An Investigation on the Role of Oxytocin in Chronic Neuropathic Pain in a Wistar Rat Model

by

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March 2023
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Abstract

**Introduction** Chemotherapy-induced peripheral neuropathy (CIPN) is a dose-limiting side effect with ineffective preventative and curative treatment, due to the condition's complexity perpetuated by the extensive central involvement, including the chronic disruption and subsequent dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Currently, only Duloxetine has been recommended as effective treatment for CIPN, which has shown individual-dependent, short-term analgesic effects, with limiting adverse effects and poor bioavailability. The neuropeptide, oxytocin, may offer significant analgesic and anxiolytic potential, as it exerts central and peripheral attenuating effects on nociception. However, it is unknown whether the intervention administered in a model of CIPN is an effective therapeutic alternative.

**Materials and Methods** The intervention was divided into two phases. Phase 1 aimed to induce CIPN in adult Wistar rats using the chemotherapeutic agent *Paclitaxel*. Mechanical (electronic von Frey filament) and thermal (acetone evaporation test and Hargreaves test) hypersensitivity testing was used to evaluate changes due to the neuropathic induction. Phase 2 consisted of a 14-day intervention period with saline (o.g.), Duloxetine (o.g.), or oxytocin (i.n.) administered as treatment. Analgesic behavioural testing was assessed throughout the intervention period. Following the intervention, anxiety-like behaviour was assessed using the elevated plus maze (EPM) and light-dark box protocols. Analysis of peripheral plasma corticosterone, peripheral plasma oxytocin, and hypothalamic oxytocin concentrations were assessed using ELISA assays.

**Results** The findings showed that we were able to successfully establish a model of chemotherapy-induced peripheral neuropathy during Phase 1, determined by the increase in mechanical and thermal nociceptive responses following chemotherapy administration. Furthermore, based on this finding, we were able to evaluate the effect of different treatments administered in the presence of CIPN. The animals treated with oxytocin displayed a significant improvement in mechanical sensitivity over the intervention phase, indicative of an improvement in nociceptive tolerance in the presence of neuropathic pain. Animals that received *Paclitaxel* and treated with oxytocin also displayed significantly greater explorative behaviour during the EPM, indicative of a reduced presence of anxiety-like behavior.
**Conclusion** Our results support the hypothesis that intranasally administered oxytocin may augment the analgesic and anxiolytic effects of duloxetine in a chemotherapy-induced peripheral neuropathy model in a Wistar rat. Administered in conjunction, oxytocin and duloxetine may provide enhanced therapeutic effects in the treatment of CIPN. Further research is necessary to establish optimal treatment and dosage requirements.

*Footnote: this thesis will employ US-based spelling and grammar notations*
Opsomming

**Inleiding:** Chemoterapie-geïnduseerde perifere neuropatie (CIPN) is ‘n dosering-beperkte newe effek, met ondoeltreffende voorkomende en genesende behandeling. Dit is as gevolg van die toestand se kompleksiteit, wat vererger word deur die omvattende sentrale meewerking, wat chroniese ontwrigting en daaropvolgende wanregulering van die hipotalamus-pituïtêre-bynieres (HPA) insluit. Tans word slegs Duloxetine aanbeveel as effektiewe behandeling vir CIPN, wat individu-afhanklike, korttermyn pynstillende effekte getoon het. Dit het egter beperkende nadelige effekte en swak biobeskikbaarheid. Die neuropeptied, oksitosien, kan aansienlike pynstillende en anxiolitiese potensiaal bied, aangesien dit sentrale en perifere verlaging van nosisepsie tot gevolg het. Dit is egter onbekend of die intervensie wat in ’n model van CIPN toegedien word, ’n effektiewe terapeutiese alternatief is.

**Materiale en Metodes:** Die intervensie is in twee fases verdeel. Fase 1 het ten doel gehad om CIPN in volwasse Wistar-rotte te induseer deur die chemoterapeutiese middel Paclitaxel te gebruik. Meganiese (elektroniese von Frey filament) en termiese (asetoon verdampingstoets en Hargreaves toets) hipsersensitiwiteitstoetse is gebruik om veranderinge as gevolg van die neuropatiese induksie te evaluateer. Fase 2 het bestaan uit ’n 14-dae intervensieperiode met saline (o.g.), Duloxetine (o.g.) of oksitosien (i.n.) toegedien as behandeling. Analgetiese gedragstoetsing is deur die intervensieperiode geevaluateer. Na die intervensie is angs gedrag geevalueer deur gebruik te maak van die verhoogde plus doolhof (EPM) en lig-donker boks protokolle. Ontleding van perifere plasma kortikosteroon, perifere plasma oksitosien en hipotalamus oksitosien konsentrasies is geassesseer met behulp van ELISA toetse.

**Resultate:** Die bevindinge het getoon dat ons ’n model van chemoterapie-geïnduseerde perifere neuropatie suksesvol kon vestig tydens Fase 1. Verder, gebaseer op hierdie bevinding, was ons in staat om die effek van verskillende behandelings wat in die teenwoordigheid van CIPN toegedien is, te evaluateer. Die bevindinge het getoon dat ons ’n model van chemoterapie-geïnduseerde perifere neuropatie tydens Fase 1 suksesvol kon vestig. Die diere wat met oksitosien behandel is, het ’n aansienlike verbetering in meganiese sensitiwiteit oor die intervensiefase getoon, wat dui op ’n verbetering in nosiseptiewe toleransie in die teenwoordigheid van neuropatiese pyn. Diere wat Paclitaxel ontvang en met
oksitosien behandel is, het ook aansienlik groter verkennings gedrag tydens die EPM getoon, wat dui op 'n verminderde teenwoordigheid van angsagtige gedrag.

**Afsluiting:** Ons resultate ondersteun die hipotese dat intranasaal toegediende oksitosien die pynstillende en anxiolitiese effekte van duloksetien kan versterk in 'n chemoterapie-geïnduseerde perifere neuropatie-model in 'n Wistar-rot. As dit saam toegedien word, kan oksitosien en duloksetien verbeterde terapeutiese effekte in die behandeling van CIPN bied. Verdere navorsing is nodig om optimale behandeling en dosisvereistes vas te stel.

*Voetnota: hierdie tesis sal gebruik maak van VS-geweeral gebaseerde spelling en grammatika notasies.*
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<tbody>
<tr>
<td>4PL</td>
<td>4 Parameters Logistics</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytrytamine</td>
</tr>
<tr>
<td>ACC</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine-vasopressin</td>
</tr>
<tr>
<td>AVP-V1a</td>
<td>arginine-vasopressin-V1a</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell leukaemia/lymphoma 2 protein</td>
</tr>
<tr>
<td>BL</td>
<td>baseline</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
</tr>
<tr>
<td>BORIS</td>
<td>Behavioural Observation Research Interactive Software</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CCI</td>
<td>chronic constriction injury</td>
</tr>
<tr>
<td>CeA</td>
<td>central nuclei of the amygdala</td>
</tr>
<tr>
<td>CeCL</td>
<td>latero-capsular part of the central nuclei</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin-gene related peptide</td>
</tr>
<tr>
<td>CIPN</td>
<td>chemotherapy-induced peripheral neuropathy</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>chlorine</td>
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<td>CNS</td>
<td>central nervous system</td>
</tr>
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<td>CORT</td>
<td>corticosterone</td>
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<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>CuZnSOD</td>
<td>copper-zinc-superoxide-dismutase</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>EPI</td>
<td>epinephrine</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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EE – environmental enrichment
EPM – elevated plus maze
fMRI – functional magnetic resonance imaging
GABA – γ-aminobutyric acid
GAS – General Adaptation Syndrome
GPCRs – G-protein coupled receptors
GPx – glutathione peroxidase
GR – glucocorticoid receptor
HPA – Hypothalamic-pituitary-adrenal
HRP – Horseradish peroxidase
HWL – hindpaw withdrawal latency
i.n. – intranasal
i.p. – intraperitoneal
IASP – International Association for the Study of Pain
IkB – inhibitory kappa B
IL-6 – interleukin 6
K+ – potassium
LC – locus coeruleus
LDB – light dark box
LTP – long-term potentiation
MAPK – mitogen-activated protein kinase
MnSOD – manganese superoxide dismutase
MR – mineralocorticoid receptor
Na2+ – sodium
NE – norepinephrine
NFkB – nuclear factor kappa B
NGF – nerve growth factor
nm – nanometer
NMDA – N-methyl-D-aspartate
NPP – Neuropathic pain
<table>
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<th>Abbreviation</th>
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<td>OT</td>
<td>oxytocin</td>
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<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PAC</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal grey</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP ribose polymerase</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDN</td>
<td>peripheral diabetic neuropathy</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PHN</td>
<td>postherpetic neuroglia</td>
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<td>PI</td>
<td>post-injection</td>
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<td>PIPN</td>
<td>Paclitaxel-induced peripheral neuropathy</td>
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<td>PVN</td>
<td>paraventricular nucleus</td>
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<td>PWT</td>
<td>paw withdrawal threshold</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>RVM</td>
<td>rostral ventromedial medulla</td>
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<td>SAM</td>
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<td>SAPK</td>
<td>stress-activated protein kinase</td>
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<td>SARM</td>
<td>selective androgen receptor modulator</td>
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<td>TMB</td>
<td>tetramethylbenzidine</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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TSP – thrombospondin
VGCC – voltage gated calcium channel
VFPC – voltage gated potassium channel
VGSC – voltage gated sodium channel
VTA – ventral tegmentum area
α2-δ1 – alpha-2/delta-1
1 Introduction

Chronic pain, defined as persisting pain for more than 3 consecutive months, is one of the leading causes of human disability and suffering (Treede et al., 2019). One of the most prevalent forms of chronic pain is neuropathic pain (NPP), defined as, “pain arising as a direct consequence of a disease or lesion of the somatosensory nervous system at either the central or peripheral level” (Treede et al., 2019). With a global estimated 6.9-10% of adults experiencing pain with neuropathic characteristics (van Hecke et al., 2014; Murnion, 2018; Tai, Yeung and Cheung, 2018), it is a crisis that requires drastic action to improve current treatment outcomes. This high prevalence is not only a representation of human suffering, but also of debilitating social and financial burden the individual, their family, and the healthcare system are confronted with (Dahlhamer et al., 2018). Various factors are associated with the high prevalence of chronic NPP, including infection, prolonged/chronic stress, nerve trauma/entrapment, excessive alcohol consumption, stroke, post-amputation, chronic inflammation, diabetes, HIV, surgical procedures, and cancer (Yan et al., 2017; Patel et al., 2018; Tai, Yeung and Cheung, 2018; Treede et al., 2019). Cancer itself is not necessarily the risk factor, but rather the extensive treatment thereof.

Chemotherapy-induced peripheral neuropathy (CIPN) is a frequently encountered complication arising from the treatment of cancer using conventional antineoplastic agents (Colvin, 2019). This condition can progress to become a dose-limiting side effect (Molassiotis et al., 2019) with ineffective preventative and curative treatment (Seretny et al., 2014) due to the condition’s complexity. During chemotherapy, 70.8% of patients reported CIPN symptoms (Bao et al., 2016), and 30% of patients presented with chronic CIPN at a 6-month follow-up post-chemotherapy treatment (Seretny et al., 2014). Currently, only duloxetine has been recommended as an effective treatment for CIPN (Colvin, 2019). However, duloxetine has only shown individual-dependent, short-term analgesic effects (di Cesare Mannelli et al., 2017), with limiting adverse effects and poor bioavailability (Chahal, Sodhi and Madan, 2020). Due to extensive central involvement (Elman and Borsook, 2016) and a plethora of associated risk factors (Li et al., 2017), less than half of patients treated with conventional analgesics experiences pain relief (Muñoz et al., 2018), and usually require the reduction or cessation of chemotherapy treatment (Niemand, Cochrane and Eksteen, 2020). CIPN is associated with a pro-inflammatory state and upregulation of anxiety, depression, and sleep disturbances (Bao et al., 2016), contributing to a decline in physical independence and significant medical costs (Molassiotis et al., 2019).
The presence of chronic pain is considered to be a self-amplifying stressor, perpetuating the dysfunctional stress response and subsequent accumulation of glucocorticoids, primarily cortisol, within the physiological milieu (Elman and Borsook, 2016). Chronic disruptions due to persistent stressors increase the risk of developing psychiatric disorders known to be associated with neuropathic pain, such as anxiety and depression (Caudle, 2016). These disorders often occur as a result of altered hypothalamic-pituitary-adrenal (HPA) axis function (Fischer, Macare and Cleare, 2017). HPA axis dysfunction is associated with chronic elevated levels of cortisol (Tang, Thomas and Larkin, 2019) and a reduced ability to cope with stressful events (Godoy et al., 2018). Subsequently, the patient experiences a prolonged deterioration in quality of life, for which effective treatment is minimal (Seretny et al., 2014).

To develop effective, long-term treatment modalities, HPA axis activity attenuation is crucial. The use of the neuropeptide, oxytocin, as an analgesic and anxiolytic has gained significant traction in recent literature (Boll et al., 2018). Known to be involved in the modulation of social behavior and downregulation of the stress response and anxiety (Neumann, 2007; Gao et al., 2015), oxytocin exerts central and peripheral attenuating effects on nociception (Tracy et al., 2015), thus providing the efficacy required to treat neuropathic pain. To our knowledge, there has only been one investigation into the nociceptive efficacy of chronically administered oxytocin in an animal model. Gonzalez-Hernandez et al (2019) demonstrated the analgesic potential using a spinal nerve ligation model. However, the therapeutic potential of this neuropeptide has not been investigated in an animal model of CIPN. Therefore, our study aimed to firstly establish a reputable model of CIPN, and thereafter evaluate any ameliorative effects facilitated by oxytocin. As oxytocin offers the most effective analgesic benefits through central pathways and is inefficient when passing the blood brain barrier (BBB) (Rash, Aguirre-Camacho and Campbell, 2014), our study utilized a method of intranasal administration to bypass the BBB (Tracy et al., 2015) with the aim to optimize the analgesic and anxiolytic effects.
2 Literature Review

2.1 Pain

2.1.1 A Brief History in Pain

In 1979, the International Association for the Study of Pain (IASP) deliberated for 2 years in order to construct the most comprehensive definition of pain, which was eventually selected as, “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (IASP, 1979). In recent years, the growing body of pain literature has identified the need for an updated definition that reflects the advances in evidence. In 2020, the definition of pain was revised to, “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (Raja et al., 2020, pp. 1977). These definitions parallel a description proposed by Melzack & Casey in 1968, who defined pain as multi-dimensional and complex, with interacting components of sensory, cognitive, psychological, and physiological in nature (Melzack and Casey, 1968; Moayedi and Davis, 2013). Prior to this definition, theories postulating an explanation for pain dated back to the 4th century BC, where Aristotle and Plato argued that pain was an emotion occurring when the stimulus was stronger than usual, not a sensory perception of the body (Dallenbach, 1939). This formed the premise of the Intensity Theory of pain, later forming the Pattern Theory, which postulated that pain occurred by intense stimulation of nonspecific receptors. When the summation of the intensity of the stimuli was sufficient to elicit a central response, pain was experienced (Goldscheider, 1894). However, this theory failed to account for the known physiological mechanisms used to support the Specificity Theory of pain (Melzack, & Wall. 1965). This proposed the concept of dedicated pain pathways consisting of specific ‘pain receptors’ located in the periphery, responsible for recognizing noxious stimuli and projecting stimulation to a ‘pain center’ in the brain. Maximilian von Frey advanced this theory by identifying distinct spots for noxious and innocuous pressure, using what is known today as von Frey filaments (von Frey, 1984).

Charles Scott Sherrington, who first coined the term ‘nociceptor’ in 1906, concluded in his studies that the function of the peripheral receptors was to determine the importance of various stimuli, either by lowering or heightening its strength (Sherrington, 1947). This discovery accounted for both the Intensity and Specificity theory, and touched on the theory of Gate Control, which revolutionized pain research (Moayedi and Davis, 2013). This theory
suggested that peripheral stimulation is analyzed at a spinal cord level before it is transmitted to the brain. The nerve fiber type and stimulus intensity are some of the factors that determine whether the transmission travels through the ‘gate’ up to the respective brain regions responsible for pain interpretation (Melzack, & Wall. 1965).

Although each of the theories discussed above have contributed to our understanding of pain perception, they have not accounted for the complexity of the pain system (Moayedi and Davis, 2013). As mentioned, Melzack, R. & Casey, K.L. (1968) described pain as multi-dimensional and complex. George Engel (1977) advanced this definition to conceptualize the biopsychosocial model of pain, which is used contemporarily within pain management therapies to describe the transition from acute to chronic pain (Tracy et al., 2015).

Under normal circumstances, an imminent threat in the immediate environment will activate the peripheral sensory neurons, which converge at the base of the spinal cord at the dorsal root ganglia. This information is then transmitted to the dorsal horn of the spinal cord and enters the ascending pain pathways, where the information is distributed to the appropriate region within the brain responsible for eliciting a stress response (Elman and Borsook, 2016). The type of stimuli received and interpreted at a supraspinal level depends on the intricacies of the structures within the spinal cord (Yan et al., 2017a).

2.1.2 Function of the Spinal Cord and Brain Regions relating to Pain Perception

The spinal cord is responsible for transmitting information from the body to the brain, and consists of outer white matter and inner grey matter. The grey matter is functionally subdivided into 3 segments, including 4 main columns/horns, 6 nuclei, and 10 laminae (I-X) (Parkinson, G. 2017; Yan et al., 2017). Afferent sensory neurons converge at the dorsal root of the spinal cord, and the neural pathways continue into the dorsal (posterior) horn which project the sensory input via ascending pathways to the appropriate brain regions (Yan et al., 2017). The ventral (anterior) horn column is comprised of motor neurons responsible for innervation of the skeletal muscle. The intermediate column and lateral horn consist of the neurons which innervate visceral and pelvic organs (Parkinson, G. 2017). The nuclei and laminae are functionally grouped into the above four regions (Figure 1). The dorsal horn consists of the marginal zone (lamina I), substantia gelatinosa (lamina II), nucleus proprius (laminae IV and V), and dorsal nucleus of Clarke (lamina VI), which are nuclei responsible for relaying pain, temperature, mechanical, and proprioceptive sensation to the brain (Xin, Bai and Liu, 2017). The interomediolateral nucleus, located in the intermediate column and lateral horn, relays visceral sensory input to the brain and transmits autonomic signals from...
The white matter contains the ascending and descending pathways (Figure 2), which enables communication between the brain and spinal cord. The ascending pain pathways include the lateral and medial spinothalamic tracts. The lateral spinothalamic tract projects to thalamus, nucleus accumbens, and amygdala (Tracy et al., 2015); while the medial spinothalamic tract projects to the limbic system, reticular formation nuclei (brain stem – arousal), superior colliculus (midbrain – orientation, sensory input into movement output), parabrachial nucleus (brainstem – relays sensory input to forebrain structures), and hypothalamus (autonomic processes and neuroendocrine stress-like output) (Elman and Borsook, 2016). Information travels to the cerebral cortex (consisting of the somatosensory, insular, and cingulate cortex), thalamus, and limbic system (amygdala, hippocampus, and hypothalamus), which are functionally linked to enable pain processing. Projection neurons predominantly from laminae I and V transmit noxious stimuli to the thalamus and amygdala, respectively (Yan et al., 2017). The thalamus transmits the pain stimulation to the somatosensory cortex, responsible for identifying the location and intensity of the pain (Elman and Borsook, 2016). The amygdala transmits to the cingulate and insular cortex, via the parabrachial nucleus in

**Figure 1** The arrangement of neurons within the spinal grey matter. Left half: laminae I to X, right half: nuclei. Created using BioRender.com, adapted from Biswas (2015).

*Poterior thoracic nucleus or Column of Clarke*
the brainstem, which interprets the emotional aspect of the stimulation (Yan et al., 2017). The cerebral cortex then interprets the nociceptive stimulus and produces the sensation of pain.

The descending pain pathways consist of the rostral ventromedial medulla (RVM), nucleus tractus solitarius, parabrachial nucleus, locus coeruleus (LC), and raphe nuclei (RN). The periaqueductal grey (PAG) forms part of both the ascending and descending pain pathways (Elman and Borsook, 2016; Zhang et al., 2020). The PAG and RVM are the primary descending pain pathways (Yan et al., 2017). These structures are vital in nociceptive transmissions; however, dysfunction of the spinal cord mechanisms has been identified as characteristics of chronic pain pathologies (Yan et al., 2017). These dysfunctions are propagated throughout the neural circuitry by altered communication between nociceptive pathways throughout the pain mechanism. In order to understand the extent of the miscommunication, an understanding of conventional communication is needed.

*PAG – periaqueductal grey; RVM – rostral ventromedial medulla; DRG – dorsal root ganglia*
2.1.3 **Action Potentials and Neurotransmission**

Neurons communicate via changes in electrical charges known as action potentials (**Figure 3**). The voltage across a neuron's membrane briefly changes due to the influx and efflux of certain ions. There are three principal phases relating to action potentials (Speller, 2021).

The resting membrane potential is the voltage expressed during a cell’s resting state, usually between $-50$ to $-75\text{mV}$ for a neuron (Speller, 2021). This is dependent on the concentration difference between intra- and extracellular ions, modulated by open ion channels and sodium-potassium-ATPase active transporter (Rash, Aguirre-Camacho and Campbell, 2014). A high potassium ($K^+$) concentration typically resides intracellularly, while greater chlorine ($Cl^-$) and sodium ($Na^{2+}$) concentrations are found outside the cell (Speller, 2021). During the resting state, the neuron is more permeable to $K^+$, which ensures the charge of the membrane remains constant. When an electrical stimulus acts on the axon hillock, it stimulates the opening of voltage-gated $Na^{2+}$ channels (VGSC). The difference between intra- and extracellular concentrations creates an electrochemical concentration gradient, allowing a rapid influx of $Na^{2+}$ ions into the neuron (Speller, 2021). This causes cell depolarization, as the potential becomes more positive. If the threshold is reached, an action potential occurs eliciting a maximal response. Once the cell has depolarized, the VGSC closes. The raised intracellular charge stimulates the opening of the voltage-gated $K^+$ channel (VGPC), allowing $K^+$ to migrate out of the cell. As $K^+$ exits, the intracellular charge decreases and repolarization occurs, restoring the original resting potential (Rash, Aguirre-Camacho and Campbell, 2014). Following each action potential is a refractory period, were the VGSC become inactive to ensure the cell returns to baseline before a subsequent action potential (Speller, 2021).
Action potentials are propagated along the neuronal axon via local current gradients (Speller, 2021). As one segment of the axon depolarizes to the required threshold and an action potential ensues, it induces depolarization to the adjacent axonal segment (Figure 4). Once this segment reaches threshold, the process is repeated. Due to the refractory period, an action potential will only travel in one direction. To allow for rapid electrical signal transmission, some neuronal axons are insulated by a myelin sheath (Tracy et al., 2015). Along the sheath, there are periodic gaps termed Nodes of Ranvier (Speller, 2021). Myelinated nociceptors are usually associated with sudden or sharp pain sensation, as the myelin sheath facilitates saltatory conduction, which enables action potentials to ‘jump’ from node to node, rapidly accelerating the conduction of the action potential down the axon. When the action potential arrives at the axon terminal, it activates and opens the voltage-gated calcium channels (VGCC) residing on the presynaptic membrane, allowing an influx on Ca\(^{2+}\) (Rash, Aguirre-Camacho and Campbell, 2014). This results in the release of the residing neurotransmitters (glutamate, acetylcholine, or neuropeptides) via exocytosis (fusion of the neurotransmitter vesicle to the presynaptic membrane), allowing the release of the neurotransmitters into the synaptic cleft, which diffuses to the postsynaptic membrane (Speller, 2021). Most nociceptive neurons communicate using glutamatergic pathways, with the majority of neuronal synapses expressing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors (Lohmann and Kessels, 2014). Once the neurotransmitters bind to the appropriate postsynaptic receptor, the electrical stimulus continues by depolarizing the postsynaptic cell to induce an action potential. In this manner, nociceptive stimuli travel through the ascending pathways to the respective brain regions (Yan et al., 2017).
Frequent synaptic activity mediates synaptic strengthening, where reoccurring activation results in synaptic plasticity. This is termed long-term potentiation (LTP), which forms part of the mechanisms underlying memory and learning (Nicoll, 2017). Synaptic activity is dependent on AMPA-receptor density, which can be influenced by NMDA receptor activation (Lohmann and Kessels, 2014). Glutamate binds to the postsynaptic AMPA receptors, resulting in activation and eventual depolarization of the postsynaptic neuron. When significant depolarization occurs, the magnesium ions inhibiting the NMDA receptors are removed, allowing a rapid influx of calcium into the cell (Nicoll, 2017). NMDA activation eventually leads to increased postsynaptic AMPA receptor expression, increasing the neurons sensitivity to glutamate, ultimately strengthening the synaptic communication (Lohmann and Kessels, 2014). Different nociceptive fiber types are associated with different action potential propagation, and the subsequent information delivered is interpreted differently within the central nervous system (Tracy et al., 2015).

2.1.4 Types of Pain Fibers
Pain can be categorized by the types of fibers transmitting the noxious stimulus. Nociceptors are divided into two major types, myelinated A-fibers (subdivided into A-δ and A-β) and unmyelinated C-fibers (subdivided into peptidergic and non-peptidergic) (Tracy et al., 2015). A-δ are responsible for mediating accurate locations of acute, rapidly conducted pain sensation, and can be further divided into Type 1 and Type 2, high-heat-threshold (>50°C) and low-heat-threshold/high-mechanical-threshold, respectively. Type 1 is typically sensitive
to mechanical and chemical stimuli, whereas Type 2 detects noxious heat stimuli (Yan et al., 2017). A-β fibers are larger in diameter and sensitive to innocuous stimuli, usually associated with light touch and pleasure. C-fibers are smallest in diameter and mediate a poorly localized stimulus of a thermal and mechanical nature. As these fibers are unmyelinated, the pain procession is slow and prolonged, associating C-fibers with chronic pain (Tracy et al., 2015). C-fibers are categorized by the substances they release. Peptidergic C-fibers release neuropeptides, including substance P (SP) and calcitonin-gene related peptide (CGRP). Non-peptidergic C-fibers are associated with non-peptide substances. Peptidergic C-fibers project predominantly to laminae I and II, whereas non-peptidergic C-fibers terminate on lamina II neurons (Yan et al., 2017). A-β fibers terminate on laminae II, III, and IV. A-δ fibers terminate on the superficial laminae I, which are neurons predominantly involved in noxious pain transmissions to the brain. Lamina V receives both noxious and innocuous stimuli via A-δ and A-β fibers, respectively (Yan et al., 2017).

Neuropathic pain is caused by a lesion or disease of the somatosensory system, including the peripheral fibers (A-δ, A-β, & C-fibers), and central neurons (Haanpää et al., 2011). It can be categorized by the location of the lesion, therefore separating into central or peripheral neuropathic pain (NPP) (Colloca et al., 2017). Central NPP can be further sub-divided into spinal cord lesions/diseases, including spinal cord injury, syringomyelia; and demyelinating diseases, and brain-associated disease, such as cerebrovascular and neurodegenerative disease (Colloca et al., 2017). Peripheral neuropathic pain, predominantly involving the A-δ, A-β, & C-fibers, is more prevalent due to the aging population, an increased global incidence of diabetes, and increased rates of cancer and the adverse effects of chemotherapy (Colloca et al., 2017).

2.1.5 The Function of Pain

The biopsychosocial model (Figure 5) is considered the most comprehensive approach to understanding pain, describing the interaction of the biological, psychological, and social components pertaining to pain enabling the formulation of a holistic intervention (Turner et al., 2021). The innovation of this model lies in its ability to differentiate between illness and disease (Tracy et al., 2015). Disease refers to anatomical, pathological, or physiological disruption to a specific structure or system within the body. Alternatively, illness is concerned with the patient’s subjective response to the presence of the disease, including the emotional distress, physical discomfort, behavioral limitations, and psychological disruptions (Turner et al., 2021). The biopsychosocial model takes into account the complexity of pain.
and the interactions between psychological, biological, cognitive, affective, behavioral, and social factors, all known to influence pain (Tracy et al., 2015). The primary function of acute pain is to evoke an appropriate response to the potentially harmful and threatening factor, and prevent or minimize physical damage (Elman and Borsook, 2016). When pain becomes chronic, the pain itself may no longer be related to the original source of tissue trauma (Tracy et al., 2015). Instead, it can be the result of varied and multidimensional neurophysiological (e.g., nociceptive, neuropathic and autonomic mechanisms), psychological (e.g., beliefs about pain, fear of further or re-injury as a consequence of movement), behavioral (e.g., altered posture or movement due to the presence of pain) and mechanical/musculoskeletal factors (e.g., the loss of protective cartilage) (Elman and Borsook, 2016). The transition from acute to chronic pain is a challenge to define, as the multi-system dysregulation associated with chronic pain (Yan et al., 2017) is a progressive deviation from the conventional pain response which develops over different time intervals.

![The biopsychosocial model](https://scholar.sun.ac.za)

**Figure 5** The biopsychosocial model – the interaction between biological, psychological, and social components in the context of chronic pain. Created using Canva, adapted from Fillingim (2017)

### 2.1.6 Mechanism of Acute Pain

A conventional pain response relies on a system of neural sensory apparatus (**Figure 6**) which begins with nociceptors, a subpopulation of primary afferent neurons located in the periphery, including A-δ- and C-fibers, which are responsible for detection of noxious
stimuli, including mechanical, thermal, and chemical in nature (Tracy et al., 2015; Yan et al., 2017). These fibers converge at the dorsal root ganglion (a cluster of sensory neuron cell bodies, which form a ganglion, located in the dorsal (posterior) root of the spinal nerve) (Elman and Borsook, 2016). The neural pathways continue into the spinal dorsal horn, where they terminate in the substantia gelatinosa. Second-order neurons are then activated, which forms the origin of ascending pain pathways within the spinal cord (Tracy et al., 2015; Elman and Borsook, 2016). Signals are propagated along the spinothalamic tract via excitatory neurotransmissions to the respective brain regions, which orchestrate an appropriate response to prevent or avoid potential tissue damage (Yan et al., 2017). The signal can be diminished or amplified before it terminates on the somatosensory cortices, as suggested by the Gate Control theory, based on the integration of internal and environmental information with pain-related affective memories and behaviors interpreted by the respective brain regions. Input from the frontal and insular cortices, hypothalamus, and amygdala converge at the PAG-RVM-dorsal horn pathway, responsible for modulating the output as either pain relief or hypersensitivity (Tracy et al., 2015; Yan et al., 2017). Therefore, the immediate attenuation or amplification of pain is perceived, and an appropriate response is then selected to remove the source of the pain and increase the probability of survival (Tracy et al., 2015). When pain transitions from acute to chronic, the nociceptive signaling is no longer an adaptive warning to prevent further injury, but a persistent sensitization of the system which ultimately results in a depreciation of the patients' quality of life (Yan et al., 2017).
Chronic Pain: Neuropathic Pain

Chronic pain, defined as recurring pain lasting for more than three consecutive months (Treede et al., 2019), is associated with maladaptive alternations to the central nervous system associated with symptoms of spontaneous pain and hypersensitivity to noxious and innocuous stimuli, otherwise known as hyperalgesia and allodynia, respectively (Elman and Borsook, 2016). In chronic pain conditions, the pain itself is no longer dependent on actual tissue damage, but is rather sustained due to multidimensional pathophysiological, psychological, and behavioral alterations (Tracy et al., 2015), topics which will be explored below. If nociceptive pain is improperly treated (Elman and Borsook, 2016), or is extensive enough to encompass nerve damage (Thuault, 2015), it can lead to the pathological persistence of pain, culminating in the development of chronic neuropathic pain (Elman and

*DRG – dorsal root ganglia

2.1.7 Chronic Pain: Neuropathic Pain

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Borsook, 2016). When the somatosensory system is damaged due to a lesion or disease, it can result in the development of neuropathic pain (Colloca et al., 2017). As the somatosensory system extends from the periphery to the central nervous system, a lesion affecting any part of the sensory pathway can produce neuropathic pain (Haanpää et al., 2011). The affected section/s of the somatosensory nervous system disrupts the conventional sensory transmissions to the spinal cord and brain, resulting in the subsequent perception of pain (Colloca et al., 2017). It is important to note that the mechanisms governing neuropathic pain are distinct from other chronic pain conditions due to the extensive central involvement, and as such must be treated using unique methods (Elman and Borsook, 2016).

When nerve injury or damage occurs, due to factors including trauma, infection, metabolic disease (i.e., diabetes), multiple sclerosis, tumor invasion (i.e., cancer), neurotoxic chemicals, or stroke (Buckley et al., 2018; Kaliyaperumal et al., 2020), it can cause pathological maladaptation of mechanisms within the peripheral and central nervous systems, altering the patient's perception of pain (Buckley et al., 2018). Peripheral maladaptations include but are not limited to immune cell proliferation, ionization channel maladaptation (i.e., ectopic discharge), and subsequent altered primary afferent activity (Yan et al., 2017). The central changes include alterations to spinal cord and supraspinal hyperexcitability, structural and functional degradation of grey matter, and adverse alterations in cortical plasticity in the spinal cord and brain (Fornasari, 2017; Yan et al., 2017).

**Peripheral Mechanism of Neuropathic Pain**

Under normal circumstances, each nociceptor has a unique threshold that must be reached before an action potential will fire, initiating the transduction of the stimulus and transmission along the nociceptive pathway (Yan et al., 2017). When tissue is damaged, there is a release of pro-inflammatory mediators from surrounding nociceptors and non-neural cells. Non-neural cells include basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts (Yan et al., 2017). When activated due to nerve injury, these cells release endogenous pro-inflammatory agents, such as neurotransmitters, peptides (i.e., SP, CGRP, bradykinin), eicosanoids, lipids (including prostaglandins, thromboxane's, leukotrienes, endocannabinoids), neurotrophins (i.e., nerve growth factor (NGF)), cytokines (i.e., tumor necrosis factor (TNF)-α, interleukin-6 (IL-6)), chemokines, adenosine triphosphate (ATP), proton, and potassium (Tracy et al., 2015; Yan et al., 2017). Serum cytokines have been identified in several neuropathic pain conditions, including lower back pain and herpes zoster. Elevated TNF-α and IL-6 levels have been associated with the
severity of neuropathy. TNF-α is a pro-inflammatory cytokine which acts as a precursor to cascade activation associated with the release of additional cytokines and growth factor inflammatory responses (Tanabe et al., 2010). It induces the release of IL-6 through the activation of the inhibitory kappa B (IkB)-nuclear factor kappa B (NFκB) pathway, p38 mitogen-activated protein (MAP) kinase and stress-activated protein kinase (SAPK) (Tanabe, K., et al. 2010). Furthermore, elevated antioxidant enzymes, namely CuZnSOD, MnSOD, and GPx, have also been observed at peak pain periods in both dorsal root ganglia (DRG) and peripheral sensory nerves (Duggett et al., 2016). This increase in antioxidant activity is a strong indication of an increase in reactive oxygen species (ROS – such as superoxide radicals and hydrogen peroxide) accumulation in the respective areas, indicative of elevated levels of oxidative stress. ROS is also affected by TNF-α signaling, which activates the NFκB pathway involved in the suppression of intracellular ROS formation. Excessive oxidative stress, in the form of an accumulation of ROS, is involved in inflammatory pain and nerve injury, and can contribute to the pathological conditions in neuropathic pain (Tanabe et al., 2010; Griffiths et al., 2018).

In addition to an accumulation of pro-inflammatory cytokines, nerve damage causes maladaptation of the ion channels in the DRG, particularly VGSCs and VGCCs (Rash, Aguirre-Camacho and Campbell, 2014). Studies have shown an aggressive aggregation of VGSC and VGCC on the neuronal membranes within injured areas (Yan et al., 2017). This contributes to neuronal plasticity, leading to increased hypersensitivity and eventual spontaneous excitability of the afferent neuron, termed ectopic discharges (Nicol et al., 2015). Subsequently, aberrant action potential discharges, which mediates the frequent release of neurotransmitters and contributes to forceful, persistent stimulation of the dorsal horn neurons, ultimately stimulating the ascending pain pathways (Yan et al., 2017b). Chronic recruitment of these pathways results in strengthening and sensitization of the connection, eventually leading to central sensitization (Chul Han, Hyun Lee and Mo Chung, 2000).

**Central Mechanism of Neuropathic Pain**
Central sensitization of the central nervous system (CNS) is the process of reaching an established state of hyperexcitability, where nociceptive information elicits an abnormally enhanced reaction (Yan et al., 2017). Nerve injury increases the stimulation of nociceptors, which results in persistent nociceptive neurotransmitter release, subsequently resulting in post-synaptic depolarization mediated through an influx of calcium via the NMDA receptor.
Central sensitization of the spinal dorsal horn is dependent on NMDA receptor mediated post-synaptic calcium influx (Li et al., 2017). C-fibers are responsible for this persistent depolarization, which results in LTP, strengthening the connection between the nociceptors and spinal dorsal horn, and altering neuronal plasticity (Yan et al., 2017).

In addition to sensitization, the generation of aberrant A-β fibers occurs. Under normal conditions, A-β fibers do not transduce noxious stimuli. However, in the presence of a nerve injury, nociceptor activity increases and new synaptic connections are formed with existing neurons (Yan et al., 2017). This phenomenon is termed ‘sprouting’, and under these conditions, A-β fibers transmit strong mechanical stimuli which terminate on lamina I and II. There is a subsequent increase in norepinephrine (NE) release (Fornasari, 2017), and propagation of the chronic stress response, leading to an increased sensitivity to noxious mechanical stimulation (i.e., hyperalgesia) (Yan et al., 2017). When a nociceptive stimulus is transmitted to the supraspinal level, based on the interpretation by the various brain regions, an inhibitory system can be activated to diminish the strength of the signal, thus reducing pain perception. This inhibitory system is comprised of γ-aminobutyric acid (GABA)/glycine inhibitory neurons in the dorsal horn (Rash, Aguirre-Camacho and Campbell, 2014). Due to the increase activation of nociceptive pathways after nerve damage, the inhibitory system becomes overstimulated, resulting in excessive receptor stimulation and subsequent impairment or termination (i.e., excitotoxicity). The loss of inhibitory effects results in poor modulation of nociceptive transmissions, resulting in hyperalgesia.

Furthermore, the partial or complete deficiency of select neurotransmitters, including serotonin/5-HT (5-hydroxytryptamine) and NE, resulting in disruptions to these pathways, have been associated with the generation and progression of NPP (Rodrigues-Amorim et al., 2020). Increases in proinflammatory cytokines within the central and peripheral nervous system directly modulates neuronal activity, increases HPA axis activity, thereby promoting monoamine reuptake, and subsequently diminishing the production of 5-HT (Nekovarova et al., 2014). As it has been demonstrated that 5-HT receptors are involved in nociceptive modulation, disturbances within the serotoninergic pathways can contribute to pronociception (Rodrigues-Amorim et al., 2020). In addition, the involvement of noradrenergic mechanisms in antinociception appears to be more significant (Obata, 2017). The prevailing mechanism associated with the mitigation of NE function, and subsequent pronociception, is mediated by α2-adrenoceptors at a spinal cord level (Nekovarova et al., 2014). These receptors, which are responsible for neurotransmitter inhibition from presynaptic neurons (Giovannitti, Thoms and
Crawford, 2015), undergo morphological alterations in the presence of nerve injury, leading to hypersensitivity and a subsequent increase in noradrenergic inhibition, resulting in enhanced nociception and NPP progression (Obata, 2017). In addition to alterations at a spinal cord level, dysregulation in these monoamine pathways emerge at a central level, specifically with regards to the phasic nature of the LC (Obata, 2017). In the presence of chronic nerve injury, it was found that the LC phasic response to noxious stimuli declined over time as a result of altered glutamatergic regulation (Kimura et al., 2015). This resulted in descending noradrenergic inhibitory pathway impairment, and a subsequent reduction of NE within the spinal cord, ultimately increasing the sensitivity response to noxious stimuli (Obata, 2017).

Finally, glial cells have been associated with the development and progression of neuropathic pain (Yan et al., 2017). Within the CNS, there are three main types: microglia, astrocytes, and oligodendrocytes. Astrocytes, the most abundant cell in the CNS, form a bridge of bidirectional communication between neuronal signaling and vasculature, which ensures coordination between neural activity, blood flow, and glucose metabolism (Guo, Ma and Pleasure, 2014). Through gap junction communication (astrocyte-astrocyte) and paracrine signaling (astrocyte-neuron/endothelial cell), astrocytes are able to regulate synaptic plasticity, neurotransmission, and metabolic function (Lee, 2015). These glial cells release gliotransmitters, which interact with neighboring neurons and endothelial cells via the astrocytic processes terminating on synaptic clefts and blood vessels, respectively (Guo, F., et al. 2014). In addition to gliotransmitter release (i.e., glutamate, GABA, & TNF-α), active/reactive astrocytes also secrete a group of glycoproteins, namely thrombospondin (TSP), involved in neuronal synaptic formation, function, and maintenance (Ikeda et al., 2010). Following neuronal damage, TSP is secreted into the extracellular matrix of the CNS and directly influences the formation of glutamate synapses. TSP upregulation has also been correlated to an increase in hyperalgesia in a rat model of neuropathic pain (Stahl et al., 2013). Glutamate release propagates nociceptive signaling, ultimately increasing synaptic activity and resulting in long term potentiation (LTP) (Kandel, E.R., et al. 2013). This glutamate-driven LTP induces morphological alterations in surrounding astrocytes, such as astrocytic processes proliferation, resulting in astrocytic plasticity and a subsequent increase in astrocyte-neuron synapse coverage and synaptic stability (Lee, 2015). Astrocyte activation and proliferation can also occur in response to changes in their environment, including chronic stress, traumatic brain injury, neurodegenerative disease, behavioral and mood
disorders, and peripheral nerve injury/trauma. These associations have implicated astrocytes in the development and maintenance of neurological disease, such as neuropathic pain (Lee, 2015; Yan et al., 2017).

Reactive astrocytes respond to these environmental changes by increasing signaling cascades, releasing proalgesic agents, and microglial recruitment and migration (Lee, 2015). Microglia, predominantly found in the spinal grey matter, are activated during the initial response to nerve trauma or injury, suggesting their involvement in the generation of neuropathic pain (Yan et al., 2017). These glial cells express a copious array of toll-like receptors (TLR), implicating their direct involvement in innate immunity (Lee, 2015). Recent evidence has shown involvement of microglia in the mechanism of chronic pain by sustained neuroinflammation, as demonstrated by a reduction in allodynia and hyperalgesia through the blockage of microglial function mediated by pharmacological analgesics binding to TLRs (Rosen et al., 2019). Chronically activated glial cells, due to sustained circulating inflammatory markers, are associated with a positive inflammatory feedback pathway modulated by an increased expression of microglia and astrocyte membrane TLRs, upregulating glial cell release of neuropeptides, chemokines, cytokines, and neurotrophins; resulting in accumulation of pro-inflammatory markers and perpetuating central sensitization (Yan et al., 2017). Consequentially, sustained glial inflammation results in neuronal toxicity and the loss of neuroprotective function, along with disruptions to the BBB. These disruptions are likely the result of altered glial cell morphology due to chronic inflammation, resulting in disturbances in the structural integrity of the BBB. These alterations lead to the progression of neurodegeneration and subsequently chronic neuropathic pain (Lee, 2015). While the mechanisms are general across neuropathic pain conditions, each condition possesses unique characteristics, contributing to the complexity associated with treatment of these conditions. The most prevalent types of neuropathic pain include postherpetic neuralgia, diabetic polyneuropathy, postsurgery neuropathic pain, multiple sclerosis, spinal cord injury, stroke, and cancer (Colloca et al., 2017). While cancer itself does not cause neuropathy, the treatment thereof leaves patients susceptible to the development of chemotherapy-induced peripheral neuropathy (CIPN), one of the most common adverse effects associated with chemotherapy (Ibrahim and Ehrlich, 2020). During chemotherapy, 70.8% of patients reported CIPN symptoms (Bao et al., 2016), and 30% of patients presented with chronic CIPN at a 6-month follow-up post-chemotherapy treatment (Seretny et al., 2014). CIPN is associated with a pro-inflammatory state and upregulation of anxiety,
depression, and sleep disturbances (Bao et al., 2016), contributing to a decline in physical independence and significant medical costs (Molassiotis et al., 2019). The mechanisms associated with the pathophysiology of CIPN contribute to its complexity.

Chemotherapy-induced Peripheral Neuropathy (CIPN)

The administration of chemotherapeutic drugs, while contributing to considerable improvements in cancer patient outcomes, induces various cellular structural and functional alterations, often resulting in progressive and sometimes irreversible toxic effects (Ibrahim and Ehrlich, 2020). The success of CIPN management depends on a multitude of factors, one being the type of chemotherapeutic drug administered, as this determines the pathological presentation and progression of the neuropathy (Staurengo-Ferrari et al., 2022). Our focus will be on the distinctive pathophysiology of Paclitaxel-induced peripheral neuropathy (PIPN).

Paclitaxel falls under the taxane subcategory of chemotherapy, predominantly used to treat breast, ovarian, and lung cancer (Staff et al., 2020). It generates one of the highest prevalence of CIPN, occurring in up to 80% of patients under administration (Hu et al., 2019). The fundamental function of Paclitaxel is microtubule stabilization, which results in impeded axonal transport due to microtubule dysfunction (Staff et al., 2020). It has been suggested that the primary cause of the PIPN includes altered electrophysiological function, impaired organelle migration and cellular signaling, mitochondrial dysfunction, innate immune response and inflammatory upregulation, and central hyperactivity (Staff et al., 2020; Omran et al., 2021).

One of the most commonly observed characteristics of neuropathic pain is central sensitization, the result of aberrant nociceptive hypersensitivity associated with a reduced action potential threshold (Yan et al., 2017). The predominant contributing factor causing this sensitization is electrophysiological alternations occurring at a central and peripheral level (Staff et al., 2020; Omran et al., 2021a). It has been shown that Paclitaxel induces these alterations by modifying the expression of receptor and voltage-gated channels within the DRG, which include an upregulated expression of calcium and sodium channels, and downregulation of potassium channels (Staff et al., 2020). Ultimately, these alterations cause a lowered threshold, resulting in more frequent firing of action potentials along these neurons, thus an increase in nociceptive stimuli transmission (Yan et al., 2017). Particular interest has been dedicated towards changes in intracellular Ca²⁺ levels, and the implications thereof. In the presence of PIPN, an increased expression of transient receptor potential
ankyrin 1 (TRPA1), known to be particularly permeable to Ca\(^{2+}\), has been found in C- and A-\(\delta\) fibers of sensory neurons, DRG, and brain regions (Sánchez et al., 2022). Activation of this channel has been associated with an accumulation of various ROS, which results in various downstream effects. Paclitaxel also directly activates toll-like receptor 4 (TLR4), which enhances intracellular Ca\(^{2+}\) and stimulates the release of various pro-inflammatory peptides, including CGRP and SP (Staff et al., 2020). Furthermore, TLR4 leads to the activation of TNF-\(\alpha\) from satellite cells located in the DRG, subsequently increasing the expression of TRPA1 and perpetuating the intracellular Ca\(^{2+}\) influx (Sánchez et al., 2022).

TLR4 facilitates the activation of various pro-inflammatory downstream signaling pathways, such as TNF-\(\alpha\), mitogen-activated protein kinase (MAPK), and nuclear factor-\(\kappa\)B (NF\(\kappa\)B). This subsequently leads to an accumulation of inflammatory cytokines in the affected areas, which has been shown to include peripheral sensory neurons, DRG, and the spinal cord (Li et al., 2017). Upregulation of pro-inflammatory chemokines and cytokines within the DRG have been associated with Paclitaxel-induced mechanical and cold hypersensitivity in a rat model (Staff et al., 2020). Furthermore, in the periphery, macrophage migration within the sciatic nerve has been correlated to Paclitaxel-induced allodynia (Staff et al., 2020). The stimulation of these pro-inflammatory pathways results in an accumulation of ROS within the DRG, and subsequent oxidative stress (Wu and Chen, 2019). Previous literature has demonstrated an amelioration of mechanical sensitivity, but not cold allodynia, through administration of ROS scavengers (Ho Kim and Mo Chung, 1992; Zhao et al., 2019). Upregulated mitochondrial and cellular endogenous antioxidant enzyme synthesis has also been demonstrated (Duggett et al., 2016) within the DRG in the presence of Paclitaxel. However, this synthesis was inadequate in diminishing the deleterious effects of excessive ROS accumulation (Zhao et al., 2019).

While oxidative stress results in numerous ramifications, a detrimental effect is induced mitochondrial dysfunction (Zhao et al., 2019). Several cells have shown dysfunction in the presence of Paclitaxel, including peripheral DRG cells. The suggested mechanism is damage via increase mitochondrial permeability and subsequent ROS production, resulting in intraxonal oxidative stress, which contributes to neuronal degeneration and eventual neuropathy (Wu and Chen, 2019; Staff et al., 2020). Literature has also identified mitochondrial dysfunction through swollen and vacuolated mitochondria in myelinated and unmyelinated sensory neurons (Staff et al., 2020), further contributing to ROS formation and a state of oxidative stress (Figure 7).
While extensive research has focused on the pathophysiology of PIPN in the periphery and DRG, emerging literature is highlighting the importance of understanding the toxic effects of Paclitaxel, directly or indirectly, on the brain (Masocha, 2016; Huehnchen et al., 2017; Omran et al., 2021). In a systematic review on the role of the brain in CIPN (Figure 8), Omran et al., (2021) identified four putative mechanisms associated with the progression of CIPN. These include (1) brain hyperactivity, (2) reduced GABAergic inhibition, (3) neuroinflammation, and (4) excitability of various signaling pathways (Omran et al., 2021a). Hyperactivity of various brain areas was identified as one of the key features of CIPN pathology, detected in various brain regions associated with pain processing. These included the somatosensory, insula, cingulate, and motor cortices, as well as the thalamus, PAG, and prefrontal cortex (PFC) (Omran et al., 2021). As all these brain areas have been associated with pain processing, it is plausible that they play a major role in the pathophysiology of CIPN (Tracy et al., 2015; Elman and Borsook, 2016; Yan et al., 2017).

Figure 7 Mechanisms of Paclitaxel pathogenesis leading to axon degeneration and painful neuropathy based on mammalian studies. (A) Isolated rodent DRG neurons and patient-derived induced pluripotent stem cells have revealed acute and chronic changes in calcium signaling in vitro. (B) In vivo studies using rodent models have shown various effects directly in sensory neurons and indirectly in skin cells, immune cells and glia. (C) Genetic studies using patient data identified polymorphisms and mutations in various pathways associated with increased risk for PIPN. Created using BioRender.com, adapted from Staff, et al. (2017).

*DRG – dorsal root ganglia; SARM – selective androgen receptor modulator; CNS – central nervous system
1 – Altered Ca$^{2+}$ signaling; 2 – microtubule dysfunction; 3 – mitochondrial swelling
Finally, maladaptations of the hypothalamic-pituitary-adrenal (HPA) axis have been identified in the pathophysiology of neuropathic pain (Bosch et al., 2007; Fischer, Macare and Cleare, 2017; Godoy et al., 2018). Elevated serum cortisol levels have been identified as a reliable HPA axis activity marker, in both human and rodent models (known as corticosterone in rodents) (Caudle, 2016). Cortisol is a glucocorticoid secreted by the adrenal glands as part of the stress response (Godoy et al., 2018). In a chronic neuropathic pain model, the pathological changes within the HPA axis due to persistent stress results in a sustained elevated concentration of serum cortisol and subsequent exacerbation of neuropathic pain (Caudle, 2016; Li et al., 2017).

2.2 Neuropathic Pain & Stress

2.2.1 A Brief History in Stress
Claude Bernard, a renowned French physiologist considered to be the father of experimental medicine, developed a concept in 1872 that formed the foundation of stress research (Robinson, 2018). Bernard observed that the body was constantly working to maintain a balanced internal environment, which he coined the ‘milieu intérieur’, translating to ‘the
environment within’ (Bernard, 1872). It was not until 1915 that Walter Bradford Cannon continued exploring Bernard’s concept of systemic regulatory mechanisms (Robinson, 2018). Cannon noted that when confronted with a stressful situation, the energy reserves which support the body at rest are drastically amplified, or completely interrupted (Godoy et al., 2018). This mobilization of energy stores enables a rapid physiological response and improves the probability of survival, which Cannon termed the ‘fight or flight’ response (Cannon WB, 1915). Cannon furthered his research and eventually formulated the definition of ‘homeostasis’ built on the earlier work of Bernard, which describes the ability of physiological systems to maintain a dynamic equilibrium in response to changes in the external environment, essential for cell survival (Cannon, 1929). Research in this field was advanced by Hans Selye, who noted similar non-specific physiological adaptations in a variety of conditions over a prolonged period of stress (Godoy et al., 2018). This discovery led to the definition of ‘General Adaptation Syndrome (GAS)’ (Selye, 1936), which describes the general pattern of the physiological response to a disturbance (Nesse, Bhatnagar and Ellis, 2016), which he later renamed the ‘stress response’ (Robinson, 2018). His premise was that prolonged exposure to stress would ultimately have a negative impact on general health, which propagated the understanding of why sustained stress becomes pathological (Robinson, 2018). Furthermore, in his later works he became a pioneer on the effects chronic stress had on the HPA axis and the role of glucocorticoids in the stress response (Selye, 1943; Selye, 1976).

This adaptive stress response was considered almost exclusively physiological until Cannon hypothesized that prolonged exposure to a persistent state of fear without the presence of a physical stressor could produce fatal outcomes (Cannon, 2015). Irrespective of this, it was only after the deleterious psychological effects induced by World War II that psychological stressors were recognized as important when describing the stress response (Robinson, 2018). Richard Lazarus was one of the first to challenge Selye’s GAS theory, as he argued that a generalized stimulus-response explanation did not accommodate the extent to which a stimulus is interpreted as stressful, and individual differences were integral to understanding the psychological stress response (Lazarus, 1966). He expanded his theory to incorporate mediators of the psychological stress response which included the individual’s personal meaning, or appraisal, of the stressor (Lazarus, 1975), as well as the coping strategies available to them (Folkman and Lazarus, 1980). Lazarus pioneered a new exploration of
stress research which considered the complex interaction between the stimulus, appraisal, and the response (Robinson, 2018).

The foundational work of Cannon, Seyle, and Lazarus, to name a few pioneers, has converged and directed modern research towards a holistic understanding of acute and chronic stress, and the implications thereof (Robinson, 2018). Ultimately, stress is an evolutionary adaptation with acute advantageous functions that natural selection deemed invaluable for survival (Nesse, Bhatnagar and Ellis, 2016).

2.2.2 Function of Stress
Although the cumulative societal perception of stress is often negative, the fundamental utility of the stress response facilitates immediate physiological advantage which heightens our chance of survival (Nesse, Bhatnagar and Ellis, 2016; Robinson, 2018). The specific function of stress is dependent on the evoking situation, as the body engages different neuronal circuitry depending on the type of stressor, such as physical versus psychological stressors (Godoy et al., 2018). On a fundamental level, conditions which disrupt homeostasis elicit a stress response, which stimulates the activation of the sympathetic nervous system and subsequent cascade of stress molecules responsible for innervating an appropriate physiological response (Chu et al., 2022). The activation of the sympathetic system leads to sudden physiological changes, such as increased alertness, vigilances, and appraisal of the situation, to maintain physiological integrity in adverse conditions (Caudle, 2016; Nesse, Bhatnagar and Ellis, 2016; Godoy et al., 2018). This response is modulated by the release of norepinephrine (NE) and epinephrine (EPI) by the adrenal medulla (Chu et al., 2022). This is the basic physiological response, best known as the ‘fight or flight’ response, which provides the overlapping functional characteristics of situational-specific stress responses (Cannon WB, 1915; Nesse, Bhatnagar and Ellis, 2016). However, the brain differentiates between physical and psychological stressors, and elicits different defensive systems accordingly, therefore making it impossible to declare a singular function of the stress response (Caudle, 2016; Nesse, Bhatnagar and Ellis, 2016). The underlying mechanisms responsible for regulating the various responses to stressors provide better insight into the exact function of each response.

2.2.3 Mechanism of the Stress Response
A physical stressor is mainly processed at the level of the brainstem and hypothalamic regions (Figure 9), and usually requires immediate systemic reaction; the first phase initiates the autonomic nervous system, considered to mediate short-term effects which are rapid and
reflexive in nature (Godoy et al., 2018). Visceral or somatic damage activates sympathetic neurons within the spinal cord, which transmits autonomic stimuli via the brainstem to the hypothalamus (Caudle, 2016). The paraventricular nucleus (PVN) of the hypothalamus then responds by releasing corticotrophin releasing hormone, which is involved in the rapid activation of sympatho-adrenomedullary (SAM) axis, considered the first phase of the stress response (Figure 10); as well as the activation of the secondary hormonal response phase, modulated by the HPA axis (Caudle, 2016; Borodovitsyna, Flamini and Chandler, 2018; Godoy et al., 2018).

Corticotrophin releasing hormone (CRH) is transmitted via direct PVN projections to the locus coerules (LC), a compact nucleus located in the pons of the brainstem, which is the primary source of NE within the CNS (Godoy et al., 2018). NE is transmitted from the LC via NE-expressing neurons projecting throughout the neuraxis (Borodovitsyna, Flamini and Chandler, 2018; Godoy et al., 2018). Projections travel down the spinal cord into the periphery, some of which terminate on the adrenal medulla, responsible for the synthesis and subsequent secretion of EPI into circulation (Godoy et al., 2018). The central release of NE is responsible for coordinating and modulating autonomic, endocrine, and neuroendocrine responses (Godoy et al., 2018), while NE and EPI released into circulation facilitate an increase in arousal, arterial pressure, heart rate, respiratory rate, cell metabolism, and energy mobilization, as well as redirects blood flow to active muscles/organs (Nesse, Bhatnagar and Ellis, 2016; Chu et al., 2022).

The activation of the PVN during the stress response not only results in the synthesis of CRH, but also vasopressin and oxytocin, all of which can act as neurotransmitters or neurohormones depending on the location and receptor they act on (Godoy et al., 2018). When CRH reaches the anterior pituitary, it stimulates the synthesis and release of adrenocorticotropic hormone (ACTH). Once released into circulation, ACTH travels to the adrenal cortex, responsible for glucocorticoid synthesis and secretion (Caudle, 2016; Fischer, Macare and Cleare, 2017). Glucocorticoids are able to bypass the BBB, enabling them to act on any brain structure expressing glucocorticoid (GR) or mineralocorticoid receptors (MR), which are ubiquitously located throughout the peripheral nervous system and CNS (Caudle, 2016; Godoy et al., 2018). The main glucocorticoid in humans is cortisol, which enters the CNS and binds to GR and MR, activating several processes involved in the stress response (Godoy et al., 2018). One of the actions of cortisol is the suppression of the HPA axis.
mediated by the binding of cortisol to receptors located on the PFC, which directly inhibits the PVN, thereby creating a negative feedback loop and terminating the stress response (Fischer, Macare and Cleare, 2017; Godoy et al., 2018). Mechanisms which mediate the stress response are strictly regulated to ensure a balance between immediate benefits and long-term adverse effects is maintained (Nesse, Bhatnagar and Ellis, 2016).

While the response to a physical stressor is predominantly autonomic, a psychological stressor elicits a physical and cognitive response (Godoy et al., 2018). Highly orchestrated integration of the limbic system and cortex allows for the generation of situation-appropriate psychological stress responses (Sibille et al., 2017). The PFC functions in decision making and working memory during the stress response by comparing the information from the immediate stressor to prior stressful encounters, which ensures a response of adequate...
magnitude is initiated (Caudle, 2016). Indirect functional connections allow for the PFC to exert an inhibitory or excitatory effect on the PVN via GABAergic or glutamatergic neurons, thus inhibiting or stimulating HPA axis activity, respectively (Godoy et al., 2018). GABAergic synapses located on the PVN are involved in the inhibition of the HPA axis (Caudle, 2016). GABA facilitates its inhibitory effect by hyperpolarizing the affected cell, reducing the probability of an action potential ensuing, thus leading to interference of the stimuli signaling (McCarson and Enna, 2014).

The PFC also projects to the amygdala, an important structure responsible for integration and consolidations of emotions, with particular involvement in the modulation of chronic pain (Li et al., 2017). The amygdala is indirectly involved in cortisol secretion via functional projections to the PVN, as well as direct connections to the periaqueductal gray (PAG), involved in the fundamental organization of the threat response (Godoy et al., 2018). Finally, the hippocampus is predominantly involved in memory formation (Lee, 2015), exerts an inhibitory effect on the HPA axis via indirect functional connections to the PVN. It also possesses excitatory projections to the PFC and amygdala, facilitating regulation of memory consolidation in response to a psychological stressor (Godoy et al., 2018). The functional integration of these systems is regulated by glucocorticoids (Caudle, 2016). Glucocorticoid
infiltrates the CNS and stimulates the activation of the glutamatergic pathway residing in the PFC, hippocampus, and amygdala. This involves an elevation in the release of the neurotransmitter glutamate, and facilitates the migration of glutamate receptors (N-methyl-D-aspartate (NMDA), AMPA to the postsynaptic neural membranes (Caudle, 2016). This serves to strengthen the adaptive stress response by enhancing the formation and consolidation of emotionally salient memories (Godoy et al., 2018). The neurotransmitter dopamine is also released in the presence of glucocorticoids, which binds to receptors on the PFC and stimulates the necessary motivation and attention needed to evaluate the stressor and elicit the appropriate behavior (Caudle, 2016). Furthermore, NE released by the LC binds to the hippocampus, which promotes AMPA receptor binding to postsynaptic neurons, increasing glutamate signaling and contributing to memory consolidation (Caudle, 2016).

Usually, the PFC will modulate the activity between the hippocampus and amygdala to ensure an appropriate response to the psychological stressor occurs. However, in the presence of a substantial psychological stressor, the activity of the amygdala and hippocampus becomes more prominent, and PFC activity is suppressed. Prolonged psychological stressors result in the loss of inhibitory projections from the PFC to the amygdala, resulting in hyperexcitability and subsequent stress-related behavioral abnormalities (Godoy et al., 2018). Therefore, it is vital that the stress response is terminated, either directly or indirectly, via the binding of glucocorticoids to their various receptors dispersed throughout the brain regions. The binding of cortisol to receptors located on the PVN results in a decrease of glutamatergic excitability and increased GABAergic inhibition, thereby directly inhibiting the HPA axis activity (Caudle, 2016; Godoy et al., 2018). Cortisol is also involved in the delayed effects of stress within the limbic-cortical structures through activation of glutamatergic, GABAergic, dopaminergic, and norepinephergic signaling systems, allowing for the restoration of homeostasis and retention of important information related to coping strategies (Godoy et al., 2018). The innervation of these systems acts as an indirect modulator responsible for suppressing HPA axis activity, and terminating glucocorticoid release (Caudle, 2016; Fischer, Macare and Cleare, 2017). A disruption of the HPA axis regulatory feedback system results in a prolonged exposure to elevated glucocorticoids, and can thus have functional consequences on the stress response circuit (Caudle, 2016). Various malfunctions can disrupt this system, resulting in an aberrant stress response. When these disruptions become chronic, there is an increased risk of the occurrence of pathological manifestations, such as depression and anxiety (Caudle, 2016). Chronic pain, which includes neuropathic pain, is associated with
these psychological disorders. Furthermore, chronic stress has been shown to be involved in the exacerbation of pain perception (Li et al., 2017).

2.2.4 Dysregulation of HPA Axis

In an environment of persistent and considerable over-exposure to stressors without sufficient recovery, structural changes within the limbic-cortical areas occur resulting in dysregulation of the HPA axis (Sibille et al., 2017; Godoy et al., 2018). Pathology of the HPA axis caused by chronic stress can contribute to debilitating neurological conditions (Caudle, 2016). In major depressive disorders there is a marker elevation in CRH, ACTH, and cortisol levels (Fischer, Macare and Cleare, 2017). Prolonged stress exposure and subsequent glucocorticoid exposure atrophies the PFC dendrites, affecting glutamatergic pathway expression and signaling (Godoy et al., 2018). NMDA and AMPA receptor expression are reduced, resulting in impaired working memory, mediated by a reduction in dopamine signaling from the mesocortical circuit (a dopaminergic connection between the PFC and ventral tegmentum) (Caudle, 2016). Dopamine dysregulation inhibits the ability of the stress circuitry to regulate the magnitude of a stressor and elicit the appropriate response, causing cognitive dysfunction within the PFC, which leads to PFC atrophy and subsequent deficits in learning (Godoy et al., 2018). Chronic stress is also associated with a reduction in hippocampal dendritic arborization and LTP as a result of a reduction of AMPA and NMDA synaptic transmission, ultimately causing spatial memory defect and associated cognitive dysfunction (Caudle, 2016; Godoy et al., 2018).

Furthermore, chronic stress has been directly associated with the exacerbation of neuropathic pain through the dual-stimulation of the central nuclei of the amygdala (CeA) (Li et al., 2017). The basolateral amygdala (BLA) receives transmission from the cortex and thalamus in relation to the chronic stress, which is responsible for sensory and emotional processing (Godoy et al., 2018). The BLA then transmits to the latero-capsular part of the central nuclei (CeCL), known to have a high expression of nociceptive neurons. In a state of chronic neuropathic pain, consistent nociceptive stimuli converge on the CeCL via the spino-parabrachio-amygdaloid ascending pathway. This relentless multidirectional input from chronic pain and chronic stress stimulates pain-related synaptic plasticity and sensitization of these parts of the amygdala, resulting in the exacerbation of pain (Caudle, 2016; Li et al., 2017). These maladaptive stress responses, mechanisms of physiological dysregulation, and neuronal functional decline, are correlated to a high frequency of persistent pain (Sibille et al., 2017). Factors which can cause HPA axis disruption include environmental conditions,
chronic pain conditions/diseases, psychosocial stressors, traumatic events/injuries, maternal care, early life experiences, and chronic stressful environments (Bosch et al., 2007; Caudle, 2016; Fischer, Macare and Cleare, 2017; Li et al., 2017). The dysregulation of the HPA axis and subsequent psychological disorders contribute to a feedforward mechanism, perpetuating the perception of pain and reducing quality of life (Li et al., 2017). This, along with a variety of additional environmental, physical, and social factors (Tai, Yeung and Cheung, 2018), makes neuropathic pain a uniquely complex condition to treat (Colloca et al., 2017).

2.3 Current Treatment

General Pharmaceutical Treatment for Neuropathic Pain

The complexity of neuropathic pain stems from the persistent symptoms which usually become drug resistant, perpetuating the prevalence of sleep disturbances, anxiety, depression, severe pain, and subsequent diminishing of the patient’s quality of life (Colloca et al., 2017). Although the use of pharmaceuticals has poor efficacy, multiple adverse effects, and inconsistent individual differences (Yan et al., 2017), there is a general consensus regarding the appropriate first-line of treatment (Fornasari, 2017). The most effective first-line of drug recommendations includes tricyclic antidepressants (TCA), serotonin-noradrenaline reuptake inhibitors (SNRI’s), and anticonvulsants (gabapentin and pregabalin) (Finnerup et al., 2015).

As the neural circuitry of pain processing mirrors that of cognition and emotion, antidepressants have been used successfully to treat neuropathic pain and its associated comorbidities (i.e., anxiety and depression) (Fornasari, 2017; Tai, Yeung and Cheung, 2018). The main analgesic mechanism of antidepressants is the inhibition of monoamine neurotransmitter (i.e., serotonin, norepinephrine, and dopamine) reuptake (Kremer et al., 2016). SNRI’s are associated with mild side effects, and have recently been introduced as a first-line neuropathic pain treatment (Finnerup et al., 2015; Khushboo, 2017). At low doses, they act by inhibiting serotonin reuptake by blocking the respective postsynaptic receptor (Khushoboo, 2017). Furthermore, at medium-high doses it can inhibit NE reuptake, and at very high doses it has been shown to inhibit dopamine reuptake (Khushboo, 2017; Stahl et al., 2013). Duloxetine is one of the most frequently used antidepressants, with strong recommendations as a first line of treatment (Finnerup et al., 2015), in particular for the treatment of chemotherapy-induced peripheral neuropathy (CIPN). The pathophysiological development and progression of CIPN is not clearly understood, and therefore severely limits the advancement of novel therapeutic alternatives (Meng et al., 2019). Due to the complexity surrounding this condition in combination with a limited understanding of the mechanisms at
play, duloxetine is the only pharmaceutical drug that’s efficacy has been sufficiently substantiated by literature. Therefore, the American Society of Clinical Oncology has recommended it as the only effective treatment option for CIPN (Staurengo-Ferrari et al., 2022).

One of the pathophysiological manifestations of NPP is partial or complete deficiency of 5-HT and NE within the CNS (Rodrigues-Amorim et al., 2020). Duloxetine’s dual mechanism of action as an SNRI, resulting in the inhibition of 5-HT and NE transporters, offers significant alleviation potential (Obata, 2017). Inhibition of these transpoters results in a reduction in the uptake of these neurotransmitters, and subsequent increase in 5-HT and NE concentrations in the synaptic cleft (Obata, 2017; Rodrigues-Amorim et al., 2020). NE then exerts analgesic effects through the inhibition of pre-synaptic α2-adrenergic receptors at a spinal cord level (Obata, 2017), ultimately resulting in nociceptive attenuation. It has also been suggested that NE and 5-HT acts by inhibiting pain through the activation of impaired descending noradrenergic inhibitory pathways projected from LC (Obata, 2017).

As previous studies have demonstrated extensive inflammatory involvement and excessive oxidative stress accumulation in the mediation of neuropathic pain, effective treatment would need to mitigate this. Meng et al., (2019) demonstrated duloxetine’s ability to partially attenuate CIPN pathological effects through neuroprotective effects associated with p38 phosphorylation inhibition and subsequent inhabitation of MAPK pathway activity, as well as NF-κB pathway mitigation (Meng et al., 2019). In addition, Lu et al., (2020) found duloxetine mediated a neuroprotective mechanism by inhibiting poly-ADP ribose polymerase cleavage (PARP) and tumor suppressor gene p53 activation, as well as through the regulation of the Bcl2 family to reverse induced oxidative stress and apoptosis (Lu et al., 2020).

Timing of treatment has also been identified as an integral variable when considering analgesic efficacy. Zhang et al., (2018) found that animals administered systematic pretreatment of duloxetine exhibited a dose-dependent reduction in spontaneous pain behaviours, as well as mechanical and thermal hypersensitivity alleviation in a model of formalin hindpaw injection. This analgesic effect was facilitated by a dose-dependent diminished expression of Fos and pERK in the spinal cord and central amygdala (Zhang et al., 2018). Furthermore, Staurengo-Ferrari et al., (2022) identified an analgesic effect mediated through the CNS descending inhibitory pathways when duloxetine was administered prior to chemotherapy treatment. Although studies have demonstrated analgesic
effects using duloxetine, it is associated with adverse effects such as nausea, somnolence, insomnia, constipation, and decrease appetite (Rodrigues-Amorim et al., 2020).

Patients who do not respond to SNRI’s are prescribed TCA’s, which have the highest analgesic efficacy as a result of their lack of selectivity and ability to act on multiple targets (Finnerup et al., 2015; Kremer et al., 2016). TCA’s produce their analgesic effect, either directly or indirectly, by inhibiting the reuptake of 5-HT and NE between nociceptors and spinothalamic neurons, thereby potentiating descending inhibitory pathways (Fornasari, 2017). TCA’s can also activate interneurons that cause the subsequent release of inhibitory substances (i.e., endogenous opioid’s & GABA), thereby indirectly inhibiting the descending pain pathways (Fornasari, 2017; Khushboo, 2017). However, the pleiotropic characteristic of TCA’s may introduce the risk of adverse drug reactions which increases the risk of cardiotoxicity, orthostatic hypotension, constipation, and urinary retention. As a result, TCA administration is dose-limiting, reducing their efficacy as a neuropathic pain treatment (Kremer et al., 2016).

Treatment of neuropathic pain using pregabalin, an anticonvulsant, is currently the only patented pharmaceutical approved adjunctive treatment for management of postherpetic neuralgia (PHN), peripheral diabetic neuropathy (PDN), fibromyalgia, and spinal cord-associated neuropathic pain (Stahl et al., 2013; Fornasari, 2017). Both pregabalin and gabapentin act as a ligand which binds to the alpha-2/delta-1 (α2-δ1) subunit of presynaptic VGCC, expressed within the CNS (Stahl et al., 2013). It has been noted that part of the pathology of neuropathic pain is an upregulation of VGCC expression on neuronal membranes residing in the injured area (Yan et al., 2017). This leads to a sustained, aberrant neurotransmission within the spinal cord, propagating nociceptor signaling through the sensory system (Fornasari, 2017) As previously elucidated (see Central Mechanism of Neuropathic Pain), activated astrocytes secrete thrombospondin (TSP), known to be upregulated in models of neuropathic pain. TSP interacts directly with α2-δ1 maintain calcium channel stability and mediate the increase in glutamate synapse, propagating nociceptive signaling (Stahl et al., 2013). The binding of pregabalin and gabapentin to α2-δ1 subunits gradually reduces the density of presynaptic glutamate synapses and calcium channel expression. The reduction of presynaptic glutamate release eventually results in the attenuation of the nociceptive signaling pathways (Stahl et al., 2013; Fornasari, 2017).
Research has also shown that acute opioid administration can suppress astrocyte-mediated TSP levels, and chronic treatment inhibits TSP gene expression and astrocyte-induced neuronal growth and synapse formation (Ikeda et al., 2010). The analgesic function of endogenous and exogenous opioids is thought to operate through immune system suppression (Rosen et al., 2019). Endogenous opioids are released by interneurons within the spinal cord in response to descending pathway activity (Fornasari, 2017). These opioids, which include β-endorphin, enkephalins, and dynorphins, binds to G-protein coupled receptors (GPCRs), located on presynaptic C- and A-δ-fiber terminals and postsynaptic second-order spinothalamic neurons. Presynaptic binding inhibits calcium channel activity thereby impeding neurotransmitter release, whereas postsynaptic binding activates potassium ion channels, resulting in hyperpolarization of the postsynaptic cell (Fornasari, 2017). Both processes block synaptic transmission, thereby reducing nociceptor stimulation to the thalamus and cortex resulting in antinociception (Fornasari, 2017). While opioid use is evidently an effective treatment for neuropathic pain, the risk of addiction, misuse, and tolerance development outweighs the analgesic benefits. They are thus considered to be a third-line treatment option (Dowell, Haegerich and Chou, 2016). Opioids should be used in extreme circumstances, such as for treating cancer-induced neuropathic pain or other exceptionally painful neuropathic conditions (Yan et al., 2017). A thorough assessment to identify risk of addiction prior to prescription, as well as ongoing monitoring during use is advised (Finnerup et al., 2015; Dowell, Haegerich and Chou, 2016).

Many of the most commonly used pharmacotherapies, such as duloxetine and pregabalin, involve inhibition of neurotransmissions, limiting their efficacy due to accessory stimulation of nociceptive neurons mediated by concomitant pro-inflammatory agents released by non-neuronal cells, such as astrocytes and microglia (Toth et al., 2010). Therefore, while pharmaceutical treatment options have produced success in selective neuropathic conditions, further investigation is being directed towards identifying new targets and treatment opportunities; as pharmaceutical use in isolation is not an effective long-term or safe treatment (Colloca et al., 2017; Xin, Bai and Liu, 2017). One endogenous neuropeptide that is emerging in the literature for its potential effective analgesic properties is oxytocin, due to its unique impact on the emotion, stress, and anxiety systems (Boll et al., 2018). As these are well-known components of chronic pain processing, the extent to which oxytocin can suppress this system is under exploration (Martínez-Lorenzana et al., 2008; Tracy et al., 2015; Xin, Bai and Liu, 2017; Boll et al., 2018; Tang, Thomas and Larkin, 2019).
2.4 Oxytocin

Oxytocin is a neuropeptide synthesized in the hypothalamus and projected to various brain regions and peripheral structures (Rash, Aguirre-Camacho and Campbell, 2014). It is involved in regulating complex social behavior, anxiety, neuroendocrine stress responses, and attenuating HPA axis activity (Tang, Thomas and Larkin, 2019). An increased interest has been directed to identifying the prospective role oxytocin has in a chronic pain management model, as it has been associated with the modulation of pain and analgesia (Xin, Bai and Liu, 2017). Oxytocin receptors have been discovered on most structures associated with chronic pain development and maintenance, including specific brain regions and ascending/descending pain pathways (Boll et al., 2018). Various mechanisms acting on the supraspinal, spinal, and peripheral structures (Figure 11) are involved in oxytocin’s capacity to improve pain tolerance and attenuate acute pain in animal models (Rash, Aguirre-Camacho and Campbell, 2014). It has been shown that the analgesic effects of oxytocin transpire at different levels within the central nervous system, and that central oxytocin exhibits different functions compared with circulating oxytocin, which is dependent on the implicated structure (Martínez-Lorenzana et al., 2008).

2.4.1 Central Analgesic Actions of Oxytocin

The paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus have been identified as the predominant structures responsible for the synthesis and release of oxytocin (Xin, Bai and Liu, 2017). These structures display projections throughout the CNS that are known to be involved in regulating acute and chronic pain responses, including the cingulate and insular cortices, medial PFC, basal ganglia, limbic system, thalamus, hypothalamus, LC, and spinal cord (Xin, Bai and Liu, 2017; Boll et al., 2018). The PVN has direct projections to the dorsal horn of the spinal cord, which are activated in the presence of a stressor, such as during a forced cold swim or restraint stress protocol, and ultimately produce antinociceptive effects (Martínez-Lorenzana et al., 2008; Xin, Bai and Liu, 2017).

Recent studies have also identified oxytocin receptor expression on the latero-capsular part of the central amygdala, which has known associations with pain-related synaptic plasticity, ascending pathway sensitization, and subsequent chronic pain exacerbation, as overstimulation of these neural connections are associated with anxiolytic symptoms typically associated with chronic neuropathic pain (Li et al., 2017). Oxytocin release has been correlated to an attenuation of fear responses, which may indicate an analgesic oxytocin
domain located within the amygdala, thus reducing the fear and anxiety-related symptoms of chronic pain (Boll et al., 2018).

The ability of oxytocin to bind to arginine-vasopressin (AVP) receptors offers another plausible mechanism of analgesia. AVP is synthesized and released by PVN neurons, and exerts its effects by binding to its respective G protein-coupled receptor, arginine-vasopressin-V1a (AVP-V1A). AVP shares a similar morphology to oxytocin, with influence in the reproductive system and possible analgesic effects on the nociceptive system (Juif and Poisbeau, 2013). Oxytocin and AVP exhibit a high degree of homology, and both can act on either oxytocin or AVP-V1A receptors (Juif and Poisbeau, 2013; Xin, Bai and Liu, 2017). AVP-V1A receptors are expressed on the PAG, where a known endogenous opioid system resides. In absence of an oxytocin receptor, or excessive oxytocin levels, oxytocin has the ability to bind to the AVP-V1A receptors and induce an antinociceptive response through descending pathway disruption, mediated by the analgesic effect of opioids (Rash, Aguirre-Camacho and Campbell, 2014). Opioid-ergic systems are also implicated in mediating the

![Central oxytocin release from the hypothalamus](https://scholar.sun.ac.za)
activation of descending antinociceptive pathways via oxytocin-induced activation of opioid receptors located in the PAG, nucleus accumbens, lateral ventricle, and raphe magnus, which results in an analgesic response (Xin, Bai and Liu, 2017).

2.4.2 Spinal Analgesic Action of Oxytocin
Alongside the functional and structural changes at a supraspinal level, maladaptation of the pain modulatory descending pain pathways, in particular the PAG and RVM, are known brain stem and spinal cord alterations occurring in a chronic pain state (Boll et al., 2018). Oxytocin fibers, which form part of the PVN descending pathway, terminate within the superficial and deep layers of the dorsal horn, which includes the substantia gelatinosa, the marginal zone, the central gray, and the intermediolateral column of the dorsal horn (Martínez-Lorenzana et al., 2008; Rash, Aguirre-Camacho and Campbell, 2014). Stimulation of this descending pathway in the presence of noxious stimuli resulted in a direct reduction of primary A-δ- and C-fiber discharge and reduced glutamate-mediated action potential propagation (Martínez-Lorenzana et al., 2008; Xin, Bai and Liu, 2017; Boll et al., 2018). As a result, oxytocin interactions with nociceptive postsynaptic neurons results in inhibition of signal transmission to the spinothalamic and anterolateral tracts (i.e., ascending pain pathways) via the marginal zone and substantia gelatinosa, resulting in an attenuation of nociceptive stimulation, thereby exerting an analgesic effect (Xin, Bai and Liu, 2017).

Evidence also suggests indirect actions of oxytocin via GABAergic system. Oxytocin release, mediated by the PVN hypothalamic projection, binds to oxytocin receptors located on glutamatergic terminals at the level of the spinal cord. A subsequent release of glutamate binds to GABAergic interneurons, releasing GABA into the sensory system (Xin, Bai and Liu, 2017). GABA then mediates synaptic inhibition of afferent nociceptive neurons A-δ- and C-fiber input, which terminate on lamina II of the dorsal horn. Oxytocin thus elicits an indirect analgesic effect by blocking nociceptive transmission (Rash, Aguirre-Camacho and Campbell, 2014; Boll et al., 2018).

2.4.3 Peripheral Analgesic Action of Oxytocin
Oxytocin is released into circulation via posterior pituitary axons, where it exerts its action as a neurohormone by binding to receptors located on various peripheral structures, including the kidneys, uterus, heart, thymus, pancreas, adipocytes, and the DRG of afferent neurons (Xin, Bai and Liu, 2017). It is suggested that oxytocin can exert a peripheral analgesic effect by binding directly to excitatory neurotransmitter nociceptor terminals, such as non-peptidergic C-fibers, and diminishing neurotransmissions (Boll et al., 2018). Oxytocin is also
involved in attenuating pro-inflammatory nociceptive signaling from peptidergic C-fibers. CGRP is upregulated in the presence of a nerve injury, thereby perpetuating stimulation of the ascending pain pathways. Oxytocin receptors are expressed on these neurons, and exogenous oxytocin is able to attenuate the intensity and frequency of neuronal stimulation by binding to the receptors, resulting in a reduced perception of pain (Xin, Bai and Liu, 2017). Finally, oxytocin can directly influence the intracellular polarity of nociceptive and non-nociceptive neurons, thereby modulating the somatosensory transmissions to the CNS (Xin, Bai and Liu, 2017). Once oxytocin binds to its respective receptor, or an AVP-V1A receptor, located on presynaptic afferent neuron terminals, it inhibits the voltage-gated potassium channels, inhibiting the action potential before threshold is reached (Rash, Aguirre-Camacho and Campbell, 2014). In addition, it also diminishes the intracellular calcium increase, which indirectly inhibits the release of neurotransmitters, subsequently attenuating the nociceptive transmissions (Xin, Bai and Liu, 2017).

2.4.4 Role of Oxytocin as Medication

It has been shown that patients with acute or chronic pain conditions have a reduced expression of central and peripheral oxytocin administration, as demonstrated in patients suffering from recurrent abdominal pain, fibromyalgia, and lower back pain (Martínez-Lorenzana et al., 2008; Xin, Bai and Liu, 2017). Furthermore, exogenous administration of oxytocin has shown to produce significant analgesic effects for these patients. For example, a terminally-ill cancer patient experienced an 88% pain reduction for 77 minutes after an intraventricular and intrathecal injection of oxytocin (Madrazo et al., 1987). In a rat model, exogenous administration of oxytocin via intrathecal injection in a spinal nerve ligation (SNL) model has been shown to reduce pain and hypersensitivity, with quicker recovery correlated to time of oxytocin delivery after nerve injury (Xin, Bai and Liu, 2017). In addition, both intrathecal administration of oxytocin and PVN-stimulated oxytocin release have been shown to interrupt LTP-mediated mechanical hyperalgesia (Xin, Bai and Liu, 2017). As LTP mediates central sensitization, a key characteristic of chronic pain, disrupting this process can produce long-lasting therapeutic benefits (Yan et al., 2017).

Although systemic administration of oxytocin (i.e., intraperitoneal or intravenous) can offer pain relief for inflammatory conditions, such as interstitial cystitis (Xin, Bai and Liu, 2017) and irritable bowel syndrome (Su et al., 2020), oxytocin is unable to cross the BBB, and therefore the primary central analgesic mechanisms are inhibited with peripheral administration (Rash, Aguirre-Camacho and Campbell, 2014). However, this obstacle may be
overcome by intranasal administration, allowing for the exogenous oxytocin to travel via the olfactory and trigeminal nerve pathway into the central nervous system (Tracy et al., 2015).

Furthermore, it has been hypothesized that manual therapy techniques stimulate the release of oxytocin into the circulatory system, thereby activating the descending pain-inhibitory pathways and producing an analgesic effect (Plaza-Manzano et al., 2014). This introduces insight into the possible indirect, non-pharmaceutical methods which may be used to manipulate the mobilization of endogenous oxytocin and subsequent relief in pain and improvement in quality of life.

2.4.5 Non-pharmaceutical treatment

While oxytocin is evident in the direct attenuation of pain processing, indirect pain-relieving mechanisms mediated through relieving anxiety, stress, and negative emotions have been explored (Boll et al., 2018). A correlation has been identified between a reduction in anxiety and negative mood, enhanced pain inhibition, and intranasal oxytocin administration in the presence of ischemic and thermal pain (Goodin et al., 2015).

Oxytocin is also known to be involved in behavioural circuitry, such as interpersonal contact, cognitive empathy, and social attachment (Boll et al., 2018). The neural activity involved in the physical experience of pain compared to observing pain in others overlaps (Bos et al., 2015), which illuminates the importance of understanding the neurological mechanism of cognitive empathy. A direct correlation between participants’ cognitive empathy for others in a painful situation and intranasal administration of oxytocin was observed under perspective-specific conditions (Abu-Akel et al., 2015). This study only used subjective measures for data acquisition. In comparison, a recent fMRI study was designed to analyze the brain regions involved in oxytocin-induced cognitive empathy when participants observed others in painful situations (Bos et al., 2015). Bos et al., (2015) observed a marked reduction in brain regions known to be involved in pain processing, including the somatosensory cortices (primary and secondary), the middle cingulate cortex, and the insular cortex, following intranasal administration of oxytocin. However, there were no implicit behavioral indices of empathy used to correlate subjective empathy to the observed results. Furthermore, neither study observed the effects of oxytocin on cognitive empathy and its relation to pain experienced by the participant. Therefore, the analgesic effect of concurrent activation of the circuits involved in cognitive empathy in the presence of chronic pain is still unknown.
Social interaction and support are associated with a reduction in pain perception mediated through supportive stress-management (Che et al., 2018). Oxytocin’s involvement in social attachment, maternal care, establishing trust, and interpersonal relations has been identified (Bernaerts et al., 2017; Boll et al., 2018). Bernaerts et al., (2017) observed an increase in attachment towards peers, a reduction in feelings of tension and anger, and an overall increase in social responsiveness and quality of life in subjects who participated in self-administration of intranasal oxytocin for a two-week period. As anxiety is a common symptom associated with chronic and neuropathic pain (Caudle, 2016; Gong et al., 2018), and the anxiolytic effect of oxytocin in the presence of chronic pain has been described in the literature (Tracy et al., 2015), the introduction of social interaction and support into a model of pain management may be crucial to its efficacy.

These findings provide insight into the importance of specific environmental conditions that may be beneficial if included in a non-pharmacological pain management model. Evidence has shown that engaging in a stimulating environment which facilitates motor, sensory, social, and/or cognitive activity in a voluntary psychologically non-stressful manner may promote neuroplasticity, as well as provide neuroprotective and restorative mechanisms (Figueiredo-Dourado et al., 2020).

Even with advances in modern medical treatment, the prevalence of debilitating chronic pain, including neuropathic pain, continues to rise exponentially (Elman and Borsook, 2016). Medications such as duloxetine, prescribed to alleviate chronic pain often lack efficacy, demonstrate poor bioavailability, and are limited due to intolerable adverse effects (Chahal, Sodhi and Madan, 2020). The need for additional, affordable, and long-term treatment alternatives aimed at enhancing pain relief and accelerating functional recovery with a reduction in drug reliance is imperative (Fornasari, 2017; Tai, Yeung and Cheung, 2018). To confront a multifaceted condition, a multidimensional approach is mandatory (Elman and Borsook, 2016). Oxytocin offers a potential cost effective, easily accessible alternative pain management option for those who cannot afford continuous medical treatment or individuals in areas with limited access to medical treatment. It will also provide an adjunct therapy to reduce monotherapy using current medication only (Brozou, Vadaluca and Zis, 2018), which often has less than 50% success rate (Muñoz et al., 2018). To determine the correlative or causative effect of pain management protocols for neuropathic pain, a clear understanding of the pathophysiology is necessary (Bushnell et al., 2015). Various animal models are used
to replicate the pathology of neuropathic pain, as the presentation of symptoms often parallel those observed in humans suffering from neuropathic pain (Bushnell et al., 2015).

2.5 Animal Models of Neuropathic Pain and Oxytocin analgesia

The animal models described below are predominantly rodent-based, as they possess a high level of similarity to human pathophysiology (Bushnell et al., 2015). Limited studies have focused on oxytocin’s analgesic potential to treat neuropathic pain (Rash, Aguirre-Camacho and Campbell, 2014; Boll et al., 2018). The majority of animal studies investigating this relationship have been conducted on acute pain scenarios (Rash, Aguirre-Camacho and Campbell, 2014; Tracy et al., 2015).

2.5.1 Neuropathic Pain Induction

1. Spinal Nerve Ligation

This model is often used to study chronic neuropathic pain as the symptoms generated closely parallel those observed in human patients with neuropathic pain (Buckley et al., 2018). The original method observed successful presentation of neuropathic symptoms in models of L5-only and L5-L6 ligation (Bennett and Xie, 1988), therefore both methods are employed in modern study designs. An incision is made lateral to the lumbar spine between L4-S2 to expose the spinous processes. Once the desired nerve/s are exposed, silk suture (3.0-6.0, depending on the study design) is tightly ligated around the nerve distal to the dorsal root ganglion and the incision is closed (Condés-Lara, Maie and Dickenson, 2005; Stagg et al., 2011; Buckley et al., 2018).

Condés-Lara, Maie and Dickenson, (2005) used a spinal nerve ligation model to induce neuropathic pain to examine the action of oxytocin in pain-related responses. Intrathecal administration of oxytocin produced a significant reduction in C-fiber post-discharge and input transmission, resulting in subsequent reduction in mechanical sensitivity (Condés-Lara, Maie and Dickenson, 2005).

2. Spared Nerve Injury (SNI)

Ligation and severing of peripheral nerves have been used as a rodent model to simulate persistent, long-lasting peripheral neuropathic characteristics as seen in humans (Vachon et al., 2013). The procedure usually targets the tibial/common peroneal nerves, and involves the surgical exposure of the concerning nerves, which are tied with suture and transected distally (Vachon et al., 2013; Li et al., 2017; Sieberg et al., 2018). Chronic mechanical allodynia and
thermal hyperalgesia are usually observed in successive months, with associated motor impairment (Vachon et al., 2013).

3. **Chronic Constriction Injury (CCI)**

   This model, similar to the spinal nerve ligation model, involves partial damage to the nerve by loose ligation of the common sciatic nerve, so that the nerve still receives blood flow and maintains sensory connection (Kazantzis et al., 2016; Casey, Atwal and Vaughan, 2017; Grégoire et al., 2020). This way, it closely mimics peripheral nerve damage, evident in the presentation of hyperalgesia and allodynia following surgery (Bennett and Xie, no date). The procedure involves the exposure of the common sciatic nerve through dissection of the biceps femoris, followed by 4 sutures (usually 4.0-7.0 chromic gut sutures) placed at 1mm intervals along the nerve (Bennett and Xie, no date; Grégoire et al., 2020).

Martínez-Lorenzana et al., (2008) used this model to produce neuropathic pain to observe the analgesic efficacy of the oxytocinergic system. Stimulation of the anterior paraventricular nucleus mediated oxytocin release in the plasma, spinal cord, and cerebrospinal fluid (CSF), which produced analgesia in a rat model (Martínez-Lorenzana et al., 2008).

### 2.5.2 Oxytocin Analgesia

1. **Central exogenous oxytocin administration**

   Oxytocin’s role in pain processing appears to function predominantly at a central level (Boll et al., 2018). Due to the ubiquitous presentation of oxytocin receptors in the CNS, studies observing oxytocin’s analgesic effect have targeted a number of different structures known to be involved in pain processing (Rash, Aguirre-Camacho and Campbell, 2014; Boll et al., 2018). The effects of exogenous oxytocin administration to the CNS have been predominantly analyzed in healthy animal models (Chul Han, Hyun Lee and Mo Chung, 2000; DeLaTorre et al., 2009; Yang et al., 2010; Gao et al., 2015), with few studies focusing on neuropathic models of chronic pain (Condés-Lara, Maie and Dickenson, 2005; Martínez-Lorenzana et al., 2008).

   Various brain regions are known to be involved in pain processing, including the hypothalamus, amygdala, and the epithalamus (Boll et al., 2018). Chul Han, Hyun Lee and Mo Chung, (2000) observed oxytocin’s involvement in nociceptive modulation via intracerebral administration to the central nucleus of the amygdala. A dose-dependent relationship between oxytocin administration and hindpaw withdrawal latency (HWL) was
observed, implicating oxytocins participate in antinociceptive pathways by binding to receptors expressed in the amygdala (Chul Han, Hyun Lee and Mo Chung, 2000). Gao et al., (2015) produced similar results by intracerebral administration of oxytocin directly into the habenular nucleus of the epithalamus, known to participate in central analgesic interactions, in healthy rats. Oxytocin significantly increased the HWL in a dose-dependent manner, supporting oxytocin’s involvement in central pain processing.

2. **Peripheral exogenous oxytocin administration**

Oxytocin is synthesized within the hypothalamus and projected to the posterior pituitary via the HPA axis, where it is then secreted into circulation and participates in various peripheral processes (Gao et al., 2015). The effects of intravenous (Juif and Poisbeau, 2013) and intraperitoneal (Tracy et al., 2015) administration of oxytocin have been observed in rodent models of acute pain. Juif and Poisbeau, (2013) identified oxytocin’s ability to attenuate action potential activity in C-type nociceptive fibers following intravenous injection of oxytocin. At low doses of oxytocin, antinociception was observed, whereas higher doses resulted in pronociception. Therefore, the biphasic ability of oxytocin is supported, indicating the possible analgesic effect at low doses of exogenous oxytocin targeting C-fiber nociceptors (Juif and Poisbeau, 2013).

As oxytocin is limited in its ability to cross the BBB, and the majority of oxytocin’s analgesic effects occur within the CNS (Tracy et al., 2015), the majority of research has been directed towards central methods of oxytocin administration. However, intranasal administration has been identified as a sufficient alternative in clinical trials, as it allows for oxytocin infiltration into the central nervous system (Abu-Akel et al., 2015; Bos et al., 2015; Bernaerts et al., 2017).

3. **Environmental Enrichment**

Environmental enrichment (EE) is an animal model used to evaluate the influence of a complex habitat including sensory, motor, cognitive, and/or social stimuli on neuroplasticity and behavioral changes at different phases of development (Cutuli et al., 2015). Is has been used to evaluate the potential physical and social analgesic effects in a chronic pain model using male Wistar rats (Figueiredo-Dourado et al., 2020), transgenerational alterations in brain function in Wistar rat offspring (Cutuli et al., 2015), and the role of oxytocin in social facilitation in an enriched environment setting in Long-Evans rats (Neal et al., 2018). This model usually consists of an enlarged cage to encourage explorative behavior, cage mates (8-
10 animals per cage), a variety of colourful toys and objects (usually rotated/replaced twice weekly), a form of physical activity (i.e., ladders, running wheel, multiple levels, etc.), and occasional additions (e.g., daily handling, additional nesting material, moving the food/water, etc.) (Cutuli et al., 2015; Neal et al., 2018; Figueiredo-Dourado et al., 2020).

In recent literature, the benefits of natural stimuli as opposed to artificial stimuli have been explored. Neal et al., (2018) introduced objects such as coconut shells, sticks, moss, and rocks, into the enriched groups’ housing, and observed the social interaction between cage mates in this environment. Central and peripheral oxytocin immunoreactivity analysis showed a significantly greater central oxytocin expression in the enriched animals compared to the controls. Additionally, circulating corticosterone (CORT) levels were inversely proportional to oxytocin levels, providing insight into oxytocin’s role in reducing stress responsivity (Neal et al., 2018). The stress-induced analgesic ability of oxytocin is well-known in the literature (Boll et al., 2018). Despite the support of the literature, the analgesic effect of oxytocin stimulated by EE in a chronic pain model has yet to be explored. Figueiredo-Dourado et al., (2020) has, however, shown a reduction in mechanical and thermal hyperalgesia in a chronic muscle pain rat model using a combination of EE and physical activity. Although reliable evidence indicating the analgesic effect of oxytocin has predominantly originated from animal studies (Rash, Aguirre-Camacho and Campbell, 2014), it must be noted that the majority of these studies were performed on normal animals in response to acute pain, and did not incorporate a chronic pain model (Tracy et al., 2015).

2.6 Problem Statement
Chemotherapy-induced peripheral neuropathy (CIPN) is a dose-limiting side effect with ineffective preventative and curative treatment, due to the condition's complexity perpetuated by the extensive central involvement, including the chronic disruption and subsequent dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Currently, only Duloxetine has been recommended as effective treatment for CIPN, which has shown individual-dependent, short-term analgesic effects, with limiting adverse effects and poor bioavailability. The neuropeptide, oxytocin, may offer significant analgesic and anxiolytic potential, as it exerts central and peripheral attenuating effects on nociception. However, it is unknown whether the intervention administered in a model of CIPN is an effective therapeutic alternative.
2.7 Hypotheses
H0: The null hypothesis states that intranasal oxytocin administration will exert no analgesic, anxiolytic, or neurochemical effect on the symptoms induced by chemotherapy-induced peripheral neuropathy.

H1: The alternative hypothesis states that intranasal oxytocin administration will provide significant analgesic, anxiolytic, and neurochemical benefits on the symptoms of chemotherapy-induced peripheral neuropathy.

2.8 Aim
The aim of the study was to; firstly, establish a model of Paclitaxel-induced peripheral neuropathy (PIPN), followed by an evaluation of the efficacy of oxytocin’s alleviating capacity as a therapeutic agent.

2.9 Objectives
Phase 1
- To establish an animal model of neuropathic pain using the chemotherapeutic agent Paclitaxel to induce peripheral neuropathy
- To verify the establishment of the animal model through the assessment of mechanical and thermal hypersensitivity testing

Phase 2
- To evaluate the efficacy of oxytocin as an analgesic over the intervention period using mechanical thermal hypersensitivity testing
- To evaluate the efficacy of oxytocin as an anxiolytic over the intervention period using anxiety-like behavioural testing
- To evaluate the effect of the intervention period on biochemical markers representative of the hypothalamic-pituitary-adrenal axis
- To compare the efficacy of the different treatments administered
3 Methodology and Materials

3.1 Animals
A total of 61 male and female Wistar rats (F = 31) were used in this study design, which was in accordance with conditions approved by Stellenbosch University Research Ethics Committee: Animal Care and Use (Addendum 1 – ACU-2021-22038). All animals were housed in standard environmental conditions, which consisted of a climate-controlled room with a temperature set at 20 ± 1°C, a relative humidity of 50 ± 10%, a constant 12-hour light/dark cycle (lights on at 06:00, off at 18:00), food and water ad libitum, dust-free wooden shavings, and environmental enrichment consisting of a red rat tunnel. The red rat tunnel remained as a permanent element in each cage, unless it needed to be changed during cage cleaning. Cages were cleaned at minimum once weekly, and more frequently as the animals grew. All procedures were conducted in strict accordance with the IASP ethical guidelines (Zimmerman, 1986) and performed at the same time of day (between 7:00 and 15:00) to avoid diurnal variations in animal responses (Zang et al., 2019).

3.2 Experimental Procedure
The study was divided into two phases. During Phase 1, animals were separated into two groups using a randomization program on Microsoft Excel 2007. The groups were defined as Saline (SAL, n = 30) and Paclitaxel (PAC, n = 31). During Phase 2, animals were further divided into six groups and underwent a 14-day intervention period (Figure 12). Pain is considered chronic if it persists for 3 consecutive months. Therefore, the duration of the intervention needed to mimic or surpass this period of time for the animals. One day for humans is equivalent to 34.8 days for an adult rat. Using this as a guide, three months for a rat is roughly equivalent to three human days. We therefore ensured the animals were of an appropriate age (two months is considered ‘adult’) before commencing the experiment.

Groups were defined as follows:

Group 1: Control (n=10)
Group 2: Paclitaxel (n=10)
Group 3: Duloxetine (n=10)
Group 4: Oxytocin (n=10)
Group 5: Paclitaxel-duloxetine (n=10)
Group 6: Paclitaxel-oxytocin (n=11)
3.3 Phase 1
Phase 1 took place over a 3-week period. The first week consisted of various baseline behavioural assessments, followed by week 2 where neuropathic pain was induced using the chemotherapeutic agent, *Paclitaxel*. Phase 1 concluded with a monitoring week (*Figure 19*).

Animals were received from the animal unit at 3 weeks of age and placed in groups of 5 per cage, separated by sex. They were left undisturbed until the age of 3 months, with food and water available *ad libitum*. As rats were group-housed, group allocation was undertaken by cage rather than individual animal to avoid contamination by coprophagia (Galley *et al.*, 2017). Each animal within the groups was then allocated an identifiable code (animals were randomly numbered by means of permanent marker lines on the base of their tails). Once the animals reached the standard weight and age appropriate for this model, baseline behavioural testing was conducted. The model required adult Wistar rats (2 months of age), weighing between 200 – 400 g. The anxiety-like behavioural testing also calls for animals to be 12 weeks or older.

3.4 Behavioural Assessment: Analgesic Testing
Following maturation and habituation, all animals underwent mechanical and thermal hypersensitivity testing (*Figure 19*) on the same day to procure baseline readings. Acclimatization in the experimental room began at 6:00, to ensure adequate time for the animals to assume a relaxed state for the assessments. The following tests were used:

3.4.1 Mechanical Allodynia – Electronic von Frey filaments
A calibrated electronic von Frey was used to assess mechanical tactile hypersensitivity to an innocuous stimulus (allodynia). The principle of the assessment is to evaluate the presence or
extent of aversive behaviour in response to a mechanical stimulus (Deuis, Dvorakova and Vetter, 2017). Three days prior to the official test, habituation training commenced. Each day, animals were placed in elevated, individual, transparent Perspex compartments (Figure 13b: 30 x 35 cm) resting on a mesh wire (Figure 13a: supplied by IITC Life Science) at 7:00. Each animal was randomly assigned to a cubicle; with randomization taking place at the beginning of every test session. A 60-minute period was conducted (González-Hernández et al., 2019). Animals were removed from the compartments at the end of the period and returned to their home cages. The transparent compartments were thoroughly wiped with 70% ethanol between every animal.

![Figure 13](image13a.png)

**Figure 13** (A) Transparent compartments resting on wire mesh grid. 5-6 animals were assessed together in one testing session (B) Electronic von Frey Anesthesiometer (IITC Life Science) (C) Electronic Von Frey. Rodents are placed individually in transparent compartments with a mesh wire floor. A rigid filament is applied perpendicularly to the hind paw. If the rodent withdraws, licks, or shakes the paw, it is considered to have had a positive response and the force (in grams) that elicited the response is recorded. Created using BioRender.com, adapted from Deius et al (2017).

On the day of testing, animals were randomly assigned to a compartment and an acclimatization period of 30-60 minutes was allowed, until alert and weight bearing on all
four paws. The testing procedure then began between 7:30 – 8:00. A single, rigid filament was applied perpendicularly to the plantar portion of the right hind paw with gradual increasing force (Figure 13c), until a paw withdrawal response was elicited (Deuis, Dvorakova and Vetter, 2017). The force (in grams) required to evoke the response was automatically recorded by the apparatus (Electronic von Frey Anesthesiometer; IITC Life Science), as well as manually recorded by the researcher. All animals were tested on the right paw before returning to the first animals’ left paw, to allow for a minimum of 5 minutes to elapse (Tai, Yeung and Cheung, 2018). Any hindpaw movements deemed as a result of reflex/locomotion were disregarded and the test trial was repeated during the following round of testing (Tai, Yeung and Cheung, 2018). Each trial lasted for an average of 10 seconds, and testing sessions lasted for an average of 45 minutes. Three baseline measurements were taken over a consecutive three-day period. These baseline measures were averaged to represent the paw withdrawal threshold (PWT).

Following the behavioural test, animals were returned to their home cages and remained in the experimental room, and all apparatus was thoroughly wiped with 70% ethanol, to prevent the transfer of stress between animals via olfactory stimulation.

3.4.2 Cold Allodynia – Acetone Evaporation Test
An Acetone Evaporation Test was used to evaluate cold hypersensitivity to an innocuous stimulus (allodynia). The principle of the assessment is to measure aversive behaviours evoked by evaporative cooling (Deuis, Dvorakova and Vetter, 2017). This assessment was performed immediately following the von Frey filaments, between 10:00 and 12:00. The same transparent compartments resting on wire mesh were used. Animals were randomly placed into the transparent cubicles and allowed 30–45-minute acclimatization. Once the animals were relaxed and weight bearing on all four paws, the test commenced. A drop of acetone (100 µL) was gently applied to the plantar surface of the left hind paw of the animal using a 1mL syringe (Figure 14a), with care taken to avoid spreading the acetone onto the animals’ fur (Deuis, Dvorakova and Vetter, 2017). Care was taken to ensure the tip of the syringe did not come into contact with the animals’ hind paw. Once applied, a stopwatch was started and the total frequency of responses was graded according to the following criteria:

1. Shaking/sudden withdrawing of paw
2. Biting/licking of paw
3. Cleaning of paw
The frequency of acetone-related behaviours exhibited over a 1-minute observation period was manually recorded by the researcher. The test was repeated thrice on the left hind paw, and the average frequency of responses was calculated. All animals within a group were tested on the left paw before returning to test the first animal, ensuring a minimum of 10 minutes elapsed before the next acetone application to the same hind paw (Vachon et al., 2013; Gong et al., 2018). Each testing session lasted for an average of 45 minutes. Following the behavioural test, animals were returned to their home cages and remained in the experimental room, and all apparatus was thoroughly wiped with 70% ethanol.

3.4.3 **Heat Hyperalgesia – Hargreaves Test**

The Hargreaves test was used to evaluate heat hypersensitivity to a noxious stimulus (hyperalgesia). The principle of the assessment is to quantify the heat threshold of the animals’ hind paw upon application of a radiant heat stimulus (Deuis, Dvorakova and Vetter, 2017). Animals were given a 2-hour respite following the acetone evaporation test (Grégoire et al., 2020). Animals were left alone in the experimental room between 12:00 and 14:00. Thereafter, the assessment took place between 14:00 and 15:00. The animal was placed in the transparent compartment on top of a glass bottom surface and left to acclimatize for 30-45 minutes, to ensure relaxation and equal weight-bearing on all four paws (González-Hernández et al., 2019). A radiant heat source (Plantar Test Analgesia Meter, IITC Life Science) was positioned underneath the mid-plantar surface of the right hind paw (Figure 14b). The time taken for a sharp withdraw from the heat stimulus to occur was automatically recorded by the apparatus, as well as manually recorded by the researcher (Deuis, Dvorakova and Vetter, 2017). This time was considered the paw withdrawal latency. The intensity of the light source was set at 30% (Ibrahim and Ehrlich, 2020) and a pre-determined cut-off time of 20 seconds was set, to prevent tissue damage (Deuis, Dvorakova and Vetter, 2017). The test was repeated thrice on the right hind paw, and the average paw withdrawal latency (PWL) was calculated. All animals within a group were tested on the right paw prior to returning to test the first animal, ensuring a minimum of 10 minutes had elapsed before the next heat application to the same hind paw (González-Hernández et al., 2019). Each testing session lasted for an average of 35 minutes. Following the behavioural test, animals were returned to their home cages and remained in the experimental room for an additional 30 minutes before being returned to their home room. All apparatus was thoroughly wiped with 70% ethanol.
Neuropathic Pain Induction

A week following the analgesic behavioural testing, animals were administered the chemotherapeutic agent *Paclitaxel*, used to generate a model of chemotherapy-induced neuropathic pain (CIPN). The first day of administration marked the start of the experimental procedure, and was denoted as Experimental Day 1 (ED 1). The antineoplastic drug was administered via intraperitoneal (i.p.) injections on alternating days (ED 1, 3, 5, 7) to mimic the conventional chemotherapy cycles (Duggett *et al.*, 2016). On the days of injection, animals were transported to the experimental room at 6:00 and allowed to acclimatize for 1 hour.

Once the animals had been transported to the experimental room, the researcher prepared the *Paclitaxel* solution. Briefly, 60 mg of *Paclitaxel* powder (*Sigma Aldrich, PHR1803, 200 mg*) was weighed and dissolved into saline solution (0.9% sodium chloride) to reach a concentration of 6 mg/mL. Thereafter, saline was gradually introduced to the solution until a concentration of 2 mg/mL was obtained. The solution was mixed using a magnetic mixer (IKA® C-MAG MS 7, Z742680), until the solvent was dissolved. At 7:00, the i.p. injection was administered (2 mg/kg fresh *Paclitaxel* solution), and the animal was immediately placed into a temporary cage separate from their littermates. Once all animals had received

![Figure 14](A) Acetone Evaporation Test. Rodents are individually placed in transparent compartments with a mesh wire floor. Acetone is applied to the left hind paw and the frequency of nocifensive response(s) was recorded (B) Hargreaves test. Rodents are placed individually in transparent compartments with a glass floor. An infrared heat source was focused on the plantar surface of the right hind paw and the time taken to withdraw from the heat stimulus was recorded. Created using BioRender.com, adapted from Deius *et al* (2017).
injections, they were transferred back to their home cages and monitored for a 30-minute period, to ensure any adverse effects were immediately attended to. Thereafter, animals were returned to their home room and left undisturbed until the next injection. Animals were observed twice daily to assess ongoing symptoms over the induction period. No animals were lost during this phase. Animals receiving Paclitaxel were administered a total dose of 8 mg/kg. The groups that received the vehicle solution underwent the same procedure. An equal concentration of saline solution was administered intraperitoneally. Once all animals had undergone the final day of injections, they were returned to their home cages and left undisturbed for a 1-week monitoring period (ED 8-13). Animals were observed twice daily using individual welfare monitoring sheets (Addendum 2) to ensure any excessive pain symptoms were identified. Monitoring throughout this project was completed under the standard ethics of the institution.

To assess whether neuropathic pain was induced, the analgesic behavioural assessments were repeated seven days following the final injection, denoted as ED 14. This protocol was used to validate whether there was a mechanical or thermal change present post Paclitaxel injection. Please refer to the protocol above (3.4 Behavioural Assessment: Analgesic Testing, pg. 66), which was replicated for the reassessment. The end of this protocol marked the conclusion of Phase 1 and the commencement of Phase 2.

3.6 Phase 2
Phase 2 consisted of a 14-day treatment period commencing on ED 15, and included the following treatments: saline solution (CTRL), Duloxetine (DUL), and oxytocin (OXY). The groups from Phase 1 were further separated into six groups using computer-generated randomization, to produce the following groups:

- Group 1: Control (n=10)
- Group 2: Paclitaxel (n=10)
- Group 3: Duloxetine (n=10)
- Group 4: Oxytocin (n=10)
- Group 5: Paclitaxel-duloxetine (n=10)
- Group 6: Paclitaxel-oxytocin (n=11)

During the 14-day period, the analgesic behavioural assessments were conducted on a weekly basis to continue monitoring for any changes in mechanical and thermal sensitivity. Please
refer to the above protocol for details. At the end of the intervention period, anxiolytic behavioural assessments were conducted, followed by euthanasia 24-48 hours later.

3.7 Treatment

During the treatment period, the respective treatment was administered daily. Animals were transferred to the experimental room at 6:00 and allowed to acclimatize for 1 hour. During the acclimatization hour, the drug solutions were prepared by the researcher. All treatment administration took place from 7:00 onwards. Once administration commenced, the animals were monitored for a 30-minute period, to ensure any adverse effects were not overlooked. Following this monitoring period, animals were either returned to their home room or remained in the experimental room, depending on what the group was scheduled for. Animals allocated to the control groups were administered saline solution via oral gavage over the treatment period (1 mL/kg).

3.7.1 Duloxetine Administration

Duloxetine is considered the gold standard treatment for chemotherapy-induced peripheral neuropathy, and will be used as a positive control over the intervention period. During the acclimatization hour, Duloxetine solution was prepared by the researcher. Briefly, 100 mg of Duloxetine Hydrochloride powder (Sigma-Aldrich, CAS # 136434-34-9) was weighed and dissolved in saline solution to reach a concentration of 15 mg/mL (Chahal, Sodhi and Madan, 2020). The solution was administered via oral gavage at 7:00 (30 mg/kg). Immediately following the administration, the animal was placed into a temporary cage separate from their littermates. Once all animals had received treatment, they were transferred back to their home cages and monitored for a 30-minute period, to ensure any adverse effects were immediately attended to, as well as to ensure any ultra-vocalization did not disturb the other animals.

3.7.2 Central Oxytocin Administration

The groups receiving oxytocin treatment were habituated for one week prior to the start of treatment (Calcagnoli et al., 2015), during the Phase 1 monitoring week. Habituation included three days of gradual introduction to the holding technique, followed by incremental intranasal administration of distilled water for a four-day period. Animals were moved to the experimental room at 6:00 and allowed to acclimatize for 1 hour. For the first three days of habituation, at 7:00 the conscious rat was held by the experimenter in a supine position with its head positioned horizontally (Figure 15a). The animal was held for a total of 2 minutes each day, but the duration of immobilization increased incrementally (Day 1: 6 x 20 seconds;
Day 2: 3 x 40 seconds; Day 3: 2 x 1 minute). A pipette was held close to the rhinarium to acclimatize the animals to its presence. Days 4-7 of habituation consisted of intranasal administration of distilled water. The animal was held in the above position and a total of 20 uL of distilled water (2 x 10 uL) was bilaterally applied using a 100 uL pipette. The solution was administered on the squamous epithelium of both the left and right rhinarium (Figure 15b), and care was taken to avoid direct contact of the pipette tip with the rhinarium, direct application into one of the nostrils, or in the proximity of the phitrum (Calcagnoli, F., et al. 2015). The solution was loaded into the pipette, and the tip was placed near the rat's nostril at a 45° angle. The contents were slowly ejected from the pipette to form a small droplet at the end of the pipette tip. This was placed around the rhinarium to allow for inhalation. Following the inhalation of this droplet, about 2-3 seconds later, another droplet of solution was ejected to be inhaled by the rat, through the other nostril. The ejection of these droplets of solution from the pipette tip was repeated and alternated between nostrils until the full volume was administered. Any snorted-out droplets were replaced. Immediately following administration, the animal was placed in a separate cage. Once all animals had undergone intranasal administration, they were returned to their home cage and monitored for a 30-minute period to ensure any adverse effects were immediately attended to, as well as to ensure any ultra-vocalization did not disturb the other animals. All animals were acclimatized to the administration method when treatment commenced.

When the intervention period commenced, the oxytocin solution was prepared every second day by the researcher during the acclimatization period. Briefly, 200 ug of synthetic oxytocin powder (Sigma-Aldrich, Lot # 103H05241 - Iyophilized powder form) was weighed and
dissolved in saline solution to achieve a final concentration of 1 ug/μL. At 7:00, the administration began. Each animal received 20 μL (2 x 10 μL into each nostril) of intranasal oxytocin solution (Neumann et al., 2013). Immediately following the administration, the animal was placed into a temporary cage separate from their littermates. Once all animals had received treatment, they were transferred back to their home cages and monitored for a 30-minute period, to ensure any adverse effects were immediately attended to, as well as to ensure any ultra-vocalization did not disturb the other animals. The remaining oxytocin solution was covered with foil and stored in 4°C until the following day’s administration. The solution was removed from the fridge an hour prior to administration, to ensure the solution was administered at room temperature.

3.8 Behavioural Assessment: Anxiolytic Testing
Following the intervention period, all animals underwent anxiety-like behavioural assessments (Figure 19) 24 hours following the completion of the final analgesic behavioural testing protocol. Anxiety-like behavioural baseline testing was not performed, as the novelty of the tests was used as an additional introduction of a stressor. Both protocols were recorded using a GoPro Hero 7 positioned directly above the apparatus. Analysis of behaviour was performed retrospectively using Behavioural Observation Research Interactive Software (BORIS), Version 7.13. Acclimatization in the experimental room began at 6:00, to ensure adequate time for the animals to assume a relaxed state for the assessments. The following tests were used:

3.8.1 Light/dark Box
This assessment is separated into three sessions, two training sessions and a testing session. Each session transpired 24 hours following the last. The principle of the assessment is to evaluate the conflict between the animal’s exploratory drive versus its aversion to bright, open spaces. Anxious animals tend to avoid the illuminated compartment (Abboud et al., 2021), therefore the percentage of time spent in the dark compartment is used as an indication of anxiety. Exploratory behaviour can be represented by the percentage of time spent in the illuminated compartment. The test session followed the same protocol as described below; expect the light/dark box was divided by a removable partition with a small aperture to allow for free access between compartments. This introduced a novel obstacle to the protocol and can be used to evaluate the animals learning and memory, by analyzing the latency to navigate the dark compartment.
On Day 1 of anxiety-like behavioural assessment, the first light/dark box training session took place at 7:00 following acclimatization in the experimental room. Assessment of all animals took place between 7:00 and 11:00. The training phase was devoid of all partitions (i.e., obstacles). The box was placed on a marked area on the experimental room floor (Figure 16) with the GoPro positioned directly above to record the entire session. To commence the session, the animal was placed on the far side of the bright compartment, facing away from the rest of the box. It then had 5 minutes to explore the box and locate the dark compartment. If the rat failed to locate the dark compartment, they will be placed in the dark compartment for 1 minute. The researcher manually recorded the number of fecal boli and urine pools, while the rest of the behaviours were recorded via video and analyzed retrospectively (Qulu, Daniels and Mabandla, 2015). Following completion of the test, the animals were transferred to a new cage until all cage mates had undergone the protocol. The entire group was then returned to their home cage and monitored for 30 minutes before being returned to their home room. The apparatus was thoroughly wiped with 70% ethanol between each animal. For a detailed explanation of each criterion, please refer to Addendum C, Table 1.

3.8.2 Elevated Plus Maze
The principle of this assessment evaluates the conflict created between a rat's exploratory drive and innate fear of open, elevated, and exposed areas (Vachon et al., 2013).
Anxious animals are expected to spend less time in the open arms (Bosch et al., 2007); therefore, the percentage of time spent in the closed arms can be used as an indication of anxiety-like behaviour. In contrast, the percentage of time spent in the open arms can be used as an indication of explorative behaviour. Total number of entries between arms can be used as an indication of locomotor activity.

The elevated plus maze protocol took place between 11:00 and 14:00 following the testing session of the light/dark box (Day 3 of anxiety-like behavioural assessment, ED 31). The maze was placed on a marked area on the experimental room floor (Figure 17), with the GoPro positioned directly above. It consisted of two open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 40 cm), connected by a center square (10 x 10 cm). The two open arms were opposite each other, and the maze was elevated to a height of 50 cm. To commence the test, the animal was placed in the center of the maze facing a closed arm (Aguggia, Suárez and Rivarola, 2013). The animal then had 5 minutes to freely explore the maze while behaviour was recorded using the GoPro (Addendum C, Figure 2). Following completion of the test, the animals were transferred to a new cage until all cage mates had undergone the protocol. The entire group was then returned to their home cage and monitored for 30 minutes before being returned to their home room. The apparatus was thoroughly wiped with 70% ethanol between each animal. For a detailed explanation of each criterion, please refer to Addendum C, Table 2.

3.9 Euthanasia
Euthanasia took place 24-48 hours following completion of the final behavioural testing. The duration between final assessment and euthanasia depended on the group in question. Animals were euthanized via decapitation using a sharp guillotine.

Animals were transported to the euthanasia area at 6:00, to allow for a 1-hour acclimatization period. This was to limit any excessive elevations in CORT levels, induced by novel or sudden stressors. Euthanasia commenced at 7:00. Immediately following decapitation, the trunk blood was collected in EDTA tubes, gently rotated, and placed on ice for the duration of the euthanasia. Various visceral organs were collected, including the heart, lungs, kidney, and liver. All organs were individually collected in 1.5 mL cyrotubes and immediately placed into liquid nitrogen following removal. The brain was dissected and several brain parts were removed, including the prefrontal cortex, hypothalamus, hippocampus, and amygdala. All brain parts were individually collected in 1.5 mL cyrotubes and immediately placed into liquid nitrogen following removal to minimize tissue degeneration.
Following the completion of euthanasia, all harvested blood and tissue samples were transported to the appropriate laboratory. All visceral organs and brain parts were removed from the remaining liquid nitrogen and immediately placed in the -80°C biofreezer for future analysis. The visceral organs have been dedicated to a 2022 Honours project titled *The Effect of Exogenous Oxytocin Administration on Visceral Organs in a Paclitaxel Rat Model*, to be completed by Ms. K-Lee Jubber (21223831).

Trunk blood was centrifuged at 1500 g for 15 minutes at 4°C. The EDTA tubes were then placed on ice while the plasma was aspirated using a P1000 pipette and aliquoted into 1.5 mL Eppendorf tubes. Once all plasma was collected and labeled, it was placed in the biofreezer and stored at -80°C until analysis. The remainders of the blood components were appropriately disposed of.

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### 3.10 Peripheral Plasma Corticosterone Analysis

Corticosterone (CORT) concentrations were measured within the plasma using commercially available rat CORT ELISA kits (*StressMarq Bioscience, Catalog # SKT-905*). This kit is a competitive ELISA (*Figure 18*), the principle of which is to quantitatively measure the concentration of a sample antigen. This relies on the competitive binding process between original antigen (sample antigen) and the pre-coated antigen present within the microplates. The CORT ELISA manufacturers’ instructions were followed to complete the assay.
A pre-experiment was performed to identify the optimal sample dilution to use for the experiment. Based on the results of this experiment, a dilution factor of 100 was selected.

To perform the actual experiment, plasma samples were thawed and kept on ice during the protocol. 5 uL of supplied Dissociation Reagent (SKC-205G), brought to room temperature, was pipetted into a 1 mL Eppendorf tube. Thereafter, 5 uL of sample was transferred to the same tube. After this, was gently vortexed and incubated at room temperature for 5 minutes (minimum), the sample was diluted with 490 uL of supplied Assay Buffer (SKC-205F) to achieve a final dilution of 1:100.

In depth, 50 uL of each standard (premixed to produce a concentration gradient) and diluted sample were pipette in duplicate into the individual wells of the supplied microtiter plate (SKC-205A, clear coated 96 well plates). The plate was pre-coated with corticosterone antibody by the manufacturers. A corticosterone-peroxidase conjugate (SKC-205D & SKC-205E) was added to the standard and sample wells. The binding reaction was initiated by the addition of the supplied polyclonal antibody (SKC-205C) to corticosterone to each well. After an hour incubation at room temperature on a plate shaker set at 600 rpm to allow for the competitive reaction to take place, the plate was washed four times using the supplied diluted wash buffer (SKC-205H). This was to ensure any excess conjugate and unbound sample was removed. The supplied TMB substrate (SKC-205I) was then added to each well, which reacted with the bound corticosterone-peroxidase conjugate. After 30 minutes quiescent incubation at room temperature, the reaction was stopped using the supplied stop solution (SKC-205J). The generated colour was then detected using a plate reader (BMG Labtech, Omega Plate Reader) measuring at 450 nm wavelength. A 4 Parameters Logistics (4PL) curve was generated using the known standard concentrations (x-values) and optical density (y-values) readings. GraphPad Prism software was used to generate the four variables needed to create the curve equation. The concentration of the unknown samples was calculated using equation to calculate the unknown x-value (the CORT concentration of the samples). After making the suitable corrections for the dilution of the sample, the CORT concentrations were analyzed for statistical significance.

3.11 Oxytocin Analysis
As CIPN affects the periphery as well as the central nervous system, we evaluated the peripheral plasma oxytocin concentration and hypothalamic oxytocin concentration as indications of circulating and central levels, respectively.
3.11.1 Peripheral Plasma Oxytocin Analysis
Peripheral oxytocin (OT) concentration was measured within plasma using commercially available rat OT ELISA kits (Elabscience, Catalog # E-EL-0029). This kit is a competitive ELISA (Figure 18), the principle of is to measure the concentration of a sample antigen. This relies on the competitive binding process between original antigen (sample antigen) and the pre-coated antigen present within the microplates. The OT ELISA manufacturers’ instructions were followed to complete the assay.

A pre-experiment was performed to identify the optimal sample dilution to use for the experiment. Based on the results of this experiment, a dilution factor of 1000 was selected for males and 500 for females.

In depth, 50 µL of each standard (premixed to produce a concentration gradient) and diluted sample were pipetted in duplicate into the individual wells of the supplied microtiter plate (clear coated 96 well plates). The plate was pre-coated with oxytocin antibody by the manufacturers. Immediately thereafter, 50 µL of Biotinylated Detection Ab working solution was added to each well. The plate was covered with a provided sealer and incubated for 45 minutes at 37°C, to allow for oxytocin in the standard/sample to compete with a fixed amount of oxytocin on the solid phase support for sites on the biotinylated detection Ab specific to oxytocin. Thereafter, the solution was decanted from each well and the plate was washed three times with 350 µL wash buffer per well, to ensure excess conjugate and unbound sample is washed away. The buffer soaked for 1 minute each wash, then it was decanted and the process repeated. Then, 100 µL of HRP Conjugate working solution was added to each well, to generate the colour compound needed to detect the antigen. The plate was covered with a provided sealer and incubated for 30 minutes at 37°C. This was to allow for the enzyme to attach to the unbound OT on the plate. The solution was decanted from each well, and the washing step was repeated five times. Thereafter, 90 µL of Substrate Reagent was added to each well in a dark room. The plate was covered with a new sealer and placed in a sleeve to protect it from the light. It was incubated for 15 minutes at 37°C. This allowed for an enzyme-substrate reaction to occur, causing the solution in these wells to turn blue in colour. The change in colour was monitored and the duration of incubation was modified based on how rapidly the blue colour was produced (no longer than 30 minutes). Finally, 50 µL of Stop Solution was added to each well in the same order as the Substrate Reagent was added, to terminate the reaction, causing the solution to turn from blue to yellow. The optical density was measured immediately using a plate reader (BMG Labtech, Omega Plate Reader).
measuring at 450 nm wavelength. A 4PL curve was generated using the known standard concentrations (x-values) and optical density (y-values) readings. GraphPad Prism software was used to generate the four variables needed to create the curve equation. The concentration of the unknown samples was calculated using equation to calculate the unknown x-value (the oxytocin concentration of the samples). The oxytocin concentrations were then analyzed for statistical significance.

### 3.11.2 Hypothalamic Oxytocin Analysis

Hypothalamic tissue was prepared according to the Elabscience Oxytocin (OT) ELISA Kit guidelines. Briefly, the hypothalamic tissue was thawed on ice and 50 mg was washed with ice-cold phosphate buffered saline (PBS) and transferred to 450 mL of PBS (0.01M, pH = 7.4; tissue weight (g): PBS (mL) = 1:9). This solution was then homogenized using Allsheng Bioprep-24R (Serial no.: 162-18493-21040001) at 4°C for 3 x 1 min cycles set at 4000 rpm. This solution was then left to rest on ice for 15 minutes. Thereafter, the solution was centrifuged using Axiology Labs High Speed Refrigerated Centrifuge (Model: BKC-TH16R; SN: 18050821XK) at 4°C, 12 580 g for 20 minutes. The supernatant was then aliquoted into 2 x 1.5 mL Eppendorf tubes and stored in the -80°C biofreezer until use.

Central oxytocin concentration was then measured within the supernatant using commercial Oxytocin (OT) ELISA kits (Elabscience, Catalog # E-EL-0029). The OT ELISA manufacturers’ instructions were followed to complete the assay.

A pre-experiment was performed to identify the optimal sample dilution to use for the experiment. Based on the results of this experiment and previous researcher’s results that have used the same kit, we decided to keep the samples undiluted for this ELISA protocol.

Supernatant was thawed at room temperature and centrifuged at 800 rpm for 1 minute at 4°C to remove any precipitate. Please refer to the above protocol for details of the oxytocin ELISA.

### 3.12 Statistical Analysis

All statistical analysis was performed on GraphPad Prism, version 8.4.3. All behavioural videos were analyzed using BORIS, Version 7.13 (Behavioural Observation Research Interactive Software). Normality and Gaussian distribution were determined using the Shapiro-Wilk normality test. All parametric data were analysed using a one-way or repeated measures analysis of variance (ANOVA) to identify significant effect of means, followed by a Tukey multiple comparison post-hoc test. These data included all analgesic (repeated
measures ANOVA: electronic von Frey, acetone evaporation, and Hargreaves tests) and anxiolytic (one-way ANOVA: all EPM variables, all LDB variables except *Latency to Enter Dark Compartment*) behavioural testing, and the oxytocin assay data (one-way ANOVA: plasma and hypothalamic data). Non-parametric data was analysed using the Kruskal-Wallis test to identify significant effect of means, followed by the Dunn’s multiple comparisons post-hoc test. These data sets included the LDB variable *Latency to Enter Dark Compartment*, as well as the CORT assay data. All data were presented as means ± standard deviation (SD). In all cases, differences were considered significant if the p-value was less than 0.05.

![Competitive ELISA reaction and protocol](https://scholar.sun.ac.za)

**Figure 18** Competitive ELISA reaction and protocol. Created using [BioRender.com](https://biorender.com), adapted from Elabscience.

(A) Add 50 uL of samples and controls to each well. Immediately add 50 uL of HRP-RBD working solution. Incubate for 60 min at 37°C.

(B) Aspirate and wash the plate 3 times.

(C) Add 90 uL of Substrate reagent. Incubate for about 15 min at 37°C.

(D) Add 50 uL of Stop Solution.

(E) Immediately read the plate at 450 nm and record the optical densities.
Figure 19 Phase 1 aimed to induce CIPN in adult Wistar rats using the chemotherapeutic agent *Paclitaxel*. Mechanical and thermal hypersensitivity testing was used to evaluate changes due to the neuropathic induction. Phase 2 consisted of a 14-day intervention period with saline (o.g.), Duloextine (o.g.), or oxytocin (i.n.) administered as treatment. Analgesic behavioural testing was assessed throughout the intervention period. Following the intervention, anxiety-like behaviour was assessed using the elevated plus maze (EPM) and light-dark box protocols.

*I. P – intraperitoneal*
4 Results

This statement aims to provide a clear description of the results obtained in this study. During Phase 1, Wistar rats were exposed to Paclitaxel. Mechanical and thermal sensitivity was measured and compared at baseline and following Paclitaxel injection for each animal. ‘Baseline’ is defined as analgesic measures prior to receiving Paclitaxel/saline injections, while ‘post-injection’ refers to measures occurring after all injections were administered. Comparisons were also made between the two groups, denoted saline (n=30) and Paclitaxel (n=31). Phase 2 consisted of a 14-day treatment period, where comparisons were made between and within the following treatment groups over the intervention period: control (n=10), Paclitaxel (n=10), duloxetine (n=10), oxytocin (n=10), Paclitaxel-duloxetine (n=10), and Paclitaxel-oxytocin (n=11).

All animals remained healthy throughout the intervention, with no evidence of alopecia or diarrhoea. No significant weight loss was observed.

4.1 Phase 1

Effect of Paclitaxel administration on mechanical hypersensitivity

Mechanical allodynia was assessed in Wistar rats using the electronic von Frey filament. The paw withdrawal latency (PWL) was used as an indication of mechanical sensitivity. The following groups were assessed: saline (SAL) & Paclitaxel (PAC). There was an overall significance when comparing baseline (BL) to post-injection (PI) measures within groups (F (1, 58) = 2066, p < 0.0001). The group which received Paclitaxel exhibited a significant increase in mechanical sensitivity from baseline to post-injection recordings ****(PAC-BL vs PAV-PI), p < 0.0001. An overall Paclitaxel-effect (F (1, 58) = 20.48, p < 0.0001) was also identified between groups. No significant difference was identified between group baseline measures. A comparison between groups’ post-injection measures exhibited a significant difference, with the Paclitaxel group demonstrating a reduction in paw withdrawal threshold (PWT), *(SAL-PI vs PAC-PI), p = 0.0123 (Figure 20).
Effect of Paclitaxel administration on thermal hypersensitivity

Thermal allodynia was assessed in Wistar rats by exposing animals to a cold stimulus. The total frequency of responses was used as an indication of thermal sensitivity. The following groups were assessed: saline (SAL) & Paclitaxel (PAC). There was an overall significance when comparing baseline (BL) to post-injection (PI) results within groups ($F_{(1, 58)} = 40.03$, $p < 0.0001$). The group which received Paclitaxel exhibited a significant increase in thermal sensitivity from baseline to post-injection recordings, ****($PAC$-BL vs $PAC$-PI), $p < 0.0001$. An overall Paclitaxel-effect ($F_{(1, 58)} = 25.88$, $p < 0.0001$) was also identified between groups. No significant difference was identified between group baseline measures. A comparison between groups’ post-injection measures exhibited a significant difference, with the Paclitaxel group demonstrating an increase in thermal sensitivity, ****($SAL$-PI vs $PAC$-PI), $p < 0.0001$ (Figure 21).
Thermal hyperalgesia was assessed in Wistar rats using a localized heat source. Paw withdrawal latency (PWL) was used as a measure of thermal hypersensitivity. The following groups were assessed: saline (SAL) & Paclitaxel (PAC). No significance was found between or within groups (Figure 22).

**Figure 21** Thermal allodynia during Phase 1 in the following groups: saline (SAL, n = 30) and Paclitaxel (PAC, n = 31). ****p<0.0001. Data are presented as mean ± SD.

**Figure 22** Thermal hyperalgesia during Phase 1 in the following groups: saline (SAL, n = 30) and Paclitaxel (PAC, n = 31). Data are presented as mean ± SD.
4.2 Phase 2

Effect of treatment and intervention duration on mechanical sensitivity

The effect of long-term treatment on mechanical allodynia was assessed using an electronic von Frey filament on Wistar rats. Repeated measures were taken during week 1 and 2 of intervention. The paw withdrawal latency (PWL) was used as an indication of mechanical sensitivity. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). There was an overall significance observed between time points ($F_{(2.628, 141.9)} = 16.97, p < 0.0001$), as well as an overall interaction-effect between time and treatment ($F_{(15, 162)} = 6.895, p < 0.0001$). A Paclitaxel-effect was observed in the animals that were administered Paclitaxel and treated with saline, indicated in the continuous increase in mechanical sensitivity over the time periods, **(PAC-BL) vs (PAC-PI), p = 0.0061; **(PAC-BL) vs (PAC-Int Wk1), p = 0.0016; *** (PAC-BL) vs (PAC-Int Wk2), p = 0.0002. Duloxetine did not have an effect during the first week of intervention. There was a duloxetine-effect found in the second week of intervention within the animals administered Paclitaxel and treated with duloxetine, as observed in the significant decrease in mechanical sensitivity, *(PAC-DUL-Int Wk1) vs (PAC-DUL-Int Wk 2), p = 0.0120. There was an overall oxytocin-effect observed, as the animals administered Paclitaxel and treated with oxytocin exhibited a significant decrease in mechanical sensitivity, *(PAC-OXY-PI) vs (PAC-OXY-Int Wk1), p = 0.0135; and intervention week 2 *(PAC-OXY-PI) vs (PAC-OXY-Int Wk2), p = 0.0344 (Figure 23).
The effect of long-term treatment on thermal alldynia was assessed using a cold stimulus, where the total frequency of responses indicated thermal sensitivity. Repeated measures were taken during week 1 and 2 of intervention. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). A significant treatment-effect was found ($F_{(5, 54)} = 8.480, p < 0.0001$), as well as a significant interaction-effect between time and treatment ($F_{(15, 162)} = 4.699, p < 0.0001$), and finally a significant time-effect ($F_{(2.827, 152.7)} = 15.65, p < 0.0001$) across the intervention period. The group administered Paclitaxel and treated with saline demonstrated an increase in thermal sensitivity throughout the intervention period, **(PAC-BL) vs (PAC-PI), $p = 0.0261$; *(PAC-BL) vs *(PAC-Int Wk1), $p = 0.0499$; ****(PAC-BL) vs (PAC-Int Wk2), $p = 0.0220$. A duloxetine-effect was observed within the group administered Paclitaxel, identified between post-injection and intervention week 1 measures, ****(PAC-DUL-PI) vs (PAC-DUL-Int Wk1), $p < 0.0001$ (Figure 24).

**Effect of treatment and intervention duration on thermal sensitivity**

The effect of long-term treatment on thermal alldynia was assessed using a cold stimulus, where the total frequency of responses indicated thermal sensitivity. Repeated measures were taken during week 1 and 2 of intervention. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). A significant treatment-effect was found ($F_{(5, 54)} = 8.480, p < 0.0001$), as well as a significant interaction-effect between time and treatment ($F_{(15, 162)} = 4.699, p < 0.0001$), and finally a significant time-effect ($F_{(2.827, 152.7)} = 15.65, p < 0.0001$) across the intervention period. The group administered Paclitaxel and treated with saline demonstrated an increase in thermal sensitivity throughout the intervention period, **(PAC-BL) vs (PAC-PI), $p = 0.0261$; *(PAC-BL) vs *(PAC-Int Wk1), $p = 0.0499$; ****(PAC-BL) vs (PAC-Int Wk2), $p = 0.0220$. A duloxetine-effect was observed within the group administered Paclitaxel, identified between post-injection and intervention week 1 measures, ****(PAC-DUL-PI) vs (PAC-DUL-Int Wk1), $p < 0.0001$ (Figure 24).
The effect of long-term treatment on thermal hyperalgesia was assessed using a localized heat source. Paw withdrawal latency (PWL) was used as a measure of thermal hyperalgesia. Repeated measures were taken during week 1 and 2 of intervention. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). A significant overall treatment-effect was identified ($F_{(5, 54)} = 5.838$, $p = 0.0002$). However, no significance was observed between specific groups (Figure 25).

Figure 24 Thermal allodynia during Phase 2 in the following groups: control (CTRL, $n = 10$), Paclitaxel (PAC, $n = 10$), duloxetine (DUL, $n = 10$), oxytocin (OXY, $n = 10$), Paclitaxel-duloxetine (PAC-DUL, $n = 10$), and Paclitaxel-oxytocin (PAC-OXY, $n = 11$). Data are presented as mean ± SD, *$p≤0.05$, **$p≤0.01$, ***$p≤0.001$, ****$p≤0.0001$.

BL – baseline; PI – post-injection; Int. Wk1 – Intervention Week 1; Int. Wk2 – Intervention Week 2
Anxiolytic Assessment

Effect of treatment and intervention duration on anxiety-like behaviour

Anxiety-like behaviour was assessed using the light dark box (LDB) post-intervention. Percentage of time spent in the light compartment is considered explorative behaviour, while percentage of time spent in the dark compartment is considered anxiety-like behaviour. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY).

A significant treatment-effect was found ($F_{(5, 55)} = 4.177, p = 0.0027$) between the groups when observing the differences in percentage of time spent in the light compartment, specifically during the training sessions (Figure 26). The animals administered Paclitaxel and treated with oxytocin exhibited a decrease in time spent in the light compartment compared to respective counterparts, *(CTRL) vs (PAC-OXY), p = 0.0198; *(PAC) vs (PAC-OXY), p = 0.0106; *(OXY) vs (PAC-OXY), p = 0.0212. No other significance was found within the training and testing session (Figure 27-30).
Assessment of explorative behaviour in the light dark box training session. Normality was confirmed using the Shapiro-Wilk test. Data are represented as mean ± SD. The neuropathic pain group receiving oxytocin as treatment demonstrated the least explorative behaviour compared to its counterparts, *p≤0.05.

Assessment of anxiety-like behaviour in the light dark box training session. Normality was confirmed using the Shapiro-Wilk test. Data are represented as mean ± SD. Greater time spent in the dark compartment is an indication of anxiety-like behaviour. No statistical significance was found.
Figure 28 Assessment of explorative behaviour in the light dark box testing session. Normality was confirmed using the Shapiro-Wilk test. Data are represented as mean ± SD. Greater time spent in the light compartment is an indication of explorative behaviour. No statistical significance was found.

Figure 29 Assessment of anxiety-like behaviour in the light dark box testing session. Normality was confirmed using the Shapiro-Wilk test. Data are represented as mean ± SD. Greater time spent in the dark compartment is an indication of anxiety-like behaviour. No statistical significance was found.
Testing Session: Latency to Enter the Dark Compartment

<table>
<thead>
<tr>
<th>Treatment Groups</th>
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<tbody>
<tr>
<td>Control (CTRL)</td>
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<tr>
<td>Paclitaxel (PAC)</td>
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<tr>
<td>Duloxetine (DUL)</td>
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<tr>
<td>Oxytocin (OXY)</td>
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<tr>
<td>Paclitaxel-Duloxetine (PAC-DUL)</td>
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<td>Paclitaxel-Oxytocin (PAC-OXY)</td>
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**Figure 30** Latency to enter the dark compartment during the testing session. Data was abnormally distributed, as identified using the Shapiro-Wilk test. Data are represented as mean ± SD. Greater latency to find the dark compartment through the obstacle is indicative of a reduction in problem solving ability and working memory. No statistical significance was found.

Anxiety-like behaviour was assessed using the elevated plus maze (EPM) post-intervention. Percentage of time spent in the closed arm was used as a measure of anxiety-like behaviour, while the percentage of time spent on the open arm was considered an indication of explorative behaviour. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). There was an overall treatment-effect between groups (F(5, 55) = 2.593, p = 0.0355). There was no significance between the duloxetine, Paclitaxel, and control groups. An oxytocin-effect was found, as the animals administered Paclitaxel and treated with oxytocin displayed significantly more time in the open arm, indicative of explorative behaviour, *(PAC) vs (PAC-OXY), p = 0.0355; *(PAC-DUL) vs (PAC-OXY), p = 0.0416 (Figure 31).
No statistical significance was observed for the percentage of time spent in the closed arms (Figure 32).

Figure 31 Explorative behaviour demonstrated in the elevated plus maze in the following groups: control (CTRL, n = 10), Paclitaxel (PAC, n = 10), duloxetine (DUL, n = 10), oxytocin (OXY, n = 10), Paclitaxel-duloxetine (PAC-DUL, n = 10), and Paclitaxel-oxytocin (PAC-OXY, n = 11). Data are represented as mean ± SD, *p≤0.05.

Figure 32 Anxiety-like behaviour demonstrated in the elevated plus maze in the following groups: control (CTRL, n = 10), Paclitaxel (PAC, n = 10), duloxetine (DUL, n = 10), oxytocin (OXY, n = 10), Paclitaxel-duloxetine (PAC-DUL, n = 10), and Paclitaxel-oxytocin (PAC-OXY, n = 11). Data are represented as mean ± SD. No significance observed.
4.4 Neurochemical Assessment

**Effect of treatment and intervention duration on neurochemical concentrations**

Peripheral plasma corticosterone concentrations were assessed in Wistar rats. The following groups were assessed: control (CTRL), *Paclitaxel* (PAC), duloxetine (DUL), oxytocin (OXY), *Paclitaxel*-duloxetine (PAC-DUL), and *Paclitaxel*-oxytocin (PAC-OXY). There was an overall treatment-effect observed ($H_{(6, 61)} = 17.59$, $p = 0.0035$). A significant decrease in the corticosterone concentration within the circulation of the animals administered *Paclitaxel* and treated with oxytocin and saline was observed when compared to the control group; **(CTRL) vs (PAC-OXY), $p = 0.0011$, *(CTRL) vs (PAC), $p = 0.0474 (Figure 33).**

![Plasma Corticosterone Concentration](image)

**Figure 33** Peripheral plasma corticosterone concentrations (pg/mL) in the following groups: control (CTRL, $n = 10$), *Paclitaxel* (PAC, $n = 10$), duloxetine (DUL, $n = 10$), oxytocin (OXY, $n = 10$), *Paclitaxel*-duloxetine (PAC-DUL, $n = 10$), and *Paclitaxel*-oxytocin (PAC-OXY, $n = 11$). Data are represented as mean ± SD, *$p\leq0.05$, **$p\leq0.01$.**

Hypothalamic oxytocin concentration was assessed in Wistar rats. The following groups were assessed: control (CTRL), *Paclitaxel* (PAC), duloxetine (DUL), oxytocin (OXY), *Paclitaxel*-duloxetine (PAC-DUL), and *Paclitaxel*-oxytocin (PAC-OXY). A significant treatment-effect was observed ($F_{(5, 47)} = 2.972$, $p = 0.0206$). The control group demonstrated a significantly increased hypothalamic oxytocin concentration compared to the oxytocin group, as well as the animals administered *Paclitaxel* and treated with duloxetine, respectively; *(CTRL) vs (OXY), $p = 0.0327$, *(CTRL) vs (PAC-DUL), $p = 0.0186 (Figure 34).**
Peripheral plasma oxytocin concentration was assessed in Wistar rats. The following groups were assessed: control (CTRL), Paclitaxel (PAC), duloxetine (DUL), oxytocin (OXY), Paclitaxel-duloxetine (PAC-DUL), and Paclitaxel-oxytocin (PAC-OXY). No significance was identified between groups (Figure 35).

Figure 34 Hypothalamic oxytocin concentrations (pg/mL) in the following groups: control (CTRL, n = 10), Paclitaxel (PAC, n = 10), duloxetine (DUL, n = 10), oxytocin (OXY, n = 10), Paclitaxel-duloxetine (PAC-DUL, n = 10), and Paclitaxel-oxytocin (PAC-OXY, n = 11). Data are represented as mean ± SD, *p≤0.05.
Figure 35 Peripheral plasma oxytocin concentrations (pg/mL) in the following groups: control (CTRL, n = 10), Paclitaxel (PAC, n = 10), duloxetine (DUL, n = 10), oxytocin (OXY, n = 10), Paclitaxel-duloxetine (PAC-DUL, n = 10), and Paclitaxel-oxytocin (PAC-OXY, n = 11). Data are represented as mean ± SD.
5 Discussion

As the prevalence of cancer rises, a corresponding increase in chemotherapy utilization is expected, subsequently resulting in an exponential rise in CIPN. Being a dose limiting condition, associated with severe impairments to one’s quality of life, the necessity to establish effective management of CIPN is vital. Currently, duloxetine is the only official recommendation for the treatment of CIPN, and is therefore considered the ‘gold standard’ pharmaceutical approach. However, it is associated with a plethora of adverse effects which escalates the overall burden to the patient. In order to identify alternative or augmentative therapeutic approaches, further research must be performed using the appropriate preclinical model. We therefore firstly aimed to establish a model of CIPN in Wistar rats, using the chemotherapeutic drug Paclitaxel. Our subsequent aim was to assess an augmented long-term treatment alternative to the conventional gold standard pharmaceutical intervention. This study was carried out in two phases, where Phase 1 was designed to establish a model of CIPN using intraperitoneal administration of Paclitaxel to both male and female Wistar rats, followed by Phase 2 which consisted of a 14-day treatment period of a vehicle solution, duloxetine, or oxytocin. We introduced synthetic oxytocin administered intranasally as a novel treatment, to investigate the potential analgesic and anxiolytic effect it provided. We evaluated the changes in mechanical and thermal sensitivity over the intervention period as an indication of the analgesic potential of the various treatments.

Our findings showed that we were able to establish a model of chemotherapy-induced peripheral neuropathy, as the animals that received Paclitaxel exhibited a significantly greater mechanical and thermal sensitivity when compared to their control counterparts. It is well-established that CIPN affects both mechanical and thermal nociceptive responses (Omran et al., 2021), resulting in a hypersensitivity to these stimuli, as we observed during Phase 1. Our findings are in agreement with Griffiths et al., (2018) and Duggett et al., (2016), who observed a delayed onset of mechanical and thermal sensitivity following the same protocol of Paclitaxel administration. We postulate that the mechanisms associated with the above-mentioned findings emulate the pathophysiology associated with Paclitaxel administration seen in literature. The putative mechanism associated with Paclitaxel-induced peripheral neuropathy (PIPN) is multifaceted, consequently manifesting in the numerous clinical symptoms. Paclitaxel has been shown to induce morphological, electrophysiological, and immunological alterations in the peripheral neurons as well as the DRG (Zang et al., 2019; Staff et al., 2020). As Paclitaxel functions as a microtubule stabilizer, axonal transport is
impeded due to microtubule dysfunction, subsequently contributing to impaired organelle migration, calcium dysregulation, mitochondrial dysfunction, excessive oxidative stress due to reactive oxygen species (ROS) accumulation, and eventual axonal degeneration (Staff et al., 2020; Omran et al., 2021). In addition, the loss of contact between Schwann cells and axolemma contributes to axonal demyelination, further impairing neuronal communication, and resulting in the typical presentation associated with neuropathic pain (Staff et al., 2020). The upregulation of Ca\textsuperscript{2+} and Na\textsuperscript{2+} voltage-gated ion channels, decrease in K\textsuperscript{+} channels, and receptor expression alterations cause fundamental aggravations in the electrophysiological mechanisms of the neuron, ultimately resulting in hypersensitivity and spontaneous activity (Li et al., 2017; Staff et al., 2020). Finally, Paclitaxel administration stimulates the activation of TLR4 within glia cells, which support and surround the DRG, resulting in the release of inflammatory cytokines (Staff et al., 2020). The above mechanisms are proposed to cause diminished neuronal function and a reduction in fiber density responsible for epidermis innervations, ultimately contributing to the assemblage of behavioural changes observed in vivo, such as mechanical and thermal sensitivity. It is also suggested that these mechanisms facilitate other associated clinical symptom presentation of neuropathic pain, including numbness, tingling, ongoing pain, and allodynia and/or hyperalgesia (Griffiths et al., 2018).

We did not observe any significant changes in thermal hyperalgesia during Phase 1. This finding is in agreement with Griffiths et al., (2018), who did not observe any evidence that a cumulative dose of 8 mg/kg Paclitaxel solution evokes heat sensitivity. A potential mechanism associated with this finding may be attributed to the various types of pain fibers affected in CIPN. Fibers found to be associated with CIPN include A-\(\beta\), A-\(\delta\), and non-peptidergic C-fibers (Duggett et al., 2016; Li et al., 2017). Studies have identified spontaneous activity of A-\(\beta\) and A-\(\delta\) fibers within the DRG contribute to CIPN symptoms(Li et al., 2017). As A-\(\delta\) fibers are categorized into Type 1 and 2, it is possible that the Type 1 fibers, responsible for high-heat responses (>50°C) are not as severely affected by low doses of Paclitaxel as Type 2, responsible for low-heat- and high-mechanical-threshold stimuli (Yan et al., 2017). C-fibers are also subcategorized into peptidergic and non-peptidergic; those responding to thermal and noxious stimuli, and those responding to mechanical stimuli only, respectively (Skirven, 2021). Duggett et al., (2016) found an upregulated accumulation of ROS within subpopulations of non-peptidergic C-fibers in a Paclitaxel-induced peripheral neuropathy (PIPN) model, with no significant change in peptidergic fibers. This finding provides evidence of the involvement of specific subpopulations of neurons, associated with
particular thermal-related symptoms tending towards cold hypersensitivity (Duggett et al., 2016). These studies support the suggested mechanism contributing to the lack of change observed in heat sensitivity. It is possible that a higher dose of Paclitaxel is required to induce neurodegeneration extensive enough to cause underlying heat perception alterations (Griffiths et al., 2018). We also noted the substantial standard deviation within both groups at both time points. This may be a contributing factor to the lack of significance found. However, clinical trials have described CIPN symptoms as “numbness, tingling, shooting or stabbing pain, burning pain, cramping, and hypersensitivity to cold temperatures” (Staff et al., 2020). Therefore, the lack of heat sensitivity would align with these clinical presentations.

To further elaborate on the progression of the CIPN model, our findings from Phase 2 showed an exacerbation of CIPN-related symptoms without administered treatment, represented by the progressive sensitivity of nociceptive responses over the intervention period. This is suggestive of the importance of timely condition management. Peak sensitivity for mechanical and thermal allodynia was observed around Day 28 following initial injection. This observation parallels the findings of Duggett et al., (2016) and Griffiths et al., (2018), who observed peak sensitivity approximately 26 days following initial Paclitaxel injection. The complex interplay between central and peripheral effects due to Paclitaxel administration contributes to the chronic progression of the neuropathy (Staff et al., 2020). One of the hallmark characteristics of chronic pain is central sensitization, caused by abnormal nociceptive hyperexcitability of the central nervous system (Yan et al., 2017a). C-fibers are responsible for persistent depolarization; via NMDA receptor mediated post-synaptic calcium influx, resulting in long-term potentiation (LTP) and neuronal plasticity (Yan et al., 2017). In the context of PIPN one of the proposed mechanisms contributing to electrophysiological disruptions is the tonic activation of NMDA receptors and an increased expression of NMDA subunits, enhancing glutamatergic pathways within the DRG (Staff et al., 2020). Duggett et al., (2016) also observed a twofold increase in ROS levels in non-peptidergic neurons at peak pain periods, suggesting that Paclitaxel evokes these responses in particular neuronal subpopulations. Furthermore, aberrant A-β fiber formation occurs in the presence of nerve injury, causing an increase in mechanical stimulation transmission and subsequent sensitivity to these stimuli, resulting in the presentation of progressive mechanical sensitivity over time (Yan et al., 2017a), as seen in our findings.

Further supporting the establishment of a CIPN model, our findings elucidate to a heightened presence of anxiety-like behaviour without treatment, a symptom often associated with
chronic neuropathic pain (Toma et al., 2017), as demonstrated by a decreased preference for open arms in the elevated plus maze (EPM). Our findings are in agreement with (Roch et al., 2022), who observed an association between PIPN and emotional disorders, demonstrated by heightened anxiety-like behaviours exhibited in the EPM and open field tests. CIPN is a disease which becomes exacerbated over time, as the central nervous system becomes increasingly involved, resulting in the increase in risk of developing severe affective disorders, such as anxiety, depression, anhedonia, and insomnia (Liu and Chen, 2014). The plausible mechanisms associated with the above finding include pathological alterations occurring in the DRG and pain processing areas within the brain, including the amygdala (Liu and Chen, 2014; Wu et al., 2022), anterior cingulate cortex (Liu and Chen, 2014), and prefrontal cortex (PFC) (Roch et al., 2022). Within the DRG, an upregulation of numerous inflammatory pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) / protein kinase B (Akt), has been identified (Li, Mai and Liu, 2014). This increases neuronal excitability via channel activation, facilitating an increase in nociception (Roch et al., 2022). These mechanisms present phenotypically as behavioural alterations, such as heightened anxiety-like behaviour and nociceptive hypersensitivity.

Interestingly, we did not observe consistent findings across the anxiety-like behavioural tests. Findings from the light dark box (LDB) inferred low levels of anxiety-like behaviour in the animals that were exposed to Paclitaxel only, as indicated by the greater time spent in the light compartment. However, this significance was only observed during the training session. Our findings are not consisted with previous literature that has found an increase in anxiety-like behaviour in models of neuropathic pain using the LDB protocol (Toma et al., 2017; Sieberg et al., 2018; Warncke et al., 2021). However, our protocol differed from these studies as no repeated tests were performed, meaning we did not have a comparison of anxiety-like behaviour pre- and post-intervention. Furthermore, there is limited research supporting the use of the LDB in this particular model of CIPN. Warncke et al., (2021) only identified an increase in anxiety-like behaviour in females receiving a high dose of Oxaplatin (30 mg/kg cumulative dose), potentially indicating that the chemotherapy agent and dosage used must be considered when selecting this specific test. In support of this, Currie et al., (2019) identified the importance of specificity regarding anxiolytic testing in a meta-analysis of CIPN in animal models. To obtain clarity regarding these results, the experimental procedure
would need to be repeated with consideration taken regarding the potential confounding variables.

We sought to further validate our behavioural changes with neurochemical measures. We did not find evidence of hyperactive hypothalamic-pituitary-adrenal (HPA) axis activity, as the corticosterone (CORT) concentration in the circulation was significantly lower when compared to the control group. Our CORT concentrations were well below what is considered baseline based on previous literature. Staurengo-Ferrari et al., (2022) found a morning baseline plasma CORT concentration of 23.22 ± 9.59 ng/mL within their groups, prior to CIPN induction. Interestingly, Kozachik and Page, (2016) found an inverse correlation between plasma CORT levels and mechanical hypersensitivity. Animals with high concentrations of plasma CORT demonstrated greater resilience to mechanical hypersensitivity, and vice versa. This may explain why we observed low plasma CORT concentrations with a reduced ability to tolerate mechanical stimuli.

We further assessed whether an intervention of duloxetine had the ability to ameliorate the adverse effects induced during Phase 1. Our findings demonstrated Duloxetine’s analgesic ability, as indicated in the attenuation of mechanical and thermal sensitivity observed during Phase 1. However, there was a delayed analgesic onset for mechanical sensitivity, with animals only exhibiting a reduction in sensitivity in the final intervention week. These findings are in agreement with Chahal, Sodhi and Madan, (2020), who observed a gradual increase in mechanical nociceptive responses in PIPN until 14 days after intervention had commenced. Thereafter, 30 mg/kg of daily duloxetine administered via oral gavage induced a significant antinociceptive effect (Chahal, Sodhi and Madan, 2020). The delay observed in the attenuating effects of duloxetine on mechanical allodynia may be the result of when the intervention was introduced. Previous studies have demonstrated more immediate analgesic effects of duloxetine when treatment commenced prior or adjacent to Paclitaxel administration (Kato et al., 2021; Staurengo-Ferrari et al., 2022). It is possible that duloxetine’s mechanism of action functions optimally when administered prior to the initiation of chemotherapy.

However, duloxetine exhibited an inclination towards ameliorating thermal hypersensitivity, as we observed a significant antinociceptive response for both thermal assessments within the first week of duloxetine treatment. It has been widely established that duloxetine has the potential to attenuate both mechanical and thermal hypersensitivity; however, dispute arises
regarding the extent of attenuation for the various types of stimuli (i.e., heat vs cold). The exact mechanisms associated with duloxetine’s thermal analgesic effects are not thoroughly understood. As this is a chronic pain model, we postulate that the well-established central disruptions which occur within the CNS in the presence of chronic pain can be applied. Therefore, various sensory brain regions may have been in a state of hyperactivity in response to painful stimuli, including the amygdala, insula, anterior cingulate cortex (ACC), and somatosensory cortex (Yan et al., 2017; Omran et al., 2021). Duloxetine may attenuate cold-induced nociceptive responses by mitigating activity in the somatosensory and insula cortex (Omran et al., 2021). Furthermore, as duloxetine has demonstrated partial neuroprotective effects through indirect MAPK pathway inhibition (Meng et al., 2019) it is plausible to infer an antinociceptive effect through the mitigation of various inflammatory markers in the spinal cord, DRG, and peripheral neurons (Li et al., 2017).

Interestingly, duloxetine did not offer an anxiolytic benefit, as demonstrated by the lack of inclination to spend time in the open arms of the EPM compared to respective counterparts. As the presence of affective disorders, such as anxiety and depression, are associated with enhanced severity of CIPN symptoms and delayed recovery (Toma et al., 2017), a viable treatment intervention must possess the ability to mitigate the manifestation of these conditions. Staurengo-Ferrari et al., (2022) identified that duloxetine’s efficacy was greater for oxaliplatin-induced neuropathy as opposed to PIPN, indicative of the importance of specificity regarding treatment. It has also been shown that duloxetine has selective efficacy, which may be due to variability in symptom development (Smith et al., 2017). Finally, duloxetine acts as a serotonin and norepinephrine uptake inhibitor, neurotransmitters which have been associated with the pathophysiology of nociception (Rodrigues-Amorim et al., 2020). However, the predominant mechanism of action in a PIPN involved upregulated glutamatergic and inhibited GABAergic pathways (Omran et al., 2021). Therefore, while duloxetine may offer partial analgesic benefits, there is a need to augment this intervention to enhance efficacy.

Neurochemical measures were used to validate our findings based on the behavioural observations. Duloxetine attenuated the stress response, as demonstrated by the diminished levels of circulating CORT when compared to our control counterpart. It must be noted that this comparison was not significant. Previous literature focused specifically on CORT changes in the presence of CIPN reported on repeated daily measures to establish a complete understanding of the neuroendocrine stress response (Staurengo-Ferrari et al., 2022).
Staurengo-Ferrari et al., (2022) demonstrated duloxetine’s ability to modulate the neuroendocrine stress axis function. Furthermore, they demonstrated duloxetine’s ability to mitigate the enhancement of the stress response in the presence of external stressors. Our results represent a snapshot in time, representative of the end of the intervention, which is likely not sufficient to generate a comprehensive representation of the physiological state of the animals. It must also be noted that when duloxetine treatment commences in relation to chemotherapy administration plays a critical role in symptom outcome (Staurengo-Ferrari et al., 2022). As our treatment was administered after PIPN was established, this may have affected the potential efficacy of duloxetine.

Finally, we sought to identify the potential analgesic or anxiolytic effects of oxytocin in an established CIPN model. We found that oxytocin provided significant nociceptive response attenuation, as exhibited in the reduction of both mechanical and cold hypersensitivity during the intervention period. It has been shown that patients with chronic pain conditions present with a reduced expression of central and peripheral oxytocin concentrations (Xin, Bai and Liu, 2017). Furthermore, exogenous administration of synthetic oxytocin is able to induce significant analgesic effects (Tracy et al., 2015). Our findings parallel those of (González-Hernández et al., 2019), who demonstrated the analgesic effects of repeated administration of exogenous oxytocin in a Wistar rat model of spinal nerve ligation. We deduce that the analgesic mechanisms of the aforementioned results can be explained by oxytocin’s potential to act at a central, spinal, and peripheral level. It has been postulated that oxytocin exerts a peripheral analgesic effect by direct binding to non-peptidergic C-fibers (Boll et al., 2018), fibers identified in the pathophysiology of PIPN (Duggett et al., 2016), thereby diminishing nociceptive transduction, particularly associated with mechanical stimuli. Furthermore, oxytocin directly inhibits $K^+$ channels and diminishes intracellular $Ca^{2+}$ increase, modulating the nociceptive signal transmission (Rash, Aguirre-Camacho and Campbell, 2014). As maladaptations of the $Ca^{2+}$ and $K^+$ channels have been intrinsically linked to PIPN progression (Toma et al., 2017), the attenuation of these mechanisms may provide significant analgesic benefits in a CIPN model.

Recently, more emphasis has been placed on the central mechanisms associated with CIPN (Omran et al., 2021). Hyperactivity in sensory brain regions, particularly the thalamus, somatosensory cortex, and periaqueductal gray (PAG), have been associated with neuropathic pain (Omran et al., 2021). Oxytocin projections have been located on various pain processing regions, including the cingulate and insular cortices, PFC, thalamus (Boll et al., 2018),
amygdala (Li et al., 2017), and PAG (Rash, Aguirre-Camacho and Campbell, 2014). Therefore, it is plausible that oxytocin exerts potential central ameliorative effects via these projections, if centrally administered. Furthermore, oxytocin may exert an antinociceptive effect on a spinal level through the inhibition of glutamatergic spinal sensory transmissions (Condés-Lara, Maie and Dickenson, 2005). As an increased expression of NMDA receptors have been associated with central sensitization (Li et al., 2017), the ability of oxytocin to diminish glutamatergic activity may strengthen its analgesic potential.

Oxytocin further demonstrated anxiolytic potential, observed in the preference to open arm activity, supplementing the analgesic behavioural results exhibited. Few studies have addressed the need for alternative interventions for emotional disorders associated with CIPN (Roch et al., 2022). Oxytocin is well-known for its anxiolytic properties, potentially mediated through action on brain areas including the amygdala, the anterior cingulate cortex, and areas of the PFC (Boll et al., 2018). Our findings are in agreement with Li, et al. (2021), who observed an attenuation of nociceptive and anxiety-like behaviour in a neuropathic model of common peroneal nerve (CPN) ligation following microinjections of oxytocin into the anterior cingulate cortex (ACC). Oxytocin reduced pre-long-term-potentiation (LTP) in the ACC of both male and female mice as well as mediated inhibitory pathway activity (Li, W.H., et al. 2021). Furthermore, evidence has suggested oxytocin indirectly facilitates excitation in the GABAergic system, mediated by PVN hypothalamic projections, offering an indirect analgesic effect (Xin, Bai and Liu, 2017). As PIPN’s mechanism has been associated with upregulated glutamatergic and inhibited GABAergic stimulation within the CNS (Omran et al., 2021), this may offer an explanation for the anxiolytic results observed.

We further sought to validate our behavioural observations with neurochemical measures. Oxytocin induced ameliorative effects on circulating corticosterone within a CIPN model, as demonstrated by the significantly lower corticosterone concentration as compared to the control counterpart. As oxytocin is a well-known antagonist of the HPA axis (Tracy et al., 2015), we speculate the above-mentioned results can be explained through these mechanisms. While there is limited research on the effects of oxytocin in a chronic pain model, studies have demonstrated the attenuating potential of oxytocin in chronic stress models. Our findings are in agreement with (Maikoo, Wilkins and Qulu, 2022) who found intranasal oxytocin administration reduced circulating corticosterone levels in a febrile seizure model. Furthermore, Windle et al., (2006) found centrally administered oxytocin significantly attenuated the plasma corticosterone concentration in an auditory stress animal model. As
oxytocin receptors have been identified on numerous brain regions and ascending/descending pathways associated with chronic pain development and progression (Boll et al., 2018), we speculate that centrally administered oxytocin may have exerted its ameliorative effects by regulating activity in affected brain regions.
6  Limitations and Future Studies

This was a preliminary study, and therefore our primary concern was establishing a working model of chemotherapy-induced peripheral neuropathy (CIPN). As a result, we did not introduce the intervention until after CIPN had been verified. Recent literature has demonstrated the importance of when intervention, in particular duloxetine, commences in relation to chemotherapy administration (di Cesare Mannelli et al., 2017; Zhang et al., 2018; Lu et al., 2020; Staurengo-Ferrari et al., 2022). It has been shown that the efficacy of duloxetine is enhanced when administered prior to the commencement of chemotherapy treatment (Staurengo-Ferrari et al., 2022). Therefore, future studies may need to account for the selected time of intervention commencement. We also did not include a treatment group treated with a combination of oxytocin and duloxetine. An evaluation of the synergistic effect of these treatments in a model of CIPN would be the ideal progression for future studies.

It has been shown that the species of animal used within a model of neuropathic pain can impact the results significantly (Liu and Chen, 2014). A meta-analysis by Currie et al., (2019) found that species did not contribute to the heterogeneity identified in results; however, the selected strain did account for heterogeneity. As this is a novel model, this variable should still be taken into consideration when planning future studies. Furthermore, sex is known to be an under-research yet fundamental variable in chronic neuropathic pain models (Currie et al., 2019). While our model did include both males and females, we did not identify considerable differences between them. We could speculate on the reason for lack of heterogeneity; however, we did not include an analysis of gonadal hormones nor the estrus cycle. Therefore, these variables could be considered for future research studies. Furthermore, it has been suggested that the anxiolytic test selected is model-specific, therefore a variety of affective disorder tests should be evaluated in this specific model, to ensure methodology optimization (Currie et al., 2019).

There are several changes that could be introduced to our methodology to optimize our outcome measures. Firstly, the duration between the final behavioural test and sacrifice may have been too long (24-48 hours). Secondly, we sacrificed in the morning, which is when the animal’s corticosterone levels would have been the lowest. As blood collection was once-off, we were only provided with a momentary snapshot of these levels. Thirdly, further research will need to be conducted to optimize the oxytocin dosage administered in a specific Paclitaxel-induced model of neuropathic pain, as Han and Yu, (2009) found a dose-
dependent relationship between centrally administered oxytocin and hindpaw withdrawal latency (HWL). Fourthly, research has shown that the vacutainer used to collect blood may interfere with the concentrations obtained through ELISA assays. Furthermore, raw versus extracted plasma results in significantly different concentrations, with raw plasma resulting in up to a four times greater perceived concentration. As we were following the ELISA kit protocol provided, raw plasma was used. Fifthly, we struggled to obtain a standard curve similar to the reference provided by the suppliers. When confronted with this issue, new kits were dispatched. However, although there was minimal improvement, the standard curves were still considerably lower than the reference offered. When queried, the suppliers offered limited assistance with regards to the potential issue. Finally, the researcher was not blinded to the intervention groups.

A crucial consideration in any preclinical study is the translational power and potential challenges thereof. The neuronal mechanisms involved in Paclitaxel-induced sensitivity likely differ between rodents and humans (Staff et al., 2020), and therefore it may be beneficial to extend research to human cells models to identify the effects of oxytocin in the presence of Paclitaxel cytotoxicity. It must also be considered that clinical symptoms are not absolute reflections of peripheral mechanisms (Omran et al., 2021). Therefore, the lack of subjective or central measures in a preclinical model could limit our comprehensive understanding of the interactive mechanisms involved in CIPN. While we measured the oxytocin concentration levels within the hypothalamus, we did not include an analysis of the dorsal root ganglion, a structure intimately involved in the development of chronic neuropathic pain (Duggett et al., 2016; Yan et al., 2017a; Staff et al., 2020). We also did not have measures of spontaneous ongoing behavioural changes (Griffiths et al., 2018), which may have broadened our understanding of how CIPN disrupts normal physiology and natural behavior, a potential corresponding measure to patient verbal reports.
7 Conclusion

Chemotherapy-induced peripheral neuropathy (CIPN) remains to be a debilitating dose-limiting side effect for patients fighting against cancer. As duloxetine is currently the only recommended treatment for CIPN management, the need for alternative or augmentative treatment modalities is imperative. Therefore, we sought to investigate the augmentative potential of the neuropeptide oxytocin in a model of CIPN. Our results support the hypothesis that intranasally administered oxytocin may augment the analgesic and anxiolytic effects of duloxetine in a chemotherapy-induced peripheral neuropathy model in a Wistar rat. Duloxetine demonstrated a greater inclination to attenuate thermal hypersensitivity as opposed to mechanical. It is plausible to postulate that oxytocin may offer the complimentary mechanisms associated with mechanical sensitivity. Administered in conjunction, oxytocin and duloxetine may provide enhanced therapeutic effects in the treatment of CIPN. With more research, it may become evident of the potential role oxytocin may play in cancer- and chemotherapy-management.
References


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Li, M.J. *et al.* (2017) ‘Chronic stress exacerbates neuropathic pain via the integration of stress-Affect-related information with nociceptive information in the central nucleus


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Addenda

Addendum 1 – Ethical Approval

REC: Animal Care and Use

Project Approval Letter

Date: 17 August 2021

PI Name: Dr L Gulu

Protocol #: ACU-2021-22038

Title: The effect of environmental enrichment on prenatal stress and neuropathic pain

Dear Dr Gulu,

Your project application, was reviewed by the Research Ethics Committee: Animal Care and Use (REC: ACU) via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics approval of projects spanning more than one year must be renewed annually through submission of a progress report (due 30 days prior to expiry date), up to a maximum of three years.

Approval Date: 17 August 2021 - 16 August 2022

Animal Species: Wistar Rats

Animal Numbers: 60

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator’s responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 22038 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the ethics help-desk at applyethics@sun.ac.za or 021 808 9003. This Committee is registered with the National Health Research Ethics Council of South Africa - registration number: 1REC-150211-007.

Sincerely,

[Signature]

Mr. WA Beukes
Coordinator: Research Ethics (Animal Use and Biosafety)
E: wabeukes@sun.ac.za
Addendum 2 – Daily Welfare Monitoring Sheet

<table>
<thead>
<tr>
<th>WELFARE MONITORING SHEET</th>
</tr>
</thead>
<tbody>
<tr>
<td>WELFARE MONITORING SHEET: One sheet per animal. Each animal to be monitored at least once daily, including weekends and public holidays, ALL monitoring to be recorded on this sheet.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SU AREC number</th>
<th>Short Study title</th>
<th>Monitoring frequency</th>
<th>Name of Monitors</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cage/Group</th>
<th>Animal No</th>
<th>Week no</th>
<th>Date of first treatment/procedure</th>
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</thead>
<tbody>
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</table>

<table>
<thead>
<tr>
<th>Baseline weight (grams)</th>
<th>Humane endpoint weight (grams)</th>
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<tbody>
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<tr>
<th>Date</th>
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<table>
<thead>
<tr>
<th>AM/PM</th>
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<tbody>
<tr>
<td>AM</td>
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<td>PM</td>
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<table>
<thead>
<tr>
<th>Weight</th>
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<tbody>
<tr>
<td>AM</td>
</tr>
<tr>
<td>PM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CLINICAL SCORING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity¹</td>
</tr>
<tr>
<td>Behaviour²</td>
</tr>
<tr>
<td>Posture³</td>
</tr>
<tr>
<td>Appearance⁴</td>
</tr>
<tr>
<td>Discharge from eyes</td>
</tr>
<tr>
<td>Grimace scale score⁵</td>
</tr>
<tr>
<td>Dehydration (tented skin, dry m/m)</td>
</tr>
<tr>
<td>Type of breathing⁶</td>
</tr>
<tr>
<td>Body condition score⁷</td>
</tr>
</tbody>
</table>

*Actions to be taken:*
If animal is showing signs of deteriorating, immediately inform the Principal Investigator

**Actions to be taken:**
If unlikely to recover and no action can be taken to alleviate its distress, the animal must immediately be euthanased

*Additional observations and notes*
**OVERALL CLINICAL SCORE**

| No discomfort/stress (0) |  |
| Distress, stress or *deterioration (1, 2 or 3) |  |
| **Distress**: Humane endpoint |  |

**STUDY SPECIFIC CLINICAL SIGNS**

- Diarrhea
- Hair loss (alopecia)
- Weight loss
- Motor Impairments

**Scoring details**

**Activity¹**: 0 = normal activity (spontaneous, active, responds to stimuli), 1 = activity decreased/increased, moves when provoked/disturbed

**Activity¹**: 2 = Inactive, little or no social interaction, moves only in response to stimulation/noise, poorly responsive when provoked, 3 = Weak, reluctant to move, poorly responsive to sound stimulation/provocation

**Behaviour²**: 0 = alert, inquisitive, social, eating, 1 = less alert, responsive or exploratory, less interactive with cage mates, 2 = No exploratory behaviour, 3 = Isolation from companions

**Posture³**: 0 = normal posture, 1 = guarded body posture, cautious slow movement, 2 = tense and/or hunched body posture, 3 = hunched body posture/crouching

**Appearance⁴**: 0 = coat clean, extremities pink or normal colour, 1 = coat dull/staring, 2 = dull coat +/- piloerection, 3 = rough staring coat/piloerection, pale extremities, gums and eyes

**Grimace scale score⁵**: Orbital tightening, nose bulge, cheek bulge, ear drooping, whisker changes, 0 = not present, 1 = moderately present, 2 = obviously present

**Type of breathing⁶**: 0 = Respiration regular rate and depth, 3 = Abnormal respiration, laboured or shallow, increased chest and/or abdominal movement, audible breath sounds

**Body condition score⁷**: 1 = emaciated, 2 = under-conditioned, 3 = well-conditioned, 4 = obese

**Overall clinical score⁸**: 0 = No discomfort or stress, 1 = mild discomfort or stress, 2 = Moderate discomfort or stress, 3 = severe discomfort or stress

**Actions to be taken**: * If animal is showing signs of deteriorating, immediately inform the Principal Investigator

**Actions to be taken**: ** If unlikely to recover and no action can be taken to alleviate its distress, the animal must immediately be euthanased
Addendum C – Anxiety-related Behavioural Testing

1. Light/Dark Box

Table 1 Light/dark Box Observation Criteria

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to Enter Dark Compartment</td>
<td>Time taken for the animal to enter the dark compartment, all four limbs enter</td>
</tr>
<tr>
<td>Time spent in the Light Compartment</td>
<td>Time spent in the light compartment, all four limbs in the compartment</td>
</tr>
<tr>
<td>Time spent in the Dark Compartment</td>
<td>Time spent in the dark compartment, all four limbs in the compartment</td>
</tr>
<tr>
<td>Grooming</td>
<td>Time spent licking, scratching, or face-washing</td>
</tr>
<tr>
<td>Supported Rears</td>
<td>Time spent where the animal uses the walls to rest its front paws while balancing on its hind paws</td>
</tr>
<tr>
<td>Free-standing Rears</td>
<td>Time spent where the animal balances on their hind paws independently with no support</td>
</tr>
<tr>
<td>Freezing</td>
<td>Complete absence of movement, tense body posture</td>
</tr>
</tbody>
</table>

Figure 1 View from GoPro, positioned directly over the box.
2. Elevated Plus Maze

Table 2 Elevated Plus Maze Observation Criteria

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to Enter Closed Arm</td>
<td>Time taken for the animal to enter closed arm for the first time (all four limbs across the threshold of the arm)</td>
</tr>
<tr>
<td>Latency to Enter Open Arm</td>
<td>Time taken for the animal to enter an open arm for the first time (all four limbs cross the threshold)</td>
</tr>
<tr>
<td>Time in Open Arm</td>
<td>Time spent in open arms (starts when all four limbs have crossed the threshold into the arm)</td>
</tr>
<tr>
<td>Time in Closed Arm</td>
<td>Time spent in closed arms (starts when all four limbs have crossed the threshold into the arm)</td>
</tr>
<tr>
<td>Open-arm Entry</td>
<td>Animal enters a open arm (all four limbs cross the threshold)</td>
</tr>
<tr>
<td>Closed-arm Entry</td>
<td>Animal enters a closed arm (all four limbs cross the threshold)</td>
</tr>
<tr>
<td>Grooming</td>
<td>Time spent licking, scratching, or face-washing</td>
</tr>
<tr>
<td>Supported Rears</td>
<td>Time spent where animal uses the walls to rest its front paws while it balances on its hind paws</td>
</tr>
<tr>
<td>Free-standing Rears</td>
<td>Time spent where animal balances on hind paws independently (with no support)</td>
</tr>
<tr>
<td>Head-dips</td>
<td>Animals head is protruded towards the floor, over the edges of the open arms</td>
</tr>
<tr>
<td>Freezing</td>
<td>Complete absence of movement, tense body posture</td>
</tr>
</tbody>
</table>

Figure 2 View from GoPro, positioned directly over the maze. (B) Diagram of EPM
Addendum D – Table of All Statistical Significance

<table>
<thead>
<tr>
<th>Comparison</th>
<th>ANOVA</th>
<th>Post Hoc</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 20 – Phase 1: Effect of Paclitaxel administration on mechanical allodynia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline vs Post-injection</td>
<td>$F_{(1, 58)} = 2066, p &lt; 0.0001$</td>
<td>(PAC-BL vs PAV-PI), $p &lt; 0.0001$</td>
<td>Pg. 83</td>
</tr>
<tr>
<td>Saline vs Paclitaxel</td>
<td>$F_{(1, 58)} = 20.48, p &lt; 0.0001$</td>
<td>(SAL-PI vs PAC-PI), $p = 0.0123$</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 21 – Phase 1: Effect of Paclitaxel administration on thermal allodynia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline vs Post-injection</td>
<td>$F_{(1, 58)} = 40.03, p &lt; 0.0001$</td>
<td>(PAC-BL vs PAV-PI), $p &lt; 0.0001$</td>
<td>Pg. 84</td>
</tr>
<tr>
<td>Saline vs Paclitaxel</td>
<td>$F_{(1, 58)} = 25.88, p &lt; 0.0001$</td>
<td>(SAL-PI vs PAC-PI), $p &lt; 0.0001$</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 23 – Phase 2: Effect of treatment and intervention duration on mechanical allodynia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>$F_{(2.628, 141.9)} = 16.97, p &lt; 0.0001$</td>
<td>(PAC-BL) vs (PAC-PI), $p = 0.0061$; (PAC-BL) vs (PAC-Int Wk1), $p = 0.0016$; (PAC-BL) vs (PAC-Int Wk2), $p = 0.0002$</td>
<td>Pg. 86</td>
</tr>
<tr>
<td>Time x Treatment</td>
<td>$F_{(15, 162)} = 6.895, p &lt; 0.0001$</td>
<td>PAC-DUL-Int Wk1 vs (PAC-DUL-Int Wk2), $p = 0.0120$; (PAC-OXY-PI) vs (PAC-OXY-Int Wk1), $p = 0.0135$; and intervention week 2 (PAC-OXY-PI) vs (PAC-OXY-Int Wk2), $p = 0.0344$</td>
<td></td>
</tr>
<tr>
<td><strong>Figure 24 – Phase 2: Effect of treatment and intervention duration on thermal alldynia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{(5, 54)} = 8.480, p &lt; 0.0001$</td>
<td>(PAC-BL) vs (PAC-PI), $p = 0.0261$; (PAC-BL) vs (PAC-Int Wk1), $p = 0.0499$; (PAC-BL) vs (PAC-Int Wk2), $p = 0.0220$; (PAC-DUL-PI) vs (PAC-DUL-Int Wk1), $p &lt; 0.0001$</td>
<td>Pg. 87</td>
</tr>
<tr>
<td>Time x Treatment</td>
<td>$F_{(15, 162)} = 4.699, p &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>$F_{(2.827, 152.7)} = 15.65, p &lt; 0.0001$</td>
<td></td>
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</tr>
<tr>
<td><strong>Figure 25 – Phase 2: Effect of treatment and intervention duration on thermal hyperalgesia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{(5, 54)} = 5.838, p = 0.0002$</td>
<td></td>
<td>Pg. 88</td>
</tr>
</tbody>
</table>
| **Figure 26 – Effect of treatment and intervention duration on anxiety-like behaviour (Light/dark**
### Box: Explorative Behaviour)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F (5, 55) = 4.177, p = 0.0027</th>
<th>(CTRL) vs (PAC-OXY), p = 0.0198; (PAC) vs (PAC-OXY), p = 0.0106; (OXY) vs (PAC-OXY), p = 0.0212</th>
<th>Pg. 89</th>
</tr>
</thead>
</table>

**Figure 31 – Effect of treatment and intervention duration on anxiety-like behaviour (Elevated Plus Maze: Explorative Behaviour)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F (5, 55) = 2.593, p = 0.0355</th>
<th>(PAC) vs (PAC-OXY), p = 0.0355; (PAC-DUL) vs (PAC-OXY), p = 0.0416</th>
<th>Pg. 92</th>
</tr>
</thead>
</table>

**Figure 33 - Effect of treatment and intervention duration on neurochemical concentrations (Plasma Corticosterone Concentration)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H (6, 61) = 17.59, p = 0.0035</th>
<th>(CTRL) vs (PAC-OXY), p = 0.0011, (CTRL) vs (PAC), p = 0.0474</th>
<th>Pg. 94</th>
</tr>
</thead>
</table>

**Figure 34 - Effect of treatment and intervention duration on neurochemical concentrations (Hypothalamic Oxytocin Concentration)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F (5, 47) = 2.972, p = 0.0206</th>
<th>(CTRL) vs (OXY), p = 0.0327; (CTRL) vs (PAC-DUL), p = 0.0186</th>
<th>Pg. 94</th>
</tr>
</thead>
</table>