THE ROLE OF PHYSALIN F IN REVERSING EXPERIMENTAL NEUROPATHIC PAIN

By

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ABSTRACT

Chronic pain continues to be an insurmountable issue in recent times, particularly due to the lack of adequate knowledge as well as the unavailability of appropriate therapeutics that target it. The increasingly common prescription of opioids as medication to treat chronic pain has ultimately contributed to a national opioid epidemic. In response to this, several strides have been made by the Center for Disease Control and Prevention (CDC) to revise guidelines pertaining to opioid prescription for chronic pain. This project specifically focuses on identifying the mechanism of action of Physalin F, a natural compound isolated from the *Physalis acutifolia* (family: Solanaceae) herb, and previously demonstrated to exhibit antinociceptive effects in models of inflammatory pain, consistent with earlier reports of its anti-inflammatory and immunomodulatory activities. Through the use of calcium imaging, it was revealed that Physalin F had a significant inhibitory effect on voltage-gated calcium channels in dorsal root ganglion. In order to replicate these findings in-vivo, Physalin F was found to reverse mechanical allodynia in both paclitaxel-induced and spinal nerve ligation-induced neuropathic pain models, further elucidating its antinociceptive behavior. Experimentation results provided a better scope on Physalin F mechanism of action as well as grounds for further investigation.

INTRODUCTION

Pain transduction is a necessary mechanism that the body requires to identify noxious stimuli that threaten homeostasis. Pain in itself is quite complex and can be largely categorized into nociceptive and neuropathic. This distinction is vital as both can be attributed to varying causes and pathways. Neuropathic pain, which is the focus of this paper, can be defined as pain associated with damage to the somatosensory system[1]. Although nociceptive pain offers evolutionary benefits, neuropathic pain largely tends to be maladaptive[1]. In addition to this, allodynia can be observed in most cases of neuropathic pain, which is the sensitization of pain achieved through the repeated stimulation of nociceptors in response to a non-painful stimulus. Neuropathic pain can be observed in instances of inflammation or metabolic disease such as diabetes[7]. While there exists a variety of treatments available for nociceptive pain such as opioids and non-steroidal inflammatory drugs, chronic pain is much harder to manage, primarily due to a lack of knowledge surrounding the pathways that drive it[1]. This has led to the use of opioids as a course of pain management for neuropathic pain, which has contributed to the opioid epidemic currently overtaking North America.

The role of prescription drugs in the lives of the average individual has seen a dramatic rise in the past two decades. The United States has been in the midst of a colossal opioid crisis for the past two decades, with approximately 2.4 million people contracting severe opioid disorder (OUD) attributed to an over-dependence on prescribed opioid medications, recreational heroine, or both[6]. Furthermore, opioid-related deaths reached a new high in 2013, surpassing deaths caused by motor vehicle accidents[6]. Since 2016, significant strides have been made by the U.S government to quell the escalating epidemic by making medication-assisted treatment such as methadone or naltrexone more accessible to the general population in order to treat an

overdose. However, the true obstacle lies within the pharmaceutical guidelines for the prescription of opioids. In response to the epidemic, the Center for Disease Control and Prevention (CDC) have revised the guidelines to lay emphasis on the avoidance of opioids in cases of chronic, non-cancerous, pain. In addition to this, the CDC recommends physicians to start opioid treatments with small dosages for patients who do qualify[6]. Although these guidelines do offer a decent foundation, there still exists a large space for improvement. A far more effective means to curb the opioid crisis, as acknowledged by the National Institute of Health, is to move away from opioid-related medications and instead identify alternate pharmacological targets through the development of non-opioid therapeutics for pain management[5].

In order to investigate potential therapeutic targets for chronic pain, it is important to first understand the process of pain conduction process. The chief players responsible for pain perception are the central nervous system (CNS) and peripheral nervous system (PNS). The PNS consists of the nerve fibers and ganglia that carry sensory information to the brain and the spinal, together known as the CNS, which works to integrate incoming sensory information and coordinate appropriate responses[1]. The fundamental mechanism of pain generation in response to noxious stimuli can be condensed into three basic steps: transduction, transmission, and modulation[1]. Transduction occurs when pain receptors, known as nociceptors, convert noxious stimuli into chemical signals. Such signals are then converted to electrical signals transmitted to the CNS via nerve fibers and, with the help of neurotransmitter, released at synaptic clefts. Modulation of these signals occurs at any part of the pathway, be it at the synaptic clefts or higher up in the CNS, through up or down-regulation. All three steps of the nociceptive pathway results in the sensation of pain[1]
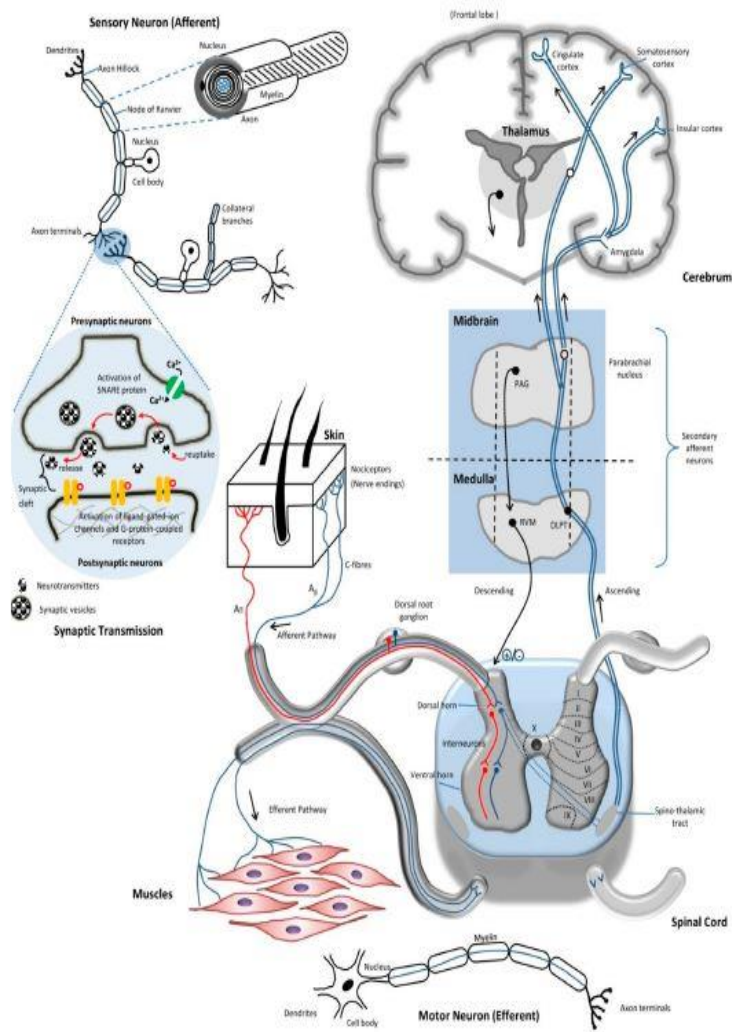
Figure 1: Schematic of nociceptive transmission involving the PNS and CNS, as well as a detailed depiction of synaptic transmission[1]

Transmission of nociceptive signals from one neuron to another is mediated by neurotransmitter release at the axon terminal and involves an action potential, as depicted in **Figure 2**. Typically, the resting potential of a cell stands at nearly -70 mV, which implies that the axon of a neuron is negatively charged at rest. This can be attributed to the higher concentration of Na^+ ions outside the cell than K^+ cells within the cell. The movement of these ions is regulated via activation of ion channels. As a result, when the pain receptors of the body are exposed to a noxious stimulus, a potential is generated within the axon. The threshold potential, which is the

potential at which all sodium channels within the axon open, is reached and allows for sodium ions to enter the axon. This results in a dramatic rise in voltage within the cell to approximately 40mV and depolarization of the axon. Once depolarization is achieved, an action potential is propagated through the axon to other neurons. Soon after, the voltage-gated Na^+ channels are restored to their inactive state, the voltage-gated K^+ channels are activated. This leads to an efflux of K^+ ions, resulting in a dramatic fall of voltage within the cell and repolarization of the axon. Resting potential is at last achieved when the voltage-gated K^+ channels are closed and the Na^+/K^+ transports reset the intracellular ion concentration[7].

The role of calcium ions and voltage-gated calcium channels becomes increasingly relevant once the action potential travels to the axon terminal. These specific channels play a key role in synaptic transmission and allow the pain stimuli to be transmitted to the adjacent neuron in the form of electrical impulses. The gap that exists between the presynaptic terminal of one neuron and the postsynaptic terminal of the adjacent neuron is known as the synaptic cleft. The synaptic cleft is involved in distinct chemical events responsible for impulse transmission. The arrival of the action at the presynaptic terminal triggers the depolarization of the presynaptic membrane via the activation of voltage-gated Na^+ channels. This is quickly followed by the activation of voltage-gated Ca^{2+} channels, which allow Ca^{2+} influx into the axon terminal. The membrane depolarization elicits the exocytosis of neurotransmitters and Ca^{2+} ions into the synaptic cleft. The released neurotransmitters continue to bind to their corresponding ligand-gated ion channels on the postsynaptic membrane, which permits the propagation of the action potential to the adjacent neuron[7].

This is pertinent to the project, as I attempt to induce depolarization in dorsal root ganglia of the spinal cord. Since depolarization is an important marker of pain transduction, it is an

excellent means to clinically observe experimental pain and test to see if the cells treated with the compound of interest inhibit depolarization either completely or to a significant degree. This inhibition indicates that the compound is successful at obstructing pain transduction and possesses potential antinociceptive properties.
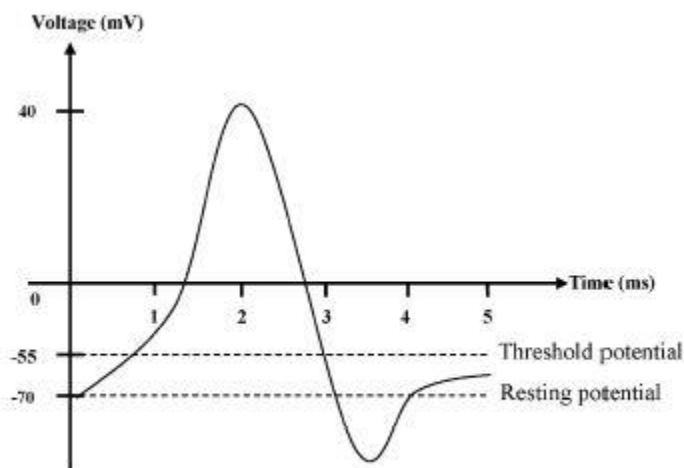
Figure 2: Components of an action potential[1]

Physalin F ($C_{28}H_{30}O_{10}$; molecular weight: 526.538 g/mol; **Figure 3)**, a derivative of the herb *Physalis acutifolia,* have been previously established to have anti-nociceptive, anti-inflammatory, and immunomodulatory properties[3]. This compound is known to inhibit the pathway of NF-KB activation; a prominent pathway responsible for the body's inflammatory response. It is also associated with pro-inflammatory factors such as tumor necrosis factor (TNF)-alpha, interleukin-6, and interleukin-12, all of which are key components of the body's mounting immune response[3]. While the compound's link to immune and inflammatory pathways can be isolated, Physalin F's antinociceptive properties are largely unknown. Recent work submitted by Villarreal and collaborates suggests that Physalin F's anti-nociceptive effects may be involved with pain transduction pathway, which was observed through its inhibition of writhing behavior generated using acetic-acid injections as well as inhibition of both early

nociceptive and late inflammatory stages of the formalin test[5]. Anti-nociceptive properties were also observed through the results of the tail-flick assay and hot-plate assay, which are indicative of the involvement of descending spinal pathways and supraspinal integrated responses respectively[3].
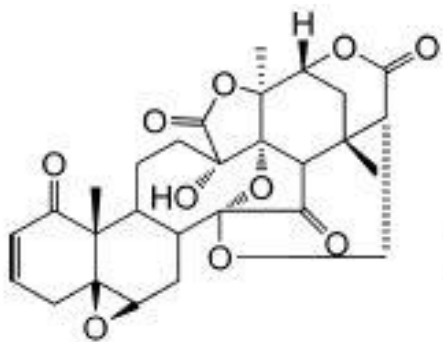
Figure 3: Chemical structure of Physalin F[3]

MATERIALS AND METHODS

Animals

The cells used for all experimentation were dorsal root ganglia extracted from female Sprague-Dawley rats. The cells were extracted according to the protocol approved by the Institutional Animal Care and Use Committee of the College of Medicine at the University of Arizona[4]. The clinical procedures were conducted in conjunction with the ethical guidelines set forward by NIH and the International Association for the Study of Pain[4]. The cells were mounted on 15mM poly-D-lysine coverslips followed by incubation with either control or compound conditions for 24 hours prior to calcium imaging.

Calcium Imaging

Calcium signaling cascades play a significant role in the propagation of pain signals within the somatosensory system. A typical cell has a resting intracellular calcium concentration

of approximately 50-100 nM, which tends to rise nearly 10-100 times higher during transmission of electrical signals[2]. In order to keep track of changes in action potential in vitro is by monitoring the activation of voltage-gated calcium channels. For this purpose, calcium imaging was the primary imaging technique used to acquire both qualitative and quantitative data. Calcium imaging is advantageous as it provides both a qualitative visualization and quantitative estimation of the action potential within the neuronal cells. This specific imaging technique employs the use of a ratiometric fluorescent dye known as Fura-2AM, which binds to intracellular calcium ions and allows for quantitative measurements of calcium influx when cells are triggered with depolarizing stimuli. Since FURA-2AM is excitable at both 340 nm and 380nm, the ratio of both wavelengths corresponds to the ratio of binding to unbound calcium ions within the cell[2]. The imaging was conducted with the help of an inverted microscope and the excitation light was produced by the Lamba-LS system[4]. Experiments were run for 8 minutes per coverslip, with cells exposed to either 40mM potassium chloride or 90mM potassium chloride, which work to activate either low or high activated voltage-gated calcium channels respectively. The two triggers are administered to the cells at 1 and 6 minutes for 15 seconds, and the peaks within these two time slots are measured and compared. Apart from the triggers, cells are exposed to Normal Tyrode's in order to maintain cell viability and resting potential.

Paclitaxel-Induced Pain Model

Adult male rats were treated with paclitaxel through an intraperitoneal administration of Paclitaxel. The rats were given a dosage of 2mg/kg every other day, adding up to a total dosage of 8mg/kg. The rats were then administered either saline (vehicle) or Physalin F (2ug/5 uL). Mechanical allodynia was achieved 10 days after initial paclitaxel administration[4]. Soon after,

paw withdrawal threshold of rats was measured before intrathecal injection of either saline (vehicle) or Physalin F, as well as an hour post injection for four successive hours. Paw withdrawal thresholds were then compared between the control rats and the Physalin F-treated rats.

**Spinal Nerve Ligation-Induced Pain Model**

Spinal nerve ligation was performed in order to induce mechanical allodynia. All adult male rats underwent intrathecal catheterization, 5 days after which all nerve procedures were conducted. Once the rats were anesthetized using 2% isoflurane in O_2, the L_5 and L_6 spinal nerves were isolated and ligated to the dorsal root ganglia, while carefully preserving left hind paw function. The animals were given 7 days of recovery before experimentation[4]. Paw withdrawal threshold was then measured 15 days after surgery.
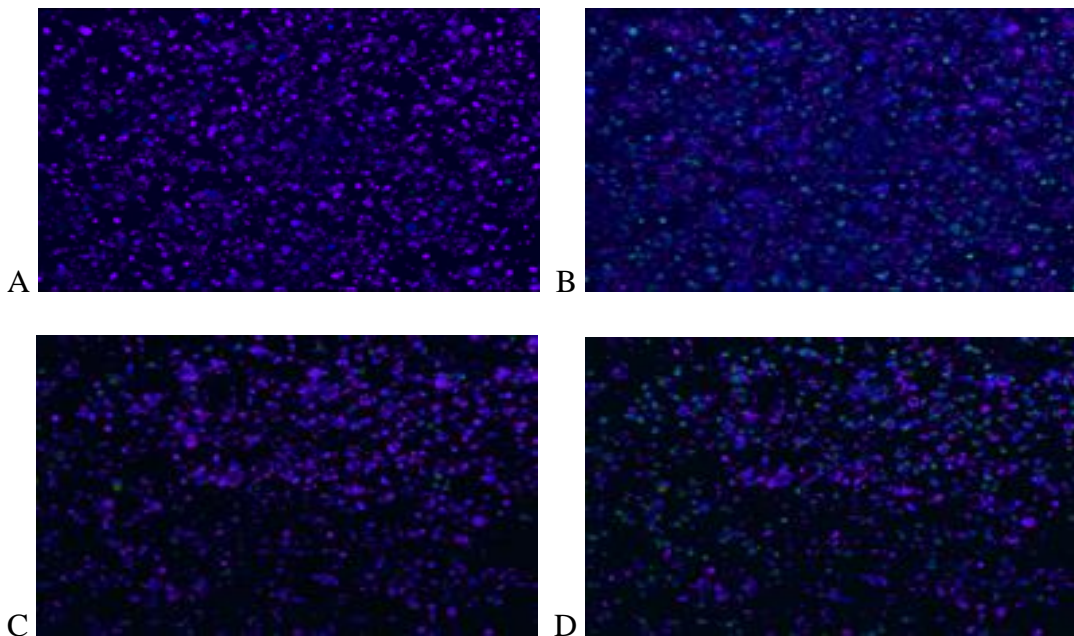
RESULTS

Calcium Imaging Analysis Data
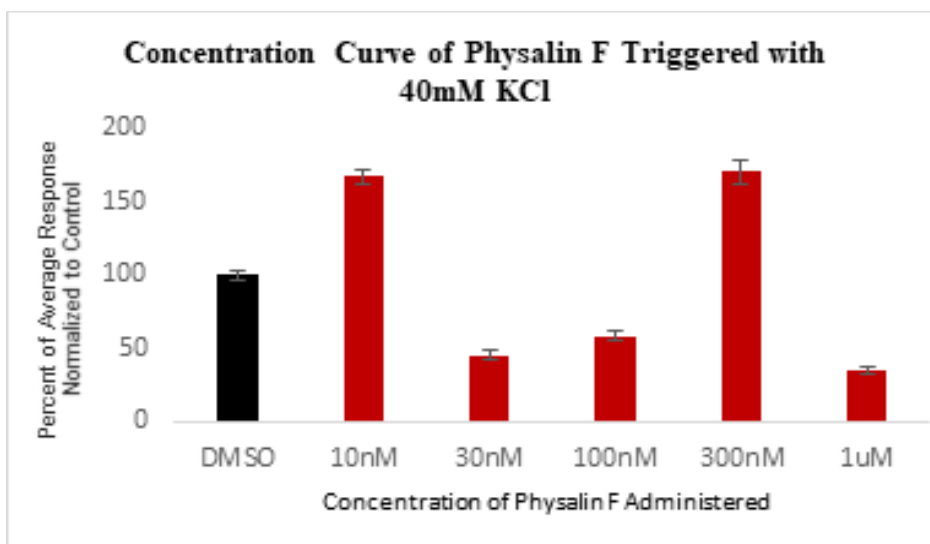
Figure 4: Average normalized response of the dorsal root ganglia when triggered with 40 mM KCl and treated with varying concentrations of Physalin F
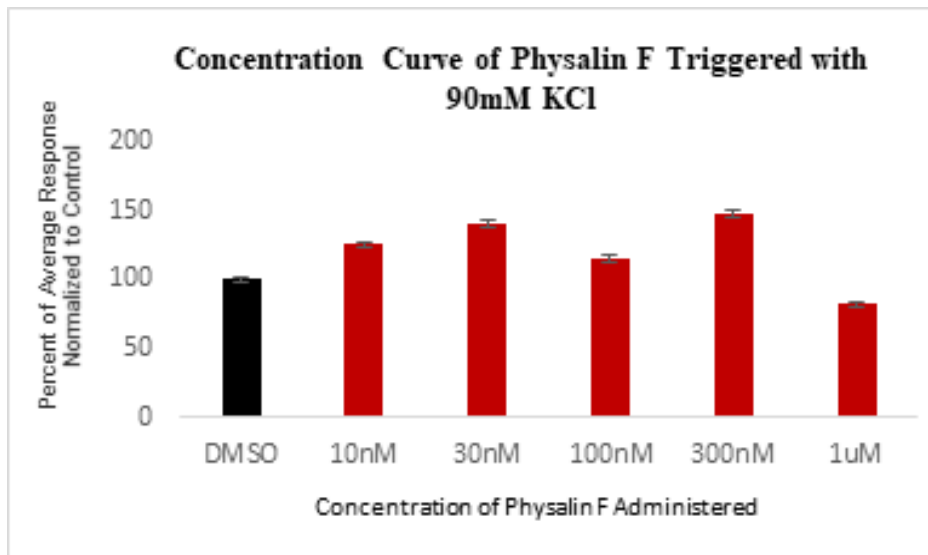
Figure 5: Average normalized response of the dorsal root ganglia when triggered with 90 mM KCl and treated with varying concentrations of Physalin F


Figure 6: Calcium imaging snapshots of dorsal root ganglia responding to buffers (A) Response of dorsal root ganglia to 40 mM KCl when treated with 0.1% DMSO (B) Response of dorsal root ganglia to 90 mM KCl when treated with 0.1% DMSO (C) Response of dorsal root ganglia to 40 mM KCl when treated with Physalin F (D) Response of dorsal root ganglia to 90 mM KCl when treated with Physalin F

Neuropathic Pain Model Data
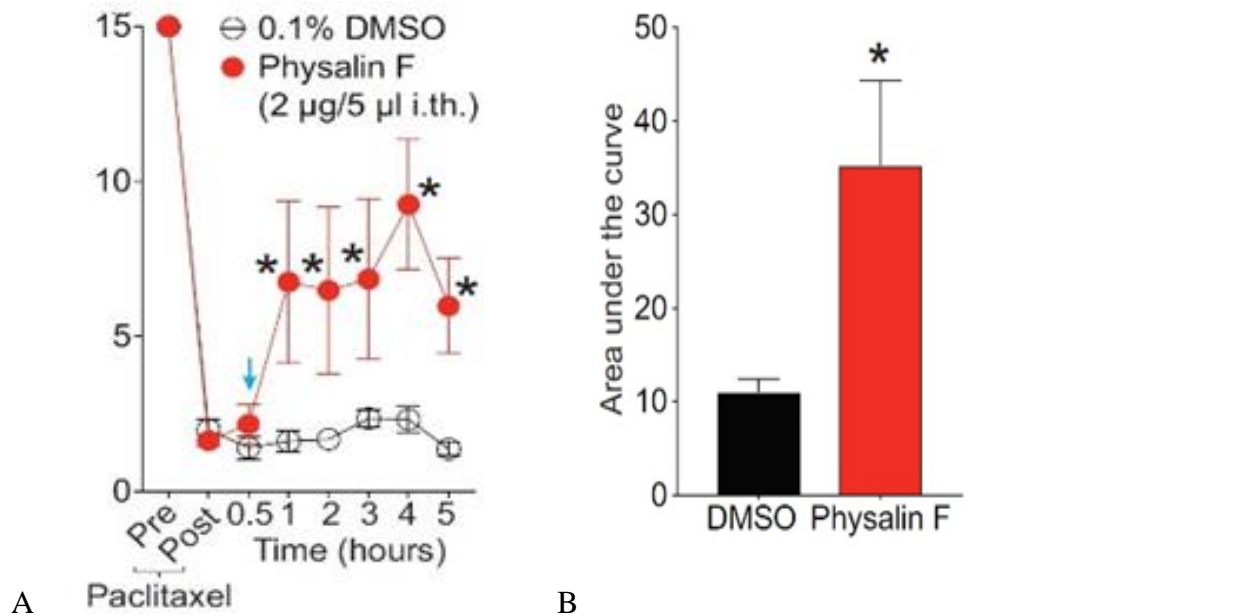
Figure 7: Paclitaxel-induced neuropathic pain model (A) Paw withdrawal threshold measured in rats treated with 0.1% DMSO and Physalin F, both pre and post injection of treatment as well as for the subsequent 4 hours[5] (B) Area under the curve measured for rodents treated with Physalin F as compared to DMSO within the window of allodynia reversal[5]
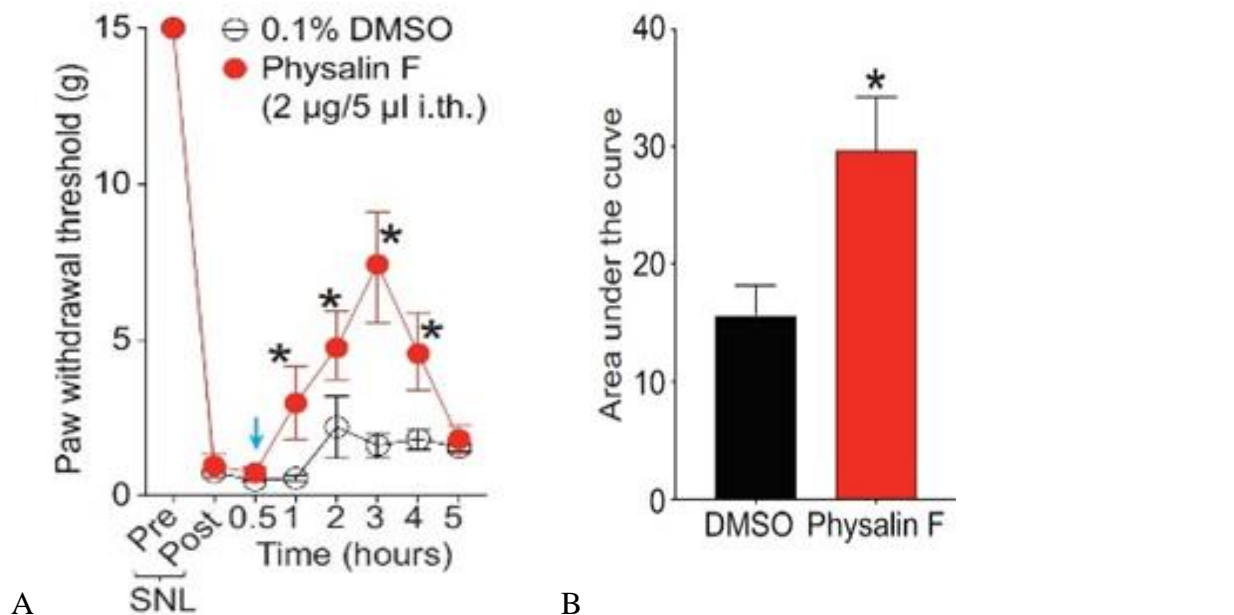
Figure 8: Spinal nerve ligation-induced neuropathic pain model data (A) Paw withdrawal threshold measured in rats treated with 0.1% DMSO and Physalin F. pre and post injection of treatment as well as for the subsequent 4 hours[5] (B) Area under the curve measured for rodents treated with Physalin F as compared to DMSO within the window of allodynia reversal[5]

DISCUSSION

Physalin F inhibits depolarization induced by calcium influx in dorsal root ganglia

Prior to experimentation, Physalin F was proposed to have antinociceptive and anti-inflammatory effects[3,5]. However, the mechanism of action remains relatively unknown. This project aims to distinguish a distinct nociceptive pathway with a targeted focus on voltage-gated Ca^{2+} channels, which are highly expressed on dorsal root ganglia and involved in pain transmission. Since nociceptive properties were previously established, this project strived to identify an effective working concentration that achieved the most effective results. In order to test the effect of Physalin F on voltage-gated Ca^{2+} channels and measure calcium influx, both 40mM potassium chloride stimulus and 90 mM potassium chloride stimulus is used to induce depolarization by activating low- and high-voltage activated calcium channels respectively. For this reason, neurons were treated with either 0.1 % DMSO or varying concentrations of Physalin F (10nM, 30nM, 100nM, 300nM, and 1uM). **Figure 3** represents the average response of the DRGs to the 40mM KCl when treated with Physalin F. According to the results, DRGs treated with 1uM Physalin F showed nearly a 50% decrease in calcium-induced depolarization as compared to the control. This indicates potential inhibition of low-voltage gated calcium channels and would require a further in-depth investigation for the replication of acquired results. Figure 4 represents the average response of DRGs to 90 mM KCl when treated with multiple Physalin F concentrations. As seen in the figure, DRGs treated with 1uM Physalin F once again exhibited significantly decreased depolarization (~20%) elicited by calcium ion influx as compared to control. This also suggests potential inhibition of high voltage-activated Ca^{2+} channels by Physalin F and offers a route for further research. **Figure 5** represents a qualitative depiction of the results as it presents FURA-2AM-mediated calcium imaging of DRGS. Figure 5a and 5b

describe DRG depolarization when treated with 0.1% DMSO and triggered by 40mM KCl and 90mM KCl respectively. Neurons presenting as purple represent a typical resting potential while neurons presenting as green indicated depolarization. As expected in the control, 90mM KCl elicits a more exaggerated response than 40mM KCl due to differences in voltage activation. **Figure 5C** and **5D** describe DRGs treated with 1uM Physalin F and triggered with 40mM KCl and 90mM KCl respectively. Qualitatively, 1uM appear to inhibit depolarization as compared to the control due to the diminished presence of DRGs imaged as green. These results indicate that Physalin F has a significant effect on voltage-gated Ca^{2+} channels in neurons.

Physalin F exhibits antinociceptive behavior in Paclitaxel-induced pain model

Calcium imaging helped reveal that voltage-gated Ca^{2+} channels are a good therapeutic target for Physalin F. In addition to this, 1uM Physalin F was established as an appropriate working concentration for further experimentation. The subsequent route of experimentation was to test the efficacy of Physalin F's antinociceptive properties in-vivo. This was first accomplished through the use of chemotherapy-induced neuropathic pain model. In this model, mechanical allodynia was induced through the administration of paclitaxel, followed by intrathecal injection of either saline (vehicle) or Physalin F, as described in the methods. According to **figure 7A**, the results indicate a significantly higher paw withdrawal threshold in rats treated with Physalin F as compared to the control rats post-injection. This increase in threshold was also sustained for four successive hours, indicating significant nociceptive behavior. Figure 7B represents this data through an area under the curve graph, which signifies a significantly higher paw withdrawal threshold observed in rats treated with Physalin F as compared to the control rats.

Physalin F exhibits antinociceptive behavior in SNL-induced pain model

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Physalin F was also tested through an SNL-induced pain model, which involves partially disrupting the sciatic nerve[5]. Once allodynia was achieved, the rats were treated with either saline (vehicle) or Physalin F. As shown in **figure 7A**, the rats treated with Physalin F showed a dramatic reversal of allodynia within a 4-hour period as compared to the control rats. These results were mirrored in **figure 7B** through an area under the curve graph which indicated a significantly higher paw withdrawal threshold among the Physalin F treated rats when compared to control. These results help conclude that Physalin F has a significant inhibitory effect on experimental neuropathic pain.

CONCLUSION

Experimentation helped revealed that Physalin F indeed had a significant effect on the voltage-gated calcium channels and exhibits antinociceptive behavior both in vitro and in vivo. Calcium imaging results revealed significantly lower responses presented by the dorsal root ganglia when treated with a 1uM concentration of Physalin F, as compared to the control. These results were reflected both quantitatively (**Figure 4 and 5**) and qualitatively (**Figure 6**). These results are substantial because of the role of calcium signaling in synaptic transmission. By targeting voltage-gated calcium channels, Physalin F affects pain transmission. The compound also reaffirmed antinociceptive behavior through both paclitaxel-induced and spinal nerve ligation-induced neuropathic pain models.

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