PAIN AND PERSPECTIVE: COMPARTMENTED CO-CULTURE TO EVALUATE SENSORY NEURON PERIPHERAL GLUTAMATE RECEPTORS

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Abstract: The neurotransmitter L-Glutamate is the primary excitatory neurotransmitter of sensory neurons in the dorsal root ganglion (DRG). These neurons may also express ionotropic glutamate receptors, causing the potential for them to be directly excited by their own release of glutamate, from a neighboring neuron, or from other tissues. Glutamate is elevated in tissues after injury or inflammation, and iGluR signaling from the periphery has been shown to increase signaling in DRG neurons and contribute to the development of chronic pain. Targeting pharmacologic intervention of sensory neuron iGluRs present in peripheral terminals may constitute an attractive alternative or augmentation to chronic pain treatment regimens. A compartmented culture system was devised to enable the co-culture of sensory neurons and keratinocyte stem cells in discrete compartments to simulate a skin tissue *in vitro*, and allow focal agonist application to peripheral terminals. Activation of peripheral receptors with focal agonist application caused the propagation of signals towards somata of neurons in a fluidically separated compartment, causing excitatory post-synaptic currents (EPSC) that were observed and recorded via voltage-clamped whole-cell electrophysiology. EPSC responses observed exhibited statistically significant differences between the respective τ values of the EPSC after respective agonist exposure. Immunofluorescent labeling and visualization of receptor expression showed that iGluR subunits are expressed in sensory neuron somata, sensory neuron peripheral processes, non-neuronal cells from the DRG, and keratinocyte stem cells. The implementation of this co-culture clamping facilitates the spatially discrete interaction of neuronal and non-neuronal cell types for the characterization of their interfaces, as well as for the discrete application of pharmacologic agents at subcellular compartments to evaluate their spatially constrained influence on activity at a cellular, and intercellular level. The spatially restricted application of agonists represents a chemotransmissive instigation of electrochemical activity in neurons for studying EPSCs, instead of electrically stimulating a presynaptic cell, and so more faithfully represents what would occur *in vivo*. The identification of the active receptors and their subunit-specific peripheral expression yield alternative therapeutic targets for chronic pain treatment.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In an ontological sense, the basis of shared experience is through interaction of each individual with elements in their environment. Throughout the realm of potential experience there may be a litany of aspects characterizing both a single stimulus and the experience of the individual via its action upon the senses. Shared experiences through common interactions and perceptions provide the substrate for interpersonal relation, but at the core the individual is bound to their experience, from which emergent phenomena may arise like pattern recognition or patterned behavioral responses to stimuli. Though the individual is inured in their own personal experience and cannot directly know the instantaneous state of another's experience, through observation of what others may evince in response to different stimuli one may deduce in what ways particular stimuli affect others. Perhaps the widest-sweeping delineation that may divide the panoply of stimuli that may play upon the senses would be between those that are noxious and those that are non-noxious. It is ultimately beneficial that the perception of noxious stimuli should be unpleasant to the individual in order to facilitate patterned avoidance behavior for potentially damaging

stimuli in order to maintain the proper functioning of the body and organs; including those same organs that generate the sensations on which the noxious stimuli exert their effects. As an example, looking at too bright a light may disrupt normal vision in the short term, and longer term exposure may damage the retina (Organisciak and Vaughan, 2010). Regarding physical sensation, the perception of noxious stimuli manifests the sensation of either itch or pain. The encoding, transmission, and perception of pain interact via multiple mechanisms within the nervous system to ensure that immediate exposures to noxious stimuli are minimized, and future exposure is avoided.

The transmission of information about physical stimuli to the central nervous system for higher order processing begins in the peripheral nervous system. The particular cells that innervate the body to facilitate this transduction of physical stimuli into discrete signals conveying somesthetic information are sensory neurons that reside in the dorsal root ganglion (DRG). Each DRG rests in an intervertebral foramen, positioned dorsolateral to the spinal cord in the spinal column and just proximal to the point at which the ventral roots join to form spinal roots. Bundled projections from the neurons within each DRG travel in parallel comprising either a central root projecting to the dorsal root entry zone of the spinal cord or a distal root projecting to respective target tissues for innervation. The counterparts for DRG neurons responsible for innervation of the face, cranial vault, and top of the head reside in a cell body mass called the trigeminal ganglion. These DRG and trigeminal ganglion neurons are all therefore called sensory neurons, and while the types of stimuli to which they are sensitive and facilitate transduction may differ, they share a common primary excitatory neurotransmitter, the amino acid L-glutamic acid or glutamate (Broman, 1994.)

SENSORY NEURONS

Sensory neurons are classified as Aα, Aβ, Aδ, or C fiber neurons depending upon several factors, including soma size, axon diameter, and degree of myelination, that ultimately influence the characteristics of their firing patterns and conduction velocities. Conduction velocity of a nerve has a positive correlation with both axon diameter and degree of myelination. Peripheral pain neurons (nociceptors) and itch neurons belong to the slower conducting Aδ and C classes and use glutamate as a neurotransmitter, but can also express metabotropic and ionotropic glutamate receptors yielding an influence of glutamate signaling on overall activity and excitability. This influence serves to aid proper signaling in the healthy state, but can exacerbate pain due to excessive signaling during injury and inflammation.

There is an increase in glutamate receptors present in peripheral neurons in response to inflammation, along with increased glutamate near inflamed tissue, leading to increased sensitivity to pain by activating glutamate receptors present on peripheral processes of neurons innervating that tissue (Carlton and Coggeshall, 1999; Jin et al., 2006; Miller et al., 2011). A prime example of glutamate release in response to injury is that glutamate increases rapidly in the basal cell layer of skin after the superficial layers are stripped away (Fuziwara et al., 2003). Evidence from *in vitro* experiments shows that keratinocytes are also capable of releasing glutamate independently, which could thereafter act on glutamate receptors on other nearby neuron projections (Fischer et al., 2009). The subcutaneous application of glutamate to skin alone is also sufficient to

induce pain, further supporting the presence of glutamate receptors in the periphery of nociceptors (Gazerani et al., 2006). Glutamate application to exposed plantar nerves increases responses to heat stimulation in Aδ and C fiber neurons (Du et al., 2001). Electrical stimulation of one thoracic DRG peripheral root increases the baseline activity of neurons in the adjacent DRG, but this effect is prevented by the peripheral application of ionotropic glutamate receptor (iGluR) antagonists (Cao et al., 2007). Taken together, these results suggest that glutamate can be released from peripheral processes of DRG neurons as well as other peripheral tissues, in addition to iGluRs being present in the peripheral processes of DRG neurons.

Sensitivities to thermal and chemical stimuli are conveyed by specific transducer receptors in DRG neurons; such as transient receptor potential vanilloid receptor 1 (*TRPV1*) for sensitivity to noxious heat and capsaicin, and transient receptor potential cation channel subfamily M member 8 (TRPM8) for sensitivity to cold and menthol (Cao et al., 2013; Bautista et al., 2007). Multiple transducer proteins may confer sensitivity to similar stimuli; such as *TRPV1*, acid sensing ion channels, and transient receptor potential cation channel member A1 (TRPA1) for sensitivity to protons (De la Roche et al., 2013). Individual nociceptors may express multiple different transducer proteins conferring sensitivity to multiple types of stimuli for a single neuron (Usoskin, et al. 2015).

The electrochemical activity instigated by transducer proteins can be augmented by the activation of glutamate receptors in peripheral processes of DRG neurons (Jin et al., 2009). While both ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs, respectively) exist in nociceptors, iGluRs regulate some key features of pain transmission. The subunits of iGluRs are divided into the three groups of α-amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Kainate, and N-methyl-D-aspartate (NMDA) receptor families. The differences in the genes that code for these families of receptors give rise to differences in the respective primary, secondary, and tertiary structures translated into transmembrane proteins causing various differences in their overall expression, localization, and function. Differences in expression of the receptor families can affect overall signaling kinetics of an individual cell across its entire body, as the kinetics for the activity of the receptors influence the kinetics of the instigation and dynamics of the current experienced by the excited cell. The subcellular localization of the receptors can influence the overall activity of an individual cell; expression at a postsynaptic terminal or one particular receptor may be excitatory while expression of the same kind of receptor at a presynaptic terminal can actually be inhibitory (Kerchner, et al. 2002). Furthermore, differences in the overall function of the proteins are numerous, despite the common primary endogenous neurotransmitter responsible for their activation. In addition to differences in the kinetics of the gating of current allowed by these receptor families, differences in selectivity of ions passed as part of those currents as well as different associated proteins on the intracellular and extracellular domains yield various effects on the immediate activity, long-term activity, and overall health of the cells in which they are expressed.

The currents experienced by the cell are dependent on the receptor families present and result from differences in the primary, secondary, tertiary, and quaternary structures formed by the fully assembled transmembrane receptors. Differences in the primary structure may arise from different gene sequences in the DNA or from post-transcriptional modification of the transcribed RNA. Substitution of a single amino acid

residue can yield different selectivity profiles for the transmembrane pores of the receptors formed as well as affect the assembly and trafficking of nascent receptors (Carlson, et al. 2000, Greger, et al. 2003,). Secondary structures formed by nascent proteins influence the tertiary assembly of the subunits and are necessary for maintaining structural integrity of the receptors as they undergo conformational changes. Differences between the families of iGluRs in their tertiary structures result in specifically distinct ligand-binding domains for different ligands, or associated intracellular or extracellular proteins, to interact with the different receptors formed. Particular proteins that associate with individual receptors may influence the trafficking of those receptors, leading to different localization profiles of those receptors within the same cell. The quaternary structures assumed by the different families of receptors correlate to their different activity states between which the receptor may alternate due to conformational changes in the orientation of the tertiary structures. These conformational changes may influence the shape of the pore opening, the availability or affinity of the ligand-binding domain, or the availability or affinity of an associated protein-binding domain.

Calcium permeable AMPA receptors in the peripheral processes of nociceptors influence chronic inflammatory pain (Gangadharan et al., 2011). Other factors involved in the induction of chronic pain include the inflammatory signals TNFα and IL-1, which likely involve NMDA receptors because NMDA receptor blockade inhibits the normal increases in expression of TNFα and IL-1β in nociceptors in response to injury (Kleinschnitz et al., 2004). Peripheral agonism of Kainate receptors on C-fiber sensory neurons causes ventral root responses comparable to withdrawal reflexes, indicating that

activation of peripheral Kainate receptors also contributes to pain sensation (Ault and Hildebrand, 1993).

Altered activity in Aδ and C peripheral neurons is implicated in the development of migraine and chronic itch as well. The pathophysiology of classic migraines has been the subject of multiple studies, although some gaps in understanding persist in the literature. Studying the influence of nociceptors on the vasculature of meninges provides a useful perspective on nociceptors' influence on vasculature in the rest of the body, which is important for understanding the processes underway in normal versus injured or inflamed tissue. The canonical factors precipitating migraine are cortical spreading depression (CSD) and increased cerebral and meningeal blood flow. CSD is a phenomenon of the nervous system characterized by a radiating depolarization of neuronal and glial cells that conducts much more slowly than normal depolarization and precludes synaptic transmission, effectively depressing the affected cortical area (Ayata, 2010). The underlying neuronal cause is thought to stem from an excess of potassium efflux due to focal hyperactivity, causing mass depolarization of, and glutamate release from, neighboring neurons that then spreads along tracts of cell bodies in cortical gray matter of the ipsilateral cerebral hemisphere (Ayata, 2010).

Unmyelinated projections into cerebral and meningeal blood vessels from trigeminal ganglion neurons comprise the main sensory component of the trigeminovascular system, which influences cerebral and meningeal blood flow and have been implicated in migraine for more than thirty years (Moskowitz, 1984). CSD elicits a delayed activation of meningeal nociceptors that can last for more than ninety minutes (Zhang et al., 2011). Activity of sensory trigeminal neurons leads to extravasation of plasma from blood

vessels in the dura mater, and the trigeminal nerve mediates hyperemia in cortical gray matter (Zhang et al., 2011b). In line with this, the matrix metalloprotease 9 (MMP-9) is activated and upregulated in response to CSD, and may mediate some of the structural changes to the blood brain barrier that permit plasma extravasation (Gursoy-Ozdemir et al., 2004; Maddahai et al., 2009). Cortical spreading depression ultimately leads to activation of central trigeminovascular neurons in the spinal trigeminal nucleus, leading to altered blood flow in cerebral and meningeal arteries (Moskowitz, 1990). This may be involved in the development of pathophysiological changes such as subcortical white matter lesions and iron accumulation in central nervous system (CNS) structures that are associated with both episodic, and more markedly, chronic migraine (Matthew, 2011).

Unfortunately, the increased frequency of migraine attacks is correlated with increased prevalence of allodynia, which may persist in between migraine attacks in those with chronic migraine (Matthew, 2011; Ashkenazi et al., 2007). Complicating this correlation, the repeated use and overuse of opioid analgesics causes opioid tolerance, the phenomenon of opioid-induced hyperalgesia, and the transition of episodic migraine sufferers towards the condition of chronic migraine (Saper and Lake, 2008). Because of the compounding nature of the progression of migraines and the tolerance building and addictive effects of opioid analgesics, modulating the activity of peripheral nociceptors that innervate cerebral and meningeal vasculature via glutamate signaling instead of targeting the anti-nociceptive circuitry of the CNS via opioid signaling could provide an attractive alternative means of decreasing durations of, or providing prophylaxis against, migraine pain.

Despite the bevy of studies on the gene expression of nociceptors, characterization of the complement of iGluRs in peripheral tissues remains incomplete. Exploiting the fact that the AMPA, Kainate, and NMDA receptors are oligomeric instead of monomeric, pharmacologic chemicals have been found that selectively modulate specific iGluRs activity based on subunit composition. Identification of iGluRs in the periphery exclusive to Aδ and C fiber neurons would yield targets for the modulation of nociceptor activity, and thus therapeutic targets for treating pain or itch. Exclusively modulating the activity of peripheral nociceptors would preclude the proclivity towards abuse and addiction common to prescribed depressive analgesics acting in the CNS, as well as maintain the proper protective function of normal pain. Conserving the nominal activity of nociceptors while preventing hyperactivity may provide a means of treating pain, without depressing neuronal activity to the point of causing numbness in the peripheral nervous system or intentionally altering signaling within the central nervous system. Modulating the activity of peripheral nociceptors could be used prophylactically in advance of traumatic medical interventions as well. Though pharmacologic agents targeting iGluRs might not immediately alleviate the perception of pain it is possible that decreased iGluR activity in sensory neurons could decrease the proportion of neurons that become chronically sensitized and hyperactive.

IONOTROPIC GLUTAMATE RECEPTORS

Since the vast majority of neurons throughout the entire nervous system utilize glutamate as a neurotransmitter, and the wide range of functionality of glutamatergic neurons both in the kinetics of firing and in the networks with which they are involved, it makes evolutionary sense for a number of glutamate receptor families and subtypes to have arisen in order to facilitate the diverse responses necessary for different neuron types in different networks and circuits. Ionotropic glutamate receptors have rapid signaling onset with concomitant rapid decay of the signal, while metabotropic glutamate receptors have slower signaling kinetics and may continue to signal for longer periods of time.

Differences in the onset or decay of signals is due to the physical and chemical dynamics of the proteins themselves after the binding of a ligand. Ligand-gated ionotropic receptors undergo a conformational change upon binding their respective ligands on the extracellular surface, which translates some portion of the tertiary structure of the protein to create an effective channel or pore in the membrane of the cell. This pore allows for the passage of ions down their respective concentration gradients, which are established by the selective movement of specific ions across the cell membrane by a host of other transmembrane proteins. Different ionotropic receptors, and indeed different functional isoforms of receptors expressed from the same gene, can exhibit selectivity for specific ions due to physical characteristics of the pore segment of the receptor.

The diameter of a pore, as well as the charges of the residues exposed on the interior surface of the pore formed, can contribute to selectivity of particular ions. Ion selectivity can be for individual ions or for ions with different charges, such as monovalent or divalent cations. The inclusion or substitution of particular amino acid residues in sequence segments comprising the internal surface of the pore of the transmembrane

receptor can contribute to the forces that will act on ions passing through the channel. The inclusion of a positively charged arginine, instead of a neutral glutamine residue, for example, in the AMPA receptor subunit GluR2 is a post-transcriptional modification that determines whether or not the receptor formed as a quaternary heteromeric structure will be permissive for calcium (Ca^{+2}) ions, or be Ca^{+2}-impermeable (Pellegrini-Giampietro, et al. 1997). Substitution of that single arginine residue instead of a glutamine residue at that individual editing site causes an alteration in the locations of charges lining the pore formed within the transmembrane receptor that leads to a selection for monovalent cations potassium (K^{+}) and sodium (Na^{+}) over the divalent cation Ca^{+2} (Pellegrini-Giampietro, et al. 1997). The importance of this comes not simply from the immediate alteration of the changes of ions in flux but also from the utilization of Ca^{+2} as a second messenger molecule within the cell. Second messenger molecules may play into multiple signaling cascades so the conductance of a second messenger like Ca^{+2} can have numerous effects on various signaling cascades that involve intracellular Ca^{+2}, such as the activation of Protein Kinase C (Newton, 1995)

Among the ionotropic glutamate receptors there are additional differences in the dynamics of signaling, whether via differences in the ligand selectivity, the selectivity of the ion channel pore, kinetic gating of the receptor, or potential secondary signaling that may occur in the presence of proteins that activate particular downstream cascades in response to the initial signal. The ligand selectivity and sensitivity are largely, though not entirely, determined before the translation of the subunits because of the primary structure giving rise to secondary and tertiary structures during the folding of the nascent protein that form ligand binding domains. The family to which the receptor subunit

belongs is genetically defined and is named after the ligands tested to which the family of receptors initially showed the highest binding affinity. Kinetics of the activity of a receptor can be influenced by post-transcriptional modification, translation of the nascent protein and the assembly of the receptor itself. Inclusion or substitution of individual residues may have diverse effects on the overall function of the receptor including changing the rate of desensitization, ligand binding affinity, or selectivity for specific ions.

BEHAVIORAL STUDIES AND PHARMACOLOGY

Pain in its normal context is caused by injury and leads to inflammation, which has long been known to cause the classic triad of rubor (redness), dolor (pain), and calor (heat) in the affected area. Edema associated with inflammation is caused by plasma extravasation from the vasculature. Redness is due to increased blood flow in an injured area that allows for the extrusion of foreign material via bleeding in injuries that break the skin. In addition to these phenomena the removal of foreign material and the cessation of bleeding are accomplished by the mobilization of immune cells from circulation to consume either foreign or dead tissue, as well as platelets to stem the flow of blood. Two major signaling factors contributing to extravasation are the neurotransmitters Substance P and calcitonin gene-related peptide (CGRP), which increase the permeability of the vasculature leading to extravasation, along with inducing vasodilation leading to the swelling and redness common to local inflammation (Weidner et al., 2000). The

neurotransmitter glutamate may also be loaded and released in vesicles along with Substance P and CGRP, leading to autaptic and ephaptic signaling of peripheral processes via their respective iGluRs in an injured area along with activation of the same or other nearby nociceptors' peripheral iGluRs (De Biasi and Rustioni, 1988; Miller, 1993).

Glutamate is loaded into vesicles in sensory neurons by *VGLUT1* and *VGLUT2*, which are present in peripheral processes of DRG neurons (Brumovsky et al., 2007). The synthesis of glutamate from glutamine is carried out in peripheral neurons by glutaminase, and the activity of glutaminase in DRG neurons is up-regulated both in expression and activity 7 days after the induction of inflammation by injection of Complete Freund's Adjuvant into the target tissue of innervation (Miller et al., 2012). The up-regulation of glutaminase is accompanied by increased thermal and pressure sensitivity in response to the induced inflammation (Miller et al., 2012). Inhibition of glutaminase activity by peripheral application of 6-diazo-5-oxo-l-norleucine decreases the number of cells labeled for Fos, which is an indicator of neuronal activity, in the corresponding superficial layers of the dorsal horn on which many of nociceptive DRG neurons synapse. (Hoffman and Miller, 2010). Glutamate is released by keratinocytes themselves upon disruption of the normal basal cell and superficial cell organization of the skin, as well as immune dendritic cells during maturation via a cystine/glutamate antiporter (Pacheo et al., 2006). These select cursory examples show that there are multiple sources of increased glutamate in tissues following injury that may act on the peripheral iGluRs to modulate nociception (Jin et al., 2006).

While the current trends in treating many types of pain rely on the prescription of depressive analgesics that affect neurons in the CNS including opioids, these have a large potential for abuse and addiction. Opioid drugs influence the activity of neurons expressing opioid receptors comprised of the δ, μ, and κ types. Neurons expressing these receptors include peripheral sensory neurons, nucleus raphe magnus neurons, periaqeductal gray neurons, ventral tegmental area dopaminergic neurons, and bulbospinal respiratory neurons (Stein, 2000; Nozazde et al., 2011; Fang et al., 1997, Veer et al., 2013; Stucke et al., 2008). The effects of opiates on the activity of these neurons in the central nervous system yield the psychotropic effects of the drugs as well as the ultimate cause of death in most instances of opiate overdose. Exclusively targeting pharmaceutical treatments to iGluRs active in the peripheral processes provides an appealing means of treating pain while mitigating the potential need for depressive analgesic intervention.

Apart from neurons in the periphery and central nervous system (CNS), other cell types may express iGluRs such as pancreatic islet cells, cardiomyocytes, and T cells (Gill et al., 1998; Weaver et al., 1996; Ganor et al., 2003). Modulation of the activity of non-neuronal or CNS neurons remains a major pitfall in pursuing the regulation of peripheral glutamate signaling (Muir and Lees, 1995). Systemic administration of the NMDA non-competitive antagonist MK-801 has analgesic effects but also causes deficits in learning and memory, as well as hyperlocomotion similar to phencyclidine (PCP) administration (Kovacic and Somanathan, 2010). The AMPA and NMDA antagonist kynurenic acid inhibits NMDA signaling by binding at the glycine site and fails to cross the blood brain barrier, which are both attractive treatment characteristics for modulating nociceptor

activity (Marosi et al., 2010). It is, however, an endogenous compound that also antagonizes the α7 nicotinic acetylcholine receptor, which is involved in the regulation of endocrine release in the lung, angiogenesis in vascular endothelial cells, and terminal differentiation of keratinocytes (Canastar et al., 2012). The systemic administration of the AMPA and Kainate antagonist LY293558 has been shown effective in treating capsaicin-induced hyperalgesia while maintaining normal pain sensation in human trials, but the side effects of headache and visual deficits indicate that it likely crosses the blood brain barrier and is therefore suboptimal for the treatment of pain, especially in cases of chronic pain where consistent administration would be needed (Sang et al., 1998). These results show the need for evaluating the different iGluR subunits in peripheral neurons, especially those receptor combinations that may be selectively expressed by Aδ and C fiber neurons. Targeting of iGluRs exclusive to Aδ and C fiber neurons requires full characterization of the quantitative presence of the iGluR subunits, and qualitative evaluation of their respective contributions to activity and excitability of the neuron. The potential benefit of pharmacologically modulating the activity of the nociceptor instead of blocking overall activity of the neuron and the transmission of pain is that it may conserve the protective function of pain in alerting the individual to injurious stimuli, while effectively treating the symptom of pain resulting from excessive nociceptor activity.

Evaluating the composition and contribution to activity of the individual iGluR subunits requires a system wherein the different segments of the nociceptors can be studied in isolation, taking into account the paracrine-like interactions that may occur between sensory neurons and their target tissues of innervation. A compartmented culture system

provides a means for studying different segments of neurons in isolation. Co-culture systems provide a means for evaluating the intercellular interactions of different types of cells including neurons and glia. Some complex microfluidic configuration systems also allow for analysis of the effects of targeted application of drugs on cultured cells, but those microfluidic systems have utilized calcium imaging techniques in order to analyze overall activity (Taylor et al., 2005; Taylor et al., 2010). While calcium imaging does portray the overall activity of a neuron, it does not facilitate the direct measurement of the current or voltage at the cell membrane. Employing a compartmented co-culture system in combination with electrophysiology may ultimately provide a means to evaluate the spatial distribution of iGluR receptor subunits as well as their respective contribution to activity of the neurons by focal application of receptor ligands. Direct measurement of the currents generated in response to the targeted application of ligands may show distinct differences in the activity of the neurons at the soma, indicating some level of signal processing prior to an impulse reaching the somatic compartment. An increased complexity of signals that might result from pre-somatic processing could provide a template for better understanding, modeling, analyzing and testing complex sensory input.

IMMUNOHISTOCHEMISTRY

Among the three classes of iGluRs, the AMPA class has 4 members, the Kainate class has 5 members, and the NMDA class has 7 members. The AMPA receptor subunits are

named GluA1-4. The NMDA receptor subunits are named NR1, NR2a-d, and NR3a-b. There has been some long standing contention in the naming of the members of the Kainate class, as GluK1, GluK2, and GluK3 were originally described in the AMPA class and given the names GluR5, GluR6, and GluR7, respectively. The two receptors originally describing the Kainate class were called KA1 and KA2, but for the sake of uniformity they are now referred to as GluK4 and GluK5.

Of the Kainate class, GluK1, GluK4, and GluK5 are present in DRG neuron cell bodies (Lucifora et al., 2006; Petralia et al., 2004). Of the AMPA class, members GluA1, GluA2, and GluA4 are present in DRG neuron cell bodies (Willcockson and Valtschanoff, 2008; Lu et al., 2002). Of the NMDA class, NR1, NR2b, NR2c, and NR2d are present in DRG neuron cell bodies (Marvizon et al., 2002).

Some evidence, via either reverse-transcriptase polymerization chain reaction (RT-PCR) or via immunohistochemistry, has been put forth suggesting the presence of the remaining receptors from all 3 classes; GluA3 from the AMPA class, GluK2 and GluK3 from the Kainate class, and NR2a, NR3a, and NR3b from the NMDA class.

While central synapses and somatic membranes of DRG neurons have been thoroughly examined for the presence and function of iGluRs, the body of evidence for receptors in peripheral projections is lacking due to lack of specificity of reagents. One paper has described population changes in overall immunoreactivity of glutamate receptors in peripheral nerve sections before and after induced inflammation (Carlton and Coggeshall, 1999). Utilizing selective antibodies for GluA1 and NR1, and an antibody for GluK1-3, Carlton and Coggeshall demonstrated increased signals for glutamate receptors after

complete Freund's Adjuvant induced inflammation in both myelinated and non-myelinated neurons as shown in Figures 1 through 4 (Carlton and Coggeshall, 1999).

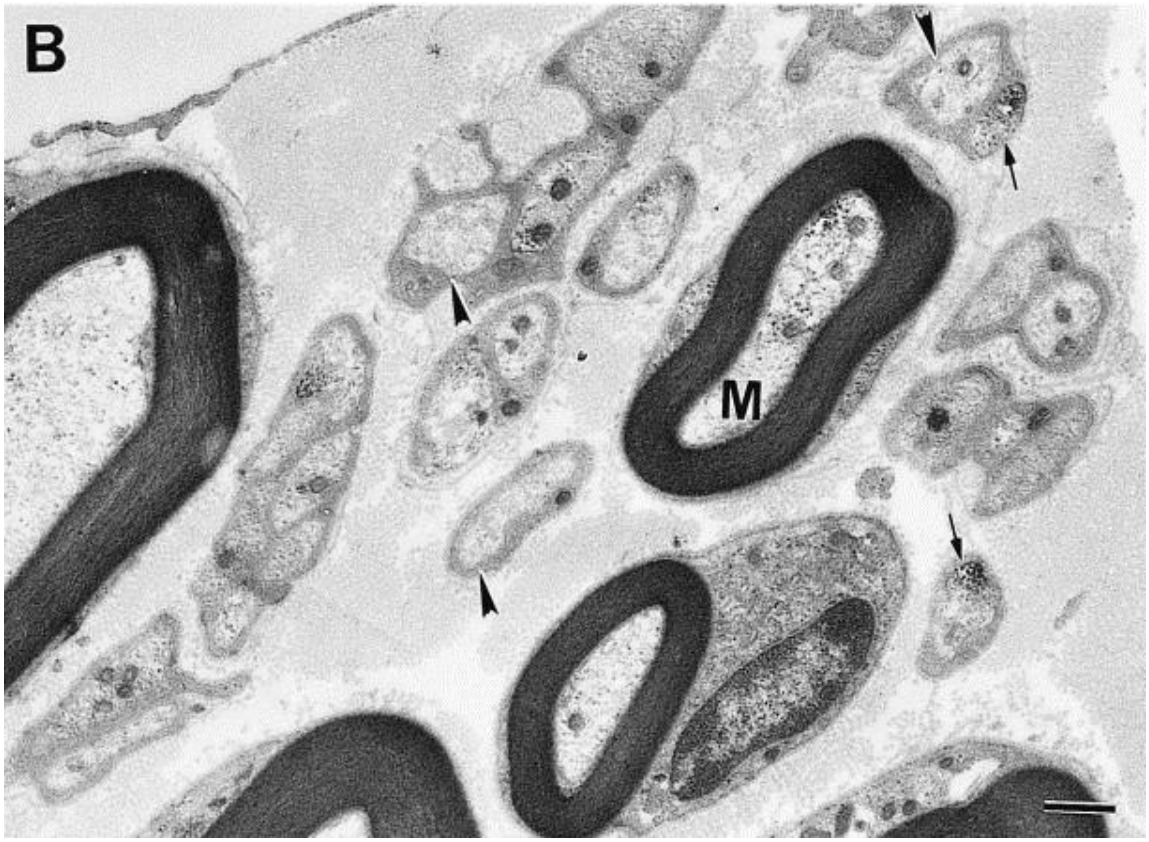

FIGURE 1: Immunoreactivity of an antibody against GluA1 in untreated cutaneous nerve cross section from plantar skin of a rat. Myelinated axons (M) and unmyelinated axons (arrows) show positive staining; whereas arrowheads indicate non-reactive unmyelinated axons. (Carlton and Coggeshall, 1999)

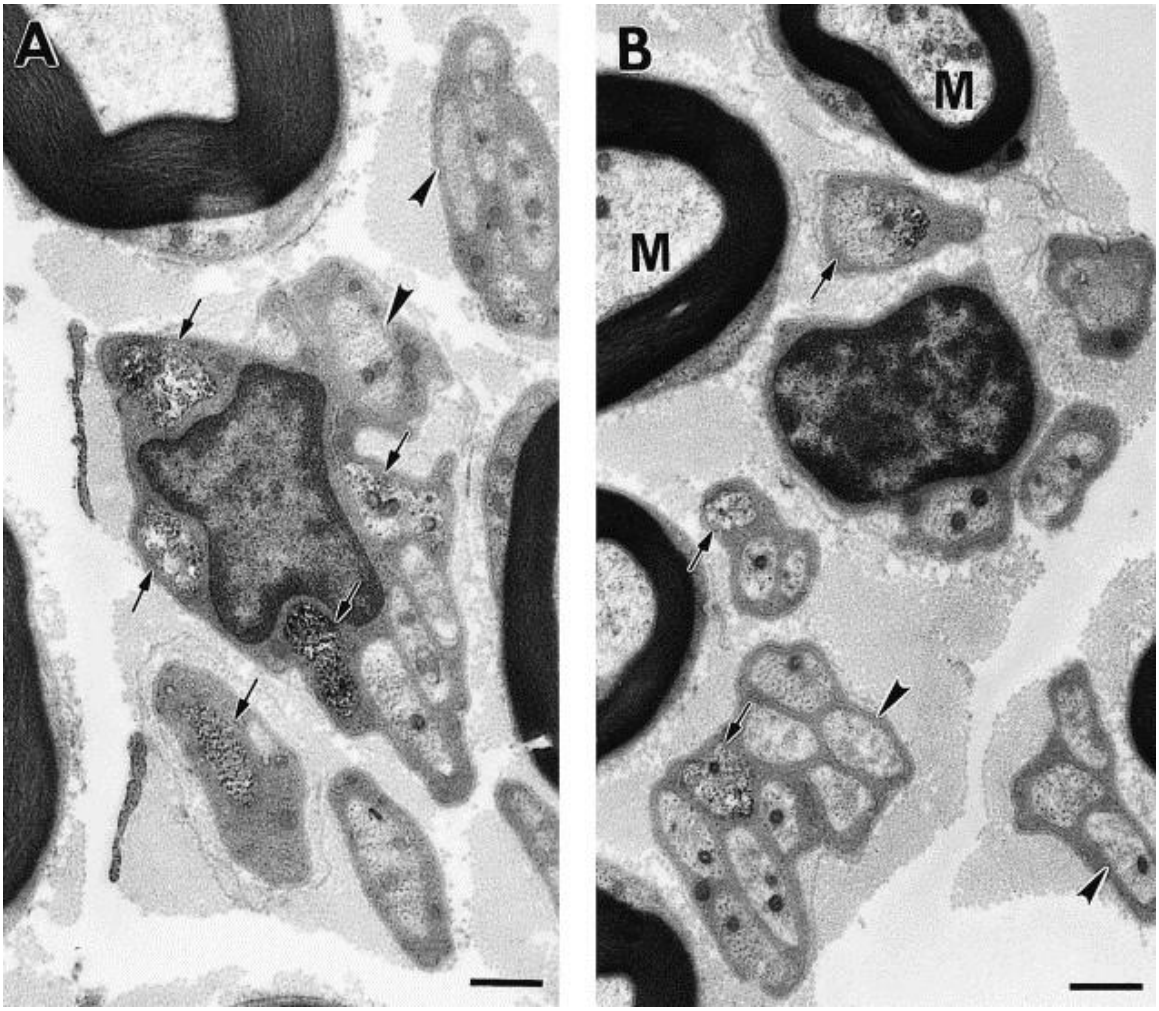

FIGURE 2: Immunoreactivity of an antibody against GluK1-3 in untreated (B) and Complete Freund's Adjuvant-induced inflammation (A) cross section of cutaneous nerve from plantar skin of a rat. Myelinated axons (M) and unmyelinated axons (arrows) show positive staining, whereas arrowheads indicate non-reactive unmyelinated axons.

(Carlton and Coggeshall, 1999)

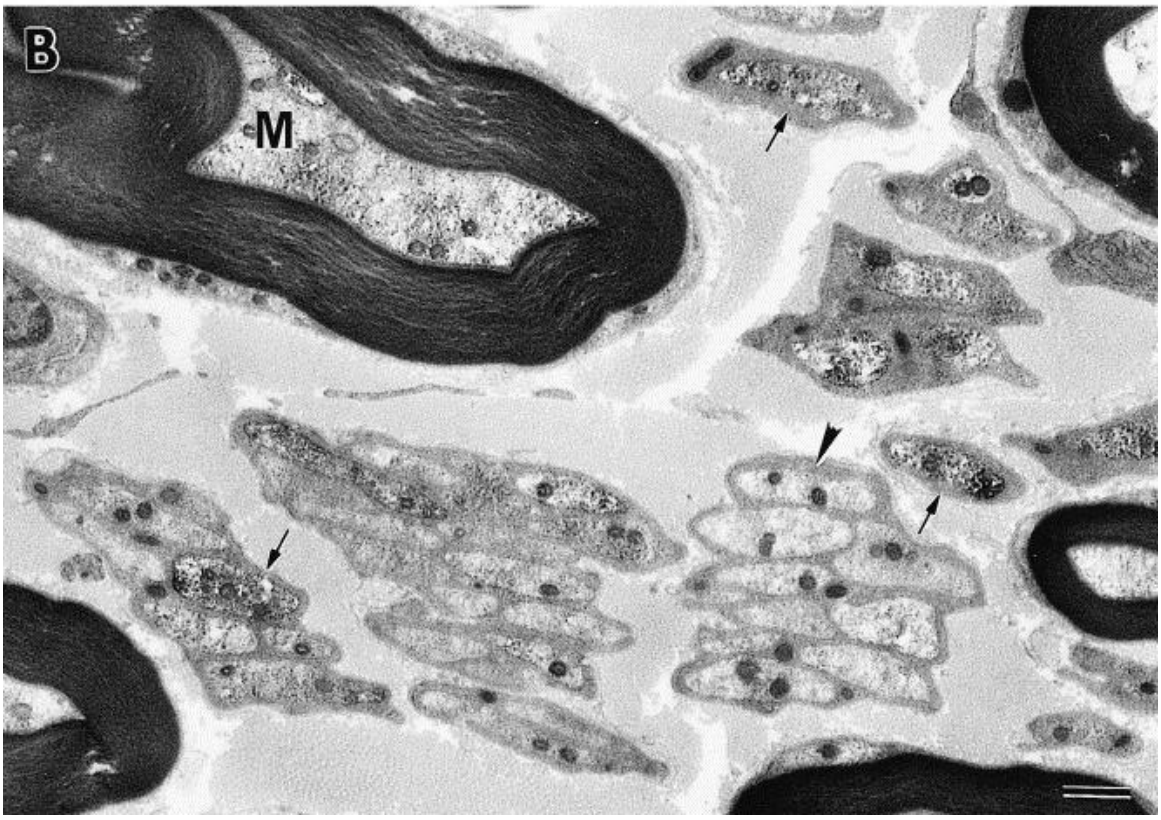

FIGURE 3: Immunoreactivity of an antibody against NR1 in untreated cutaneous nerve cross section from plantar skin of a rat. Myelinated axons (M) and unmyelinated axons (arrows) show positive staining; whereas arrowheads indicate non-reactive unmyelinated axons. (Carlton and Coggeshall, 1999)

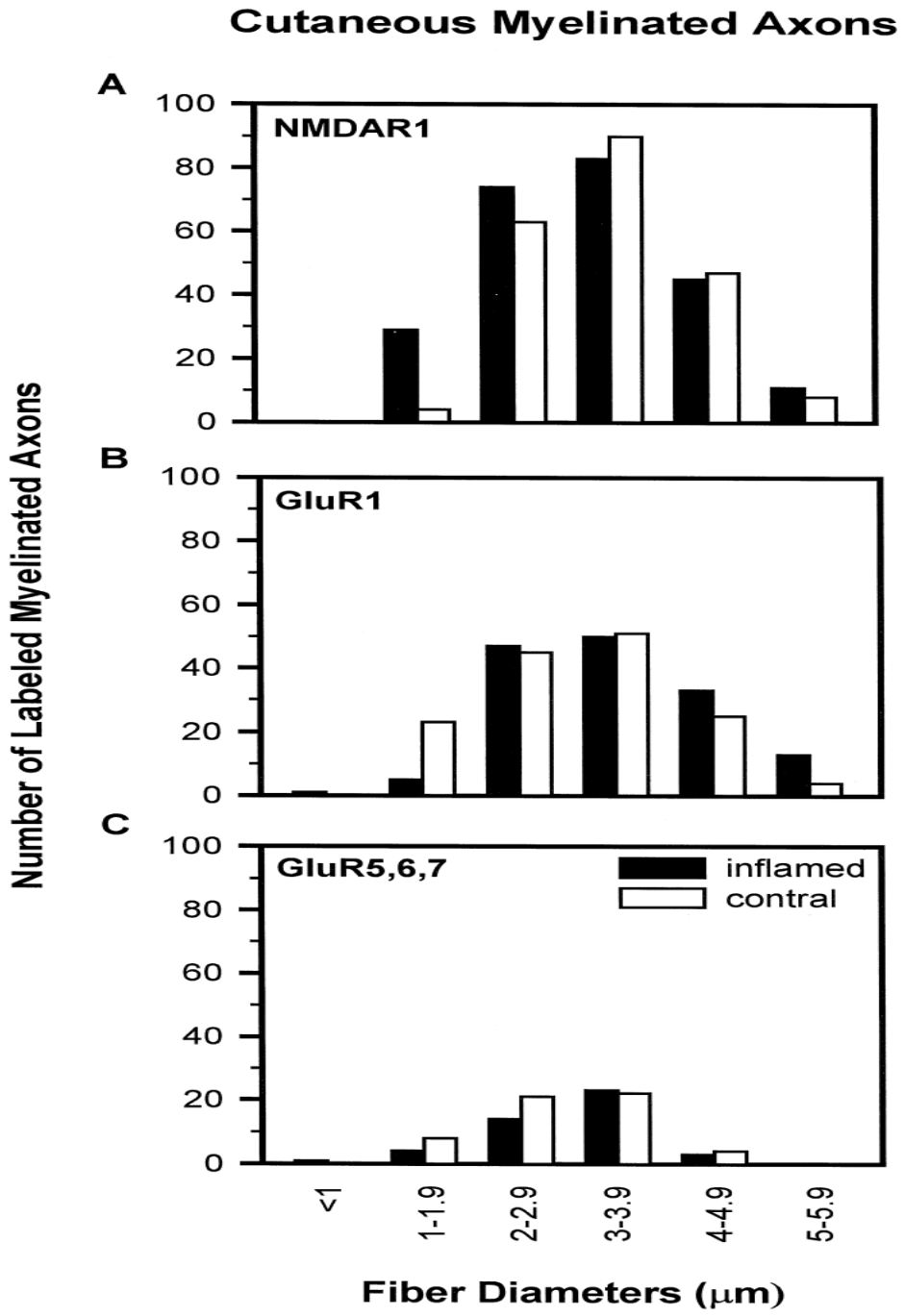

FIGURE 4: Comparison of NR1, GluA1, and GluK1-3 staining in Complete Freund's Adjuvant treated and contralateral untreated cross sections of cutaneous nerves. Fibers are separated by size to show greater increases in smaller diameter Aδ fiber neurons.

(Carlton and Coggeshall, 1999)

This early study provides compelling evidence for the presence of iGluRs in peripheral processes of DRG neurons that could potentially be targeted for the treatment of pain in inflammation. Technological advances since the publication of this study, however, allow for a more specific and comprehensive quantification of the presence of iGluRs in peripheral processes with the advent of novel, more selective antibodies that can be used to refine the quantification of the iGluRs present in peripheral processes of sensory neurons.

Fully characterizing the types of receptors present in order to provide a more complete understanding of which iGluRs contribute to peripheral pain and itch may yield more ideal targets for pharmacologically treating pain and itch without exerting effects on the CNS. In order to verify which receptors are present in peripheral processes it is important to perform spatially constrained immunofluorescent experiments to evaluate the expression of different receptor subunits in different segments of sensory DRG neurons. The combination of reagents and techniques used here provide compelling evidence for which receptors are present in what proportions in the periphery, and how they might contribute to activity and excitability.

ELECTROPHYSIOLOGY

Electrophysiology has been employed to study the activity of iGluRs of DRG neurons at their respective somata as well as the activity of their dorsal horn neuron targets in the spine. AMPA, Kainate, and NMDA receptors all instigate depolarization in DRG

neurons, but the outcome of receptor activation varies with receptor composition and location (Lee et al., 2004). The influence of the individual receptor subunits on neuronal activity depends on factors including the ionic permeability and subcellular localization in the membrane. Kainate applied to the central axon of a dorsal root ganglion depresses activity elicited by a stimulating electrode, whereas application to the ganglion itself may not (Agrawal and Evans, 1986). The depression of evoked activity is attributed to the increase in conductance of monovalent cations due to the activation of the non-NMDA (Kainate and AMPA) receptors when applied to the central processes (Huettner, 1990). The sensitivity of the DRG neurons to kainate is also substantially higher than the sensitivity of freshly dissociated cortical neurons from the rat cerebrum (Huettner, 1990). Activation of presynaptic Kainate receptors on central terminals of DRG neurons decreases neurotransmitter release, indicated by the decreased subsequent depolarization of the dorsal horn target neuron (Lee et al., 2004; Huettner et al., 2002). Figure 5 shows a decrease in peak amplitude responses from a dorsal horn neuron in response to electrically stimulated depolarization of a DRG neuron on a microisland in the presence of kainate.

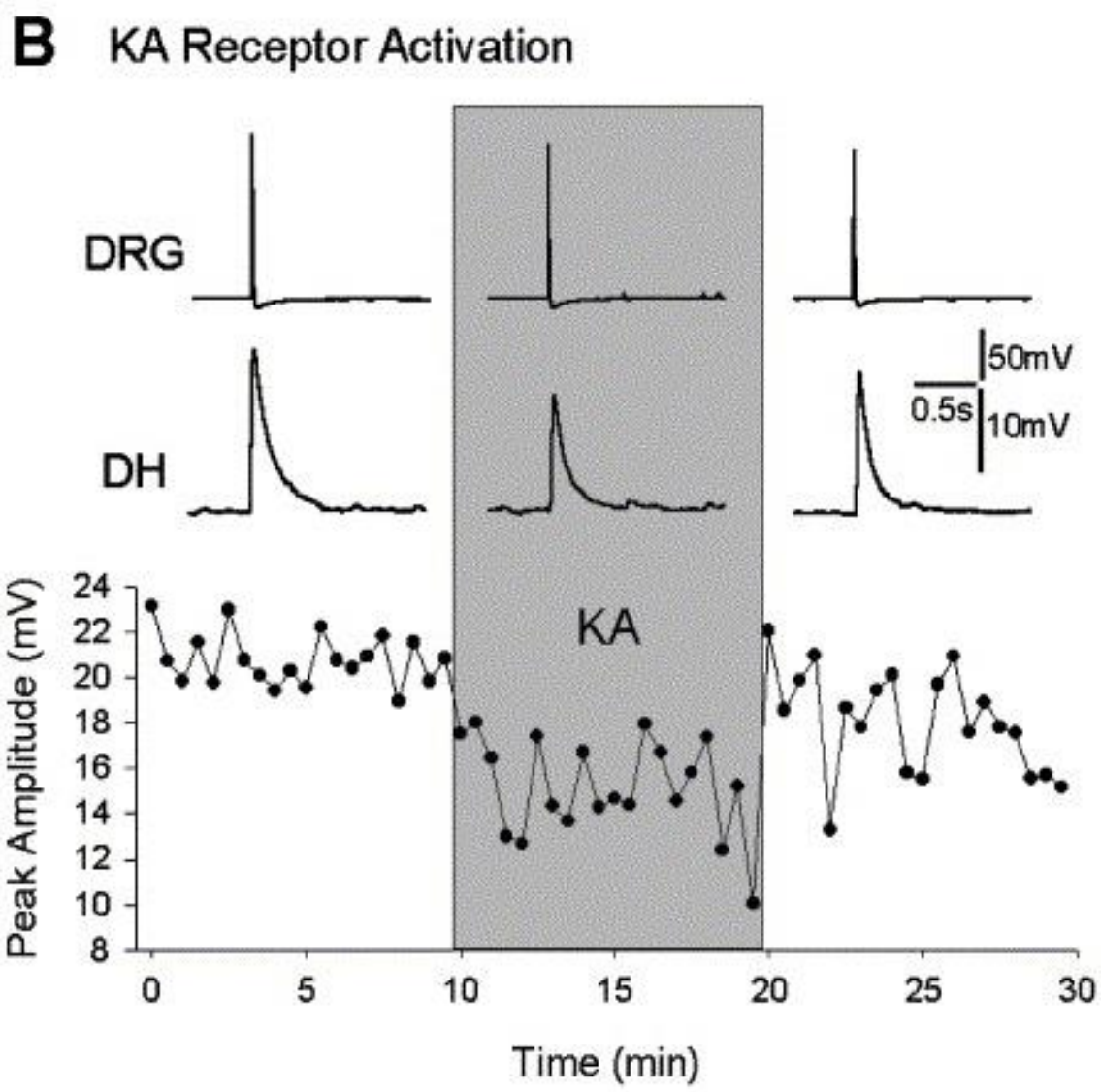

FIGURE 5: Representative traces from an electrically stimulated DRG neuron and the response of its paired dorsal horn neuron, with and without application of kainate, along with peak amplitude values recorded over time with repeated application and subsequent recovery after washout.

(Lee et al., 2004)

The inhibitory effect of kainate on excitation of dorsal horn neurons is attributed to the presence and activity of GluK1 on the pre-synaptic central terminals of DRG neurons (Kerchner et al., 2002). While the absence of GluK2 on the post-synaptic dorsal horn neuron causes the inhibitory effect of kainate on dorsal horn neurons to decrease, this influence is nullified by the selective pharmacological targeting of GluK1 on the presynaptic membrane of the DRG neuron (Kerchner et al., 2002). Figure 6 depicts the comparison of the depression of NMDA receptor mediated responses in dorsal horn neurons to excitation of a nearby DRG neuron with a stimulating electrode in the presence of continuously applied kainate and a GluK1 selective agonist, (RS)-2-Amino-3-(3-hydroxy-5-tert-bu-tylisoxazol-4-yl)propanoic acid (ATPA) (Kerchner et al., 2002).

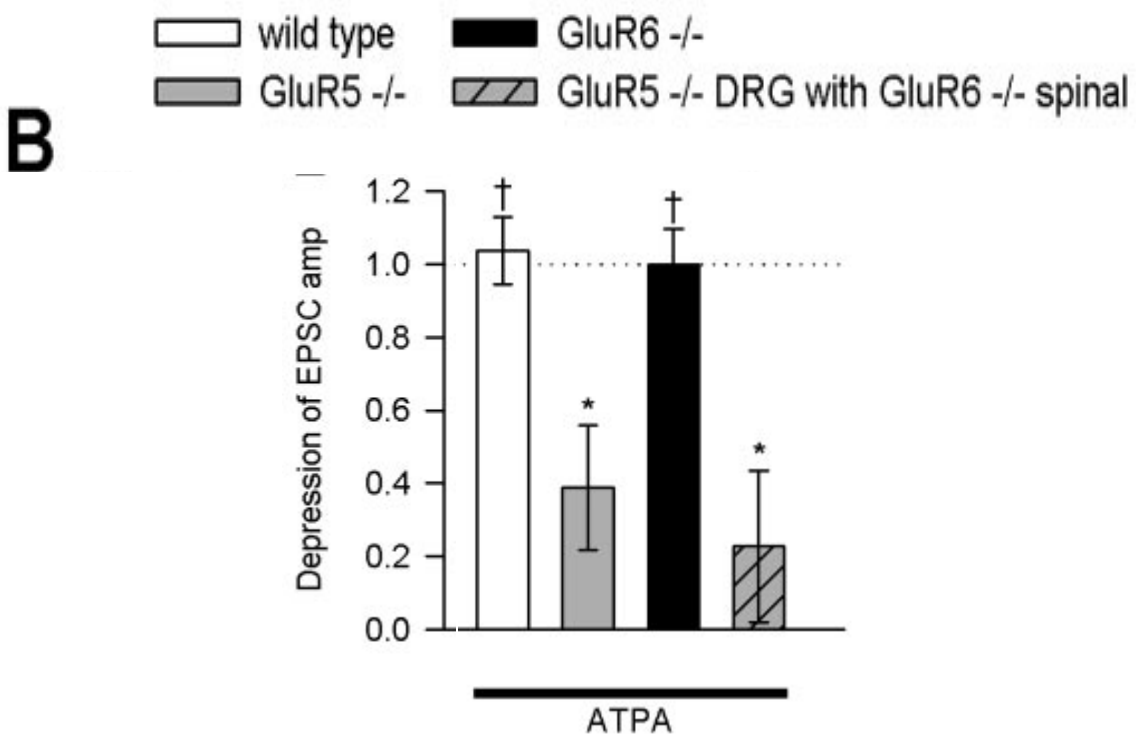

FIGURE 6: This excerpted figure depicts the comparison of depression of dorsal horn NMDA receptor mediated responses to excitation by electrical stimulation of a nearby DRG neuron; both cells are co-cultured from a rat with all Wild-Type receptors (white bar), with GluK1 receptors knocked out (grey bar), with GluK2 knocked out (black bar), or with GluK1 missing from DRG neurons and GluK2 missing from spinal neurons.

(Kerchner et al, 2002)

GluK1 is expressed as three splice variants, dubbed GluK1a-c, which have different characteristic tissue distributions and protein interactions (Vesikansa et al., 2012). When GluK1c complexes with GluK4 or GluK5 its localization to distal dendritic processes of hippocampal neurons is increased, and the GluK1c splice variant may contribute to the functional differences between Kainate receptors that increase or decrease neurotransmission in response to depolarization (Vesikansa et al., 2012).

The Kainate family of receptor subunits is not the only family to exhibit a decrease in current during activation, the NR3a and NR3b subunits of the NMDAR family are both dominant-negative subunits that cause decreased current when transfected into *HEK 293* cells and evaluated electrophysiologically (Nishi et al., 2001).

Differences in ion permeability between iGluRs also greatly affect the potential consequences of receptor activation. NMDA receptors are permeable to calcium (Ca^{2+}), which is an important signal for synaptic plasticity among other functions. The AMPA receptor subunit GluA2 is subject to RNA editing, which substitutes an arginine residue in place of a glutamine residue within the channel pore which makes it much less permeable to Ca^{2+} (Paschen et al., 1996). Interestingly, activation of presynaptic AMPA and Kainate receptors in DRG neurons depress the responses of their dorsal horn neuron synaptic targets, and those DRG neurons that contain AMPA receptors do so more effectively than their Kainate receptor-only counterparts; glutamate acting on peripheral glutamate receptors, however, increases signaling (Lee et al., 2004; Cao et al., 2007).

The NR2b selective antagonist, Ifenprodil, has been shown to decrease the normal NMDA receptor mediated response to application of NMDA and glycine, indicating its

presence and functionality in rat DRG neurons (Li et al., 2004). It should be noted, however, that these data are not based on peripheral application of pharmacological agents and so the activity of iGluRs in the periphery remains unclear. Since electrical stimulation of one thoracic DRG peripheral root increases the baseline activity of neurons in the adjacent DRG, and this effect is prevented by the peripheral application of iGluR antagonists, it follows that iGluRs on peripheral terminals are functional and influence the activity of an individual neuron as well as the adjacent neurons (Cao et al., 2007).

There are known differences in levels of expression and activity of iGluRs attributable to co-expression, post-translational modification, RNA editing, subcellular localization, and protein interactions. Because these factors may ultimately determine which iGluRs are present in which segments of neurons, as well as how they function, further research must be done to elucidate exactly which combinations of iGluRs are present and active in the peripheral processes of DRG neurons, as well as the consequences of the activation of each type of receptor in this context. It is evident that iGluRs of different composition or location can have different, even contrary effects on the activity of neurons. In order to characterize fully those differences in activity it is important to perform a series of electrophysiological experiments to evaluate the influence of the different iGluR subunits on activity and excitability over time and in different contexts, in this case the influence of peripheral iGluR subunits on overall activity of DRG neurons as measured via electrophysiology at the soma.

CHAPTER II

MODEL DEVELOPMENT

Plating of the cells and assembly of the culture systems used for this study constitute a significant deviation from previous protocols and therefore warrant a thorough procedural description. The invention of compartmented culture in the 1970s facilitated a novel system for discretely evaluating spatially segregated phenomena within different segments of neurons, wherein the dissociated neurons are placed in a central chamber and can grow projections into lateral chambers with minimal fluid exchange between the two. This compartmentalization of environs allows for discrete study of the morphology of neurons, their projections, their juxtaposition among neighboring neurons, and potential associations with other cells, as well as spatially restricted exposure to substances that could occur in whole tissue. The endeavor was to co-culture sensory neurons along with keratinocytes in a configuration that would allow characterization of the distal processes and free nerve endings of the sensory

neurons in association with keratinocytes in order to view a simulated 'innervated skin tissue.'

CULTURE SUBSTRATUM

One primary concern in the development of a model incorporating the co-culture of neurons and the keratinocytes they would innervate is the mobility of the cells themselves. While the compartmented culture configuration originally invented facilitates the discrete isolation of compartments while allowing the penetration of neuron processes from one compartment into another, some means of simultaneously maintaining the integrity of the fluid barrier while enabling the penetration of the projections from neurons and preventing the migration of keratinocytes became necessary. The original substrate for compartmented culture as outlined by Campenot in 1977 is rat tail collagen.

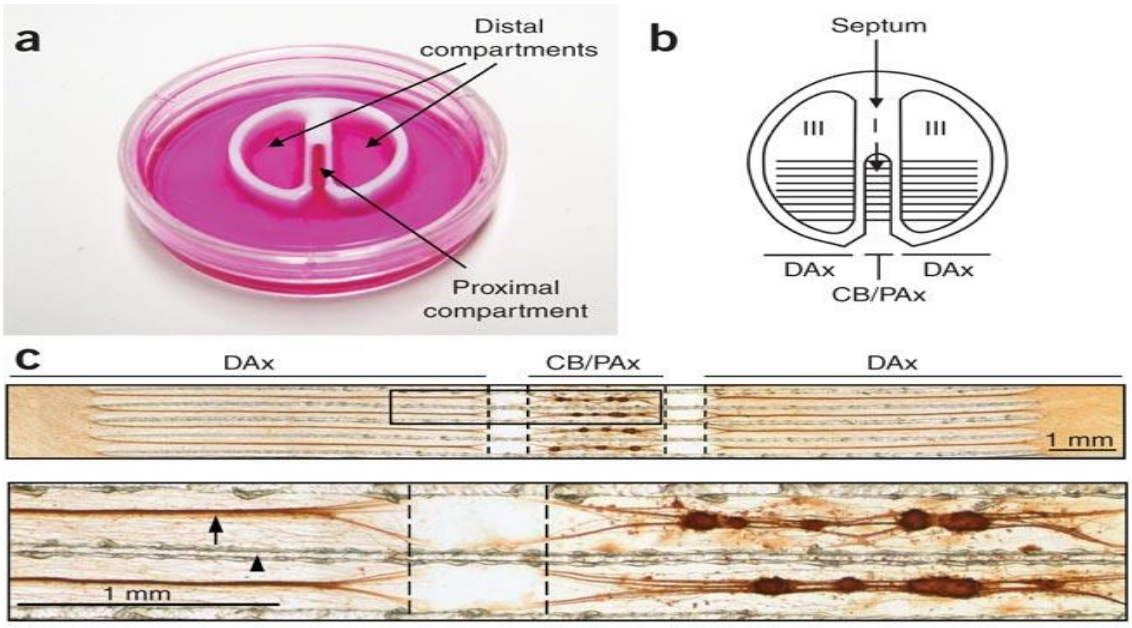

FIGURE 7: This figure depicts a picture and a diagram of the original Campenot chamber compartmented culture assembly configuration in A and B, and representative images of neurons cultured in the compartmented system, C. Clearly evident in the images in C are ganglionized neuronal cell bodies and their projections extending in either direction towards the distal compartments. In this the last image portraying this configuration, the area near the center of the image outlined by dashed lines shows where the Campenot chamber divider, grease strip, and septum fluid interfaced with the collagen culture substrate. In this area, the neuronal projections that penetrate the barrier do so by uncoupling from each other and spreading out laterally to traverse the compressed gelatinous layer underlying the septum. The neuronal projections can be seen on either side of this area to be 'cabled' together, where processes in proximity to each other in individual lanes have become juxtaposed and attached to each other along their course within the lane, as marked by an arrow. A channel scratched into the substrate is indicated by an arrowhead.

Collagen represents a substantial component of healthy dermal layers, and facilitates the migration and maturation of keratinocytes and their progenitors (Decline and Rouselle, 2001; O'Toole et al., 1997; Pilcher et al., 1997; Woodley et al., 1988; Nickeloff et al., 1988; Zhang et al, 1996). This migration accompanies proliferation of dermal progenitor cells in the skin to facilitate wound healing in healthy skin, and as such has been studied for applications pertaining to wound healing and to combat the effects of aging on healthy skin (Martin, 1997). Because a collagen substrate facilitates the migration of keratinocytes, the avenues for preventing the migration of keratinocytes resolved to blocking the migration of keratinocytes across collagen, or attempting to recreate compartmented culture with a substitution of the original collagen substrate.

Pursuing the avenue of blocking the migration of keratinocytes across collagen, potential methods considered were restrained to chemical intervention, eliminating physical means so as to not interfere with the compartmented co-culture assembly and maintenance. Potential chemical mediators of inhibiting migration included; antibodies or small molecules targeted to inhibit with protein interactions required for migration, mitotic inhibitors, and changes to the substrate that would interfere with migration.

Mitotic inhibitors are used in various cell culture protocols in order to select for different cell types or to influence the rates of clonal expansion via metabolic or DNA replicative interference (Liu et al., 2013). Because they are useful in influencing the rate of reproduction and clonal expansion of a cell culture, mitotic inhibitors could be a potential means of cordoning off the keratinocyte population within a specific chamber so that the neurites projecting from the neuronal chamber might reach them without expansion and migration causing the proliferation of keratinocytes in the neuronal chamber. The

inclusion of this type of chemical mediator would, however, constitute a significant divergence from what would be expected to occur in normal healthy tissue without any previously proven influence on or inhibition of migration itself.

The inclusion of interfering antibodies or small molecules that might disrupt the protein interactions necessary to facilitate keratinocyte migration would be more ideal than mitotic inhibitors. Peptide hydroxymates inhibit the activity of matrix metalloprotease enzymes, which are necessary for the migration of keratinocytes across collagen (Pilcher, et al. 1997). Particular antibodies targeted to beta-integrin 2 and laminin 5 have been shown to interfere with the signaling of those proteins and to inhibit the migration of keratinocytes in vitro (Zhang and Kramer, 1996; Decline and Rousselle, 2001). Inclusion of these antibodies would likely inhibit the migration of keratinocytes, however the inhibition may not be complete and would be prohibitively costly, induce unintended immune responses from the various cell types present, and possibly be dose dependent. Small molecules that would interfere with the signaling of these proteins necessary for migration would not be likely to introduce immune related molecules into the media, but may cause other unintended consequences with regards to their metabolism or other mechanisms of action by which they may affect other signaling pathways.

Because of the preceding issues, the most appealing avenue for inhibiting migration is alteration to the substrate originally outlined for compartmented culture. There are four commonly used substrates for neuronal culture in the relevant literature; collagen, poly-lysine, poly-ornithine, and laminin. Careful consideration was given in the process of selecting a culture substrate because of the divergent interactions possible between the substrate types and the keratinocytes stem cells and sensory neurons being co-cultured.

In addition to the interactions necessary for culture by which the cells may adhere to the substrate molecules, other interactions between the cells and the substrate molecules could yield unintended consequences jeopardizing the health and stability of the cells individually and the culture system overall.

Collagen has been widely used in cell culture for diverse cell types, including neuronal culture for electrophysiology (Peacock et al., 1973). Specific protein interactions are required for the migration of keratinocytes over a collagen substrate, and so altering the tertiary structure of the collagen by denaturing it could provide one avenue for inhibiting keratinocyte migration without affecting the growth of neurites projecting from the neuronal compartment. Collagen may be denatured by multiple avenues, but one consistent and repeatable method is by vapor exposure to ammonium hydroxide, which causes the normally parallel chains of collagen to bend or kink across each other (Iversen et al., 1981). This means of altering the substrate to inhibit keratinocyte migration without disrupting neurite growth is an attractive option. Regarding the interactions of this substrate type with neurons, however, cultured rat sensory neurons have a tendency to aggregate and reganglionize prior to effectively detaching from a collagen substrate over time, whether or not the collagen has been denatured. Because of the time required in culture to obtain plates with numerous neurite projections from the neuronal to the keratinocyte compartment, even denatured collagen proves an inadequate substrate choice.

Poly-ornithine has been widely used in neuronal culture for electrophysiology; among other applications (Soussou, et al. 2007). Poly-ornithine and poly-lysine are both polycations, enabling them to form complexes with DNA (Ramsay, et al. 2000). Poly-

ornithine is able to complex with plasmid DNA at lower concentrations than poly-lysine (Ramsay, et al. 2000). The propensity to form complexes with DNA has previously been exploited as a component in a procedure for the efficient transfection of keratinocytes using poly-ornithine after a DMSO treatment to disrupt the integrity of the cell membrane (Nead and McCance, 1995). The presence of DMSO in the freezing media of the keratinocyte cell line used in this culture system makes the use of poly-ornithine as a culture substrate a suboptimal choice because of the potential for inadvertent transfection of any DNA that could be present in the keratinocyte compartment media during the period of culture.

Laminin has been used as a substrate for multiple types of cell culture as well, including for electrophysiology (Lindsay, 1988). Laminin has previously been shown to inhibit human keratinocyte migration over a collagen substrate (Woodley, et al. 1988; Nickoloff et al. 1988). Interaction of laminin-5 has specifically been shown to inhibit keratinocyte migration through interaction with the α3 integrin receptor, as was shown by blocking the expression of the receptor via antisense oligonucleotide incubation (O'Toole, et al. 1997). The relatively high cost of laminin and the discrepancies over whether to use it as a substrate or to add it to the media on a collagen substrate to inhibit keratinocyte migration make it a potentially useful, but less than ideal substrate choice.

Poly-lysine has also been used for various cell culture protocols, including some for neuronal culture and electrophysiology (Malin et al., 2007). While poly-lysine is a polycation like poly-ornithine, the affinity of poly-lysine for DNA in order to form condensed structures is significantly lower so the chance of inadvertent transfection of the keratinocytes is a less significant drawback (Nead and McCance, 1995).

The effect of poly-lysine on the migration of keratinocytes is drastic. Culturing keratinocytes on poly-lysine effectively inhibits migration across the substrate (Sadowski et al., 2003). Creating a scratch in a field of keratinocytes in culture on poly-lysine, without removing the poly-lysine, creates a gap that remains unfilled throughout the entire duration of culture. This demonstrates the drastic decrease in mobility of keratinocytes when compared with other substrate types. The widespread use of poly-lysine in the culture of neurons and inhibition of migration of keratinocytes, along with the normal morphology, attachment, and growth of the cells cultured, make poly-lysine an ideal substrate for the compartmented co-culture system outlined here. Because there are no reported gross morphological deformities in the cells cultured on poly-lysine, the cultures generated may more faithfully represent the nature and interactions of the cultured cells as they would exist in tissue. By ensuring that all cells successfully attach, cultures can be reliably generated with a consistent initial health and density of plated cells that attach and begin the culturing process. The normal growth and clonal expansion of cells on poly-lysine substrate, in combination with its inhibition of keratinocyte migration, have the cumulative effect of preventing movement of keratinocytes into the neuronal compartment without inhibiting their expansion to form confluent monolayers similar to an epithelial sheet.

CULTURE MEDIA

Careful research and thorough testing were required to arrive at a combination of media formulations that faithfully simulate the environs of the cells in their respective tissues and support the health of the cells for the time required in culture. While more widely used formulations like F12 and DMEM support the short term survival and neurite outgrowth of sensory neurons, B27 Neurobasal Electrophysiology Kit media formulation is promoted by the manufacturer to be better optimized for neuronal cultures for electrophysiology compared to some other media formulations. Since the introduction of B27 Neurobasal Media (B27N) there has been a slight alteration to the formulation resulting in a higher concentration of cysteine within the media (Hogins et al., 2011). While cysteine might not be considered an excitatory amino acid, it has been shown to activate NDMAR- mediated currents at relatively high concentrations (Hogins et al., 2011). The alteration to the formulation of B27N resulted in cysteine content that surpasses the concentration required to induce NDMA receptor mediated currents (Hogins et al., 2011). Because excessive NMDAR signaling contributes greatly to excitotoxicity the suitability of B27N is lessened because of the effect of NMDAR mediated currents consistently induced due to the concentration of cysteine. The survival of cells for the time needed in culture is greatly attenuated because of this excitotoxicity, however inhibiting the signaling of NMDAR even in the presence of elevated cysteine levels dramatically improves the viability of the neurons over an extended culture time period. Inhibition of NMDAR activity is relatively easily accomplished pharmacologically, as many NMDAR antagonist and NMDA subunit-specific antagonists have been introduced, with various differences in their affinity, selectivity, and dissociation constants. Some NMDAR antagonists, like MK-801, may inhibit activity of

the NMDARs but bind so tightly to the receptor the dissociation of the antagonist and receptor via washout is far too slow for an electrophysiological recording session. If such an antagonist were used then cells exposed to the antagonist would be unresponsive during a long recording time. The D-enantiomer of the compound 2-amino-5-phosphonopentanoic acid (D-AP5) is an effective NMDAR antagonist that dissociates from the NMDAR relatively quickly and so makes an ideal candidate for culture of NMDAR expressing neurons with B27N. Because the antagonist does dissociate fairly rapidly the cells antagonized by D-AP5 can be rendered responsive by its effective washout within a matter of minutes, making it an ideal antagonist for culture for this electrophysiological study.

The manufacturer of B27N also produces another formulation of the media that is especially optimized for *in vitro* electrophysiology, the B27N Electrophysiology Kit (B27EK). The manufacturer of the media propounds that neurons cultured using this media exhibit increased spontaneous firing rates as well as increased current amplitudes. Because of the nature of the experimentation being undertaken, where focal application of agonist at a peripheral terminal and the resulting impulses are recorded to evaluate receptor activity, B27EK constitutes an ideal medium for culturing neurons for this electrophysiological study, so long as the protracted cysteine-instigated NMDAR activity can be mitigated. The inclusion of D-AP5 in the media markedly improved the health and viability of the cultures in a dose dependent manner, seeming to reach its maximal effect around 200 μM. For the cultures used in this experiment D-AP5 was added to the B27EK at a concentration of 250 μM.

Sensory neurons collected from the DRG in rats less than 2 days old are dependent on nerve growth factor for survival (Malin et al., 2007). To circumvent nerve growth factor dependence animals were not used for neuronal harvest until day 8 in accordance with an established protocol (Mintz et al., 1992). Exposure to nerve growth factor also increases the expression of *TRPV1* in DRG neurons and increases thermal hypersensitivity via their peripheral activation (Eskander, et al. 2015). In order to better simulate the environment of the DRG, specific growth factors were added to the B27EK promote the overall health and mitigate alterations to the receptor expression patterns of the cells. Nerve growth factor was added at a concentration of 20 ng/µl, while Brain-derived neurotrophic factor and Glial-derived neurotrophic factor were each added to a respective final concentration of 5 ng/µl. Central Septum fluid contained a higher concentration of Nerve growth factor, at 100 ng/µl, along with epidermal growth factor, also at 100 ng/µl. The growth factors included in the central septum fluid were included in order to promote the growth of peripheral processes from the neuronal compartment into the keratinocyte compartment.

The component added to the B27EK that facilitates gelatinization of the media, especially the central septum fluid, is methylcellulose at a concentration of 0.4% w/v in accordance with the original protocol for compartmented cultures (Campenot, 1977).

DRG COLLECTION

Sprague Dawley rats from Charles River Labs were used for all experiments. All animals were handled and treated in compliance with IACUC standards, and all possible efforts were made to minimize any suffering and the total number of animals used for experimentation. Animals ranging from 8-13 days old were used for electrophysiological recordings and immunocytochemical experiments. Animals aged 6-8 weeks were used only for immunocytochemical experiments. Adult animals were anesthetized with isoflurane before decapitation while immature animals were sacrificed by decapitation. Removal of the tail allowed for introduction of scissors up the length of the remaining vertebral column along the line of the spinous processes (Figure 8A). Rat-toothed forceps were then used to flay the two pieces of back skin laterally, and to remove some of the longitudinal spinal musculature near the vertebral column in immature rats (Figure 8B). In adult rats, a scalpel was used to cut along the lateral edge of the vertebral column through the longitudinal spinal musculature down to the level of the ribs (Figure 8C).

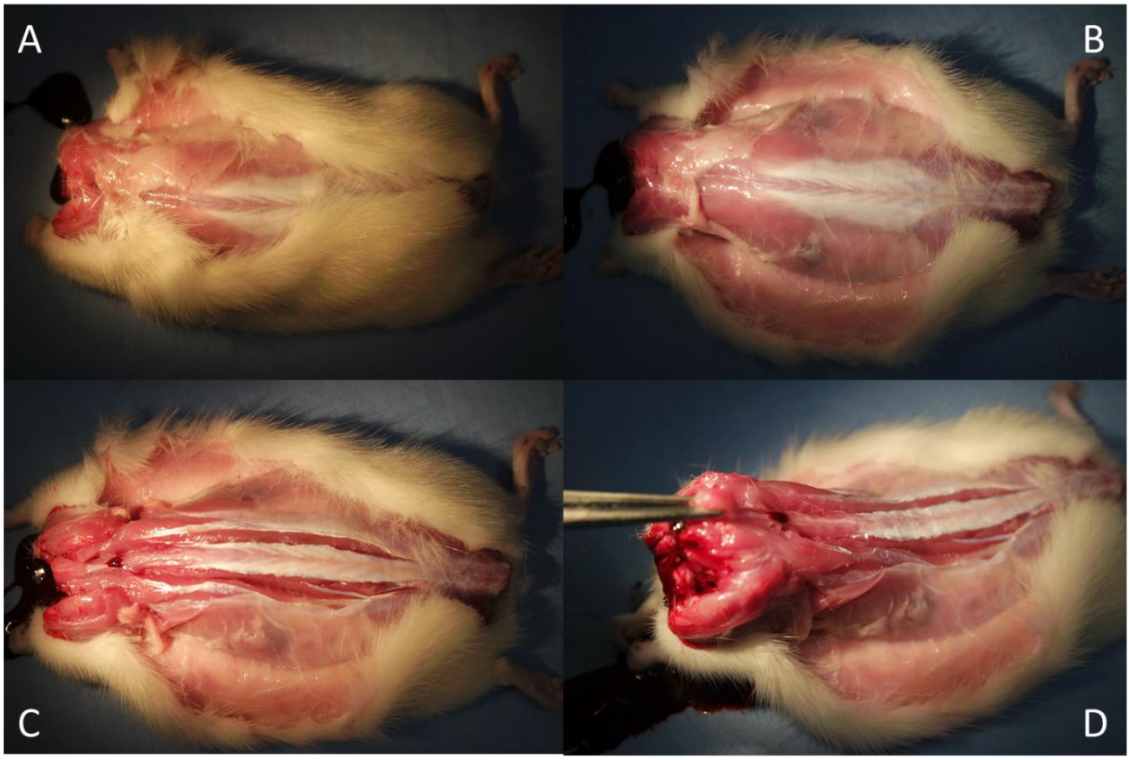

FIGURE 8: This figure depicts the gross dissection of the rat and removal of tissues for the resection of a portion of the spinal column. Surgical scissors for the removal of large tissue sections and a fresh scalpel blade to cut through the musculature allowed for the easy removal of a rostral portion of the spinal column. Panel A shows a juvenile rat with head and tail removed, while Panel B shows the back skin flayed open. Panel C shows the musculature just lateral to the spinal column cut through with a scalpel, and Panel D shows the spinal column lifted at the rostral end with large rat-toothed forceps.

Grasping the rostral end of the vertebral column with rat-toothed forceps (Figure 8D), scissors were used to cut through the ribs and remaining tissue, just lateral to the vertebral column, in order to free the rostral portions of the vertebral column before transecting the spine within the lumbar region at approximately the level of the L3 vertebra (Figure 9). The liberated portion of the spine was then moved to a 100 mm Petri dish for further dissection. The harvested vertebral column was axially segmented with surgical scissors in two places targeting the prominent cervicothoracic and thoracolumbar spinal curvatures, yielding 3 longitudinal sections, the first cut was at approximately T1 and the second cut was at approximately T8. Separating the spinal column portion into these smaller fragments allowed for easier and more organized bisection of the fragments because the size of the scissors required to make longitudinal cuts in the spinal column fragments were larger than the spinal canal. This size discrepancy caused a tendency for the spinal column to rotate with respect to the scissors when the entire spinal column portion was cut along its length.

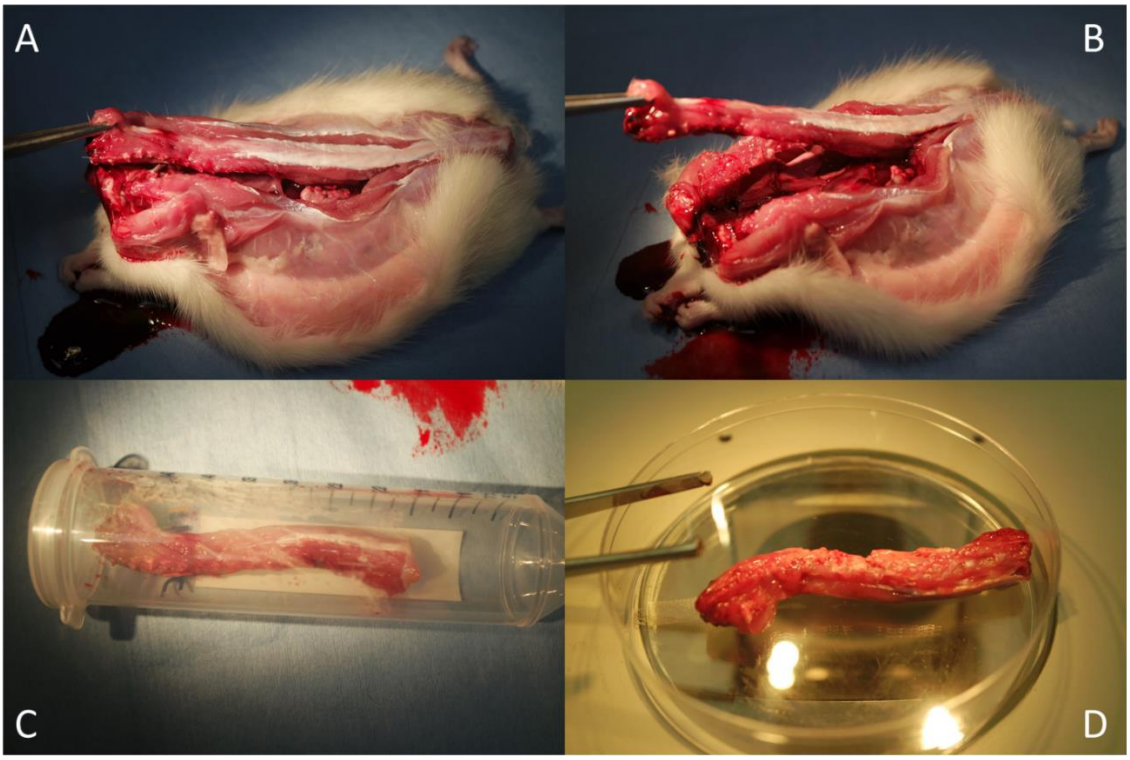

FIGURE 9: This figure depicts the removal of a rostral portion of the spinal column, which completes the gross dissection portion of the harvest and dissociation procedure. Further dissection involved in harvest was performed in a sterile laminar flow hood to prevent microbial contamination of the DRGs. Panel A shows the spinal column after cutting through the ribs on one side of the spinal column, while Panel B shows the spinal column lifted after cutting both sides of the ribcage. Panel C shows the spinal column removed for further dissection, and Panel D shows the spinal column fragment placed on a 100 mm Petri Dish in a sterile hood.

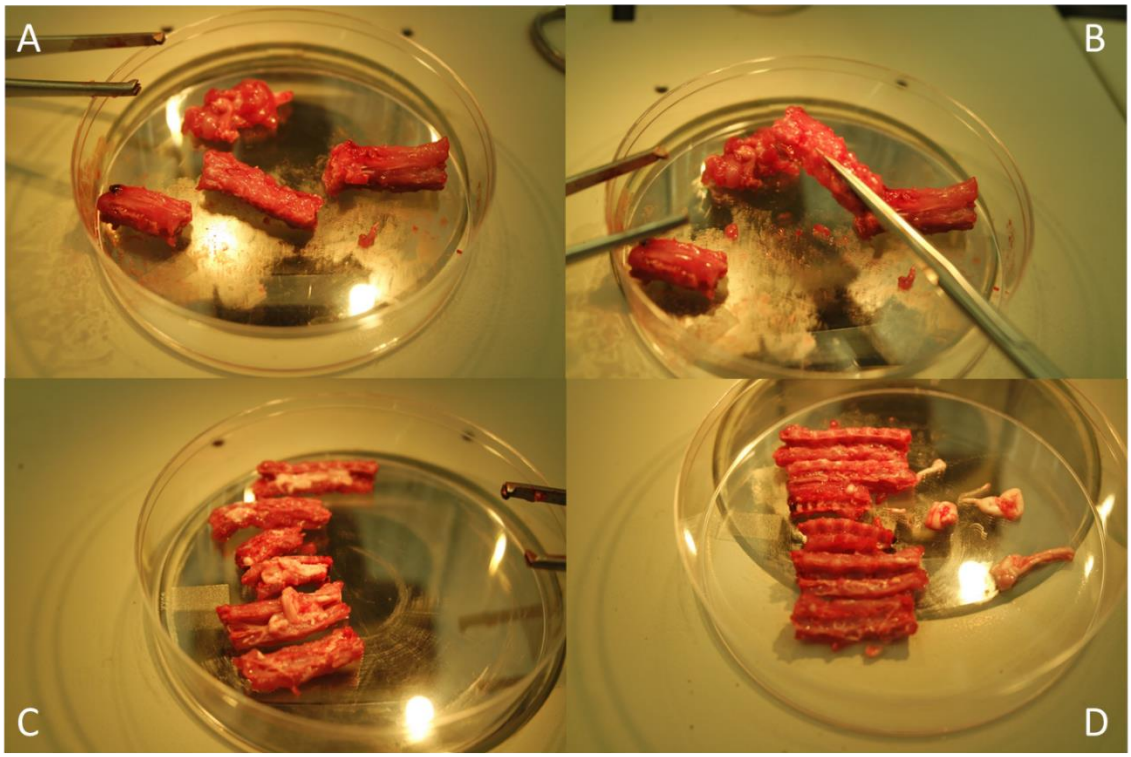

FIGURE 10: This figure depicts the preparation of the spinal column tissues for DRG harvest. Excess spinal musculature was removed prior to axial cuts sectioning the column portion into three longitudinal fragments. Each longitudinal fragment was laterally bisected with two cuts along the ventral and dorsal surfaces. These bisected fragments were laid with the spinal canal facing upwards to maintain cleanliness and allow easy access for the removal of the spinal cord, meninges, and DRGs. Panel A shows the spinal column fragment cut into 3 segments, while Panel B shows one of the segments being cut longitudinally. Panel C shows the three segments laid open with the spinal canal facing upwards, and Panel D shows those same segments with the remaining spinal cord tissue removed.

The three sections were then cut rostrocaudally with small straight scissors on both the dorsal and ventral surfaces to yield two bilaterally symmetrical halves for each longitudinal section. At this point the spinal cord was carefully removed from the spinal canal and the remaining proximal roots were clipped to allow removal of the intact DRG. The remaining meninges were scraped along the interior surface of each vertebral segment with 45˚ #5 forceps and the DRGs were then lifted out in the case of pups, or after clipping the remaining spinal nerve with small spring-loaded scissors in the case of juveniles. The difference in the amount of connective tissue between the juveniles and pups was the cause for the differential handling in this phase of the extraction. Grasping and gently pulling on the proximal rami of the DRGs was sufficient to break the distal rami in pups, while that force was only enough to tease the DRG out of the intervertebral foraminae in the juvenile animals. Once the distal rami were exposed in the juvenile animals a quick and clean cut with surgical scissors was sufficient to liberate the DRG entirely.

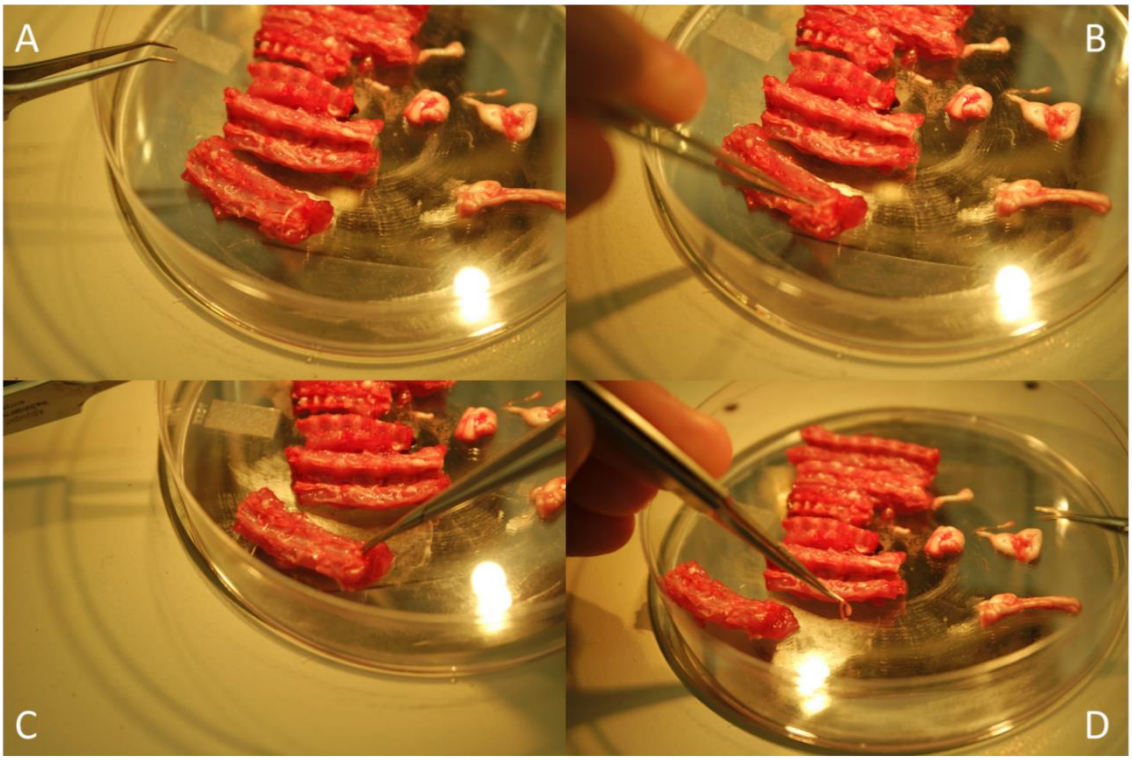

FIGURE 11: This figure depicts the removal of an individual DRG from one of the spinal fragments. A pair of #5 forceps were used to tease the DRG up out of the intervertebral foramina by gently pulling on the proximal ramus and spinal nerve while small scissors were used to cut through the distal rami to liberate the DRG from the spinal fragment. Panel A shows one of the DRGs teased out of the intervertebral foramen, while Panel B shows forceps grasping the proximal ramus of the DRG. Panel C shows scissors inserted into the intervertebral foramen to clip the spinal nerve, and Panel D shows the DRG grasped by forceps after removal from the spinal column.

DISSOCIATION

Harvested DRGs were initially placed into a 60 mm Petri dish containing 5 ml of a Collagenase Type IV bath (0.15% w/v) on ice until all had been collected from the vertebral column fragments. The DRGs were then individually trimmed of the remaining root fragments and meninges to obtain clean DRGs and replaced in a fresh 5 ml of collagenase solution in another 60 mm dish on ice until all harvested DRGs had been trimmed. Once all DRGs were trimmed the dish was moved to an incubator at 37˚C and 5% CO2 containing a VWR Standard Analog Shaker and agitated in the dish at a speed setting of 3, corresponding to approximately 120 rpm.

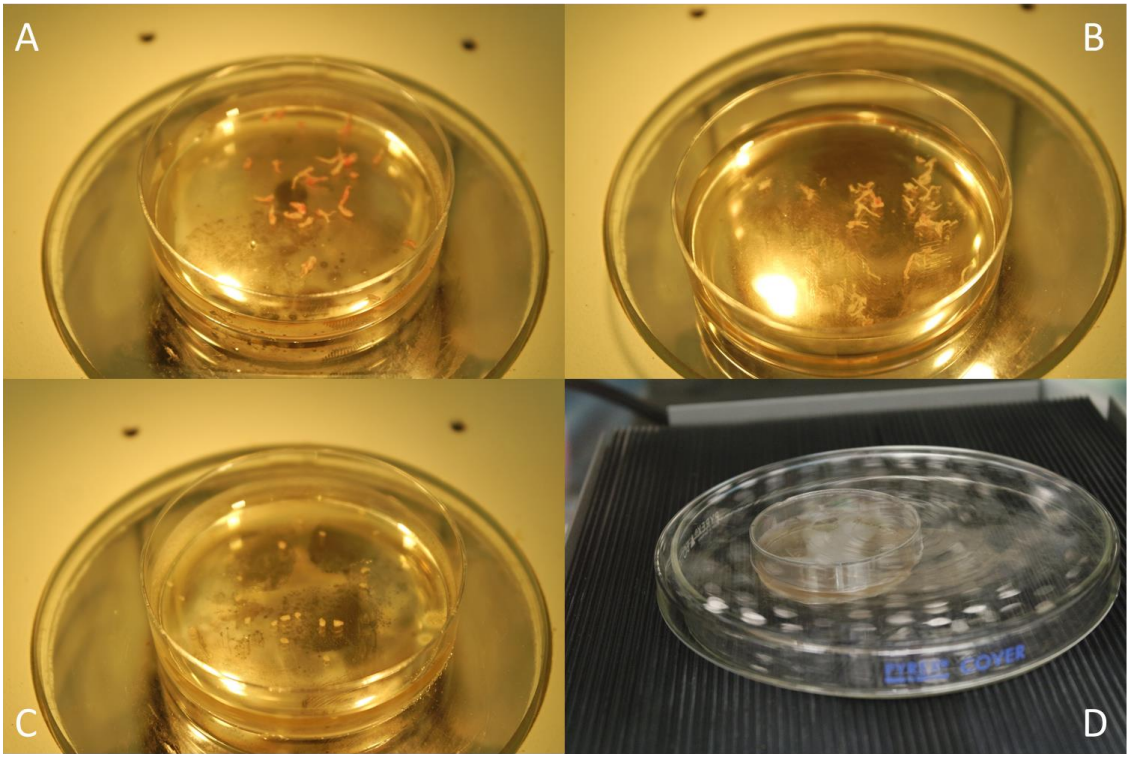

FIGURE 12: This figure depicts the trimming and dissociation of the harvested DRGs. Individual DRGs were placed in 0.15% w/v Collagenase Type 1 in HBSS, and the proximal and distal rami were cut away to minimize extraneous tissue undergoing enzymatic digestion. After trimming away the rami, the DRGs were placed into a dish containing fresh 0.15% Collagenase in HBSS and set on a shaking platform in a cell culture incubator. Panel A shows harvested DRGs sitting in 0.15% w/v Collagenase Type 1, while Panel B shows all of the DRGs from an individual rat. Panel C shows those same DRGs after removal of the proximal and distal dorsal root as well as the ventral root fragments. Panel D shows the same dish placed on a shaker in a cell culture incubator.

At the end of this digestion, the cells were transferred to a Falcon 5 ml round bottom polystyrene tube and the remaining collagenase solution was decanted and replaced with 1 ml complete neuronal compartment media for trituration. Trituration was sequentially performed with 8 repetitions through a 1000 µl micropipette, followed by 4 repetitions with a 9 inch glass Pasteur pipette. Care was taken to minimize the introduction of bubbles into the media being triturated. After trituration the media containing the cells was passed through a 40 µm sterile cell filter, in order to remove large debris and cells. The process of filtering the cell suspension increased the proportion of small and medium diameter neurons, which comprise the proportion of DRG neurons that are nociceptive. After passing through the cell strainer, the solution containing the cells was removed to another 5ml Falcon tube and 10µl of the suspension was taken for counting. An aliquot of the cell suspension was then taken to obtain approximately 300,000 cells and diluted into 2 ml of complete medium for a final plating concentration of around 150,000 cells/ml.

CELL PLATING AND CULTURE ASSEMBLY

Cells were plated on Poly-D-Lysine coated 35 mm plastic Petri dishes, on which a solution of 0.1 mg/ml Poly-D-Lysine was applied to each dish for 30 minutes before rinsing thoroughly with DNAse and RNAse free water and allowing to dry overnight. A custom plate scratching rake was derived from the suggestions outlined by Campenot (Campenot et al., 2009). The rake is comprised of 14 #00 stainless steel insect pins

(BioQuip) fixed in parallel on a plexiglass holster with epoxy. The regular point shape, spacing, and size of the pins make the spacing between the points of the pins relatively consistent at approximately 200 nm. Three parallel sets of scratches by the rake were etched into the bottom of the culture dish, effectively removing the poly-lysine laid down as a substrate and leaving lanes of poly-lysine approximately 200 nm wide, with precipitous substrate-free channels dividing the lanes. The lanes and channels introduce some linearity to the culture system so that projections from the neurons tend to grow in parallel along the lanes, instead of growing in a disorderly configuration purely based on chemotactic growth signals. The benefits of this linearity are two-pronged. First, the projections from the neurons growing along the lanes can direct them towards the keratinocyte compartment; increasing the total number of projections extending from neural somata in the neuronal compartment into the keratinocyte compartment. Second, the spatial isolation of potential growth directions of the projecting neurites by the lanes etched into the substratum groups sets of neurons and their respective projections together. This grouping facilitates the focal application of agonist on individual lanes along which lie linearized fields of neuronal projections in association with keratinocytes.

After the parallel scratches were introduced, a small line of septum fluid (approximately 12.5 µl) is applied perpendicular to the direction of the scratches. The septum fluid consisted of the normal neuronal media with the addition of increased nerve growth factor and epithelial growth factor.

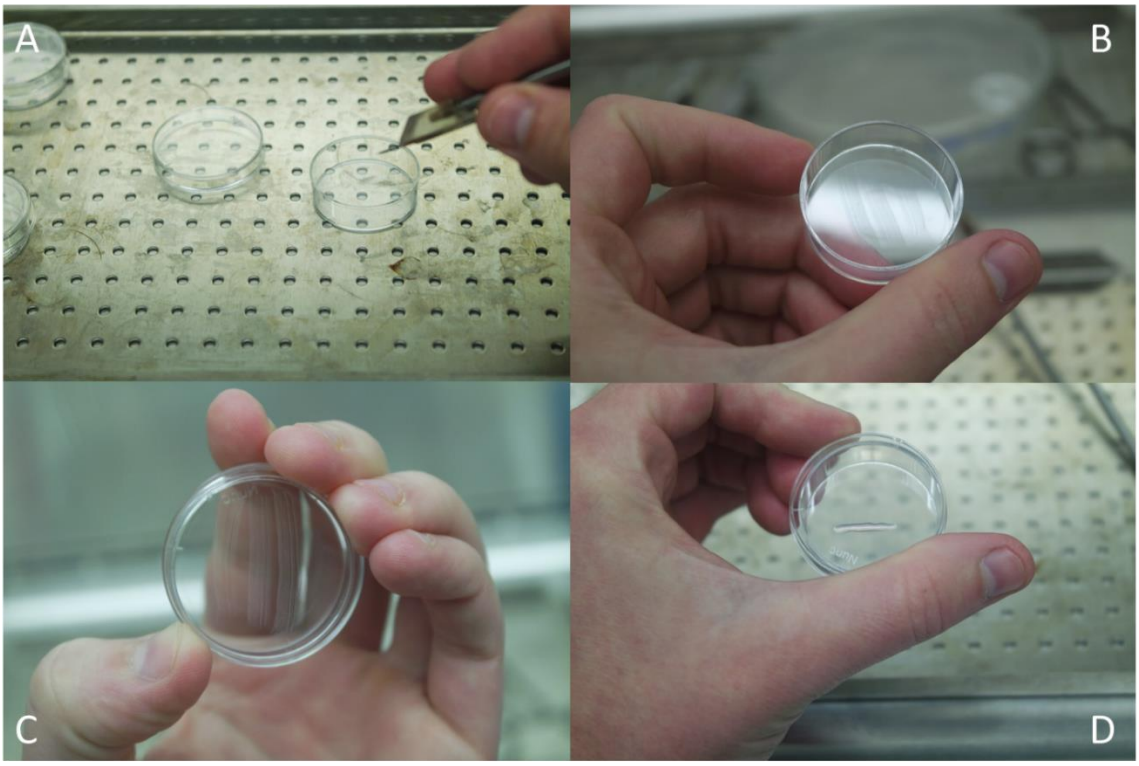

FIGURE 13: This figure depicts the initial steps of the assembly process of the compartmented co-culture systems. Initial scratches made with a rake were perpendicularly overlaid with central septum fluid. A flattened strip of grease extruded through a crimped 18-gauge was floated across the top of the fluid strip and tapped to balance the height of fluid atop the plate. Panel A shows the custom rake created to scratch the poly-D-lysine substrate, while Panels B and C show the parallel sets of scratches on the culture plate. Panel D shows the strip of central septum fluid laid across the sets of scratches.

A moderately flattened 18 gauge needle, having been crimped with pliers, was used to lay a strip of high-vacuum silicone grease atop the septum fluid. The importance of crimping the aperture of one of the 18-gauge needles is to broaden the surface area of the deposited grease strip in contact with the septum fluid, allowing it to float better during compartmented culture assembly atop the septum fluid strip through which the neuron projections will grow to the keratinocyte fields. The needles used for grease deposition were affixed to glass 2 ml luer-lok syringes, in order that they might be regularly autoclaved.

Curved forceps were used to grasp the middle septum of the culture divider and the forceps were laid flat with the divider facing upwards. A regular 18 gauge needle was used to apply high-vacuum silicone grease to the underside of the divider. Once the grease was laid down on both surfaces, the poly-lysine dish turned upside down with the lid removed and tapped lightly to ensure that the septum fluid was distributed evenly and placed onto the upturned, grease-coated surface of the divider. The placement of the dish onto the divider was with the central septum of the divider placed close to the septum fluid, but not touching it. Once the divider was affixed to the culture plate, the plate was pressed against the divider to ensure complete seal of the grease lining the edges of the divider. The plate was simultaneously pulled towards the septum fluid in order to create a complete seal between the two compartments with the septum fluid, the grease lying atop the septum fluid, and the central septum of the divider itself.

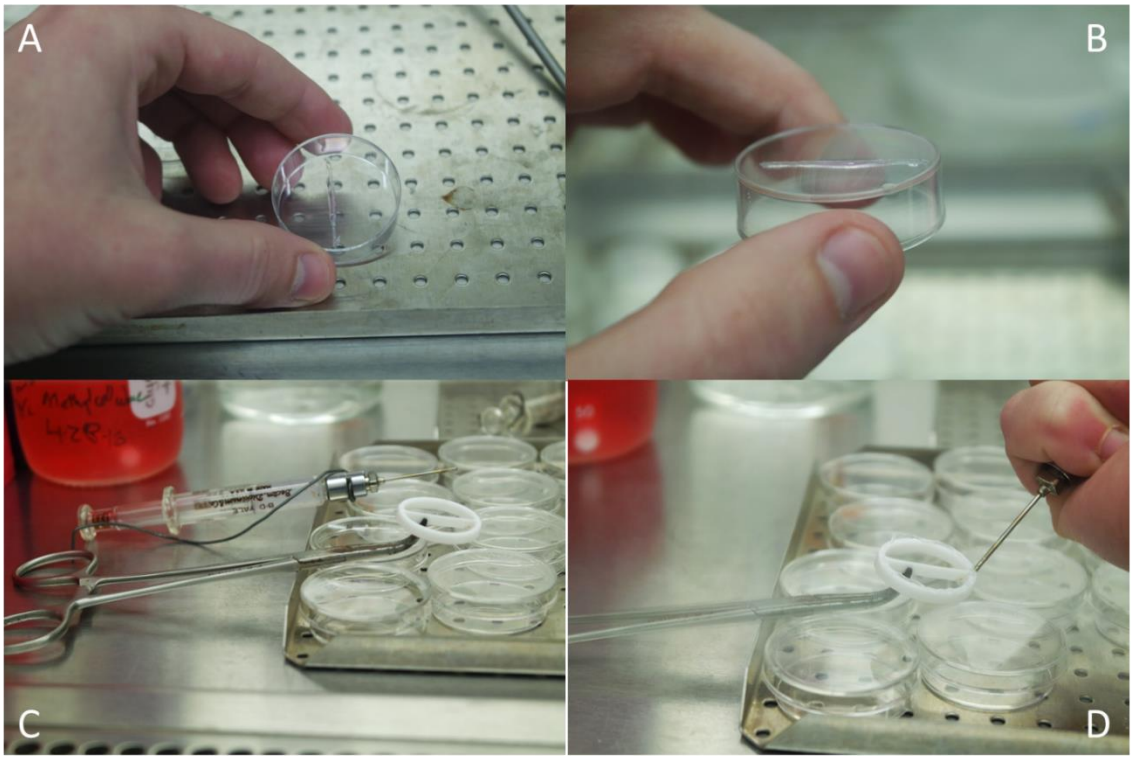

FIGURE 14: This figure shows the intermediate steps involved in the creation of compartmented co-culture systems. After the fluid level resting between the flattened grease strip and scratched substrate is made even a Teflon Campenot divider was firmly grasped by a curved baby mixter hemostat and laid forceps side down. Grease was applied to the upturned face of the Teflon Campenot divider with an 18-gauge needle around the periphery of the divider. Once the outer circle of the was complete the culture plate was pressed onto it with the septum fluid in close proximity to the central septum. Panel A shows the septum interface grease strip having been laid down, while Panel B shows the plate rotated to highlight the configuration of grease laid atop the septum fluid. Panel C shows the culture divider gripped with forceps and resting face up for grease application, and Panel D shows the grease being applied to the divider.

The final step in the assembly of the compartmented culture system was to apply another strip of grease towards the top of the central septum of the divider on the neuronal compartment side. That strip of grease serves two functions via its interaction with the neuronal media. The first is to act as an insulator of sorts, to retain heat and counteract the radiating effect of the thin central septum. The second was as a surface for interaction with the media to distribute more of the fluid towards the central septum as the Teflon divider is fairly hydrophobic. It is important to distribute the fluid in this way because sensory neurons have been shown to settle out according to thermal gradients at the initial plating of a dissociated culture (Malin et al., 2007). Sensory neurons tend to attach in the warmer areas of the dish in which they are plated (Malin., 2007). Using the strip of grease towards the top of the divider as an insulator increases the heat close to the central septum, increasing the proportion of neurons that attach near the central septum. Distributing as much of the fluid towards the central septum of the divider as possible increased the number of neurons that attached to the poly-lysine substrate in close approximation to the central septum, increasing the total number of neurons possessing projections that crossed the central septum to enter the keratinocyte fields. Once the plates were assembled in this manner, they were placed in an incubator at 37ºC for 5 minutes while keratinocyte stem cells were being thawed from liquid nitrogen storage.

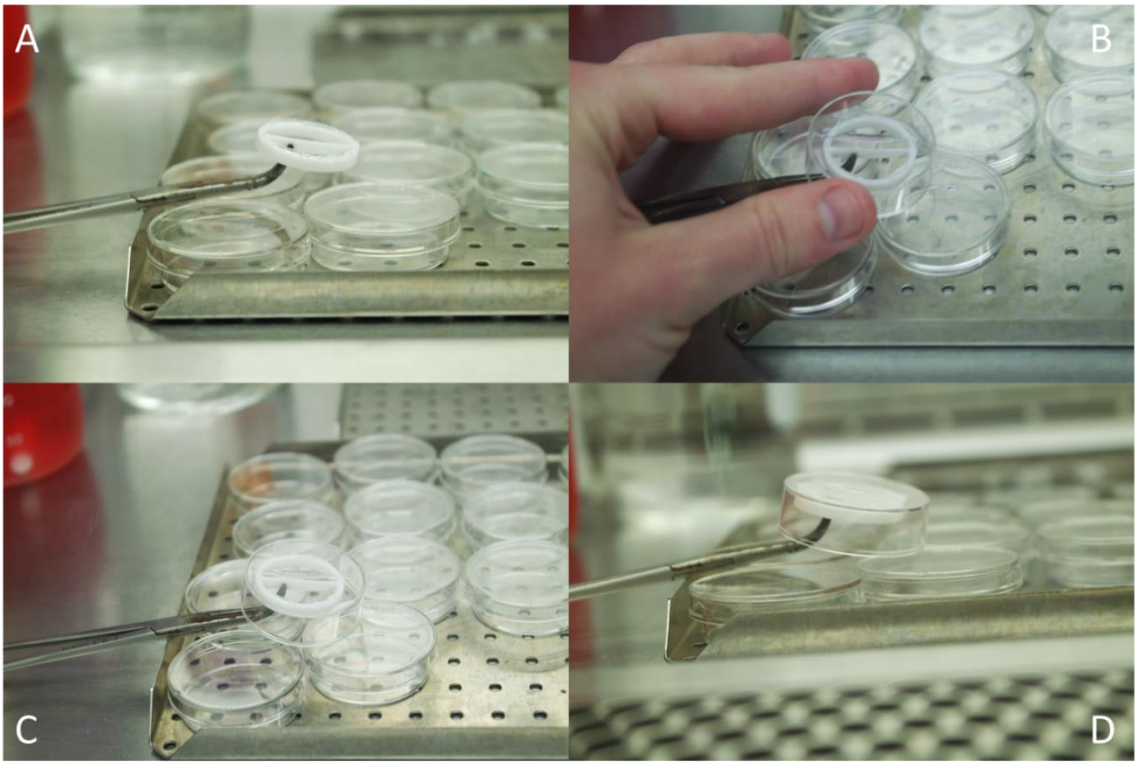

FIGURE 15: This figure depicts further steps in the assembly of the compartmented co-culture system. The translation of the culture plate across the Teflon Campenot divider created a relatively strong and wide strip of fluid underlying the central septum to act as a barrier and prevent fluid exchange between the two compartments. Panels A and B show the divider with grease applied and the culture plate pressed onto it, while Panels C and D show the culture plate with divider applied from two different angles.

Keratinocyte stem cells were plated at a density of approximately 125,000 cells per ml in manufacturer supplied keratinocyte stem cell culture serum-free media that contained 0.4% methylcellulose. Approximately 200 µl of media containing keratinocyte stem cells was plated in the keratinocyte compartment, and then the plates were returned to the incubator while the neuronal dissociation was completed so the sensory neurons could be plated on the other side. The time required for the mechanical dissociation of the DRG neurons was approximately 15 minutes. The keratinocyte cell suspension was plated carefully in a semicircular pattern tracing the interior of the keratinocyte compartment, with the most distal portion and one proximal side plated first to minimize fluid movement towards the central septum as much as possible.

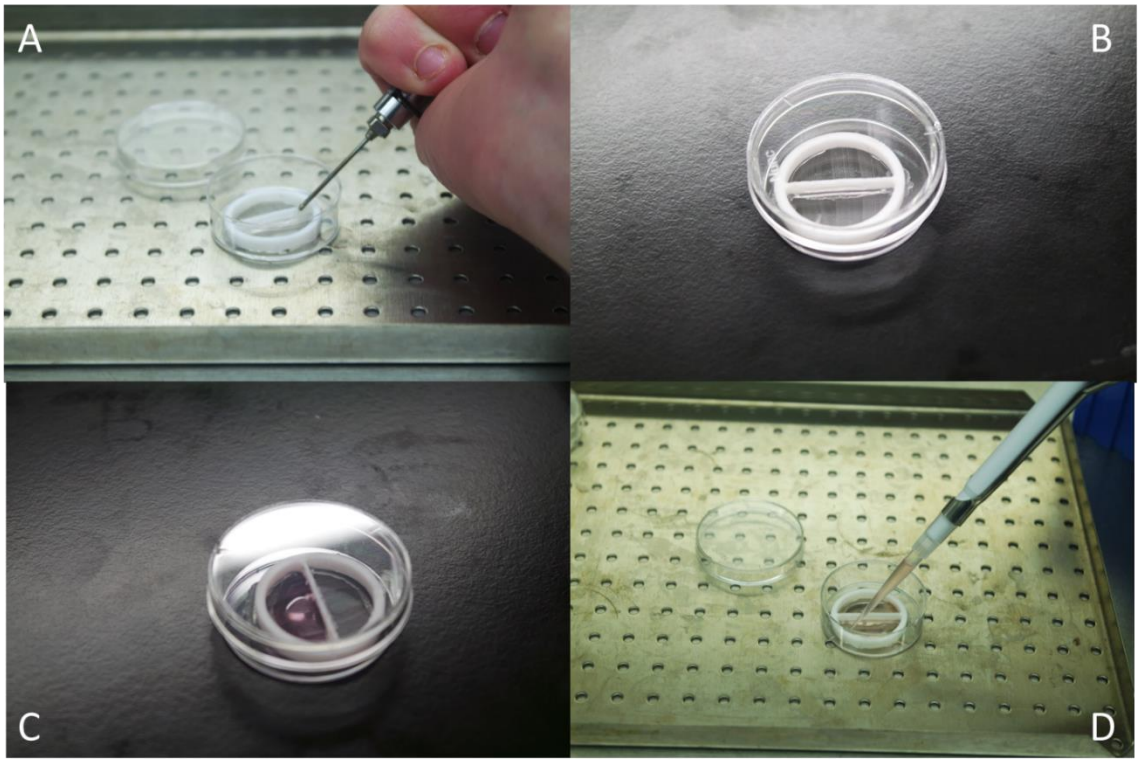

FIGURE 16: This figure depicts the plating of keratinocyte stem cell suspension into the assembled compartmented co-culture systems. Approximately 200 µl of cell suspension containing approximately 90,000 was plated for each culture. Culture plates were returned to the incubator to warm as the trituration of the neuronal cells proceeded. Panel A shows the application of the top grease strip to the neuronal compartment side of the central septum, while Panel B shows the assembled compartmented culture prior to cell plating. Panel C shows the culture with the Keratinocyte Stem Cell solution plated to one side of the divider, while Panel D shows the DRG suspension being plated.

The neuronal cell suspension was carefully plated in a semicircular patter tracing the interior of the compartment in the same manner as the plated keratinocyte suspension, however the benefit of the interaction of the media with the grease strip near the top of the central septum required careful addition near to it to ensure full contact without disrupting the barrier underlying the central septum. Dissociated DRG cells were plated at a density of approximately 150,000 cells per ml, with care taken to ensure contact of the neuronal plated media with the insulating grease strip towards the top of the central septum to counteract the hydrophobicity of the Teflon and distribute as much of the fluid in close approximation to the central septum as possible. After the plating of the neurons the cultures were returned to the incubator at 37 ºC.

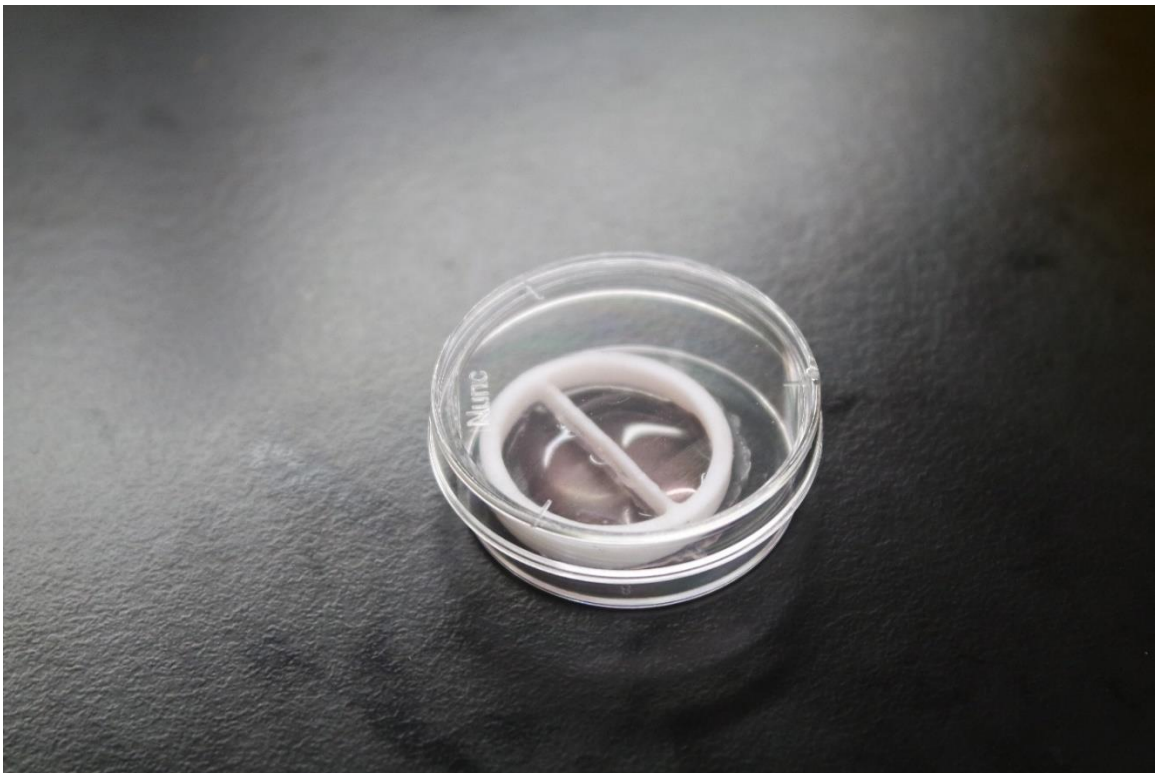

FIGURE 17: This figure shows a completely assembled compartmented co-culture system. The keratinocyte compartment is on the right in this image and the strip of grease near the top of the central septum on the neuronal compartment side is visible and its interaction with the fluid in the neuronal compartment is evident in this photo.

The day after plating approximately 50 µl of the neuronal media was drawn off to remove some of the cells which had not attached overnight. After that approximately 250 µl of neuronal media was added to the neuronal compartment, followed by 300 µl of keratinocyte media to the keratinocyte compartment, followed by another 250 µl of neuronal media into the neuronal compartment. Because the volume of the media on the day of plating was significantly lower than the volume of media present during the culture period, addition of the fluids during the first media change were accompanied by tilting the co-culture dish so that the compartment to which media was being added was lower than the opposing compartment to minimize any differences in pressure at the central septum that might disrupt the barrier separating the two compartments prematurely. Fluids were changed sequentially and slowly in order to prevent a premature collapse of the gelatinous layer of septum fluid underlying the central septum. Ultimately the collapse of that gelatinous layer was intentionally brought about by overloading bath solution in the neuronal compartment so care was taken to add and remove fluids slowly avoid unintentional collapse.

The structural integrity of the barrier underlying the central septum is of paramount importance for this compartmented co-culture, because it is the basis of maintaining discrete environs for the different cell types cultured. The initial media change on the day following plating was the point at which the greatest number of cell cultures were discarded because of a collapse of the gelatinous barrier. The failure rate of the assembled co-culture systems was relatively low compared to that of the original protocol for the assembly of compartmented culture systems. In our hands the failure rate for this assembly method was less than 10%, while the failure rate for the original assembly

protocol was as high as 40%. The depth of the central septum fluid was relatively constant from one culture plate to the next following the protocol outlined here as well. The original assembly protocol proscribed the formation of the barrier as a droplet compressed underneath the grease applied to the Teflon divider. The amount of central septum fluid was constant (12.5 µl) for the assembly in this protocol and the translation of the Teflon divider relative to the culture plate in this assembly method did not compress the central septum fluid. The area underlying the septum where the septum fluid was laid to form the barrier was significantly larger than that of the original media droplet compression method devised by Campenot. This increase in the interface area under the central septum, which constitutes the compartmental barrier, translates to an increased area across which neuron projections from one compartment can extend to reach the distal compartment. The increased area available for neural projections to cross from their respective proximal compartment to a distal compartment comprises an increase in the potential throughput of projections that can be evaluated experimentally. These alterations to the assembly process increase the number and variety of experiments that can be performed on spatially discrete segments or populations of cells, as well as the variety of substrates on which compartmented culture can be performed. One serendipitous coincidence that facilitated the compartmented co-culture of sensory DRG neurons and keratinocyte stem cells is the difference in migration capability of the different cell types across a poly-D-lysine substrate. While other types of cells may display such differences in their respective abilities to migrate across the substrate, further adaptations and refinements will be necessary to fully exploit the compartmented co-culture of many relevant pairings of cell types.

Extreme caution was taken in all culture assembly and handling steps to reduce the risk of contamination because of the lack of antibiotics in the culture media. Stripette pipettes were used instead of micropipettes in accordance with the suggestions outlined by Campenot (Campenot et al., 2009). All cell cultures were housed in VWR 5.3A Symphony incubators at 37 ˚C and 5% CO2 inside of the humidified chamber. Media was changed in the cultures on the 4th and 7th days in culture, and thereafter as needed until their use in electrophysiology or immunocytochemistry.

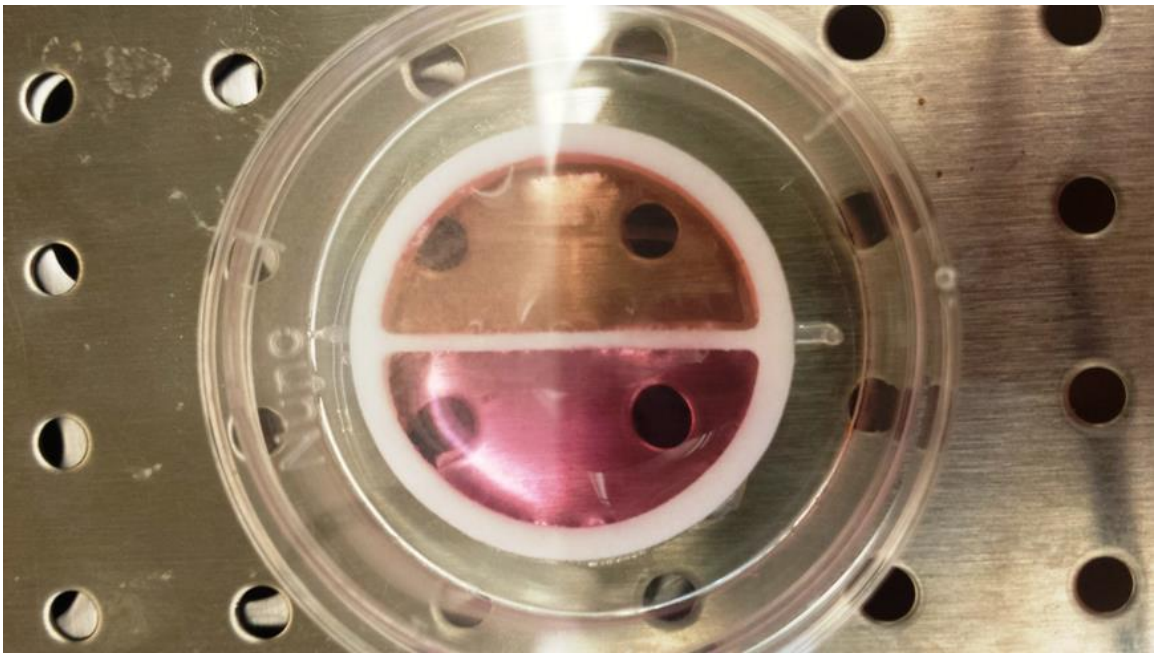

FIGURE 18: This image displays a completely assembled compartmented co-culture system after the first media change. The 1mm central septum along with the grease strip and strip of serum fluid prevent the admixture of the media from the two separate compartments. In this image the neuronal compartment is oriented towards the bottom of the image, while the keratinocyte compartment is oriented towards the top. Slight differences in the colors of the media for the respective compartments facilitated easy assurance of compartmentalization of the fluids, as is evident by the discrete boundary of color shown above.

CHAPTER III

IMMUNOFLUORESCENCE

In order to evaluate the receptor expression at different segments of sensory neurons, immunofluorescent staining was employed to facilitate the specific identification of individual receptor subunits present in a compartmented co-culture. Understanding which receptor subunits are expressed in the peripheral segments and peripheral terminals would not only further overall understanding of the mechanisms by which sensory neurons function, but may enable selective pharmacologic intervention targeting of specific receptor subunits in peripheral segments of sensory neurons without acting on neurons in the central nervous system.

Compartmented co-cultures were removed from the incubator and rinsed with phosphate-buffered saline (PBS) prior to fixation with 4% formaldehyde in PBS for approximately 15 minutes. Following fixation, the plates were rinsed again with PBS before incubating with primary antibodies, which were in a PBS solution containing 0.3% Triton X-100, 0.5% Bovine Serum Albumin, and 0.5% polyvinlypyrrolidone. Primary antibodies were used at a concentration of 1:100, 1:250, or 1:500 following preliminary immunocytochemical experiments to evaluate their respective performances and their subsequent stratification into working concentration groups.

Individual antibodies raised against each of the subunits in the AMPA and Kainate ionotropic glutamate receptor families were used. For the AMPA family the antibodies and their respective concentrations used were; a rabbit anti-GluA1 polyclonal antibody (AB1504, Millipore) at 1:250, a mouse anti-GluA2 monoclonal antibody (MAB 397, Millipore) at 1:250, a goat anti-GluA3 polyclonal antibody (sc-7613, Santa Cruz Biotechnology) at 1:250, and a rabbit anti-GluA4 polyclonal antibody (AB1508, Millipore) at 1:100. For the Kainate receptor family the following were used; a goat anti-GluK1 polyclonal antibody (sc-7617, Santa Cruz Biotechnology) at 1:100, a rabbit anti-GluK2 polyclonal antibody (ab53092, Abcam.com) at 1:100, a goat anti-GluK3 polyclonal antibody (ab82148, Abcam.com) at 1:100, a goat anti-GluK4 polyclonal antibody (sc-8917, Santa Cruz Biotechnology) at 1:100, and a goat anti-GluK5 polyclonal antibody (sc-8915, Santa Cruz Biotechnology) at 1:100. Antibodies for NMDA receptor subunits NR1, NR2a, NR2b, NR2c, NR2d, NR3a, and NR3b were used as well. For the NMDA receptor family the following antibodies were used; a goat anti-NR1 polyclonal antibody (sc-1467, Santa Cruz Biotechnology) at 1:100, a goat anti-NR2a polyclonal antibody (sc-1468, Santa Cruz Biotechnology) at 1:250, a goat anti-NR2b polyclonal antibody (sc-1469, Santa Cruz Biotechnology) at 1:100, a goat anti-NR2c polyclonal antibody (sc-1470, Santa Cruz Biotechnology) at 1:100, a goat anti-NR2d polyclonal antibody (sc-1471, Santa Cruz Biotechnology) at 1:100, a rabbit anti-NR3a polyclonal antibody (07-356, Millipore) at 1:500, and a rabbit anti-NR3b polyclonal antibody (07-351) at 1:250. The expression of ionotropic glutamate receptor δ 1 and 2 was not analyzed because the binding of a ligand by those receptor subunits has not been shown to cause any opening of an ionotropic channel. A rabbit polyclonal antibody against *TRPV1* was used at a concentration of 1:500 to demonstrate the presence of *TRPV1* in peripheral processes, which would confer sensitivity to capsaicin and indicate a nociceptive input. While *TRPV1* may be expressed by keratinocytes, differences in morphology between epithelial cells and neuronal projections and the use of neuron cytoskeletal markers facilitated the identification of neuronal processes expressing *TRPV1*. A mouse-derived antibody

(mab1637, Millipore) against β-Tubulin 3 (BT3) was used to identify neuronal processes, and very clearly delineated between putative neuronal and non-neuronal structures. In those instances where multiplex staining with the mouse monoclonal BT3 was not possible due to overlap in antibody host species, a rabbit polyclonal anti-BT3 antibody or a sheep-derived pan-Tubulin antibody was used. All the BT3 and Tubulin, as well as the TRV1 antibodies, were used at a working concentration of 1:500.

Because of the range of the array of primary antibodies being employed to compare iGluR expression in the compartmented co-culture system, consideration was taken to ensure that the antibodies selected had been successfully used in previous studies to evaluate expression of their respective iGluRs. Although not all antibodies that were available at the time of selection had examples of use for immunofluorescent experiments in the relevant literature, those antibodies selected for this study that did not have relevant examples of use in immunofluorescent experiments did have examples in Western Blotting. Priority was given to the respective performances of antibodies at selectively highlighting their respective targets, however consideration was also paid to which antibodies had been used in conjunction within individual previous publications.

The antibody for GluA1 has been used for immunohistochemical analysis of GluA1 versus GluA2 expression (Hagahari et al., 2011). The antibody for GluA2 has also been used for immunocytochemical experiments to evaluate its expression levels in neuronal processes (Qiu et al., 2012). Importantly, all four of the antibodies selected for the evaluation of AMPAR expression have been used in an individual experiment to evaluate their respective expression in the spinal cords of wobbler mutant mice (Bigini et al., 2006).

The GluK1 antibody selected for this study was used to evaluate GluK1 receptor expression as compared to the Kainate family receptor subunits in immunohistochemical staining of

hippocampal astrocytes after experimentally induced status epilepticus (Vargas et al., 2014). The GluK2 antibody, GluK3 antibody, and GluK5 antibody were all utilized in an individual study via immunohistochemical experiments to evaluate their respective expression in the mouse cochlea (Peppi et al., 2012). The GluK4 and GluK5 antibodies were also utilized in the same study to immunohistochemically evaluate their expression in dendrites in the mouse retina (Lee et al., 2012)

The NR1, NR2b, NR2c, and NR2d antibodies selected for this study have all been used in a single study to immunohistochemically evaluate somatic expression of their respective receptor subunits in rat vagal afferent neurons in the nodose ganglia of rats (Czaja et al., 2006). The antibody for NR2a has been used to evaluate its expression and interaction with NR1 and NR2b in mouse cerebral cortex via Western Blotting experiments on subcellular fractions (Wang et al., 2012). The antibody for NR3a selected for this study has also been used successfully in Western Blotting experiments on subcellular fractions of membranes to evaluate its expression and interactions (Henson et al., 2012). The antibody selected to target NR3b has been used to characterize the subunits expression in rat forebrain, cerebellum, and spinal cord (Wee et al., 2008).

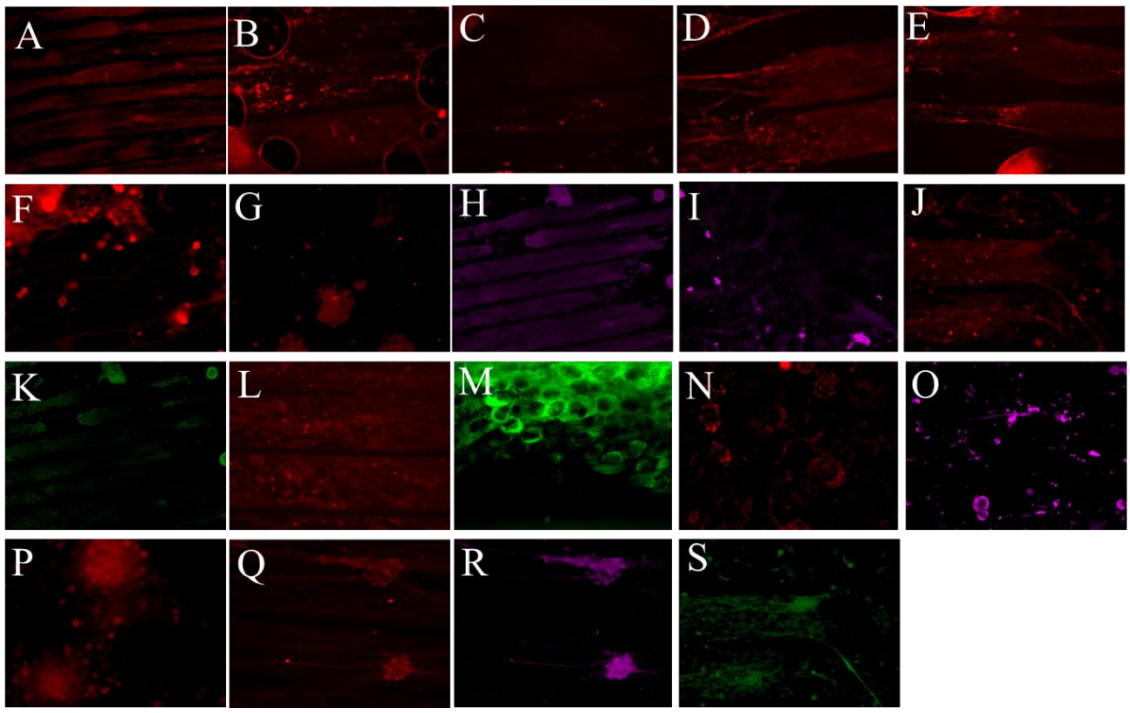

FIGURE 19: This figure shows a representative image for each of the primary antibodies used to show their respective and comparative performances. The staining in these images shows both somatic staining and staining in processes of the respective receptors at the working concentrations used to generate the other images generated for this study. The antibodies highlighting their respective targets correspond to the lettered panels as follows; A: NR1, B: NR2a, C: NR2b, D: NR2c, E: NR2d, F:NR3a, G:NR3b, H: GluA1, I: GluA2, J: GluA3, K: GluA4, L: GluK1, M: GluK2, N: GluK3, O: GluK4, P: GluK5, Q: pan-Tubulin, R: BT3, S: TRPV1.

After the addition of the PBS containing Triton X-100, Bovine Serum Albumin, and polyvinylpyrrolidone, along with primary antibodies, the compartmented co-culture plates were incubated at 4º C for two days, on the 3rd day this solution was removed and the plates were rinsed with PBS before applying the secondary antibodies. Secondary antibodies were donkey-raised anti-mouse, anti-rabbit, or anti-goat antibodies conjugated to Alexafluor 488, Alexafluor 555, or Alexafluor 647 (ThermoFischer Scientific.) Secondary antibodies were used at a dilution of 1:1000 in PBS containing 0.3% Triton X-100, 0.5% Bovine Serum Albumin, and 0.5% polyvinylpyrrolidone. The secondary antibody solution was incubated over the culture plates for 1 hour at room temperature in the dark. Following the secondary antibody incubation, plates were washed with PBS for 10 minutes and then incubated with the nuclear stain (4',6-diamidino-2-phenylindole) DAPI for 15 minutes. After the DAPI incubation, the plates were rinsed with PBS for 10 minutes 3 successive times before removing all fluid in preparation for mounting. Prolong Gold Anti-Fade reagent was used to sandwich a glass coverslip onto the plates, protecting and preserving the activity of the secondary antibody-conjugated fluorophores. Each plate was coated with 30 µl of Prolong Gold Anti-Fade reagent and then a glass coverslip was laid on top of the plate to sandwich the culture between the glass and plastic to facilitate clear viewing with the confocal microscope for immunofluorescence.

Single images or stacks of images through a field of view were acquired using an epifluorescent microscope or a Leica confocal microscope, respectively. Multiple images of the same field of view at different focal lengths allowed for the deconvolution of the images acquired with the confocal microscope to better resolve the individual images as well as compile multiple focal length images into single images. Images were processed using the FIJI ImageJ software suite along with the DeconvolutionLab software plugin. Image sequences from individual fluorescence channels were deconvoluted using the default settings for the software plugin with

no external PSF file required. The type of deconvolution procedure run for all image sequences was the Tikhonov-Miller process. The stack of images through the field of view were then collapsed to a 2 dimensional image combining all depths through the field of view using the "Z Project" stack function of ImageJ. After the deconvolution and 2 dimensional reduction processes, images were colorized and overlaid to show colocalization of different proteins in the same subcellular compartments. Wide field images of the culture plates to display the compartmentalization of the system and restricted fields of different cells were acquired using an epifluorescent microscope at 4X or 10X magnification.

Expression of iGluRs was not solely restricted to neuronal structures, subunit-specific anti-iGluR primary antibodies showed reactivity in putative keratinocytes in the distal compartment where keratinocyte stem cells were plated. Some iGluR staining also seemed to localize more to the distal ends of neuronal projections in close association with keratinocyte fields.

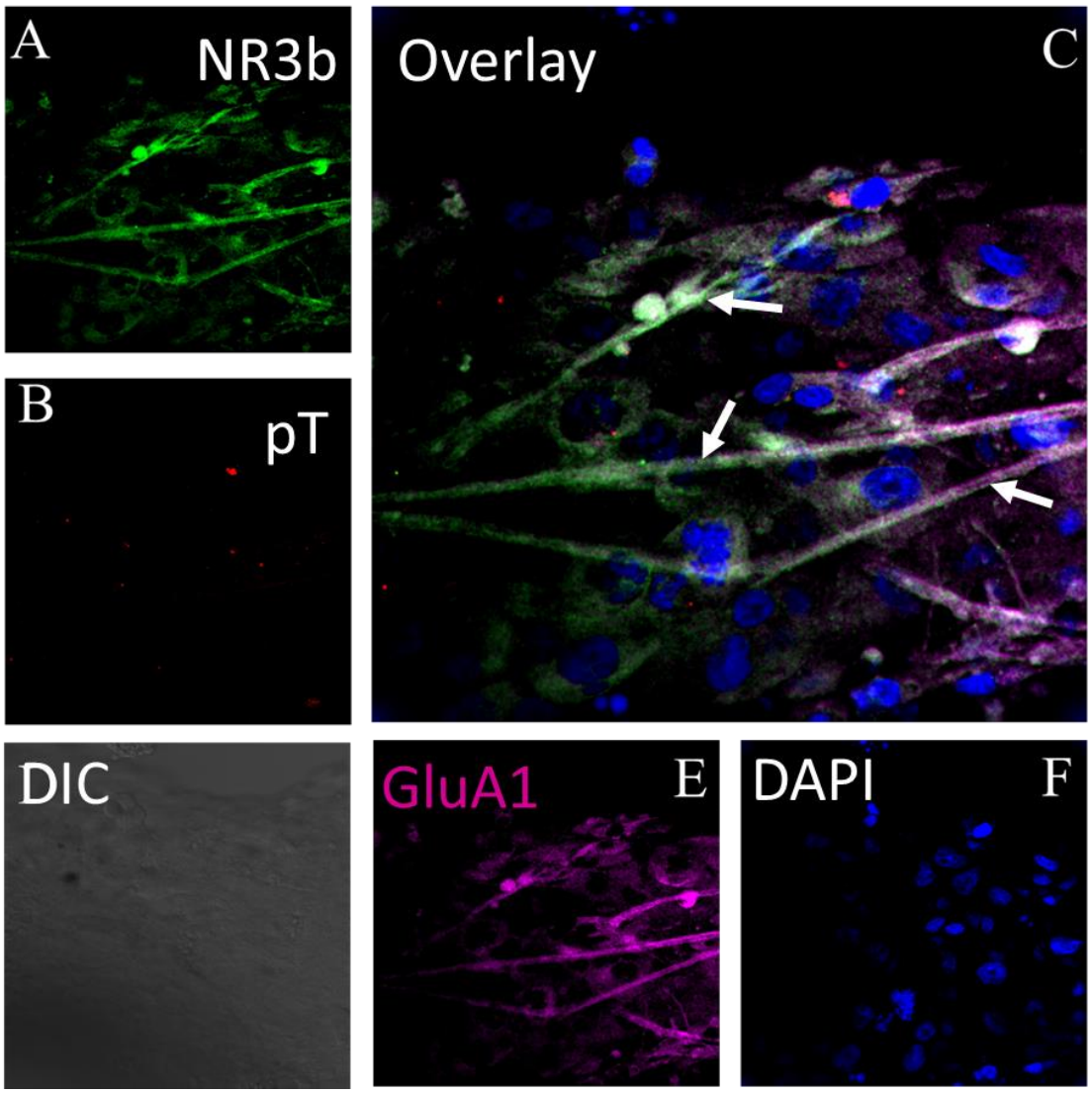

FIGURE 20: This figure shows GluA1(magenta) along with NR3b (green), pan-Tubulin (red), and DAPI (blue) along three possible sensory neuron processes in a keratinocyte stem cell field.

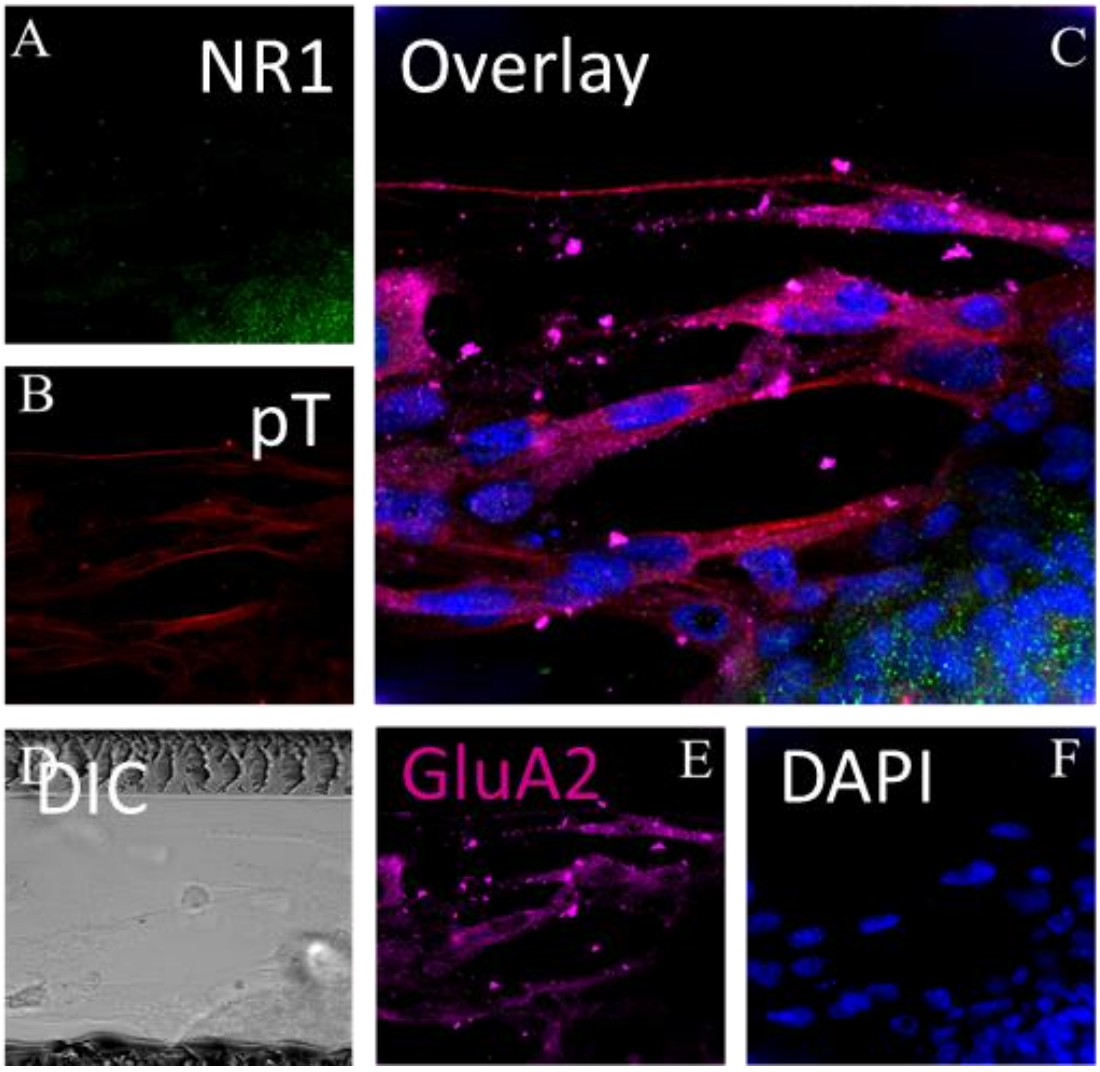

FIGURE 21: This figure GluA2 (magenta) along with NR1(green), pan-Tubulin (red), and DAPI (blue) near a possible sensory neuron process in a keratinocyte stem cell field.

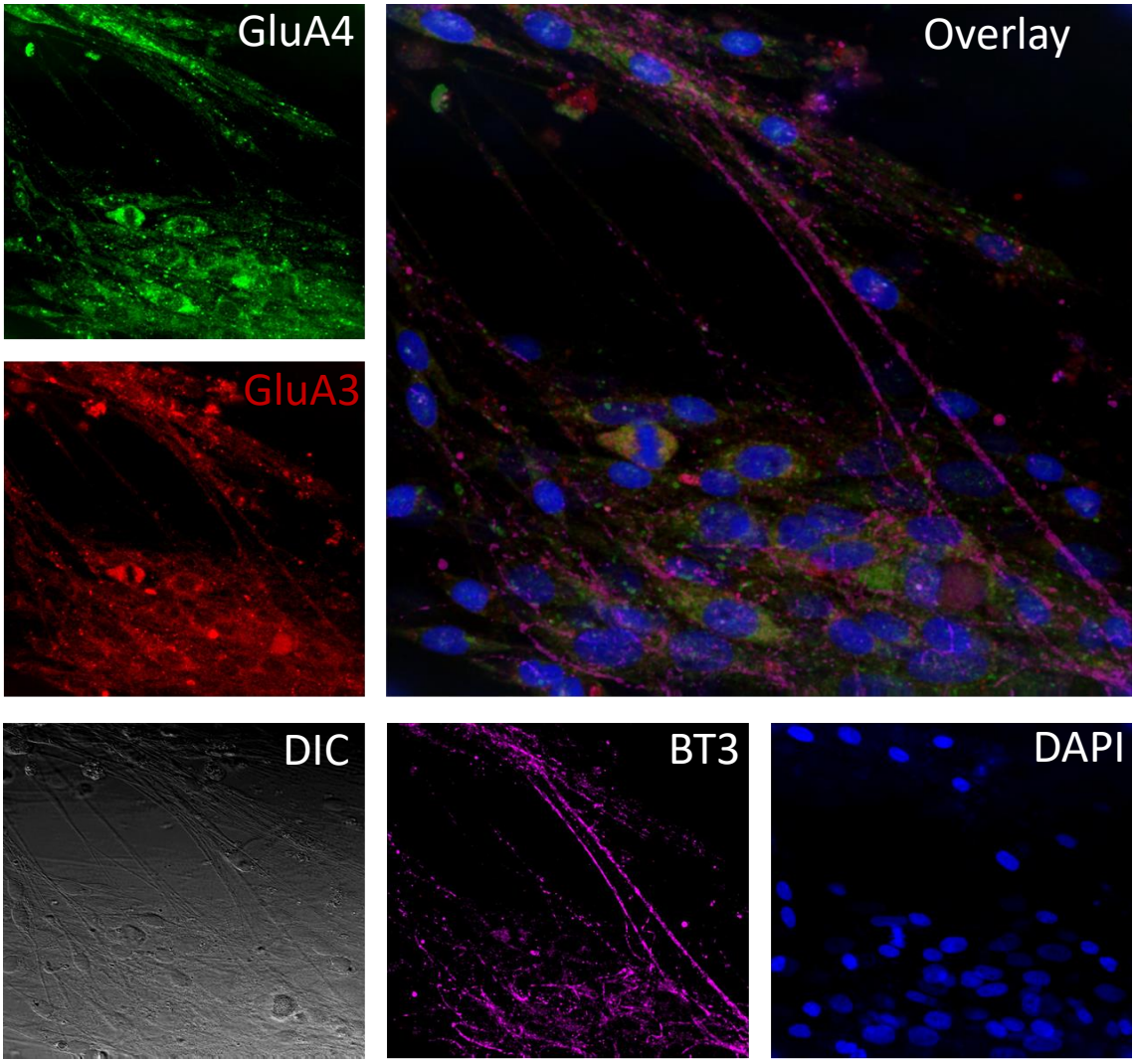

FIGURE 22: This figure shows GluA3 (green) and GluA4 (red), along with BT3 (magenta) and DAPI (blue) along several sensory neuron processes extending into the keratinocyte stem cell compartment.

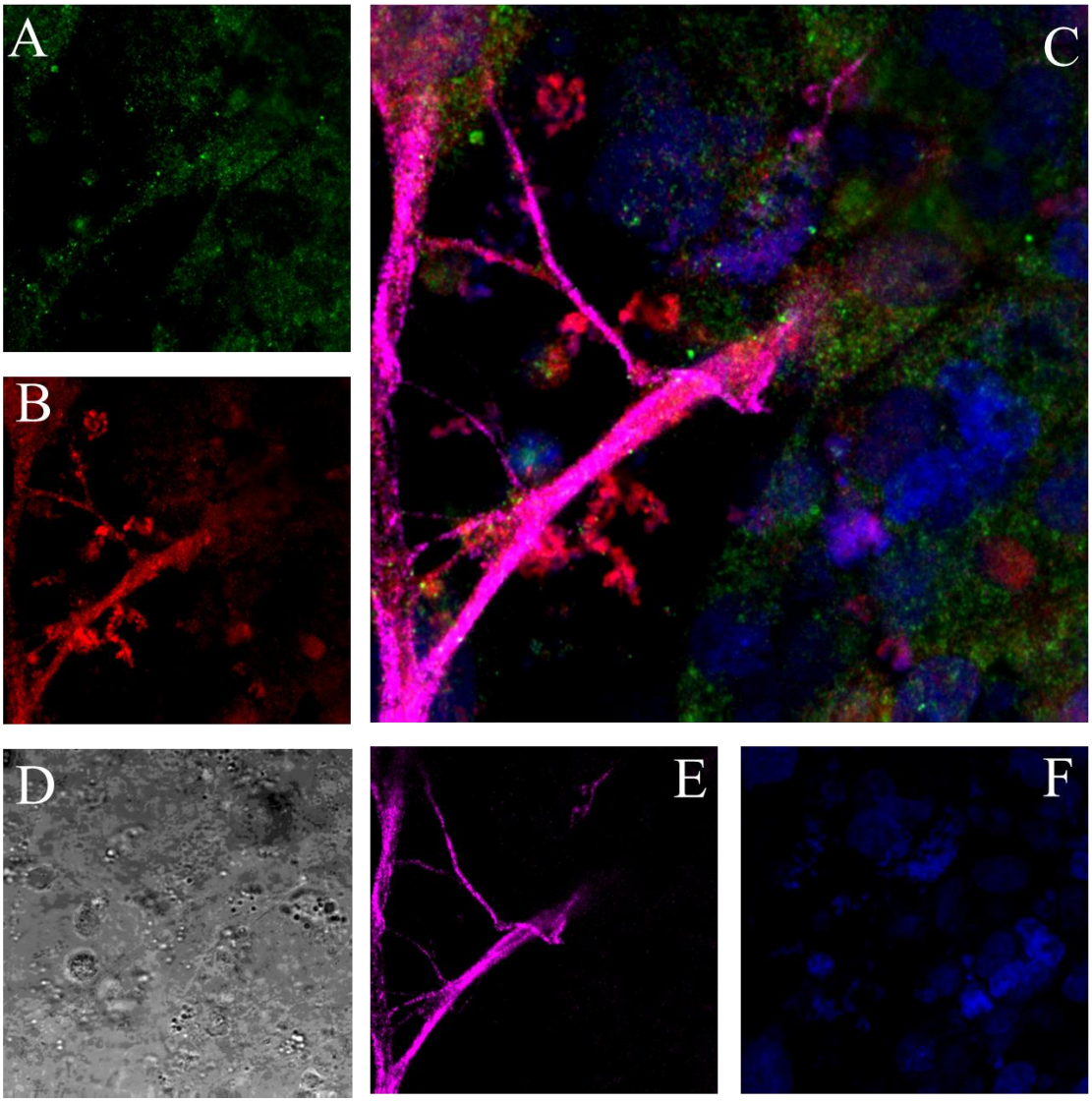

FIGURE 23: This figure shows GluA4 (red) and NR1 (green), along with BT3 (magenta) and DAPI (blue) at a potential branch point of multiple neuronal processes extended into the keratinocyte stem cell compartment.

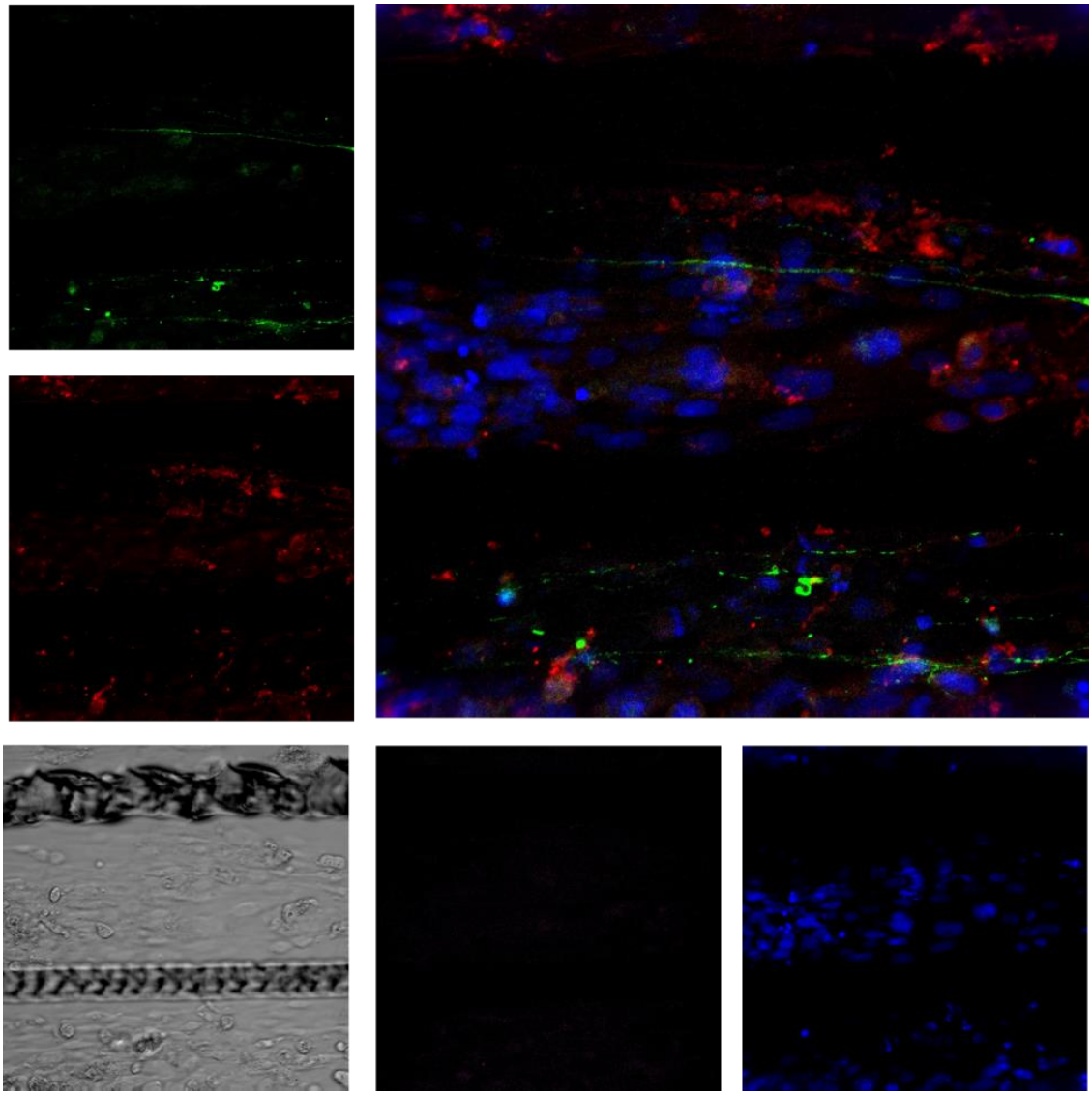

FIGURE 24: This figure shows GluK1(red) along with cytokeratin (magenta), DAPI (blue), and BT3(green) around a sensory neuron process extended into the keratinocyte stem cell field.

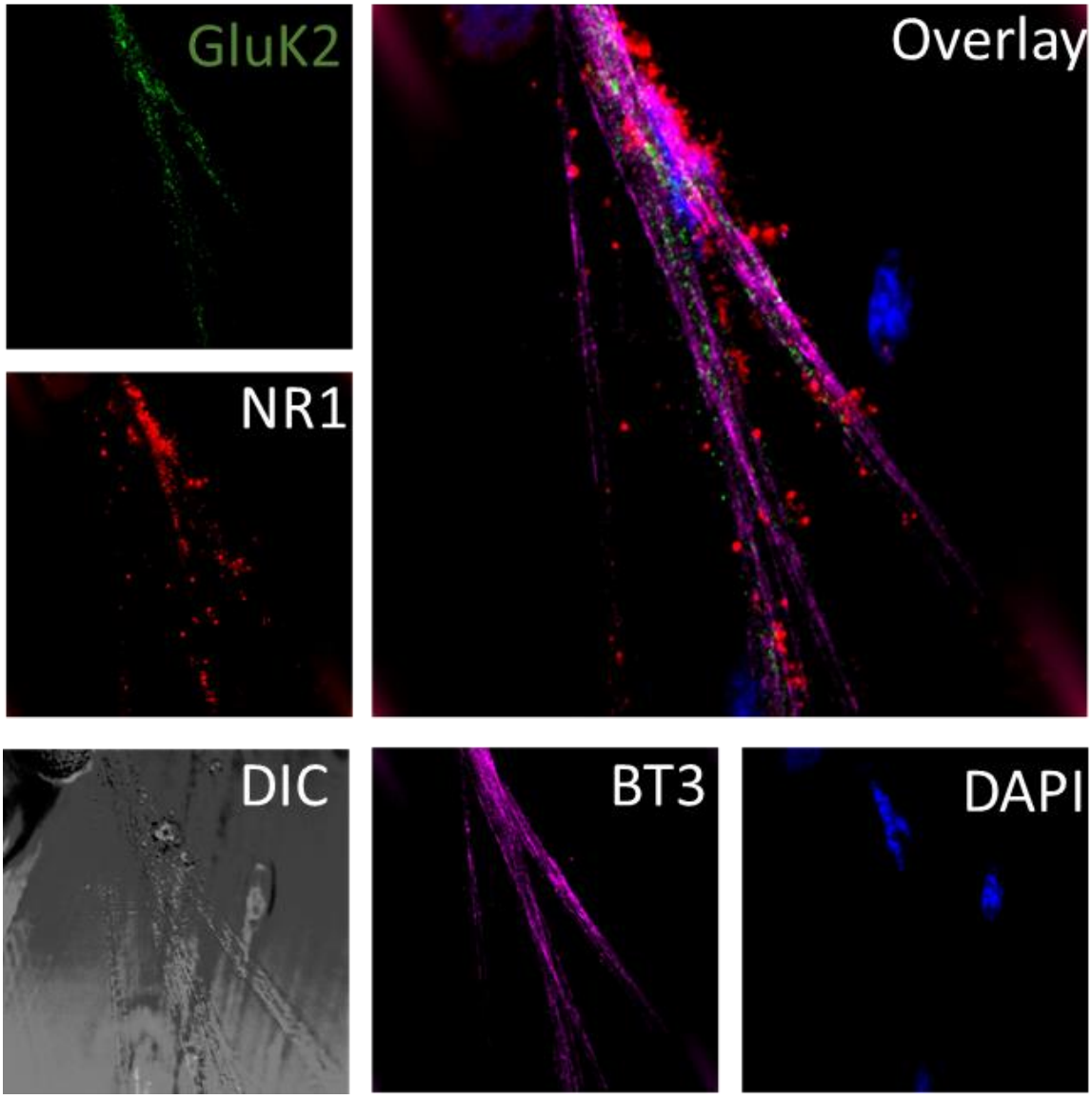

FIGURE 25: This figure shows GluK2 (green) along with NR1 (red), BT3 (magenta), and DAPI (blue) along a potential neuronal process extending into a keratinocyte stem cell field.

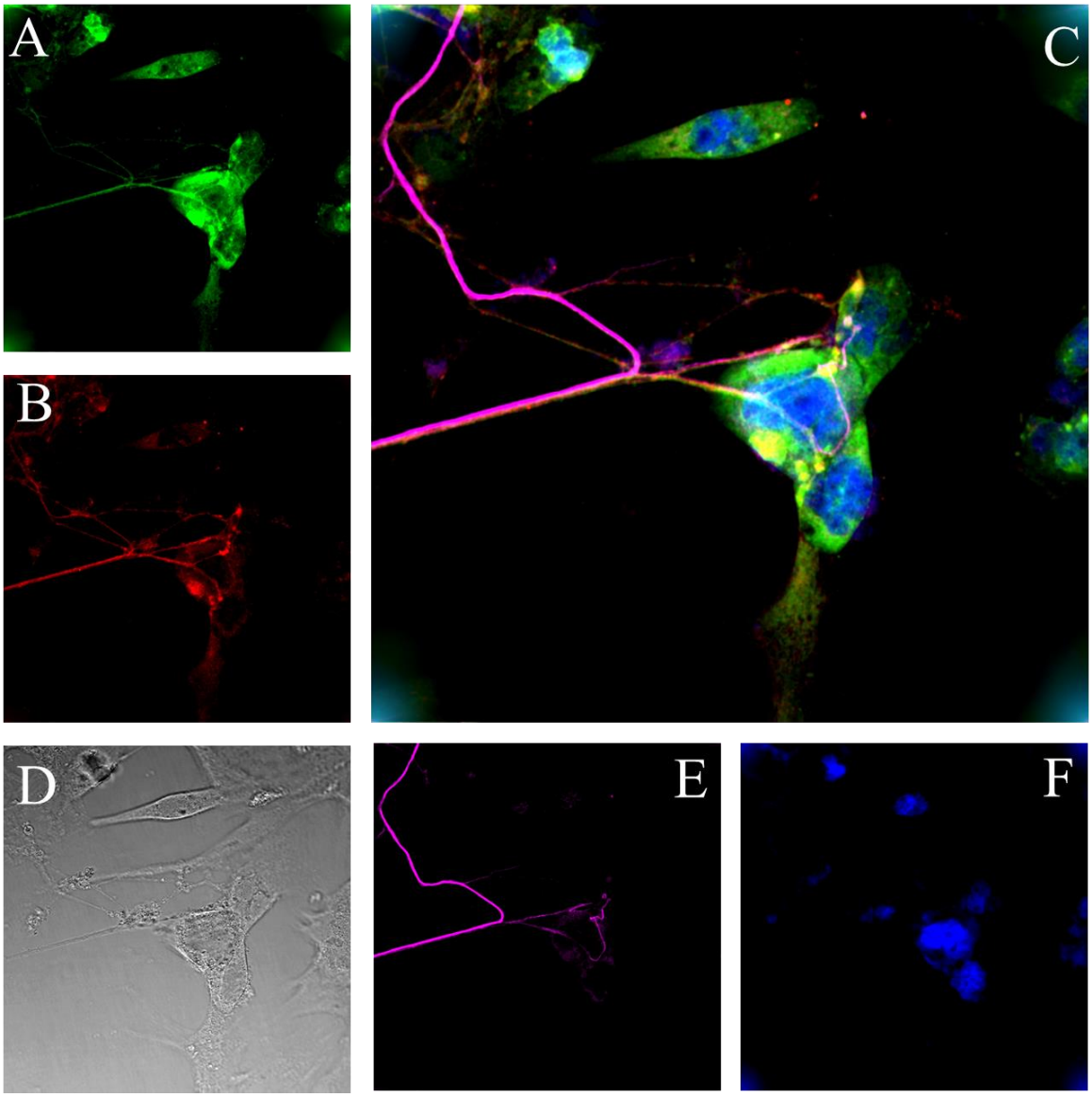

FIGURE 26: This figure shows GluK3 (red), along with TRPV1 (green), BT3 (magenta), and DAPI (blue) along a sensory neuron process in the keratinocyte stem cell field.

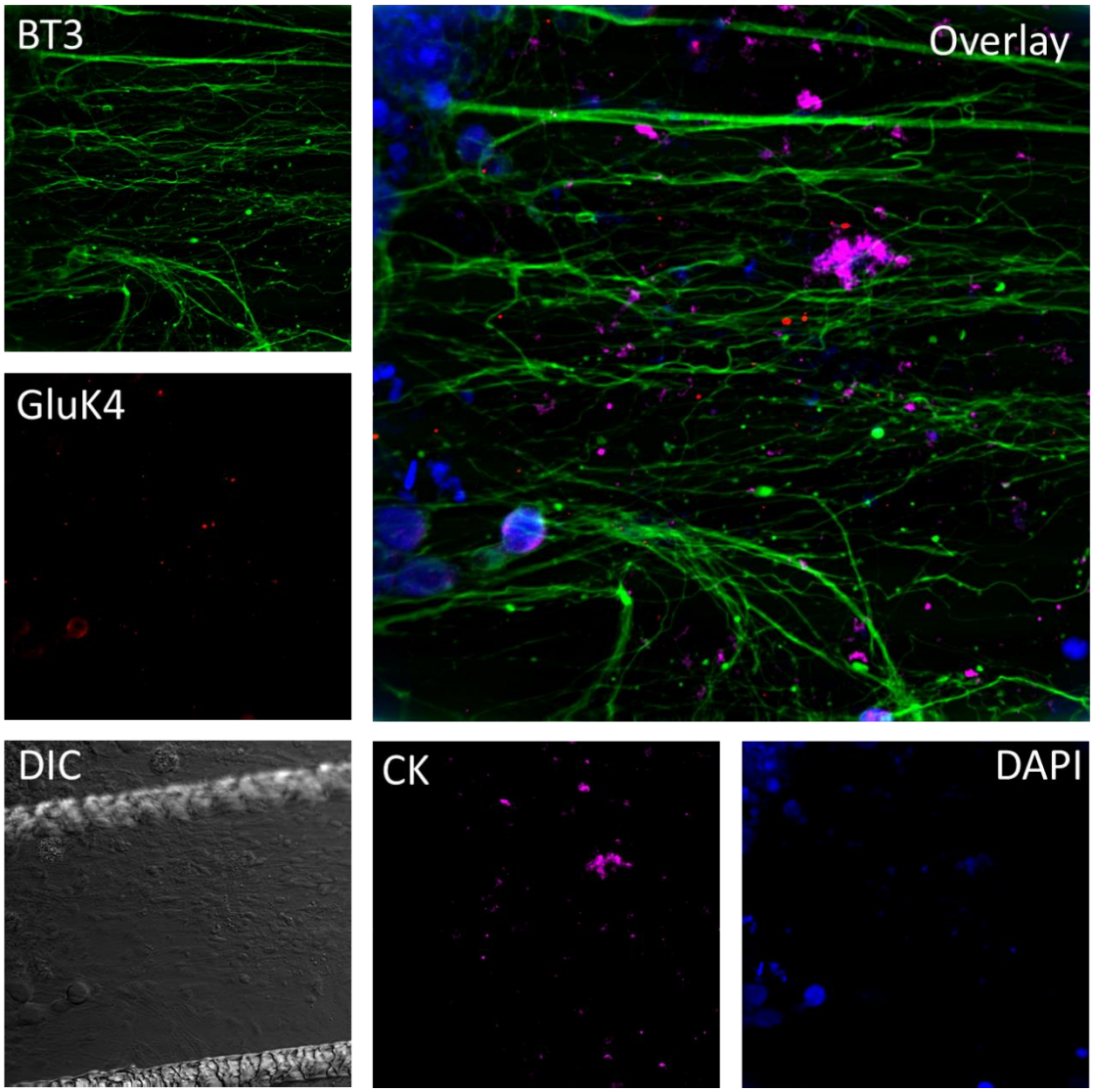

FIGURE 27: This figure shows GluK4(red) along with cytokeratin (magenta), DAPI (blue) and BT3(green) around sensory neurons and their processes at the septum barrier.

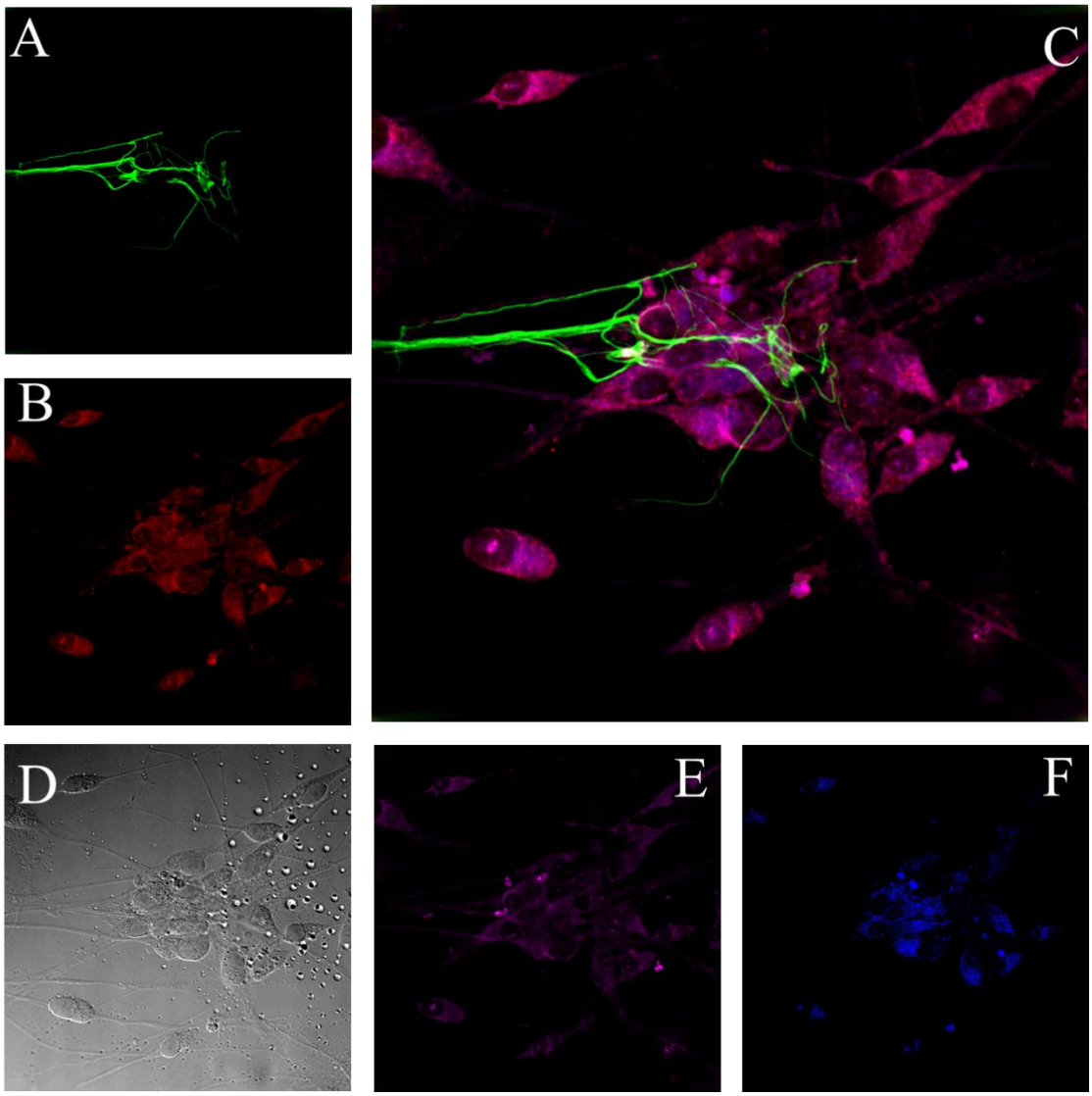

FIGURE 28: This figure shows GluK5 (magenta), along with BT3 (green) cytokeratin (red) and DAPI highlighting sensory neuron processes that have extended into the keratinocyte stem cell compartment and appear to possess complex interfaces with keratinocytes.

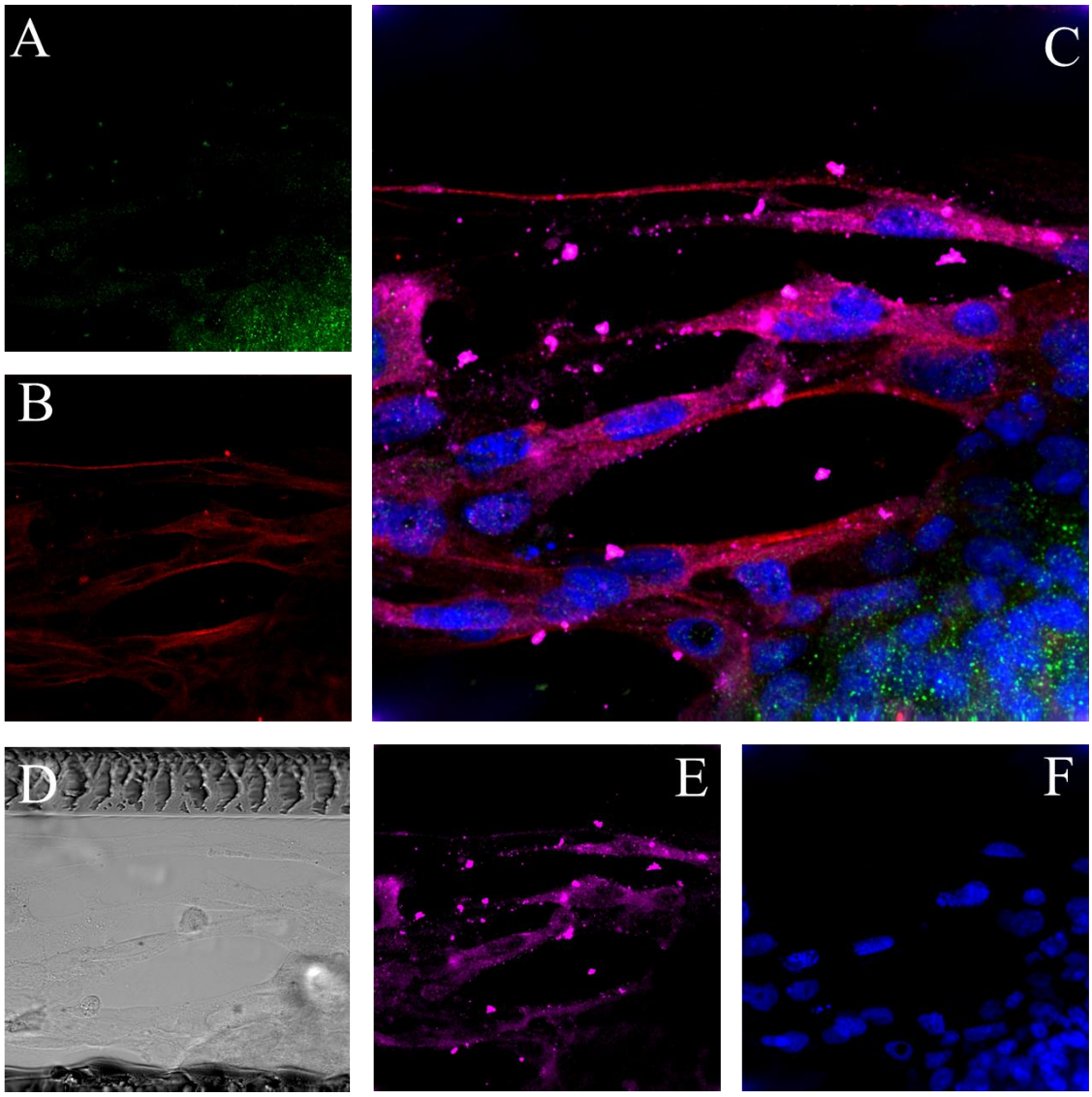

FIGURE 29: This figure shows NR1 (green) along with GluA2 (magenta), pan-Tubulin (red), and DAPI (blue), along neuron processes extending into the keratinocyte stem cell field.

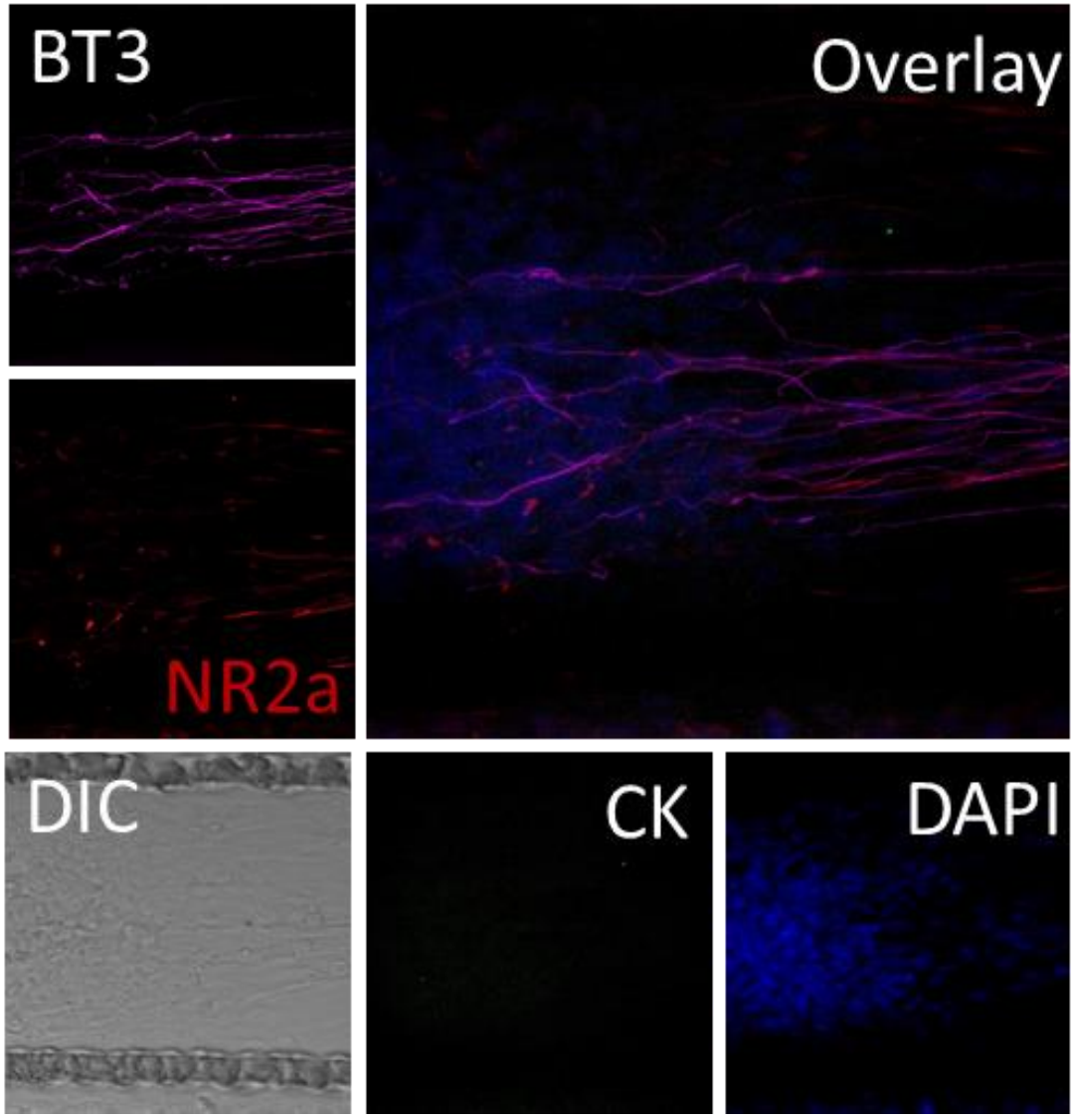

FIGURE 30: This figure shows NR2a (red), along with cytokeratin (green), BT3 (magenta), and DAPI (blue) highlighting sensory neuron processes extending into the keratinocyte stem cell compartment.

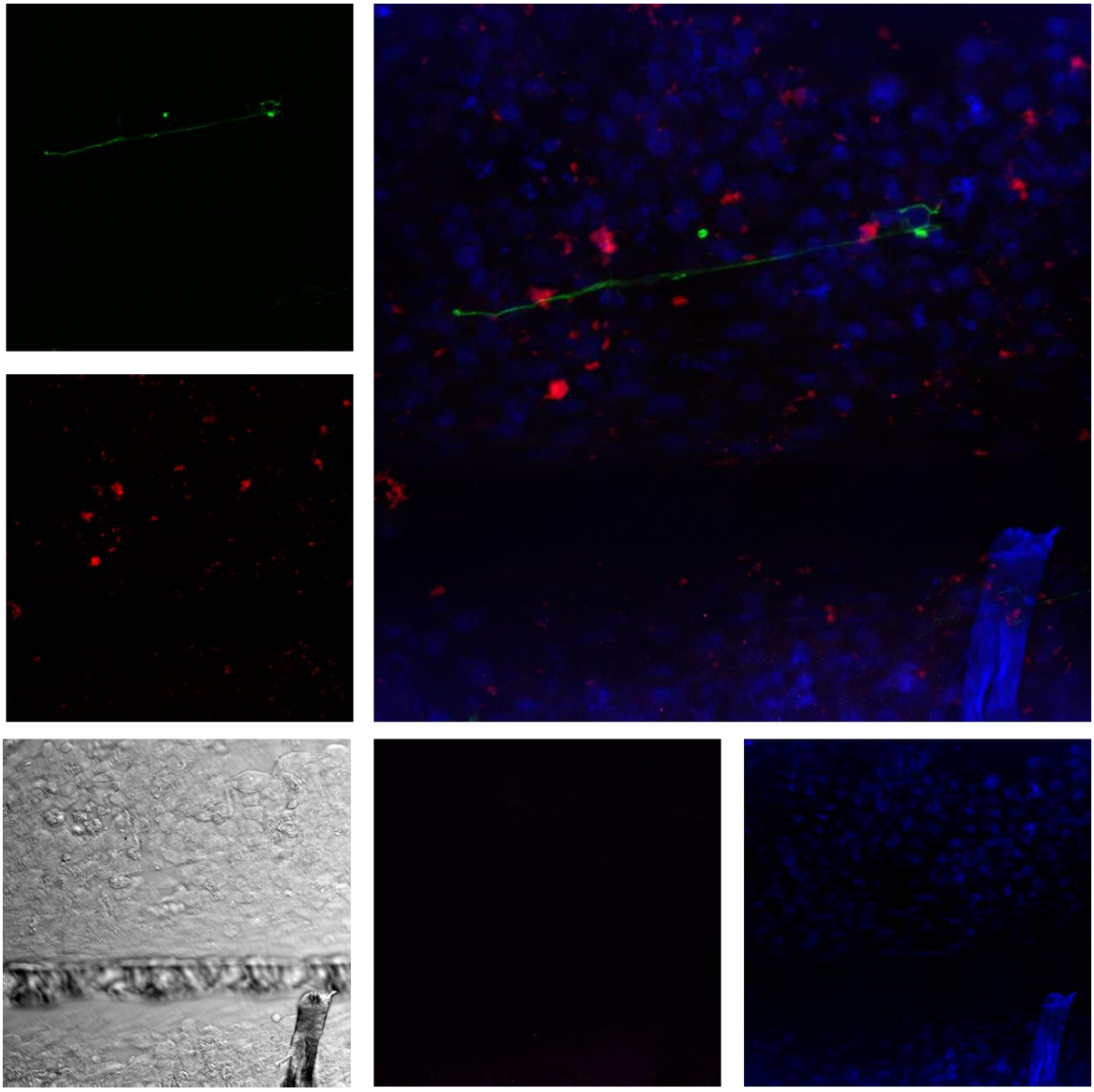

FIGURE 31: This figure shows NR2b (red), along with BT3 (green), cytokeratin (magenta), and DAPI (blue) highlighting a sensory neuron process that has extended into a keratinocyte stem cell field.

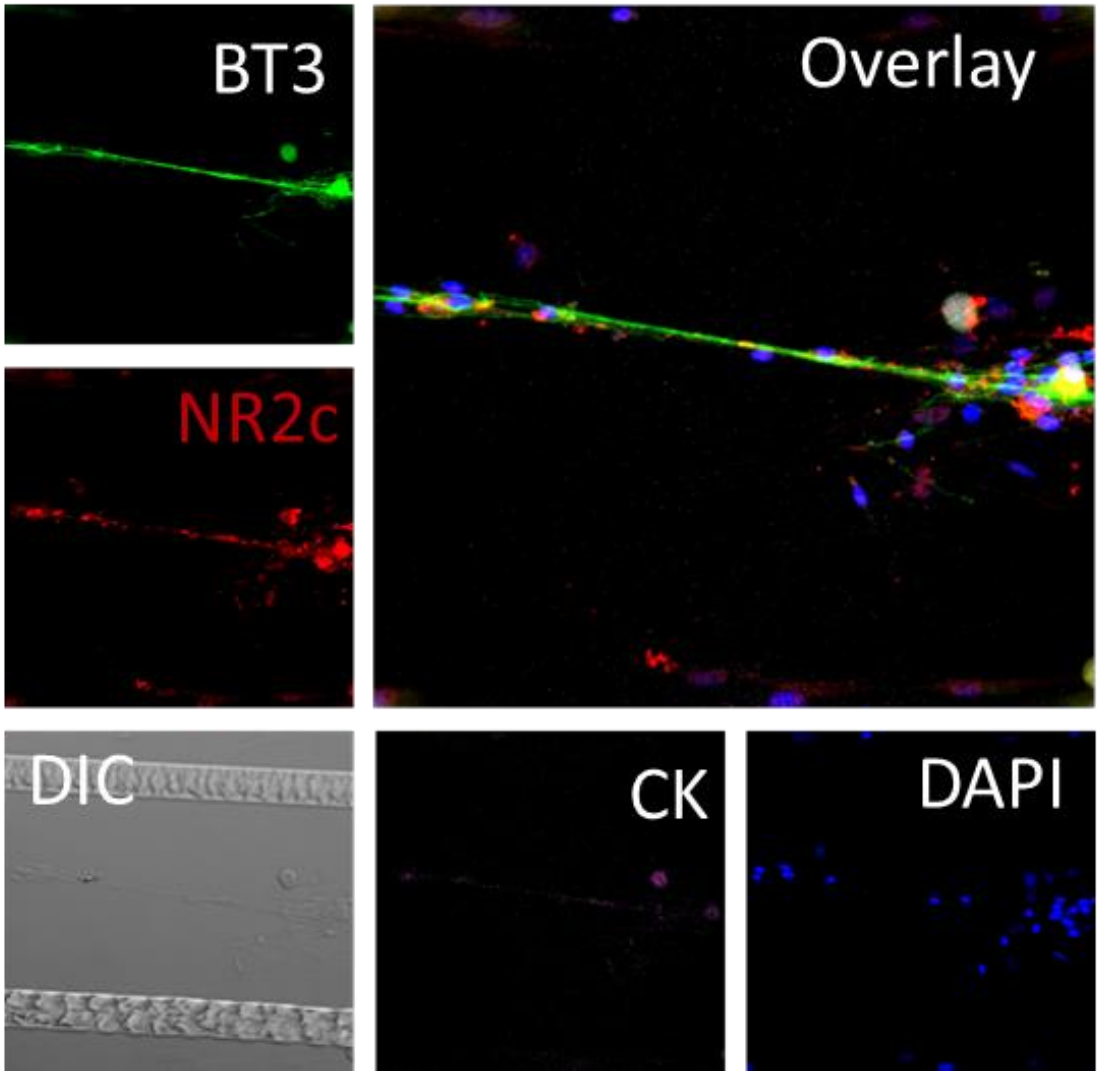

FIGURE 32: This figure shows NR2c (red), along with BT3 (green), cytokeratin (magenta), and DAPI (blue) highlighting a sensory neuron process that has extended into a keratinocyte stem cell field.

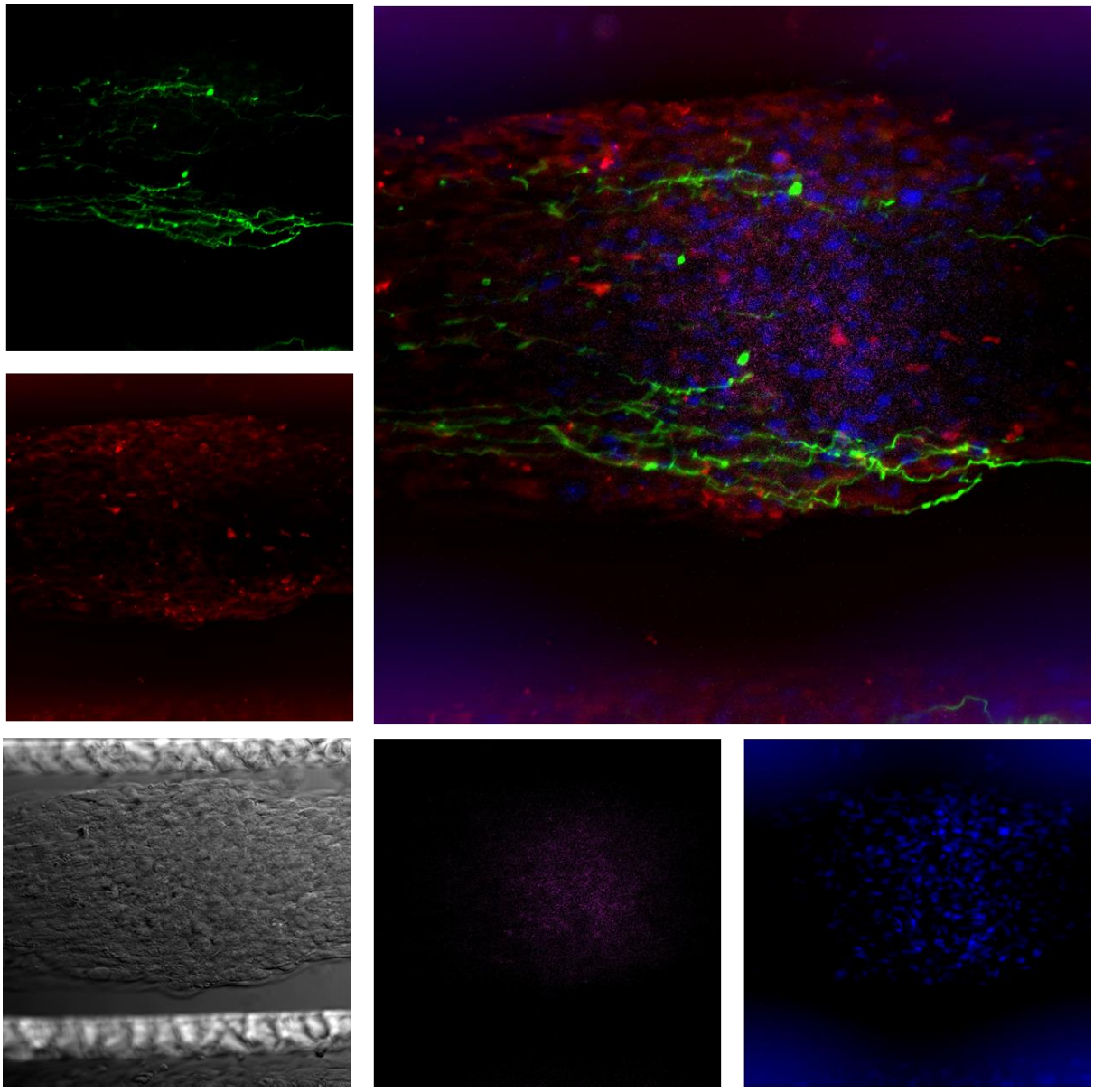

FIGURE 33: This figure shows NR2d (red), along with BT3 (green) cytokeratin (magenta), and DAPI (blue) highlighting multiple processes that have extended from the neuronal compartment into a keratinocyte stem cell field in the keratinocyte compartment.

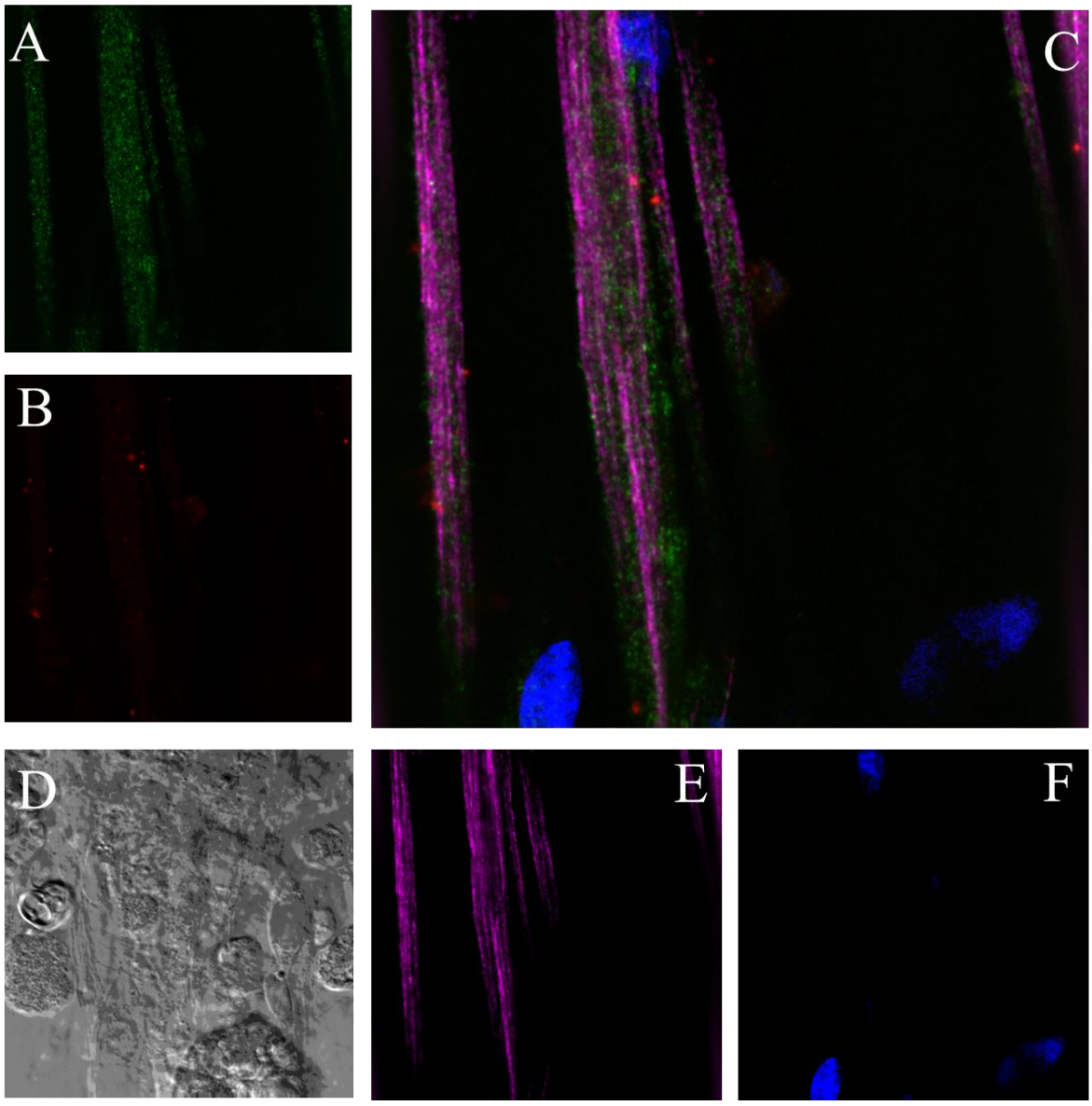

FIGURE 34: This figure shows NR3a (green) along with GluA3 (red), BT3 (magenta), and DAPI (blue) highlighting a peripheral sensory neuron process that has extended from the neuronal compartment into the keratinocyte stem cell compartment.

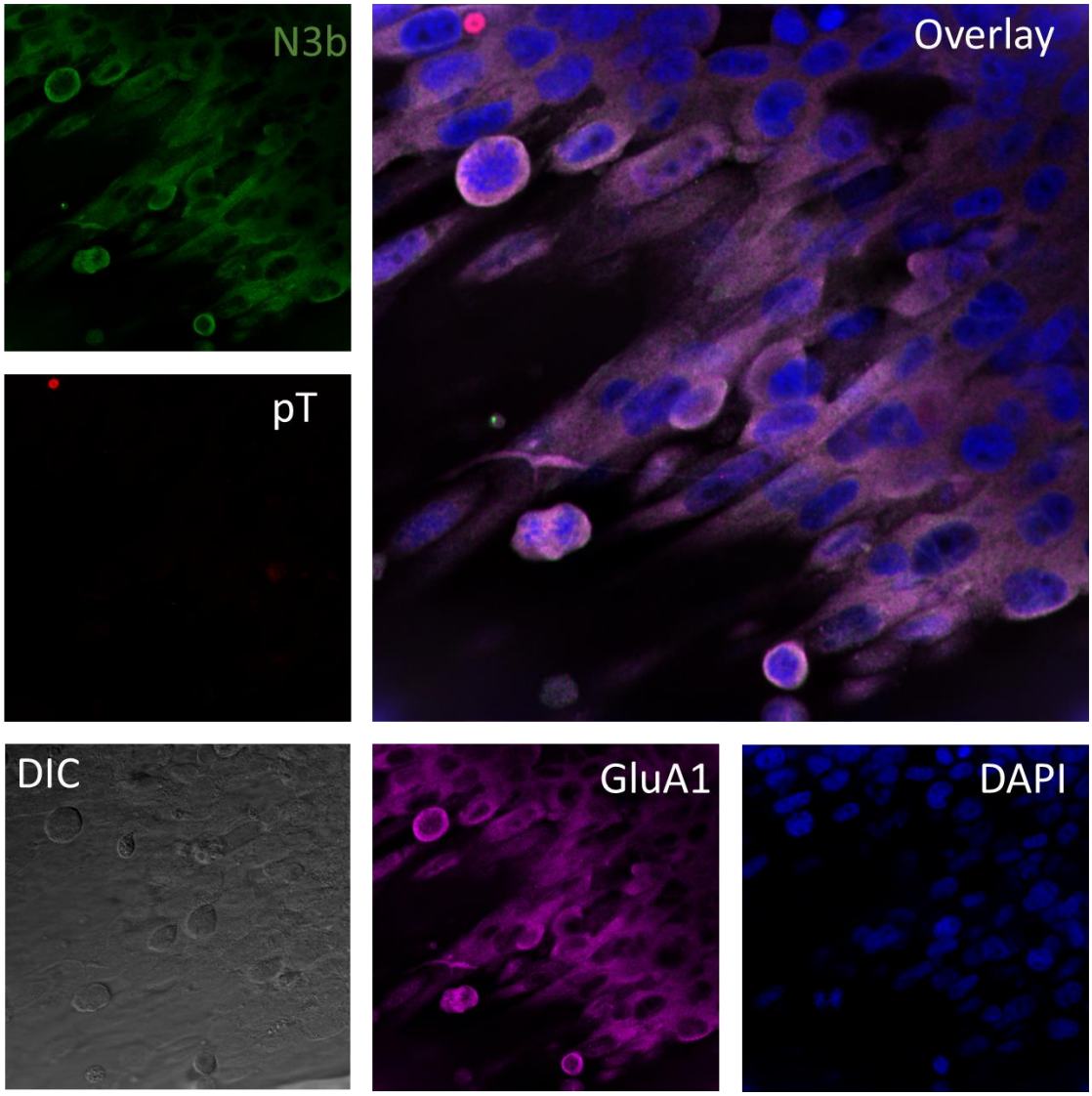

FIGURE 35: This figure shows the expression of NR3b (green), along with GluA1 (magenta), pan-Tubulin (red), and DAPI (blue) highlighting a neuron process that has extended from the neuronal compartment into the keratinocyte stem cell compartment.

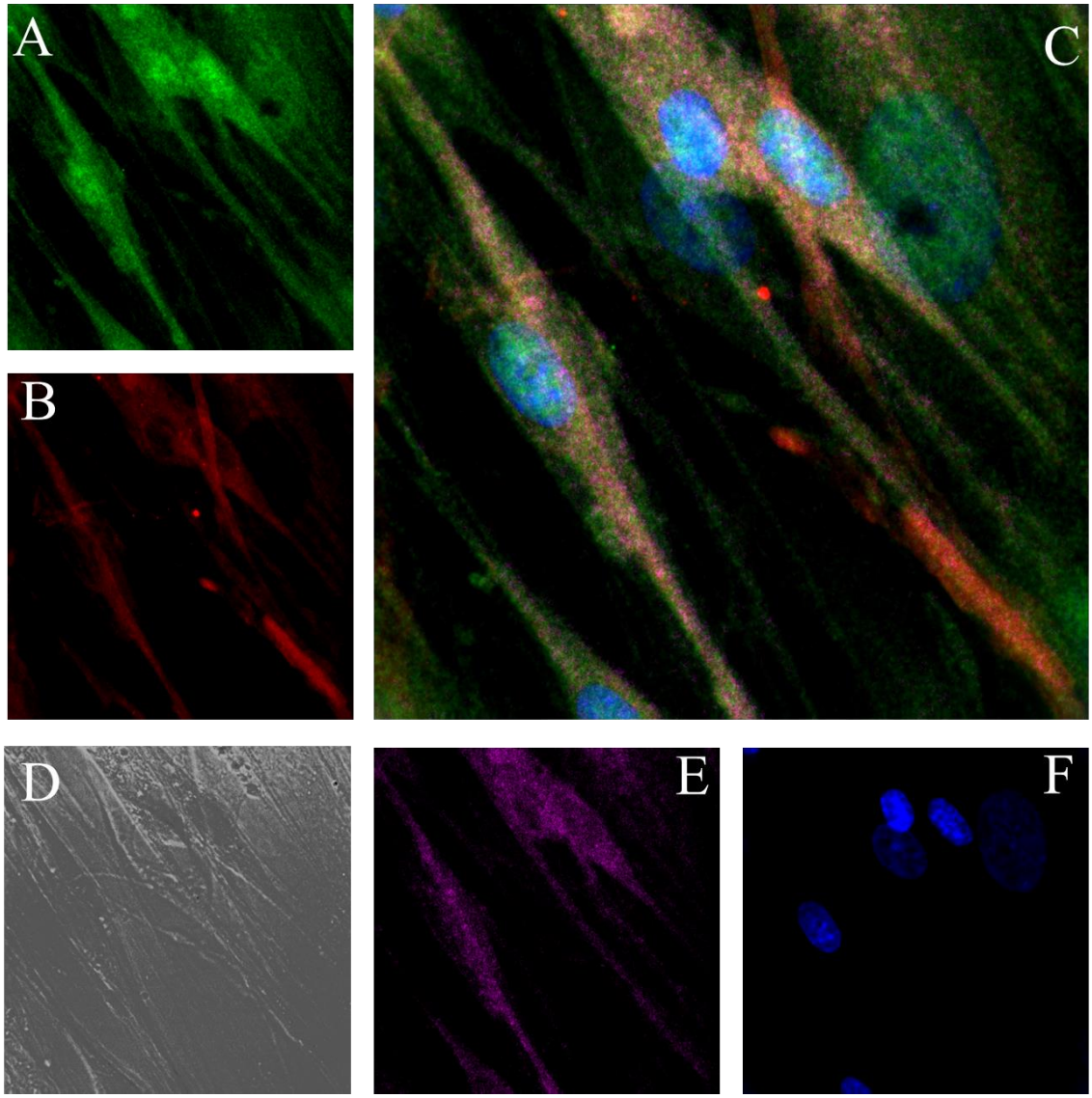

FIGURE 36: This figure shows GluA1 (green), GluK1 (red), and NR1 (magenta), along with DAPI (blue). Imaged on the keratinocyte side of the divider these represent putative neuronal processes and glial cells, where neural processes appear as relatively straight lines separate from those bodies staining with the nuclear stain DAPI.

Subunit-specific identification of iGluRs expressed in individual sensory neurons and present at different sub-cellular compartments is of paramount importance in pursuing pain treatments targeted to peripheral iGluR activity, whether the chronic pain is due to inflammation or injury. The expression of different iGluR subunits at different sub-cellular compartments may affect the overall activity of a sensory neuron differently because of the specific kinetics of the receptors as well as their differing ion conductances. While iGluRs are excitatory in a broad sense, the differences between receptors cause for signals to be comparatively different depending upon the subunits comprising each functional receptor. Some receptor subunits may permit or block the passage of Ca^{2+} ions through their pores, influencing not only the amplitude or duration of current during activation, but directly affecting the influx and amount of cytoplasmic Ca^{2+}, a secondary messenger, within the immediate area. Pharmacologically inhibiting activity or decreasing peripheral process expression of those iGluRs that are permissive to Ca^{2+}, or that have been shown to have longer firing kinetics, could have the ultimate influence of decreasing the overall neuronal tone and firing rate. Increasing peripheral process expression of receptors that are impermeable to Ca^{2+} could prevent the activation of those peripheral processes from being subjected to extreme influxes of the secondary messenger ion Ca^{2+}, which might in turn mitigate the increase in neuronal firing frequency and sensitivity that are hallmarks of sensory nerve activity in chronic pain conditions.

While normal iGluR activity is necessary for the proper functioning of an organism, the contribution to chronic pain via excessive activation of these sensory neuron peripheral receptors in chronic pain conditions presents an attractive target for pharmacologic intervention. The subunit-specific identification of iGluR composition in peripheral processes of sensory neurons presented herein outlines a road map of which receptors should be preferentially targeted for developing treatments for chronic pain outside the central nervous system.

CHAPTER IV

ELECTROPHYSIOLOGY

Compartmented co-cultures were removed from the incubator after 10-15 days in culture and the plates were placed on a stage with a bath application source, a gravity fed perfusion and piezo-motor translation system, along with a headstage for mounting a glass capillary electrode to take elctrophysiological recordings. Application of each agonist used was restricted to the keratinocyte compartment so that the agonist could only reach projection segments that had crossed the central septum by the application of excess bath solution into the neuronal compartment and vacuuming of the excess bath solution from the keratinocyte compartment. This configuration of bath solution application caused a constant flow from the neuronal compartment, under the central septum of the Campenot chamber divider, into the keratinocyte compartment. This flow disrupted the intermittent flow of agonist solution exiting the perfusion array, preventing the movement of agonist solution from the keratinocyte compartment into the neuronal compartment as well as causing a rapid dilution of the agonist solution exiting the perfusion array. The rapid dilution of the agonists applied is important for maintaining the health of the neurons without excessive stimulation of their processes from stray agonist activating

neurons in lanes apart from the cell from which recordings were taken. The disruption of the flow of agonist exiting the perfusion array by the counter-flow of bath introduced was confirmed to prevent the movement of agonist solution into the neuronal compartment by visualization of a 2% w/v fluorescein solution, both under white light and with mercury bulb fluorescent illumination. Comparing the appearance of red solution in the neuronal compartment both when the bath was applied versus when it was off, provided proof that the counter-flow system prevented agonist solution from reaching the neuronal compartment in high enough concentration to activate the soma. To further validate the counter-flow preventing agonist reaching the neuronal compartment the system was viewed under wavelength filtered magnesium bulb illumination, to show whether low concentrations of agonist solution flowing into the neuronal compartment because the fluorescent signal would be visible in lower concentrations than the dye in white light. Under both illumination sources, the absence of directed bath counter-flow allowed fluorescein solution exiting the perfusion system to visibly pass under the barrier into the neuronal compartment, while the application of the bath counter-flow prevented fluorescein solution from the perfusion system to appear in the neuronal compartment.

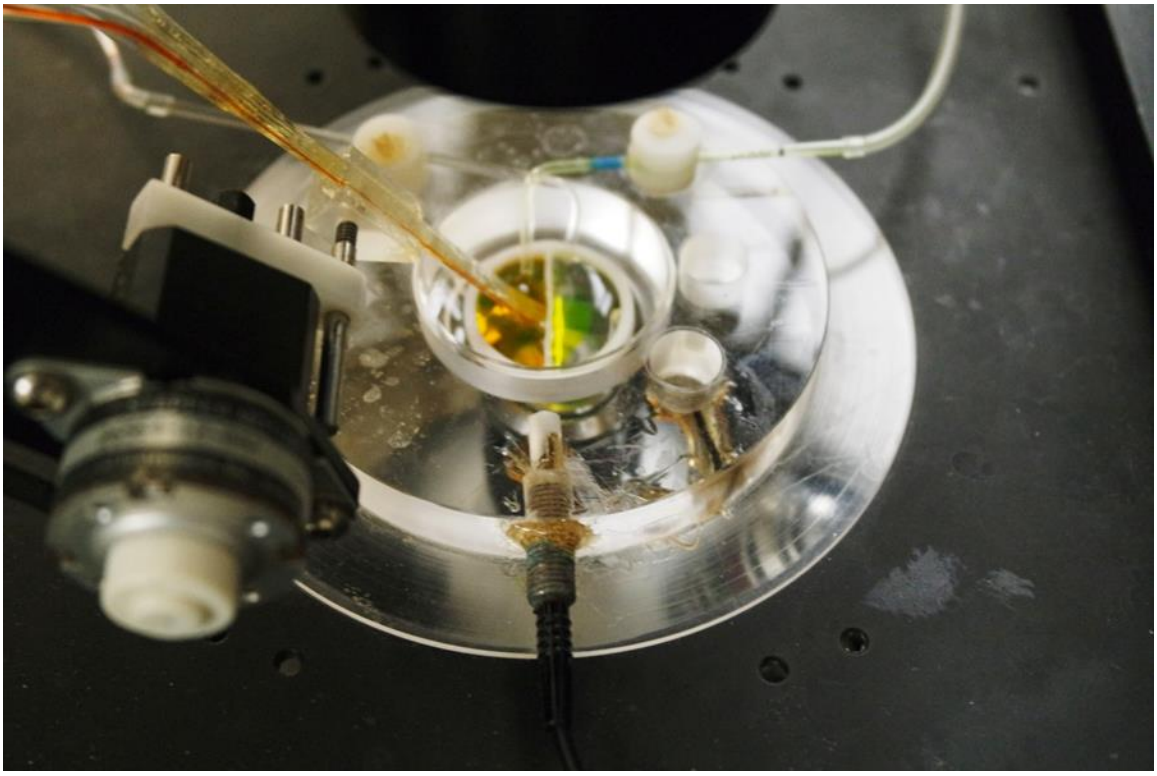

FIGURE 37: This image shows the configuration of the recording equipment for recording current from the cultured DRG sensory neurons. The bath solution is clear and is being introduced on the right in this image, corresponding to the neuronal compartment. The red solution being introduced to the left compartment in close approximation to the central septum via gravity-fed perfusion array is 2% w/v fluorescein. In this image the bath has been turned off briefly to show how fluid fed from the perfusion system would distribute under the central septum and into the neuronal compartment without the directional flow consistently repelling it from the central septum. When bath is applied, none of the dye solution crosses into the neuronal compartment. Fluorescein was chosen to visualize the respective flows under visible light and fluorescent conditions.

Elicited responses from individual neurons could vary in the timing and the amplitude of the current recorded dependent upon the configuration of the connectivity and order of the neurons being activated. Sensory DRG neurons are capable of generating both orthodromic (peripheral process to soma) and antidromic (central process to soma) impulses (Wall and Devor, 1983; Laszlo, 2013). This bidirectional functionality in signaling further complicates the potential configurations and interpretability of the results obtained. Through analysis of the characteristics of the currents produced some evidence may be garnered in support of correlation of specific response types with particular configurations. Multiple types of responses were observed, with different characteristics exhibited by each that could correspond to different configurations of cell-to-cell activation.

There are five potential configurations for the activation of neurons within the compartmented co-culture system to generate the responses observed. The most common response types correlate with 2 prospective configurations and represent the cohort of responses included in statistical analyses for this study. These 2 prospective configurations generated consistent signals of the same type that resembled EPSCs from other studies. These responses exhibited relatively small amplitudes with rapid return to baseline that fit a single logarithmic function curve. The other type of response observed resembled a more classic action potential.

The first configuration of responses seen is one where the peripheral application of agonist activates a peripheral process of an individual neuron, depolarizing that neuron and causing it to conduct a current towards the neuronal compartment, where it synapses on the cell from which recordings are being taken. The second configuration would be of multiple processes from multiple neurons extending into the keratinocyte compartment, being stimulated by the peripheral

agonist exposure and conducting respective impulses towards the neuronal compartment, where they might synapse on the same neuron being recorded and generate multiple or compound current impulses within a single recording window. A third configuration may be such that a cell being recorded possesses the peripheral process being activated. The fourth configuration is of a neuron possessing multiple branches of processes that cross the central septum that may synapse one to another. The fifth configuration is one where a neuronal soma may have penetrated through the barrier into the keratinocyte compartment. While the movement of neuronal somata into the keratinocyte compartments was comparatively rare, the capability of neurons to migrate across poly-D-lysine did allow for the occasional neuron to cross under the barrier. This would cause the elicited response to be instigated by agonist exposure at a somatic compartment instead of along a process, before conducting a current to the neuron from which the recording is being taken. The activation of a somatic compartment of a presynaptic neuron located in the keratinocyte compartment could lead to a synaptic transmission, in turn leading to an EPSC exhibited by the neuron being recorded on the neuronal side, however this configuration of activation would cause a consistent latency to responses seen across multiple exposures to a given agonist. There being multiple exposures to each individual agonist facilitated the comparison of latencies to responses. No cells recorded that showed such consistent latencies were included in the data-set subjected to statistical analyses. If the neuron from which recordings were taken was directly activated along one or more peripheral processes in the keratinocyte field then the speed of conductance would cause for there to consistently be a very low latency to the first responses seen across multiple exposures of multiple agonists, in addition to an increase likelihood for the initial response to be an action potential, as the process carrying the signal and the somatic compartment from which recordings were taken were the same. All responses included in the data-set subjected to statistical analyses displayed a latency of at least 80 milliseconds after the initiation of agonist exposure.

An action potential could be induced by the direct activation of a peripheral process of a specific neuron from which the current traces were recorded. An action potential could also result from temporal summation of multiple coincident EPSC impulses at a given time (Kidd and Isaac, 2001). Temporal summation of individual EPSCs is a phenomenon whereby EPSCs quantitatively integrate when they occur in rapid succession to drive the potential at the neuronal membrane towards a depolarized state. Once a depolarizing threshold is reached by the cumulative action of enough EPSCs, other channels in the neuronal membrane are activated and cause an action potential to occur.

Because EPSC currents tend to be quantal the recorded traces could potentially be analyzed to differentiate between direct activation of a neurons peripheral process and EPSCs resulting from presynaptic neuron neurotransmitter release, but that would require a large number of neurons for comparison. Although EPSCs are quantal the kinetics of the current observed in a post-synaptic cell are influenced by the firing properties of the pre-synaptic cell as well as the amount of neurotransmitter released in addition to the complement of receptors on the post-synaptic cell (Takahashi et al., 1995) Though the type of recording undertaken herein had no precedent from which to glean what exactly the responses might look like, the differences in pre-synaptic cell physical characteristics and receptor expression facilitated distinct differences in response kinetics to different low-concentration agonist exposures.

While a recorded neuron whose peripheral process is directly activated by the peripheral application of agonists may show classic action potentials as the impulse conducted towards the soma would be from its own peripheral process, there may also be EPSCs resulting from synaptic transmission onto the soma from neighboring neurons' projections seen as well. While action potentials were seen in some neurons, the number of recordings obtained did not allow for statistical analysis to determine whether the action potentials were the result of direct activation of the peripheral processes or the summation of multiple EPSCs driving the membrane potential

past the threshold for action potential. The delineating characteristic between those neurons that may or may not be directly activated by the peripheral application of agonists might be the presence of overlapping EPSCs and action potentials. Those EPSCs that may occur in the midst of an action potential would be masked by the large current of the action potential, but action potentials resulting from temporal summation can show a characteristic inflection or hump in the depolarizing current. This inflection in the trace of the depolarizing current likely results from the arrival of one or more EPSCs due from synaptic transmission events. An example of such a hump indicating that an action potential might be driven by EPSC summation is presented in the following figure.

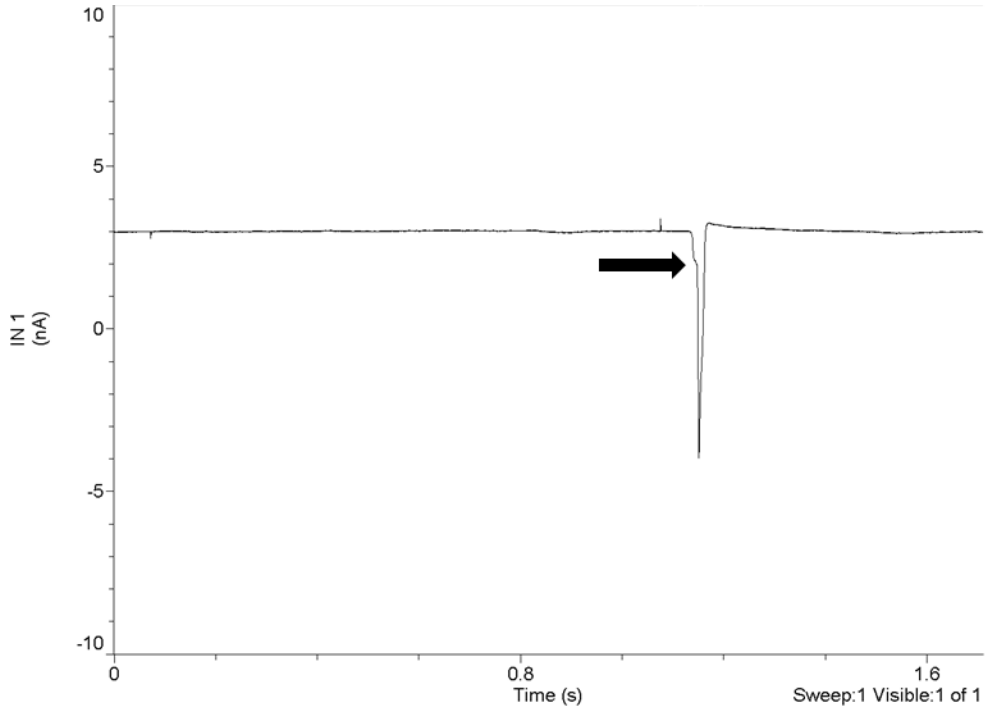

FIGURE 38: This figure depicts an action potential response observed from a recorded neuron that may be due to the temporal summation of multiple coincident EPSCs arriving in rapid succession. The characteristic depolarization, repolarization, and hyperpolarization currents of a classic action potential are brought about by the rapid arrival of a final EPSC at the cell body. The arrow shows the point of inflection in the depolarizing current indicating the arrival of the final EPSC and driving the cell into an action potential response instead of repolarizing and returning to baseline.

The arrival of the final EPSC impulse may have driven the membrane potential past the action potential threshold and caused an action potential to occur instead of the return to baseline that would be characteristic of an EPSC. The importance of the chemotransmissive origin of the signals reported herein should not be neglected. While a multitude of electrophysiological studies have evaluated EPSCs from various types of neurons in the context of electrode stimulation of presynaptic cells, or of fiber bundle activity in response to focal chemical exposure at one end of the fiber bundle, this method allows for characterization of EPSC responses in individual cells after focal, peripheral exposure of selective agonists in order to evaluate the influence of ligand-gated receptors along processes. This method could therefore be used to evaluate the effects of multisynaptic events on activity patterns of recorded neurons in response to complex trains of EPSCs arriving at a cell soma. This cell culture system also allows for the discrete co-culture of diverse cell types in discrete media compartments with defined components. The perfusion flow and bath counter-flow component facilitates focal application of ligands to ligand-binding ionotropic receptors to evaluate spatially constrained activation of receptors along neuronal processes and the overall influence on activity at the neuronal soma.

EQUIPMENT AND VALIDATION

The equipment used for electrophysiology including the following; a Nikon PSM-1120 microscope inside a Faraday cage on a Newport VW-3648-OPT floating table, an attached Nikon HB-10101AF mercury light, a Burleigh PCS-5000 micromanipulator mechanism, a Warner Instrument Co. VC-6 Channel Valve Control, a Warner Instrument Co. SF-77B Perfusion Fast-Step motor, an custom flow-pipe with gravity-fed perfusion system, a Brownlee Precision Model 440 Instrument Amplifier, a Hameg HM507 Analog/Digital Oscilloscope, an Axon Instruments

AXOPATCH 200A Integrated Patch Clamp Interface, an Axon CNS Molecular Devices DIGIDATA 1440A Data Acquisition System, pCLAMP Clampex software for acquisition and ClampFit software for analysis on a Dell Optiplex 745 desktop. All statistical tests run on the data acquired were performed in GraphPad Prism software. The bath solution was composed of 125mM $CsMeSO_3$, 15 mM CsCl, 5 mM Cs BAPTA, 10 mM HEPES, 3 mM $MgCL_2$, and 0.5 mM $CaCl_2$. Agonists for the iGluR families of receptors as well as for *TRPV1* were dissolved in the same solution as the bath for experimental recording sessions, except for the NMDA agonist and control solutions, which each contained only 0.5 mM Ca^{+2} and no Mg^{+2}. Capsaicin was dissolved in dimethyl sulfoxide (DMSO) instead of water prior to addition to the bath solution for recordings. Because of this the control solution that was introduced immediately prior to the application of capsaicin solution via the perfusion system contained the same concentration of DMSO, in order to mitigate any effect of DMSO itself on the responses recorded. Bath solution, without Ca^{+2} and Mg^{+2}, was heated to approximately 60 ˚C and placed into a vacuum chamber to degas for at least 30 minutes before preparing solutions. The agonist AMPA was used at a concentration of 200 μM. The Kainate receptor-specific agonist SYM-2081 was used at a concentration of 250 nM. The agonist NMDA was used at a concentration of 60 μM and in the NMDA solution the amino acid Glycine was also present at a concentration of 5 μM. The *TRPV1* specific agonist capsaicin was used at a comparatively higher concentration of 3 μM in order to ensure activation of *TRPV1* receptors present to identify putative nociceptive neurons. Relatively low concentrations of iGluR family-specific agonists were used to ensure that only cells expressing high levels of the receptors would respond while a higher concentration of capsaicin was used to ensure activation of processes from all *TRPV1* expressing neurons in the area of agonist application. Only neurons showing EPSCs in response to the peripheral application of capsaicin were included in the statistical analyses of response characteristics, in order to evaluate activity from only those neurons receiving nociceptive input.

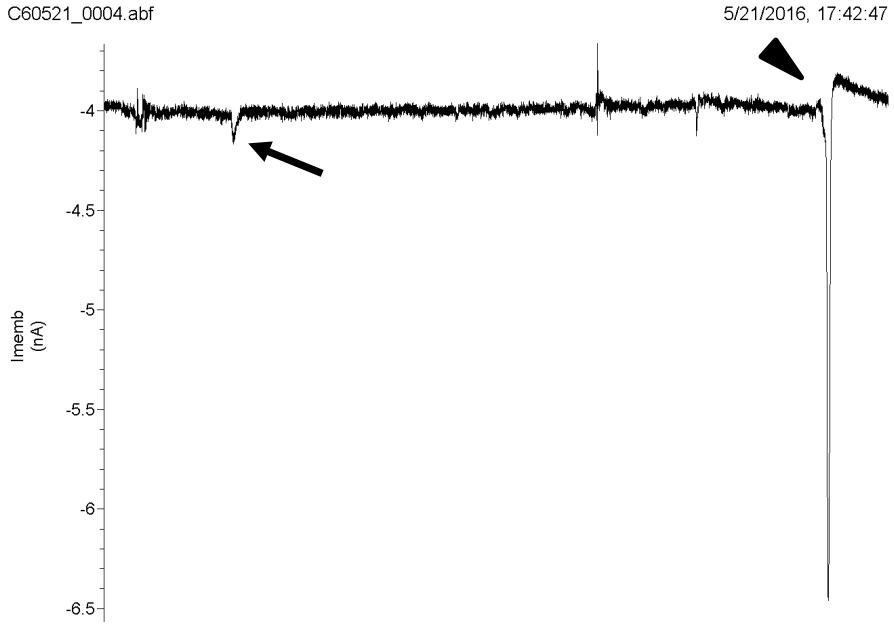

FIGURE 39: This figure displays the current trace recording from an individual neuron, designated according to the recording nomenclature as 60521C, showing both an EPSC and a classic action potential in response to peripheral exposure to AMPA. The current exhibited on the left, indicated by an arrow is characteristic of an EPSC in that it contains a sharp deflection away from zero and a quick, though logarithmic return to baseline. The action potential on the right, indicated by an arrowhead shows a sharp and large depolarization current, and repolarizing current that goes past the baseline yielding a hyperpolarizing effect.

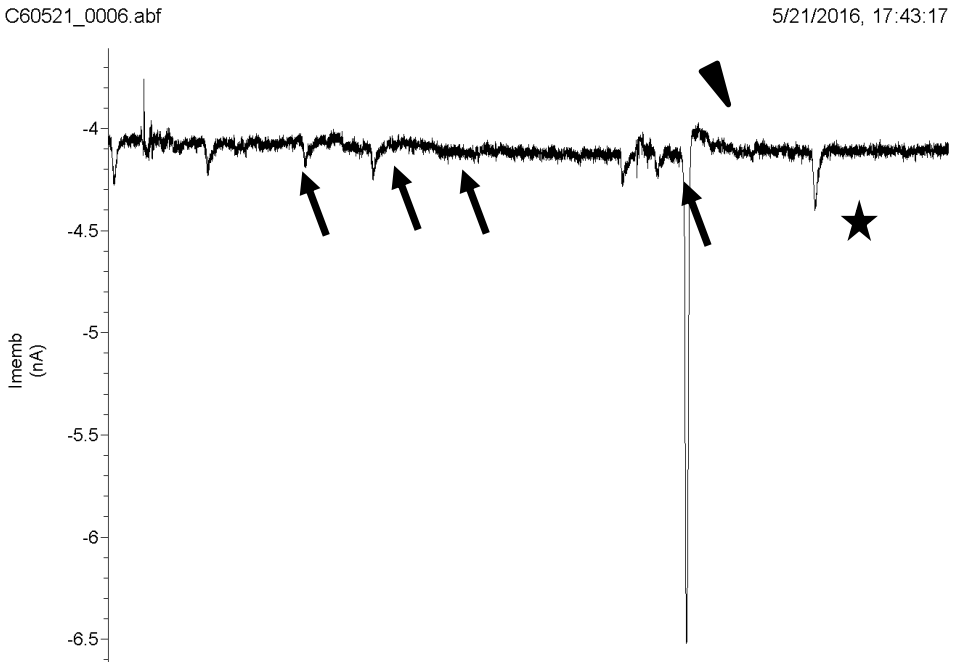

FIGURE 40: This figure portrays the currents recorded from neuron 60521C in response to peripheral application of the Kainate receptor-specific agonist, SYM 2081. Of note are the multiple EPSCs visible in the trace, indicated by arrows, in addition to the action potential, indicated by an arrowhead, seen in the right half of the recording. The quantal nature of EPSCs is evident by comparing the height of the EPSCs seen here. Those EPSCs occurring before the action potential are likely caused by individual synaptic transmission events. The EPSC seen after the action potential is indicated by a star and appears to be twice the height of the other EPSCs and therefore likely reflects the quantal nature of EPSCs and represents activity caused by two simultaneous synaptic transmission events.

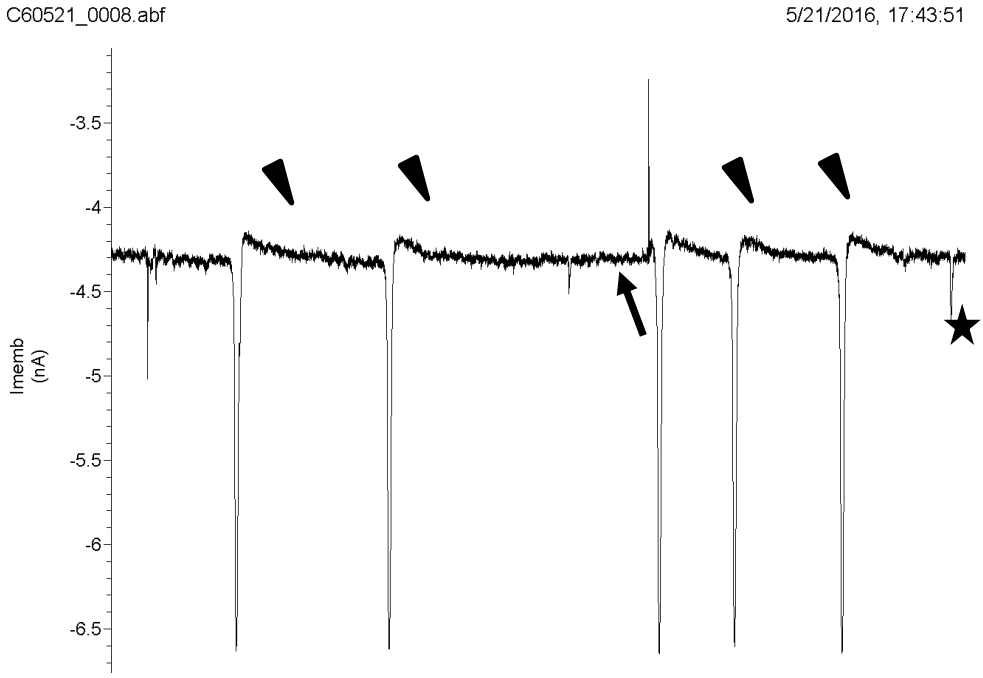

FIGURE 41: This figure displays the current traces recorded from neuron 60521C in response to peripheral application of NMDA. The first downward deflection and the individual upward deflection in the trace were caused by electrical noise from the stepper motor and valve control of the drug perfusion system. Individual EPSCs are seen in the central portion and on the far right of the recording, the EPSC in the center, indicated by an arrow, likely resulted from one synaptic transmission event, while the EPSC on the far right, indicated by a star, likely resulted from two coincident synaptic events. The five action potentials, indicated by arrowheads each display prominent and rapid depolarization and repolarization currents along with the characteristic after-hyperpolarization.

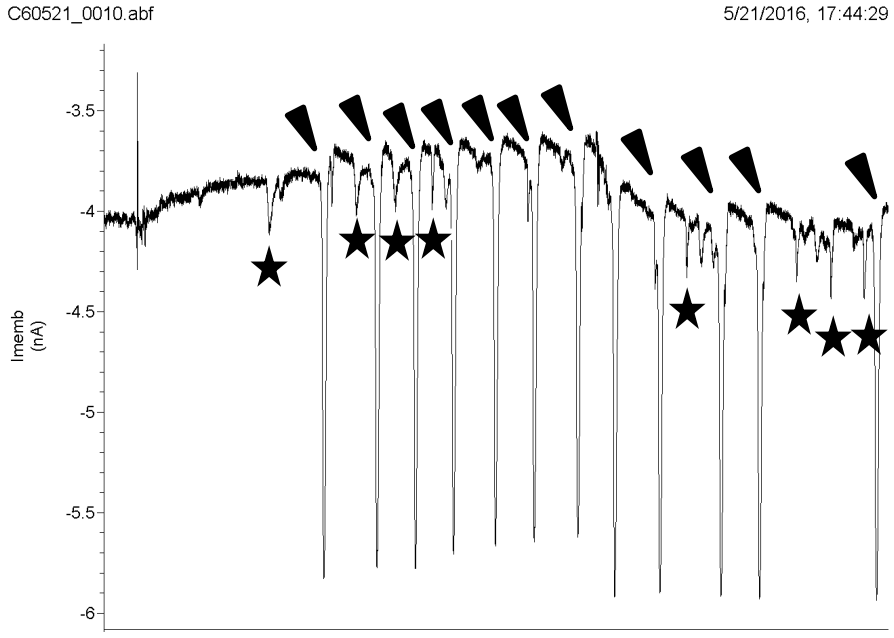

FIGURE 42: This figure depicts the current trace recorded from neuron 60521C in response to peripheral application of the TRPV1-selective agonist, capsaicin. Individual EPSCs, indicated by stars and likely resulting from synaptic transmission events, are seen in addition to the 12 action potentials likely resulting from direct activation of a peripheral process of the recorded neuron or summation from synapses from one or more presynaptic neurons. Each individual action potential displays a rapid depolarization and repolarization current, along with a characteristic after-hyerpolarization

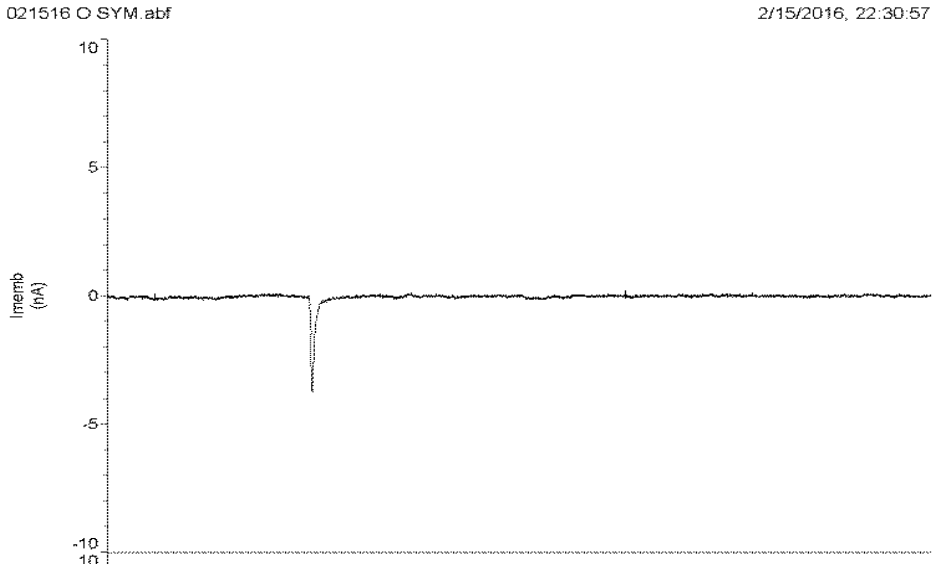

FIGURE 43: This figure shows a representative trace recording of current in a volt-clamped sensory neuron resembling an EPSC likely mediated by Kainate receptors in response to focal application of the Kainate receptor-specific agonist SYM 2081 along the peripheral processes extended underneath the central septum and into the keratinocyte compartment. The relatively simple logarithmic curve allowed for consistent isolation of response components across all recordings to characterize and compare them.

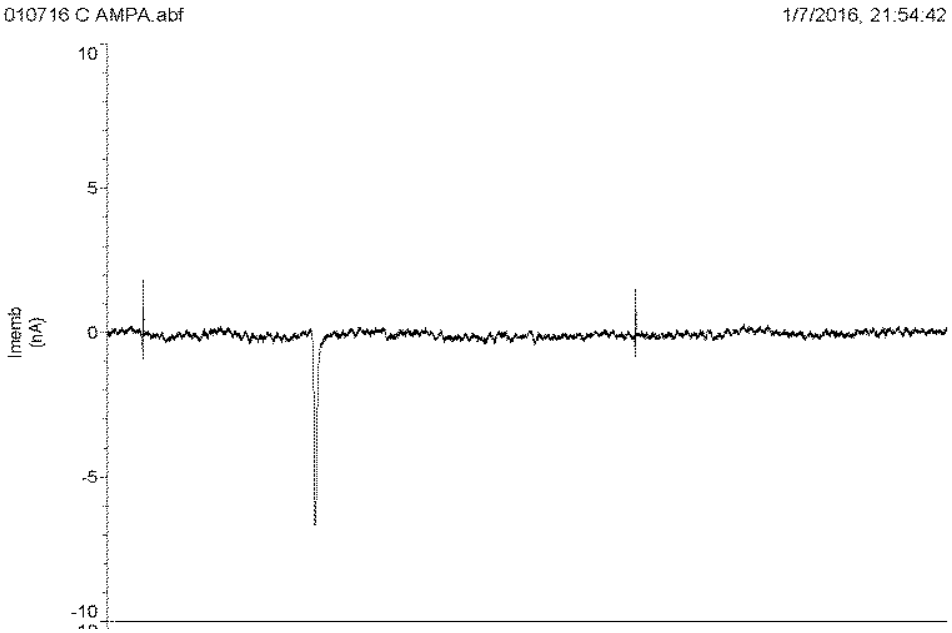

FIGURE 44: This figure shows a representative trace recording of current in a volt-clamped sensory neuron resembling an EPSC likely mediated by AMPA receptors in response to focal application of AMPA along the peripheral processes extended underneath the central septum and into the keratinocyte compartment.

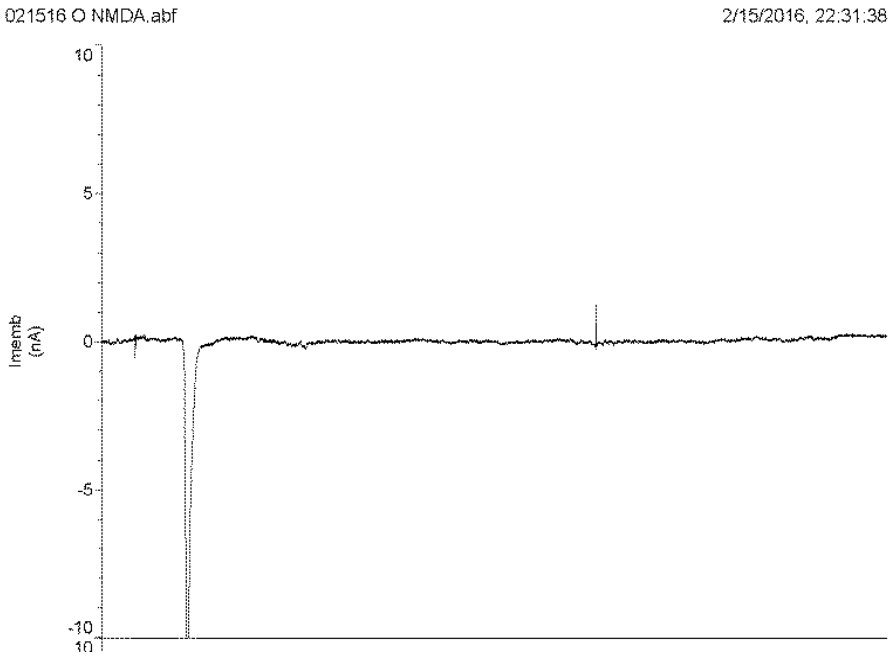

FIGURE 45: This figure shows a representative trace recording of current in a volt-clamped sensory neuron resembling an EPSC likely mediated by NMDA receptors in response to focal application of along the peripheral processes extended underneath the central septum and into the keratinocyte compartment.

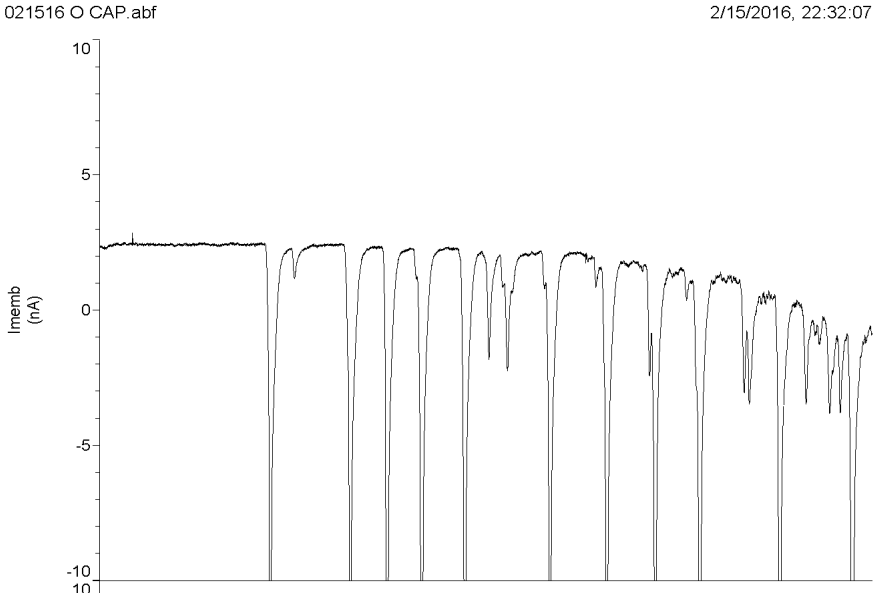

FIGURE 46: This figure shows a representative trace recording of current in a volt-clamped sensory neuron resembling an EPSC likely mediated by TRPV1 receptors in response to focal application of the TRPV1 receptor-specific agonist capsaicin along the peripheral processes extended underneath the central septum and into the keratinocyte compartment. These responses portray the differences in size of current resulting from the quantal summation of individual EPSCs. No classic action potential is present in this record, as is evident by the complete lack of after-hyperpolarization currents on any of the responses seen.

Different concentrations of selective agonists have been used in various different studies to evaluate the presence and activity of iGluRs in particular cells and subcellular compartments. Preliminary evaluations of family-specific iGluR agonists were performed to determine what concentrations consistently elicited responses while being effectively blocked by family-specific antagonists. Those lower concentrations were then used throughout the course of experimentation for the iGluRs. The *TRPV1* specific agonist capsaicin was used at a higher concentration in accordance with what is reported in literature and to more effectively saturate the receptors present. Using lower concentrations of family-selective agonists for the iGluRs decreases the likelihood of receptor cross-activation, where an agonist that is selective for one iGluR family at a low concentration when used at a higher concentration activate iGluRs from a different family. Decreasing the likelihood of cross-activation was important for the evaluation of family-specific iGluR presence and activity in peripheral processes because even if the number of fibers activated overall is decreased, the specificity of observed responses should be improved.

The importance of different activity patterns elicited by selective pharmacologic agents is that there may be some signal processing prior to the arrival of any signal reaching the somatic compartment. Signal processing has been shown to occur in dendritic processes via electrophysiological evaluation, wherein spatial and temporal summation of impulses may or may not cause the cumulative depolarization of a large process on which multiple dendritic processes may converge (Magee, 2000; London and Hausser, 2005). This signal processing is in contrast to the disproven phenomenon of dendritic filtering, whereby more distant synaptic sites would be less effective at eliciting depolarization of the somatic compartment than would more proximal synapses (Magee and Cook, 2000). While the distance of a dendritic synapse does not determine the proclivity to depolarize to the point of action potential threshold of the cell, the spatial restriction of receptor expression may cause an individual neuron to respond differently to exposures to the same agonist ad different sub-cellular compartments (Magee and Cook, 2000).

The differences in signaling observed via the method of compartmented co-culture may represent another means of evaluating complex signal integration from a stimulated set of neural processes. Multiple neurons may have processes in the same lane in this compartmented co-culture that express significant numbers of one of the iGluR families. Alternatively, a single neural process may contain substantial numbers of multiple iGluR families, and thereby be responsive to multiple types of selective agonists. Individual neurons are indeed capable of expressing active receptors from multiple iGluR families (Patneau and Mayer, 1991)

The EPSCs elicited from the cell being recorded, as well as the preceding neural activity of the cells synapsing on it, may differ slightly in the recordable kinetics observed due to the complement of receptors present. The dynamics of EPSCs displayed by the cell from which recordings were taken are dependent on the receptors present at a post-synaptic, but may also be influenced by the dynamics of the impulse transmitted by the pre-synaptic cell. The abundance of receptors, as well as their individual gating and current kinetics profiles influence the current activity ultimately displayed by the post-synaptic cell. The iGluR receptors desensitize rapidly, with Kainate receptors being the fastest to desensitize, followed by AMPA, and then NMDA receptors. The *TRPV1* receptor, however, desensitizes much more slowly. While the iGluRs desensitize on the order of milliseconds, *TRPV1* desensitizes over the course of approximately twenty seconds.

The cumulative effect of these differences in receptor expression levels and the kinetics of the currents that they permit, in combination with the agonist concentrations used, is that responses recorded from the post-synaptic cell appear with distinct characteristics in response to an extended agonist exposure along peripheral processes. Stimulation with family-specific iGluR agonists at the peripheral terminals of the neurons that did respond caused depolarization that propagated towards the central processes and somatic compartment on processes from fewer neurons than did stimulation with the *TRPV1* agonist, capsaicin. In addition to more cells being

responsive to exposure to capsaicin than to any of the family-specific iGluR agonists, the comparatively slower desensitization and relatively higher saturation of agonist increased the number of impulses received in train from a single agonist exposure sweep. While impulses elicited in response to a single second exposure to the family-specific iGluR agonists often arrived either individually or separated by hundreds of milliseconds, impulses elicited in response to capsaicin often caused multiple responses in rapid succession. The trains of responses to capsaicin were occasionally so rapid that the current was unable to completely return to baseline before the initiation of the next current impulse in the train. This pattern of activity would sometimes cause a change in the resting current in the cell even in the absence of rapid enough depolarizing currents to instigate a classic action potential.

DATA ANALYSIS

The three characteristics of the recorded responses analyzed were the latency to onset of impulse, the amplitude of current permitted, and curve fitted to the time course to return to baseline current. The latency to impulse and the amplitude of currents permitted as a part of the EPSCs did not statistically significantly differ between responses to different specific iGluR agonists as measured by ANOVA, however the curves fit to the time course to return to baseline after current impulses showed statistically significant differences between types of agonist exposures. Cells responding to capsaicin were included for comparison of the three different quantitative measures of elicited response kinetics. For those recordings in which multiple responses were seen, the first response was evaluated, so that only one current response was characterized from each one second agonist exposure. Responses from the different agonist exposures were individually evaluated and fit with a logarithmic curve in ClampFit. The characteristics of latency to

depolarization, amplitude of the response, and the τ constant were generated within ClampFit based upon the logarithmic curve fitted to the recorded current trace. Using the responses to capsaicin exposure and consequent *TRPV1* activation to define a population of putatively nociceptive neurons in the culture the responses to the family agonists showed statistically significant differences in their τ values, and a post-hoc Bonferroni test showed that there were statistically significant differences between both of the AMPA and Kainate, when compared to the NMDA responses.

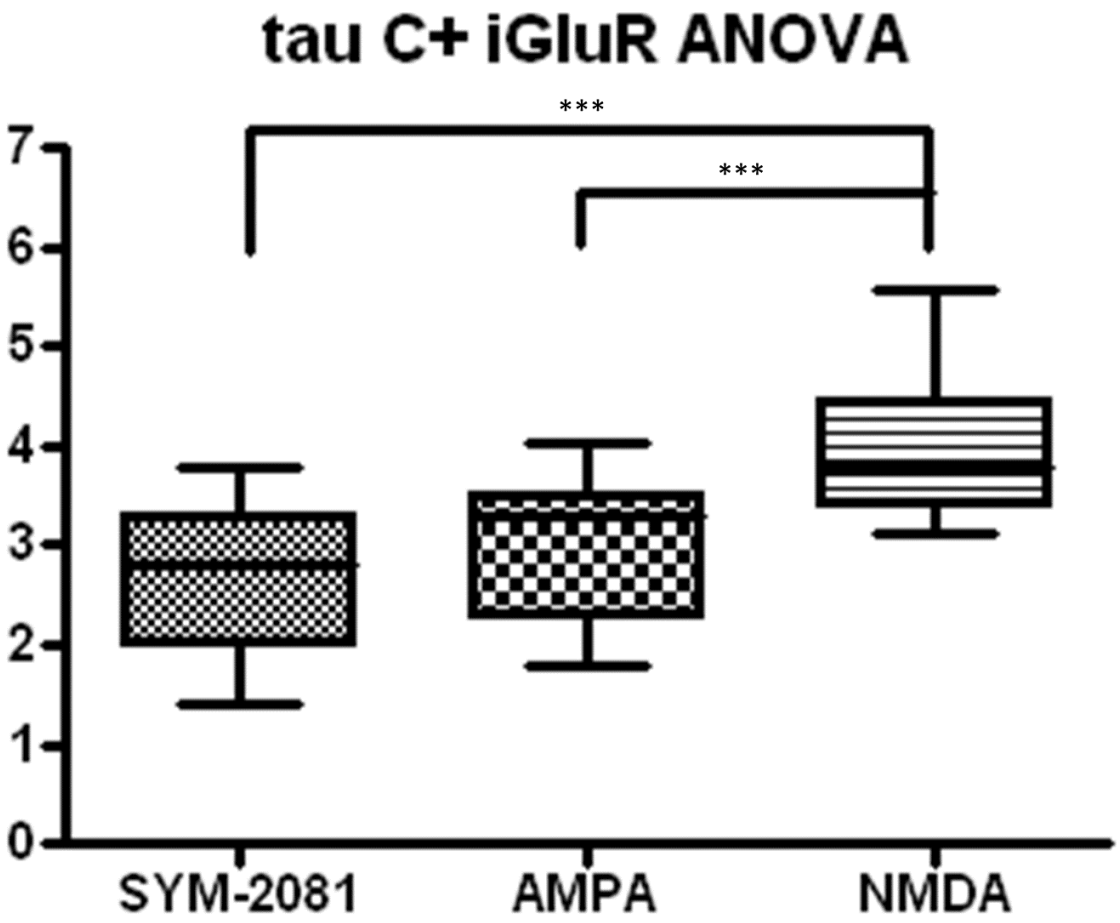

*FIGURE 47: This figure displays the graphical results of the ANOVA test run on the tau values generated from the curves fitted to first responses from all agonist iGluR agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. The mean is shown as a midline within the box plotted for each iGluR family-specific agonist, the box plotted for each agonist represents the 25th to 75th percentiles of values generated from the responses recorded. The bars extending above and below each box plotted represent the actual range of values within each category. A One-Way ANOVA showed statistically significant differences between categories ($p<0.0001$). A post-hoc Bonferroni test showed statistically significant differences between SYM-2081 vs. NMDA and AMPA vs. NMDA, as indicated by asterisks where $p<0.001$ is represented by ***.*

tau Capsaicin-responsive iGluR ANOVA				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	16.74			
R squared	0.3963			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	0.5227			
P value	0.77			
P value summary	Ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	16.33	2	8.166	
Residual (within columns)	24.88	51	0.4879	
Total	41.21	53		
Bonferroni's Multiple Comparison Test	Mean Diff.	T	P value	95% CI of diff
SYM-2081 vs AMPA	-0.3308	1.381	P > 0.05	-0.9239 to 0.2622
SYM-2081 vs NMDA	-1.271	5.518	P < 0.001	-1.842 to -0.7009
AMPA vs NMDA	-0.9405	4.082	P < 0.001	-1.511 to -0.3701

Table 1: The above table displays the numerical results of the ANOVA test run on the tau values generated from the curves fitted to first responses from all agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. In addition to the ANOVA test a post-hoc Bonferroni test was performed to evaluate whether the individual response values from each category significantly differed from the other respective categories.

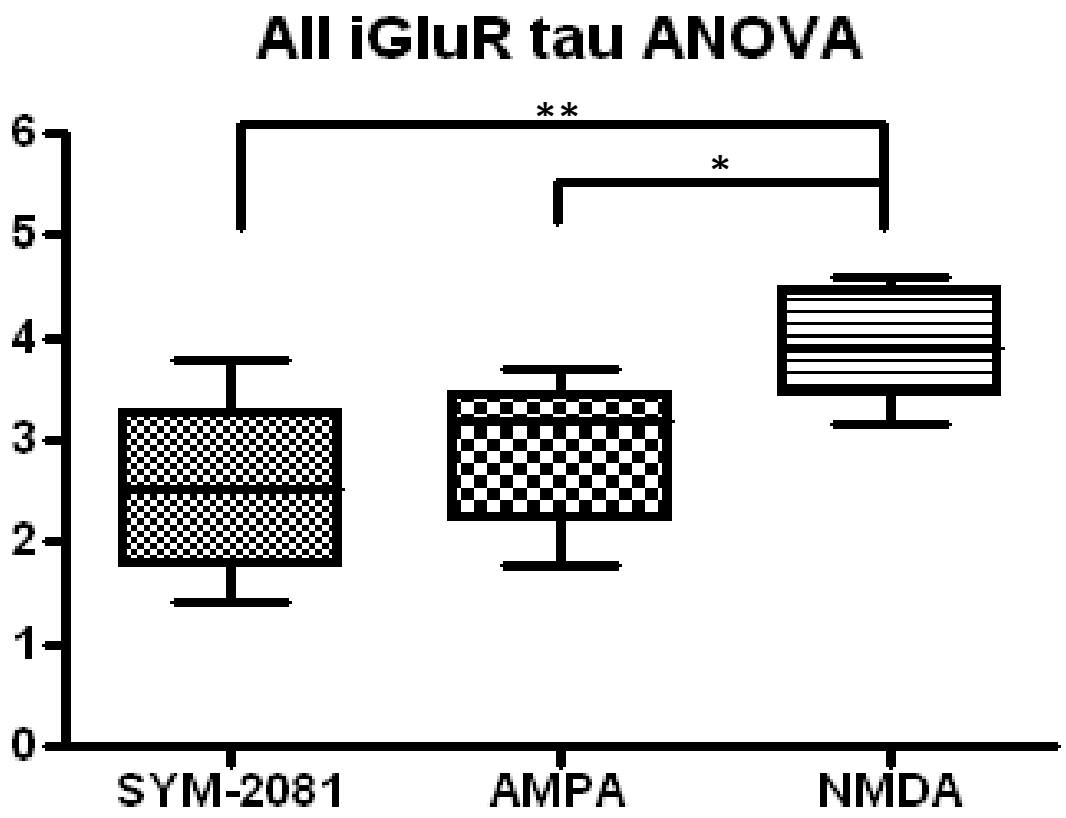

*FIGURE 48: This figure displays the graphical results of the ANOVA test run on the τ values (measured in milliseconds) generated from the curves fitted to first responses per agonist application for those neurons patched that responded to ALL of the iGluR family-specific agonists as well as the TRPV1-specific agonist, capsaicin. The mean is shown as a midline within the box plotted for each iGluR family-specific agonist, the box plotted for each agonist represents the 25th to 75th percentiles of values generated from the responses recorded. The bars extending from each box plotted represent the full range of values within each category. A One-Way ANOVA showed statistically significant differences between categories (p=0.0031; F-Value 7.696). A post-hoc Bonferroni test showed statistically significant differences between SYM-2081 vs. NMDA, and AMPA vs. NMDA, as indicated by asterisks where p<0.01 is represented by ** and p<0.05 is represented by *.*

total iGluR TAU ANOVA				
One-way analysis of variance				
P value	0.0031			
P value summary	**			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	3			
F	7.696			
R squared	0.423			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	1.331			
P value	0.5141			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	8.333	2	4.166	
Residual (within columns)	11.37	21	0.5414	
Total	19.7	23		
Bonferroni's Multiple Comparison Test	Mean Diff.	T	P value	95% CI of diff
SYM-2081 vs AMPA	-0.368	1	P > 0.05	-1.325 to 0.5890
SYM-2081 vs NMDA	-1.393	3.786	P < 0.01	-2.350 to -0.4356
AMPA vs NMDA	-1.025	2.785	P < 0.05	-1.982 to -0.06766

Table 2: This table displays the numerical results of the ANOVA test run on the tau values generated from the curves fitted to first responses from all agonist applications for those neurons patched that responded to ALL of the iGluR family-specific agonists as well as the TRPV1-specific agonist, capsaicin. In addition to the ANOVA test a post-hoc Bonferroni test was performed to evaluate whether the individual response values from each category significantly differed from the other respective categories.

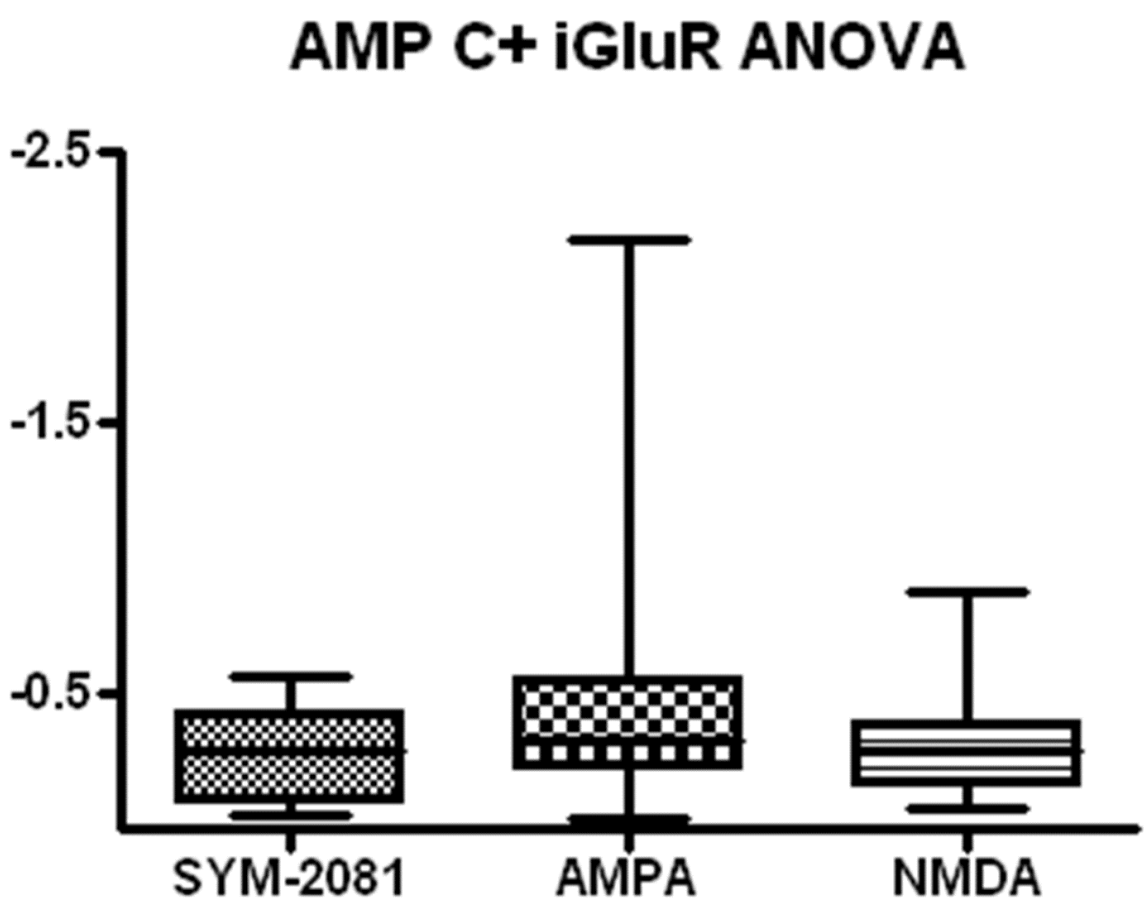

FIGURE 49: This figure displays the graphical results of the ANOVA test run on the maximum amplitude values generated from the curves fitted to first responses from all agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. The mean is shown as a midline within the box plotted for each iGluR family-specific agonist, the box plotted for each agonist represents 25th to 75th percentiles of values generated from the responses recorded. The bars extending above and below each box plotted represents the actual range of values within each category.

AMP C+ iGluR ANOVA				
One-way analysis of variance				
P value	0.1402			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	2.042			
R squared	0.07415			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	22.11			
P value	P<0.0001			
P value summary	***			
Do the variances differ signif. (P< 0.05)	Yes			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	0.4032	2	0.2016	
Residual (within columns)	5.034	51	0.0987	
Total	5.437	53		
Bonferroni's Multiple Comparison Test	Mean Diff.	T	P value	95% CI of diff
SYM-2081 vs AMPA	0.1973	1.831	P > 0.05	-0.06944 to 0.4641
SYM-2081 vs NMDA	0.02292	0.2212	P > 0.05	-0.2336 to 0.2795
AMPA vs NMDA	-0.1744	1.683	P > 0.05	-0.4309 to 0.08216

Table 3: This table displays the numerical results of the ANOVA test run on the maximum amplitude values generated from the curves fitted to first responses from all agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. In addition to the ANOVA test a post-hoc Bonferroni test was performed to evaluate whether the individual response values from each category significantly differed from the other respective categories.

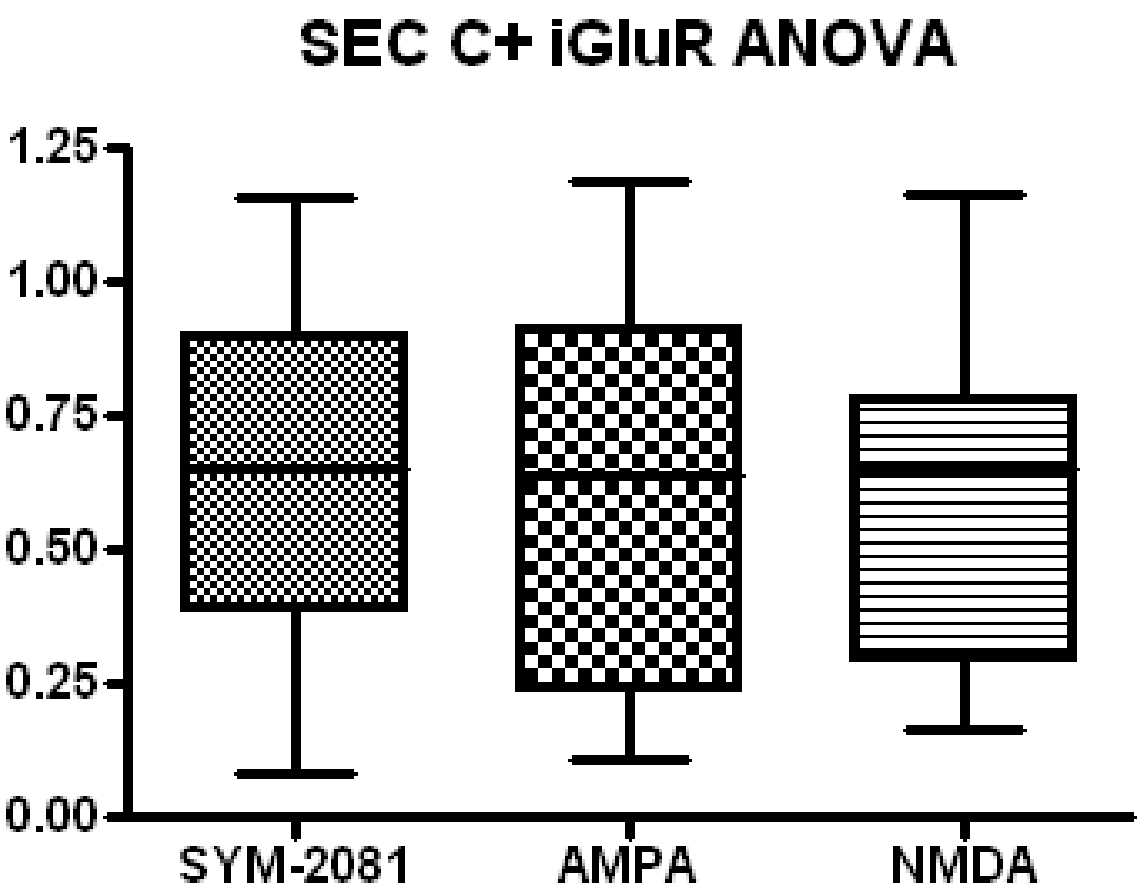

FIGURE 50: This figure displays the graphical results of the ANOVA test run on the latency to the first responses from all agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. The mean is shown as a midline within the box plotted for each iGluR family-specific agonist, the box plotted for each agonist represents the 25th to 75th percentiles of values generated from the responses recorded. The bars extending above and below each box plotted represents the actual range of values within each category.

SEC C+ iGluR ANOVA				
One-way analysis of variance				
P value	0.9703			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	0.03018			
R squared	0.001182			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	0.3255			
P value	0.8498			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	Df	MS	
Treatment (between columns)	0.006403	2	0.003201	
Residual (within columns)	5.41	51	0.1061	
Total	5.416	53		
Bonferroni's Multiple Comparison Test	Mean Diff.	T	P value	95% CI of diff
SYM-2081 vs AMPA	0.02383	0.2133	P > 0.05	-0.2527 to 0.3004
SYM-2081 vs NMDA	0.0231	0.215	P > 0.05	-0.2429 to 0.2891
AMPA vs NMDA	-0.000728	0.006776	P > 0.05	-0.2667 to 0.2652

Table 4: This table displays the numerical results of the ANOVA test run on the latency to the first responses from all agonist applications for those neurons patched that showed current impulses in response to the TRPV1-specific agonist, capsaicin. In addition to the ANOVA test a post-hoc Bonferroni test was performed to evaluate whether the individual response values from each category significantly differed from the other respective categories.

It is important to note that the differences between response categories that were recorded from a single cell are between categories of EPSC responses of a post-synaptic cell instigated by a pre-synaptic cell and therefore are not necessarily mediated by iGluR family receptors on the post-synaptic cell. The pre-synaptic cell is the neuron responsible for the chemical instigation of activity at peripheral processes on the distal side of the barrier, as well as the propagation of current impulses across the barrier from the keratinocyte compartment into the neuronal compartment.

SIGNIFICANCE AND RATIONALE

The characteristic differences in the τ values seen as statistically significant between the responses to different iGluR family agonists were likely due to the activity in the presynaptic neuron being mediated mostly by the activation of individual iGluR families of receptors. It is likely that those distal processes of the presynaptic cell that were activated by the Kainate, AMPA, or NMDA specific agonists expressed those same receptors at a high level and influenced the characteristics of EPSCs in the post-synaptic cell being recorded. The relatively low concentration of family-specific iGluR agonists as compared with the more saturating concentration of the *TRPV1*-specific agonist, capsaicin, may have influenced the number of cells responding to the iGluR vs. *TRPV1* agonists as well as some of the variability of the characteristics compared between categories of elicited responses. For example, the τ values within responses to application of capsaicin may have exhibited higher variability than the other agonist-elicited responses because a more diverse population of presynaptic neurons expressing *any TRPV1* receptors along a peripheral process was activated than within each population of iGluR-responding neurons. A higher number of cells may have responded to *TRPV1* than the

iGluR family-specific agonists not because the presynaptic cells acting on them had *TRPV1* without iGluR expression, but because the concentration of *TRPV1* being perfused was closer to saturating the receptors present on the peripheral processes exposed to agonist perfusion. Differential co-expression of iGluRs within the population of neurons expressing *TRPV1* may indicate some selective patterned activation corresponding to different neuronal types. Different neuronal types co-expressing *TRPV1* and different iGLURs could facilitate unique activity patterns for neurons belonging to different characteristic populations and functional modalities. Continuing the use of compartmented clamping of DRG neurons may facilitate the identification of more categories of response types to be identified via statistical analyses of their respective kinetic properties. Combining this with an increased number of receptor-specific agonists may facilitate the identification of correlations between iGluR expression patterns and individual sensory neuron modalities. Continuing the use of compartmented co-culture and counter-flow clamping with a broader array of specific agonists could show which specific compose the receptors present in the peripheral processes of sensory neurons that contribute to signaling and could therefore present potential targets for pharmacologic intervention for the treatment of pain. Continuing counter-flow clamping with other neuronal types may show the influence of receptors in different sub-cellular compartments, to better understand how chemically instigated signals along processes influence activity at the neuronal soma.

CHAPTER V

CONCLUSION

Evaluation of the composition and contribution to activity of the receptors present and active at different segments of the neurons may lead to new avenues for chronic pain treatment, in addition to expanding the understanding of the normal functioning of sensory neurons. While the purely scientific implications of better understanding the spatial relationships between receptor distribution, agonist exposure, and elicited electrochemical activity are important in their own right, the economic and societal costs of chronic pain bolster the necessity for discovering new targets for treatment of chronic pain. New treatments may augment or replace some of the tools already widely employed to manage chronic pain, but careful consideration must be given to the mechanisms by which those treatments work in order to reduce the potential number of complications and detrimental side-effects experienced by those already in pain. One important component relevant to pursuing new avenues of pain treatment is a more complete and detailed understanding of the multiple aspects of signaling exhibited by first order sensory neurons in the dorsal root ganglion. These neurons are of a pseudounipolar morphology, which distinguishes them from other neuronal cell types and may affect their signaling characteristics, including the

potential for orthodromic as well as antidromic impulse propagation and some filtering due to impedance mismatch that can occur at the T-junction (Debanne, 2004). The potential impedance mismatch is due to the differences in conductance of the peripheral and central branches of the process attached to the neuronal soma (Sundt et al., 2015). While the impedance mismatch should decrease the number of action potential impulses experienced by the neuronal somata, the increase in firing rates that occurs in the instance of chronic pain could still cause an increase in the number of action potentials reaching the somata even as far more action potentials might fail to pass the T-junction (Debanne, 2004).

Substantial evidence has been gathered as to the sensitivity of sensory neurons to specific agonists and antagonists at the soma and central processes, along with description of the firing characteristics of these neurons and their aggregate bundles bidirectionally, in an orthodromic or antidromic direction. Evidence for electrochemical activity instigated in the peripheral processes by receptor-specific agonists, and specific identification of the receptor subunits present in the peripheral processes has not yet been published. Using electrophysiological techniques adapted to a compartmented co-culture system simulating an innervated skin tissue facilitates the characterization of the sensory neuron somatic electrochemical activity instigated by peripheral application of specific agonists. Using chemicals that display a high degree of selectivity as agonists for one family of receptors over another facilitates an evaluation and characterization of the types of signals experienced by a post-synaptic cell from pre-synaptic cells expressing functional ligand-binding receptors of multiple types. Agonist application restricted to a distal subcellular compartment such as a peripheral process of a sensory neuron instigates a current impulse that propagates along the process towards the cell body residing in a neuronal compartment. Upon reaching the neuronal compartment a current impulse within a neuron can cause synaptic transmission onto post-synaptic cells. Voltage-clamping a post-synaptic neuronal cell body in the neuronal compartment enables the recording of the EPSC impulses exhibited by

the cell due to synaptic transmission from pre-synaptic neurons after the stimulation of their respective peripheral processes with exposure to various agonists restricted to the distal compartment. This system provides for the analysis of individual and summed EPSCs, action potentials, and multisynaptic events from sources more physiologically relevant than electrode-stimulated presynaptic cells. Although the cells are dissociated and have been in culture for an extended period, the focal application of agonists on peripheral process, instead of direct depolarization with an electrode, represents a stimulation that more faithfully replicates the chemotransmission that takes place to instigate electrochemical phenomena in sensory neurons.

One unique characteristic that facilitated the development of this compartmented co-culture is the fact that primary sensory DRG neurons are able to attach to, survive on, and grow projections across a poly-D-lysine substrate. While keratinocytes are capable of attaching to, surviving on, and even clonally expanding over a poly-D-lysine substrate, they are effectively unable to migrate across it. The relevant and convenient effect of this is that keratinocytes or keratinocyte stem cells plated in one delineated or compartmented area of a poly-D-lysine substrate are effectively incapable of escaping from the area in which they are plated. Because of the differences in migration capability between these types of cells, plated keratinocyte stem cells are free to clonally expand in the compartment in which they are plated while plated DRG sensory neurons in the neuronal compartment are able to extend projections that may penetrate the gelatinous barrier separating the two compartments and interact with the keratinocyte stem cells in the distal compartment. The interaction of the cells in co-culture while maintaining their spatial segregation effectively simulates a sort of innervated skin tissue *in vitro*. The process of compartmented co-culture clamping allows for the evaluation of various characteristics of the peripheral terminals in a system where they can be more easily isolated and individually analyzed for specific characteristics, since classical immunohistochemical and electrophysiological

techniques may have their respective pitfalls or incompatibilities with the various types and spatial relationships of cells and extracellular components present in such tissues.

The delineation of cell types between compartments because of the differences in migration capability over the substrate persists throughout the duration of a long term culture. The maintenance of that delineation allows for the keratinocyte stem cells to expand within their compartment and across the lanes in which they originally settle to form confluent monolayers of cells, as well as for neurons to extend a substantial number of processes, some of which then penetrate into the keratinocyte compartment to richly innervate each available lane etched into the culture substrate.

The application of the *TRPV1*-specific agonist, capsaicin, was used to electrophysiologically identify neurons receiving nociceptive input from at least one pre-synaptic cell innervating the distal compartment. For compartmented co-culture clamping, neurons that possessed projections extending into the keratinocyte compartment were stimulated along their peripheral processes by focal application of agonists within the keratinocyte compartment via a perfusion and bath counter-flow system. The constant application of the bath solution into the neuronal compartment and its constant removal from the keratinocyte compartment created a consistent counter-flow of bath solution from the neuronal compartment under the central septum of the Campenot chamber divider. The introduction of the bath solution into the neuronal compartment occurred at a rate of approximately 2 ml/min while the introduction of agonists into the keratinocyte compartment occurred at a rate of approximately 0.4 ml/min. Because the flow of the bath solution from the neuron compartment into the keratinocyte compartment occurs at a much greater flow rate than the introduction of agonist there is no flow of the agonist solution from the perfusion system that reaches the neuronal compartment, meaning that any elicited activity results either from spontaneous discharge, synaptic activity from the neuronal compartment, or from peripheral agonist activation of receptors along the peripheral processes

extending into the keratinocyte compartment. Spontaneous discharge and synaptic activity from within the neuronal compartment are possible, and could be mediated by neurotransmitter release from the neurons themselves or from the glial cells present in the neuronal compartment. The introduction of bath solution contributing to the maintenance of a unidirectional counter-flow from the neuronal compartment effectively and constantly diluted the solution within that compartment minimized the probability of erroneous paracrine activation of receptors. The difference in the activity pattern elicited in response to capsaicin also demonstrated that the activity observed was unlikely to originate from paracrine signaling within the neuronal compartment itself. It could be possible that individual EPSCs in response to glutamate receptor agonists could be interpreted as arising from paracrine signaling within the neuronal compartment. The trains of EPSCs elicited in response to the distal application of capsaicin during a single second exposure demonstrated that not only were responses generated due to the focal peripheral application of agonists instead of paracrine signaling, but that the exposure to agonists at a concentration capable of instigating activity was maintained only during the brief window of application of agonist solution via the perfusion system into the keratinocyte compartment along the peripheral processes of neurons.

Determining the receptors responsible for activation of peripheral processes, as well as the overall contribution to the characteristics of the signals generated, may facilitate the characterization of signal processing that occurs prior to the arrival of current impulses at a somatic compartment. Better understanding the relationships of the spatial characteristics of individual neurons, the subcellular distribution of the ligand-binding receptors responsible for the instigation of their activity, and the other neurons on which they may synapse will be integral to better understanding and modeling neuronal circuitry. Better understanding and modeling sensory neuron circuitry will be indispensable in engineering more complex and adaptable sensor systems for various purposes. Increases in the efficiency, sensitivity, and adaptability of sensor systems may have

implications in areas ranging from environmental monitoring sensors, to complex feedback systems, to integrating novel biomechanical augmentations with existing neuronal networks.

Furthering the understanding of the initial step in pain transmission is significant, but it is not the only important consequence of the development of compartmented co-culture clamping. The *in vitro* model system of an innervated tissue because of the compartmented co-culture, and the evaluation of peripheral and axonal ligand-gated ion channel activation yield a means of evaluating somatic activity from peripheral process receptor activation. This *in vitro* system thereby facilitates the study of excitability of the peripheral processes of sensory neurons in the context of isolated peripheral inflammatory mediator exposure or pharmacologic intervention. Compartmented Co-Culture Clamping thereby facilitates a reliable assay to test novel pharmaceuticals to evaluate their efficacy in a simulated innervated tissue context. This system comprises, therefore, an intermediary research component in between validation of ligand binding and receptor activation and systemic administration and evaluation of behavioral responses. By evaluating potential effects of novel pharmacologic agents on the peripheral processes and terminals of sensory neurons in a simulated skin tissue, the throughput for testing novel compounds may be increased and the necessity of subjecting research animals to experimental treatments may be reduced. This implementation of this system will help to facilitate study of the initial steps of pain transmission without having to subject intact animals to experimental treatments or induction of disease models.

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VITA

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