This work is dedicated to my father, Ravi Atmaramani, and mother, Sonia Atmaramani
DEVELOPMENT OF AN IN VITRO PHENOTYPIC ASSAY FOR SCREENING CHRONIC PAIN THERAPEUTICS

by

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Dorsal root ganglia (DRG) are a collection of first order sensory neurons involved in the perception of innocuous as well as noxious stimuli, and the conduction of corresponding signals to the spinal cord. Upon suprathreshold stimulation, sensory fibers from the DRG are depolarized, transmitting signals originating in the periphery in the form of all-or-nothing action potentials to be processed by the central nervous system. The DRG play a critical role in the manifestation of chronic pain, a disorder affecting 100 million people throughout the US. Enhanced DRG excitability is a hallmark feature of chronic pain, which can be induced by exposure to cytokines in vivo and modeled in vitro. Currently, there is a critical technology void in our ability to screen for potential chronic pain therapeutics. Phenotypic screening approaches that focus on cell behavior endpoints such as excitability have been previously limited to single cell measurements (e.g., patch clamp) or extracellular recording from cultured embryonic tissue. Furthermore, unlike the in vivo scenario, these in vitro approaches do not spatially segregate axonal processes from cell bodies and, therefore, increasingly rely on cell soma responses and do not allow selective axonal manipulation. Our novel approach will leverage a combination of
adult mouse DRG neurons cultured on multi-well microelectrode arrays (MEAs) with specially fabricated microchannels to allow long-term monitoring of phenotypic activity from spatially and chemically segregated DRG cell bodies and axons. To date, the only related prior effort has focused on embryonic derived DRG cultures, which require neurotrophic factors to maintain viability in culture. These factors (e.g., nerve growth factor) are known to play a role in inflammatory pain by causing sensitization through the increased expression and trafficking of transient receptor potential cation channel subfamily V member 1 (TRPV1). Therefore, there is a need for alternative, more physiologically relevant in vitro models for the study of sensitization of DRG neurons. Firstly, we developed and described methodologies for the culture of spontaneously active adult DRG sensory neurons on MEAs. The spontaneous and stimulus evoked activity is characterized over chronic time periods in vitro which are consistent with pharmacological interventions in the context of a screening paradigm. Additionally, we demonstrate that the observed activity can be modulated with inflammatory cytokine interleukin-6 (IL-6), giving rise to persistent hyperexcitability in vitro. As a next step, to enable translation as a high-content screening (HCS) platform an assay quality indicator, such as the Z’-factor was determined. A robust version, which leverages the insensitivity of parameters such as the median and median average deviation in the context of extracellular electrophysiological recordings from DRG neurons is developed. Additionally, a hit detection methodology is assessed based on putative compounds to assess our ability to identify true hits in the assay. Lastly, using a PDMS-based microfluidic culture system, we illustrate the utility of compartmentalized cultures for the study of the axonal microenvironment independent of the cell body.
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CHAPTER 1
INTRODUCTION

1.1 Vision

Chronic pain is a complex and challenging medical and public health concern affecting 100 million Americans and representing an estimated cost of $560-635 billion/year (Mills et al. 2016). Pharmacological management of pain has predominantly relied on the use of Nonsteroidal anti-inflammatory drugs (NSAIDS) and opioids. In spite of their perceived efficacy, opioid abuse which is comprised of clinical dependence, addiction, and severe side-effects (Sehgal et al. 2012; Mills et al. 2016), is now considered a national epidemic. Therefore, there is a critical and unmet need to develop and implement novel non-opioid therapeutics that ameliorate pain with increased efficacy and reduced side effects.

Since the completion of the Human Genome Project in 2003, significant advances have been made in genomics and molecular biology, enabling elucidation of pathological mechanisms by identifying associated specific genes and the corresponding proteins. This mechanistic approach, termed target-based drug discovery, has led the design of drugs that are specific to molecular targets, which play a role in disease pathogenesis. However, prior to a target-based approach, phenotypic screening was used commonly to identify novel compounds in cell-based assays or animal models that modulate a specific cellular or organismic behavior (Szabo et al. 2017). In contrast to target-based approaches, phenotypic screening does not require a hypothesis concerning a specific biological target and instead considers complex cellular-level behaviors, which are central to pathology. Interestingly, for neurodegenerative disorders, phenotypic
screening assays have proved more successful in recent years in the drug discovery process compared to target-based approaches, with examples including Memantine for the treatment of mild to moderate Alzheimer’s disease and Varenicline for nicotine addiction (Swinney and Anthony 2011). Despite detailed understanding of mechanistic pathways and identification of targets, the efficacy of target-based approaches has been limited, thus driving renewed interest at phenotypic assays (Pruss 2010). The present study develops and demonstrates a cell-based phenotypic screening assay using adult dorsal root ganglion (DRG) sensory neurons cultured on microelectrode arrays (MEAs) for screening novel therapeutics for the treatment of chronic pain using neuronal excitability as a phenotypic end point.

The innovation of this research is the use of adult tissue rather than embryonic-derived tissue, quantifiable alterations in neuronal excitability with inflammatory cytokines associated with inflammatory chronic pain in vivo, proof of concept screening of analgesics with known mechanisms of action, and extension of this system towards compartmentalized cultures allowing selective axonal and somal manipulation.

1.2 Significance

1.2.1 Public Health Significance

Chronic pain is a complex and challenging disorder, affecting 30% of the United States population and representing an estimated cost of $560-635 billion/year (Ashburn and Staats 1999; Mills et al. 2016). The condition affects the physiological and cognitive functioning of patients and decreases their quality of life. The situation becomes dire for aged populations with 75-85% of elderly in U.S. care facilities suffering from chronic pain (Kaye et al. 2010). Current pharmacological management relies on opioid analgesics, with 58 opioid prescriptions written
for every 100 Americans, making it the most commonly prescribed class of medication for the treatment of chronic pain. (Sehgal et al. 2012). However, pharmaceutical opioids have been widely associated with drug overdose deaths and a parallel increase in addiction, which poses serious concerns of its use as a viable therapeutic approach (Ballantyne and LaForge 2007; Sehgal et al. 2012; Volkow et al. 2017). Therefore, there is an urgent and unmet clinical need to develop novel therapeutics with increased efficacy and reduced side effects. Our central goal is to explore the development and implementation of a phenotypic based drug screening approach to enable screening and potential discovery of alternative, non-opioid treatments for chronic pain.

1.2.2. Phenotypic Assays as Basis for Drug Discovery

Prior to the introduction of target-based drug discovery in the 1990s, physiological and phenotypic assays were the foundation of pharmaceutical drug discovery. With the completion of the Human Genome Project, significant advances were made in molecular biology and genomics, enabling the study of the molecular basis of pathologies. As a result, this led to the widespread adoption of target-based approaches, allowing rational design of therapeutics and offering a platform to test their effects on a specific and isolated drug target that is directly implicated in the pathology. Unfortunately, the yield of this strategy in developing novel drugs has been limited despite an increase in pharmaceutical R&D investments (Sams-Dodd 2005, 2013; Paul et al. 2010). Interestingly, during a time where target-based approaches have dominated the vast majority of drug discovery programs, there has been a renewed interest in phenotypic strategies, as they have been responsible for more first-in-class small molecule drugs approved by the U.S. Food and Drug Administration (FDA) (Swinney 2013; Zheng et al. 2013). The critical difference
between target-based and phenotypic methods is based in the cell behavior related metrics, with phenotypic methods relying on measures that are integrative in nature such as structural morphology and biochemical characteristics (neurite outgrowth, protein expression, cell death etc.). In contrast, target-based strategies often involve isolated targets and lack the physiological context of phenotypic screens. An ideal drug discovery platform should incorporate relevant physiological processes associated with the pathological condition. This is especially true for complex diseases such as chronic pain, where numerous signaling pathways and processes are involved. Another significant advantage of phenotypic screens is that they do not require prior knowledge or a specific hypothesis regarding the drug’s target, overcoming a major disadvantage of target-based strategies. In the present proposal, we focus on the development and demonstration of a phenotypic screening paradigm, which leverages the bioelectrical activity of nociceptive neurons, specifically neurons derived from the DRG, for screening therapeutics for the treatment of chronic pain.

1.3 Innovation

Our novel approach will leverage a combination of murine derived adult mouse DRG neurons cultured on multi-well MEAs and specially fabricated microchannels to allow long-term monitoring of phenotypic activity from spatially and chemically segregated DRG cell bodies and axons. With adult DRG neuron excitability as a high-content phenotypic end point, we will demonstrate sensitization of these neurons with known inflammatory cytokines, screen against putative compounds to assess the overall assay quality, and extend this paradigm to compartmentalized structures to elucidate axonal versus somal contribution to hyperexcitable states in this model.
1.3.1 **DRG Neuronal Excitability as a Phenotypic end point**

The DRG are a cluster of sensory neuronal cell bodies that have both a central axonal branch and a peripheral branch that innervates target tissue such as muscle, skin, and viscera (Basbaum et al. 2009). A subpopulation of DRG neurons, termed nociceptors, detect specific modalities of sensory information such as thermal, mechanical, or chemical stimuli and reliably transfer them to the central nervous system in the form of all-or-nothing action potentials (Woolf and Ma 2007). The type of stimulus that is transduced, the speed and fidelity with which it is conducted, and transmitted depends on the type of sensory neurons. There are two major types of sensory neurons. The first class are medium diameter myelinated neurons (Aδ) which respond to and mediate acute or “fast” localized noxious stimuli. Aδ fibers differ from large diameter myelinated and rapidly conducting Aβ fibers that encode innocuous mechanical stimulation. The second class of sensory neurons are slow conducting C-fibers called nociceptors which sense poorly localized “second” or slow pain.

Under normal physiological conditions, nociceptor DRG neurons are “quiet” and exhibit low spontaneous bioelectrical activity where exposure to noxious stimuli triggers bioelectrical activity. However, the process of peripheral sensitization, which may be mediated by pro-inflammatory cytokines secreted at the site of injury or caused due to disease, leads to phenotypic plasticity of these cell types and increased intrinsic spontaneous and evoked activity (Woolf and Salter 2000; Davidson et al. 2014; Dib-Hajj 2014; Djouhri et al. 2015). These maladaptive changes in excitability contributes, at least in part, to the establishment and maintenance of chronic pain states (Waxman 1999). Peripheral sensitization is a complex process involving modulation and modification of molecular pathways, transducer receptors/ion
channels, and neighboring cells. Ultimately, the increased neuronal plasticity is mainly due to dysfunction of receptor/ion channels or associated regulatory proteins, leading to alterations in intrinsic biophysical properties or cell-surface expression of channels. Several ion channels such as sodium channels, voltage gated and two-pore potassium channels, hyperpolarization-activated cyclic nucleotide-gated channels, and voltage gated calcium channels have been implicated to undergo dysregulation due to sensitizing agents at the site of injury. Recent studies have also demonstrated that satellite glial cells, which envelope individual sensory neurons, may contribute to hyperexcitability via intracellular coupling mediated at gap junctions (Dublin and Hanani 2007). Therefore, to establish a more relevant physiological context for a phenotypic screening platform would benefit from a stable culture of both sensory neurons and satellite glial cells.

1.3.2. Extracellular Recording as the Basis of High Content Screening

Electrophysiological recordings of excitable tissue is a well-established method to study the functional and phenotypic outcome of a wide range of neurotransmitter modulators and neurotoxins (Knaack et al. 2013; Schmidt et al. 2013; Charkhkar et al. 2014a; Black et al. 2017). Patch clamp is the gold standard technique used in neuropharmacological studies, where the effect of pharmacological compounds can be determined on single ion channels, receptors, and other bimolecular processes. Although there are high-throughput patch clamp techniques, these are usually limited to the study of single ion channels, which are artificially expressed in cell lines (Dunlop et al. 2008). In contrast to patch clamp, extracellular recording using MEA technology relies on the detection of extracellular action potentials by small, typically metallic, electrode sites in close contact with excitable cell bodies and/or processes. From the pioneering work of Gross and Pine (Gross et al. 1977, 1985; Pine 1980), MEA based systems have been used extensively to
make electrophysiological measurements from neuronal preparations *in vitro* and has become the basis of biosensors (Gross et al. 1995; Pancrazio et al. 1999, 2003; Selinger et al. 2004; Martinoia et al. 2005), neuropharmacological applications (Pancrazio et al. 2001; Xiang et al. 2007; Novellino et al. 2011), and for the fundamental study of neuronal networks (Selinger et al. 2004). Typically, embryonically derived biological preparations form intact neuronal networks *in vitro*, giving rise to all-or-nothing extracellular action potentials. The biophysical principle governing extracellular action potentials is well understood and depends on current sources and sinks in proximity to microelectrodes consistent with volume conductor theory (Heinricher 2004). The occurrence and ability to resolve more than one characteristic waveform on a single recording microelectrode sites is not uncommon, where differences in the amplitude, shape, and time course of waveforms can be used to distinguish units that correspond to putative single active neuronal sources (Humphrey and Schmidt 1977).

MEAs offer a platform from which spontaneous and evoked activity can be readily acquired and monitored in a non-invasive, long-term, and label free manner from interconnected neuronal networks *in vitro* from multiple microelectrode sites simultaneously (Pancrazio et al. 2001). Measurement of mean firing rates, bursting activity, and correlated activity between units can be used as the quantitative basis for analysis and serves as a high-content format of data acquired from MEA recordings. Previously, MEA devices have been considerably low throughput, available in the form of single wells with only 32-64 microelectrode sites, compared to 384-well formats used for intracellular calcium measurements as an endpoint (Stacey et al. 2018a). With the advent of novel microfabrication techniques, visualization/analysis software, and data acquisition technology, MEAs have been extended to multi-well systems with 768 electrodes divided across
12, 48, or 96-well plates allowing for much higher throughput for pharmacological studies. For pharmaceutical ventures in drug discovery, these systems have influenced the way in which cardiac toxicity assays are performed \textit{in vitro} (Qu and Vargas 2015; Rast et al. 2016; Zhang et al. 2016; Abi-Gerges et al. 2017). In the present proposal, we have successfully cultured murine derived adult DRG neurons on multi-well MEAs. Fig 2. Illustrates spontaneous activity from adult DRG neurons in multi-well format of MEAs where at least 40% of the 768 electrodes show distinguishable spiking activity with an excellent signal to noise ratio.

1.3.2 Importance of Adult DRG as a Tissue Source for Screening

For neural tissue preparations on MEAs, embryonically derived neuronal cultures have been used extensively in prior studies (Gross et al. 1985). A recent study exploring the feasibility of culturing DRG neurons on MEAs relied on embryonic sources and demonstrated emergence of spontaneous activity \textit{in vitro} (Newberry et al. 2016a). However, the use of embryonic preparation as a tissue source from a phenotypic assay standpoint for peripheral pain is problematic for numerous reasons. First, to maintain viability in culture, embryonic DRG neurons require considerably high doses of nerve growth factor (NGF, 100 ng/ml), which have been shown to cause sensitization in adult DRG neurons (Winston et al. 2001; Tsantoulas et al. 2013). Therefore, embryonic tissue may not be an amenable source to study the effects of NGF induced sensitization mechanisms. Second, the expression profiles of integral sodium ion channel subtypes such as Nav1.7/Nav1.8 known to play a role in several modalities of chronic pain differ significantly among adult and embryonic tissue. Other transcriptional differences include mRNA associated with axonal transport, vesicle trafficking, and axonal protein synthesis (Gumy et al. 2011). The important feature in our present proposal is the reliance on adult murine DRG neurons as the source of excitatory tissue to establish
a more physiologically relevant context for a phenotypic screening assay as it relates to chronic pain.

1.4 Overview and Objectives

The research strategy is aimed to leverage the use of adult DRG tissue on MEAs to develop and demonstrate an *in vitro* DRG cell-based screening assay. We first hypothesized that DRG neurons derived from adult (4-6 weeks old) mice cultured on MEAs will enable stable and non-invasive recording of extracellular action potentials from a largely nociceptive population. A stable and persistent baseline level of spontaneous activity allows the acquisition of an end point that is relevant to pathology (excitability) and long-term recording is advantageous for pharmacological intervention in a screening paradigm. We then asked if baseline level of spontaneous activity can be modulated by relevant agonists and increased to a hyperexcitable state by known inflammatory mediators. Sensitization of DRG neurons by inflammatory mediators is a hallmark of chronic pain, and the ability to recapitulate a hyperexcitable phenotype *in vitro* is central to implement a physiologically relevant context for screening. Next, we asked what quality metric can be applied to the current assay format to interpret the overall performance of the assay to detect positive “hits” or compounds which reduce the observed hyperexcitability. A statistical parameter is required to evaluate assay performance in terms of the signal dynamic range (difference between the sample signal and negative control) and the associated variance across experimental wells to guide reproducibility, reliability, and robust screening. Lastly, we hypothesized that peripheral axons may play a direct role in DRG sensitization and ask under what conditions can axonal processes be chemically and physically isolated from the soma to allow distinct interrogation of the axonal component. The key questions and hypothesis will be achieved by a series of objectives. In
Objective (1) adult mouse derived DRG neurons are cultured in vitro on a multi-well MEA platform to recapitulate spontaneous and/or evoked responses to known inflammatory mediators involved in chronic pain. Integral to this, a stable baseline level of spontaneous activity will be demonstrated from DRG cultures on MEAs from which activity can be modulated in response to inflammatory cytokines and pain mediators. Sensitization caused by incubation with inflammatory cytokines such as IL6, NGF, and PGE2 will be tested in terms of changes in spontaneous activity and evoked responses to known chemical agonists (capsaicin and bradykinin) and/or response to temperatures of noxious range (42°C). Objective (2) focuses on the assay’s ability to detect positive hits from analgesic compounds known to attenuate hyperexcitability and to subsequently calculate a $Z’$-factor to validate the quality of the assay. The $Z’$-factor is a dimensionless quantity, which describes the overall quality of an assay and does not require the use of test compounds. Therefore, it will serve as a statistical parameter to evaluate the overall performance of the assay and guide the reproducibility and reliability of the assay. Objective (3) will extend this paradigm to a compartmentalized microfluidic culture system that allows spatial and chemical separation of somal component and associated axonal processes, to selectively sensitize and stimulate nociceptive axons and elucidate their role in hyperexcitability as it pertains to assay configuration.
References


CHAPTER 2

ADULT MOUSE SENSORY NEURONS ON MICROELECTRODE ARRAYS EXHIBIT INCREASED SPONTANEOUS AND STIMULUS EVOKED ACTIVITY IN THE PRESENCE OF INTERLEUKIN-6*

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Abstract

Following inflammation or injury, sensory neurons located in the dorsal root ganglia (DRG) may exhibit increased spontaneous and/or stimulus-evoked activity, resulting in chronic pain. Current treatment options for peripherally-mediated chronic pain are highly limited, driving the development of cell- or tissue-based phenotypic (function-based) screening assays for peripheral analgesic and mechanistic lead discovery. Extant assays are often limited by throughput, content, use of tumorigenic cell lines, or tissue sources from immature developmental stages (i.e., embryonic or post-natal). Here, we describe a protocol for culturing adult mouse DRG neurons on substrate-integrated multiwell microelectrode arrays (MEAs). This approach enables multiplexed measurements of spontaneous as well as stimulus-evoked extracellular action potentials from large populations of cells. The DRG cultures exhibit stable spontaneous activity from 9 to 21 days in vitro (DIV). Activity is readily evoked by known chemical and physical agonists of sensory neuron activity, such as capsaicin, bradykinin, PGE2, heat, and electric field stimulation. Most importantly, we demonstrate that both spontaneous and stimulus-evoked activity may be potentiated by incubation with the inflammatory cytokine interleukin-6 (IL6). Acute
responsiveness to IL6 is inhibited by treatment with a MNK1/2 inhibitor cercosporamide. In total, these findings suggest that adult mouse DRG neurons on multiwell MEAs are applicable to ongoing efforts to discover peripheral analgesic and their mechanisms of action.

**New and Noteworthy**

This work describes methodologies for culturing spontaneously active adult mouse DRG sensory neurons on microelectrode arrays. We characterize spontaneous and stimulus-evoked adult DRG activity over durations consistent with pharmacological interventions. Furthermore, persistent hyperexcitability could be induced by incubation with inflammatory cytokine IL6 and attenuated with cercosporamide, an inhibitor of the IL6 sensitization pathway. This constitutes a more physiologically relevant, moderate throughput in vitro model for peripheral analgesic screening as well as mechanistic lead discovery.
2.1 Introduction

The Dorsal root ganglia (DRG) are collections of first order sensory neurons involved in the perception of innocuous as well as noxious stimuli. Upon supra-threshold stimulation of peripheral nerve terminals, afferent fibers from the DRG are depolarized, propagating signals in the form of all-or-nothing action potentials toward the spinal cord to be processed by the central nervous system. After an injury, inflammatory cues enhance spontaneous and stimulus-evoked activity in primary afferents (White et al. 2005; Davidson et al. 2014; Djouhri et al. 2015; Kim et al. 2016). These changes are mediated by complex signaling cascades involving myriad pro-inflammatory mediators such as interleukin-6 (IL6) and nerve growth factor (NGF). Their mechanisms of action impact many cellular processes. However, our understanding of their effects on sustained neuronal activity are incomplete owing to the technical challenge of obtaining reproducible long-term electrophysiological recordings.

Chronic pain is the most common cause of long-term disability in the world (Insitute of Medicine (US) Committee on Advancing Pain Research, Care 2011), yet pharmacological treatment options for chronic pain are limited and problematic. The most prevalent approach involves prescription opioids which are associated with high incidence of adverse effects, most prominently, addiction (Volkow et al. 2017). Modern analgesic discovery has been largely driven by the identification of molecular targets or pathways associated with a particular pathology where modulation may then impact disease progression (Bleicher et al. 2003). However, a complementary or even alternative approach has emerged with the advent of cell- or tissue-based phenotypic screening assays. Rather than focusing on the activity of a molecular target, phenotypic (or function-based) assays capture complex cellular-level behaviors or traits that are physiologically relevant to a pathology without
relying on the identification of a specific drug target or a corresponding hypothesis concerning its role in pathology.

To date, *in vitro* data regarding primary sensory neuron excitability and sensitization has been principally derived from single-cell patch clamp recordings, and has provided tremendous insights into the changing electrophysiological profiles of DRG neurons after exposure to inflammatory cytokines (e.g., (Wang et al. 2007; Ke et al. 2012; Fischer et al. 2017)). However, patch clamp methods are invasive, incompatible with long-term measurements, and low throughput. Substrate-integrated micro-electrode arrays (MEAs), however, enable label-free, long-term measurements of action potentials from large populations of cells, potentially making it a suitable platform for function-based pharmacological and toxicological screening paradigms (Xiang et al. 2007; Johnstone et al. 2010).

To date, a single study has made use of primary DRG neurons cultured on microelectrode arrays to characterize spontaneous and chemically evoked activity. A limitation of this work is reliance on embryonic tissue (Newberry et al. 2016a). While embryonic tissue is readily amenable to *in vitro* culture due to increased viability (Eide and McMurray 2005), there are significant disadvantages driven by widespread changes in gene expression that emerge later in development. These include changes linked to the voltage-gated Na channel Nav 1.8/1.9 (Benn et al. 2001), mRNA associated with axonal transport, vesicle trafficking, and axonal protein synthesis (Gumy et al. 2011). Additionally, dissociated embryonic and neonatal cultures of DRG neurons show little or no apparent NGF-induced sensitization to capsaicin (Zhu, W.; Oxford 2011), unlike adult DRG *in vivo* and *in vitro*. 
Here, we report the first detailed study of spontaneous and evoked activity using adult murine DRG neurons cultured on substrate-integrated MEAs. Spontaneous action potentials (or spikes) emerged within 24 h of culture, reaching a peak mean spike rate at 9 days in vitro (DIV). Increased activity was reproducibly evoked by both chemical and physical agonists (capsaicin, bradykinin, temperature increase, electric field stimulation) by DIV 7 and spontaneous activity could be transiently decreased by relatively high frequency electrical stimulation (≥ 100 Hz). Most importantly, both spontaneous and evoked activity was potentiated by short- and long-term incubation with inflammatory cytokine IL6 and persistent sensitization to capsaicin (following IL6 washout) was induced by ≥ 24 h incubation with the cytokine. The acute effects of IL-6 were diminished in the presence of the MNK1 inhibitor cercosporamide. In total, these findings suggest that adult DRG cultures on multi-well plates of microelectrode arrays may enable a stable, moderate throughput, high content platform for screening peripheral analgesics and conducting mechanistic studies pertaining to sensory neuron hyperexcitability.

2.2 Results

2.2.1 Culture and population

To determine whether dissociated adult mouse DRG cultures would develop stable spontaneous activity profiles and warrant further nociceptive studies, we dissected and cultured DRG from 4-6 week-old male ICR-CD1 mice onto multi-well MEAs as well as glass-bottomed dishes for morphological and immunocytochemical (ICC) characterization. The presented data represent recordings from 144 microelectrode wells prepared from dissection from 24 mice. Figure 2.1 A shows neuronal subtype ICC staining based on overlaps of CGRP, IB4, and NF200 expression. ICC indicated that approximately 95% of the cellular population by DIV 15 was non-neuronal,
and most likely a combination of fibroblasts, satellite glia and Schwann cells. Of the neuronal population, approximately 88% were found to be either CGRP or IB4 positive (nociceptive). Furthermore, we have measured the cross sectional area of neuronal cell types and found approximately 97% to be of the ‘small’ or ‘medium’ gauge, based on previous size-based characterizations of DRG cell subtypes (Newton et al. 2001). These data suggest that we have cultured a largely nociceptive neuronal population and indicates that we were able to culture with sufficient density and viability for long-term MEA recordings.

Figure 2.1: Immunocytochemistry and size-based discrimination of neuronal subtypes. (A and B) CGRP positive cells (blue) indicate peptidergic C-fiber neurons. IB4 positive (green), NF200 negative cells indicate non-peptidergic C-fiber neurons. IB4 positive, NF200 positive (red) cells indicate Aδ fiber neurons. NF200 (only) positive cells indicate Aβ fiber neurons. Scale bar represents 250 µm. (C) Automated measurements of cross-sectional area suggest a large percentage of small and medium gauge neurons, indicating a primarily nociceptive cell population.
2.2.2 Spontaneous activity

After a single DIV, action potentials could be readily recorded with excellent signal-to-noise ratios (SNR, 12.3 ± 0.8) and manually sorted into single units based on characteristic waveform shapes (Figure 2.2 B and C). Individual recording sites on microelectrode arrays in contact with neural tissue, both in vivo and in vitro, often detect more than one unit, or distinct bioelectrical signal source. To determine whether our analysis of individual recording channels would reflect single

![Image](image_url)
cells (single units), waveforms were sorted in 12 representative wells on DIV 15. Only 29 out of 384 active channels exhibited more than one unit (7.6 %), indicating that under our culture conditions the recordings from individual microelectrode sites likely correspond to a single bioelectric source. Therefore, all additional analyses were carried out with the assumption that channel recordings represented single cells. While the SNR did not significantly change over time, the spontaneously active electrode yield increased from 2.1 to 26 % between DIV 1 and 9 (Z (768) = 5.4, p = 7.9E-8, two-sample proportion test). Over the same duration, the mean firing rate per well increased from 0.05 ± 0.01 to 0.99 ± 0.35 Hz (Z(12) = 3.8, p = 1.4E-4, Mann-Whitney Test). However, neither the mean firing rate nor the active electrode yield changed significantly between DIV 9 and 21 (Figure 1.2F) (χ² (12) = 0.79, p = 0.38, Kruskall-Wallis ANOVA), suggesting the establishment of a stable baseline activity over time courses sufficient for pharmacological studies. The observed mean firing rates were relatively low as compared to those reported in studies of cortical (Charkhkar et al. 2014a), ventral horn motor neurons (Black et al. 2017), but not embryonic DRG cultures (1.14 ± 0.12 Hz, (Newberry et al. 2016a)), and were significantly skewed toward lower firing rates. Of the recorded units, 72.5% were found to have firing rates at or below 0.1 Hz, while only 1.5% of units had firing rates of greater than 5 Hz (Figure 2.2E). Importantly, however, this range of spontaneous firing rates is consistent with previously reported spontaneous firing of nociceptors from the L4/L5 DRG in vivo, where observations have ranged between 0.01 and 1.3 Hz (Djouhri 2006), with a high percentage of neurons exhibiting little-to-no spontaneous activity (range: 4.8 - 6.8%) (Liu et al. 2000). The relatively minor reduced firing rate and skewness in our distribution as compared to embryonic DRG recordings may be attributed to methodological
differences in analysis, including active channel criterion, or to intrinsic firing rate differences due to tissue derivation at different stages of cellular maturity (Gebhart et al. 2009).

Additionally, we observed distinct differences in the firing patterns across recording channels. Figure 2.3 shows representative raster plots and inter-spike interval (ISI) histograms of 4 spontaneously active electrodes from a single well. Overall, the ISI coefficient of variation (CV) was $1.62 \pm 0.03$ for spontaneous activity on DIV 12. This is in contrast to reported CVs for embryonic cultures ($0.91 \pm 0.05$, DIV 14) and suggests a higher incidence of intrinsic bursting (Svirskis and Rinzel 2000; Stiefel et al. 2013) in adult cultures. However, we observed only 1.3% of active electrodes exhibiting synchronous or correlated activity. To determine whether these correlated electrode recordings were a single neuron extending processes across multiple electrodes or were representative of functional connections, we treated wells in which correlated activity was observed with either synaptic vesicle blocker BoNT/A (100 ng/ml, 24 h incubation) or gap junction inhibitor carbenoxolone (50 µM, 10 min incubation). In only 3 cases (out of 10) did we observe any reduction in correlation or firing rates following treatment with carbenoxolone.

Figure 2.3: Spontaneously active DRG neurons exhibit various firing patterns in vitro. (A) Representative raster plots from 4 spontaneously active electrodes within a single well exhibiting different firing patterns and (B) associated inter-spike

24
(0 cases for BoNT/A) suggesting that the few functional connections which exist may be mediated by gap junctions but not by synaptic transmission.

2.2.3 Evoked activity

To determine whether adult DRG cultures were sensitive to known chemical and physical agonists of sensory neuron activity, they were treated with 10 nM capsaicin, 100 nM bradykinin, 1 μM prostaglandin E2 (PGE2), or were subject to increased temperature or electric field stimulation.

2.2.4 Capsaicin responsiveness

Figure 2.4A shows representative raster plots from 6 electrodes responsive to 10 nM capsaicin within a single well. In total, 27% of previously active channels responded to 10 nM capsaicin and the well-wide mean firing rate was found to increase from 1.1 ± 0.2 to 2.6 ± 0.5 Hz ($Z(101) = 4.84$, $p = 1.3E-6$, Mann-Whitney Test). Both the percentage of responsive electrodes and the evoked mean firing rates were found to increase with increased capsaicin concentration. 27, 36.6, and 39.1% of electrodes were found to be responsive to 10, 100, and 1000 nM capsaicin, respectively ($Z(101) = 6.4$, $p = 1.9E-10$ for 1000 nM, Two-Sample Proportion Test). These values are highly consistent with previous reports of colocalization of neuronal markers in the DRG, including TRPV1 (Price and Flores 2007), but are on the lower end of previously reported values of capsaicin responsiveness in vitro (Gold et al. 1996; Tsantoulas et al. 2013; Malsch et al. 2014). These differences are likely attributable to approaches in cell culture (embryonic vs. adult, lumbar only vs. lumbar and cervical DRG), methodology (calcium imaging vs. patch-clamp vs. MEA), and/or definition of responsiveness.
2.2.5 Temperature sensitivity (42 °C)

Following baseline recordings, calibrated temperature increases were induced via the recording system’s stage plate heater. Approximately 38.9% of channels were responsive at 42 °C or during the temperature rise. Overall normalized mean firing rates were observed to significantly increase, reaching a steady, elevated state after reaching 42 °C (Figure 2.4B), and returned to baseline levels following stage plate cooling. These data suggest that calibrated stage-plate heating may be used as a measure of sensitivity for adult DRG neurons in vitro and, interestingly, that functional temperature sensitivity of these cultures lies well below levels typically defined as noxious (42 °C).

Figure 2.4: (A) Representative raster plots from 6 10 nM capsaicin-responsive electrodes within a single well (left). Capsaicin concentration responsiveness as a percentage of active electrodes (right). (B) Representative raster plots from 6 temperature-responsive electrodes within a single well (left). Normalized mean firing rate during calibrated temperature increase from 37 to 42 °C.
°C). This is likely due, though not investigated here, to conserved expression of cation channels with sensitivity at or below 40 °C, such as transient receptor potential vanilloid 4 (TRPV4).

2.2.6 Bradykinin and PGE2 responsiveness

Treatment with either 100 nM bradykinin or 1 μM PGE2 resulted in significant increase in mean firing rate on 43 and 35% of previously active channels, respectively. In contrast to capsaicin, the onset of bradykinin and PGE2 evoked activity was relatively slow, reaching its mean peak at 60 and 70 sec, respectively (10 sec bins), while capsaicin-evoked activity reached its mean peak within 10 sec. Moreover, the time required for return to baseline was also increased, requiring 2 min and 12.2 min, respectively, while capsaicin-treated wells returned to baseline activity within 40 sec.

2.2.7 Electric field stimulation

Electric field stimulation was carried out using recording electrodes with previously observed spontaneous activity. Single biphasic pulses were found to be ineffective at eliciting activity within the range of amplitude/duration parameters available through the Axion Maestro system. However, we observed differential responsiveness (both activation and inhibition) on a large subset of stimulated microelectrodes (83.3%) in response to pulse trains of 10 cathodic-leading biphasic square pulses (1.2 V, 750 μs phase duration) at or above 100 Hz. 54.8% exhibited activation only (evoked activity), 11.9% exhibited inhibition only (reduced activity as compared to baseline), and 16.7% exhibited activation followed by inhibition (Figure 2.5A and B). For stimulated channels exhibiting inhibition, the mean time to return to baseline was 19.2 ± 2.8 sec. For stimulated channels exhibiting activation, the mean time to return to baseline was 3.0 ± 0.4 sec.
To determine whether activation or inhibition outcomes were associated with nociceptive cell types, we followed electrical stimulation experiments with 1 μM capsaicin treatment. Figure 2.5C shows the percentage of electrodes exhibiting different responses to electrical stimulation which were also responsive to capsaicin. While the relative number of capsaicin-responsive electrodes was low (between 3 and 8), these data suggest that differential responsiveness to electrical stimulation is not indicative of neuronal subtype.

Figure 2.5: Electrical stimulation at frequencies > 100 Hz elicits differential responsiveness. (A) Representative raster plots from two stimulated electrodes exhibiting either activation (top) or inhibition (bottom) of activity. Scale bar represents 2 sec. Inset (red square) illustrates cathodic-leading biphasic waveform plus artifact removal. (B) Percentage breakdown of electrodes which were either activated, inhibited, both, or neither (n = 42). (C) Percentage of
2.2.8 Short- and long-term excitability changes in the presence of IL6

To determine whether primary adult DRG culture activity could be potentiated by inflammatory cytokines, recordings were carried out in the absence and presence of 100 ng/ml IL6 at 0 and 48 h time points relative to IL6 addition. Thirty-minute baseline recordings were followed by either 3 h or 30 min continuous recordings in the presence of IL6 or vehicle (water). Figure 2.6A shows short-term (0-3 h) responsiveness to IL6, which was observed on 41% of previously active channels. Overall, median firing rates significantly increased from 0.02 to 0.15 Hz over the 3 hours of IL6 exposure (Z(203) = 10.4, p = 3.5E-25, Mann-Whitney Test). Additionally, median ISI CVs also significantly increased from 1.13 to 2.29 in the presence of IL6 (Z(203) = 12.3, p = 1.6E-34), suggesting an increase in intrinsic bursting behavior. However, no significant

Figure 2.6: Spontaneous sensory neuron activity is significantly increased with short- and long-term incubation with IL6. (A) Normalized mean firing rate for electrode subset found to be responsive to IL6 (41% of previously active electrodes. Red line indicates addition of 100 ng/ml IL6. (B) Firing rates before (Pre), immediately following (0–3 hr) and 48 hr following IL6 addition. (C) Firing rates before and immediately following IL6 addition in the presence of vehicle (water) or MNK1/2 inhibitor cercosporamide. *, **, *** indicates p
differences were observed in terms of mean spike amplitude (40.6 ± 5.3 vs. 43.5 ± 2.7 μV, Z(203) = 0.17, p = 0.87, Mann-Whitney Test). After 48 h incubation, IL6-treated cultures still exhibited both increased spontaneous firing rates (Z(203) = 4.8, p = 1.52E-6, Mann-Whitney Test) and increased ISI CVs (Z(103) = 3.0, p = 0.002, Mann-Whitney Test) as compared to baseline, suggesting long-term hyperactivity in the presence of IL6 (Figure 2.6B).

Previous work from our group (Moy et al. 2017) demonstrated reduced short-term responsiveness to IL6 in cultures prepared from mice lacking eIF4E phosphorylation. To determine whether IL6-induced hyperexcitability could be reduced by eIF4E phosphorylation inhibitors, adult DRG cultures were exposed to a 30-min pre-treatment with either 100 nM cercosporamide (a potent Mnk and JAK3 pathway inhibitor) or medium plus equal volumes of vehicle (water) 30 min before treatment with IL6. Vehicle-pretreated wells exhibited significant increases in spontaneous median firing rate following IL6 exposure (Z(37) = 2.14, p = 0.03, Mann-Whitney Test) whereas those pretreated with cercosporamide exhibited a significant decrease in median firing rate Z(80) = 0.61, p = 1.4E-9, Mann-Whitney Test) (Figure 2.6C). However, there were no significant differences in terms of ISI CVs. Moreover, there was no significant difference in either median firing rate or ISI CV following 48 h incubation with IL6.

To determine whether capsaicin-evoked activity may be potentiated by IL6 incubation, cultures were exposed to a low concentration (10 nM) of capsaicin before and after IL6 incubation. The number of electrodes which were responsive to 10 nM capsaicin significantly increased (Z(69) = 2.8, p = 4.8E-3, Two-Sample Proportion Test) after 48 h IL6 incubation (from 23 to 38), but not in the case of vehicle (19 to 17). Additionally, we tested three periods of IL6 incubation and washout (3, 24, and 48 h), all followed by 10 nM capsaicin treatment. We found that both 24 and
48 h incubation with IL6 was sufficient to caused increased capsaicin responsiveness, but not 3 h incubation (Figure 2.7A). These data suggest that there is a minimum incubation time necessary to elicit persistent changes in hypersensitivity to chemical stimuli in vitro.

![Graph showing the effect of IL6 incubation on capsaicin responsiveness.](image)

**Figure 2.7: Incubation with IL6 causes increased capsaicin and temperature responsiveness.** (A) Active electrodes responsive to capsaicin prior to treatment (Pre-) and following 3, 24, or 48 hr incubation with IL6 or vehicle. All treatment recordings (Post-) were carried out 48 hr after baseline. (B) Active electrodes responsive to 42 °C following 48 hr incubation with IL6 or vehicle. (C) Representative fluorescence images of immunocytochemistry staining for capsaicin- and temperature-sensitive cation channel TRPV1 (left, red) and neuronal marker NeuN (blue, middle). Scale bar represents 100 μm. (D) Percentage of TRPV1-positive neurons following 48 hr incubation with IL6 or vehicle. **, *** indicates p < 0.01 and p < 0.001.

To determine whether temperature-evoked activity may be potentiated by IL6 incubation, adult DRG cultures were exposed to 42 °C following 48 h incubation with IL6. Temperature
responsiveness was increased following 48 h IL6 incubation. 64% of baseline active electrodes were found to respond to temperature increase (42 °C) in wells treated with IL6, whereas only 38.9% of channels in untreated wells were found to be responsive to the same temperature increase (Z(299) = 3.65, p = 2.6E-4, Two-Sample Proportion Test, Figure 2.7B). Temperature variations did not appear to cause significant shifts in rise time or peak firing rate between treatment groups.

To determine whether increased capsaicin and temperature responsiveness was related to expression of TRPV1, targeted ICC was carried out following 48 h incubation in either 100 ng/ml IL6 or vehicle. Figure 2.7C shows representative labeling for TRPV1 and NeuN expression in neurons treated with IL-6. We found a significant increase of TRPV1-positive cells following IL6 incubation (Z(488) = 6.0, p = 2.7E-9, Two-Sample Proportion Test, Fig 2.7 D).

In total, these data suggest that short- and/or long-term incubation with inflammatory cytokine IL6 induces persistent changes in both spontaneous and stimulus-evoked activity in adult DRG cultures in vitro. Additionally, these changes in stimulus-evoked activity may be due to increased membrane expression of TRPV1.

2.3 Discussion

We have carried out the first detailed characterization of spontaneous and stimulus-evoked recordings from adult mouse DRG neurons in vitro using a multiwell MEA platform for up to 21 days. Importantly, we have demonstrated differential excitability, both in terms of spontaneous firing rates/patterns and responsiveness to external stimuli, in the presence and absence of the inflammatory mediator, IL6. IL6 was chosen as previous studies have demonstrated both short- and long-term effects of IL6 incubation on cultured DRG neurons (Obreja et al. 2005; Andratsch et al. 2009; Melemedjian et al. 2010; Ebbinghaus et al. 2015) and targeting IL6 or its receptor may
lead to the discovery of novel therapeutic interventions targeting pathological pain states (Zhou et al. 2016). It is worth mentioning that the model presented here does not preclude the study of other inflammatory mediators known to alter excitability *in vivo* or *in vitro*, nor does it preclude the use of other tissue sources, including primary DRG from previously injured animals. DRG extracted from animals subjected to chronic constriction (Study and Kral 1996), thoracic spinal injury (Bedi et al. 2010) and chemotherapy induced neuropathy have been shown to retain their hyperexcitable phenotype in vitro (Li et al. 2017).

Previous studies have reported little-to-no spontaneous activity from adult murine DRG neurons cultivated from uninjured animals for MEA or patch-clamp recordings (Kitamura et al. 2005; Kayano et al. 2013; Newberry et al. 2016a) without the addition of relatively high concentrations of nerve growth factor (NGF). This highlights the ability of MEAs to capture sporadic or low-frequency spontaneous activity over time courses incompatible with whole-cell patch clamp recordings. Additionally, we have developed our adult DRG culture protocols specifically for MEA recording. Previously published protocols, which were developed for patch-clamp electrophysiology, called for the addition of mitotic inhibitors upon seeding to limit the proliferation of non-neuronal support cells. Support cells, including satellite glial and Schwann cells, have been previously shown to promote long-term adhesion as well as increased intrinsic activity and functional connections in mixed cultures of both spinal motor neurons (Ullian et al. 2004) and retinal ganglion cells (Pfrieger 1997). Therefore, we have added relatively low concentrations of mitotic inhibitor (8.75 ng/ml uridine, 3.75 ng/ml 5-fluoro-2-deoxyuridine) only after 5-7 days in culture, when we observed a confluent population of non-neuronal support cells.

With the ability to consistently monitor nociceptor activity on MEAs, our work provides a potential
means to dissect the contribution of individual cell types within this complex milieu of support
cells to nociceptor activity. This problem is particularly attractive given the recent resurgence of
interest in intercellular communication in relation to the neuroimmune interface.

We demonstrate the use of the MEA platform for pharmacological studies and extend this work to
evaluate safe and efficient electrical stimulation parameters in vitro. Electrical stimulation of DRG
or whole nerve has found preclinical and clinical applications for transient antinociceptive effects
(Kent et al. 2018). For this study, we were unable to match parameters previously reported for
randomized human clinical trials (20.8 Hz, 915.4 μA (Deer et al. 2017)) without potentially
damaging the electrodes and/or tissue. Shockingly, we observed distinct classes of responsiveness
to electrical stimulation, none of which were found to exclusively coincide with nociceptor subtype
(capsaicin responsiveness). This outcome may be attributable, instead, to the stimulated organelle
and the relative location of the stimulating electrode. In a disorganized culture with axonal
processes, it is not trivial to interpret signals as originating at the soma, axon, or dendrite (Obien
et al. 2015). In fact, our observations of morphological culture development in combination with
the increasing development of spontaneous activity suggests that we are recording from a
combination of soma and axons. Since stimulation and recording were carried out on the same
electrodes, this also suggests that we are stimulating a subpopulation of both soma and axons. This
argues for the future development of compartmentalized cultures, wherein the soma and axonal
processes may be electrically accessed separately. Previous studies have used compartmentalized
cultures to optically, electrically, and chemically interrogate soma versus axons/dendrites
(Tsantoulas et al. 2013). A similar system may be used to test stimulation parameters on axons
versus soma to further optimize efficiency and safety of electrical modulation devices.
In summary, we have characterized a spontaneously active in vitro adult mouse DRG model using substrate-integrated multi-well MEAs. We have demonstrated stable, long-term spontaneous activity and have generated a pharmacological profile consistent with a nociceptor-rich culture which is sensitized by inflammatory mediators known to play a role in chronic pain development. In total, these findings suggest that adult mouse DRG on MEAs may serve as a potential model for peripheral analgesic and mechanistic lead discovery.

2.4 Materials and Methods

2.4.1 Reagents

Laminin, Poly-D-lysine (PDL), polyethyleneimine, Hank’s balanced salt solution (HBSS), collagenase, trypsin, glial-derived neurotrophic factor (GDNF), uridine and 5-fluoro-2-deoxyridine (FRDU) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s Medium: Nutrient Mixture F-12 (DMEM/F12) + Glutamax, and DNase were purchased from Thermo Fisher Scientific (Waltham, MA). Complete cell medium refers to DMEM/F12 + Glutamax, 10% FBS, 1% penicillin/streptomycin, and 5 ng/ml GDNF.

2.4.2 Primary DRG extraction and culture

This study was carried out in accordance with the recommendations of the University of Texas at Dallas’ Institutional Animal Care and Use Committee (IACUC). The protocol was approved by the UTD IACUC. Euthanasia was conducted in accordance with AVMA Guidelines for the Euthanasia of Animals. Adult ICR(CD-1) mice (Envigo, 4 – 6 weeks old) were anesthetized with 3% isoflurane and euthanized by cervical dislocation. DRGs were dissected as previously described (Sleigh et al. 2016). Briefly, the spinal column was isolated and hemisected, and the spinal cord carefully removed to expose the DRG. DRG were individually isolated from
central/peripheral roots and placed in ice-cold HBSS. DRG were transferred to dissociation solution, which consisted of collagenase (2 mg/mL) and DNase (0.1 mg/mL) and incubated for 40 minutes at 37°C. After 40 minutes, trypsin (0.025%) was added and the solution was incubated for an additional 5 minutes at 37°C. The tissue was then triturated with a fire-polished glass Pasteur pipette until solution appeared homogeneous. The solution was passed through a 70 µM nylon filter and the volume was tripled by adding DMEM/F12 plus 10% fetal bovine serum to quench trypsinization. The cells were collected by centrifugation (10 min at 600x g) and the resulting pellet was re-suspended in fresh complete medium. 5-10µL of medium containing an estimated 10,000 cells were seeded at the center of each MEA device or glass-bottomed plate. The plates were incubated at 37°C and 10% CO₂ for 30 minutes to allow cells to adhere, and then each well was carefully flooded with 600 µL of fresh, pre-heated complete medium. Cell cultures were maintained at 37°C, 10% CO₂, and 95% humidity. 100% media exchanges were performed after the first 24 hours and every alternate day afterward. After non-neuronal cells reached 90% confluence (DIV 5-7), media was supplemented with mitotic inhibitors uridine (17.5 µg/mL) and 5-fluoro-2-deoxyridine (7.5 µg/mL).

2.4.3 Multi-well Microelectrode Array Plate Preparation

DRG cells were cultured on 12-well MEA devices from Axion Biosystems for electrophysiological recording as well as multiwell glass-bottomed culture plates for immunocytochemical (ICC) characterization. The day before dissection, the center of each well of the MEA devices was coated with polyethyleneimine (0.1%); glass-bottom wells were coated with PDL (50 µg/mL). Plates were incubated at 37°C and 10% CO₂ overnight. The following morning, plates were rinsed three times with sterile double-deionized water and allowed to dry. 10 µL of
laminin (20 µg/mL) was added to the center of each well and incubated at 37°C for two hours. Laminin was removed immediately prior to cell seeding.

### 2.4.4 Extracellular Recordings and Stimulation

Extracellular voltage recordings were acquired from each multiwell plate every alternate day, beginning on day *in vitro* (DIV) 3, using 12-well MEA plates (768 total electrodes) and the Axion Maestro multi-well plate recording system (Axion BioSystems, Atlanta, GA). All baseline recordings were carried out for 30 min at 37°C and 5% CO₂. Continuous data were acquired simultaneously at 12.5 kHz per electrode and filtered using a 1-pole Butterworth bandpass filter (200 – 3000 Hz). Individual spikes were detected by filtered continuous data crossing of a ± 5.5 σ adaptive threshold. Unless otherwise stated, an active electrode was defined by at least 5 spikes per baseline recording session constituting a characteristic waveform (single-unit). Additional analysis (e.g., mean firing rate, synchrony index) was carried out using NeuroExplorer software (Nex Technologies, Madison, AL) in combination with Axion’s Axis Metric program. In order to avoid unanticipated long-term changes in baseline activity due to repeated pharmacological treatments, separate cultures were designated for spontaneous and evoked recordings.

Electrical stimulation and recording was performed using Axion’s Stimulation Studio in combination with software solutions provided by AxIS 2.3. Briefly, 7 to 8 active electrodes in each well (N = 42 electrodes) were selected and stimulated with a 100 Hz cathodic-leading biphasic square pulse train (x10 stimuli) with an amplitude of 1.2V and pulse duration of 750 µs/phase. To determine if the number of units stimulated were nociceptors, 10 nm, 100 nm, or 1 µM capsaicin treatment was performed after the electrical stimulation events. Unless otherwise stated, all data
associated with electrical stimulations were visualized and analyzed in Axion’s NeuralMetric Tool software.

2.4.5 Pharmacological Exposure

All compounds were reconstituted in either DMSO, ethanol, or DI water at ≥ 100X working concentrations. Preliminary experiments indicated that full bath exchange caused short term, but significant changes in baseline activity. Therefore, all pharmacological compounds were added as high concentration, low volume boluses to the corners of wells and mixed once with a 100 µl pipette. Prior to addition of compounds, a 30 minute baseline recording was performed simultaneously for all experimental, vehicle, and control conditions. To establish a baseline for evoked responsiveness to chemical agonists, adult DRG at DIV 10-16 were exposed to either 100 nM Bradykinin or 10 nM capsaicin for 5 minutes. Next, a 100% medium exchange was performed, and cells were incubated with either 1µM Prostaglandin E2 (PGE2) for 10 minutes, or 100 ng/ml interleukin 6 (IL6) for a period of 3hr, 24hr, or 48hr. To test cytokine mediated evoked responsiveness after incubation with PGE2 or IL6, cells were challenged with 100 nM bradykinin or 10 nM, 100nM, and 1µM capsaicin respectively. In addition, temperature increase on naïve and IL6 treated cells was performed using the Environmental Control module in AxIS 2.3. Briefly, after a 30 minute baseline recording at 37°C, the temperature was increased incrementally at approximately 0.5 degree/min to 42°C while changes in activity was captured via simultaneous recordings.
2.4.6 Immunocytochemistry

Primary adult DRG cultures at DIV 11-13 were fixed with 4% paraformaldehyde for 10 minutes, washed thrice with ice-cold PBS, and permeabilized with 0.25% Triton X-100 for 30 minutes at room temperature (RT). Non-specific binding sites were blocked with 10% normal goat serum (NGS) for 2 hours and primary antibodies against Neurofilament-200 (1:1000, Sigma, St. Louis, MO), isolectin B4 (IB4) (1:1000, Thermo Fisher Scientific, Waltham, MA), calcitonin-gene related peptide (CGRP) (1:1000, Peninsula Laboratories, San Carlos, CA) were diluted in PBS with 10% NGS and incubated overnight at 4ºC. Following day, cells were incubated with species-specific secondary antibodies for 1 hour at RT. TRPV-1 staining following incubation with IL6 or naïve groups was performed using primary antibodies against TRPV1 (1:1000, Neuromics, Edina, MN) and NeuN (1:500, Abcam, Cambridge, MA) diluted in PBS + 10% NGS and incubated for 2 hours at RT. Samples were incubated with species-specific secondary antibodies for 1 hour at RT. Unless stated otherwise, nuclei in all samples were visualized using 1 µg/ml DAPI (Vector laboratories).

2.4.7 Fluorescence microscopy and Image Analysis

All epifluorescence and confocal imaging was performed at 20x magnification using an inverted microscope (Nikon Ti eclipse, Nikon, Tokyo, Japan) and epifluorescent light sources (Lumencor, Beaverton, OR). To determine the percentage of distinct neuronal subtypes in the existing culture preparation, three regions of interest (ROI) were acquired in separate wells (N=6). To quantify the expression of TRPV-1 after treatment with IL6 or in naïve preparations, three defined ROIs were used to visualize a minimum of 50 neuronal soma in a single field of view (N=3 for treatment, N=3 for naïve). All multi-channel images were processed in ImageJ (NIH, United States). Briefly,
a user defined threshold was applied to each channel and neuronal soma were manually counted based on maximum intensity projections. Cell counts were further analyzed, quantified, and plotted using OriginPro software, (OriginLab Corporation, Northampton, MA, United States).

2.4.8 Statistical Analysis

To be considered for analysis, only active electrodes were chosen based on a criteria of an electrode having a mean firing rate of greater than 1 spikes/min. In the case of responsiveness to chemical agonists (bradykinin and capsaicin), an electrode qualified as responsive if the peak firing rate after treatment was at least twice that of the peak mean firing rate during baseline. All statistical analysis and data visualization was performed in OriginPro 2017 (OriginLab Corp., Northampton, MA). Prior to group or pair-wise tests, Shapiro-Wilk normality tests were performed. Pair-wise tests were carried out using Mann-Whitney Test or Two-Sample Proportion Test. In all cases, P < 0.05 was considered as statistically significant. Statistics are reported as $Z(n$-number) = $z$ metric, p-value, type of statistical test. In cases where n numbers were unequal between treatment groups, the lower n number is reported. Well-wise parameters are presented as mean ± standard error of the mean (SEM) unless stated otherwise.
References


CHAPTER 3

EXPRESSION AND FUNCTIONAL PROFILES OF SODIUM CHANNEL SUBTYPES
NAV1.7 AND NAV1.8 ARE MAINTAINED IN ADULT DORSAL ROOT GANGLION
NEURONS IN VITRO

Abstract

Sensory neurons respond to noxious stimuli by relaying information from the periphery to the central nervous system via all-or-nothing action potentials. We focus on the role of Nav1.7 and Nav1.8. Both are conserved voltage-gated sodium channels that facilitate inflammatory pain in mammals. The ability to screen compounds that interfere with voltage-gated sodium channels using cell-based screening assays of fully mature sensory neurons may enable therapeutic identification assuming that key channels present in vivo are maintained in vitro. Prior electrophysiological work in vitro utilized acutely dissociated tissues. While Nav 1.7 and 1.8 are expressed and functional in these experiments, the use of damaged sensory neurons is problematic for modeling mechanisms of pain signaling. A potential alternative involves substrate-integrated multi-electrode arrays. They permit long-term non-invasive measures of neural spike activity and are well suited for assessing persistent sensitization consistent with chronic pain. Here, we demonstrate that addition of two inflammatory mediators associated with chronic inflammatory pain, NGF and IL-6, to adult DRG neurons increases their firing rates on multi-electrode arrays in vitro. Nav1.7 and Nav1.8 proteins are readily detected in cultured neurons and contribute to evoked activity. Yet, blockade of Nav1.8 has a more profound impact on thermally evoked firing after treatment with IL-6 and NGF relative to Nav1.7. This work
underscores the utility of multi-electrode arrays for pharmacological studies of sensory neurons and may facilitate discovery and mechanistic analyses of anti-nociceptive compounds.

3.1 Introduction

Nociceptor cell bodies reside in the dorsal root ganglion (DRG). Their fibers innervate the dermis, internal organs, and the viscera. In addition to a critical role in detection of noxious external stimuli in their peripheral receptive field, nociceptors transmit information from the periphery to the dorsal horn of the spinal cord in the form of all-or-nothing action potentials (Okuse 2007). The generation and propagation of such action potentials are driven by voltage-gated sodium channels (VGSCs) (Sulayman D. Dib-Hajj et al. 2010). Three VGSC α-subunits are preferentially expressed in C-nociceptive subpopulations of small-medium diameter DRG neurons, namely, Nav1.7, Nav1.8, and Nav1.9 (Patil, Hovhannisyan, and Akopian 2018). As a result, the dysregulation of Nav1.7 and Nav1.8 subtypes in specific pain modalities has been linked to the maintenance of inflammatory pain states (Levinson, Simon R., Luo, Sonjiang, Henry 2012).

Inflammatory mediators released at the site of injury result in peripheral activation and sensitization of nociceptors due to their effects on a multitude of ion channels, including sodium channels (H. J. Gould et al. 1999). Under chronic pain conditions, maladaptive changes in sodium ion channel subtypes in nociceptive populations have been extensively documented in pre-clinical models of inflammatory pain in vivo. Injury generally results in increased accumulation of Nav1.7 and Nav1.8 protein (J. A. Black et al. 2004; Sulayman D. Dib-Hajj et al. 1999; H. J. Gould et al. 1999; Harry J. Gould et al. 1998; Strickland et al. 2008; Tamura, Nemoto, Maruta, et al. 2014). In the acute phase, mitogen-activated protein kinases (MAPKs),
stimulated by pro-nociceptive cytokines, have been shown to directly modulate nociceptive excitability by either altering the gating properties, in the case of Nav1.7 (Stamboulian et al. 2010), or current density, in the case of Nav1.8 (Hudmon et al. 2008). These findings suggest that pharmacological disruption of Nav1.7 and Nav1.8 post-translational modification, activity, or expression may ameliorate specific pain phenotypes. Consistent with this notion patients with loss of function mutations in the voltage-gated sodium channel Nav1.7 are insensitive to pain (Cox et al. 2006). Similarly, knockdown of Nav1.8 in pre-clinical models can reverse a specific model of neuropathic pain (Lai et al. 2002).

Several lines of evidence have demonstrated the conserved expression of Nav1.7 and Nav1.8 in DRG cultures in vitro which provide a promising approach to probe single ion channel function and contribution to hyperexcitability induced by inflammatory mediators (Sulayman D. Dib-Hajj et al. 2010; Krafte and Bannon 2008; Linley et al. 2010). These approaches have largely used dissociated small- and medium-gauged naïve rodent DRG neurons for single cell patch-clamp recordings which have provided excellent insight into the electrophysiological profiles of Nav1.7 and Nav1.8. However, most recording sessions are made in acutely dissociated tissue (14-20 hours after harvest) at room temperature with a short exposure time to the specific chemokine under investigation (Belkouch et al. 2011; Gu et al. 2015; Patil, Hovhannisyan, and Akopian 2018; Qiu et al. 2016; Schink et al. 2016; Tamura, Nemoto, Onizuka, et al. 2014). As a result, patch-clamp measurements are incompatible with long-term measurements, low-throughput, and strictly fail to recapitulate the in vivo mechanisms of channel dysregulation which occur over chronic time periods (Cai et al. 2016; Chang et al. 2018; Sulayman D. Dib-Hajj et al. 1999; H. J. Gould et al. 1999; Harry J. Gould et al. 1998; Jarvis et al. 2007; Li et al. 2018; Salas et al. 2015;
Substrate integrated microelectrode arrays (MEAs), alternatively, provide non-invasive and long term measurements recorded simultaneously from a large population of cells. The use of an MEA platform may allow the development of in vitro phenotypic screening assays that utilize cellular excitability for screening potent small molecule inhibitors against “pain” channels and support efforts in identifying analgesic compounds (Bourque et al. 2018; Martínez et al. 2019; Melli 2014; Rana et al. 2017; Sidders et al. 2018; Stacey et al. 2018; Vincent and Feldman 2008).

Embryonic DRG neurons have been successfully cultured on substrate integrated microelectrode arrays (MEAs) and develop spontaneously active networks (Newberry et al. 2016). However, reliance on embryonic tissue is problematic due to the differential expression and/or responsiveness of ion channel subtypes to pharmacological treatments (Benn et al. 2001; Newberry et al. 2016). More recently, we have demonstrated that adult DRG neurons cultured on multi-well MEAs are spontaneously active and are responsive to treatment with inflammatory cytokines such as interleukin-6 (IL-6), eliciting increased spontaneous activity and stimulus-evoked responses (B. J. Black et al. 2018; Moy et al. 2017). Therefore, in the present study, we have extended the use of adult DRG cultured on MEAs to investigate the role of Nav1.7 and Nav1.8 after chronic exposure to IL-6 and NGF in vitro. We demonstrate that adult DRG neurons cultured for extended periods in vitro retain expression of relevant VGSCs to pain and bioelectrical activity shows modulation to specific antagonists, an effect distinct from that observed using cultured cortical neurons. Our results demonstrate that the histiotypic profiles of Nav1.7 and Nav1.8 are maintained in in vitro adult DRG cultures. Both subtypes are required for sensitization by IL-6 and NGF but display differential effects on thermally evoked firing events.
The study highlights the utility of adult DRG cultures on MEAs as a potential phenotypic screening tool for antagonists to sodium channels that are relevant to pain.

3.2 Materials and Methods

3.2.1 Primary adult DRG culture

All surgical procedures were carried out in accordance with the University of Texas at Dallas’s Institutional Animal Care and Use Committee. Institute for Cancer Research (CD-1) male mice (4-6 weeks old; Envigo) were utilized for DRG tissue extraction as described in detail previously (Sleigh et al. 2016). Briefly, mice were deeply anesthetized with 3% isoflurane and euthanized via cervical dislocation. Spinal columns were extracted, hemisected, and DRGs were isolated from central/peripheral roots and stored in ice-cold Hanks Balanced salt solution (HBSS). Tissue was dissociated as previously described with minor modifications (Black et al. 2018). An enzyme buffer consisting of 2 mg/ml collagenase and 0.1 mg/ml DNase was added to pooled tissue and allowed to incubate at 37°C for 40 minutes. After 40 minutes, 100 μL of 1X papain solution was added and the tissue was gently triturated using a fire polished pasture pipette. The cells were isolated via centrifugation (300g for 10 minutes) and resuspended in fresh medium consisting of DMEM/F-12+glutamax + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (PS) + 5 ng/ml GDNF. 10,000 viable neurons were plated on multi-well MEAs (Axion BioSystems, Atlanta, GA) pre-treated with 50 μg/ml poly-D-lysine (overnight) followed by surface coating of 20 μg/ml laminin (2 hours). Cultures were maintained at 37°C, 10% CO₂, and 95% humidity and medium was exchanged every 48 hour. After non-neuronal populations reached confluence of approximately 90%, medium was supplemented with mitotic
inhibitors uridine (17.5 μg/ml) and 5-fluoro-2’-deoxyuridine (7.5 μg/ml) for the remainder of the culture.

### 3.2.2 Primary embryonic cortical culture

Murine derived cortical networks were derived from embryonic age (E15-E18) mice. Time pregnant female mice (ICR-CD1, Envigo RMS Inc, Indianapolis) were deeply anesthetized followed by euthanasia via cervical dislocation. Individual embryos were obtained via cesarean section, removed from amniotic sacs, and stored in ice-cold HBSS. Cortical neurons were dissected and dissociated from 3-6 embryos as described in detail previously (Charkhkar et al. 2014a). Briefly, frontal cortices were surgically sectioned and pooled in an enzyme buffer consisting of 0.1 mg/ml DNAase and 100 μL of 1X papain reconstituted in HBSS and incubated at 37°C for 30 minutes. Tissue sections were homogenized via mechanical trituration using a fire polished pasture pipette and cells were isolated via centrifugation (300g for 10 minutes). 90,000 viable cells were plated on pre-treated multi-well MEAs as described previously and maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 5% horse serum, 5% FBS, and 1% PS. Medium was exchanged at 48 hour after which serum was removed to prevent over proliferation of non-neuronal cells and maintained by 50% medium exchanges for at least 21 days in vitro.

### 3.2.3 Extracellular recordings

Spontaneous and evoked extracellular recordings were performed with 48-well plate MEAs (Axion Biosystems, Atlanta, GA) using the Axion Maestro MEA recording system (Axion Biosystems, Atlanta, GA) as described previously (Black et al. 2018). Briefly, extracellular voltage recordings were carried out at 12.5 kHz sampling rate from a total of 768 available
substrate integrated microelectrodes. Continuous data were filtered using a 1-pole Butterworth band pass filter (200-3000 Hz) and individual spikes were detected using a 5.5 $\sigma$ adaptive threshold method. For analysis, only electrodes were considered for analysis when a mean firing rate of at least 1 spike/min was detected during the recording session. Additional analysis was carried out in NeuroExplorer (Nex Technologies, Madison, AL) and AxIS Metric.

3.2.4 Pharmacology

Before exposure, all pharmacological compounds were reconstituted in either complete medium, water, or DMSO at a stock concentration of $\geq$100X. Before addition of compounds, a baseline recording of 30 minutes was acquired. For exposure to IL-6 and NGF or vehicle (water), bolus volumes were added simultaneously to treatment groups (100 ng/ml IL-6 + 100 ng/ml NGF) and spontaneous recordings were acquired at the following discreet time points: 3 hour, 48 hour, and 72 hour. At each time point, a temperature challenge was introduced using the Environmental Control system in AxIS 2.3. Briefly, at the end of the associated 30 minute baseline recording, the temperature was increased at 0.5°C/min until 42 °C and held at 42°C for 5 minutes, while recording changes in activity. To determine the pharmacological responsiveness to Nav1.7 (Huwentoxin-IV 30 nM), Nav1.8 (A-803467 300 nM), and Nav1.1/1.3 (ICA-121431 23 nM) blockers, respective compounds were added to treatment groups that were previously incubated with IL6 or vehicle for 72 hours followed by a 10 minute recording session.

3.2.5 Immunostaining and image analysis

Post treatment with either IL-6 or vehicle, DIV13-15 primary DRG cultures were fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT) followed by a triple wash with ice-cold PBS. Cells were permeabilized by 0.3% Triton X-100 for 30 minutes at RT. Following
permeabilization, non-specific binding sites were blocked with 2% normal goat serum (NGS) for 30 minutes, and samples were incubated with primary antibodies [Anti-NeuN (1:500, Abcam), Anti-Nav1.7 (1:200, Abcam), Anti-Nav1.8 (1:500, Abcam)] for 18 hours. The following day, species specific secondary antibodies were incubated for 1 hour at RT. Confocal images were acquired at 20X magnification using an inverted Nikon Eclipse Ti (Nikon, Tokyo, Japan) microscope. To determine the percentage of Nav1.7 and Nav1.8 expressing neurons in IL-6 or vehicle treated groups, a minimum of three regions of interest (ROI’s) were selected from a total of n=6 wells (n = 3 IL-6, n = 3 vehicle). Multi-channel images were processed in ImageJ (NIH). Briefly, a user defined threshold was applied to all channels and neuronal somata was manually counted based on maximum intensity projections. For the normalized mean fluorescence intensity of associated Nav1.7 and Nav1.8 expression post-treatment with IL-6 (or vehicle), circular ROI’s were drawn across a minimum of n = 60 somata, and the signal intensity of Nav1.7 and Nav1.8 was measured using ImageJ (NIH). For all experiments, only presumptive nociceptors were selected (diameter ~ 20 µm), and cells were identified as positive for Nav1.7 and/or Nav1.8 if the mean fluorescence intensity was ≥2.5x then the average intensity of the background.

3.2.6 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA from DIV13 DRG cultures treated with IL-6 (n= 6 wells) or vehicle (n= 6 wells) was isolated and purified using the GeneJET RNA Purification Kit (Cat#: K0731, Thermo Scientific). RNA concentration was determined using Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer. 1 µg of total RNA was reverse transcribed to cDNA using ImProm-II Reverse Transcription System (Cat#: A3800, Promega), according to the manufacturer's
instructions. The cDNA was amplified by a set of sequence-specific primers (Custom Oligos Service, Sigma Aldrich) and detected with SYBR Green reagent (Cat#: 1725120, BioRad) using a CFX Connect Real-Time PCR Detection System (Cat#: 1855200, BioRad). The qRT-PCR reactions were performed in triplicate. The cycling conditions included a denaturing step at 94 °C for 1 min, followed by annealing at a primer-specific temperature for 1 min, and elongation at 72 °C for 45 s. The primer sequences used are as follows: Nav1.7 (Scn9a) forward TCCTTTATTTCATAATCCCAGCCTCAC, Nav1.7 (Scn9a) reverse GATCGGTTCCGTCTCTCTTTGC, Nav1.8 (Scn10a) forward ACCGACAATCAGAGCGAGGAG, Nav1.8 (Scn10a) reverse ACAGACTAGAAATGGACAGAATCACC, Gapdh forward CTAGGACTGGATAAGCAGGGC, Gapdh reverse GCCAAATCCGTTCACACCCGA. Fold changes in gene expressions were quantified using the ΔΔCt method, where Ct is defined as the threshold cycle (Livak and Schmittgen, 2001). Values were normalized to Gapdh.

3.2.7 Statistical Analysis

All statistical tests and visualization of data sets were carried out in OriginPro 2019 (OriginLab, Northampton, MA). To compare differences between two sample proportions, a test of proportions z-test was used. A 2-sample t-test was performed to compare means of two groups to test the hypothesis of no difference between treatment and vehicle groups. For all conditions, a p value < 0.05 was considered as statistically significant. All descriptive statistics are represented as mean ± standard error of the mean (SEM).
### 3.3 Results

#### 3.3.1 IL-6 and NGF increase spontaneous and stimulus evoked excitability

To assess changes in excitability of adult DRG neurons *in vitro* in response to known inflammatory mediators, DIV13-DIV15 cultures were treated with a combination of 100 ng/ml IL-6 and 100 ng/ml NGF or vehicle and spontaneous and evoked activity were assessed at 3 hour, 48 hour, and 72 hour time periods. Combinatorial treatment of IL-6 and NGF led to increased instances of spontaneously active neurons in terms of active electrodes (IL-6+NGF: 3-, 48-, 72 hour: 13.5%, 14.8%, 15.9%; Vehicle: 3-, 48-, 72 hour: 7.8%, 7.3%, 7.6%; Test of proportions z-test, $p < 0.05$, Figure 3.1A.) and greater number of spikes compared to vehicle treated groups (IL-6+NGF: 3-, 48-, 72 hour: 0.94 ± 0.17, 1.02 ± 0.14, 1.39 ± 0.15; Vehicle: 3-, 48-, 72 hour: 0.29 ± 0.10, 0.41 ± 0.19, 0.59 ± 0.16; Two-sample t-test, $p < 0.05$; Figure 3.1B.).

To determine whether nociceptor-evoked response to noxious temperature is potentiated by IL-6 and NGF, we measured the percentage of temperature responsive electrodes (mean firing rate $\geq$ 2 x baseline).

![Figure 3.1](image-url)

Figure 3.1: IL-6 and NGF induce acute and persistent increase in excitability and thermally-mediated evoked responses in adult DRG neurons *in vitro*. (A) Percentage of active electrodes post treatment with 100 ng/ml IL6 + 100 ng/ml NGF or vehicle over 3-72 hour period. (B) Log spike count per well post treatment over 3-72 hour period. (C) Percentage of temperature responsive electrodes post treatment over 3-72 hour period. Responsiveness to temperature was defined as an electrode exhibiting a mean firing rate (MFR) of $\geq$ 2x that of baseline. Temperature stimulus was introduced in the form of base plate heating to a
2x of baseline at 42°C) and compared treatment to vehicle groups. We observed a significant increase in thermally evoked responses in IL-6 and NGF treated groups at 3-72 hour periods (IL6+NGF: 3-, 48-, 72- hour: 11.9%, 13.3%, 15.9%; Vehicle: 3-, 48-, 72 hour: 7.0%, 7.6%, 8.8%, Test of proportions z-test, p < 0.05; Figure 3.1C.). These data suggest that IL-6 and NGF elicit significant changes in excitability and may mediate thermal hypersensitivity at acute and persistent time periods in vitro.

**3.3.2 IL-6 and NGF-mediated hyperexcitability is attenuated by Nav1.7 and Nav1.8 antagonists**

To determine if sensitization by NGF and IL-6 requires Nav1.7 and Nav1.8, adult DRG neurons incubated with IL-6 and NGF for 72 hours were treated with 30 nM huwentoxin IV (HWTX – IV) (Nav1.7 blocker) or 300 nM A-803467 (Nav1.8 blocker). Additionally, 23 nM ICA-121431,
a potent inhibitor of central nervous system (CNS) specific Nav1.1/1.3 channel subtypes, was administered as a negative control. At nanomolar concentrations, both HWTX-IV and A-803467 were potent at inhibiting spontaneous DRG activity (HWTX-IX: 83 ± 9% inhibition, n = 6 wells; A-803467: 65 ± 9%, n = 6 wells, p < 0.05 compared to negative control, ICA-121431; Figure 3.2 A, C.). In contrast, inhibition of CNS specific Nav1.1/1.3 channel subtypes by ICA-121431 had little to no effect on spontaneous DRG activity (Figure 3.2A, C.). To further elucidate subtype-selective modulation of nociceptive-specific ion channels in our DRG tissue preparation, DIV21 embryonic cortical networks were administered with HWTX-IV, A-803467, and ICA-121431. As expected, ICA-121431 caused strong inhibition of spontaneous cortical activity (92 ± 3% inhibition, n ≥ 3 wells), whereas A-803467 failed to significantly reduce activity (p = 0.10 compared to vehicle DMSO, n ≥ 3 wells; Figure 3.2B.). Additionally, both HWTX-IV and A-803467 reduced 35% of previously temperature responsive channels to 20% and 4%, respectively in DRG cultures; both statistically significant reductions (p = 0.03 and p < 0.0001, respectively, Test of proportions z-test; Figure 3.3A-D). Taken together, these data indicate that the pharmacological profiles of Nav1.7 and Nav1.8 are maintained in adult DRG neurons in
We find that both channels contribute to IL-6 and NGF-mediated increase in hyperexcitability and stimulus evoked responses.

3.3.3 Nav1.7/1.8 expression is unchanged after 72 hour IL-6 and NGF treatment

Next, we asked whether the observed increase in excitability induced by IL-6 and NGF treatment is due to increased accumulation of Nav1.7/1.8 protein. DIV 13-15 adult DRG neurons were incubated with 100 ng/ml IL-6 and 100 ng/ml NGF for 72 hours, and Nav1.7/1.8 expression was measured via immunostaining (Figure 2.4A.). Small DRG neurons (≤ 30 μm) with a signal intensity of Nav1.7/1.8 above a define threshold (≥2.5x mean background intensity) was 52%
and 65% after IL-6 and NGF treatment (Figure 3.4B). The percentage of Nav1.7/1.8 positive neurons in treatment groups did not increase compared to vehicle (Vehicle, Nav1.7: 51%, Nav1.8: 72%; p = 0.79, p = 0.03, respectively; Figure 3.4B). Furthermore, quantification of the mean fluorescence intensity revealed a similar expression of the sodium channel subtypes in treatment and vehicle groups (IL6+NGF, Nav1.7: 3.16 ± 0.41 A.U., Nav1.8: 3.50 ± 0.18 A.U., Vehicle, Nav1.7: 3.75 ± 1.21, Nav1.8: 4.20 ± 1.71, n = 3 wells, p = 0.46, p = 0.52, respectively; Two sample t-test). The observed absence of modulation of ion channel abundance post treatment with IL-6 and NGF was further supported by mRNA expression studies. Using quantitative real-time PCR (qRT-PCR) (Figure 2.4C), we found that Nav1.7/1.8 transcripts were not significantly different between treatment and vehicle groups (IL6+NGF, Nav1.7: 0.51 ± 0.38, Nav1.8: 1.46 ± 0.18, Vehicle, Nav1.7: 1.19 ± 0.1, Nav1.8: 1.08 ± 0.02, n = 6 wells, p = 0.2, p = 0.09, respectively; Two sample t-test; data not shown). Therefore, the data suggest that both

Figure 3.4: Nav1.7/1.8 expression is maintained in adult DRG neurons in vitro and is unchanged by treatment with IL-6 and NGF. (A) Representative immunofluorescence images of adult DRG neurons stained for Nav1.7 (top left; red), Nav1.8 (top right; green), and neuronal marker NeuN (bottom left; blue). (B) Percentage of Nav1.7, Nav1.8, and Nav1.7/1.8 co-expressing neurons following exposure to IL-6 and NGF for 72 hours or vehicle. (C) mRNA expression of Nav1.7 and Nav1.8 normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) following exposure to IL6 and NGF for 72 hours or vehicle. Errors are expressed as mean ± STD.
mRNA and protein expression of Nav1.7/1.8 were unchanged after acute (72 hours) treatment with IL-6 and NGF. We conclude that expression levels are unlikely to explain increased activity of Nav1.7/1.8 after sensitization by NGF and IL-6.

3.4 Discussion

The present study demonstrates that the expression and pharmacological profiles of VGSC subtypes, Nav1.7 and Nav1.8, are maintained in adult DRG neurons in vitro and may partially contribute to the acute and persistent changes in excitability and thermally evoked responses post treatment with IL-6 and NGF. Additionally, we have demonstrated that such maladaptive hyperexcitability is readily abrogated by pharmacological blockade of Nav1.7 and Nav1.8. The observed increase in excitability is unlikely related to regulation of Nav1.7 and Nav1.8 expression as the mRNA and protein levels are unaltered after sensitization.

The role of Nav1.7 and Nav1.8 in inflammatory pain and thermal hyperalgesia has been previously demonstrated in vivo using pre-clinical models. For example, ablation of the gene that encodes Nav1.7, Scn9a, (Nassar et al. 2004) or conditional knock out Nav1.7-expressing Nav1.8 neurons reduced inflammation-mediated pain behavior induced by formalin and Complete Freund’s Adjuvant (CFA). Furthermore, the presence of thermal hyperalgesia was diminished in Nav1.7 null-mice Similarly, Nav1.8 null mice display attenuation of NGF-induced thermal hyperalgesia (Kerr et al. 2001). Consistent with in vivo findings, we report that pharmacological antagonists of Nav1.7/1.8 led to a decrease in thermally-mediated evoked responses. This result is perhaps surprising given the pleiotropic nature of the mediators given their effects on cap-dependent and Poly(A)-dependent translation(Barragán-iglesias et al. date unknown; Melemedjian et al. 2010; de la Peña, June Bryan I; Song, Jane J;Campbell 2019). In contrast to
several prior reports, we find that NGF and IL-6 have no impact on the expression of Nav1.7/1.8. CFA and NGF result in an increase in the expression of Nav1.7 and Nav1.8 \textit{in vivo} (Gould et al. 1998; Tate et al. 1998; Dib-Hajj et al. 1999; Iii et al. 2004; Strickland et al. 2008) (Iii and Levinson 2000). However, the time of administration (24 hr – 1 week) and concentration (5 µg NGF) differ from the present \textit{in vitro} exposure (100 ng/ml NGF). Moreover, the inflammatory state induced by CFA may include a myriad of inflammatory cascades and mediators including serotonin, histamine, and adenosine, which may not be recapitulated \textit{in vitro}. While chronic exposure to inflammatory mediators has been shown to modulate protein accumulation, we favor a model wherein the acute effects of nociceptive sensitivity are due to direct activation of the ion channel isoforms. For example, it has been shown that 100 ng/ml acute incubation (30 min) of NGF with DRG neurons increased an initial response to capsaicin and brief pre-treatment with anisomycin causes rapid increased in Nav1.8 current density in a p38-dependent manner (Shu and Mendell 1999; Hudmon et al. 2008). This direct activation, presumably due to phosphorylation of TRPV1 via MAPK, is fully consistent with the present findings where acute modulation of spontaneous activity was readily observed after a 3 hour treatment with IL6 and NGF. Additionally, it has been shown that addition of IL-6 to cultured nociceptors result in a direct association of ERK and Nav1.7, modulating hyperexcitability by direct phosphorylation of Nav1.7 (Stamboulian et al. 2010; Yan et al. 2012; Black et al. 2018).

The present findings also highlight the utility of adult DRG neurons as a tissue source for screening potential therapeutics that specifically block Nav1.7 and Nav1.8. Prior studies have utilized embryonic DRG tissue which may not be amenable to study NGF-induced sensitization, as high doses of the neurotrophic factor are strictly required to maintain viability in culture.
Moreover, the expression profiles of Nav1.7 and 1.8 are significantly different among adult and embryonic tissue (Gumy et al. 2011). Notably, culture of dissociated sensory neurons and the maintenance of gene expression profiles in vitro is non-trivial. For example, Roland et al. (1997) reported that dissociated cultures of embryonic DRG neurons expressed trkA and trkC receptors dissimilar to those found in intact DRG explants, suggesting differential expression of genes that are repressed under physiological conditions which may manifest in vitro (Roland H. Friedel, Harald SchnurRch, Jutta Stubbusch 1997). Similar dysregulation of receptor expression in vitro has been observed in aortic endothelial cells (Brunner and Kukovetz 1991) and neuronal cells (Roskoski et al. 1985). In contrast, the mRNA expression profiles of Nav1.7 and Nav1.8 in the present study were maintained for at least 13 days in vitro, and are consistent with profiles in acutely dissociated adult mouse DRG neurons shown previously (Chang et al. 2018). Additionally, our histological study demonstrated a high percentage of Nav1.8 expressing neurons in combination with Nav1.7 (60-70%) which is consistent with previous observations in DRG tissue (Dib-Hajj et al. 2010; Black et al. 2018; Patil et al. 2018), wherein 88% of cultured DRG tissue were described as nociceptive (CGRP + IB4 positive). This suggests that our present culture methods provide a reliable platform for the study of Nav1.7 and Nav1.8 for acute and chronic measurements. In conclusion, the data suggest a clear role of Nav1.7 and Nav1.8 in IL-6 and NGF mediated hyperexcitability. They also highlight the utility of phenotypic screens using adult DRG neurons subjected to inflammatory mediators that promote chronic changes in physiological activity.
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CHAPTER 4

ADAPTATION OF ROBUST Z’-FACTOR FOR ASSAY QUALITY ASSESSMENT IN MICROELECTRODE ARRAY BASED SCREENING USING ADULT DORSAL ROOT GANGLION NEURONS*

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Abstract

Background

Cell-based assays comprising primary sensory neurons cultured in vitro are an emerging tool for the screening and identification of potential analgesic compounds and chronic pain treatments. High-content screening (HCS) platforms for drug screening are characterized by a measure of assay quality indicator, such as the Z’-factor, which considers the signal dynamic range and data variation using control compounds only. Although widely accepted as a quality metric in high throughput screening (HTS), standard Z’-factor are not well-suited to indicate the quality of complex cell-based assays.

New Method

The present study describes a method to assess assay quality in the context of extracellular recordings from dorsal root ganglion (DRG) sensory neurons cultured on multi-well microelectrode arrays. Data transformations are applied to electrophysiological parameters, such as electrode and well spike rates, for valid normality assumptions and suitability for use as a sample signal. Importantly, using transformed well-wide metrics, a robust version of the Z’-factor was applied, based on the median and median absolute deviation, to indicate assay quality and assess hit identification of putative pharmacological compounds.
Results

Application of appropriately scaled data and robust statistics ensured insensitivity to data variation and approximation of normal distribution. The use median and median absolute deviation of log transformed well spike rates in computing the Z’-factor revealed a value of 0.61, which is accepted as an “excellent assay.” Known antagonists of nociceptor-specific voltage-gated sodium ion channels were identified as true hits in the present assay format under both spontaneous and thermally stimulated conditions.

Comparison with Existing Methods

The present approach demonstrated a large signal dynamic range and reduced sensitivity to data variation compared to standard Z’-factor used widely in HTS.

Conclusion

Overall, the present study provides a statistical basis for the implementation of a HCS platform utilizing adult DRG neurons on microelectrode arrays.

Key words: microelectrode arrays, dorsal root ganglion, Z’-factor, assay quality, high content screening

4.1 Introduction

The development of effective screening paradigms for chronic pain treatment and analgesic lead discovery is an active area of research (Black et al. 2019). Despite detailed understanding of several mechanistic pathways, the discovery of effective, non-addictive first-in-class analgesics remain limited (Taneja et al. 2017). This may be due to contemporary methods of drug discovery, which rely primarily on molecular target-based assays wherein a specific receptor or target is identified, modulated, and validated in an animal model. However, target-based
strategies have resulted in limited yield for early drug discovery processes (Sams-Dodd 2005, 2013; Woolf and Ma 2007; Munos 2009; Sapunar et al. 2012; Krames 2014). An alternative approach, high-content phenotypic screening (HCS), leverages physiologically relevant functional and/or morphological endpoints, and is widely used in target validation and lead optimization; providing insight into complex biological processes underlying a specific disease phenotype (Giuliano et al. 1997; Arraste and Finkbeiner 2005; Dragunow 2008; Bickle 2010; Zanella et al. 2010; Jain et al. 2012). This type of approach is especially suitable for screening candidate therapeutics for chronic pain; where signaling complexity, due to a multitude of biomolecular pathways which act in concert, pose a significant challenge for therapeutics targeting specific molecular pathways. However, a significant challenge in translating HCS data is establishing the overall quality of a given assay (Azegrouz et al. 2013).

Our group is exploring the utility of cell-based assays using dorsal root ganglion (DRG) neurons in vitro. Nerve injury or inflammation may cause enhancement in DRG neuron excitability (Woolf and Ma 2007; Sapunar et al. 2012; Krames 2014). Importantly, DRG neuron hyperexcitability may be induced in vitro by incubating cells with proinflammatory cytokines and/or neurotrophic factors and be measured in the form of all-or-nothing action potentials using substrate-integrated microelectrode arrays (MEAs) (Sorkin et al. 1997; Junger and Sorkin 2000; Ferrari et al. 2014; Black et al. 2018). While recent pain-related HSC findings have been reported, assessment of assay quality has been limited in most cases.

One measure of assay quality, defined by Zhang et al. (1999), is the $Z'$-factor, which has been widely adopted for high throughput screening (HTS). In the present study, we examined the $Z'$-factor metric for cell-based screening assays using adult DRG neurons cultured on MEAs.
Normality assumptions, implicit in the use of the standard Z’-factor, are assessed for microelectrode-wise and well-wide spiking metrics and data transformation approaches are applied. We show a robust form of Z’, based on median values and median absolute deviations, computed for background and temperature-evoked signal using lidocaine treatment as a negative control. A hit identification method is described using pharmacological compounds with a known mechanism of action under baseline and thermally-evoked conditions.

4.2 Experimental Methods

4.2.1 Primary adult DRG culture

All surgical procedures were carried out in accordance with the University of Texas at Dallas’s Institutional Animal Care and Use Committee. Institute for Cancer Research (CD-1) male mice (4-6 weeks old; Envigo) were utilized for DRG tissue extraction as described in detail previously (Sleigh, Weir, and Schiavo 2016). Briefly, mice were deeply anesthetized with 3% isoflurane and euthanized via cervical dislocation. Spinal columns were extracted, hemisected, and DRGs were isolated from central/peripheral roots and stored in ice-cold Hanks Balanced salt solution (HBSS). Tissue was dissociated as previously described with minor modifications (Black et al. 2018b). An enzyme buffer consisting of 2 mg/ml collagenase and 0.1 mg/ml DNase was added to pooled tissue and allowed to incubate at 37 °C for 40 minutes. After 40 minutes, 100 μL of 1X papain solution was added and the tissue was gently triturated using a fire polished pasture pipette. The cells were isolated via centrifugation (300 g for 10 minutes) and resuspended in fresh medium consisting of DMEM/F-12+glutamax + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin (PS) + 5 ng/ml GDNF. 10,000 viable neurons were plated on multi-well MEAs (Axion BioSystems, Atlanta, GA) pre-treated with 50 μg/ml poly-D-lysine (overnight)
followed by surface coating of 20 μg/ml laminin (2 hours). Cultures were maintained at 37°C, 10% CO2, and 95% humidity and medium was exchanged every 48 hour. After non-neuronal populations reached confluence of approximately 90%, medium was supplemented with mitotic inhibitors uridine (17.5 μg/ml) and 5-fluoro-2’-deoxyuridine (7.5 μg/ml) for the remainder of the culture.

4.2.2 Spontaneous and Evoked extracellular recordings using in vitro microelectrode arrays

Spontaneous extracellular recordings were performed with 12-well plate MEAs (Axion Biosystems, Atlanta, GA) using the Axion Maestro MEA recording system (Axion Biosystems, Atlanta, GA) as described previously (Black et al. 2018b). Briefly, extracellular voltage recordings were carried out at 12.5 kHz sampling rate from a total of 768 available substrate integrated microelectrodes. Continuous data were filtered using a 1-pole Butterworth band pass filter (200-3000 Hz) and individual spikes were detected using a 5.5 σ adaptive threshold method. Thermally-evoked extracellular recordings were performed using the calibrated stage plate heater. Briefly, the temperature was increased from 37°C to 42°C at approximately 0.5 °C/min and held at 42°C for an interval of 5 min while evoked activity was recorded.

4.2.3 Pharmacology and screening

All pharmacological compounds were reconstituted in either complete medium, water, or DMSO at a stock concentration of ≥100X. Before addition of compounds, a baseline recording of 30 minutes was acquired. For exposure to IL-6 and NGF or vehicle (water), bolus volumes were added simultaneously to treatment groups (100 ng/ml IL-6 + 100 ng/ml NGF) for 48 hours. To determine the signal of the negative control (background) data, >50 μM lidocaine was
administered to respective treatment groups. The negative control is defined as the control compound which produces the minimum signal (Zhang, Ji-Hu, Chung, Thomas, Oldenberg 1999; Sui and Wu 2007). To determine the pharmacological responsiveness to Nav1.7 (Huwentoxin-IV 30 nM), Nav1.8 (A-803467 300 nM), and Nav1.1/1.3 (ICA-121431 23 nM) blockers, respective compounds were added to treatment groups that were previously incubated with IL6+NGF or vehicle for 48 hours followed by a 10 minute recording session.

4.2.4 Data Analysis

Microelectrodes were considered for analysis when a mean firing rate of at least 1 spike/min was recorded during a 30 minute baseline recording. Mean firing rate (Hz) and total spikes/well were determined using NeuroExplorer (Nex Technologies, Madison, AL) and AxIS Metric (Axion Biosystems, Atlanta, GA) as described previously (Black et al. 2018b). All statistical tests and visualization of data sets were performed using OriginPro 2019 (OriginLab, Northampton, MA). A two-sample t-test was used to compare means of two groups to test the null hypothesis between treatment and vehicle groups.

4.3 Results

4.3.1 Normality Assumption and Log Transformation

The design of a phenotypic, cell-based assay, requires consideration of the physiological end point which is relevant to pathology. Neuronal excitability is an especially important end point for chronic pain, since maladaptive changes, particularly in the context of neuropathic and inflammatory states, alter the bioelectrical properties of DRG neurons (Basbaum et al. 2009; Krames 2014, 2015). Such alterations result in a decrease in the threshold and/or increase in the magnitude of responsiveness to sensory stimuli triggering increased generation of action
Adult DRG neurons cultured on MEAs exhibit spontaneous extracellular action potentials. Typically, recordings from embryonic DRG neurons exhibit stable baseline activity at DIV 11-14, consisting of at least 50% active microelectrodes per well (16 microelectrodes/well – 48 wells) (Newberry et al. 2016). Similar observations have been made for adult DRG neurons, wherein peak activity is observed at DIV9 and stable for at least 21 days, with an active microelectrode yield of 25% per well. (Black et al. 2018). Figure 4.1(b) shows filtered continuous extracellular recordings from three representative microelectrodes. A data metric which is readily extractable from such recordings is the mean firing rate (MFR), defined as the number of spikes over a specified time period. However, the MFR of DRG neurons shows substantial variability when compared to electrical activity recorded from cortical networks (Charkhkar et al. 2014b) and spinal motor neurons (Black et al. 2017), and may represent a skewed data set. A bias towards low MFR is consistent with previous in vitro observations made by Newberry et al. (2016) and Black et al. (2018), as well as with the observed spontaneous firing of nociceptors from the L4/L5 DRG in vivo (Djouhri 2006; Djouhri et al. 2015). This lack of normality may reflect the intrinsic variance associated with a heterogeneous cell population (Woolf and Ma 2007).
Since the Z’-factor has been developed under the assumption of a normal distribution and best reflects assay quality for data sets which do not violate normality (Sui and Wu 2007), we first sought to assess the distribution of microelectrode MFR. The frequency distribution (n = 792 active microelectrodes (> 1 spike/min) from n = 5 plates) of adult DRG neuron cultures at day in vitro (DIV) 15-17 revealed an excessively right skewed distribution (skew = 5.85, kurtosis = 55.7) (p = 0, Shapiro-Wilk normality test) which significantly deviated from the standard normal distribution. On performing a log transformation of the microelectrode MFR, the data approximates a normal distribution on a log scale, however, the hypothesis that the sample was

![Figure 4.2](image)

Figure 4.2: Well-wide metrics assume a normal distribution on a log scale (a) Frequency histogram of total spike count/well from n = 5 separate cultures of adult DRG neurons consisting of n =53 wells. Each well consists of a total of 64 available recording electrodes. (b) Normal Q-Q plot of total spike/count (c) Log transformed spikes/well (d) Normal Q-Q plot of the transformed well-wide metrics
drawn from a normally distributed population was still rejected (p = 1.99 E^{-15} Shapiro-Wilk normality test), suggesting an overly-dispersed data set.

An alternative approach involves the use of the total spike count/well as a sample signal. Essentially, well-wide metrics represent the total combined signal from all available active microelectrodes in a given well (64 microelectrodes/well) and may be less sensitive to per microelectrode firing rate differences. Similar to the non-transformed microelectrode MFR described previously, the total spike count/well assumed a non-normal (p = -5.78E-8, Shapiro-Wilk Normality test) and an excessively right skewed distribution (skew = 1.71, kurtosis = 2.75) (Figure 4.2(a), (b)). However, the transformed data assumed a normal distribution on the log scale (skew = - 0.30, kurtosis = - 0.07; p = 0.157, Shapiro-Wilk Normality Test) (Figure 3.2(c)). This is readily apparent from the comparison of the standard normal distribution and the well-wide spike count distribution, wherein, the data values and the reference normal distribution have a linear relationship (Figure 4.2(d)). The data suggest that transformed well-wide metrics may serve as more appropriately scaled data for assay characterization and hit identification.

4.3.2 Robust form of the Z’-factor

To assess the potential of MEA-based DRG recordings as a screening paradigm, the widely used Z’-factor (Zhang et al. 1999), based on mean and standard deviations (STD) was tested as an assay quality indicator. Both transformed microelectrode MFR and transformed spike count/well signals were compared to lidocaine-treated wells (>50 µM) as the negative control. In Figure 4.3(a), representative MFR of active microelectrodes (n > 200 active microelectrodes) are plotted and compared against lidocaine treated (background) MFRs. The Z’-factor computed for the transformed microelectrode MFR sample signal revealed a Z’-factor value of -1.7. A Z’ value <
0 indicates an assay which cannot be used for any deterministic identification of compounds, since there is no separation band and the distributions of the sample and background overlap (Figure 4.3(a)). In contrast, well-based metrics revealed a value of 0.23. The use of well-wide metrics result in increased signal dynamic range and lowered data variation (Figure 4.3(b)). However, this represents a “double assay” (0.5 < Z < 0, separation band is too small) and may be ineffective for use in a screening paradigm.

Although the well-wide sample and negative controls illustrate a large signal dynamic range as observed previously, the Z’-factor is extremely sensitive to data variability. One approach is the use of robust statistics which include measures such as median and the median average deviation (MAD) and is commonly used in HTS data analysis for its insensitivity to outliers. To this end, a robust form of Z’-factor was applied for well-wide metrics using the median and MAD of the transformed spike count/well:

Figure. 4.3: The application of Z’-factor as an indicator of assay quality to sample signals using transformed electrode MFR and transformed well-wide metrics (a) Sample and background (lidocaine > 50 µM) signal of log transformed electrode MFR (n = 204 active electrodes). (b) Sample and background signal of log transformed spike count/well (n=53 wells, 64 electrodes/well).
\[ Z'_M = 1 - \frac{3MADc^+ + 3MADc^-}{|Mc^+ - Mc^-|} \]

Where \(MADc^+\) and \(MADc^-\) refer to the median average deviation of the positive and negative controls and \(Mc^+\) and \(Mc^-\) refer to the median of the positive and negative controls, respectively. The robust \(Z'\) (\(Z'_M\)) revealed a value of 0.61 for well-based metrics, which is accepted as an “excellent assay” (Zhang et al. 1999).

### 4.3.4 The effect of well-exclusion criteria on \(Z'\)-factor

Considerations regarding the number of active electrodes per well are imperative, since the collection of microelectrode derived firing rates directly contribute to the total spike count per well, and therefore, the acquired sample signal. To determine the minimum number of active electrodes required per well or an active electrode criterion, first a frequency histogram of the number of active microelectrodes as a function of well percentages was determined (\(n = 5\) cultures, \(n = 52\) wells) (Figure 3.3). On further analysis on the relationship of an exclusion criteria and the effect on the \(Z'_M\) factor, a score of > 0.5 (“excellent assay”) was observed at a minimum requirement of 2 active electrodes/well (Figure 3.4). While the \(Z'_M\) factor continually increases and trends towards an “ideal assay” with more active microelectrodes, the number of available wells drops significantly to impact assay practicality (Figure 3.4). Based on prior studies of DRG cultures on MEAs, where stable baseline activity typically comprises of 25-50% of active microelectrodes (Newberry et al. 2016; Black et al. 2018), the data suggest that 4 active microelectrodes/well is suitable as the sample signal, provides a \(Z'_M\) factor of > 0.5, and may
forecast the use of 48-well plate formats, with 16 microelectrodes/well for assay implementation.

4.3.5 Hit detection and identification methodology

The main goal of screening assays is to be able to identify putative “hits”. Such hits in a screen represent outliers which have large differences from the average sample signal and have a desired physiologically relevant mode of action to the pathology under investigation. Typically, conventional cut-offs for assays configured for inhibition/antagonist screening set thresholds of 3 STD below the mean sample signal or the robust version of 3 MAD for hit detection which represents a type I error rate (false positive) of 0.0013 (Wu et al. 2008; Goktug et al. 2013). Since the $Z'_M$ leverages the use of the median as an estimate of the signal, intuitively, a hit threshold can be defined as a compound with a signal below 3 MAD for an inhibition/antagonist type assay which aims to detect compounds that reduce the

Figure 4.4: Assessment of well exclusion criteria and the effect on the quality of the assay. $Z'_M$ factor is plotted as a function of the electrode exclusion criteria and the frequency of encountering a given number of active electrodes per well.
observed hyperexcitability of DRG neurons. In addition to the selection of a hit limit, the quality of the assay also affects confirmation rates. We have previously discussed the use of exclusion criteria to improve the $Z'_M$ factor which directly increases the median value and lowers the absolute deviation. Another complementary approach is to introduce an external stimulus to induce neuronal excitability which may improve signal intensity compared to stochastic spontaneous activity. Adult DRG neurons have been previously demonstrated to undergo IL6-mediated increase in thermally evoked responses at noxious temperature range, an effect mediated by vanilloid receptor, TRPV1 (Basbaum et al. 2009; Black et al. 2018). Therefore, we sought to determine the hit quality under spontaneous and thermally stimulated conditions.

Preferentially expressed voltage gated sodium ion channel subtype Nav1.7 and Nav1.8 were inhibited with known ion channel blockers in sensitized adult DRG cultures. Briefly, DRG

Figure 4.5: Identification of putative hits with known mechanism of action in spontaneous and thermally stimulated adult DRG cultures. (a) Representative raster plot from 4 active electrodes at 37°C (spontaneous activity) and 42°C (thermally evoked). (b) Detection of hits in the assay at baseline spontaneous activity (37°C) and (c) thermally evoked (42°C) using 30 nM Huwentoxin IV and 300 nM A-803467 to inhibit nociceptive specific sodium ion channel Nav1.7 and Nav1.8, respectively. Red dashed lines indicate hit detection thresholds. The upper limit corresponds to median + 3MAD (5.38) and lower limit corresponds to median – 3MAD (2.52).
neurons pre-incubated for 72 hrs with pro-inflammatory cytokines, IL-6 and NGF, were treated with 30 nM Huwentoxin IV (HWTX-IV) (Nav1.7 blocker) or 300 nM A-803467 (Nav1.8 blocker) under baseline (37°C) and noxious (42°C) temperature range. Calibrated stage plate heating to 42°C induced reliable and reproducible increase in the firing rates of the available active microelectrodes (Figure 4.4(a)). At nanomolar concentrations, HWTX-IV and A-803467 were potent at inhibiting spontaneous DRG activity (HWTX-IX: 83 ± 9% inhibition, n = 6 wells; A-803467: 65 ± 9%, n = 6 wells, p <0.05 compared to negative control (DMSO)) and identified as positive hits in the assay under spontaneous and thermally-evoked formats (Figure 4.4(b), (c)), as the median spike count/well for both compounds were reduced beyond the defined lower cut-off from the sample signal (37 °C: HWTX M = 0.48, A-803467 M = 1.72, 42°C: HWTX M = 0.69, A-803467 = 1.27). Although both spontaneous and thermally-evoked assay formats accurately identified hits, it is important to note the latter resulted in a $Z'_M = 0.74$ above that which was observed at 37 degrees C ($Z'_M = 0.61$). Interestingly, we observed a decrease in the MAD in thermally-stimulated conditions which may be due to a greater contribution of nociceptive-specific subtypes which are primarily temperature sensitive.

### 4.4 Discussion

Primary DRG neuron-based assays may have a significant impact in identifying novel therapeutic compounds for ameliorating chronic pain conditions (Melli 2014). Under normal physiological conditions, DRG neurons exhibit limited spontaneous activity *in vitro* and *in vivo*. However, nerve-injury and inflammation cause cells to become hyperexcitable; playing a major role in the generation and maintenance of chronic pain. Therefore, *in vitro*
phenotypic assays using peripheral sensory neurons which leverage cellular excitability as an endpoint are a useful tool for identifying compounds which attenuate DRG hyperexcitability. However, a significant challenge in the development of phenotypic-screening assays using DRG neurons is the lack of quantitative statistical approaches to validate the quality of the assay which is an indispensable step in assay validation (Chai et al. 2015). In the present study, we describe a robust statistical approach to determine the assay quality of a MEA-based adult DRG neuron screening platform.

First, we assessed the distribution of the microelectrode mean firing rates and well spike counts as sample signals to determine whether the normality assumption, implicit in the widely used and accepted assay quality indicator Z’-factor, was maintained. Both raw sample values of microelectrode-wise and well-wide metrics assumed excessively right skewed distributions. Data transformation schemes, based on log transformations, generated a normal distribution for well-wide metrics which was further used as an endpoint for computing the Z’-factor, as described previously by Zhang et al. (1999). Standard Z’-factor, based on the mean and STD, produced a poor Z’-factor value (<0.5). In contrast, a more robust version, consisting of the median and MAD parameters of the signal, revealed a Z’-factor of 0.6, a range suggesting an excellent assay; described as having a large signal dynamic range and low variance.

To further establish a standard for DRG/MEA-based screening platforms, we investigated the dependency of Z’ factors on minimum active microelectrode criteria. In short, we asked the questions “How many active microelectrodes did we typically observe per well given a set of culture conditions?” and “How does pooling data from wells with different active
microelectrode numbers effect our assay quality?” A minimum of 2 active microelectrodes per well were required to produce a Z’-factor > 0.5, and therefore, only 30% of the data acquired were omitted (where a well showed <2 active microelectrodes). This is especially important, since it allows the use of scaled-up multi-well MEA plates; for example, 48-well plates which consist of 16 microelectrodes per well. The MEA-based assay was robust and reproducible at detecting nociceptive-specific sodium ion channel antagonists as true hits under stable baseline (37°C) and thermally evoked stimuli (42 °C). Prior studies developing sensory neuron-based phenotypic screens have used chemical and physical antagonists to evoke membrane depolarization when screening chemical libraries (Rana et al. 2017; Stacey et al. 2018b; Martínez et al. 2019). For example, Stacey et al. (2018) employed veratridine, a non-specific sodium channel agonist to evoke neuronal depolarization in iPSC-derived sensory neurons. However, large concentrations of veratridine were required to induce neuronal excitability, which directly affected the tested potency of sodium channel blockers in the assay and, therefore, limited the classes of nociceptor-specific compounds which may be detected. Electrical field stimulation has been used as an alternative approach to induce cellular excitability and record intracellular calcium transients, demonstrating stable assays with Z’ > 0.5 (Jägervall et al. 2015). However, these studies relied on calcium transient imaging, which is a surrogate measure of action potential generation. Furthermore the ability to resolve the effects on neuronal subpopulations may be contaminated due to calcium elevation in adjacent glia (Suadicani et al. 2010). In the present study, temperature sensitivity was used as a stimulus to evoke cellular excitability which improved the Z’ factor compared to those computed under baseline spontaneous activity. More importantly, since nociceptors
are known to respond noxious thermal stimuli, temperature-based conditions may be more physiologically relevant while screening compounds.

**Competing Interests**
The authors have no competing interests to declare.

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CHAPTER 5
INVESTIGATING THE FUNCTION OF PERIPHERAL AXONS IN NOCICEPTORS
USING AN IN VITRO MICROFLUIDIC CULTURE SYSTEM

5.1 Introduction
The major role of peripheral axons is the conduction of all-or-nothing action potentials from peripheral sensory neurons to the central terminals in the dorsal root ganglion for transmission to the central nervous system (CNS) (Esposito et al. 2019). In the case of nociceptors, noxious stimuli is encoded by nerve endings and transmitted along the pain axis to the CNS (Sapunar et al. 2012). Plasticity along multiple sites in this pathway has now been widely implicated in the maladaptive changes occurring in pathological pain states. Increasing evidence suggests that nociceptive axons are not nascent to such plasticity changes and actively participate by local expression of ion channels, receptors, and signal transduction molecules through axonal mRNA translation machinery (Price and Geranton 2010). Notably, the peripheral terminals, not the soma, are exposed to a myriad of inflammatory cytokines during inflammation and/or nerve injury, and may contribute to the peripheral hyperexcitability of DRG neurons (Black et al. 2019). Therefore, a more detailed understanding of the role of axons, independent of the soma, in nociceptive plasticity is required.

The ability to physically and chemically isolate peripheral axons for genetic and or pharmacological manipulation may elucidate the role of axons in the maladaptive changes occurring in hyperexcitable nociceptors under inflammatory conditions. However, typical in vitro neuronal cultures are “mixed”, wherein, no physical and chemical isolation of axonal processes is present, and the principle measurements rely increasingly on that of the cell soma.
responses. However, advances in microfluidic cell culture platforms have overcome these challenges (Neto et al. 2014; Habibey et al. 2015; Malishev et al. 2015; van de Wijdeven et al. 2018). Microfluidic structures allow the culture of neuronal cell bodies in a distinct compartment, where growing axons extend across microchannels interconnecting a separate distal compartment. By physical and chemical isolation of the soma and axonal compartments, microfluidic systems allow spatial and temporal control over the axonal microenvironment allowing separate characterization of cellular components. Prior studies have utilized DRG neurons in microfluidic structures for imaging and biochemical manipulations, however, have relied primarily on neonatal tissue, which we and others have previously demonstrated to not be suitable for the study of NGF-mediated sensitization (Tsantoulas et al. 2013). Notably, the limited use of adult tissue is the slow growth of axonal processes in vitro compared to NGF-stimulated embryonic cultures. To compensate, smaller length microchannels have been used to improve axonal crossings from adult tissue to mirror optimal culture conditions from neonatal tissue. An inherent disadvantage of such design considerations is the increased instances of non-neuronal cell migration across smaller length microchannels. We have previously demonstrated, as a proof of principle, that pro-migratory adult myoblasts can readily migrate across microchannels of 100 μm, contaminating the axonal compartment (Atmaramani et al. 2019), which may affect the interrogation of axonal responses.

Here, we report optimization of adult DRG neurons cultured in a microfluidic culture system, which allows isolation of adult DRG axons and maintains fluidic isolation between the soma and axonal chamber. Calcium imaging is used to study the ability of axons to respond to relevant agonists and to generate and propagate signals to cell bodies in the soma chamber.
Additionally, we also demonstrate the use of this culture preparation to investigate axonal excitability following IL-6 sensitization restricted to the axonal compartment to directly assess the role of axons in IL-6 mediated sensitization of DRG neurons.

5.2 Materials and Methods

5.2.1 Fabrication of PDMS microfluidic chambers

Polydimethylsiloxane (PDMS)-based microfluidic chambers were fabricated as described previously with minor modifications (Atmaramani et al. 2019). All microfluidic devices had two identical chambers designated as the soma and axonal chambers with constant dimensions (3000 μm wide × 100 μm tall) that were connected by a series of parallel ladder microchannels that were orthogonal to the soma and axonal chambers. The spacing between ladder channels was either 50 μm, while the length of the connecting ladder channels was designed to be 500 μm. A standard two-step photolithography technique was used to construct the multilevel photoresist templates utilized for generating the PDMS microchannels. In the first step a ~10 μm thick layer of KMPR 1005 negative photoresist (MicroChem, Westborough, MA, USA) was patterned on the silicon wafer (University Wafers, Boston, MA) to serve as the template for the connecting microchannels. This layer was formed by spin coating (1500 RPM) 4ml of photoresist for 30 s, followed by baking at 100°C for 5 min, and then exposure to ultraviolet (UV) light at 335 mJ/cm². After exposure, the wafer was post-baked for 2 min and then developed for 2 min using a SU-8 developer (MicroChem, Westborough, MA, USA). The wafer was then rinsed with 20 mL isopropyl alcohol (IPA) and dried under nitrogen stream. In the second step, a ~130 μm thick layer of photoresist (KMPR 1050) was deposited by spin coating at 1300 RPM for 30 s followed by baking at 100°C for 20 min. The wafer was aligned and then exposed to UV light at 1831
mJ/cm² to pattern the taller main channels (proximal/distal). The wafer was then baked at 100°C for 6 min, developed for 5 min using SU-8 developer, rinsed with 20 mL IPA, and dried under a stream of nitrogen. Prior to pouring PDMS over the photoresist template, the wafer was first coated with 10 μL of (Tridecafluoro-1, 1, 2, 2-TetraHydrooctyl) Methyl dichlorosilane (Geleste Inc, Morrisville, PA, USA) by vapor deposition for 4 h under vacuum to improve the release of cured PDMS from the photoresist template. The PDMS microfluidic devices were fabricated using previously described methods (Alsmadi et al. 2017). Briefly the curing agent and base were mixed in a 1:10 ratio, (Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI, USA), poured over the photoresist template, and degassed for 30 min. The wafers then were placed in an oven at 80°C for at least 1 h to cure. The channels were then removed from the mold, and an outlet port was punched directly into the proximal and distal chamber to form an “open well” structure using a sterile 6 mm whole punch. The PDMS stamps were sealed to glass cover slips that were pre-sterilized with a 70% ethanol solution for 30 min and then with a triple DI water rinse. PDMS stamps were permanently sealed to the glass coverslips by first treating the surface of the PDMS and the coverslip with plasma cleaner at high radio frequency (RF) for 1 min.

5.2.2 Microfluidic device preparation

Microfluidic devices were coated by the introduction of 10–20 μL of 50 μg/mL Poly-D-lysine (PDL) into soma and axonal chambers and incubated overnight at 37 °C. Following PDL coating, the devices were washed three times with sterile deionized water and subsequently treated with 20 μg/mL laminin for 1–2 h at 37 °C. Before introducing the cells into the devices,
excess non-adherent laminin was removed from the inlet ports of both chambers and replaced with Hank’s balanced salt solution (Sigma Aldrich, St. Louis, MO, USA).

5.2.3 Assessment of gradient generation

To investigate the generation of the neurotrophic gradient and the maintenance of chemical and/or fluidic isolation in the microfluidic chambers, 10 μM FITC-conjugated dextran was introduced as a surrogate molecule in the soma chamber. FITC was allowed to diffuse over an 8 hour period and the visualization of the gradient formation was carried out using time lapse imaging of both chambers every 30 minutes. For the quantification of the gradient generation due to the diffusion of FITC through the microchannels, three sections spanning the length of all microchannels were acquired every 30 min for a total duration of 8 h. Individual images were stitched in ImageJ using the 2D stitching plugin. Line intensity profiles were acquired across microchannels and were defined from the boundary of the proximal to distal chamber with a total length of 100 μm. Data were plotted using OriginPro software (OriginLab Corporation, Northampton, MA, USA).

5.2.4 Isolation and culture of adult DRG neurons

Adult DRG neuron isolation and culture methodology was adapted from Black et al. (2018) and performed as described previously (chapter 2, section 2.1, pg 70). After dissociation and isolation of the cell pellet, cells were resuspended in fresh 10-20 μL of TG medium. Approximately, 4-5 x 10^5 viable neurons were plated per device in the soma chamber of the microfluidic device and allowed to adhere for 30 minutes. Following adhesion, complete TG medium was added to both soma and axonal chambers. To induce axonal outgrowth across the microchannels and into the axonal chamber, a neurotrophic gradient was maintained as described
previously. Briefly, from DIV 0-1, both axonal and soma chambers were maintained in media containing 100 ng/ml of each mouse nerve growth factor and human glial derived neurotrophic factor (hGDNF). From DIV 1-3, 50 ng/ml in the soma chamber, and 100 ng/ml in the axonal chamber of each growth factor was maintained. From DIV 3 onwards, 25 ng/ml in the soma chamber and 100 ng/ml in the axonal chamber was maintained and medium was exchanged every 48 hours.

5.2.5 Calcium imaging

Post 6 DIV, media was removed and the calcium indicator Fluo-4, AM, (5 μM, Thermofisher) was loaded in both chambers for 30-45 minutes at 37°C. Following staining, both chambers were washed once with HBSS before imaging commenced. Microfluidic chambers were directly mounted on the stage of an inverted microscope (Nikon Ti eclipse, Nikon, Tokyo, Japan) equipped with a humidified chamber (OKOLAB USA Inc., San Francisco, CA, USA) maintained at 37 °C and 10% CO₂. The imaging regime consisted of acquiring fluorescence images (488 nm excitation) every 500ms for 2 minutes. Unless otherwise stated, physically and chemically isolated axons were stimulated with 100 nM capsaicin reconstituted in complete growth medium. Following axonal stimulation, a follow up stimulation was carried out in the soma chamber. In all experiments, axonal stimulation was performed first, while soma stimulation was performed at the end of the assay to avoid desensitization or cell death due to calcium induced cytotoxicity as a result of direct soma stimulation. Each drug addition was followed by a complete wash out and 10 minute incubation period. To ensure chemical and fluidic isolation during the assay, the soma chamber was maintained in 100 μL HBSS and the axonal chamber was maintained in 50 μL total volume.
5.2.6 IL-6 sensitization of axons

For IL-6 sensitization of axons, adult DRG neurons were prepared as described previously. After DIV 3, NGF supplemented media was removed from both soma and axonal chambers and replaced with media containing anti-NGF (1:50, Sigma). Following 24 hours of NGF deprivation, cultures were assigned to two experimental groups: axons treated with 100 ng/ml IL-6 and axons continued in anti-NGF for an additional 48 hours (negative control). At DIV6, axonal responses to 100 nM capsaicin were tested using calcium imaging as described previously (section 2.6).

5.2.7 Immunocytochemistry

At DIV 6 adult DRG cultures in the microfluidic chambers were fixed with 4% paraformaldehyde reconstituted in sterile PBS for 30 minutes at room temperature (RT). Since fixation was carried out without removal of the PDMS microfluidic structures, differential volumes were maintained in the compartments to ensure fixation of the axons in the microfluidic channels. Following fixation, both chambers were washed three times for 5 minutes each with PBS and stored in PBS + 0.1% sodium azide at 4°C for atleast 24 hours. The next day, cells were permeabilized with 0.2% triton in PBS solution for 30 minutes at RT and blocked with 10% NGS for 2 hours. Primary antibody solution was reconstituted in 10% normal goat serum (NGS) and added to both chambers and placed at 4°C overnight on a plate rocker. Next, chambers were washed with PBS and treated with secondary antibodies for 1 hour at RT. The following primary antibodies were used: anti mouse β3tubulin (1:1000, Sigma), rabbit anti-peripherin (1:2000, Sigma), rabbit anti-calcitonin gene related peptide (CGRP, 1:2000, Sigma). Secondary antibodies were goat anti-rabbit Alexa 405 or goat anti-rabbit Alexa 488. For visualization of isolectin B4
(IB4), cells were incubated with 1:500 FITC-conjugated IB4. After staining, microfluidic structures were imaged directly using confocal microscopy (Nikon Ti eclipse, Nikon, Tokyo, Japan).

5.2.8 Image analysis

Unless otherwise stated, all fluorescence and bright field imaging was carried out using an inverted microscope all epifluorescent images were acquired using epifluorescent light sources (Lumnecor, Beaverton, OR, USA). Post processing of calcium imaging data was carried by first defining regions of interest (ROI) of cell bodies with an approximate diameter of 30 μm to limit analysis to small-medium gauge neurons which are presumptive nociceptors. A positive calcium response was attributed to a neuron when the mean fluorescence intensity was greater than three standard deviations above the mean of the baseline calculated from a 30 second window before drug application.

5.3 Results

5.3.1 Fluidic isolation and neurotrophic gradient generation across microchannels

Microfluidic systems allow fluidic isolation between the soma and axonal chambers which is established by a volume difference between chambers. As a result, the fluidic pressure generated at the microchannels minimizes diffusion of molecules. This is especially important for the generation of a stable neurotrophic gradient to stimulate axonal growth across microchannels into the axonal chamber. To characterize the temporal presence of the chemotactic field across the microchannels, FITC-conjugated dextran (molecular weight = 40,000 kDa) was used as a growth factor mimetic. To quantify the diffusion process, 10 μM FITC was introduced into the axonal chamber and the soma chamber was maintained in cell medium to model whether
concentration gradient would be established across the microchannels into the soma chamber where DRG cell bodies are maintained. The gradient generated was visualized as fluorescence intensity profiles due to diffusion of FITC across the microchannels.

Figure 5.1: Generation of chemical gradients across microchannels. (a) Normalized mean fluorescence intensity of FITC-conjugated dextran (molecular weight = 40,000 kDA) as a function of microchannel length at different time points. (i) Denotes the boundary of the soma chamber (ii) Denotes the boundary of the axonal chamber where FITC was introduced. Inset displays a representative image of 4 microchannels (width = 1.5 μm) at the 4 h time point which demonstrates the generation of a gradient of FITC molecules from axonal (top) to soma chamber (bottom). (B) Representative images of FITC-conjugated dextran diffusion profile across microchannels of different widths at the 4 h time point. (a) 20 μm width microchannels. (b) 10 μm width microchannels. (c) 1.5 μm width microchannels.

The FITC molecules diffused into the soma chamber immediately after introduction and a linear gradient was established in approximately 1 h and was maintained for at least 8 h (Figure 5.1(a)). No fluorescence intensity was detected in the soma compartment via fluorescence microscopy after establishment of a gradient. Notably, we observed a differential gradient dynamic as a function of microchannel width. For microchannels of 20 μm width we observed an exponential gradient and microchannels less than 3 μm we observed a nearly linear gradient (Figure 5.1(b)). Similar observations have been made for straight linear microchannels of width 20 μm and is dependent
on the permittivity allocated by wider dimensions that allow faster diffusion. Taken together, the data suggest that stable chemical gradients are readily established and maintained in the fabricated microfluidic structures and gradient strength can be controlled by constricting the width of the microchannels.

5.3.2 Characterization of adult DRG neuron preparations in microfluidic chambers

As a first step, we sought to create and optimize culture conditions to isolate the axonal component of adult DRG neurons in the microfluidics-based culture platform. Having empirically established the fluidic integrity and chemical isolation of the soma and axonal

![Schematic](image1)

Figure 5.2: Isolation of axons in adult DRG neuron cultures in microfluidic-based culture platforms (a) Schematic representing soma (left) and axonal (right) chambers interconnected via microchannels (5 μm width and 500 μm length). (b) Representative bright field imaging of the soma and axonal chambers at 6 DIV. Axonal outgrowth is evident by 24 hours and extensive growth is observed until 6 DIV. Horizontal scale bar represents 100 μm (c) Axonal crossing can be monitored by the use of a fluorescent tracer, Dil, applied to the axonal chamber. Dil is taken up by crossing axons and retrogradely transported to the soma. (d) Isolated axons were identified by staining with β-III tubulin. Horizontal scale bar represents 100 μm. (e) Axons stained with peripherin, a marker which is preferentially expressed by peripheral sensory neurons. Horizontal scale bar represents 250 μm.
chamber, we first sought to stimulate axonal growth across the microchannels using a
neurotrophic gradient as described previously elsewhere (Tsantoulas et al. 2013). DRG neurons
were plated in the soma chamber (Figure 5.2(a)) and axonal crossings were monitored over 6
DIV. Axonal crossings in the distal chamber were identified as early as 24 hours and by 6 DIV a
large proportion of crossings were visible across all microchannels (Figure 5.2(b)). Axonal
growth across the microchannels was attributed to the concentration gradient of the neurotrophic
factors generated as well as the hydrostatic pressure maintained via differential volumes in the
chamber. To quantify the percentage of neurons extending axons, a fluorescent tracer (Dil)
(1/200) was added to the axonal chamber for 2 hours. As a lipophilic membrane stain, Dil was
readily incorporated into the membrane of axons and retrogradely transported to the cell soma
where the stain is accumulated resulting in a maximized signal. At 6 DIV, we found 80 ± 3 % (n
= 3 devices, n > 100 cells/device) of the cell bodies positively stained with Dil with the majority
of cells being of small-to-medium gauge neurons (Figure 5.2(c)). This observation was further
verified by immunocytochemistry which revealed that crossing axons were positive for βIII-
tubulin, expressed in the axons of mature neurons, (Figure 5.2(d)) and the small sensory neuron
marker peripherin (Figure 5.2(e))

5.3.3 Calcium imaging-based model to assess functional axonal responses

Physically and chemically isolated axons can be probed by relevant agonists to measure the
baseline excitability using calcium imaging. Calcium imaging is a reliable and stable tool which
allows the measurement of neuronal excitability by reporting intracellular calcium influx during
electrical events (Anand et al. 2006; Chen and Huang 2017; Chen et al. date unknown).

Typically, in vivo, action potentials are initiated in the nerve ending of sensory axonal fibers in
response to a stimulus in the peripheral receptive field. This signal is transduced and transmitted by axons to the soma for transmission to the central nervous system. Therefore, we postulated that stimulation of the isolated peripheral axons with a relevant agonist will result in local axonal depolarization and propagation of action potentials to soma which can be monitored as a calcium influx using a calcium indicator. Exposure of the axonal compartment to 100 nM capsaicin led to an increase in the recorded calcium intensity at the soma (Figure 5.3 (A)) and was represented by an increase in the calcium signal recorded as a function of time (Figure 5.3(B)). In comparison, a greater proportion of responsiveness on a shorter time order was observed when 100 nM

![Figure 5.3: (A) Representative images of calcium responses to 100 nM capsaicin applied in the axonal chamber. (B) Representative traces of normalized fluorescence intensity of calcium influx post-axonal stimulation as a function of time from defined regions of interest (ROI). (C) Representative images of calcium response following 100 nM capsaicin applied in the soma chamber. (D) Representative traces of normalized fluorescence intensity of calcium influx post-soma stimulation as a function of time from defined ROI.](image-url)
capsaicin was applied directly to the soma chamber (Figure 5.3 (C), (D)). To quantitatively assign a positive calcium response after soma or axonal stimulation, cell bodies in the soma after drug application were regarded as “responders” to 100 nM capsaicin if the mean fluorescence intensity was greater than 3 times the standard deviation above the baseline recorded 30 seconds prior to drug application. We found 70% of positive responses in the soma compartment after application of 100 nM capsaicin and > 90% responders after direct application to the soma. These data suggest that the imaging of increase in intracellular calcium levels is a reliable and sensitive approach to assess axonal function in microfluidic cultures.

5.3.4 Axonal sensitization in the presence of IL-6

To determine whether isolated peripheral axons could be sensitized in the presence of inflammatory cytokines, we investigated the effect of chronic (48 hours) exposure to IL-6, an inflammatory cytokine with a demonstrated neuroinflammatory role in the development of chronic pain (Milligan and Watkins 2009; Krames 2014). Briefly, adult DRG neurons in microfluidic devices were prepared as described previously, except for the addition of anti-NGF antibodies at DIV 3 for 24 hours. Following NGF deprivation, cultures were divided into two experimental groups: one experimental group received 100 ng/ml of IL-6 in the axonal chamber for 48 hours and the control group was continued in anti-NGF antibodies until DIV 6. At DIV 6, capsaicin-evoked responsiveness significantly increased the magnitude of calcium responses in cultures treated with IL-6 from 1.03 ± 0.007 to 1.15 ± 0.006 (two sample unpaired t-test, p < 0.0001, n = 27 and n = 26 from n = 2 devices) (Figure 5.4(A)). Similarly, the number of positive responders to capsaicin were significantly increased from 51% to 80% post chronic exposure to IL-6 (test of proportions, p = 0.02) (Figure 5.4 (B)). Taken together, the results suggest that
chronic, 48 hour exposure to exogenously administered IL-6 in the axonal microenvironment potentiates local axonal responses to capsaicin; an effect independent of the soma.

**Figure 5.4:** Local treatment of the axonal microenvironment with inflammatory cytokine IL-6 enhances capsaicin-evoked responsiveness. (A) Magnitude of the calcium transient recorded post-exposure to 100 nM capsaicin in vehicle and IL-6 treated groups. Data are represented as the mean fluorescence intensity of the ROI selected at soma compartment and normalized to control. (B) Quantification of the number of positive responders as a proportion of total neurons post-exposure to 100 nM capsaicin in vehicle and IL-6 treated groups.

**5.4 Discussion and Conclusion**

In the present study, a microfluidic-based system is developed for the culture and study of nociceptive axons *in vitro* from adult DRG neurons. Firstly, we demonstrated that maintenance of differential volumes and geometric considerations of the microchannels interconnecting the soma and axonal chamber create positive pressure preventing diffusion between chambers, thereby, maintaining fluidic isolation. This enabled chemical interrogation of the axonal microenvironment independent of the cell body. Secondly, we demonstrated physical isolation of axonal processes from > 80% of the cell bodies seeded in the soma chamber which
expressed classical markers of mature, nociceptive nerve endings. Thirdly, using calcium imaging, the axonal microenvironment is stimulated by relevant agonists and the functional read out is performed via propagated signals reaching the cell body as a surrogate of axonal activation. Lastly, we demonstrate that axons may play a direct role in peripheral sensitization via local administration of IL-6 to the axonal microenvironment. This effect recapitulates the in vivo scenario, wherein, peripheral nerve endings of nociceptors are in intimate contact with the inflammatory milieu after nerve injury and/or inflammation.

Geometric considerations of the microchannels is essential in maintaining fluidic custody of the soma and axonal chamber. We show that the present channel dimensions (5µm x 500 µm) was sufficient in preventing diffusion of FITC-dextran for at least 8 hours. However, additional studies are required to extend this time period to assess long term chemical separation to guide microchannel design. Moreover, stimulating growth of axons across depends on the stable generation and maintenance of a neurotrophic gradient. A linear gradient was readily formed and maintained across the microchannel length which played a crucial role in enticing axons to cross the microchannels into the distal chamber. An additional challenge in preparation of primary adult tissue in vitro is the presence, proliferation, and migration of support cells, such as Schwann cells and fibroblasts. Initial plating of DRG neurons in the soma compartment cause proliferation and migration of such support cells across microchannels leading to contamination of the distal axonal compartment. To prevent this, a detailed study from our group has previously demonstrated that cell migration across microchannel width of 5 µm and 500 µm length is impeded (Atmaramani et al. 2019). In contrast, cells traversed easily across wider and shorter channels, elucidating that cell migration is severely impeded under confinement. Therefore, the
present design, inhibited support cell migration in the axonal chamber and did not require the addition of mitotic inhibitors to axonal microenvironment.

We sought to assess the functional role of axonal excitability in terms of axonal activation and propagation of the signal independently of the soma using a calcium indicator. Axonal stimulation with 100 nM capsaicin produced a significant increase in the soma calcium response, which recapitulates the generation and propagation of bioelectrical signals in vivo in a controlled and accessible microenvironment. While calcium imaging is an important tool in probing neuronal excitability, calcium-based activity is a surrogate measure of neural activity due to slow kinetics relative to changes of the membrane potential. Moreover, since calcium is an important secondary messenger mediating numerous intracellular processes, the read out is a global response and cannot be readily tied to action potential firing rates or patterns. Long term monitoring of cellular excitability is not amenable to calcium-based fluorescence dyes due to phototoxicity or photobleaching under long exposure times. Future studies will assess the development of an integrated system, combining substrate integrated MEAs with PDMS-based microfluidic structures to allow recording electrodes to interface directly with soma and axonal chambers to allow long term, non-invasive, and high content measures of neuronal excitability.

Chronic pain conditions can cause significant maladaptive changes rendering peripheral nerve fibers hyperexcitable (Wilson et al. 2007). We investigated the role of peripheral axon mediated changes in hyperexcitability by treating chemically and physically isolated axons with 100 ng/ml of IL-6 for a chronic time period. The results indicated an increased number of positive responders with an increased magnitude of calcium intensity in response to capsaicin. Our previous studies have demonstrated an increased protein expression of TRPV1 in cell bodies
of DRG neurons in vitro after 48 hour incubation with IL-6 (Black et al. 2018). Therefore, it is highly likely that enhanced responses to capsaicin may be mediated by an increased local expression of TRPV1 in peripheral axons.
References


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6.1 Conclusions

Chronic pain is a complex disorder that is refractory to the current medical standard of care and has been estimated to affect 1.5% of the general population. Pharmacological interventions to treat chronic pain is suboptimal and often ineffective, relying heavily on the use of prescription opioids, which have been implicated in a high incidence of abuse and overdose deaths. More importantly, new evidence suggests that long-term use of opioids may increase the perception of pain leading to opioid-induced hyperalgesia, thereby creating a positive feedback loop for the need of increased dosages. Therefore, there is an urgent and unmet clinical need for the development of safe, effective, and non-addictive therapeutic compounds for the management and treatment of chronic pain. Current approaches have traditionally involved a target-based approach—a “druggable” target is first identified and compounds that modulate this target are screened and optimized during lead discovery phase. However, the signaling complexity associated with chronic pain pathology impedes the usefulness of such a method. An antagonistic approach, namely, phenotypic screening, has seen renewed interest, wherein a specific target or a hypothesis of a mechanism of action is not required. Instead, compound libraries are screened against a relevant cell-based model with a phenotypic end point which is physiologically relevant to the disease.

Pain sensing neurons or nociceptors located in the DRG are first order neurons involved in the sensing, transduction, and propagation of noxious stimuli via all-or-nothing action potentials. Maladaptive changes in nociceptor excitability is a hallmark feature of chronic pain
which is induced and maintained by the release of inflammatory cytokines and pain mediators at the site of injury. MEAs enable long term, non-invasive, and quantitative measurements of neuronal excitability and signaling via recording of extracellular action potentials. The overarching goal of the present study was to develop and demonstrate a DRG neuron cell-based phenotypic screening assay of acute and chronic nociception using adult DRG neurons on multi-well MEAs. The research primarily focused to: (1) determine whether murine derived adult DRG neurons cultured on MEAs develop baseline spontaneous activity, (2) recapitulate a hyperexcitatable phenotype in the presence of inflammatory cytokines, (3) represent a viable phenotypic screening platform by adhering to assay quality metrics, (4) and to explore and examine the role of peripheral axons independent of the cell body.

In Chapter 2, an in vitro culture methodology was described and developed for spontaneously active adult DRG neurons cultured on multi-well MEAs. We cultured a largely nociceptive population (~88%) and characterized the baseline spontaneous activity and stimulus-evoked activity over time periods consistent with pharmacological interventions. Most notably, acute and persistent hyperexcitability was successfully induced by inflammatory cytokine IL-6. Taken together, the results suggested that adult DRG neurons on MEAs constitute a physiologically relevant, moderate-throughput, and high content assay for analgesic lead discovery. Chapter 3 extends the use of the assay to assess the role of Nav1.7 and Nav1.8, conserved sodium ion channel subtypes, which have been implicated in inflammatory pain. We first demonstrated that combinatorial treatment of IL-6 and NGF induce persistent increase in firing rates and thermally evoked activity. We observed the conserved expression of Nav1.7 and Nav1.8 in in vitro cultures of adult DRG neurons and implicated their contribution to the
spontaneous and evoked responses via pharmacological blockade. In parallel, cortical cultures were insensitive to specific antagonists of Nav1.7 and Nav1.8, highlighting the sensitivity of the assay to detect positive compounds with a known mechanism of action. Taken together, we demonstrated that important “pain” channels, Nav1.7 and Nav1.8, are conserved in long term cultures of adult DRG neurons, directly contribute to the increased hyperexcitability, and highlight the use of the current assay format to screen compounds that specifically block nociceptive-specific sodium ion channel subtypes.

In Chapter 4, we described a robust statistical method to assess the quality of the DRG-cell based assay in the context of extracellular recordings. First, data transformations were applied to electrophysiological recordings to adhere to requirements for normality and suitability for use as a sample signal. Using well-wide total spikes, a robust version of the $Z'$-factor, based on the median and median absolute deviation, was applied to indicate the quality of the assay and assess hit identification of putative compounds. The robust $Z'$-factor based on the median log transformed signal revealed a value of 0.61, indicative of an “excellent assay.” Known modulators of nociceptive-specific receptors were identified as hits in both an agonist and antagonist assay format. The approach allowed a large signal dynamic range, reduced sensitivity to data variability, and reduced the requirement of number of electrode samples/well. Overall, the present method provided a statistical basis for implementation of HCS platform for a DRG-neuron cell-based assay.

Lastly, in Chapter 5, we extended the use of the assay to implement a microfluidic culture-based system to physically and chemically isolate DRG axons and combine a variety of imaging, targeted pharmacology, and functional assessment. We first used FITC-dextran as a
chemical mimic to demonstrate chemical isolation of the soma and axonal chamber. No detectable fluorescence was detected in the soma chamber for > 8 hours and a concentration gradient was generated in < 1 hour. These results suggested that the axonal microenvironment can be stimulated independent of the soma. Using a neurotrophic gradient, > 80% of neurons in the soma were observed to extend axons across microchannels which were positive for markers such as β-III tubulin and peripherin, indicating the presence of mature axons belonging to small diameter DRG neurons (nociceptive). The platform was then used to test the ability of isolated peripheral axons to respond to chemical agonists, transduce the signal, and propagate action potentials to the soma using calcium imaging. We found > 90% and > 70% of positive calcium responses at the soma, when the soma and axonal compartment were stimulated with 100 nM capsaicin, respectively. Thus, we described how the axonal microenvironment can be chemically and physically separated from DRG cell bodies and interrogated with a combination of pharmacological and imaging-based tools in a microfluidic culture platform.

6.2 Future work

The present research provides detailed insights on the development and implementation of a complex high content cell-based assay to screen therapeutics for chronic pain. However, future studies may further enhance the relevance of the model as well as include integrative measures of cellular phenotypic end points.

6.2.1 Human primary tissue source for screening

While murine-derived cultures of DRG neurons are extremely useful for the study of the molecular, cellular, and bioelectrical properties underlying the sensing and transduction of stimuli in the pain axis, notable disadvantages in the translatability of this cell model have been
noted (Mogil 2009). The ability to procure and culture human donor DRG tissue as the cell model for phenotypic screening is undeniably the most clinically and physiologically relevant method to assess drug efficacy when identifying potential analgesics for human use. Progress towards developing in vitro cultures of human DRG neurons involve the study of genetic/molecular repertoire, electrophysiological signatures, morphological classifications, and myelination and regenerative capacities (Davidson et al. 2015; Valtcheva et al. 2017; Zhang et al. 2017; Rostock and Schrenk-siemens 2018). Increasing evidence suggests species differences in the expression of ion channels, receptors, and signal transduction molecules between human and murine DRG. For example, immunoreactivity to neurofilament 200 in human DRG neurons is not restricted to large diameter neurons. Additionally, nociceptor-specific markers such as TRPV1, CGRP, and P2X3 are restricted to expression in small-to-medium gauge neurons in murine DRG but present in all sizes in human tissue (Haberberger et al. 2019). Perhaps most importantly, voltage gated sodium ion channel subtypes, Nav1.7 and Nav1.8, which are regarded as important pain channels due to their established role in chronic inflammatory pain, have a higher expression in human nociceptors (Rostock and Schrenk-siemens 2018; Zhang et al. 2019). The discrepancy in the expression profile and bioelectrical properties do not necessarily reduce the importance of rodent DRG-based assays to model hyperexcitability in vitro, since the effect of several pain-related molecules are highly conserved across species (Anand et al. 2006). While the prospects of using human derived tissue to model hyperexcitability in vitro is an exciting avenue to explore, the use of human tissue poses inherent disadvantages for use in in vitro phenotypic screening. Scarce availability of tissue from donors, patient-to-patient genetic variance, and low viability under chronic culture conditions have limited their use as a model for
high throughput screening. Therefore, a human induced pluripotent stem cell model of peripheral sensory neurons cell-based assay may be a promising opportunity to develop a scalable, translatable, and reproducible phenotypic screening model for chronic pain.

6.2.2 Human induced pluripotent stem cells as a cell model

Human induced pluripotent stem cells (hiPSCs), derived from somatic cells such as skin or blood cells, can be reprogrammed back into a developmental state (embryonic) which allows the directed differentiation to any human cell type. Recent efforts in the generation of hiPSC peripheral sensory neurons (PSN) demonstrate conserved genetic expression of canonical sensory markers (Jain et al. 2012) and has also been implemented in imaging-based HTS paradigms (Vojnits, K., Mahammad, S., Collins, T., Bhatia 2019; Stacey et al. 2018b; Martínez et al. 2019). While significant advancements in terms of functional responses (Guimarães et al. 2018) and release of neuropeptides, most studies have failed to unequivocally demonstrate stable baseline excitability and sensitization to known inflammatory cytokines. One significant limitation is the disparity in the differentiation protocol used across studies which give rise to genetic variability (Schwartzentruber et al. 2018). Our experiments with the culture of adult tissue have highlighted the possible role of support cells, leading to high instances of functional spontaneous activity by maintaining relatively a high confluence of non-neuronal cell types (satellite glia, Schwann cells, and fibroblasts). The role of non-neuronal cells has been previously established in inflammatory pain maintenance by intraganglionic paracrine signaling between the DRG soma and adjacent satellite glial cells. Therefore, future studies will aim to incorporate hiPSC PSNs with satellite glial cells (hiPSC-based DRG tissue mimic) on multi-well MEAs to produce a model with the greatest human relevancy. In addition to investigating spontaneous and
a sensitized phenotype, RNA sequencing can be leveraged to confirm expression profiles consistent with functionally mature nociceptors.

6.2.3 Long-term non-invasive interrogation of adult DRG neurons on integrated microfluidic MEA platform

Compartmentalized cultures of DRG neurons have been investigated only in a few cases with neuronal excitability as a phenotypic end point, wherein, somal and/or axonal activity is monitored via surrogate measures, such as calcium activity or directly through patch-clamp electrophysiology (Tsantoulas et al. 2013; Jocher et al. 2018). While manual patch-clamp (MPC) is the gold standard for measurements of cellular excitability, commercial or standard designs of microfluidic structures are yet to be customized to incorporate patch-clamp electrodes. In addition to design challenges, MPC is not suitable for long-term measurements, technically challenging for axonal recordings, and provide lower throughput in recording from multiple cell bodies simultaneously (Yajuan et al. 2012). The integration of single and/or multi-well MEAs with microfluidic systems may allow non-invasive, long-term, and label-free measurements of extracellular action potentials using substrate-integrated recording electrodes (Figure 6). While PDMS-based microfluidic devices have been used previously with MEAs to investigate sensory neuron responses to chemical agonists (Tsantoulas et al. 2013), chemotherapeutic agents (Gornstein et al. 2018), and temperature (Pearce et al. 2005). However, to date, no prior study has investigated compartmentalized cultures on MEAs. Advancements in microfabrication techniques allow innovative design capabilities of electrode configurations, wherein, recording and stimulation electrodes of distinct surface area and geometry can be spatially configured in the soma and axonal chamber, as well as, incorporated within microchannels. Recording
Electrodes in microchannels may allow investigation of the conduction velocity of traversing axons, which is especially important for sensory neurons as they can be discriminated and classified based on conduction velocity of the peripheral axons. Future studies will aim to design custom MEAs which readily integrate with PDMS-based microfluidic systems with recording electrodes in the soma chamber, electrodes within microchannels, and stimulating and/or recording electrodes in the axonal chamber. Notably, the use of transparent substrates will not preclude the use of calcium indicators and can integrate simultaneous monitoring of calcium transients and electrophysiological recordings, which may allow exploration of non-neuronal and immune cell signaling in peripheral sensitization.

Figure 5.1: Integration of PDMS-based microfluidic structures on single well MEAs. (A) Concept flow diagram of assembling microfluidic chambers on a substrate-integrated single well MEA. Recording electrodes on MEAs in distinct chambers can resolve detection and recording of extracellular action potentials in designated axonal and somal compartment. (B) Preliminary design considerations of microelectrodes integrated with microfluidic structures demonstrate spatial configuration of recording electrodes separately in axonal/somal chamber and microchannels.
References


BIOGRAPHICAL SKETCH

Rahul Atmaramani was born in Dubai, United Arab Emirates. After completing his schoolwork at Cambridge International School in Dubai, Rahul enrolled at Southeast Missouri State University (SEMO) in Cape Girardeau, Missouri where he pursued a bachelor’s degree in biomedical sciences with a minor in engineering physics and psychology. During his time at SEMO, he joined the Nano-Bio Lab as an undergraduate research assistant under the mentorship of Dr. Santaneel Ghosh. Along with pursuing his academic degree, Atmaramani worked as an undergraduate researcher from 2013-2016 before joining the PhD program in the Bioengineering Department at The University of Texas at Dallas.
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PROFESSIONAL RESEARCH EXPERIENCE

Graduate Research Assistant
The University of Texas at Dallas, Neuronal Networks and Interfaces Lab, Dallas TX (2016-2020)
• Excellent multidisciplinary skills demonstrated by direct contributions to lead discovery in chronic pain, using dorsal root ganglion neuron cell-based assays, resulting in interdepartmental collaborations and $1.6 million in grant funding.
• Designed, planned, and executed preclinical and clinical feasibility of DRG stimulation with Abbott Neuromodulation by elucidating the role of SK-type calcium channels in stimulation-mediated analgesia.
• Creative and advanced strategist utilizing advanced statistical methods in analyzing large data sets as demonstrated by the optimization of existing parameters to handle electrophysiological data.
• Active scientific member on several research teams providing experimental support, justification for study design, and developing professional protocol documentation.
• Excellent and efficient communicator of scientific research and concepts culminating in 10 peer-reviewed journal articles and >10 presentations at national conferences.
• Directly collaborated with industry professionals (Abbott) and government agencies (NCATS, NIH) on research projects to develop innovative applications of existing technologies and methodologies.
• Independently guided, developed, and executed design of experiments, drafted executive summaries for technical and non-technical personnel, and made professional scientific recommendations for future directions and studies.

Undergraduate Research Assistant and Lab Manager
Southeast Missouri State University, Nano-Bio Lab, Cape Girardeau, MO (2012 – 2016)
• Developed, synthesized, and characterized drug delivery systems for immune oncology research to improve delivery of novel therapeutics to region of interest in the central nervous system.
• Investigated cell attachment and proliferation using physical and chemical cues to enhance neuronal function, metabolism, and directed differentiation for in vitro cell cultures.
• Demonstrated leadership skills by managing key scientific goals and deliverables, recruiting and mentoring undergraduate and graduate research personnel, and training via tailored workshops in cell-techniques.

RESEARCH and TECHNICAL SKILLS
Tissue engineering/Cell and Molecular Biology: Culture of primary and hiPSC-derived neurons, in vitro and in vivo electrophysiology, multi-color flow cytometry, FACS, qRT-PCR, Western Blot
Functional cell-based assays: multi-well microelectrode array (MEA), calcium imaging (Hamamatsu, Omniplex, Axion Maestro), in vitro neuropharmacology (Mechanism of action/target: GPCRs, kinases, ion channels)
Imaging: immunocytochemistry, immunohistochemistry, epifluorescence, confocal microscopy
Software and Data Analysis: Word, Excel, PowerPoint, GraphPad Prism, OriginPro, ImageJ, MATLAB, Image processing algorithms, NIS Elements, NeuroExplorer, FlowJo, Biostatistics

EXTRAMURAL FUNDING SOURCES
• NIH HEAL UG3/UH3 RFA-TR-19-003
  Title: Development of a Function-based Platform for Peripheral Analgesic Lead Discovery In Vitro
  Role: Graduate Research Assistant
  Award amount: $1,667,733
• Abbott Investigator-initiated Pre-clinical Award
  Title: Examining the role of Ca-dependent K channels in mediating effects of electrical stimulation in sensitized DRG model
  Role: Graduate Research Assistant
  Award amount: $31,400

PEER-REVIEWED PUBLICATIONS


**SELECT PRESENTATIONS AND PROCEEDINGS (**indicates presenting author**)**

**Atmaramani, R., Black, B., Pancrazio, J. Expression and functional profiles of sodium channel subtypes Nav1.7 and Nav1.8 are maintained in adult dorsal root ganglion neurons in vitro. *Texas Pain Research Consortium and BMES annual meeting.* (2019) (Poster presentation).**


Rihani R., Kim, H., Atmaramani R., Black, B., Pancrazio, J., Ware, T. Liquid Crystal Elastomer as a Novel Material for Neural Interfaces, *Neural Interfaces Conference* (2018). (Poster presentation)


**HONORS, AWARDS, AND FELLOWSHIPS**

- Jonsson Family Graduate Fellowship in Bioengineering (2018). *Merit-based fellowship for graduate students in biomedical engineering*
- Funding for Results Award (2014). *Awarded for excellence in research (Physics and Engineering Physics)*
- Grants and Research Funding Committee Award (2013). *Awarded for excellence in research (Physics and Engineering Physics)*
• Alpha Chi Sigma (2013). *Induction honoring outstanding performance in chemical sciences*
• The Outstanding Freshman Award (2013). *Awarded for academic excellence in Chemistry.*