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Modelling chronic pain in the F11 cell line

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Declaration

I declare that the work presented in this thesis has not been submitted for any degree or diploma at this, or any other university and that the work described herein is my own with the following exceptions:

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Abstract

The current study addressed the effects of the cytokine *tumor necrosis factor alpha* (TNF-α) on the *dorsal root ganglion* (DRG) derived F11 cell line. In both humans and rodents, increased expression of TNF-α is found in states of inflammatory and neuropathic chronic pain. TNF-α's pain eliciting effects are mediated by its ability to sensitize DRG sensory neurons by upregulation and sensitization of a variety of ion channels and receptors. Research on pain and the development of novel analgesics may be accelerated by the use of immortal cell lines that resemble natural DRG neurons in their functioning and their response to inflammatory mediators. In DRG neurons extracted from animals, TNF-α treatment alters a variety of processes commonly implicated in chronic pain. It increases calcium influxes induced by pain eliciting agonists and it increases spontaneous firing and agonist induced firing. TNF-α also induces apoptosis in DRG neurons and alters neuritogenesis. However, the effects of TNF-α on the DRG-derived F11 cell line remain to be elucidated. Therefore, the current study analysed TNF-α's effect on four end points in undifferentiated and forskolin differentiated F11 cells. Firstly, cell viability was measured by MTT assays. Secondly, neurite outgrowth was measured in microscopic images using ImageJ and NeuronJ. Thirdly, fluorescent Ca2+ imaging using Fluo-4 was performed to measure Ca2+ influxes in response to ATP, bradykinin and capsaicin. Lastly, a multi-electrode array was used to measure baseline spiking activity and spiking activity in response to ATP, bradykinin and capsaicin.

In the current study, TNF-α treatment did not significantly alter cell viability, spiking activity and agonist induced Ca2+ influx. TNF-α did significantly reduce neurite outgrowth, but this effect was inconsistent. The current results suggest that the F11

cell line may not lend itself as an appropriate model in studying the underlying mechanisms of TNF-α mediated neuropathic and inflammatory pain. However, future studies are needed to replicate the current results.

List of commonly used abbreviations

Tumor necrosis factor alpha (TNF-α)

Tumor necrosis factor receptor 1 (TNFR1)

Tumor necrosis factor receptor 2 (TNFR2)

Dorsal root ganglion (DRG)

Transient receptor potential cation channel subfamily V member 1 (TRPV1)

Chronic constriction injury (CCI)

Lipopolysaccharide (LPS)

Protein kinase A (PKA)

Protein kinase C (PKC)

Extracellular signal-regulated kinases (ERK)

Ribonucleic acid (RNA)

Adenosine triphosphate (ATP)

C-Jun N-terminal kinases (JNK)

Mitogen-activated protein kinase (MAPK)

Nerve growth factor (NGF)

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Introduction

1.1 SENSORY PROCESSING

The human body has 31 ‘mixed’ nerves originating from the spinal cord. These nerves are responsible for the transmission of both sensory input and motor output between the spinal cord and the periphery (Ahimsadasan & Kumar, 2018). Depending on what level in the spinal cord they originate from, spinal nerves are called thoracic, sacral, coccygeal or cervical. These nerves, which emerge from the intervertebral foramen, are comprised of a dorsal root consisting of afferent sensory neurons, and a ventral root consisting of efferent motor neurons. As the dorsal root emerges from the spinal cord, it travels to the dorsal root ganglion (DRG), which marks the location of the majority of the body’s sensory neurons (Purves et al. 2001). These neurons play an important role in the transduction, conduction and transmission of neural responses related to both noxious and innocuous stimuli, and they are responsible for nociception (perception of harmful stimuli), mechanoreception (e.g. perception of pressure) and proprioception (perception of body position). The different functions are performed by sensory neurons of varying morphology (Steeds, 2009). Mechanoreception and proprioception are performed by low threshold sensory neurons, which have large cell bodies connected to myelinated Aα and Aβ fibers. On the other hand, nociception is carried out by high threshold sensory neurons with small cell bodies, which are connected to either Aδ fibers with some degree of myelination or C fibers which lack myelination. C fibers make up the majority of nociceptors. Due to their lack of myelination, they have slow conduction

velocity and cause a dull, burning pain. Myelinated Aδ fibers on the other hand, have high conduction velocity and their activation causes a sharp, well-localized pain. Nociception can be classified as visceral or somatic (Yam et al., 2018). Somatic nociception is generally well-localized and is a result of peripheral nociceptor activation (e.g. in the skin) without damage to the central nervous system (CNS) or peripheral nerves. Visceral nociception, is poorly localized and is a result of activation of nociceptors of pelvic, abdominal or thoracic viscera.

The nerve endings of nociceptors are free nerve endings that innervate peripheral tissues and express a variety of receptor ion channels that respond to stimuli of noxious intensity such as heat, acid or mechanical stimuli (Figure 1). The activation of these ion channels leads to an influx of ions, which depolarizes nociceptors and can initiate action potentials to be sent down their axons (Dubin and Patapoutian, 2010). Moreover, ATP released from damaged cells may also activate nociceptors. The process of detecting noxious stimuli and damage to cells is known as *transduction*.

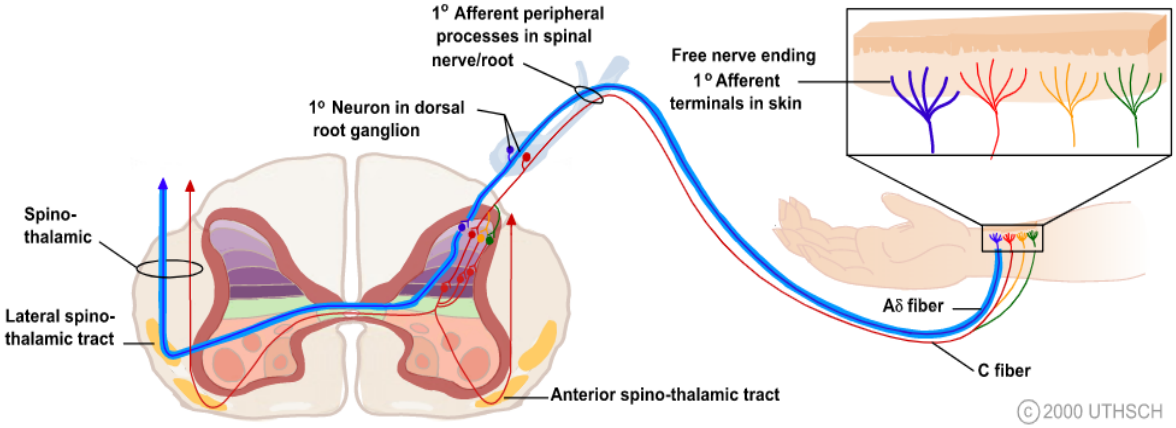


Figure 1 (Nba.uth.tmc.edu, 2019). Nociceptors have free nerve endings with ion channels responsive to noxious stimuli. When these channels are activated, the influx of ions depolarizes these first-order neurons and causes action potentials to propagate towards the spinal cord, where first-order neurons synapse onto second-order

neurons that transmit the signal further towards the brain. However, the DRG can act to enhance or block propagation of action potentials before they reach the spinal cord.

Nociceptors are called first-order neurons, because they function at the very start of sensory processing. They are also pseudo-unipolar, meaning that instead of having one axon and one dendrite, they have one axon that is separated into two branches by a T-junction. The distal branch extends from the periphery towards the DRG, while the proximal branch extends from the DRG into the spinal cord. If the activation threshold is reached, a pain signal in the form of action potentials propagates from peripheral tissues towards the spinal cord, a process known as *conduction*. However, before reaching the spinal cord, the DRG can act to block, decrease or enhance propagation of action potentials (Krames, 2014).

When the pain signal reaches the dorsal horn of the spinal cord, synaptic *transmission* passes the signal on to second-order neurons. In the case of Aδ fibers, this process occurs in laminae I and V of the spinal cord, and is facilitated mostly by glutamate binding to NMDA receptors (Steeds, 2009). For most C fibers, transmission occurs in lamina II and is facilitated by sensory neuropeptides such as Substance P, which binds to neurokinin (NK-1) receptors.

The second order neurons then propagate the pain signal towards the brain through ascending neural pathways such as the spinothalamic and spinoreticular tracts (Yam et al, 2018). When the signal reaches the thalamus, third order neurons propagate the signal further towards the somatosensory cortex, where the signal may or may not be interpreted as pain. The distinction between nociception and the experience of pain is therefore an important one. While nociception describes the transduction, conduction and transmission of noxious stimuli, the experience of pain is more complex and is

the result of several systems. For example, high order cognitive systems such as the amygdala are capable of influencing the perception of pain (Seno et al, 2018), which helps explain why pain perception varies between people and can be mood-dependent.

Nociception itself is also modulated in several ways. For example, descending inhibitory tracts, which are abundant with endogenous opioids, can block nociception in the spinal cord by inhibiting substance P (Ossipov, 2010). Similarly, interneurons of the substantia gelatonisa can inhibit synaptic transmission between first and second order neurons in the spinal cord (Wall, 1980).

In addition to short-term mechanisms that modulate pain processing and nociception, there are numerous mechanisms that modulate nociception and pain perception in the long-term. As the next section will discuss, first-order sensory neurons plays an important role in long-term pain modulation.

1.2 CHRONIC PAIN

In healthy people, the perception of pain is adaptive in that it promotes recovery and teaches us which stimuli and behaviors lead to harm. However, in certain cases pain can be chronic or pain can persist even after nerve or tissue damage has been resolved. Chronic pain can be inflammatory or neuropathic in nature (Xu, Yaksh, 2011). Chronic inflammatory pain either directly or indirectly relates to tissue damage and the consequent inflammation process, while neuropathic pain relates to damage to central or peripheral neurons. However, neuropathic pain has been found to have inflammatory elements, while inflammation can result in nerve damage, making the distinction less clear cut than was previously thought (Sommer, Leinders and Üçeyler, 2017).

Nonetheless, both types of chronic pain are characterized by an intensification of pain response (*hyperalgesia*) and the presence of pain response to stimuli that are normally harmless (*allodynia*). Examples of conditions characterized by chronic pain include *complex regional pain syndrome* (CRPS), rheumatoid arthritis and multiple sclerosis (MS). Chronic pain is a current healthcare crisis, and it is estimated that 19% of adult Europeans experience moderate to severe chronic pain, greatly affecting the quality of their working and social lives (Breivik et al., 2006).

While the origin of chronic pain is complex and involves multiple physiological systems, one important factor in its aetiology is the sensitization of DRG neurons, meaning these neurons convert to a state in which they are more easily excited than normally. Sensitization of sensory neurons may be due to mechanisms in the central nervous system (*CNS*) as well as the peripheral nervous system (*PNS*), and at this point it remains unclear which nervous system plays a more essential role in chronic pain (Leung and Cahill, 2010). In both the CNS and the PNS however, it is thought that pro-inflammatory mediators such as serotonin, bradykinin, chemokines and cytokines sensitize sensory neurons to stimuli and increase nociceptive transmission (Abdulkhaleq, 2018). These mediators are released by a variety of cells, including immune cells and glial cells. For example, peripheral tissue damage can lead to the activation of mast cells, neutrophils and macrophages, which in turn release mediators that either directly activate nociceptors, or indirectly through the synthesis of other mediators (e.g. prostaglandins) (Figure 2). In the long-term, these mediators are also capable of activating signalling cascades that sensitize nociceptors, thus reducing their threshold for activation and causing an increase in spontaneous firing. Moreover, while it was traditionally thought that DRG nociceptors are polymodal

(responsive to several types of stimuli), recent evidence suggests that these neurons are modality specific under normal conditions but may convert to a polymodal state during inflammation (Emery et al., 2016). This conversion to a polymodal state is likely another factor contributing to chronic pain.

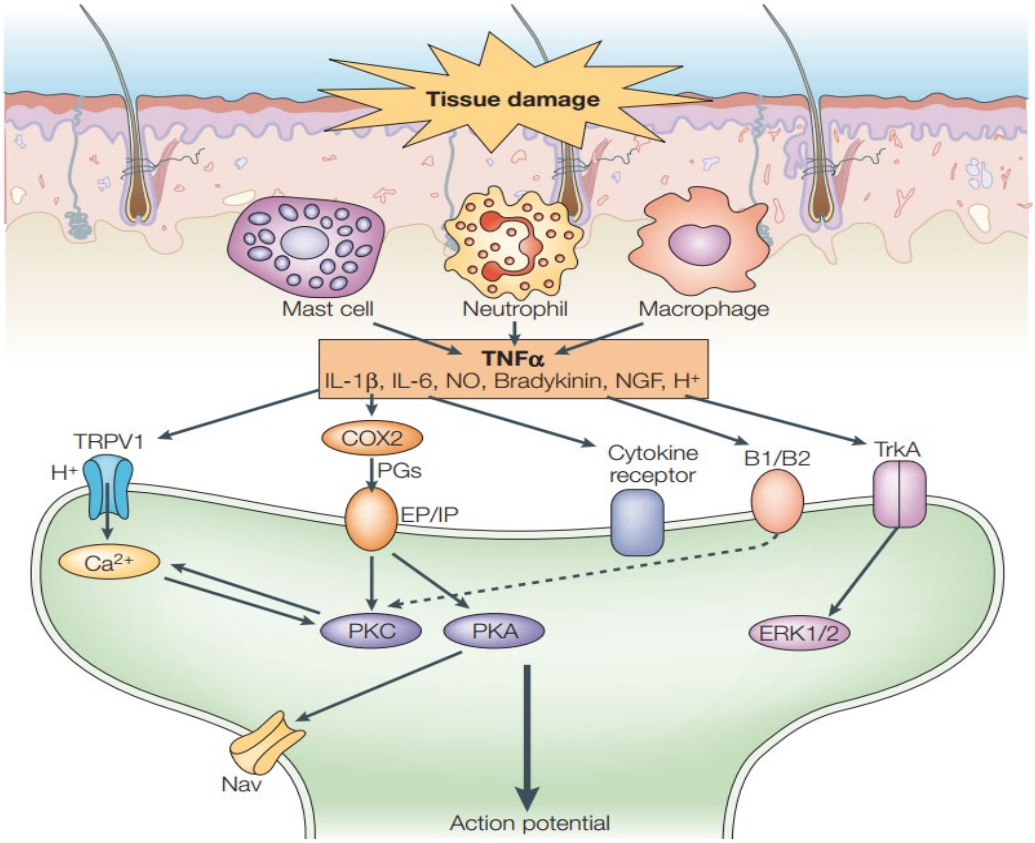


Figure 2. After tissue damage or nerve injury, activated immune can release inflammatory mediators that either directly or indirectly activate DRG nociceptors. Moreover, inflammatory mediators activate signalling cascades that reduce the activation threshold of nociceptors and increase spontaneous firing, which contributes to chronic pain (Marchand, Perretti and McMahon, 2005).

In addition to immune cell mediated release of inflammatory mediators at peripheral nerve terminals, similar processes happen at the DRG cell bodies and also in the CNS at spinal level (Krames, 2014). In the spinal cord, injury and inflammation can lead to the activation of microglial cells that release inflammatory mediators. This

inflammatory environment can modulate transmission of pain by affecting presynaptic neurotransmitter release at the terminals of first-order neurons. In addition, the postsynaptic excitability of second-order neurons may also be modulated.

At the level of the DRG cell bodies, immune cells and inflammatory mediators can easily cross the layer of *satellite glial cells* (SGC's) that engulf the cell bodies. SGC's also have receptors for inflammatory mediators and are capable of releasing mediators. The next section will discuss inflammatory mediators involved in chronic pain.

1.3 INFLAMMATORY MEDIATORS AND CHRONIC PAIN

1.3.1 MEDIATORS

Numerous inflammatory mediators have found to be involved in chronic pain and the sensitization of DRG neurons. Examples include prostaglandins, ATP, bradykinin, *tumor necrosis factor alpha* (TNF-α) *and nerve growth factor* (NGF).

TNF-α, which is most central to the current study, is a cytokine member of the TNF superfamily. This family consists of trans-membrane proteins involved in a variety of processes including inflammation, cell differentiation and apoptosis (Chu, 2013). TNF-α binds to two receptors: TNFR1 and TNFR2, also called p55/60 and p75/80 respectively, after their mass in kilo Daltons. Under normal conditions, TNFR1 activation leads to recruitment of TNFR1 associated death domain protein (TRADD), which initiates apoptosis and inflammation (Tseng et al., 2018). Apoptotic cell death is implicated in chronic pain conditions, arthritis being an example of this. In vitro

studies show that TNF-α can induce apoptosis in DRG neurons (Zhang, Yang and Luo, 2018).

Many of TNF-α's effects on nociceptors are mediated by TNFR1 activation and subsequent phosphorylation of Mitogen-activated protein kinase (MAPK) pathways mediated by p38, ERK and cJun N-terminal kinases (JNK).

Moreover, TNFR1 signalling has also been found to be crucial in the early development of nociceptors, and its activation is capable of influencing nociceptor neurite outgrowth and the innervation of targets (Wheeler et al., 2014). Alterations in neurite outgrowth are also implicated in inflammatory and neuropathic disorders. For example, impaired axonal regeneration is seen in diabetes. On the other hand, increases in neurite outgrowth may cause chronic pain by creating novel nociceptor innervations, one example being the coupling of sensory neurons with sympathetic neurons DRG seen in CRPS (Gibbs et al., 2008).

What underlies TNFR1 mediated alterations in neurite growth is the receptor's ability to regulate NGF, which in turn regulates signalling pathways critical for cell survival, excitability and neurite growth. During injury and inflammation, NGF itself also acts as an inflammatory mediator, and nociceptors abundantly express TrkA receptors, at which NGF binds.

Activation of the second TNF-α receptor, TNFR2, leads to recruitment of TNFR associated factor 1 (TRAF1) and TRAF2, which have homeostatic functions such as regeneration of tissue and cell survival. TNFR2 and TNFR1 activation therefore seem to have opposing functions.

Part of TNF-α's effects is mediated by its ability to induce cyclooxygenase 2 (COX-2) (Shishodia, Koul and Aggarwal, 2004). This enzyme, just like its constitutively

expressed relative COX-1, is known to induce prostanoids such as prostaglandins, which act as inflammatory mediators. Inhibition of COX enzymes is therefore the mode of action of many non-steroidal anti-inflammatory drugs (NSAIDS). Several types of g-protein coupled receptors for prostaglandins are expressed in the nerve terminals of DRG nociceptors. The binding of prostaglandins at these receptors leads to an increase in intracellular Ca2+ and the activation of secondary messengers such as protein kinase A (PKA) and protein kinase C (PKC) (Okuse, 2007).

Nociceptors also express receptors for bradykinin. This mediator binds to two g-protein coupled receptors, B1 and B2, the former of which is only expressed after inflammation (Fischer, Mak and McNaughton, 2010). Both receptors signal through Gq and Gi mechanisms, leading to recruitment of diacylglycerol (DAG) and phospholipase C (PLC) and subsequent release of Ca2+ from intracellular stores.

Lastly, the earlier mentioned ATP is another mediator involved in chronic pain. ATP binds to purinergic receptors, of which 3 subtypes exist: the g-protein coupled P1 and P2Y receptors, and the ionotropic P2X receptors. Similar to the receptors of other mediators, purinergic receptors signal by increasing intracellular Ca2+.

1.3.2 INVOLVEMENT IN CHRONIC PAIN

There is plentiful evidence that inflammatory mediators and their receptors are involved in states of chronic pain and the sensitization of DRG neurons. For example, acute topical application of TNF-α in rat DRG neurons has shown to increase the spontaneous firing rate of nociceptors and increase their sensitivity to electrical stimulation (Zhang et al., 2002). Interperitoneal injections of TNF-α in rats also directly produce hyperalgesia, as evidenced by decreased latency in tail flick tests (Watkins et al., 1995). Moreover, animal models of neuropathic pain, such as

chronic constriction injury (CCI) of the sciatic nerve, have shown to increase TNF-α *immunoreactivity* (IR) in rat DRG neurons (Schäfers et al., 2003). Similarly, animal models of inflammation, such as the administration of toxin chemicals like carrageenan, Freund's adjuvant and *lipopolysaccharide* (LPS), commonly lead to increased expression of TNF-α and its receptors in DRG cells (Li, 2004). In humans, increased TNF-α levels may also be especially relevant in neuropathy that is accompanied by pain. One study compared nerve biopsies between patients suffering from painful or non-painful neuropathies, and found that IR for TNF-α in the patients' schwann cells was much higher if they suffered from a painful neuropathic condition rather than a painless condition (Empl et al., 2001).

Similar results are found for other mediators. In rats, the intraplantar administration of bradykinin lowers the pain threshold to thermal stimuli, indicating sensitization of nociceptors (Schuligoi, Donnerer and Amann, 1994). Similarly, intraplantar administration of NGF and in rats produces thermal hyperalgesia to heat (Andreev et al., 1995), and intraplantar injection of ATP causes mechanical allodynia in rats (Tsuda et al., 2000).

These studies show that inflammatory mediators can produce pain behaviours and can sensitize sensory neurons. Moreover, the success of pharmacological agents that antagonize inflammatory mediators provides further evidence for their involvement in chronic pain states. For example, antagonists selective for certain purinergic receptors have shown to decrease sensitization in animal models of inflammation and neuropathic pain (Donnelly-Roberts et al., 2007).

In addition, CCI model induced hyperalgesia/allodynia in rats can successfully be combatted with antibodies that neutralize TNF-α (Sommer, Schmidt and George,

1998). In the years following such results, several drugs that inhibit TNF-α obtained clinical approval, including certolizumab pegol (Cimzia), infliximab (Remicade) and adalimumab (Humira) (Gerriets & Khaddour, 2019). Inflammatory and autoimmune diseases such as Crohn's disease, ulcerative colitis and rheumatoid arthritis are currently being treated with these medications.

1.4 SENSITIZATION MECHANISMS

Several proposals have been made for mechanisms by which TNF-α and other inflammatory mediators can sensitize peripheral nociceptors and increase nociceptive transmission in the CNS. Both in the PNS and CNS, secondary messengers downstream of inflammatory mediator activation may sensitize and upregulate ion channels crucial in nociception.

1.4.1 PERIPHERAL SENSITIZATION

1.4.1.1 TRPV sensitization

Inflammatory mediators have been shown to modulate activity of vanilloid transient receptor potential channels (*TRPV*). This receptor family identifies 6 receptors (TRPV1-6) that play a role in the perception of noxious stimuli (Liedtke, 2006). TRPV receptors are cation channels that let Ca2+ ions flow into cells when activated. TRPV1 may be of particular relevance in chronic pain. In addition to being activated by capsaicin, the ingredient that gives spicy food its burning sensation, this receptor is activated by acidic environments and temperatures above 43°. TRPV1 receptors are abundant in the nerve endings of DRG nociceptors. In DRG neurons extracted from mice and rats, it has been found that long term (48h) application of TNF-α increases the amount of DRG neurons expressing IR for the TRPV1 receptor

(Hensellek et al., 2007). In the same study, fluorescent Ca2+ imaging experiments found that TNF-α treatment increased the proportion of neurons that responded to capsaicin with a transient Ca2+ influx, which further confirms that TNF-α upregulated the expression of functional TRPV1 receptors. U126, a highly selective inhibitor of *extracellular signal-regulated kinases* (ERK), abolished the TNF-α induced increase in TRPV1, indicating ERK signalling is necessary for TNF-α induced TRPV1 upregulation. A different study also found that TNF-α increased the amplitudes of ionic currents activated by capsaicin (Constantin et al., 2008), suggesting that in addition to upregulating TRPV1 expression, TNF-α can increase TRPV1 sensitivity to capsaicin.

Similar results are found for other inflammatory mediators. As mentioned previously, bradykinin sensitizes nociceptors to heat stimuli, which are detected by TRPV1. Likewise, in-vitro patch-clamp experiments found that ATP enhanced capsaicin-evoked currents and decreased the temperature threshold for activation of TRPV1 receptors, to a point where normal temperatures would lead to TRPV1 activation (Tominaga, Wada and Masu, 2001).

While inflammatory mediators all signal through different pathways, one feature they have in common is that kinases downstream of their signalling cascades can phosphorylate target sites on TRPV1 (Figure 3) (Huang, Zhang and McNaughton, 2006). Depending on the kinase, this phosphorylation may increase TRPV1 activation in a number of ways. For example, PKC activation increases the probability that TRPV1 channels open, while PKA activation increases TRPV1 activity by reversing desensitization. A third example is the *Proto-oncogene tyrosine-protein kinase* (Src). When this kinase phosphorylates its TRPV1 target, it

promotes the trafficking of TRPV1 receptors from intracellular vesicles to the cell membrane.

All these mechanisms may cause hyperalgesia and/or allodynia by sensitizing nociceptors to stimuli detectable by TRPV1, such as heat or acid. Moreover, TRPV1 sensitization has also been implicated in mechanical hyperalgesia induced by inflammatory mediators (Jones, 2005), even though TRPV1 receptors are not sensitive to mechanical stimuli under normal conditions. This exemplifies the overall importance of TRPV1 sensitization in states of chronic pain.

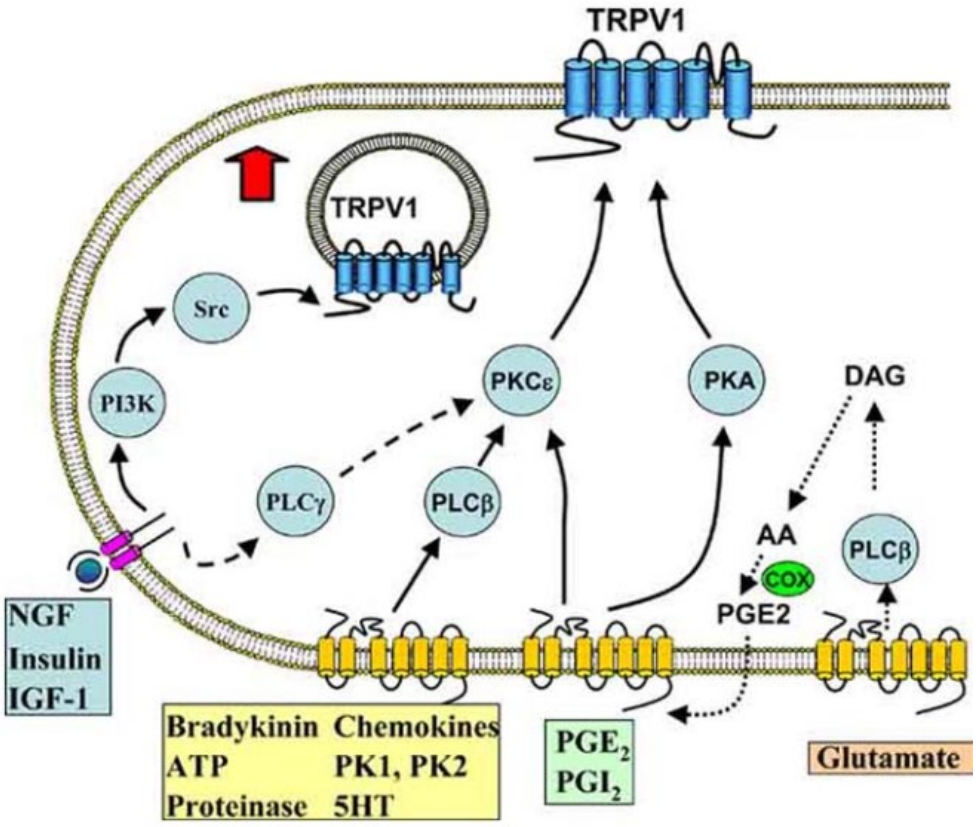


Figure 3. *TRPV1 sensitization (Huang, Zhang and McNaughton, 2006).* Binding of inflammatory mediators to their receptors activates a variety of signalling cascades. Downstream of these signalling cascades, kinases phosphorylate target sites on TRPV1 receptors, leading to TRPV1 sensitization and trafficking of TRPV1 from intracellular vesicles to the cell membrane.

1.4.1.2 Voltage gated ion channels

In addition to sensitizing TRPV1, inflammatory mediators are capable of modulating the activity of voltage gated ion channels for sodium and potassium. When activated, these channels lead to influx/efflux of ions that can depolarize/polarize neurons and thus regulate action potentials. While these channels can be distinguished by their permeability to sodium or potassium, there are many subtypes that play a differential role in regulating neuronal excitability.

When activated, voltage-gated sodium channels (VGSC) can depolarize neurons and initiate action potentials. DRG neurons express several of VGSC subtypes: NAV 1.3, NAV1.1, and NAV1.6-1.9 (Rush, Cummins and Waxman, 2007). Noteworthy is that NAV1.5, NAV1.8, NAV1.9 are resistant to the tetrodotoxin, which is a neurotoxin commonly used to block sodium channel activity. One study found increased mRNA and protein level expression of NAV1.3 and NAV1.8 in DRG neurons after motor fiber injury (He et al., 2010). Since the increases in channel expression were highly co-localized with TNF-α, a subsequent experiment was done to test TNF-α's involvement. It was found that in absence of injury, the administration of recombinant rat TNF-α was sufficient to upregulate the sodium channels. Similarly, the release of prostaglandins and NGF has been found to modulate NAV1.8 expression (Okuse, 2007).

Under normal conditions, the high threshold of DRG nociceptors ensures that pain is only perceived when a painful stimulus is actually present and is of high intensity. However, increased expression in VGSCs can increase spontaneous firing and can reduce the firing threshold (Bridges, Thompson and Rice, 2001), both of which can induce allodynia and hyperalgesia.

Another study found that inhibition of p38 strongly reduced TNF-α mediated increases in tetrodotoxin-resistant sodium channel (*TTX)* currents, indicating activation of p38 kinases mediates TNF-α's effect on TTX-resistant sodium channels (Jin, 2006). TNF-α's ability to increase sodium currents may also be mediated by its ability to induce COX enzymes. Prostaglandin synthesis by COX enzymes can increase cyclic AMP levels in nerve terminals, which in turns increases sodium currents (Schäfers et al., 2003).

While sodium channels are important in the induction of action potentials, Voltage-gated potassium channels (VGKCs) are important in repolarization of excited neurons, and therefore also play a crucial role in managing neuronal excitability. After application of the CCI model in rats, one study found reduced gene expression for several VGKS (Kim et al., 2002). Patch clamp experiments have also shown that acute TNF-α application can reduce potassium currents in a dose dependent-manner (Liu et al., 2008). The ability of inflammatory mediators to modulate expression of VGKS provides another route by which they can increase excitability of DRG nociceptors.

1.4.1.3 Reciprocal sensitization

While inflammatory mediators may modulate activity of TRPV1 and voltage gated ion channels, another important feature is the ability of inflammatory mediators to modulate expression of one another. For example, expression of the B1 bradykinin receptor has been found to be modulated by TNF-α and other cytokines (Campos, Souza and Calixto, 1999). Another example is the finding that extracellular ATP increases TNF-α expression in rat microglia (Hide et al., 2002). The ability of

inflammatory mediators to increase expression of other mediators and their receptors can further exacerbate the sensitization of neurons.

1.4.2 CENTRAL SENSITIZATION

Inflammatory mediators also cause chronic pain through mechanisms in the CNS. In the spinal cord, an inflammatory environment can modulate the transmission between first and second order neurons. Indeed, the injection of TNF-α into the spinal cord of rats has shown to enhance the response of dorsal horn neurons to stimulation of first-order C-fibers (Reeve et al., 2000). Moreover, spinal cord injections of TNF-α and other cytokines like Interleukin-1β and Interleukin-6 produce hyperalgesia to heat stimuli in rats. In spinal cord neurons, TNF-α enhances currents induced by NMDA receptors and increases the expression and trafficking of AMPA receptors (Kawasaki et al., 2008). Since glutamate released by Aδ nociceptors binds at these receptors, their increased expression can strengthen the synaptic connection between first and second order neurons. Moreover, the binding of prostaglandins (which are inducible by TNF-α) at postsynaptic neurons leads to activation of PKA and subsequent phosphorylation of NMDA receptors, thus sensitizing these receptors to glutamate (Latremoliere and Woolf, 2009). Intense peripheral noxious stimuli may also lead to bradykinin production in the spinal cord (Wang, 2005). Through PKC, ERK and PKA signalling, bradykinin can sensitize AMPA and NMDA receptors and increase their trafficking and expression, thus strengthening synaptic connections in the spinal cord.

1.5 IN VITRO MODELS OF SENSORY NEURONS

As the previous sections highlighted, the release of inflammatory mediators alters many cellular processes that contribute to chronic neuropathic and inflammatory pain. In addition to increasing the excitability of sensory neurons by modulating receptors and ion channels, mediators can influence cell survival and axonal innervation of targets. To gain understanding of the underlying mechanism that mediate these processes, it is common practice to study cultures in which sensory neurons are extracted from animals. For example, DRG neurons can be extracted from rodents and chick embryos (Powell, Vinod and Lemons, 2014).

However, there are several drawbacks to extracting DRG neurons from animals. It is technical, time consuming and subject to ethical dilemmas since it requires the use of animals. In addition, the extraction of DRG neurons and the severing of peripheral axons can induce functional changes such as increased neuronal excitability, increased expression of the TrkA receptor and downregulation of potassium channels (Yin, Baillie and Vetter, 2016). Moreover, the heterogeneous nature of DRG neurons further complicates matters, since cells may express different ion channels and therefore show inconsistent responses to drugs.

In response to these mentioned drawbacks, researchers can substitute primary DRG neurons with immortal cell lines derived from sensory neurons. The use of cell lines is cost efficient and provides an unlimited supply of cells, which is ideal for high throughput studies of pharmacological agents (Kaur and Dufour, 2012). However, research on cell lines is based on the assumption that such cells mimic many of the characteristics and behaviors found in their primary equivalent. Unfortunately, this is often not the case, as many cell lines lack certain receptors or respond differently to

drugs or inflammatory mediators compared to their primary parents. Cellular processes processes such as cell survival, proliferation or the formation of neurites may also be altered in cell lines. Therefore, in order for cell lines to be useful in studying these underlying mechanisms, it is important to ascertain similarities and differences between cell lines and the primary cells that they model after.

One example of a sensory neuron cell line is 50B11, which is derived from embryonic rat DRG's (Chen et al., 2007). When differentiated with forskolin, one study described these cells as having many features characteristic of nociceptive neurons. They produced long neurites, generated action potentials and expressed functional TRPV1, as indicated by a capsaicin induced Ca2+ influx. Other examples of sensory neuron cell lines include hybrid cell lines derived from both rat DRG's and mouse neuroblastoma, such as F11 and ND7/23. These cell lines contain chromosomes of both rats and mice. Similar to 50B11 cells, one study on ND7/23 cells concluded that these cells share many features with primary DRG cells, such as expression of substance P, prostaglandin receptors and several types of voltage gated calcium channels (Mitani et al., 2016). Another cell line sometimes used as model for sensory neurons is the human derived SH-SY5Y cell line, which was originally isolated from a bone marrow biopsy of a child with neuroblastoma.

As previous sections discussed, many of the ion channels and g-protein coupled receptors implicated in DRG sensitization signal by increasing intracellular levels of Ca2+ ions. Modulation of these receptors is a potential therapeutic strategy. Therefore, it is useful to assess the expression of these receptors in cell lines, and to assess

	ND7/23		F11		50B11		SH-SY5Y	
	pEC_{50}	% F_{Max}	pEC_{50}	% F_{Max}	pEC_{50}	% F_{Max}	pEC_{50}	% F_{Max}
Acetylcholine	–		–		–		6.7 ± 0.2	29.6–46.4
ATP	–		4.9 ± 0.1	26.0–41.7	4.9 ± 0.1	1.5–6.8	–	
Bradykinin	8.3 ± 0.1	12.6–16.2	11.4 ± 0.1 8.7 ± 0.1	47.2–69.3	–		7.4 ± 0.3	4.1–5.1
Choline	–		–		–		5.1 ± 0.2	45.6–71.0
Histamine	–		–		–		5.8 ± 0.09	12.3-23.7
Neurotensin	–		–		–		–	
Nicotine	–		–		–		5.5 ± 0.04	13.4–29.0
Trypsin	–		7.4 ± 0.2	22.6–38.2	–		7.0 ± 0.1	27.6–47.8

Figure 4. Table from Vetter & Lewis (2010). Comparison of different cell lines in endogenous Ca2+ response. % Fmax = maximum fluorescence response.

how cell lines respond to agonists at these receptors. Using fluorescent Ca2+ imaging, one study explored endogenous Ca2+ signalling in a number of cell lines, including ND7/23, SH-SY5Y, 50B11 and F11 (Vetter & Lewis, 2010).

Prior to testing, cells were differentiated with either NGF (ND7/23, F11) or forskolin (50B11). Interestingly, there was much diversity in agonist-induced Ca2+ responses between the different cell lines (Figure 4). While SH-SY5Y responded to a large amount of agonists with a transient increase in Ca2+, 50B11 and ND7/23 cells only responded to ATP and bradykinin, respectively. F11 cells were responsive to ATP, bradykinin and trypsin. In all tested cell lines, Ca2+ influx was absent in response to noradrenaline, glutamate, GABA, neurokinin A/B, substance P, adenosine, dopamine and most notably, capsaicin. For 50B11, this contradicts the previously mentioned study in which capsaicin induced Ca2+ influx was found. Likewise, the lack of Ca2+ influx in ND7/23 cells in response to exogenous substance P is surprising given the previously mentioned finding that these cells endogenously express substance P.

These results point towards the complexity of using in vitro cell lines as models of sensory neurons. Studies on these cell lines often find contradicting results, and it is often unclear why this is the case. Potentially, serial passaging or differences in experimental conditions may alter the characteristics and functioning of cells.

A different way to study receptor expression is to analyze RNA transcripts. Using this method, one study concluded that F11, ND7/23 and SH-SY5Y cells lacked many markers of nociceptive sensory neurons, including expression of TRPV1 (Yin, Baillie and Vetter, 2016). Moreover, the characteristics of the cell lines did not match any specific subset of DRG neurons. However, the cell lines did express markers for unmyelinated and myelinated neurons, and also expressed markers for voltage-gated potassium, sodium and calcium channels, although to a lesser extent than natural DRG neurons.

The cell line central to this study is the aforementioned F11 cell line. In addition to previously mentioned findings, these cells resemble DRG neurons in a number of other ways. They exhibit constitutive action potentials similar to those of DRG neurons (Platika et al., 1985) and express functional KCNQ2/3 channels (Jow et al., 2006), as well as functional μ and δ opioid receptors (Fan et al., 1992). Also, application of menthol in F11 cells has shown to inhibit the activity of TTX-R Na+ channels, confirming not only that F11 cells express TTX-R channels, but also that they express the menthol sensitive TRPM8 channel (Gaudioso et al., 2012). F11 cells also express functional prostaglandin receptors and are capable of synthesizing and releasing substance P (Francel et al., 1987).

While these results highlight ways in which F11 cells resemble sensory neurons, it has yet to be studied how F11 cells respond to an inflammatory environment caused by TNF-α. In primary DRG cultures, TNF-α alters neurite outgrowth and can induce apoptosis. Both of these processes have been implicated in pain disorders. Moreover, TNF-α mediates pain by sensitizating/upregulating a variety of voltage gated ion channels, as well as ion channel receptors and g-protein coupled receptors. Through

modulation of voltage gated ion channels, TNF-α can reduce the activation threshold of DRG nociceptors and increase spontaneous firing. Moreover, by modulating receptor ion channels and g-protein coupled receptors, many of which signal through calcium, TNF-α can sensitize nociceptors to agonists, leading to increased agonist induced firing.

The current study explored whether the F11 cell line responds similarly to an inflammatory environment induced by TNF-α. By measuring intracellular calcium levels in response ATP, bradykinin and capsaicin, it was attempted to determine if TNF-α sensitized or upregulated receptors for these agonists in F11 cells. Moreover, by measuring baseline spiking activity and spiking activity in response to the agonists, it could be determined if TNF-α decreased the activation threshold of F11 cells and increased agonist induced firing. Also, by means of MTT assays and NeuronJ it was explored whether TNF-α altered neurite outgrowth and cell viability, the latter of which would give an indication if TNF-α triggers apoptosis in F11 cells. If TNF-α's effects on F11 cells are similar to its effects on DRG neurons, then this suggests the F11 cell line may be an appropriate tool in studying the underlying mechanisms of TNF-α mediated chronic pain. The cell line may then also be useful in high throughput studies of novel analgesics and anti-inflammatory substances.

1.6 HYPOTHESIS

In the current study, it was expected that TNF-α treatment in F11 cells would reduce neurite outgrowth and cell viability. Moreover, by sensitization and upregulation of ion channels and receptors, TNF-α was expected to increase agonist induced Ca2+ influx and spontaneous and agonist induced spiking activity.

Materials & Methods

2.1 MATERIALS

Reagent/product	Source	Catalog #
12 well cell culture plate	Sarstedt	83.3921
2-Propanol (anhydrous, 99.5%)	Sigma-Aldrich	278475
48 well cell culture plate	Sarstedt	83.3923.005
6 well cell culture plate	Sarstedt	83.3920.005
Adenosine 5′-triphosphate (ATP) disodium salt hydrate	FLAAS	FLAAS
Bradykinin acetate salt	Sigma-Aldrich	B3259
Capsaicin	EMD Millipore Corp	211275
Dulbeco's Modified Eagle's Medium – High Glucose (With 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture)	Sigma-Aldrich	D6429
Fluo-4 AM, cell permeant	Thermo Fisher Scientific	F14201
Fetal Bovine Serum	Sigma-Aldrich	12103C
Forskolin	Sigma-Aldrich	F6886
Maxi-Mix III Rotary Shaker type 65800	Thermolyne	
MEA2100-System	Multi Channel Systems	
MTT solution	Sigma-Aldrich	M2003
N6,2′-O-Dibutyryladenosine	Sigma-Aldrich	D0627

3′,5′-cyclic monophosphate sodium salt		
Recombinant human TNF alpha protein	Alomone labs	T-100
RPMI-1640 Medium (With L-glutamine and sodium bicarbonate)	Sigma-Aldrich	R8758
Trypsin-EDTA	Sigma-Aldrich	T3924
Victor3 1420 multilabel counter	PerkinElmer	

2.2 METHODS

2.2.1 DEFROSTING CELLS

F11 cells were stored at -80°C in 10% DMSO. A cryovial containing the frozen cells was removed from storage and immediately placed it into a 37°C water bath. Cells were quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath until a small bit of ice was left in the vial. In a laminar flow hood, the contents of the vial were transferred to a centrifuge tube to which 9ml of pre-warmed complete growth medium was added. The cell solution was centrifuged at approximately 200 × G for 3 minutes. After centrifugation, the supernatant was removed to waste and the cell pellet was gently resuspended in 1 ml of complete medium before transfer into a T75 culture flask. 12 ml of complete medium was added to the flask and the flask was placed into the incubator.

2.2.2 CELL CULTURE CONDITIONS

F11 cells were routinely cultured in Sarstedt T75 flasks in a humidified incubator at 37°C, 5% CO_2. The culture medium consisted of *Dulbecco's Modified Eagle's Medium* (DMEM) supplemented with 5% *fetal bovine serum (*FBS) and 2% of a *glutamine-penicillin-streptomycin* solution. Culture medium was changed every 2 days, and cells were split when approximately 60-90% confluent according to the subculturing protocol described in the next section.

2.2.3 SUBCULTURING

If cells were to be split or harvested for an experiment, 5 ml of pre-heated trypsin was added to the flask and the flask was incubated for 5 minutes to allow detachment of cells. When cells were >80% detached, 10 ml of culture medium was added and the entire 15 ml cell suspension was transferred to a 15 ml falcon tube. The falcon tube was then centrifuged at approximately 200 × G for 3 minutes. After centrifugation, the supernatant was removed to waste and the cell pellet was gently resuspended in 1 ml of complete medium. Cells were then split 1:6 and added to a new T75 flask. However, if cells were to be seeded onto plates for experiments, cells were counted with a hemocytometer after the pellet was resuspended. After establishing cell count, the cell suspension was diluted to reach the desired cell concentration and cells were seeded onto plates or the wells of the multi electrode array. All experiments were performed using three biological replicates (different plates) and three technical replicates (different wells) for each condition. Experiments were performed using cells of passages between 23 and 45.

2.2.4 Differentiation Pilot Study

The endpoints in the current study were measured both in undifferentiated and differentiated F11 cells. Prior to the experiments, a differentiation pilot study was carried out to determine the appropriate differentiation protocol for subsequent experiments. F11 cells were seeded at a density of approximately 5000 cells/cm2 on coverslips in a 6 well plate, with 3 coverslips in each well. Cells were seeded in culture medium consisting of DMEM supplemented with 5% FBS and 2% glutamine-penicillin-streptomycin solution. After a 24 hour attachment period, the wells were exposed to differentiation medium of varying composition (Table 1). The differentiation process was tracked for 7 days and microscopic images were taken daily. Differentiation medium was replenished every 2 days. In subsequent experiments, cells were differentiated using DMEM, 1% FBS, 2% glutamine-penicillin-streptomycin solution and forskolin (30 µM).

Table 1. Differentiation pilot study

DMEM, 1% FBS, 2% GPS	DMEM, 1% FBS, 2% GPS, 0.5 mM DBCAMP	DMEM, 1% FBS, 2% GPS, 30 µM forskolin
DMEM, 1% FBS, 2% GPS	DMEM, 1% FBS, 2% GPS, 1 mM DBCAMP	DMEM, 1% FBS, 2% GPS, 15 µM forskolin

*Each table cell represents one well in a 6 well plate. *GPS* = glutamine-penicillin-streptomycin solution

2.2.5 MTT Assay

To measure cell viability, cells were seeded on 48 well plates with a density of approximately 5000/cm2, in DMEM, 5% FBS and 2% glutamine-penicillin-streptomycin solution. After a 24 hour attachment period, the medium was changed to reflect the appropriate condition. In cultures that were not to be differentiated, the composition of medium remained unchanged for control wells but TNF-α (10 ng/ml)

was added to experimental wells. In cultures that were to be differentiated, after the 24 hour attachment period the medium was changed to a differentiation medium consisting of DMEM, 1% FBS, 2% glutamine-penicillin-streptomycin solution and Forskolin (30 μM). After 5 days of differentiation, TNF-α (10 ng/ml) was added to the medium. For both differentiated and undifferentiated cultures, MTT assays were performed after 24, 48 and 72 hours of TNF-α treatment.

The MTT assay protocol was as followed. 200 μl of MTT solution was added to the wells and the plates were incubated for approximately 1.5 hour to allow the formation of Formazan crystals. After the incubation period, 200 μl of 2-propanol was added to the wells as solvent for the Formazan crystals. To facilitate the dissolving process, the plates were placed on a Maxi-Mix III Rotary shaker for 20 minutes. Subsequently, the contents of the wells were transferred to a 96 well plate, because there was no software protocol available for the reading of absorbance in 48 well plates. Absorbance was then read at 550 nm using a Victor3 spectrophotometer.

2.2.6 NEURITE OUTGROWTH

To measure neurite outgrowth, microscopic images were taken of the same cultures in which metabolic activity was measured. Daily images were taken starting after the 24 hour attachment period for undifferentiated cultures, and starting after 5 days of differentiation in the case of differentiated cultures. Subsequently, ImageJ software, along with a plugin called NeuronJ, was used to analyse neurite outgrowth between TNF-α treated wells and control wells. The NeuronJ plugin facilitates tracing and quantification of neurites, and does not require cells to be stained. Per condition, all cells were counted and all neurites were traced. The neurites were averaged to

acquire mean neurite length, which could then be compared with Student's t-test between conditions to assess the effect of TNF-α on neurite outgrowth.

2.2.7 CA2+ IMAGING

F11 cells were seeded on poly-l-lysine coated coverslips in 12 well plates, at a density of approximately 5000 cells/cm2, in a growth medium of DMEM, 5% fetal bovine serum and 2% glutamine-penicillin-streptomycin solution. Ca2+ imaging was done at 0, 24 or 48 hour of TNF-α treatment (10 ng/ml). In undifferentiated cultures, imaging started after a 24 hour attachment period. In differentiated cultures, imaging started after 5 days of differentiation with Forskolin (30 μM). Ca2+ imaging was recorded in response to ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). The imaging procedure was as followed. Coverslips were washed three times with Krebs-Ringer's solution (119 mM NaCl, 2.5 mM KCl, 1.0 mM NaH_2PO_4, 2.5 mM CaCl2·2H2O, 1.3 mM MgCl2·6H2O, 20.0 mM HEPES, 11.0 mM C6H12O6) , which is a Ca2+ imaging buffer. Subsequently, Fluo-4 (10 μM, AM ester form) dissolved in dimethyl sulfoxide (DMSO) was added onto the coverslips. Fluo-4 is a dye that increases in fluorescence when Ca2+ is bound. After 40 minutes of incubation at 37°, coverslips were washed three more times with Krebs-Ringer's solution. Then, another 20 minutes of incubation allowed for complete dye de-esterification. Coverslips were then placed in the imaging chamber, which was loaded with 180 μl of Krebs-Ringer's solution. The chamber was then mounted on the stage of a zeiss axiovert 200 microscope. Responses were measured with excitation at 488 nm and emission at 510 nm. Using ocular software, 200 frames were imaged, and agonists were added at the 100^{th} frame.

Fluosnnap was used to identify regions of interest (ROI) and to calculate the fluorescence response per ROI over 200 frames. Changes in fluorescence were expressed as ΔF/F to correct for baseline fluorescence. The change in fluorescence between the 100th frame and the 150th frame was used per ROI to establish the response to the agonist. Data were only included if the absolute peak response for a given ROI exceeded a 2% change from baseline, to exclude ROI's that had very low baseline Ca2+ activity and would therefore be unlikely to respond to agonists. Subsequently, results of each biological replicate were combined to obtain the mean response, average peak response, area under the curve and full half-maximum width (FHMW) for each condition. Statistical analysis was done using ordinary one-way ANOVA's with Dunnett's as post hoc.

2.2.8 ELECTRICAL ACTIVITY

Electrical activity in F11 cells was measured using a MEA2100 multi electrode array (MEA). To prepare the MEA for culture, the MEA was left overnight in 1% Terg-A-Zyme. Subsequently, the MEA was rinsed with de-ionized water and placed into a 100 mm polystyrene petri dish. F11 cells were then seeded into the wells at a density of 5000 cells/cm2. After cell adherence was achieved, visual inspection ensured that cells sufficiently covered the electrodes. Subsequently, the dish was flooded with differentiation medium consisting of DMEM supplemented with 1% FBS, 2% glutamine-penicillin-streptomycin solution and forskolin (30 μM). Cells were differentiated for 3 days, after which TNF-α was added to the medium. Electrical activity was recorded after 48 hours of TNF-α treatment.

During recordings, the MEA was transferred to the recording preamplifier. Seven minutes of waiting time allowed for cells to adjust to room temperature and for

electrical activity to stabilize. Then, baseline recordings were taken as well as recordings in response to ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Recordings were performed using Multi Channel Systems software. Data were analysed to establish spiking activity per minute. Spikes were counted if the electrical activity (in picovolts) at a certain time was four standard deviations from the average activity in the preceding four milliseconds. Using this method, baseline spiking activity and spiking activity in response to agonists could then be calculated for TNF-α treated wells and control wells. Spiking activity was compared between conditions using Student's t tests.

Results

3.1 DIFFERENTIATION PILOT STUDY

A differentiation pilot study was performed to determine the appropriate differentiation protocol for subsequent experiments (Figure 5). F11 cells were differentiated with serum stripped medium or serum stripped medium supplemented with varying doses of DB-CAMP (1/0.5 mM) or forskolin (30/15 µM).

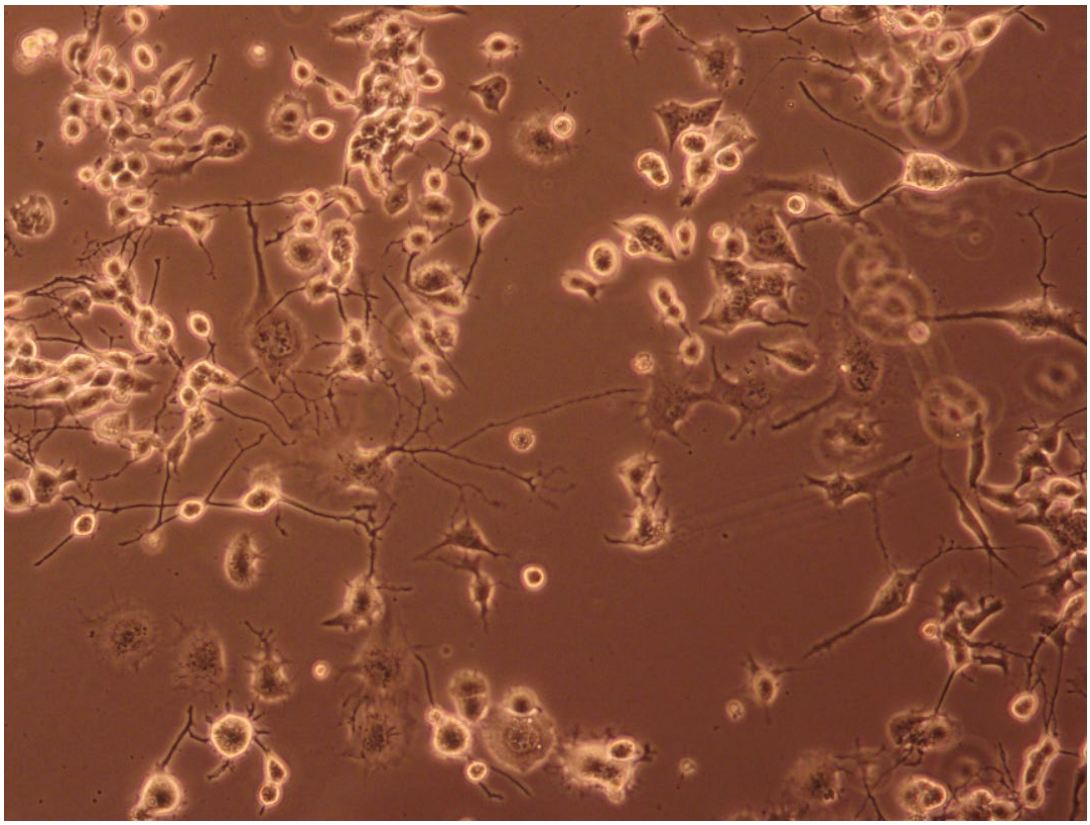


Figure 5. Differentiation pilot study. F11 cells were differentiated with serum stripped medium or serum stripped medium supplemented with DB-CAMP (1/0.5 mM) or forskolin (30/15 µM). Image shows forskolin (30 µM) differentiated cells.

The differentiation process was tracked for 7 days and microscopic images were taken daily. While all differentiation methods induced neurite outgrowth, this was

most apparent for cells treated with 30 µM of forskolin. Therefore, cultures in subsequent experiments were differentiated using forskolin at 30 µM.

3.2 CELL VIABILITY

In order to test whether TNF-α influences cell viability in F11 cells, MTT assays were performed after 24, 48 or 72 hours of TNF-α (10 ng/ml) treatment (Figure 6). The assays were performed both in undifferentiated cultures and in cultures that were differentiated with forskolin (30 µM) for 5 days before the addition of TNF-α.

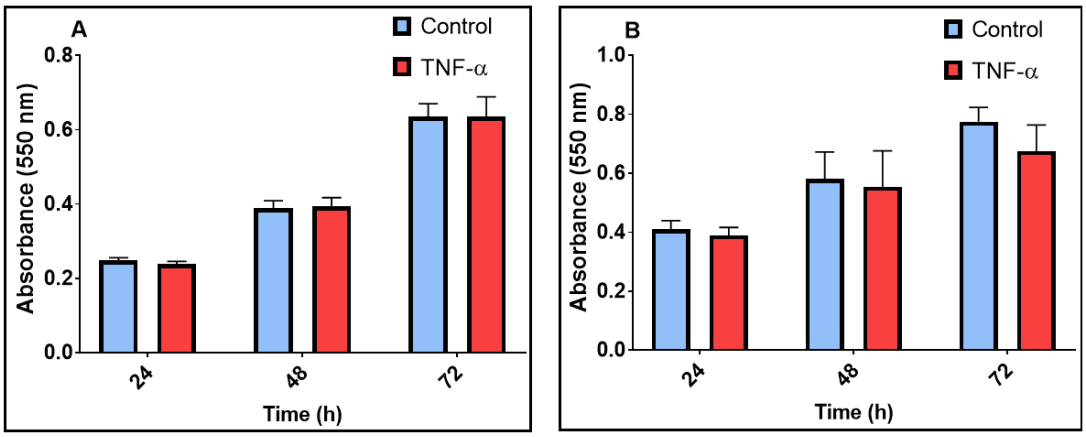


Figure 6. Cell viability. Data are expressed as mean absorbance + SD (n = 3). **A.** Undifferentiated F11 cells were cultured in medium containing TNF-α (10 ng/ml). MTT assays to measure cell viability were performed after 24, 48 and 72 hours. **B.** F11 cells were differentiated with forskolin (30 µM) for 5 days. Subsequently, the differentiation medium was supplemented with TNF-α (10 ng/ml). MTT assays were performed 24, 48 and 72 hours after addition of TNF-α.

TNF-α treatment had no significant influence on absorbance in undifferentiated cultures **(A).** In differentiated cultures **(B)**, a difference in absorbance emerged after 72 hours of TNF-α treatment, with TNF-α treated cultures seeing a reduction in absorbance. However, this difference did not reach statistical significance at α = 0.05. These data suggest that TNF-α at 10 ng/ml does not alter the viability of F11 cells in the given timeframe.

3.3 NEURITE OUTGROWTH

To test the influence of TNF-α on neurite outgrowth in F11 cells, microscopic images were taken in undifferentiated and differentiated F11 cultures after 0, 24, 48 and 72 hours of TNF-α treatment. Pictures were analyzed using ImageJ and NeuronJ. Figure 7 shows a sample image of the neurite tracing procedure.

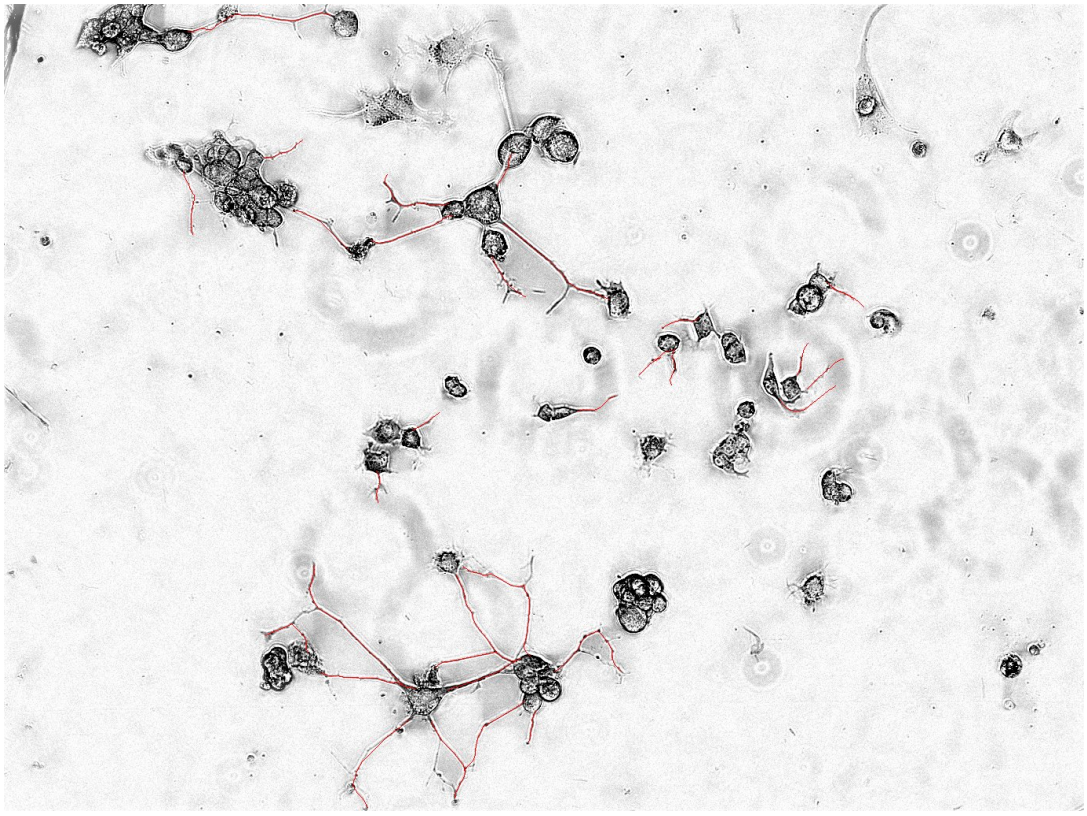


Figure 7. Sample image of neurite outgrowth measure. The ImageJ plugin NeuronJ allows for semi-automatic tracing of neurites. In this image, traced neurites are colored in red. NeuronJ automatically stores data on the length of the neurites in µm.

Table 2 shows quantitative data on neurite outgrowth and cell count in undifferentiated F11 cells. The increase in cell count over time confirms that these cells were proliferating. Despite not being in differentiation medium, a small

percentage of these cells developed neurites. Neurites of any length were counted. Mean neurite length was obtained by averaging the average neurite length per culture (n = 3). Likewise, mean standard deviation of neurite length was obtained by averaging the average SD per culture. At 72 hours, there was a significant reduction in mean neurite length in TNF-α treated cultures compared to control cultures (Student's t-test, p=0.0251, t=3,491 df=4). At 0, 24 or 48 hours no difference in mean neurite length was found between conditions, and at no time point was there a significant difference in mean standard deviation of neurite length.

Time (hour)	**Condition**	**Cell count**	**Cells with neurites**	**Cells without neurites**	**Neurite count**	**Sum neurite length (µm)**	**Mean neurite length (µm) ± SD of mean**	**Mean SD of neurite length (µm) ± SD of mean**	**Max neurite length (µm)**
0	Control	810	74 (9.1%)	736 (90.9%)	96	3150.2	32.4 (± 4.9)	11.3 (± 1.5)	80.3
	TNF-α	640	90 (14.1%)	550 (85.9%)	112	3828.6	34.3 (± 6.3)	12.5 (± 1.3)	87.9
24	Control	836	76 (9.1%)	760 (90.9%)	96	3150.2	32.4 (± 4.9)	11.3 (± 1.5)	80.3
	TNF-α	712	88 (12.4%)	624 (87.6%)	112	3828.6	34.3 (± 6.3)	12.5 (± 1.3)	87.9
48	Control	1543	87 (5.6%)	1429 (92.6%)	94	3767.9	39.7 (± 2.9)	17.5 (± 2.1)	130.7
	TNF-α	1620	105 6.5%)	1515 (93.5%)	122	4731.4	39.7 (± 8.5)	19.3 (± 2.4)	133.2
72	Control	2510	197 (7.8%)	2313 (92.2%)	152	5882.3	38.3 (± 2.4)	16.1 (± 3.0)	163.2
	TNF-α	2679	286 10.7%)	2393 (89.3%)	168	5175.0	31.6 (± 2.3*)	13.5 (± 1.2)	104.6

Table 2. Neurite outgrowth data. The effect of TNF-α (10 ng/ml) on neurite outgrowth was measured in undifferentiated F11 cells. ImageJ software and the NeuronJ plugin were used to trace neurites and obtain cell count.

As expected, neurite outgrowth was much more pronounced in differentiated cultures (Table 3), indicated by greater percentages of cells that produces neurites. Since a large number of these cells develop miniature neurites, only neurites longer than 20 µm were included for analysis. At 24 hours of TNF-α treatment, mean neurite length

was significantly reduced in TNF-α treated cultures compared to control cultures (Student's t-test, p= 0,0043, t = 5,839 df = 4). At 48 and 72 hours, no significant difference in mean neurite length was found. Surprisingly, mean neurite length was actually larger in TNF-α treated cultures at 48 hours compared to control cultures. At no time point was there a significant difference in mean SD of neurite length.

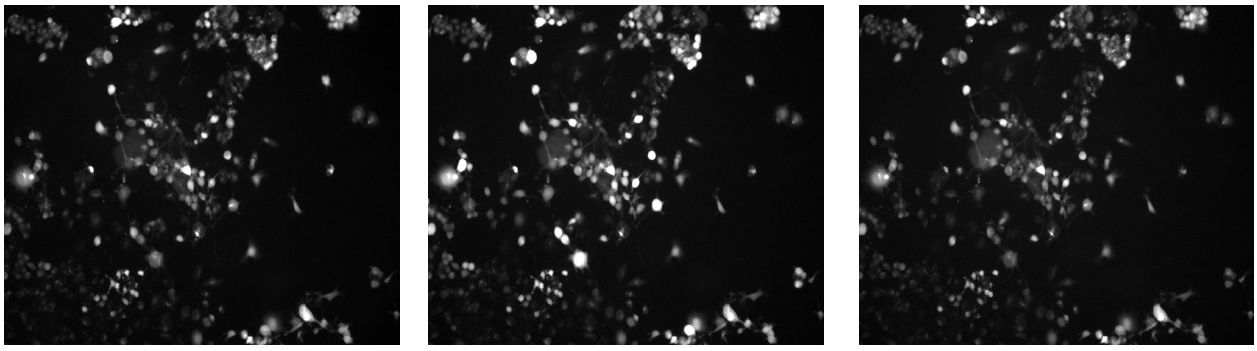
Time (hour)	**Condition**	**Total cell count**	**Cells with neurites**	**Cells without neurites**	**Neurite count**	**Sum neurite length (µm)**	**Mean neurite length (µm) ± SD of mean**	**Mean SD of neurite length (µm) ± SD of mean**	**Max neurite length (µm)**
0	Control	1137	312 (27.4%)	825 (72.6%)	445	42298	94.5 (± 5.9)	51.6 (± 8.4)	504
	TNF-α	1184	326 (27.5%)	858 (72.5)	456	42008	93.5 (± 10.4)	55.1 (± 14.9)	459.3
24	Control	1905	336 (17.6%)	1569 (82.4%)	416	39016	93.8 (± 3.8)	48.7 (± 2.5)	507.1
	TNF-α	1585	339 (21.4%)	1246 (78.6%)	429	33923	79.9 (± 1.6)**	45.8 (± 4.2)	418.5
48	Control	1041	302 (29.0%)	739 (71.0%)	401	50637	126 (± 5.5)	72.8 (± 9.6)	544.9
	TNF-α	1015	320 (31.5)	695 (68.5%)	437	58238	137.9 (± 26.5)	87.4 (± 13.1)	963.7
72	Control	1436	338 (23.5)	1098 (76.5%)	516	68141	130.6 (± 20.7)	80.8 (± 25.6)	632.7
	TNF-α	976	270 (27.7	706 (72.3%)	497	59801	124.9 (± 21.1)	73.5 (± 22.8)	569.9

Table 3. Neurite outgrowth data. Neurite outgrowth was measured in differentiating F11 cells. After 5 days of differentiation, TNF-α (10 ng/ml) was added to the differentiation medium. Daily microscopic pictures were taken starting the moment TNF-α was added. ImageJ software and the NeuronJ plugin were used to trace neurites and obtain cell count.

3.4 CA2+ IMAGING

3.4.1 IMAGING PROCEDURE

To explore Ca2+ response, F11 cells were loaded with the Fluo-4 dye. Binding of Ca2+ to this dye leads to an increase in its fluorescence properties, which gives an approximation of intracellular Ca2+ activity. Figure 8 shows an example of this procedure. Before the addition of the agonist **(A)**, cells show small oscillations in Ca2+ activity. Then, the addition of an agonist **(B)** leads to a transient influx of Ca2+ ions into the cells, indicated by an increase in fluorescence. Subsequent to this influx, Ca2+ activity returns to a more quiescent state **(C)** resembling baseline activity.



A **B** **C**

Figure 8. Sample of Ca2+ imaging procedure. **A.** Fluorescence before addition of agonist (frame 0 - 100) **B.** The addition of bradykinin provokes a transient influx of Ca2+, indicated by an increase in fluorescence (frame 100 – 150). **C.** Following the influx, Ca2+ activity returns to a more quiescent state (frame 150 - 200) resembling baseline activity.

3.4.2 PROPORTION OF ACTIVE AND RESPONSIVE CELLS

Table 4 shows Ca2+ imaging data on undifferentiated F11 cells, and describes the proportion of active cells and the proportion of cells responsive to agonists. The ‘cell count’ resembles all regions of interest (ROI’s) defined using Fluosnapp software.

Only ‘active’ cells were used in data analysis. Cells were considered active if their peak fluorescence response exceeded a 2% change from baseline.

This filter aimed to exclude cells that had very low constitutive Ca2+ activity and were therefore unlikely to respond to agonists. It was set at 2% because a preliminary analysis concluded that a higher percentage filters would exclude too much data, rendering statistical analysis impossible.

Cells were considered ‘responsive’ to an agonist if their peak fluorescence response exceeded a 10% change from baseline. The mean % of responsive cells was obtained by averaging the average % of responsive cells per culture (n = 3). This made it possible to statistically compare the amount of responsive cells in untreated cultures (time 0) with the amount of cells responsive in cultures treated with TNF-α for 24/48 hours.

Agonist	Time (h)	Cell count	Active cells	Cells responsive to agonist	Mean % of responsive cells + SD
ATP (10 µM)	0	465	78	22	24.5% (± 26.4%)
	24	241	17	4	22.5% (± 3.5%)
	48	396	132	20	17.6% (± 15%)
Bradykinin (20 µM)	0	363	143	132	86.4% (± 12.3%)
	24	458	90	10	30.7 % (± 50%)
	48	863	84	23	37.5% (± 30.8%)
Capsaicin (100 µM)	0	324	59	0	0% (± 0%)
	24	510	70	28	25% (± 35.6%)
	48	550	222	28	9 % (± 13.2%)

Table 4. Proportion of active cells and agonist response. Fluorescent Ca2+ imaging in response to three agonists was done in undifferentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n=3). The cell count represents ROI's defined using Fluosnnap. Cells were considered active if their peak fluorescence responses exceeded a 2% change from baseline, and cells were considered responsive to an agonist if peak response exceeded a 10% change from baseline.

However, using one-way ANOVA's with Dunnett's post hoc tests, no statistically significant differences were found in the percentage of responsive cells between untreated cultures and cultures treated with TNF-α for 24 or 48 hours for any of the agonists. Nonetheless, it is noteworthy that a response to capsaicin was only observed in TNF-α treated cultures. In cultures that were not treated with TNF-α (time 0), not a single cell was capsaicin responsive. In cultures treated with TNF-α for 24 hours, 28 cells were capsaicin responsive, while there were only 11 more active cells in these cultures.

An opposite trend was seen for bradykinin: the percentage of bradykinin responsive cells was much larger in untreated cultures (86.4%) then in cultures treated for 24 (30.7 %) or 48 (37.5%) hours.

Table 5 shows the same data for forskolin differentiated F11 cells. Similarly, for none of the agonists was there a significant difference in the percentage of responsive cells between untreated cultures and cultures treated with TNF-α for 24/48 hours. However, the same trends that were present in undifferentiated F11 cells were also present in differentiated cells. The percentage of cells responsive to bradykinin was highest in untreated cultures (51.3%) and lowest in cultures that were TNF-α treated for 48 hour (32.9%). On the other hand, the percentage of cells responsive to capsaicin was lowest in untreated cultures (12.5%) and highest in cultures treated with TNF-α for 48 hour (24.9%)

Agonist	Time (h)	Cell count	Active cells	Cells responsive to agonist	Average % of responsive cells + SD
ATP (10 µM)	0	465	262	3	1.6%(± 1.8%)
	24	241	102	57	27.4% (± 44.6)
	48	396	324	9	2.3% (± 3.5)
Bradykinin (20 µM)	0	363	342	175	51.3% (± 40.5%)
	24	458	412	189	46.6% (± 38.3%)
	48	863	784	229	32.9% (± 13.6%)
Capsaicin (100 µM)	0	324	263	34	12.5% (± 1.0%)
	24	510	431	68	16.7% (± 13.4%)
	48	403	279	185	24.9% (± 42.4%)

Table 5. Proportion of active cells and agonist response. . Fluorescent Ca2+ imaging in response to three agonists was done in differentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n=3). Cell count represents ROI's defined using Fluosnnap. Cells were considered active if their peak fluorescence responses exceeded a 2% change from baseline, and cells were considered responsive to an agonist if peak response exceeded a 10% change from baseline.

3.4.3 ANALYSIS OF AGONIST RESPONSE

For each cell (i.e. ROI) identified with Fluosnnap, the change in fluorescence between the 100th frame and the 150th frame was used to establish Ca2+ response to the agonists. Cells were only included for analysis if their peak fluorescence response exceeded a 2% change from baseline. Results of each replicate culture (n = 3) were combined to obtain mean fluorescence response, average maximum response, the area under the curve (AUC) and the full half-maximum width (FHMW) for each agonist. This allowed for statistical comparison between non-treated F11 cultures and

F11 cultures treated with TNF-α for 24 or 48 hours. Data were analyzed by one-way ANOVA's with Dunnett's as post-hoc.

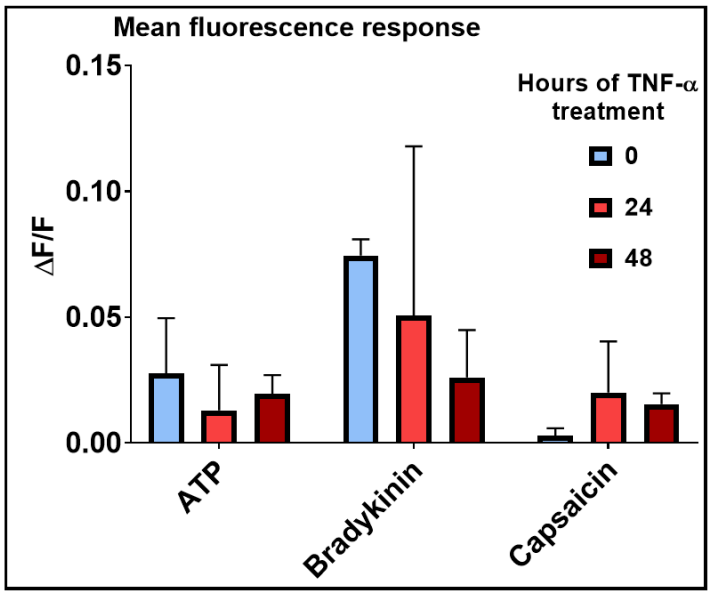


Figure 9. Mean fluorescence response . Fluorescent Ca2+ imaging was done in undifferentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n = 3). The bar graphs shows mean fluorescence response after addition of ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Data are expressed as mean + SD

TNF-α was not found to significantly affect mean fluorescence response (Figure 9 & 10), average maximum response (Figure 11 & 12), AUC or FHMW (not shown) for any of the agonists. However, in undifferentiated F11 cells (Figure 9), the mean fluorescence response caused by bradykinin was highest in untreated cultures and lowest in cultures treated with TNF-α for 48 hours. On the other hand, the mean fluorescence response caused by capsaicin was lowest in untreated cultures and higher in TNF-α treated cultures. In differentiated F11 cells (Figure 10), the same pattern emerged for bradykinin, but not for capsaicin, for which the mean fluorescence response was essentially equal regardless of treatment.

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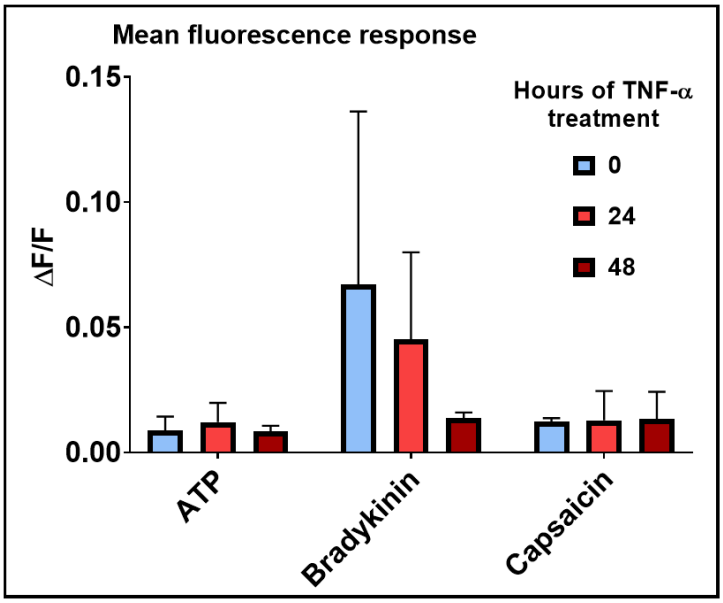

Figure 10. Mean fluorescence response . Fluorescent Ca2+ imaging was done in differentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n = 3). The bar graphs shows mean fluorescence response after addition of ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Data are expressed as mean + SD.

Data on the average maximum fluorescence response to the agonists in undifferentiated (Figure 11) and differentiated (Figure 12) cells also resemble the same patterns.

It is also noteworthy that for all agonists, the mean fluorescence response and average maximum fluorescence response were higher in undifferentiated cultures compared to differentiated cultures, even though differentiated cultures had a greater number of cells that were considered responsive to the agonists (as seen in Table 4/5). This suggests that differentiation increased the amount of cells responsive to the agonists, but that many of these cells had weak fluorescence responses to the agonists.

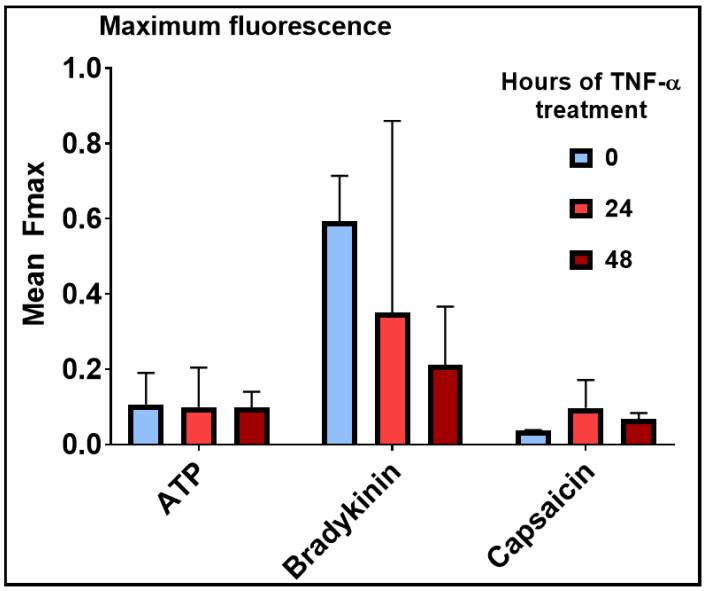

Figure 11. Maximum fluorescence response. Fluorescent Ca2+ imaging was done in undifferentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n = 3). The bar graphs show the average maximum fluorescence response of all cells after addition of ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Data are expressed as mean + SD.

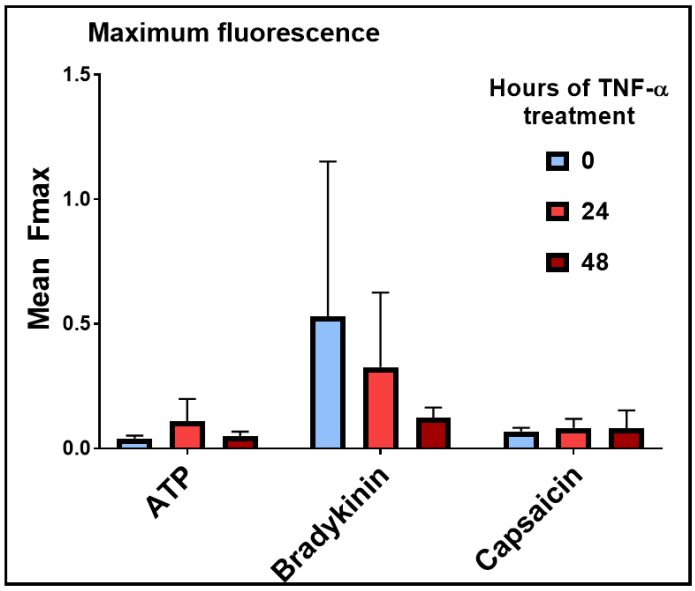

Figure 12. Maximum fluorescence response. Fluorescent Ca2+ imaging was done in differentiated F11 cells exposed to 0, 24 or 48 hours of TNF-α (10 ng/ml) treatment (n = 3). The bar graphs show the average maximum fluorescence response of all cells after addition of ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Data are expressed as mean + SD.

3.5 ELECTRICAL ACTIVITY

A MEA2100 multi electrode array (MEA) system was used for assessment of electrical activity in forskolin (30 μM) differentiated F11 cells. Figure 13 shows sample images of the array and the data output. The data were analyzed to obtain spiking activity per minute. Electrical activity (in picovolts) at any given point was counted as a spike if it were four standard deviations away from the average electrical activity in the preceding four milliseconds. Figure 14 shows data on baseline spiking activity in control wells and TNF-α treated wells.

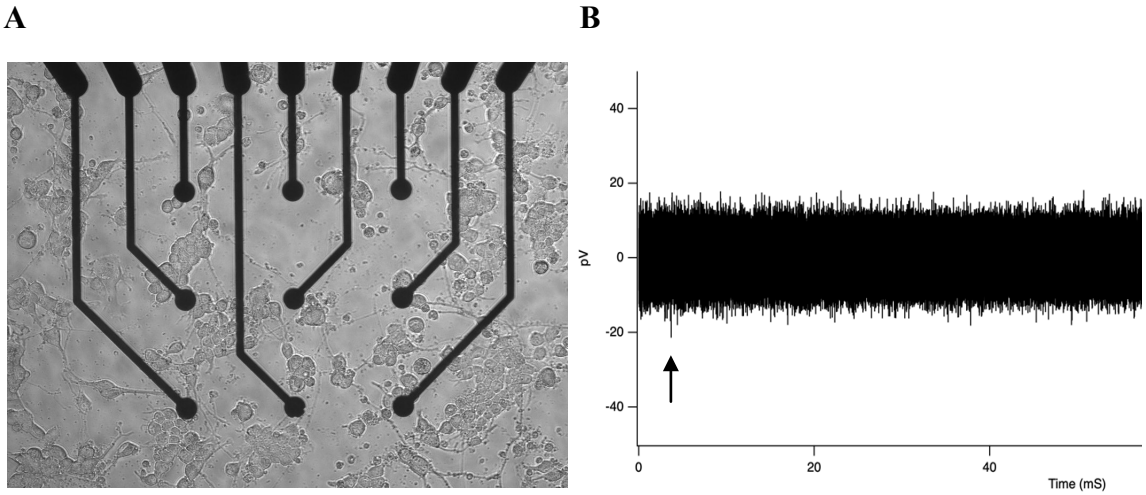

Figure 13. *Sample images of MEA* **A.** Microscopic image of multi electrode array connecting to differentiated F11 cells **B.** Sample data of electrical activity in picovolts of one electrode. Arrow indicates a potential spike.

As can be seen, baseline spiking activity was greater in TNF-α treated wells than in control wells. However, this difference did not reach statistical significance at α = 0.05. Electrical activity was also measured in response to ATP**,** bradykinin and capsaicin (Figure 15). These data were corrected for the baseline activity seen in figure 14. As can be seen, bradykinin and capsaicin provoked spiking activity, indicated by deviations from the 100% baseline. ATP however, did not provoke spiking activity. Moreover, there were no significant difference in spiking activity

between TNF-α treated wells and control wells for any of the tested agonists, although spiking activity in response to bradykinin and capsaicin was lower in TNF-α treated wells compared to control wells.

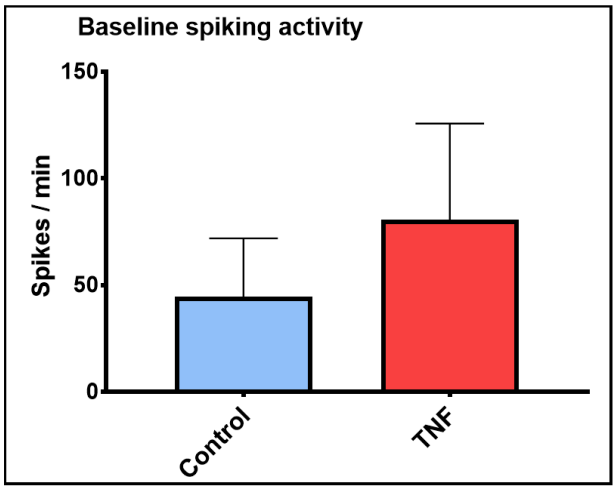

Figure 14. Baseline spiking activity in F11 cells. Electrical activity was measured in forskolin differentiated F11 cells. Data are expressed as spikes per minute + SD.

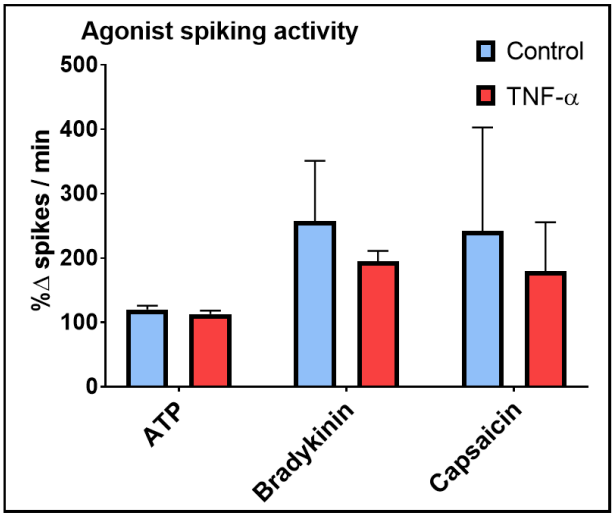

Figure 15. Agonist provoked spiking activity in F11 cells. Electrical activity was measured in forskolin differentiated F11 cells activity in response to ATP (10uM), bradykinin (20uM) and capsaicin (100 uM). Data are expressed as %Δ spikes per minute compared to baseline activity (Figure 14) + SD. Baseline activity is 100%.

Discussion

Chronic pain is thought to arise in part due to peripheral sensitization of DRG nociceptors. During injury and inflammation, the release of TNF-α and other inflammatory mediators can sensitize nociceptors by modulating the activity and expression of a variety of ion channels and receptors. Moreover, inflammatory mediators also influence cellular processes commonly involved in chronic pain states, such as cell survival and axonal growth to peripheral targets.

Chronic pain is a widespread healthcare crisis, and current treatment options are not always efficacious. Therefore, the use of immortal cell lines that resemble DRG neurons may be very valuable in the study of pain and the underlying cellular mechanisms. Such cell lines may also be used for high throughput studies of novel analgesics. One candidate is the F11 cell line. This DRG derived cell line has been described as having features similar to DRG nociceptors. However, while the effects of TNF-α on isolated DRG neurons are relatively well established, it is unclear what the effects of TNF-α are on the F11 cell line. If an inflammatory environment evoked by TNF-α elicits similar effects in F11 cells as in isolated DRG neurons, then this may validate the F11 cell line as a tool for studying the underlying mechanisms of inflammation and chronic pain.

Therefore, the current study analysed the effects of TNF-α in F11 cells on 4 endpoints: calcium signalling, spiking activity, neurite outgrowth and cell viability. All tests were performed both in undifferentiated F11 cells and forskolin differentiated F11 cells.

A pilot study confirmed that F11 cells produced extensive neurites in the presence of serum stripped culture medium supplemented with forskolin (30 μM). This is

congruent with other studies that often differentiate F11 cells with a similar dose of forskolin.

Apoptotic cell death is implicated in chronic pain states, and TNF-α can induce apoptosis in DRG neurons. Therefore, it was expected in the current study that TNF-α would reduce cell viability of F11 cells. However, the current study found no conclusive evidence that TNF-α altered cell viability in undifferentiated and differentiated F11 cells. While TNF-α treatment over longer time periods or at a higher dose could potentially alter F11 cell viability, the current data suggest F11 cells may not be useful in studying TNF-α mediated apoptosis.

In DRG neurons, TNF-α regulates neurite outgrowth, which is important in regulating axonal regeneration and the innervations of nociceptors. In the current study, TNF-α reduced neurite length in both differentiated and undifferentiated F11 cells, but this effect was inconsistent and no strong conclusions could be drawn from the current data.

Inflammation in DRG neurons leads to sensitization and increased expression of receptors for ATP, bradykinin and capsaicin, which all signal by increasing intracellular calcium levels. Therefore, in the current study it was expected that TNF-α treatment in F11 cells would increase Ca2+ influx induced by these agonists. However, fluorescent calcium imaging experiments found no evidence for this. TNF-α did not significantly alter the percentage of F11 cells responsive to the agonists, nor did it significantly alter the mean fluorescence response, average peak fluorescence response, AUC or FWHM. However, a few findings should be noted. Firstly, forskolin differentiation increased the amount of constitutively Ca2+ active cells and it increased the amount of cells responsive to the agonists. However, mean

Ca2+ influx and average maximum Ca2+ influx was smaller for all agonists in differentiated cells, suggesting many of new these newly responsive cells showed weak Ca2+ responses to the agonists.

Secondly, in the current study capsaicin provoked Ca2+ responses similar to those of ATP, suggesting the cells expressed functional TRPV1 receptors. This contradicts other studies on F11 cells in which capsaicin does not provoke Ca2+ influx, and it also contradicts studies in which TRPV1 RNA transcripts were found to be absent from F11 cells. It is unclear why this is, but differences in experimental parameters could play a role.

Thirdly, capsaicin response was essentially absent from undifferentiated F11 cells that were not TNF-α treated, while in TNF-α treated undifferentiated cells there was a capsaicin response. The fact that capsaicin response was only present in undifferentiated cells if they were TNF-α treated may suggest that TNF-α increased the expression of TRPV1. This would be congruent with studies on TNF-α treated DRG neurons. Nonetheless, capsaicin response was very low regardless of differentiation or TNF-α treatment, suggesting the F11 cell line may not be very useful in studying pharmacological agents that modulate TRPV1. The low Ca2+ response to ATP also suggests F11 cells may not be very useful in studying purinergic receptors.

A fourth trend was that in both undifferentiated and differentiated cultures, there was a tendency for TNF-α treated cultures to have a lower percentage of cells responsive to bradykinin. Mean Ca2+ responses and average maximum Ca2+ responses to bradykinin were also lowest in TNF-α treated cultures. This is surprising given that TNF-α is known to upregulate bradykinin receptors. Nonetheless, Ca2+ response to

bradykinin was large, which is congruent with previous Ca2+ imaging studies in F11 cells. Therefore, F11 cells may be useful in studying potential analgesics/ant inflammatory drugs that antagonize bradykinin receptors.

In DRG neurons, TNF-α increases spontaneous firing and agonist induced firing by modulating activity and expression of voltage gated ion channels, ligand-gated receptors and g-protein coupled receptors. In the current study, TNF-α increased spiking activity in differentiated F11 cells, which may represent a reduction of activation threshold by modulation of voltage gated ion/potassium channels. However, this increase was not statistically significant.

Moreover, spiking activity was present in response to bradykinin and capsaicin, but essentially absent in response to ATP, which further indicates that purinergic receptor expression is low in this cell line. Also, TNF-α did not significantly alter spiking activity in response to any of the agonists. In fact, TNF-α treated wells saw a decrease in bradykinin and capsaicin provoked spiking activity. These results do not suggest that TNF-α sensitized or upregulated capsaicin or bradykinin receptors.

In conclusion, the discrepancy between TNF-α's effects on primary DRG neurons and F11 cells suggest that the F11 cell line may not be an appropriate model to study the underlying mechanisms of TNF-α mediated chronic pain. TNF-α had no effect on F11 cell viability and its effects on neurite outgrowth were inconsistent. Moreover, TNF-α did not sensitize these cells to pain eliciting agonists and it did not reduce the activation threshold of the cells.

However, to the experimenter's knowledge there have been no prior studies in which F11 cells were treated with TNF-α. Therefore, future replication studies are necessary to verify the current results.

Moreover, the results of the current study are subject to limitations. Firstly, all experimental techniques used in this study were novel to the experimenter, which could undermine the quality of the data due to inexperience. Secondly, it is possible that the images taken for measuring of neurite outgrowth were not fully representative of their condition, which could lead to statistical artefacts. Thirdly, during the MTT assays, the formazan crystals were difficult to dissolve and required pipetting of the liquid to facilitate dissolvement. Moreover, since no software was available to read absorbance in 48 well plates, the contents of the plates were first transferred to 96 well plates, which further increased the odds of pipetting error.

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