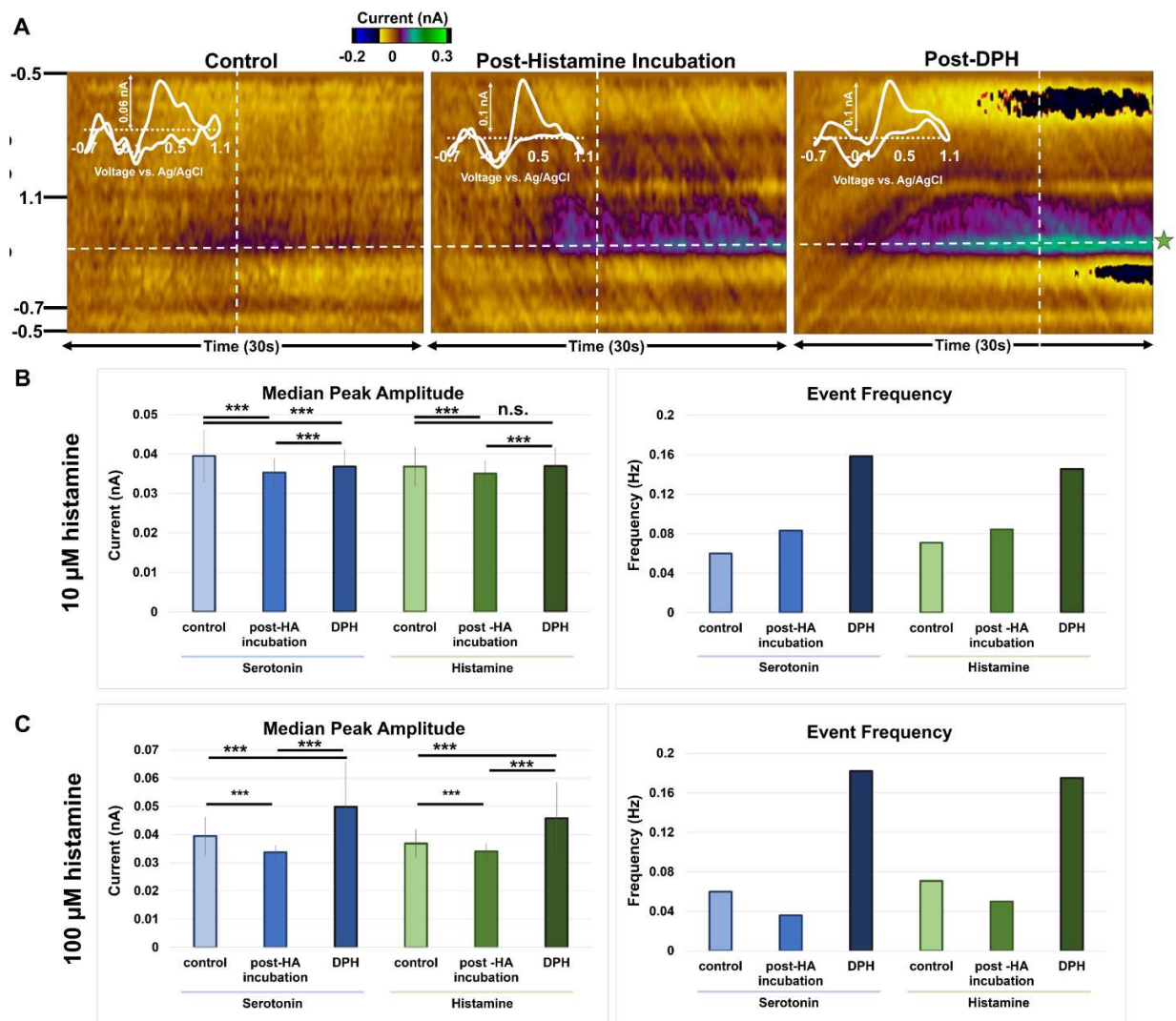


Figure 5.4. Fibroblasts differentially release neurochemicals when pre-incubated with histamine. (A) Representative color plots of histamine release from fibroblasts in HBSS before (left), after an acute (60 minutes) incubation with 100 μ M histamine (middle) and after 10 μ M DPH was added to the incubated cells (right). Inset CVs are taken at the vertical line in each color plot. Current vs. time traces extracted at the oxidation potentials for histamine (green star) and serotonin were analyzed *via* peak detection program written in python and compared in panels B and C. (B) Median peak amplitude (left) and event frequency (right) of serotonin (blue) and histamine (green) release before (light color), after incubation with 100 μ M serotonin (medium color) and post-incubation with 10 μ M DPH (dark color). The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** p <0.001 (C) Median peak amplitude (left) and event frequency

(right) of serotonin (blue) and histamine (green) release before (light color), after incubation with 10 μ M serotonin (medium color) and post-incubation with 10 μ M DPH (dark color).). The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** $p < 0.001$.

However, when pre-incubating fibroblast cultures with 100 μ M serotonin and then adding DPH during the second hour of FSCV collection, we observed the counter effect to histamine pre-incubation. Serotonin and histamine signalling significantly decreased in amplitude after incubation, which was further reduced with the application of DPH (serotonin: control: 0.039 ± 0.007 nA, post-100 μ M incubation: 0.033 ± 0.002 nA, $p < 0.001$; post-100 μ M incubation+DPH: 0.033 ± 0.002 nA, $p < 0.001$; post-incubation vs. post-DPH: $p = 0.38$) (histamine: control: 0.037 ± 0.005 nA, post-100 μ M incubation: 0.034 ± 0.003 nA, $p < 0.001$; post-100 μ M incubation+DPH: 0.033 ± 0.002 nA, $p < 0.001$; post-incubation vs. post-DPH: $p < 0.001$). Additionally, the signal frequency was significantly reduced for both serotonin and histamine after incubation and again with the application of DPH (serotonin: control: 0.060 Hz, post-10 μ M incubation: 0.022 Hz, post-10 μ M incubation+DPH: 0.022 Hz) (histamine: control: 0.071 Hz, post-10 μ M incubation: 0.043 Hz, post-10 μ M incubation+DPH: 0.021 Hz). These data show that the application of exogenous histamine and serotonin have contradictory effects on the spontaneous activity of dermal fibroblasts.

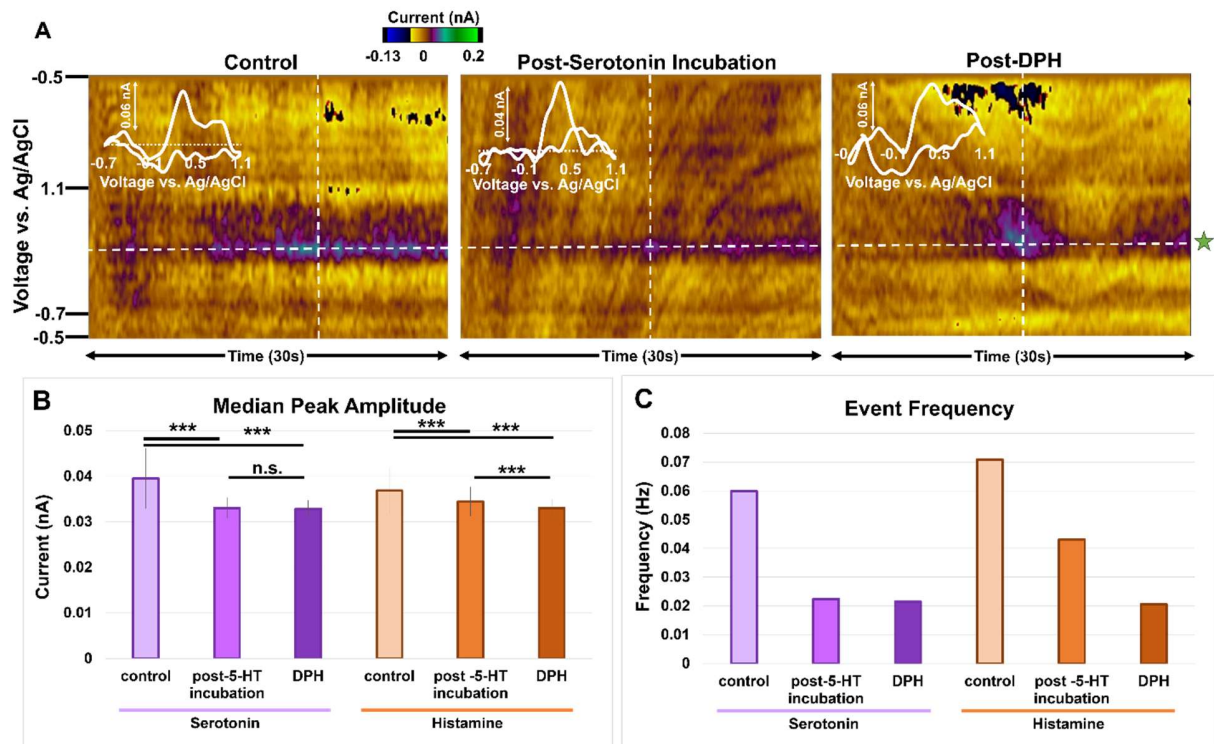


Figure 5.5. Fibroblasts differentially release neurochemicals when pre-incubated with serotonin. (A) Representative color plots of histamine release from fibroblasts in HBSS before (left), after an acute (60 minutes) incubation with 100 μ M serotonin (middle) and after 10 μ M DPH was added to the incubated cells (right). Inset CVs are taken at the vertical line in each color plot. Current vs. time traces extracted at the oxidation potentials for histamine (green star) and serotonin were analyzed *via* peak detection program written in python and compared in panels B and C. (B) Median peak amplitude of serotonin (purple) and histamine (orange) release before (light color), after incubation with 100 M serotonin (medium color) and post-incubation with 10 μ M DPH (dark color).). The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** p <0.001 (C) Event frequency of serotonin (purple) and histamine (orange) release before (light color), after incubation with 100 M serotonin (medium color) and post-incubation with 10 μ M DPH (dark color). The data did not fit a normal distribution, so a non-parametric (Whitney-Mann) test was performed to determine statistical significance, *** p <0.001.

Discussion

In this study, based on the findings presented in Chapter 4, we characterized the spontaneous activity of human dermal fibroblasts in culture at bevelled CFMs with FSCV. We first observed spontaneous serotonin release in ORS cells, keratinocytes and fibroblasts at cylindrical CFMs (i.e. bulk sensing). From this we characterized the spontaneous activity of fibroblasts with single-cell resolution using bevelled CFMs. We first observed spontaneous release of histamine and serotonin and that increased in response to the H₁ receptor antagonist, diphenhydramine. Then, we applied bafilomycin from which we observed a partial decrease in signal frequency, but no cessation of release. Finally, we ascertained the effect of exogenous histamine and serotonin on neurochemical release mechanisms, where they were differentially altered after acute incubation with high concentrations of serotonin and histamine. From this exploratory study of the spontaneous activity of dermal fibroblasts we were able to develop hypotheses surrounding the mechanisms controlling neurochemical signalling and designed experiments for future investigation.

We observed a significant increase in signal frequency for both serotonin and histamine upon antagonism of the H₁R with DPH. This response to DPH was conserved when cells were incubated with excess histamine. Receptor antagonist agents, like DPH, would normally inhibit firing, however when these agents act on autoreceptors, cellular signalling persists and/or increases as blocking the autoreceptor removes the negative feedback control for the cell. From these data, we hypothesize that the H₁R acts as an autoreceptor on the fibroblast that senses histamine in the extracellular space and, when blocked, a secondary, compensatory mechanism is triggered to increase release frequency. It has been previously reported that the H₁R mediates the response of fibroblasts to exogenous histamine through collagen detection, but the regulatory role of the H₁R as an auto- or heteroreceptor was not distinguished⁵⁴. Our results confer the integral role of the H₁R in regulating fibroblast function, and point to its function as an autoreceptor, however its exact actions are yet to be understood.

As we determined through the application of bafilomycin, the release of both neurochemicals from fibroblasts was not ATP-dependent. In contrast, ORS cells in the hair follicle were found to have vesicles, which was demonstrated by ceased signalling upon application of bafilomycin¹⁷². Therefore, it is unlikely that fibroblasts secrete neurochemicals from vesicles, instead they could be released from secretory granules, like in mast cells¹⁷⁶. To validate this notion, a future plan is to apply a degranulation drug to dermal fibroblasts in the future. Additionally, in response to all pharmaceutical interventions, we observed similar trends (both increased or decreased) for histamine and serotonin release. Therefore, this suggests that serotonin and histamine are co-released from the same secretory granules.

For FSCV at CFMs, current amplitudes are similar for histamine in the μM range as for serotonin in the nM range^{77, 78}. Based on the relatively equal peak currents, this indicates that the concentration difference between serotonin and histamine released from fibroblasts are in 1000-fold variance, just like in the brain^{77, 78}. From this it can be inferred that the reuptake of serotonin is more efficient than for histamine. Additionally, the application of 100 μM serotonin resulted in a decrease in signalling that was enhanced with DPH. For this we believe that the concentration of serotonin was too high, and likely induced cytotoxicity. To validate this claim we plan to evaluate cell viability at various concentrations of exogenous serotonin in fibroblasts.

These ideas and proposed mechanisms are summarized below in Figure 5.6.

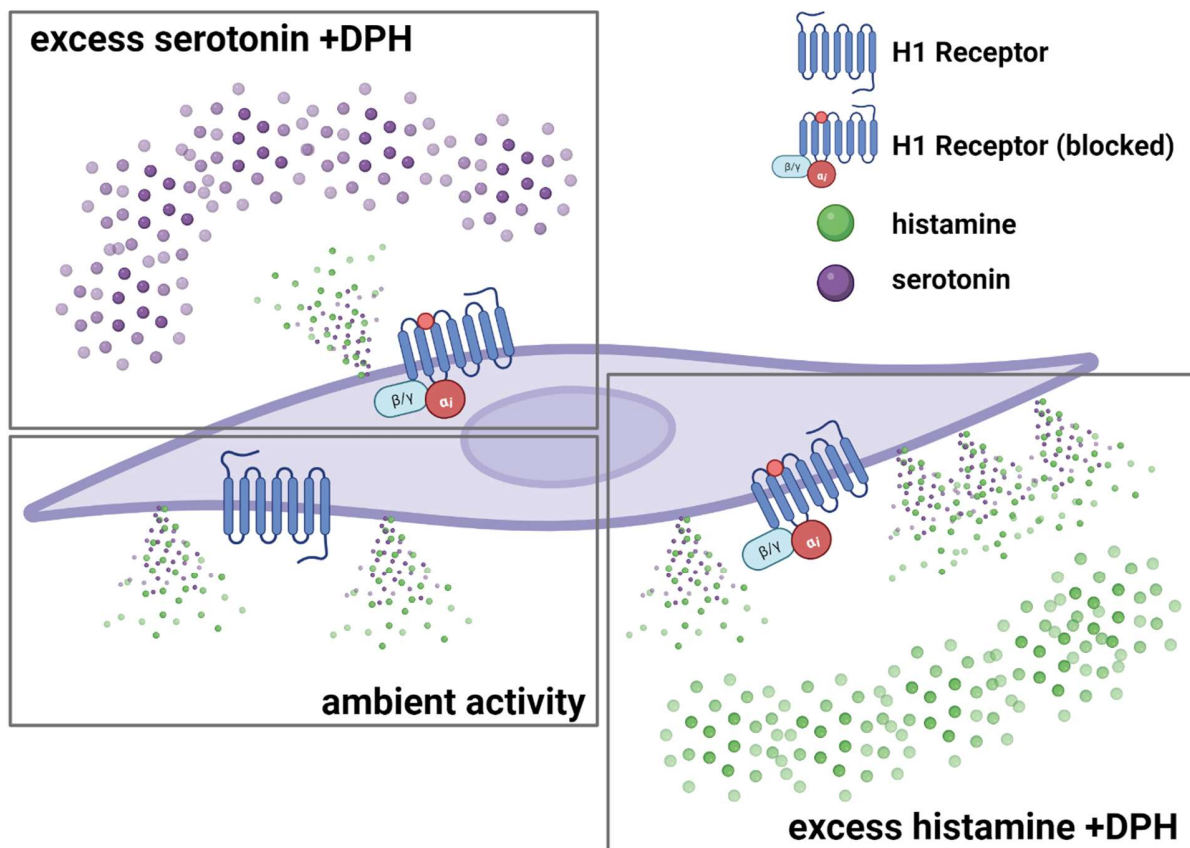


Figure 5.6. The co-release of histamine and serotonin from dermal fibroblasts is mediated by the H1 receptor. Histamine and serotonin are spontaneously co-released from fibroblasts in culture, which is increased when the H₁R is antagonized with DPH. In the presence of histamine and DPH, signalling increases, whereas excess serotonin dampens activity. Made using Biorender.com.

Conclusion

In sum, this exploratory study found that dermal fibroblasts spontaneously release histamine and serotonin, and that the H₁R regulates the fibroblast response to extracellular histamine. We began to elucidate biological mechanisms regulating this release and found a potent increase in neurochemical release upon antagonism of the H₁ receptor. However, unlike the ORS cells and keratinocytes described in Chapter 4, neurochemical release did not appear to be vesicular, rather we postulate storage and release is *via* secretory granules. Experimental data to support this notion should be a focus of future work. Finally,

the differential response to diphenhydramine after acute incubation with excess histamine and serotonin indicates that these neurochemicals have a major role in the regulation of fibroblast signalling. For this, more work will be done to elucidate the exact role of neurochemical release from fibroblasts as it pertains to skin physiology. Overall, this data is an important piece in understanding intracellular signalling of neurochemicals within the skin and lays the foundation for future work elucidating signalling dysfunction in the skin to neurological diseases.

6. Chapter 6 Co-Detection of Serotonin and Insulin in Real-Time with Fast Voltammetry

The work presented in this chapter was done in collaboration with the Salem laboratory at Imperial College London. The data presented here was performed by Brenna Parke with the assistance from Dr. Kinga Suba or Ben Hansen from the Salem group for Figure 6.7.

Abstract

Diabetes mellitus is a widespread endocrine disorder that is a major burden on global healthcare systems, characterized by chronically elevated blood glucose levels that causes cardiovascular, eye and kidney damage. Diabetes can be broadly differentiated into two types: type 1 (autoimmune destruction of beta cells) and type 2 (failure of pancreatic beta cells to produce enough insulin). Homeostatic regulation of blood sugar levels is controlled *via* islets of Langerhans, where insulin is released from beta cells in response to high levels of glucose and with the contrary mechanism being glucagon release from alpha cells. Recently, serotonin has been identified as an integral regulatory messenger within islet physiology. Serotonin is co-released with insulin from beta cells and suppresses glucagon release from alpha cells. Still, while much is known of the pathophysiology of diabetes, the mechanism leading to beta cell dysfunction is still to be uncovered. A major obstacle in studying islet physiology is the lack of tools to detect insulin sensitively and selectively in real-time. Measurement techniques typically utilized in diabetic research are biological assays, analytical separation techniques and fluorophore-mediate detection of calcium dynamics. Electrochemical methods have been developed for insulin detection but are not commonly employed as their temporal and qualitative characteristics do not exceed that of established techniques. However, fast scan cyclic voltammetry (FSCV) is an electrochemical technique that employs fast scan rates to monitor redox reactions in real-time (typically 10 Hz), for which serotonin is well-characterized. Thus, in this work, we aimed to develop an FSCV method for real-time co-detection of serotonin and insulin. We

first optimized a FSCV waveform for insulin and serotonin co-detection at carbon fiber microelectrodes. Then, we characterized sensor performance at this waveform in terms of sensitivity and stability for both analytes. Finally, the sensors were applied to biological models of insulin release (INS-1 cells and islets). With this work we offer a novel FSCV methodology for temporally resolved insulin and serotonin co-detection at carbon fiber microelectrodes. This technique fills a large measurement gap in diabetes research, and, in the future, can be used to directly probe intra-islet insulin dynamics and assess functionality of stem cell-derived beta cells.

Introduction

Diabetes mellitus is a prevalent metabolic disease that affects over 422 million people worldwide¹⁷⁷. Diabetes is characterized by elevated circulating blood glucose, which damages blood vessels leading to damage in the heart, eyes and kidneys. The high blood sugar levels that characterize diabetes are a result of a disturbance in insulin secretion or action. Insulin is the only hormone in the body capable of lowering blood glucose, and it acts on numerous tissues to enhance intracellular glucose uptake. Diabetes can broadly be split into two types. Type 1 diabetes (10% cases worldwide¹⁷⁸) is the result of autoimmune destruction of the insulin-producing beta cells, resulting in absolute insulin deficiency and lifelong exogenous insulin injections¹⁷⁹. Type 2 diabetes (90% cases worldwide¹⁷⁸) results from the failure of pancreatic beta cells to produce enough insulin to overcome peripheral insulin resistance¹⁷⁷.

All types of diabetes are related to either the destruction or functional failure of insulin-producing beta cells. Arguably, a major barrier to finding a cure for diabetes has been the difficulty of directly studying these beta cells. Insulin production in the pancreas occurs in endocrine cells situated in millions of (100 μm) islets of Langerhans that are dispersed throughout the exocrine tissue. These highly vascularized and innervated islets mainly contain (60%) insulin-producing beta cells, but also several other hormone-producing cell-types including alpha cells which produce glucagon and delta cells which produce

somatostatin (Figure 6.1). Glucagon release is a counter regulatory response to low blood sugar levels and signals to the liver to increase glucose output and is, therefore, an important regulator of a homeostatic system tuned to maintain normal blood sugar levels in a narrow range (4-6 mM)¹⁸⁰. The full intricacy of the body's glucose regulatory system remains to be fully elucidated. The islets themselves are complex functional units. For example, serotonin has recently been found to be integral in blood glucose regulation as it co-released with insulin from beta cells and acts on alpha cells through the 5HT_{2A} receptor to inhibit glucagon secretion¹³. Serotonin levels have also been found to regulate beta cell mass^{181, 182}. While serotonin is emerging as an important messenger in beta cell function, its exact role in the aetiology of diabetes is unknown. Furthermore, peripheral factors including insulin clearance by the liver and peripheral insulin sensitivity, as well as glucose absorption are important too. In fact, measures of peripheral (blood) levels of glucose and insulin are so compounded by various biological factors, that their relation to intrinsic islet insulin secretion is difficult to extrapolate. Therefore, the direct measurement of insulin from islets (or immortalized insulin-producing cell lines like INS-1 cells) remains a central requirement in diabetes research.

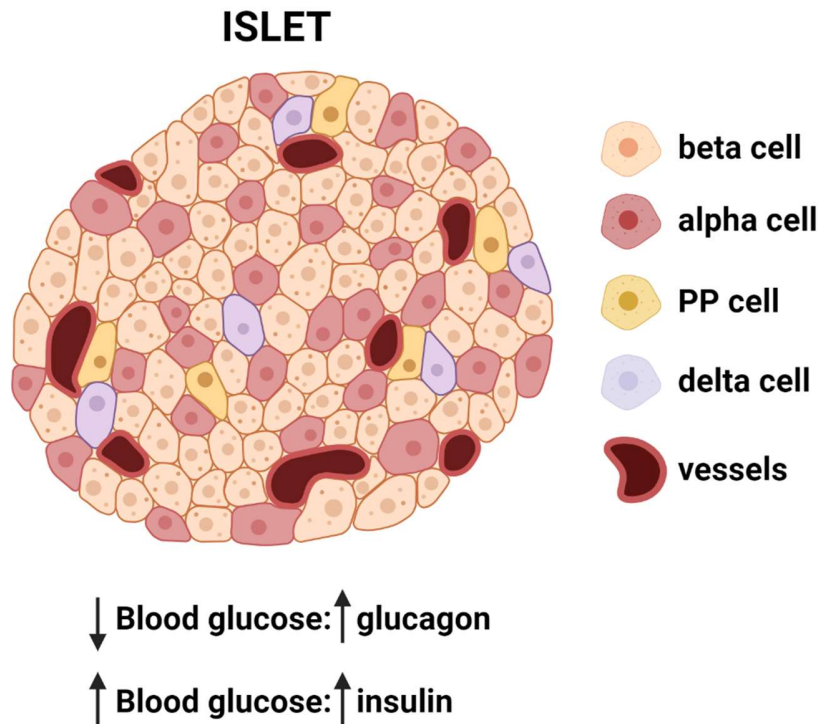


Figure 6.1. Physiological makeup of pancreatic islets of Langerhans. Islets are made up of beta cells (orange) that release insulin in response to high blood glucose, alpha cells (red) that release glucagon in response to low blood glucose, pancreatic polypeptide (PP) cells (yellow) that release PP after eating to regulate satiety, delta cells (purple) that release somatostatin to modulate alpha and beta cell activity, and blood vessels.

It is of vital importance to develop novel tools capable of measuring insulin secretion in real-time and with sensitivity that could be applied to single islets. Islet biologists typically measure insulin secretion in perfusion experiments that pool hundreds of islets in order to collect enough effluent at high enough temporal resolution (minutes) to perform immune assays for insulin or detect with liquid chromatography/mass spectrometry. Alternatively, proxy markers of insulin secretion may be measured using fluorophores, for example, genetically modified mouse lines developed to encode the calcium fluorophore GCaMP under the control of the insulin promoter have been used to study single beta cell dynamics¹⁸³ (calcium imaging acquired at a minimum of 3 Hz). However, there are many examples of a

dissociation between calcium signalling and actual insulin release¹⁸⁴. Furthermore, studying human cells with fluorophores requires preloading with dyes which do not penetrate uniformly and avidly scavenge oxygen which impedes cellular respiration.

Thus, non-invasive, real-time and highly sensitive measurements of insulin secretion from islets remains an unmet need. This is required for *ex vivo* study of donor islets but importantly is becoming even more pressing for the development of beta cell replacement technologies. This century has witnessed advances in the production of stem cell derived beta cells with the goal of re-transplantation into patients living with type 1 diabetes as a 'cell-based' cure¹⁸⁵. Central to speeding up the development of optimized protocols or advanced biomaterials to improve the maturation, function and longevity of these cells is the ability to measure their insulin secretion. Since it is not currently possible to rapidly measure insulin secretion from stem cell-derived beta cell clusters at different stages of differentiation or in different conditions, such advances are fundamentally slowed. Before detailing the proposal for an electrochemical probe for the measurement of insulin secretion at the cellular level, it is worth considering first the structure, electrochemical features and secretion biology of insulin and its closely related counter regulatory hormone glucagon.

Insulin and glucagon are both peptide hormones comprised of amino acids. Insulin consists of two amino acid chains, an A-chain and B-chain, linked by disulfide bridges. In beta cells, insulin is synthesized as pre-proinsulin, then cleavage of the amino terminal signal sequence results in proinsulin folded with disulfide bridges and subsequent cleavage of the connecting polypeptide produces insulin¹⁸⁶. Insulin is then packaged in large dense core vesicles readily available for release in beta cells. In this work, we aim to develop an electrochemical tool with the speed, sensitivity, specificity and sensitivity to detect exocytotic insulin and serotonin release in real-time. Electrochemical methods have been developed to detect insulin oxidation (are discussed below) which mechanistically occurs *via* its four tyrosine residues. However additional electroactive amino acids (cysteine, histidine and methionine¹⁸⁷) are present within the insulin

structure but have not been found to contribute to insulin's oxidation mechanism. When probing islets, in addition to insulin, we also expect an electrochemical signature for glucagon release from alpha cells. In pancreatic alpha cells, preproglucagon is processed by the PC2 enzyme to produce glucagon¹⁸⁸. Resultant glucagon is then packaged into peptide vesicles for exocytosis upon stimulation. Glucagon oxidation occurs *via* its tyrosine and tryptophan residues and has been sensed at carbon nanotubes and glassy carbon sensors using amperometry¹⁸⁹. While a similar oxidative signature is expected for glucagon an insulin, their differential release based on exogenous glucose levels indicate that their release will not be simultaneous.

As mentioned, electrochemical methods have been developed for insulin detection. Several groups have modified glassy carbon electrodes to measure insulin with cyclic voltammetry¹⁹⁰⁻¹⁹³. Notably, Snider *et al.* applied their carbon nanotube-modified glassy carbon electrode, with insulin detection as low as 1 μ M, to sense bulk insulin secretion from ~100 islets using a microphysiology chamber¹⁹⁴. However, as cyclic voltammetry employs scan rates less than 1 V/s, a single measurement can take as little as several seconds to acquire. Amperometry has been used to detect insulin oxidation with no temporal constraints at surface-modified carbon microelectrodes in detecting insulin pulses from isolated beta cells⁷¹, however with amperometry the oxidative currents cannot be qualified making the method unsuitable to probe complex biological systems. An adaptation of cyclic voltammetry, fast scan cyclic voltammetry (FSCV), has been used for real-time (10 Hz) detection of monoamines at carbon fiber microelectrodes (CFMs) for qualitative and quantitative detection in one measurement.

In this work we sought to develop a novel electrochemical measurement protocol to directly probe insulin and serotonin signalling in real-time at carbon microelectrodes using FSCV. Serotonin redox is well-characterized with FSCV⁷⁷, as its fast electron transfer kinetics allow for detection with scan rates up to 1000 V/s. Thus, we first optimized several waveform parameters based on the relatively slower transfer kinetics of insulin. Then, adapted the holding potential and modified the CFM surface for peak-resolved co-detection of insulin and serotonin. Sensor performance at the newly optimized waveform was assessed

for both insulin and serotonin in terms of sensitivity and stability towards both molecules. Finally, we apply our novel FSCV methodology to biological models of insulin release for the first direct, real-time measurements of insulin exocytosis. This novel measurement technique has the potential to revolutionize diabetes research, as real-time insulin dynamics and the role of its co-secreted messenger, serotonin, have yet to be fully uncovered. Furthermore, this technology can be applied in the future to assess functionality of stem cell-derived beta cells and correlate islet signalling dynamics with peripheral insulin levels.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Synthetic insulin (100 IU/mL, Humulina, Eli Lilly and Company, Indianapolis, IN, USA) was used for all FSCV experiments. 10X phosphate buffered saline (PBS) was purchased and diluted to 1X PBS for experimentation ($\text{pH } 7.4 \pm 0.005$). Krebs'-Ringer buffer solution was prepared fresh for each experiment (10 mM HEPES, 2 mM NaHCO_3 , 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 and 1.5 mM CaCl_2) and adjusted to $\text{pH } 7.4 \pm 0.005$. As HEPES was found to interfere with FSCV measurements when scanning the potential up to 1.3 V after initial waveform optimization occurred, a solution of Hank's balanced salt solution (HBSS) was used for subsequent experiments. HBSS was prepared fresh at pH-adjusted to $\text{pH } 7.4 \pm 0.005$ based on a published formulation¹⁹⁵ (Thermo Fisher product number: 14025092; 140 mg/L CaCl_2 , 100 mg/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 400 mg/L KCl, 60 mg/L KH_2PO_4 , 350 mg/L NaHCO_3 , 8000 mg/L NaCl, 48 mg/L Na_2HPO_4).

FSCV

Carbon fiber microelectrodes, flow injection analysis and FSCV data acquisition (Chapter 2, Figure 2.2) and analysis were performed as described in chapter 2. Descriptions below are specific to this work.

Flow Injection Analysis

For this work FIA was used to develop a waveform for insulin and serotonin co-detection. Calibration solutions of insulin and serotonin were prepared fresh each day in salt buffer (HBSS, Krebs' or PBS) prior to experimentation. All points were repeated in triplicate for each electrode. For repeated injections of serotonin or insulin at CFMs over several hours, solutions were prepared fresh prior to experimentation. Stability curves were generated by giving a 10 second bolus injection once every two minutes until the desired time and injection number was reached. Nuances of each experiment are denoted in the results section.

Cell Culture

INS-1 cell culture

Frozen cells from the INS-1 832/3 linewere gifted by Dr. Benjamin Jones at Imperial College London. The immortalized cell line was thawed and placed into a T25 tissue culture flask with RPMI-1640 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (p/s) (Thermo Fisher, Waltham, MA, USA) and 10% fetal bovine serum (FBS) (Thermo Fisher, Waltham, MA, USA). The cultured dishes were maintained in an incubator at 37 °C with 5% CO₂/95% O₂. Cells were passaged into new tissue flasks (T25 or T75) every 3-4 days based on confluency. Prior to FSCV experiments, cells were pre-incubated in low glucose RPMI-1640 medium (2 mM D-glucose) for 8-12 hours. After pre-incubation, cells were washed twice with phosphate-buffered saline (PBS), centrifuged and re-suspended. The suspended cells were plated onto 35 mm petri dishes and incubated in the same low glucose RPMI-1640 medium for 1-2 days based on confluency. Dishes were used for FSCV experiments when 70-90% confluency was reached. For FSCV experiments, the RPMI-1640 medium was replaced with 2 mL PBS. Dishes were then secured onto the platform of an inverted light microscope (Olympus, Tokyo, Japan). A freshly prepared Ag/AgCl pseudo-reference electrode was placed at the edge of the dish so that it contacted the buffering solution.

A cylindrical CFM was positioned $\sim 3\text{-}5\text{ }\mu\text{m}$ above a dense population of INS-1 cells using a quadrupole micromanipulator (QUAD, Sutter Instruments, Novato, CA, USA). The CFM was equilibrated to the environment by applying the insulin-optimized waveform with a neutral holding potential (0 to 1.3 to 0 V at 400 V/s) at 60 Hz for 10 minutes then at 10 Hz for 10 minutes. FSCV files during zero and high (20 mM) glucose conditions were acquired until 10 minutes of data were recorded, then the electrode was moved to a separate location. This procedure was repeated for 6 locations across 2 dishes, where each condition was acquired at 3 separate locations.

Animal Husbandry

Male C57BL/6 mice (18-25 g) were group-housed in individually ventilated cages (21-23 °C, 12-hour light/dark cycle) with *ad libitum* access to food and water. Animal work was performed under the project license PA03F7F07A (held by Dr. Isabelle Leclerc) at Imperial College London (CBS animal facility). All procedures were performed in compliance with the UK Home Office Animals (Scientific Procedures) Act 1986).

Islet Isolation

For pancreatic islet isolation, donors were sacrificed (Schedule 1) and the peritoneum was excised to expose the internal organs. Under a stereomicroscope, the sphincter of Oddi was clamped and the common bile duct was identified. Then, a 30G needle was used to perfuse the pancreata with 3-5 mL of collagenase (1 mg/mL collagenase NB 8 broad range (S1745601 Universal Biologicals) dissolved in serum-free RPMI-1640 medium (Thermo Fisher, Waltham, MA, USA) filtered using a $0.45\text{ }\mu\text{m}$ diameter filter) *via* the common bile duct. Once fully sufflated, the pancreas was excised and placed in a tube on an ice bath. Excised pancreata within the tubes were incubated in a 37 °C water bath for 10 minutes. Then the pancreata were washed with 15 mL of serum-free RPMI-1640. Once added, the tube was shaken vigorously for 20 seconds then centrifuged for 1 minute at 1000 rpm. The tubes were placed on ice to

prevent over-digestion and the supernatant was removed. After gently dislodging the pellet, 15 mL of serum-free medium was added, and the tube was centrifuged again for 1 minute at 1000 rpm. This washing procedure was repeated twice more. After the samples were washed, the supernatant was discarded, and the pellets were resuspended in 4 mL of Histopaque-1119 (Merck) and transferred to a 15 mL falcon tube. Without intermixing, 4 mL of Histopaque-1083 (Merck) then 4 mL of serum-free RPMI-1640 medium were added dropwise. The tubes were centrifuged at slow deceleration for 20 minutes to prevent intermixing between the three layers. Isolated islets settled at the interface of the upper and middle layers, which were aspirated into a 1 mL pipette then placed into a new cell-suspension petri dish with 10 mL of RPMI-1640 medium supplemented with 10% FBS and 2% p/s.

For FSCV experiments, 5-6 islets were plated into a 35 mm petri dish with 2 mL of PBS. The experimental setup was as described for the INS-1 cells, however here the bevelled CFMs were placed 3-5 μ m above the apex of the chosen islet. The bevelled CFM was equilibrated to the environment by applying the insulin-optimized waveform with a negative holding potential (-1.0 to 1.3 to -1.0 V at 400 V/s) at 60 Hz for 10 minutes then at 10 Hz for 10 minutes. FSCV files during zero and high (20 mM) glucose were acquired until 15 minutes of data were recorded for each condition. This procedure was repeated for a new dish of islets, with a total of 7 islets probed for this work.

Results

FSCV waveform optimization for insulin and serotonin co-detection at cylindrical CFMs

As mentioned, insulin is a small globular protein comprised of two chains of amino acids linked by disulfide bridges. Out of the 20 natural amino acids, 5 are electroactive: histidine, cysteine, methionine, tryptophan and tyrosine. The Ross lab characterized the electrochemical signatures of each electroactive amino acid at CFMs with FSCV where only tryptophan and tyrosine produced Faradaic oxidation peaks on the forward scan of their potential window (-0.4 V to 1.45 V to -0.4 V at 400 V/s) at 1.06 V and 1.13 V, respectively¹⁸⁷.

The amino acid sequence of insulin contains all the electroactive amino acids except for tryptophan¹⁹⁶ and is known to oxidize at its four tyrosine residues¹⁹³ (validated in Figures 6.11 and 6.12). Based on these works, we expected insulin to oxidize at a similar potential to that of tyrosine (~1.0 V).

We initially applied a wide potential window scanning from a highly negative potential (-1.0 V) to a positive switching potential above 1.0 V (1.3 V) for carbon surface renewal, to mitigate potential surface fouling. Waveform parameters were optimized by injecting insulin to CFMs using FIA. As seen in Figure 6.3A, the resultant color plot from a 1 μ M insulin injection has three distinct peaks: two oxidation peaks at 1.0 V and 0.65 V and a reduction peak at -0.5 V. The appearance of the oxidation peak at 0.65 V ~0.5 s after the 1.0 V peak, indicates the peak at 0.65 V is a secondary oxidation of tyrosine. There are several mechanistic steps to fully oxidize tyrosine¹⁹⁷, and here we theorize the peak at 1.0 V is from the initial radicalization of tyrosine ($Y^{\bullet+}$) and the peak at 0.65 V from a second oxidation. Regardless, as insulin oxidizes at 1.0 V within this potential window and shifts more positive in Ross's work, we opted to maintain the switching potential at 1.3 V throughout this work.

Next, using the same potential window, the scan rate was varied in increments of 100 starting from 100 V/s until the signal was no longer detectable. The maximum current for 1 μ M insulin boluses peaked at a 400 V/s scan rate (Figure 6.2). As the peak currents plateaued after 400 V/s, this was determined as the optimal scan rate for insulin oxidation with FSCV. Additionally, plotting the log of the scan rate vs log of the current response had an R^2 of 0.9972 which indicates insulin adsorbs to the CFM (Figure 6.2)¹⁹⁸. As a high current response was obtained for 1 μ M insulin (125.9 ± 0.4 nA), we characterized the response to insulin at this waveform in terms of sensitivity and stability (Figure 6.3). While a highly stable and sensitive response was observed at this waveform (Figure 6.3B), the secondary oxidation peak (0.65 V) significantly interferes with that of serotonin (~0.6 V). Next, because the switching potential and scan rate are limited by the position of the primary oxidation peak and insulin's electron transfer kinetics, we altered the holding potential to exclude the secondary oxidation peak from the signal.

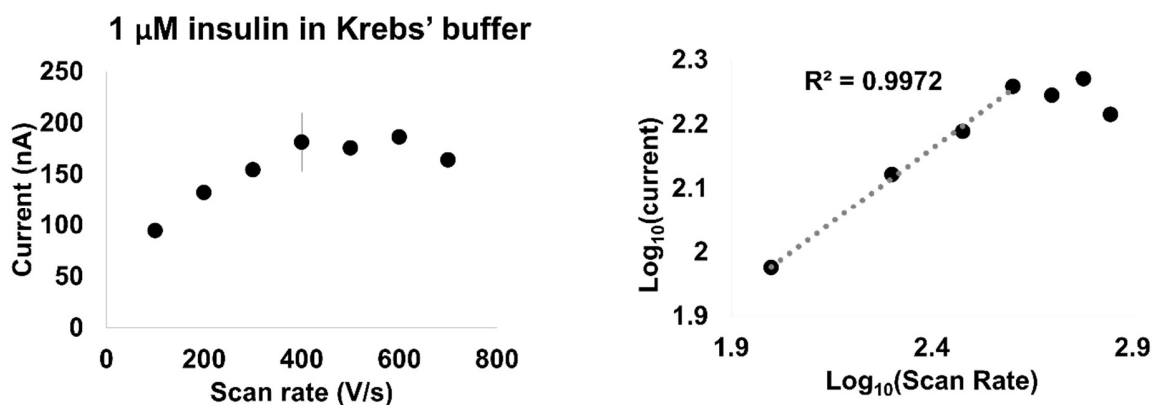


Figure 6.2. Optimized scan rate for insulin detection at bare cylindrical CFMs. FIA injections of 1 μ M insulin in Krebs' buffer were used to evaluate the effect of scan rate on insulin oxidation. The scan rate for the potential window of -1.0 V to 1.3 V to -1.0 V was varied from 100 to 700 V/s in increments of 100 until the cylindrical CFM (n=3 injections per scan rate from 1 CFM) was overloaded with capacitive current on the oscilloscope. The current from the primary oxidation peak was plotted against the scan rate (left). The log of the current and scan rate was then plotted (right) to determine whether insulin oxidation was adsorption or diffusion driven (based on the R^2).

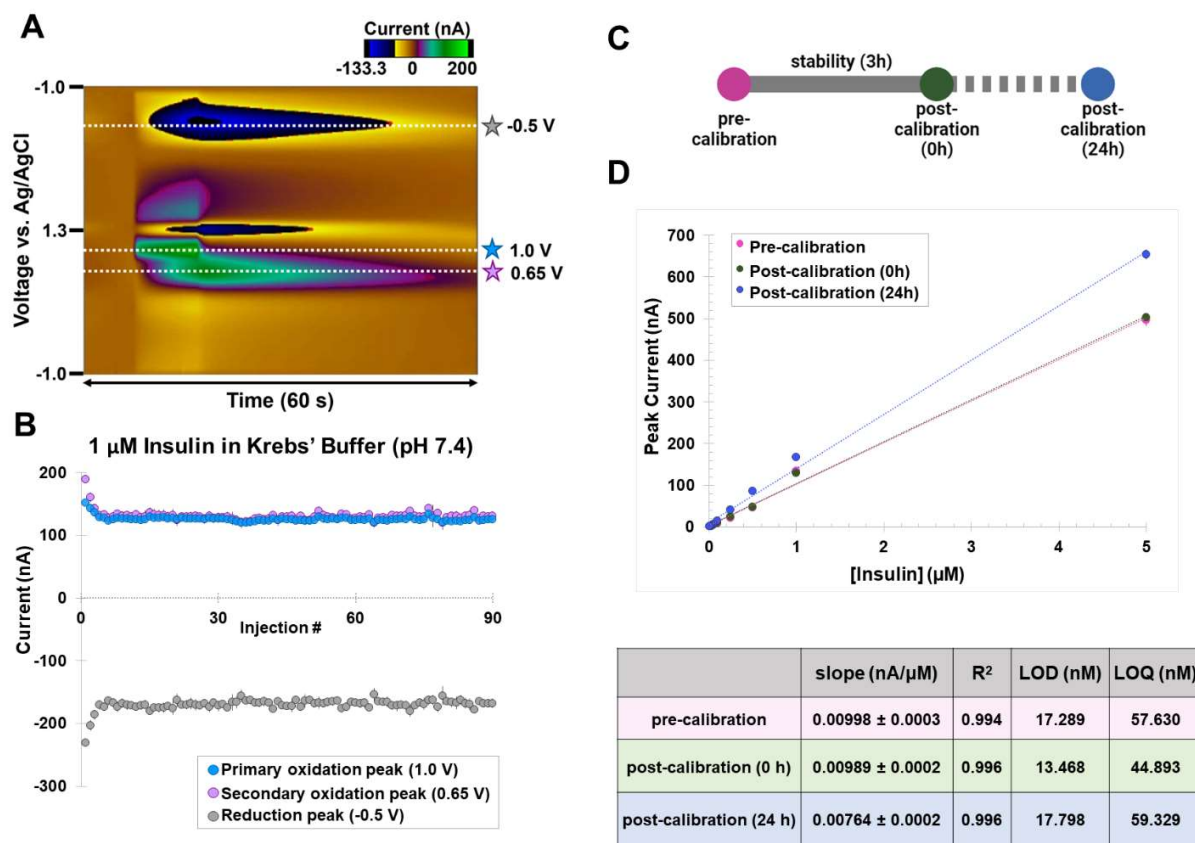


Figure 6.3. CFM (bare, cylindrical) performance for insulin detection using a negative holding potential. (A) representative color plot of a 1 μM insulin injection (FIA) in Krebs' buffer. The primary oxidation peak is denoted with a blue star, the secondary peak with a purple star, and the reduction peak with a gray star. (B) CFM response to repeated injections of 1 μM insulin in Krebs' buffer over 3 hours with an injection occurring every two minutes. The peak currents from the primary oxidation (blue), secondary oxidation (purple) and reduction (gray) peaks are quantified over the injection period from $n=5$ electrodes. (C) Illustrative depiction of when the electrodes from panel B were calibrated to insulin. An insulin calibration curve was applied prior, immediately after, and 24 hours after the 3 hours of repeated injections were applied. (D) Calibration curves taken before ('pre-calibration', pink), immediately after ('post-calibration (0h)', green) and 24 hours after ('post-calibration (24h)', blue) 3 hours of repeated injections ($n=5$ electrodes). Associated analysis parameters are outlined in the table below.

We assessed the effect of holding potentials on insulin oxidation at CFMs with FSCV using 0.5 μM injections of insulin in salt buffers (Krebs' and HBSS). We applied holding potentials from -1.0 to 0.4 V and found that

a 0 V holding potential significantly reduced the presence of the secondary oxidation peak (Figure 6.4B) compared to the -1 V potential (Figure 6.4A), while preserving peak sharpness. However, as the secondary peak was not fully eradicated from the color plot, we explored polymeric surface modification. We utilize a robust protocol for electrochemical deposition of NafionTM, a cation exchange polymer, onto CFM surfaces in our laboratory⁷⁷. As such, we injected insulin onto Nafioned CFMs with the expectation that the secondary oxidation peak would increase, as the Y^{•+} would be attracted to the negative sulfoxyl groups, and thus justify the purchase of an anion exchange polymer. Instead, to our surprise, the secondary peak was absent at Nafioned CFMs (Figure 6.4C). It is likely that the Y^{•+} adsorbs to the Nafion rather than the carbon, which explains the exclusion of the secondary peak. With the exclusion of the secondary oxidation peak of insulin, we were confident that resolved insulin and serotonin oxidation peaks could be achieved at Nafioned CFMs using a 0 V to 1.3 V to 0 V at 400 V/s waveform (insulin-optimized waveform). Next, we characterized the performance of Nafioned CFMs at the insulin-optimized waveform in terms of sensitivity and stability.

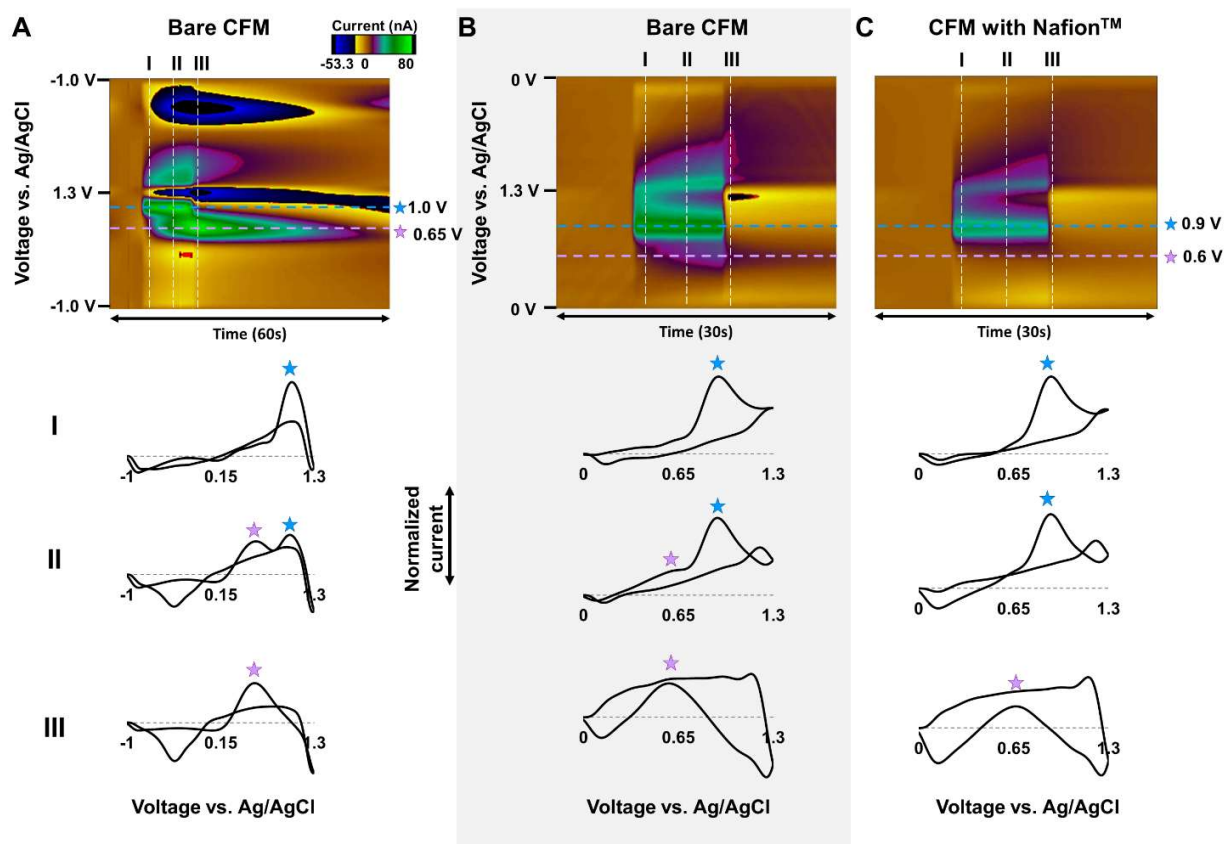


Figure 6.4. Waveform and surface modifications exclude the persistent secondary oxidation peak of insulin. All color plots are representative FIA injections of 500 nM insulin in various buffers. The blue dashed lines and stars indicate the main oxidation peak at ~0.9 V while the purple indicate the interfering by-product of insulin oxidation at ~0.6 V. Cyclic voltammograms (CVs) taken at the beginning (9.0 s, I), in the middle of (14.0 s, II) and after (19.0 s, III) the ten-second injection. Corresponding CVs with normalized current (white dashed lines) are below their respective color plots with oxidation peaks indicated by blue and purple stars. (A) Insulin in Krebs' buffer injected onto a bare CFM. The holding potential was set to -1.0 V. (B) Insulin in HBSS injected onto a bare CFM. The holding potential was set to 0 V. (C) Insulin in HBSS injected onto a CFM coated with Nafion™. The holding potential was set to 0 V.

We assessed the performance of CFMs in response to insulin and serotonin at bare vs. Nafioned CFMs using the insulin-optimized waveform. Sensitivity was determined by calibrating CFMs with and without a thin coating of Nafion™ to insulin and serotonin in Hank's Balanced Salt Solution (HBSS) at pH 7.4 (as an undesired artefact in Kreb's buffer was attributed to the presence of HEPES (Figure 6.5)) using FIA (Figure

6.6 A,C i-iv). An enhanced current response to insulin was observed with Nafioned CFMs (Figure 6.6D). Then, the stability of the CFM to insulin and serotonin was observed through repeated injections of 1 μ M serotonin and insulin with one injection every two minutes. We observed a decay in current over time for insulin (First 5 injections: 33.44 ± 4.16 nA, last 5 injections: 16.29 ± 2.09 nA, $n=25$ injections across 5 electrodes, two-tailed paired t-test: $p=0.01$; $-51.3 \pm 0.1\%$ loss in current) (Figure 6.6E) and serotonin (First 5 injections: 86.10 ± 20.94 nA, last 5 injections: 36.22 ± 6.39 nA, $n=30$ injections across 6 electrodes, two-tailed paired t-test: $p=0.02$; $-51.4 \pm 5.5\%$ loss in current) (Figure 6.6F). This loss in signal has previously been described for serotonin measurements at Nafioned CFMs¹¹² and was found to stabilize during the typical time frame for FSCV experiments (1-2 hours).

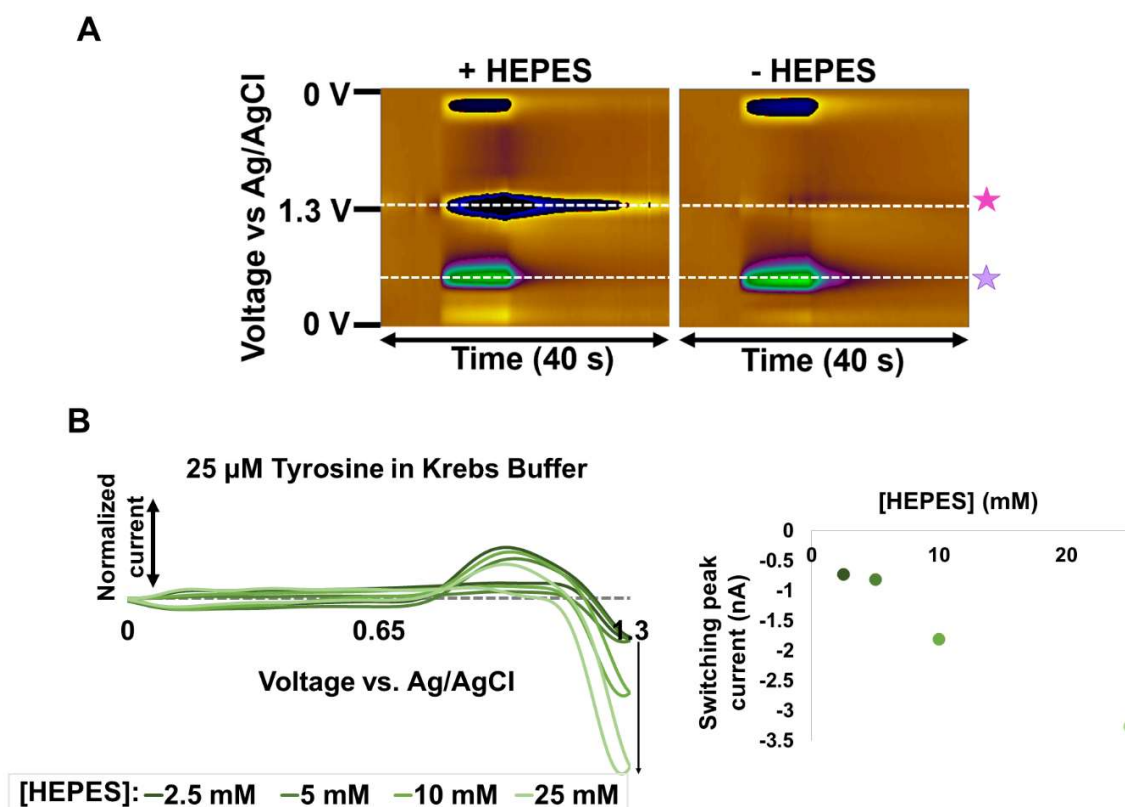


Figure 6.5. HEPES negatively affects insulin detection at CFMs. (A) 1 μ M serotonin injected onto cylindrical CFMs in PBS with (left) and without (right) 10 mM HEPES. The purple star denotes the serotonin oxidation peak, and the pink

star denotes the capacitive peak resultant from HEPES. (B) CVs extracted from color plots where 25 μM tyrosine in Krebs' buffer dosed with 2.5, 5, 10, and 25 mM HEPES (dark to light green denotes low to high concentrations) (left) (n=1 CFM). The switching peak was increasingly negative with increasing HEPES concentration (right).

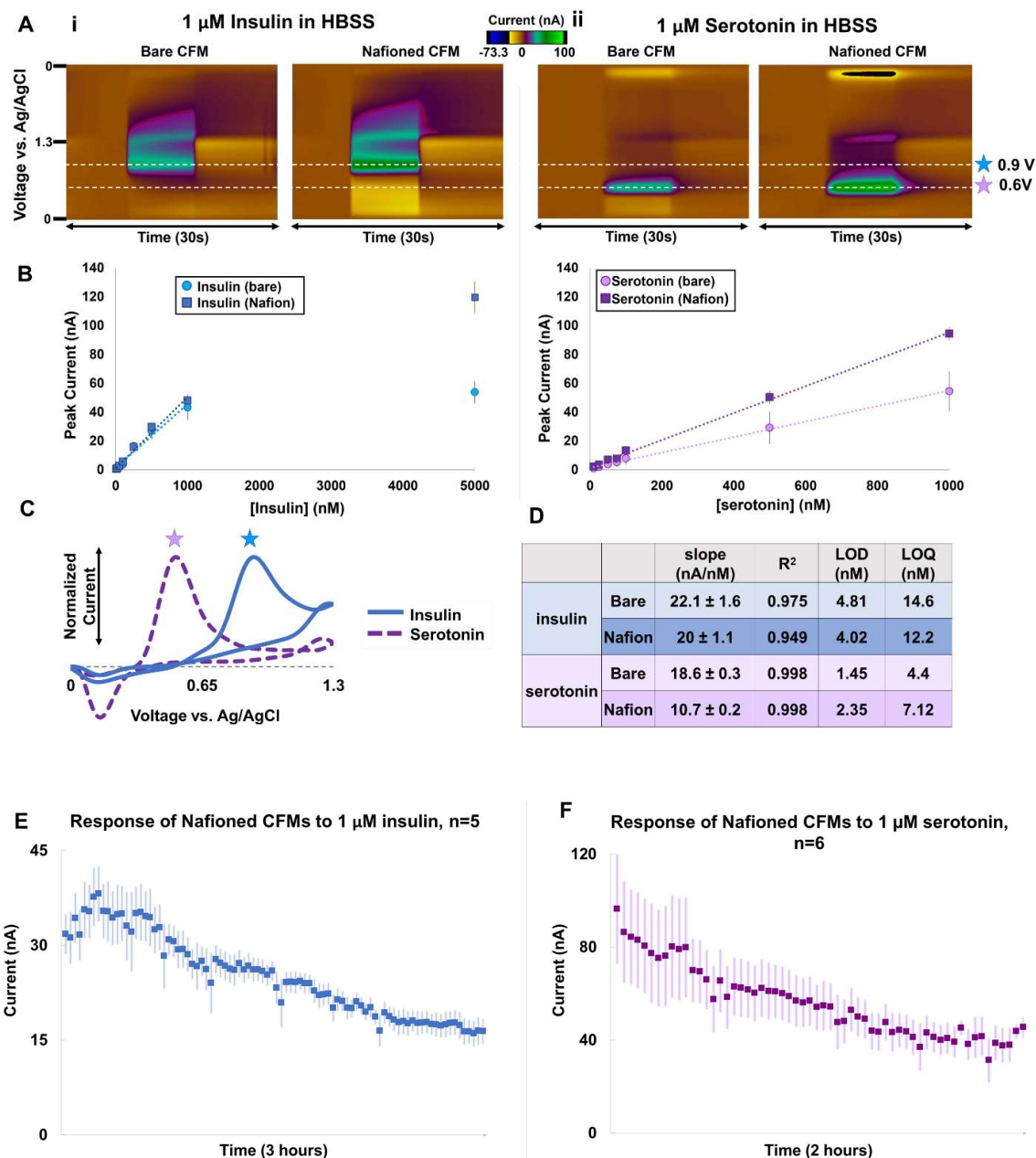


Figure 6.6. Waveform and surface modifications exclude the persistent secondary oxidation peak of insulin. (A) Representative color plots of insulin (i) and serotonin (ii) at bare (left) and Nafioned (right) CFMs. The oxidation

current for insulin is denoted with a blue star and a purple star for serotonin. (B) Comparative calibration curves for insulin (blue, i) and serotonin (purple, ii) with (dark color, squares) and without (light color, circles) NafionTM. (C) Overlapped CVs of serotonin (purple dashes) and insulin (blue) at Nafioned CFMs. The oxidation peaks are denoted by stars. (D) Metrics for the calibrations in panel B including slope, R^2 , limit of detection (LOD) and limit of quantification (LOQ). (E) Repeated injections of 1 μ M insulin (blue, i, n=5 electrodes) over 3 hours and serotonin (purple, ii, n=6 electrodes) over 2 hours at Nafioned CFMs with one injection occurring every two minutes.

Together, these data show that applying the insulin-optimized waveform at Nafioned CFMs results in sensitive insulin and serotonin detection as well as relatively stable peak currents over time. We next employed the sensor to biological systems of insulin secretion.

Real-time insulin detection from cells

To test the ability of our sensor to detect exocytosis of insulin and serotonin, we probed two types of insulin-secreting cells: an immortalized line of rat beta cells (INS-1) and mouse-derived pancreatic islets. For INS-1 measurements, a dish of cells was mounted on an inverted microscope and then the CFM was lowered 3-5 μ m above a cluster of cells (Figure 6.7A). After baseline measurements in a glucose-free buffer (phosphate-buffered saline (PBS)) were taken, the cells were stimulated with 20 mM glucose to elicit insulin release. Chemical stimulation was performed by spiking in a concentrated solution of glucose to bring the total volume of the dish to the desired concentration. We observed spontaneous (pre-glucose), stimulated and post-stimulation current spikes on the color plots at the oxidation potential of ~ 0.4 - 0.6 V (Figure 6.7B). The signals from INS-1 cells were validated against CVs from FIA injections of insulin (Figure 6.7E). The currents were converted to concentration from the calibration curves in Figure 6.6B. Quantitative analysis showed that stimulated insulin release was slightly higher than spontaneous activity (spontaneous: 0.959 ± 0.014 nM, n=4 events across 6 locations from 2 cell dishes; glucose-stimulated: 0.990 ± 0.007 nM, n=2 across 2 locations from 2 cell dishes, two-tailed unpaired t-test: $p=0.111$) and post-stimulation releases were of similar amplitude to the spontaneous, pre-glucose transients (pre-glucose: 0.959 ± 0.014 nM, n=4

events across 6 locations from 2 cell dishes; post-glucose: 0.959 ± 0.010 nM $n=5$ events across 6 locations from 2 cell dishes, two-tailed unpaired t-test: $p=0.997$) (Figure 6.7G). As INS-1 cells are known to solely secrete insulin and the oxidation currents from the cells overlapped with insulin signal obtained *via* FIA, we were confident that the sensors indeed captured insulin release from INS-1 cells in real-time. We next applied our sensors murine pancreatic islets to assess performance in a more complex biological model of insulin secretion.

The experimental set-up for the islets was like that for the INS-1 cells, where 3-5 islets were placed in a dish of PBS at pH 7.4 and the CFM was lowered to ~ 3 μm above the apex of a single islet (Figure 6.7C). Baseline measurements were taken in a glucose-free environment and insulin secretion was again stimulated with 20 mM glucose, as in the INS-1 cells. Prior to and in response to chemical stimulation, we observed oxidation events at the same potential for insulin oxidation (Figure 6.7D). These oxidative events were normalized against CVs from insulin injected into a flow cell (Figure 6.7F), and the overlap of the oxidation peaks confirmed their identity to be insulin. The peak currents from each event were converted to concentration as above and averaged (Figure 6.7H). The spontaneous events were not significantly different in peak concentration from stimulated (spontaneous: 0.9396 ± 0.0015 nM, $n=6$ events 5 islets; glucose-stimulated: 0.945 ± 0.002 nM, $n=7$ events from 7 islets, two-tailed unpaired t-test: $p=0.071$). Together, these data show that employing the insulin-optimized waveform at CFMs is suitable for qualifying and quantitating insulin secretion from islets in real-time.

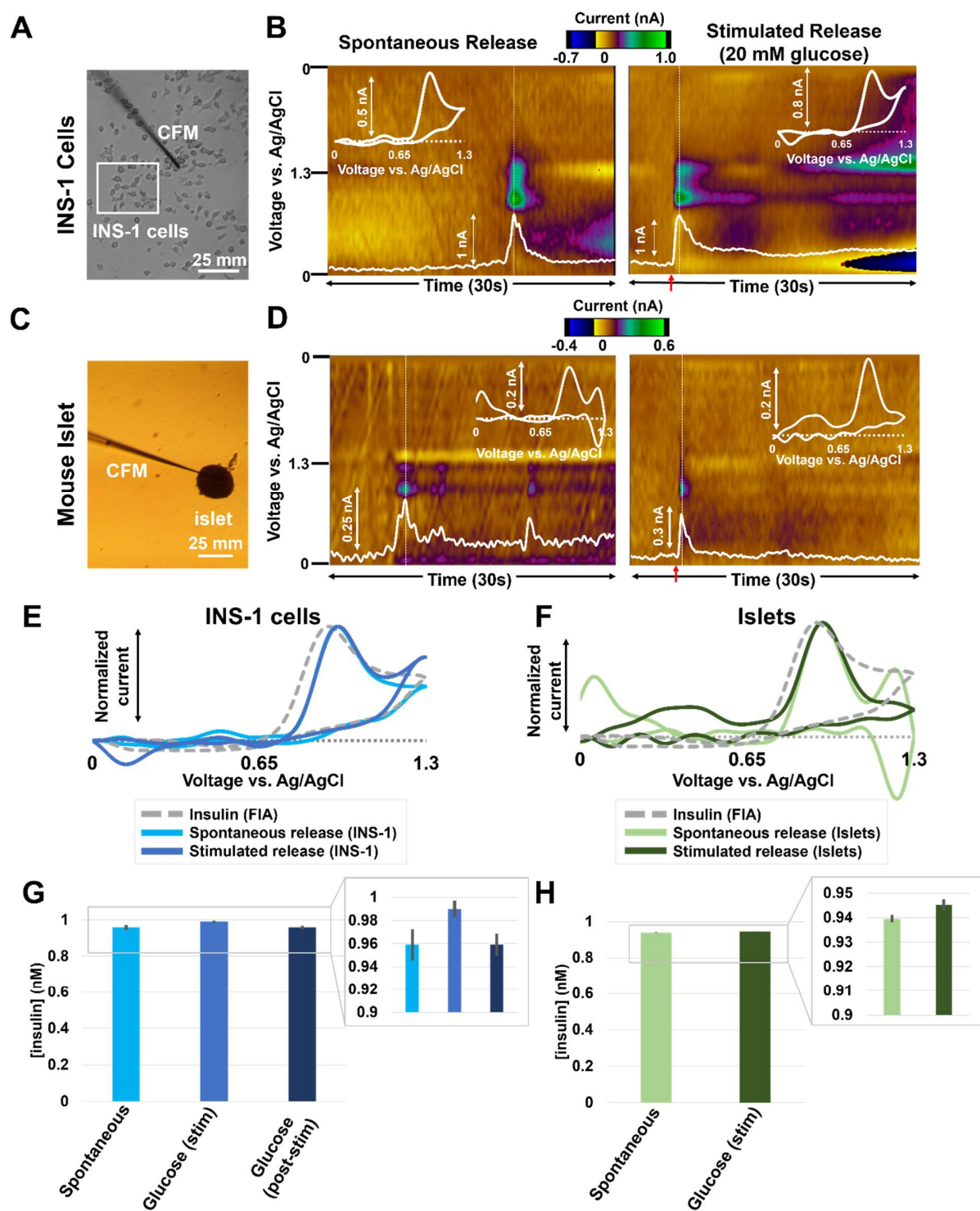


Figure 6.7. Real-time detection of insulin release from INS-1 cells and pancreatic islets. (A) Image of an INS-1 cells in culture with a Nafioned, cylindrical CFM placed 3-5 μ m above the cells. (B) Representative color plots of

spontaneous (left) and glucose (20 mM)-stimulated release (right). The glucose application is denoted by the red arrow. Inset CVs are taken from the white vertical line in each color plot. (C) Image of an islet in culture with a Nafioned, cylindrical CFM placed 3-5 μm above the cells. (D) Representative color plots of spontaneous (left) and glucose (20 mM)-stimulated release (right). The glucose stimulation is denoted by the red arrow. Inset CVs are taken from the white vertical line in each color plot. (E) Cyclic voltammograms (CVs) from insulin in a flow cell (FIA, gray dashes), a spontaneous (light blue) and a glucose-stimulated (dark blue) release from INS-1 cells in panel B. The oxidative currents are normalized to the maximum. (F) CVs from insulin in a flow cell (FIA, gray dashes), a spontaneous (light green) and a glucose-stimulated (dark blue) release from INS-1 cells in panel B. The oxidative currents are normalized to 1. (G) Averaged peak concentration of insulin from spontaneous (light blue, n=6 events across 6 locations in 2 cell dishes), glucose-stimulated (blue, n=2 events across 2 locations in 2 cell dishes), and spontaneous events in a high glucose (post-stim) environment (dark blue, n=5 events across 6 locations in 2 cell dishes) from the INS-1 cells. The outset gray box is the same chart with the y-axis re-scaled to visualize the height differences. Unpaired t-tests showed no significant change in peak concentration between the three groups. (H) Averaged peak concentration of insulin from spontaneous (light green, n=6 events from 5 islets) and glucose-stimulated (dark green, n=7 events from 7 islets) events measured from the islets. The outset gray box is the same chart with the y-axis re-scaled to visualize the height differences. Unpaired student's t-tests showed no significant change in peak concentration between the three groups.

In addition to the insulin peaks in both cell preparations, we observed non-Faradaic current fluctuations. The source of the non-Faradaic current fluctuations is likely due to an electrochemical phenomenon caused by the variable height of the CFM in reference to the cells. The CFM is cylindrical, and with the lowermost portion of the carbon fiber positioned above the cells at an angle there is a variable diffusion distance to the carbon surface. As dictated by Fick's second law of diffusion, the current flux is proportional to the diffusion distance and area¹⁹⁹. Additionally, when electroactive species adsorb and exchange electrons at the CFM surface, the capacitive background is altered from its initially subtracted state and therefore visible in the color plot. This, in combination with a variable diffusion distance, results in non-

Faradaic current fluctuations. To avoid this phenomenon, we opted to use CFMs bevelled at a 45° angle. When these CFMs are placed in solution at a 45° angle, the carbon surface is planar to the cells and the diffusion distance is fixed, eliminating the non-Faradaic current flux. As described in chapter 2, bevelled CFMs are made using a different type of carbon fiber, so we evaluated the response of bevelled CFMs to insulin and serotonin.

Waveform parameters are not transferrable between Cylindrical and Bevelled CFMs

Bevelled CFMs are fabricated with a different type of carbon fiber (P-55) to the cylinders (T-650), therefore we characterized the response to insulin at these CFMs using the insulin-optimized waveform to determine any signal variations between fibers.

Initial FIA injections showed a loss of the Faradaic oxidation current for insulin (Figure 6.8A). Bevelled CFM fabrication requires physical smoothing (polishing) of the carbon surface, losing the microroughness needed to promote adsorption of redox-active species. To roughen the surface of macro-electrodes, they are buffed in a paste made of alumina powder, however this process would break the delicate bevelled CFMs. Instead, we electrochemically roughened the bevelled CFMs to generate an adsorptive surface by applying a wide potential window (-1.2 V to 1.3 V to -1.2 V at 400 V/s) at 60 Hz for 10 minutes prior to the normal cycling paradigm on the insulin-optimized waveform. This procedure restored the presence of the Faradaic oxidation peak for insulin on the color plot (Figure 6.8B).

When evaluating the stability of the current response to insulin, we observed a significant loss in current over two hours (First 5 injections: 4.143 ± 0.19 nA, last 5 injections: 0.588 ± 0.114 nA, $n=5$ injections across 1 electrode, two-tailed paired t-test: $p<0.001$; -87% loss in current) (Figure 6.8C,D). We hypothesized that although the secondary oxidation peak was excluded from the signal by holding at 0 V, the by-product persisted on the carbon surface, taking up active sites. This notion is supported by the stable peak currents observed from repeated insulin injections over 3 hours when applying a holding potential of -1 V (First 5

injections: 137.1 ± 5.0 nA, last 5 injections: 128.8 ± 4.6 nA, $n=25$ injections across 5 electrodes, two-tailed paired t-test: $p=0.034$; $-6.0 \pm 1.8\%$ loss in current) (Figure 6.3), where the secondary oxidation peak visibly decayed in the color plot. Therefore, to mitigate the significant signal loss at bevelled CFMs, we opted to use a -1 V holding potential to oxidize the secondary peak off with each potential sweep, generating a clean carbon surface.

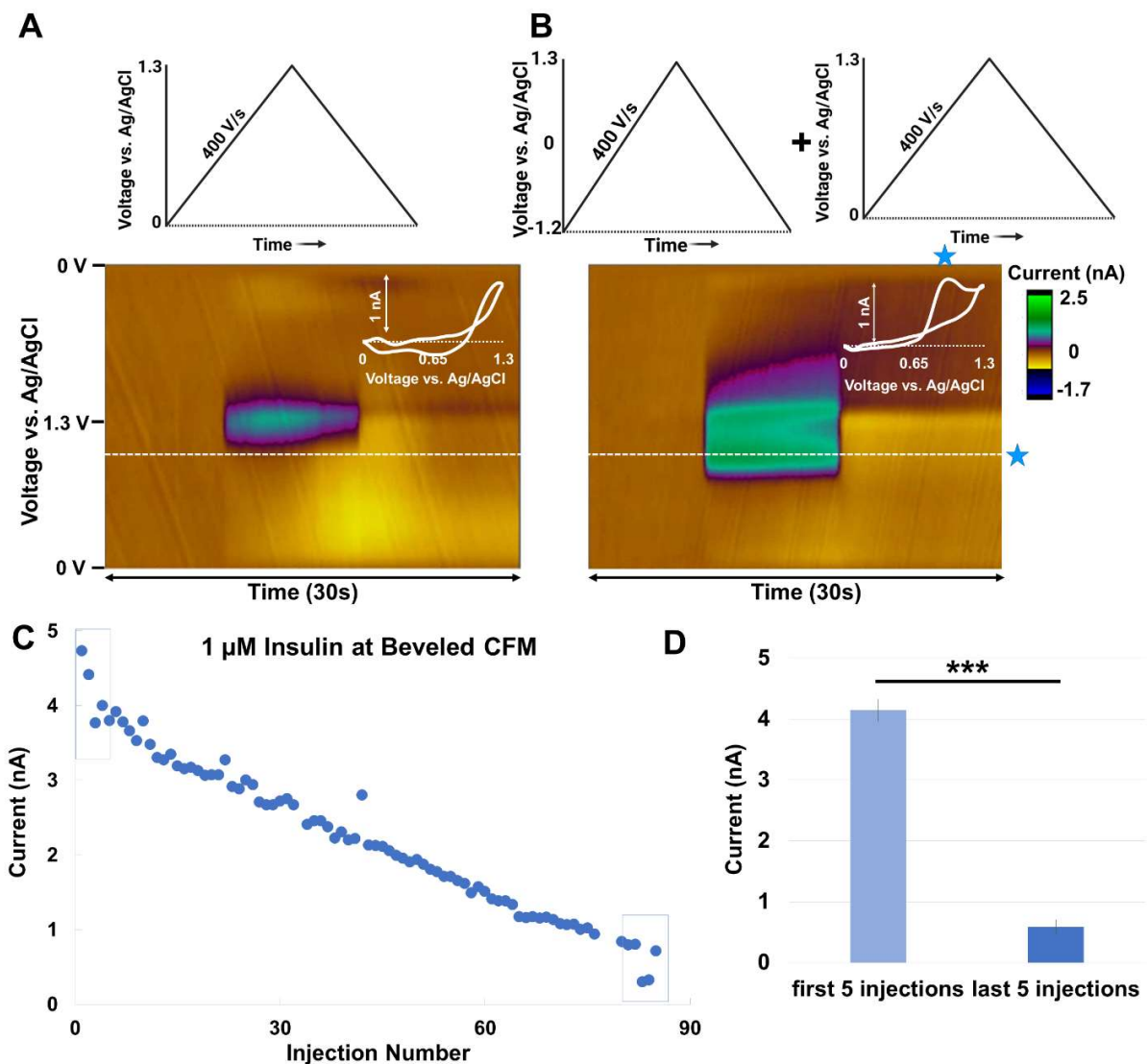


Figure 6.8. Current signal for insulin is diminished at bevelled CFMs using the insulin-optimized waveform. (A) Representative color plot of 1 μ M insulin bolus injected onto a bevelled CFM cycled normally on the insulin-optimized waveform. The inset cyclic voltammogram (CV) indicates the loss of the oxidation peak. (B) Representative color plot

of a $1\ \mu\text{M}$ insulin bolus injected onto a bevelled CFM cycled first on the polymer waveform (to electrochemically etch the surface) then normally on the insulin-optimized waveform to equilibrate the CFM to the solution. The inset CV indicates the restoration of the oxidation peak (blue star). (C) Repeated $1\ \mu\text{M}$ insulin injections to a bevelled CFM over 3 hours (one injection every two minutes). $n=1$ CFM, therefore error bars are not reported. (D) The average of the first 5 injections (light blue bar) and the last 5 injections (dark blue bar) from the data in panel C. Error bars are the standard error of the mean (SEM). Statistical significance was determined by a paired t-test, *** $p<0.001$.

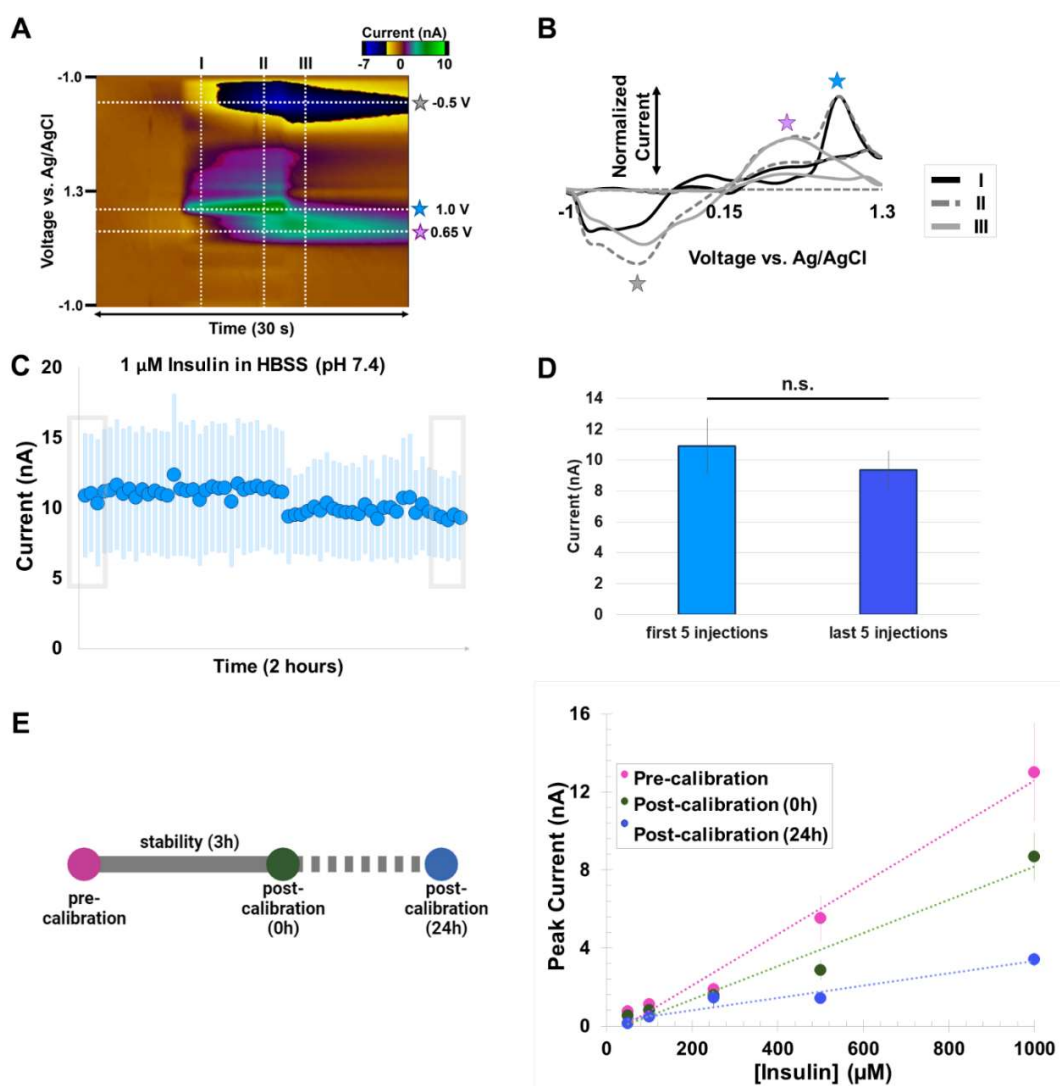


Figure 6.9. Characterization of bevelled CFMs using the extended insulin waveform. (A) Representative color plot of a $1\ \mu\text{M}$ injection of insulin in HBSS at pH 7.4 using FIA. The primary oxidation peak is denoted by a blue star at 1.0

V, the secondary oxidation peak denoted by a purple star at 0.6 V, and the reduction peak denoted by a gray star at -0.5 V. (B) CVs taken over the 10 second injection of insulin from the color plot in panel A. The first CV (I, black) with the primary oxidation peak, the second CV (II, dark gray dashes) has both the primary and secondary oxidation peaks, and the third CV (III, light gray) taken after the injection ceased shows only the secondary oxidation peak. (C) Repeated injections of 1 μ M insulin to bevelled CFMs (n=6) over 3 hours, where an injection occurs once every two minutes. The gray boxes denote the first (left) and last (right) 5 injections of the experiment. Error bars represent standard error of the mean (SEM). (D) Average of the first (light blue) and last (dark blue) 5 injections from the experiment in panel C. A paired t-test showed no significant current loss over 3 hours (p=0.349). (E) The bevelled CFMs were calibrated before ('pre-calibration', pink), immediately after ('post-calibration (0h)', green), and 24 hours after ('post-calibration (24h)', blue) the three hours of repeated insulin injections shown in panel C. Made using Biorender.com.

Table 6.1. Metrics for the calibration curves in Figure 6.6E

	slope (nA/nM)	R ²	LOD (nM)	LOQ (nM)
pre-calibration	0.01313 $\pm$ 0.00096	0.9843	170.0	566.5
post-calibration (0 h)	0.008503 $\pm$ 0.000966	0.9627	264.8	882.5
post-calibration (24 h)	0.003169 $\pm$ 0.00463	0.9399	340.2	1134

We assessed the performance of bevelled CFM performance to detect insulin and serotonin using the extended insulin waveform (-1 V to 1.3 V to -1 V at 400 V/s). Like the cylindrical CFMs, insulin exhibited two oxidation peaks: a primary peak at 1.0 V and a secondary peak at 0.65 V that appears ~0.5 seconds after the primary peak, and a reduction peak at -0.5 V (Figure 6.9A,B). Repeated injections of 1 μ M insulin over 2 hours resulted in a stable response (10.53 $\pm$ 0.47 nA, n=360 injections across 6 electrodes) with no significant loss in current (First 5 injections: 10.92 $\pm$ 1.82 nA, last 5 injections: 9.36 $\pm$ 1.24 nA, n=30 injections across 6 electrodes, two-tailed paired t-test: p=0.349; -6.4 $\pm$ 5.9% loss in current) (Figure 6.9C,D).

Sensitivity over time was determined by calibrating the bevelled CFMs to insulin immediately prior to, immediately following, and 24 hours after the 2 hours of repeated insulin injections. The sensitivity was highest for the pre-calibration (0.01313 ± 0.00096 nA/nM) with successive losses immediately after (0.008503 ± 0.000966 nA/nM, $p=0.098$, paired t-test) and 24 hours following (0.003169 ± 0.00463 nA/nM, $p=0.164$, paired t-test) the stability injections (Figure 6.9E). Additional metrics for these calibration curves are shown in table 6.1, where increases in the limits of detection and quantification were also observed with sequential calibrations. This loss in sensitivity over each calibration indicates that the small surface area decreases over time, as the carbon surface sloughs off at both the holding and switching potentials of this waveform.

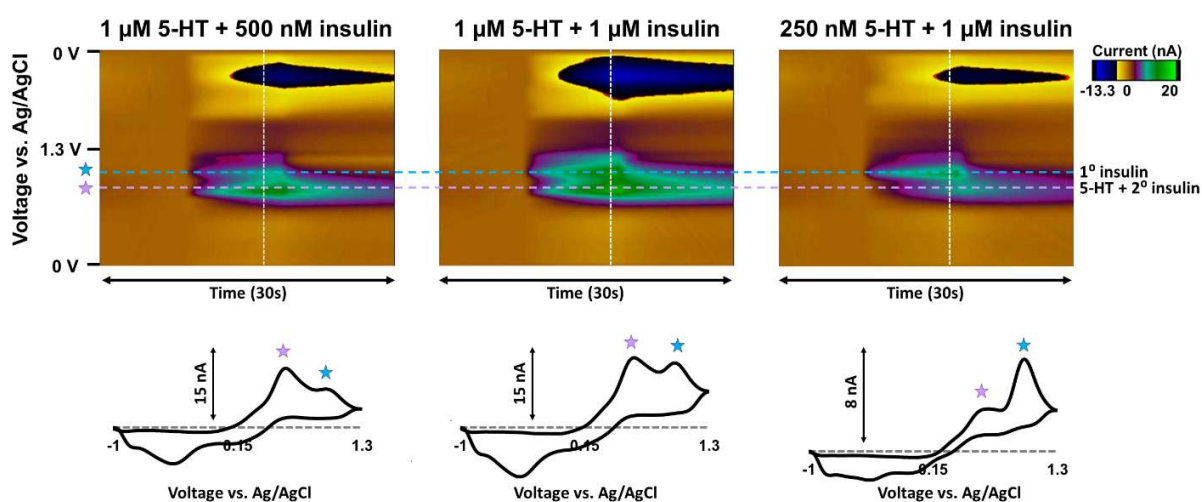


Figure 6.10. Serotonin oxidation is convoluted by the secondary insulin oxidation peak at bevelled CFMs.

Representative color plots (top) from mixed FIA injections of serotonin and insulin at bevelled CFMs. The CVs associated with injection (white vertical lines) are below the color plot from which they came. The primary oxidation peak for insulin (1° insulin) is denoted by blue stars, and the position for the secondary oxidation peak for insulin and for serotonin (as they overlap) is denoted by purple stars on the color plots and associated CVs below the plots. These injections represent serotonin concentration and oxidative currents being higher than insulin (left), equal to insulin (middle) and lower than insulin (right).

While sensitivity was lost over time, a -1 V holding potential resulted in a more stable current response over time compared to the cylindrical CFMs. Still, the presence of the secondary oxidation peak overlaps with serotonin oxidation at this waveform. Injections of various concentration ratios of serotonin and insulin at a bevelled CFM is shown in Figure 6.10, where the serotonin peak overlaps with the secondary oxidation peak of insulin. While serotonin can be seen when the currents are higher than for insulin, it is completely overtaken when serotonin is lower in concentration. Future work to mitigate this issue will include the mathematical exclusion (deconvolution) of the secondary peak from the signal or mathematical modelling, using Fick's law of diffusion, to exclude the non-Faradaic current fluctuations from the color plots.

Finally, we assessed whether glucagon could be electrochemically differentiated from insulin at cylindrical, Nafioned CFMs using the wide potential window (-1.0 V to 1.3 V to -1.0 V at 400 V/s). As shown in Figure 6.13, we observed similar oxidative signatures for glucagon as for insulin. However, the reduction peaks are resolved between the two species. It is likely that in a more complex biological system, insulin and glucagon can be electrochemically differentiated, but more work discerning the signature for glucagon at CFMs on different waveforms will need to be performed.

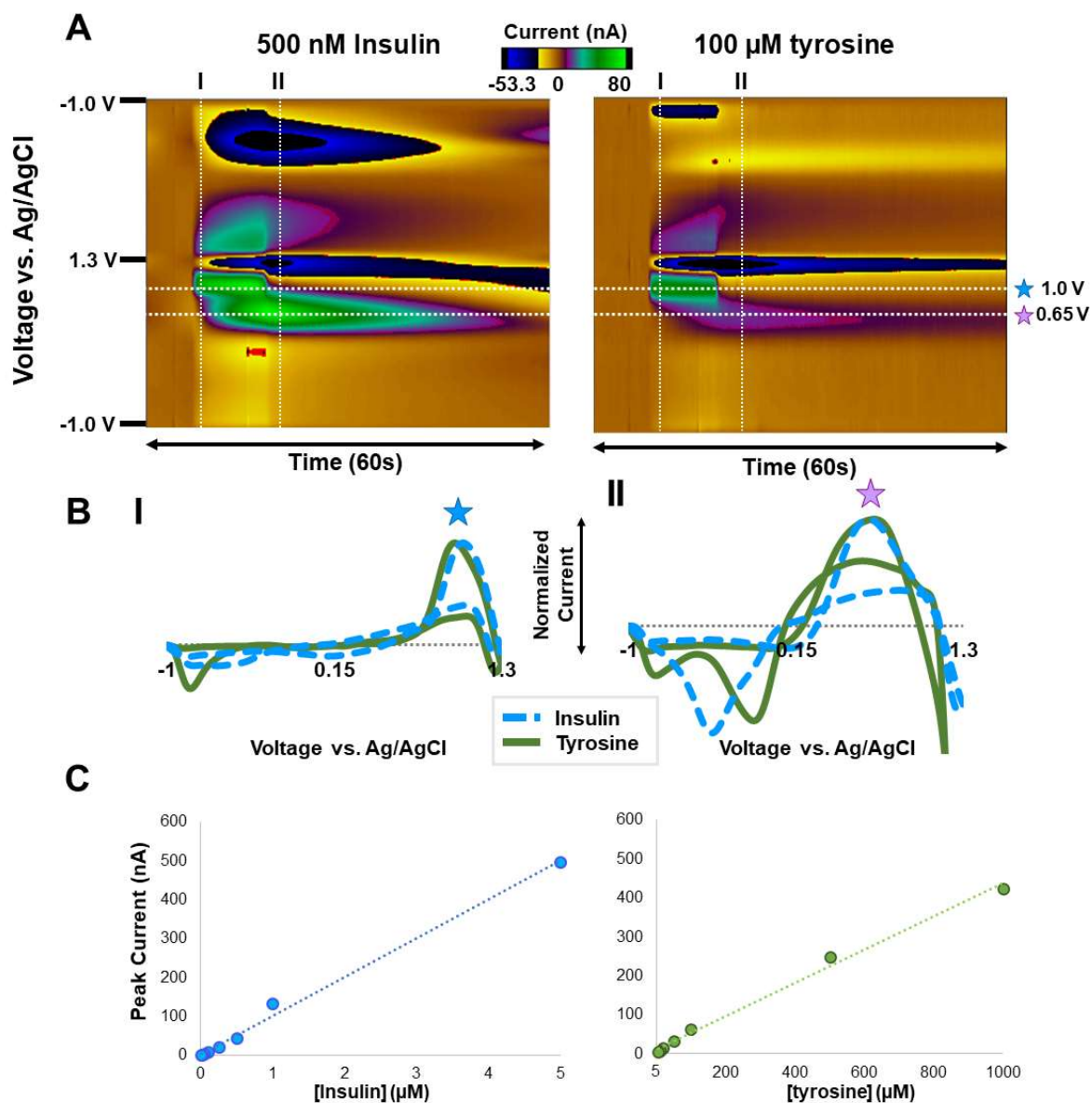


Figure 6.11. The mechanism of insulin redox is due to oxidation of its four tyrosine residues. (A) Color plots of 1 μ M insulin and 100 μ M tyrosine 10 second bolus injections to a cylindrical CFM using FIA. The primary oxidation peaks are denoted by the blue star at 1.0 V and the secondary oxidation peaks by the purple star at 0.65 V. (B) CVs of insulin (blue dashes) and tyrosine (green) overlaid with current normalized to the primary (I, blue star) and secondary (II, purple star) oxidation potentials. The CVs are taken from the vertical lines at different time points in the color plots in panel A (C) Calibration curves of insulin and tyrosine in Krebs's buffer.

Table 6.2 Metrics for the calibration curves in Figure 6.11C.

	slope (nA/ μ M)	R^2	LOD (μ M)	LOQ (μ M)
Tyrosine	2.33 ± 0.08	0.994	19.84	66.13
Insulin	0.00998 ± 0.0003	0.994	17.29	57.63

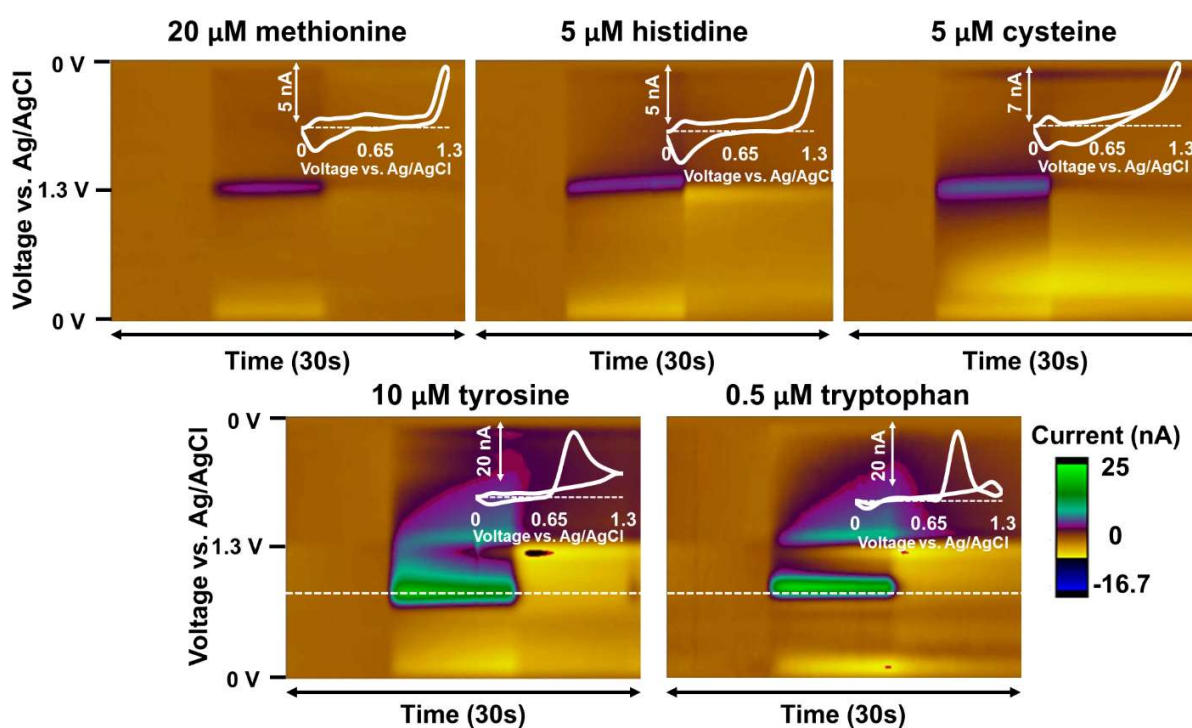


Figure 6.12. CFM response to electroactive amino acids on the insulin-optimized waveform. Representative color plots of 10-second bolus injections of 20 μ M methionine, 5 μ M histidine, 5 μ M cysteine, 10 μ M tyrosine and 0.5 μ M tryptophan at cylindrical CFMs in PBS. Inset CVs are taken in the middle of the injection event.

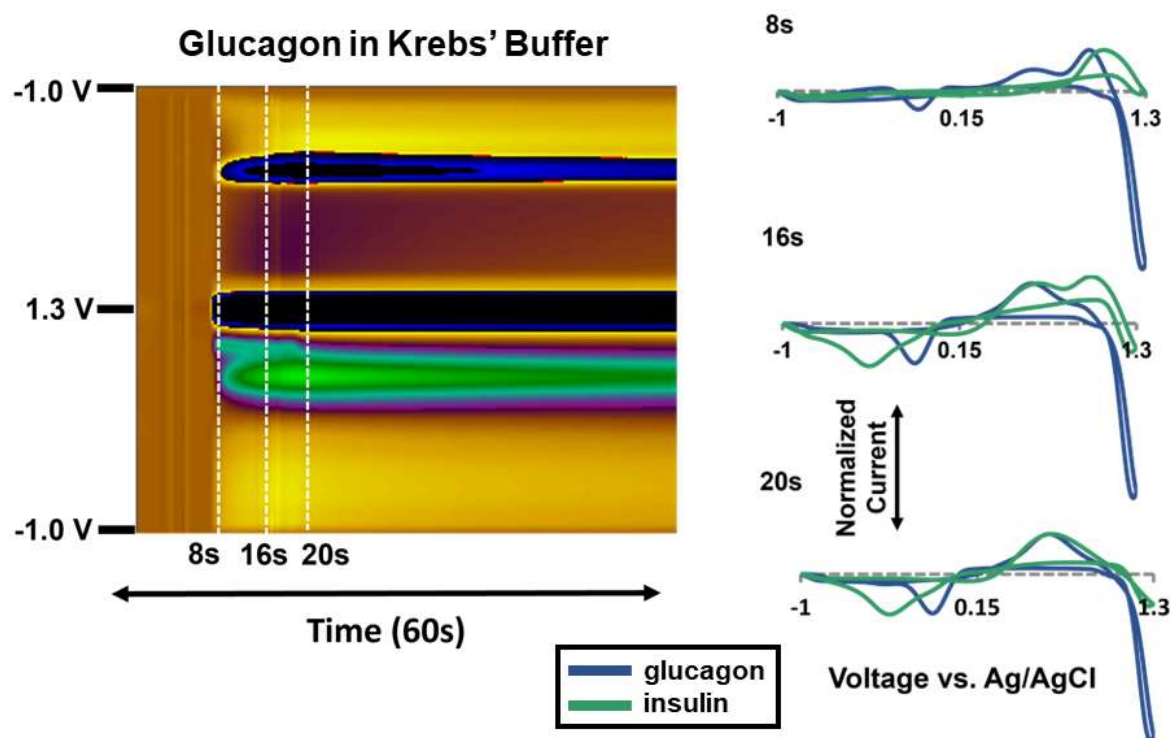


Figure 6.13. Glucagon in Krebs' buffer has a similar electrochemical signature to insulin. (A) A representative color plot from a bolus injection (FIA) of glucagon (60 nM) in Krebs' buffer at a CFM using the extended insulin waveform (-1 V to 1.3 V to -1 V at 400 V/s). (B) CVs taken at different time points from the color plot in panel A. The currents are normalized and CVs from the glucagon injection (green) are overlaid with ones taken at the same time points from a 1 μ M insulin injection (blue)

Discussion

Insulin signalling is dysregulated in the pathophysiology of diabetes mellitus, and recent reports have shown serotonin to be co-released with insulin from beta cells, acting as a regulatory messenger on alpha cells. Measurement techniques, including electrochemical sensors, fail to selectively capture insulin signalling in real time. In this work we present novel FSCV methodologies for insulin and serotonin co-detection at carbon fiber microelectrodes. Waveform parameters were altered between cylindrical and bevelled CFMs for optimal insulin detection however the serotonin signal was convoluted by the secondary insulin peak, making it unsuitable for co-detection. We characterized the performance of both sensors in terms of sensitivity, selectivity and stability for insulin and serotonin. Finally, we applied our sensors to insulin-secreting biological systems for the first real-time measurements of insulin release with FSCV.

Our data coincides with previous findings that the electroactivity of insulin is due to the oxidation of its four tyrosine residues. We found CFMs to be much more sensitive to insulin (nM range) than tyrosine (μ M range), which is not surprising as 4 tyrosine residues contribute to the signal. However, the 233-fold difference in sensitivity towards insulin over tyrosine (Figure 6.11), indicates that other mechanisms might be contributing to the sensitivity towards insulin. In addition to tyrosine there are 4 other amino acids that are electroactive: methionine, cysteine, histidine and tryptophan. Weese-Myers *et al.* demonstrated the current response towards all 5 electroactive amino acids at CFMs using FSCV, where cysteine, histidine and methionine all exhibited non-Faradaic current peaks at the switching potential¹⁸⁷. We also observed non-Faradaic switching peaks for these amino acids using the insulin-optimized waveform (Figure 6.12), ruling out the contribution of these residues to our Faradaic current response. We postulate that the combination of tyrosine with the other electroactive residues could contribute to the enhanced response to insulin, or insulin has enhanced adsorption to the CFM surface compared to free tyrosine in solution.

Altering the holding potential for the final waveform optimization demonstrated nuances of the redox mechanism of insulin. The initial potential sweep between -1 V and 1.3 V resulted in three distinct peaks on the CV: the primary oxidation peak at 1.0 V, a secondary peak at 0.65 V and a reduction peak at ~0.5 V. We opted to maintain the peak potential at 1.3 V to promote carbon surface renewal, and scan rate studies clearly showed 400 V/s is optimal based on the current response. Then, as the secondary oxidation peak overlaps with serotonin, we altered the holding potential to exclude this peak from the insulin signal. A holding potential of 0 V at a NafionTM CFM resulted in enhanced current response and an isolated primary oxidation peak for insulin. It is well-documented that NafionTM enhances the CFM response to serotonin, however it is less clear as to why NafionTM, a negatively charged polymer, enhances insulin currents while excluding the positively charged radical ($Y^{\bullet+}$) formed from the initial oxidation of insulin¹⁹⁷. The rationale for this is evidenced by the stability curves at different holding potentials (-1 V and 0 V), with and without NafionTM at cylindrical and bevelled electrodes. The insulin signal was most stable at bare CFMs when holding at -1 V (~6% signal loss), where the secondary peak was present and clearly oxidized off the surface at bevelled and cylindrical electrodes. Then, when both electrode types were coated with NafionTM and held at 0 V, the secondary peak was excluded from the color plot, but there was also significant current loss over time (bevelled: -87% over 2 hours and cylindrical: -51% over 3 hours). It is likely that the NafionTM attracts insulin to the carbon surface to enhance the current response, and after the primary oxidation occurs the by-product then adsorbs to the NafionTM rather than the carbon surface. Then, with each waveform application, the NafionTM - $Y^{\bullet+}$ is stripped from the surface, revealing more active sites for insulin to adsorb to. The proposed oxidation mechanism for tyrosine is outlined in Figure 6.14. The faster current decay at bevelled vs. cylindrical electrodes is due to the disparity in their surface areas. Bevelled electrodes have approximately 110 μm^2 of exposed carbon compared to the 2400 μm^2 cylinders cut to 150 μm have, a 22-fold discrepancy. Therefore, for the co-detection of serotonin and insulin at CFMs, a thin layer of NafionTM is needed to mitigate surface fouling of the positive radical formed from insulin oxidation.

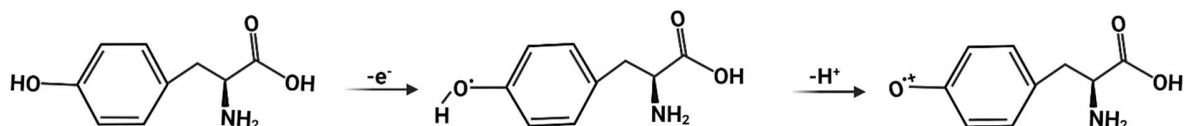


Figure 6.14. Proposed oxidation mechanism of tyrosine at CFMs. Initially, an electron is lost, forming a radical species, which is designated in this work as the primary oxidation peak on the CVs. Then, a hydrogen ion is lost (secondary oxidation peak), leaving a positively charged radical to adsorb to the carbon surface. Made with Biorender.com.

We observed biological insulin releases, spontaneous and glucose-stimulated, at cylindrical CFMs from INS-1 cells and pancreatic islets. However, non-Faradaic current fluctuations accompanied the Faradaic current spikes, which indicates an unstable background current. For INS-1 and islet measurements, the cylindrical CFM was positioned at an angle ($\sim 30\text{--}45^\circ$) so that the lowermost point was a few microns above the cells. The angle of the cylinders to the cells created a variable diffusion distance to the electrode. When analytes adsorb to and oxidize at the carbon surface, this alters the background current from the initial subtraction, and non-Faradaic currents appear in the color plots most often at the switching potentials. According to Fick's second law of diffusion, the current flux is related to the distance an analyte crosses to the electrode surface. When analytes reach the electrode surface at different time points this causes constant changes in the capacitive background resulting in significant non-Faradaic current flux. In sum, the variable diffusion distance induces the interfering non-Faradaic current flux in the color plots from cells. To mitigate this issue, we opted to fabricate CFMs bevelled at a 45° angle so that the planar carbon surface is at a fixed distance from the point of release.

During the characterization of insulin and serotonin at bevelled CFMs with the optimized insulin waveform (0 V holding potential), we worked through several measurement challenges. The oxidation peak for

insulin was lost at bevelled CFMs, leaving only a switching peak on the color plot, but was regained when a wide potential window (the polymer waveform¹⁰¹) was applied prior to cycling on the insulin waveform. Adsorption of electroactive species is promoted by the microroughness of the carbon surface, which is lost during the bevelling process. The application of the polymer waveform electrochemically etched the carbon to reestablish a rough surface for insulin adsorption. Then, repeated injections of insulin over a 2-hour window resulted in a significant loss in current when holding at 0 V and a stable response when holding at -1 V. From this, the limited surface area of these electrodes requires the positive tyrosine radical to be oxidized off the surface. However, the secondary oxidation peak overtakes serotonin. Future work in co-detecting insulin and serotonin at bevelled electrodes (holding at -1 V) may require the secondary oxidation peak to be mathematically deconvoluted from the signal.

Several salt buffers were used throughout this work: Krebs', PBS and HBSS. Krebs' buffer is used for calcium imaging of islet cultures, however large negative switching peaks can be seen in all color plots and CVs taken in Krebs' buffer. The extreme switching peaks were observed for insulin, tyrosine and serotonin measurements. For Krebs' buffer, the pH is maintained by HEPES, a buffering molecule that is utilized in salt buffers for cellular measurements as it promotes ATP signalling and is not cytotoxic. When adding HEPES to PBS the negative switching peak was present, and when HEPES was removed the peak was eliminated color plot, evidenced in Figure 6.5. As this phenomenon was not found in any of the other work in this thesis (chapters 4 and 5) where we added 10 mM HEPES to HBSS for histamine and serotonin FSCV measurements where the waveform was scanned up to 1.1 V, we concluded that HEPES must interfere with the CFM response when scanned up to 1.3 V. For the remainder of the work, we used HBSS without any additive buffering molecules, which resulted in normal switching peaks associated with normal FSCV processes.

Some initial work was performed to investigate potential interferents. The islet is comprised of several cell types: ~60 % beta, ~20% alpha and the remaining delta, gamma and epsilon cells²⁰⁰. Based on the cellular

makeup of the islet, the main interferent of concern for insulin detection is glucagon released from alpha cells. Glucagon is a small peptide hormone comprised of amino acids, including tyrosine and tryptophan²⁰¹. As glucagon has tyrosine and tryptophan residues, we indeed observed that it oxidizes at similar potentials to insulin when applying the -1 V holding potential (Figure 6.14). Despite overlapping oxidation peaks, glucagon reduces at a higher potential (-0.2 V) and, physiologically, insulin and glucagon are released at different blood sugar conditions so it isn't expected that alpha and beta cells will be active at the same time. In addition to glucagon, somatostatin (delta cells) and pancreatic polypeptide (PP, gamma cells) are potential interferents to detecting insulin from islets. However, the cell population of both types are less than 10% of the islet makeup, and based on the low islet signals observed in this work it is likely that they won't be released in large enough quantities to be detected at the CFM. Future applications of this technology to more complex tissues such as the brain or pancreas should consider additional compounds that could hinder insulin and serotonin detection.

Conclusion

With this work, we present novel FSCV methodology for the real-time co-detection of insulin and serotonin at CFMs. A waveform was first optimized for insulin and serotonin at cylindrical CFMs, where a neutral holding potential and the use of NafionTM as a sacrificial polymer were integral in excluding an interfering by-product of insulin oxidation for peak-resolved serotonin and insulin measurements. Cellular insulin release from INS-1 and pancreatic islets was detected at cylindrical CFMs using the newly optimized waveform. However, interfering non-Faradaic current fluctuations at cylindrical CFMs led us to fabricate bevelled electrodes to mitigate the issue. Characterization of insulin at bevelled CFMs showed that a negative holding potential was needed for stable and sensitive current responses, reestablishing the presence of insulin's secondary oxidation peak. Future work will require mathematical intervention to either subtract the secondary oxidation peak to reveal serotonin oxidation, or data filtering to mitigate the capacitive current fluctuations at cylindrical CFMs. Regardless, we present a robust FSCV protocol to probe

insulin and serotonin secretion in real-time. This is the first qualitative, quantitative, and most temporally resolved detection method for insulin, which can be adapted for *in vivo* applications such as the brain or within the pancreas.

Chapter 7 Summary and Future Work

Serotonin and histamine are both implicated in the aetiology of depression. However, their respective roles in the pathophysiology of this disease are not well characterized. As such, the lack of peripheral biomarkers for depression has sparked debate as to the validity of serotonin and histamine in the physiology of depression. In previous works, we have used FSCV to probe serotonergic signalling in the brain in the context of depression, notably finding that serotonin is indeed affected by antidepressant pharmaceuticals^{22, 85} and ambient serotonin is reduced in animals that exhibit depressive phenotypes⁹¹. From these works we have shown that serotonin is not to be excluded in depression. Instead, we hypothesize here, rather, that the theories of depression are synergized by both the immune system and HPA axis. To support this idea, in this dissertation, we probed biological models of serotonin and histamine release throughout the body (skin, hair, brain and pancreas) that share an immune/HPA axis with the brain.

In chapter 1 I discussed how the physiological interconnectivity between the serotonin, cytokine and neurogenesis hypotheses of depression lie with the immune system and HPA axis. These hypotheses are thought to be separate, and researchers design their studies to usually support one. Future experimentation should rather be geared instead towards finding links between the three schools of thought *via* measurements in peripheral systems that share an immune and/or HPA axis with the brain.

The general methods in chapter 2 delineated several important theories underlying the experimental procedures used throughout this thesis. FSCV is a niche electrochemical technique that is difficult conceptually and experimentally. The adaptability of CFMs in terms of dimensions, shape and surface coatings has made them an idyllic choice for probing biological systems using FSCV. To expand the boundaries of electrochemical sensing, it is imperative that new carbon sensors be fabricated so that *in vivo* measurement can be made in peripheral systems such as the skin and endocrine system.

In chapter 3, I configured a new portable FSCV hardware system for a niche collaboration in Belgium where I made the first histamine measurements in tissue slice preparations. I found that the peak potential of histamine oxidation significantly downshifts in an oxygenated buffer required for tissue measurements. Then travelled to Belgium to set up an FSCV system in our collaborator's lab where I made the first histamine measurement in tissue slice preparations. With this work we offer a protocol in which high-histamine measurements can be made in the brain. Future studies geared toward understanding histaminergic dysfunction in the brain should indeed use our protocols for probing the histamine's involvement in psychiatric illnesses with origins of dysfunction within the striatum.

In chapters 4 and 5, in collaboration with the Higgins' lab, I used FSCV to identify and characterize neurochemical release from human hair and skin cells. Previously, neurochemicals were postulated to be signalling molecules within the skin and hair follicle, but our work showed the first direct evidence of serotonin and histamine release. We determined that serotonin and histamine were the chemical mediators of tactile information from the outside world to sensory neurons in the brain. This work led us to also show the skin and hair cells spontaneously release serotonin and histamine, and we extensively explored this in dermal fibroblasts. These works show the power of FSCV and uncovered important chemical signalling mechanisms within human skin and hair cells. Future studies should be geared towards understanding the differences in hair follicle and skin signalling between patients with psychiatric illnesses and those without. The implications of uncovering variations in skin/hair signalling could lead to the first peripheral biomarker for depression and ultimately be used a patient-specific indicator of pharmaceutical efficacy. Building on this work, future studies should be designed to build data sets to show the chemical signalling of skin and hair cells alone and in co-culture. Ultimately, building up how the cells signal together will give a better understanding of *in vivo* signals from the skin. Additionally, far into the work, it would be interesting to determine chemical-based thresholds to diagnose depression from both a network of

neurochemical signalling data in the skin of depressed and non-depressed patients and personalized thresholds.

Finally, I expanded the scope of FSCV and offered a new method to the diabetic research community in chapter 6 with the development of FSCV methodology for real-time insulin and serotonin co-detection. Previously, measurement techniques have failed to directly capture insulin signalling in real-time, leaving a large gap in the diabetic research community. However, I adapted FSCV parameters to co-qualify and quantify insulin and serotonin oxidation a single measurement with high temporal resolution. Additionally, I demonstrated that FSCV can detect insulin release from biological models of insulin secretion. The measurement of insulin with FSCV has the potential to be transformative in diabetes research. A gap in research for diabetes has been in characterizing the functionality of stem cell-derived beta cells, where their transplantation in type 1 diabetic patients could be curative. Applying our FSCV paradigm to beta cells would allow researchers to identify functionality along the maturation pathway. Additionally, the expansion of FSCV towards other hormones of interest in diabetes such as pancreatic peptide, glucagon, glucagon-like peptide 1 and glucose-dependent insulintropic polypeptide would lead to a total physiological picture of glucose-dependent signalling in the endocrine system. A reasonable future goal for this work would be to make insulin detection with FSCV more accessible. Currently, the hardware, CFM fabrication and software for FSCV require expertise to utilize and fabricate. However, for insulin detection with FSCV it would be most useful to many diabetic researchers. Therefore, the development of an 'FSCV kit' or widespread education and start-up materials would be beneficial to expand the technology to those characterizing insulin pulsatility in the context of diabetes.

In total, this work contributes important knowledge to the research fields of neurochemistry, FSCV, skin and diabetes and lays the foundation for future work probing disease pathologies where serotonin and histamine are dysregulated. We pushed measurement boundaries in new (to FSCV) biological models of serotonin and histamine signalling to expand our understanding of physiological connections within the

context of depression. These models can later be probed for their response to various stimuli relevant to perceived touch. With the work presented in this dissertation along with the accessibility to human cells in conjunction, we are making strides towards personalized, high throughput studies to understand peripheral serotonin release in the context of depression.

In the future, the continuation of this work should be geared towards developing devices to culture and probe primary and stem cell-derived cells for personalized medicine applications. A plethora of 'on-a-chip' devices have been published in recent years, where cells and tissues are placed on a fluidic platform (micro- or macro-) as external physiological models. To bring the central hypothesis of this work together, the co-culture comprised of peripheral endothelial, endocrine, immune and neuronal cells on a microfluidic device would provide a useful physiological model to probe serotonergic dysfunction in depression. Indeed, the future development of fluidic devices with FSCV capabilities would not only make FSCV more accessible, but also generate platforms for high throughput studies with applications to any disease with a redox-active biomarker.

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