ROLE OF SELECTED MUSCARINIC, NICOTINIC AND OPIOIDERGIC DRUGS IN PAIN REGULATION IN THE *TACHYORYCTES SPLENDENS*.

A thesis submitted in partial fulfilment of the degree of Masters of Science in Comparative Animal Physiology.

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DECLARATION

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DEDICATION

To the Thuo's family, for your support, encouragement, patience and understanding that gave me the courage to pursue this work to the very end. Cheers!

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

APPENDICES

LIST OF ABBREVIATIONS

5-HT	5-Hydroxy-tryptamine
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxalone propionate
cAMP	Cyclic adenosine monophosphate
CCK	Colecystokinin
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
GABA	Gamma-aminobutyric acid
IASP	International Assosiation for the Study of pain
iNOS	Isoenzyme Nitric oxide synthase
L-NAME	L-N-Nitroarginine Methyl Ester
LTP	Long-term potentiation
mGluR	Metabotropic glutamate receptors
mAChR	Muscarinic acetylcholinergic receptors
MIAs	Mechanically insensitive afferents
nAChR	Nicotinic acetylcholinergic receptors
NK-1	Neurokinin-1
NK-2	Neurokinin-2
NKA	Neurokinin-A
N-MDA	N-Methyl-D- Aspartate
NO	Nitric oxide
NS	Nociceptor specific
NSAIDs	Non steroidal anti-inflammatory drugs
PAG	Peri-aqueductal gray

S.E.M	Standard error of the mean
Secs	Seconds
SG	Substantia gelatinosa
SP	Substance P
STT	Spinothalamic tract
WDR	Wide dynamic range

ABSTRACT

Little is known about nociception in the East African root rat *Tachyoryctes splendens*. This therefore prompted further exploration of nociception and antinociception in this species with specific attention to pain modulation by the cholinergic and opioidergic neurotransmitter systems. Three nociceptive tests, namely the formalin-, tail flick-, and the acetic acid induced writhing tests were used to study the nociceptive and antinociceptive effects of selected cholinergic (oxotremorine-muscarinic receptor agonist and epibatidine-nicotinic receptor agonist) and opioidergic drugs (morphine-μ-receptor agonist). Oxotremorine (10, 20, and 60 μg/kg dose levels), epibatidine (1, 3 and 10 μg/kg dosage levels) and morphine (1, 3, and 6 mg/kg dosage levels) were administered systemically. Atropine, mecamylamine and naloxone which are their respective blockers were used for antagonistic reactions. A total of one hundred and twenty East African root rats were used in the experiments.

In the formalin test, a monophasic (0-5 minutes) pain behavioral response characterized by biting, licking and favoring of the injected limb was observed. The behavioural response in the late phase (> 5 Minutes) was insignificantly different ($P \geq 0.05$) from that of the controls. Oxotremorine at the selected doses (30 or 60 μg/kg) induced a statistically significant ($P \leq 0.05$) dose-dependent reduction in the mean time spent licking/biting the injected paw in the early phase of the formalin test. The median effective dose was 21 μg/kg. The effect of oxotremorine (30 μg/kg), on the mean time spent licking/biting the injected paw was reversed by atropine.

Epibatidine (3 or 10 μg/kg) caused a statistically significant ($P \leq 0.05$) reduction in the mean time spent in licking/biting the injected paw in the formalin test. The median effective dose was 4.5 μg/kg. Co-administration of mecamylamine with epibatidine (3 μg /kg), significantly increased the mean time spent in licking/biting the injected paw.

Morphine (3 or 6 mg/kg) caused a statistically significant ($P \leq 0.05$) decrease in the mean licking/biting response. The mean effective dose was 4.5 mg/kg. The effect of morphine (3 mg/kg) was reversed by the administration of naloxone (2.5 mg/kg).

Lower doses of oxotremorine, epibatidine and morphine had no effect on the mean licking/biting behaviour.

In the tail flick test, with a sensitivity setting of 10, beam at 8 and a cut-off time of 10 seconds, no tail flick was observed even after increasing the cut off period to 20 seconds. The acetic acid induced writhing test also did not cause any observable nociceptive behavior.

In conclusion, the present data uniquely showed that the formalin test induces a monophasic pain behavior in the East African root rat and secondly, this species appears to have a functional nociceptive system sensitive to cholinergic and opioidergic analgesics.

CHAPTER ONE

1.0 INTRODUCTION

Nociception, the detection of tissue-damaging stimuli is evident in a number of different phyla including birds and mammals (Walters, 1996), and all animal species down to the protozoan posses distinctive behavioural responses to noxious stimulation (Dennis and Melzack, 1983). The ability to detect such stimuli and take action to minimize their effects represents a major selection pressure in animal phylesis (Dennis and Melzack, 1983). The physiology and organization of pain pathways also appears to be highly conserved across all vertebrate taxa and all vertebrate species. The complexity of nociceptive systems, which ultimately produce pain, has increased during evolution as a result of the pressure to avoid organic lesions or their aggravation (Walters, 1994). Animals which lack this nociceptive ability e.g those with various forms of congenital insensitivity to intense stimuli have greatly reduced life expectancies (Dennis and Melzack, 1983).

Pain has been defined by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, 1979). Pain constitutes an alarm that ultimately has the role of helping to protect an organism since it triggers reactions and induces learned avoidance behaviors which may decrease the stimulus causing pain and as a result, the organism avoids the nociceptive stimulus. Different species of animals respond differently to noxious stimuli and such a difference also exists within members of the same species. There is evidence indicating that sex (Bodman *et al*., 1988), age (Kavaliers and Hirst, 1983), developmental changes (Hamm and Kinsley, 1988) and geographical areas (Innes and Kavaliers, 1987) do influence nociception in animals. This difference is in part, determined by the complexity of the central nervous system of any given animal (Stevens, 1992).

Nociceptive and behavioural responses to aversive and stressful stimuli in mammals may also be modulated by various endogenous and exogenous factors (Amit and Galina, 1986).

The East African root rat is distributed throughout the highlands of Ethiopia, Central and Eastern Africa. These rodents belong to the mammalian order Rodentia. Their systematic position is not well established since they are cryptic and as such, requires further investigation. Little is known about its biology and in particular pain perception and regulation (Towett and Kanui, 1995), and this therefore prompted further exploration of nociception and antinociception in this root-rat with specific attention to the cholinergic and opioidergic modulation. There is evidence that cholinergic drugs may be potentially good analgesics (Dulu *et al.,* 2014) and more research is required on this line.

To the best of our knowledge, only one study has been done to investigate pain perception and regulation in the *Tachyoryctes splendens* (Towett and Kanui, 1995). This root rat is fossorial just like the naked mole rat (*Heterocephalus glaber)* on which more studies have been done and has shown unique and remarkable features in regard to pain perception and regulation (Park *et al*., 2008). Although the two species of rats are fossorial, they remarkably differ morphologically, socially and in their geographical distribution.

The main objective of this study was to evaluate, using the formalin-, tail flick- and the acetic acid induced writhing tests, the antinociceptive effects of oxotremorine (a muscarinic receptor agonist), epibatidine (a nicotinic receptor agonist), morphine (a mu-opioid agonist), atropine, mecamylamine and naloxone which are their respective antagonists in the East African root rat. It was hypothesised that the cholinergic and opioidergic systems have a role in pain regulation in the East African root rat.

The study was expected to provide additional information on the physiology and pharmacology of pain modulation in the East African root rat. The study has contributed additional information on pain systems in this fossorial rodent.

The results obtained generated basic information that in a future perspective will improve the health and welfare of these animals. The biology of pain in the *Tachyoryctes splendens* appeared remarkable just like that of the naked mole rat which is unique among mammals. Lastly, this study boosted knowledge about the cholinergic and opioidergic involvement in antinociception, which is an essential field for exploring the high potential of development of pain treatments for both animal and human use.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The East African root rat

The East African root rats belong to the mammalian order Rodentia. Taxonomists have placed this species into either the family *Rhizomyidae* (Allen, 1939; Kingdon, 1974; Baskevich *et al*., 1993) or *Muridae* (Ellerman, 1941). This root rat is considered as one of the 14 species of the genus *Tachyoryctes* (Allen, 1939; Baskevich, *et al*., 1993). However, Kingdon, (1997) recognized 11 species of the genus *Tachyoryctes* in the Family *Rhizomyidae*. According to Nowak (1999), the total number of species in the genus *Tachyoryctes* is not clearly known. Many taxonomists agree that the East African root rat belongs to the species *splendens*.

Tachyoryctes splendens is a hairy, aggressive and solitary subterranean root rat (Delany, 1986). Its external morphology is basically rat-like, cylindrical with small eyes and ear pinnae, short limbs and tail, broad feet and large prominent incisors (**Plate 1)**. These are modifications for underground life (Kokiso and Bekele, 2008). It weighs between 160 to 280g (Kingdon, 1974; Nowak, 1999). It is distributed throughout the highlands of Ethiopia, Kenya, Rwanda, Burundi, Uganda, Northern Tanzania and some parts of Zaire and Somalia (Jarvis and Sale, 1971; Kingdon, 1997, Nowak, 1999). It inhabits medium to high altitudes (Rahm, 1969; Yalden *et al*., 1976; Sewnet and Bekele, 2003) and prefers open habitats like grasslands, wooded savanna with scattered trees and cultivated areas with loose soil. The root rat mainly feeds upon underground roots, rhizomes, tubers, as well as stem bulbs and grasses. It also stores food at a nesting chamber for adverse conditions and destroys crops grown in high potential areas (Kokiso and Bekele, 2008).

The physiology of nociception and antinociception in the East African root rat has not been studied adequately. To the best of our knowlegde, there is only one single study where the analgesic properties of morphine, a prototype narcotic analgesic, and pethidine, a synthetic narcotic analgesic was evaluated (Towett and Kanui, 1995). It was reported in this study that the *Tachyoryctes splendens* had a higher thermal threshold than other rodents. Interestingly, it was also reported that instead of producing analgesia, morphine and pethidine caused an increase in sensitivity to thermal stimulus. The marked reduction in the response latency was reversed when naloxone was administered with either of the opiates. The authors concluded that exogenous stimulation of the opioid system causes hyperalgesia rather than analgesia and that opiates are not suitable for relieving pain similar to that induced by thermal algesimetry in the *Tachyoryctes splendens*.

In a recent study, oxotremorine and epibatidine, which are direct acting cholinergic drugs, were reported to produce atropine and mecamylamine-reversed potent antinociceptive effect in the tail flick, formalin and hot plate tests in the naked mole rat (Dulu *et al*., 2014). Prior to this study the role of the the same drugs on analgesia in the *Tachyoryctes Splendens* was not known. Cholinergic effects are mediated through muscarinic and nicotinic receptors that have been shown to be widely distributed throughout the central nervous system in many animal species (Nicoll *et al*.,1990).

Plate 1: The East African root rat (*Tachyoryctes splendens*) is rat-like and hairy, has short limbs and prominent incisors.

2.2 Pain and pain terminologies

2.2.1 Definition of pain

The ability to detect potentially injurious stimuli is what gives rise to pain which usually has not only a sensory component but also an emotional experience. The IASP defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey and Watson, 1979). In non human subjects, pain is defined as an aversive sensory experience caused by actual or potential injury that elicits protective and motor vegetative reactions, resulting in learned avoidance and may modify species specific behaviour, including their social behaviour (Millan, 1999; Le Bars *et al*., 2001).

Pain serves three functions; to warn the individual of existence of real tissue damage, to warn the individual of the probability that tissue damage is about to occur by realizing that a stimulus has the potential to cause such damage and to warn a social group of danger as soon as it exists for anyone of its members (Dennis and Melzack, 1983). Behaviors resulting from

pain can facilitate other fundamental biological functions such as maintenance of tissue regeneration (inflammation and healing). Moreover animals with deficits in their nociceptive systems have shorter life span and even minor injuries can lead to catastrophic consequences (Caterina *et al*., 2000; Le Bars *et al*., 2001). Pain perception is a subjective experience in that it differs not only in different subjects, but also in the same subject at different times and in differnt situations (Rosland, 1991; Hole and Tjolsen, 1993).

2.2.2 Pain terminologies

These terminologies were initially defined by a group of reseachers led by Merskey (1986), and later re-writen by Merskey and Bogduk (1994).

A **noxious stimulus** is one which has the potential to, or is damaging to normal tissue, has an intensity and quality sufficient to trigger reflex withdrawal, autonomic responses, and pain, collectively constituting what is refered to as the nociceptive reaction. A **nociceptor** is a specilaized receptor or nerve ending that detects pain in the body. **Analgesia** is the absence of pain in response to stimulation which would normally be painful, while **hypoalgesia** is diminished pain response to a normally painful stimulus. **Hyperesthesia** is an increased sensitivity to stimulation, excluding the special senses. **Hyperalgesia** is increased response to painful stimulus. It is usually assosiated to inflammation or tissue injury. Hyperalgesia can be induced by heat, exposure to ultraviolet radiation or injection of hyperalgesic substances such as prostaglandins, histamine, bradykinin, capsaicin etc, into the skin. **Allodynia** is a pathological condition in which pain sensation is elicited by a stimulus that is normally non-painful. It is due to activity in non-nociceptive, fast conducting, thinly myelinated A-beta afferents, which evoke pain following inflammation or nerve injury.

Pain threshold is defined as the first barely perceptible pain to appear in an instructed subject under a given condition of stimulation. In humans, it is usually revealed by a verbal expression and measured in terms of lowest intensity of stimulus that will evoke it. In

animals, reflex responses to presumed pain are used to measure pain threshold. The primary pain related responses are autonomic, somatic motor and motivational affective (Melzack and Casey, 1968). These include the more obvious signs such as lameness, biting and scratching at an irritation site, or obscure signs such as inappetence, lassitude and dysuria. Pain threshold can be influenced by a number of factors, including diurnal variation, race, sex, age, circulatory change, skin temperature, trauma, anxiety and fear (Beecher, 1957, Rosland, 1991; Hole and Tjolsen, 1993). Analgesic agents are capable of altering pain threshold in animals and humans. Hyperalgesia, sweating, fatigue and high partial presure of carbon dioxide do also influence pain threshold in both humans and animals (Beecher, 1957).

Pain tolerance level is the greatest level of pain which a subject is prepared to tolerate.

2.3 Peripheral mechanisms of pain/nociception

According to the IASP, the term nociception, derived from the Latin nocere meaning "to hurt/harm," is defined as the process that neural information about actual or potential tissue damage can be detected, transduced, encoded and transmitted from the site of origin to the higher brain centers where it is perceived as pain. There is considerable evidence that the skin is provided with a set of nerve endings whose specific function is to be amenable to noxious stimuli (Sherrington, 1903). These neural apparatus responsible for detecting a noxious stimulus are nociceptors. They are peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root and trigeminal ganglia. Nociceptors convey their information through the primary afferent neurons, which project to the central nervous system. The function of primary nociceptive afferents is to transduce chemical, thermal or mechanical energy into action potentials and transmit information about the intensity of nociceptive stimuli (Price, 1999). Transmission involves the release of neurotransmitter substances by nociceptive afferent neurons onto second order neurons of the spinal cord, which in turn transmit nociceptive information over the ascending pathways (Fürst, 1999).

Nociceptors have characteristic thresholds or sensitivities that distinguish them from other sensory nerve fibres. They can be directly activated by noxious heat, pressure or irritant chemicals, or activated after being sensitised during tissue injury, inflammation, ischaemia or low pH (Almeida *et al*., 2004).

There are two types of first order nociceptive afferent nerve fibres based on anatomical and functional criteria: A-delta (δ) and C-fibres (Burgess and Perl, 1967). Zotterman (1933) found that Aδ fibers and C-afferent fibers could account for the first and second pain respectively. Further research has shown that stimulation of Aδ fibers produced localized sharp sensation of pain called "first pain", whereas blocking of Aδ fibers and stimulating C-fibers produced an unbearable summating pain that is diffuse, burning and less well localized (Giordano, 2005).

Aδ fibers are small (1-5 µm in diameter), thinly myelinated, rapidly conducting (5-30 m/sec) neurons (Djouhri and Lawson, 2004). They have small receptive fields and specific high threshold channels that are activated by high intensity thermal or mechanical input. Aδ fibers are further classified into type I and type II fibers. Type I fibers are responsive to high temperatures (52 - 56ºC) and are insensitive to capsaicin. Type II Aδ fibers are sensitive to lower temperatures (40 - 45 ºC) and are sensitive to capsaicin. The responses of this heat-sensitive A-delta fibers sub serve the rapid, painful reaction to first exposure of noxious heat and the ability to discriminate thermal (nociceptive) sensation according to intensity. (Lawson, 2002)

The C-fibres are unmyelinated and thereby thinner (0.25– 1.5 µm in diameter) with slower conduction velocity (0.4 – 1.4 m/s) (Djouhri and Lawson, 2004). They have larger receptive fields than the Aδ fibres and constitute majority of cutaneous nociceptive innervation. C-fibers are activated by mechanical, thermal and/or chemical stimuli hence given the term polymodal for this characteristic (Perl, 2007). The natural stimulus of some nociceptors is

difficult to identify. Such receptors are refered to as ‘silent’ or ‘sleeping’ nociceptors and are responsive only when sensitized by tissue injury (Schmidt *et al*., 1995; Gold and Gebhart, 2010).

Both Aδ and C-polymodal nociceptive neurons can undergo sensitization. After damage to the skin or during inflammatory conditions, such neurons can become spontaneously active, have lowered thresholds to thermal and mechanical stimuli and show enhanced responses to suprathreshold and subthreshold stimulation (Price, 1999; Woolf and Ma, 2007). Tissue damage causes the production and accumulation of ions, peptides, lipids and proteins such as growth factors and cytokines in the inflammed tissue. These factors have been known to sensitise nociceptors to noxious stimuli (Levine and Reichling, 1999). Bradykinin and neurotrophic factors such as nerve growth factor can also sensitize nociceptors to noxious stimuli (Lewin and Mendell, 1993; Julius and Basbaum, 2001). Sensitization of nociceptors is one of the key mechanisms that drives and maintains persistent and chronic pain (Gold and Gebhart, 2010).

2.4 Spinal mechanisms of pain

The spinal cord is the first relay site in the transmission of nociceptive information from the periphery to the brain. The terminals of primary afferent fibres terminate in the dorsal horn of the spinal cord which is organized into different laminae, extending from the superficial to the deep dorsal horn (Rexed, 1952). Most nociceptive Aδ- and C-fibers terminate superficially in laminae I–II, with a smaller number reaching deeper laminae (Todd, 2002). Intrinsic neurons of the dorsal horn promote the interaction of the afferent and efferent nociceptive stimuli and are also responsible for their transfer to supraspinal structures. According to Price (1999), there are three main types of neurons in the dorsal horn; these are the projection neurons, excitatory and inhibitory interneurons. The projection neurons relay nociceptive information to the brain while the excitatory interneurons relay information to the projection interneurons,

other interneurons and motor neurons which mediate spinal reflexes. The inhibitory interneurons modulate the transmission of nociceptive information (Giordano, 2005; Heinricher *et al*., 2009).

There are two types of projection neurones involved in nociception; nociceptive-specific (NS) neurons and the wide dynamic range (WDR) neurons. The NS cells are mostly found superficially and synapse with Aδ- and C-fibres only (Sorkin and Carlton, 1997). These cells fire action potentials when a painful stimulus is detected at the periphery. The WDR cells receive input from all three types of dorsal horn neurons, and therefore respond to the full range of stimulation, from light touch to noxious heat, and chemicals. WDR cells fire action potentials in a graded fashion depending on stimulus intensity, and also exhibit 'wind-up,' a short-lasting form of synaptic plasticity (D'Mello and Dickenson, 2008). During wind-up, repetitive stimulation of WDR neurones induces an increase of their evoked response and post-discharge with each stimulus (Dickenson and Sullivan, 1987). With respect to noxious mechanical stimuli, NS neurons are classified as maintenance cells because they exhibit a prolonged response time in relation to the initial stimulation. In contrast, the WDR are classified as adaptive neurons because their time response ends right after the end of the initial stimulus (Almeida *et al*., 2004). Only the NS have the ability to code the intensity of the stimulus and are possibly responsible for the sensation of pain caused by sustained mechanical stimuli, also contributing to the acute sensation of pain (Willis and Westlund, 1997).

The excitatory or inhibitory interneurones can increase or decrease the response of NS and WDR cells, thus influencing the output of the dorsal horn. There is evidence that some non-neuronal cell types within the spinal cord such as astrocytes and microglia are able to influence pain transmission through the dorsal horn, particularly under pathological conditions (Coyle, 1998; Watkins *et al*., 1997).

2.4.1 The Gate Control Theory

Painful stimuli transmitted to the spinal cord are modulated at the level of the dorsal horn by the dorsal horn neurons. Melzack and Wall (1965) proposed the "gate control theory," a theory that has since been widely revised (Melzack, 1999; Giordano, 2005).

At the dorsal horn of the spinal cord three components of the gate control interact. They are the sensory afferents, the segmental cells and the descending controls. The segmental cells are cells within the central nervous system whose role is to select and compute combinations that terminate on them. Melzack and Wall (1965) proposed that the nociceptive information reaching the substantia gelatinosa (SG) acts as a gating mechanism to control the afferent input before it affects the spinal nociceptive neurons (T cells) located in the dorsal horn. The inhibitory effect of SG neurons on nociceptive transmision is influenced by the activity of primary afferents. Stimulation of large fibers enhances the inhibitory effect of SG neurons, while stimulation of small diameter fibres (Aδ and C-fibres) reduces it (Melzack, 1999). For the gate to operate properly, it was suggested that an ongoing activity in Aδ and C-fibres, a stimulus evoked activity and a relative balance of activity in large verses small fibres need to be present. It was postulated that tonic activity in small fibres would keep the gate partly open, while large fibres would close the gate thus limiting the output from the nociceptive neurons. Descending pathways are also capable of altering the gate, by probably setting the excitability level of both the pre and post synaptic mechanisms (Melzack, 1999).

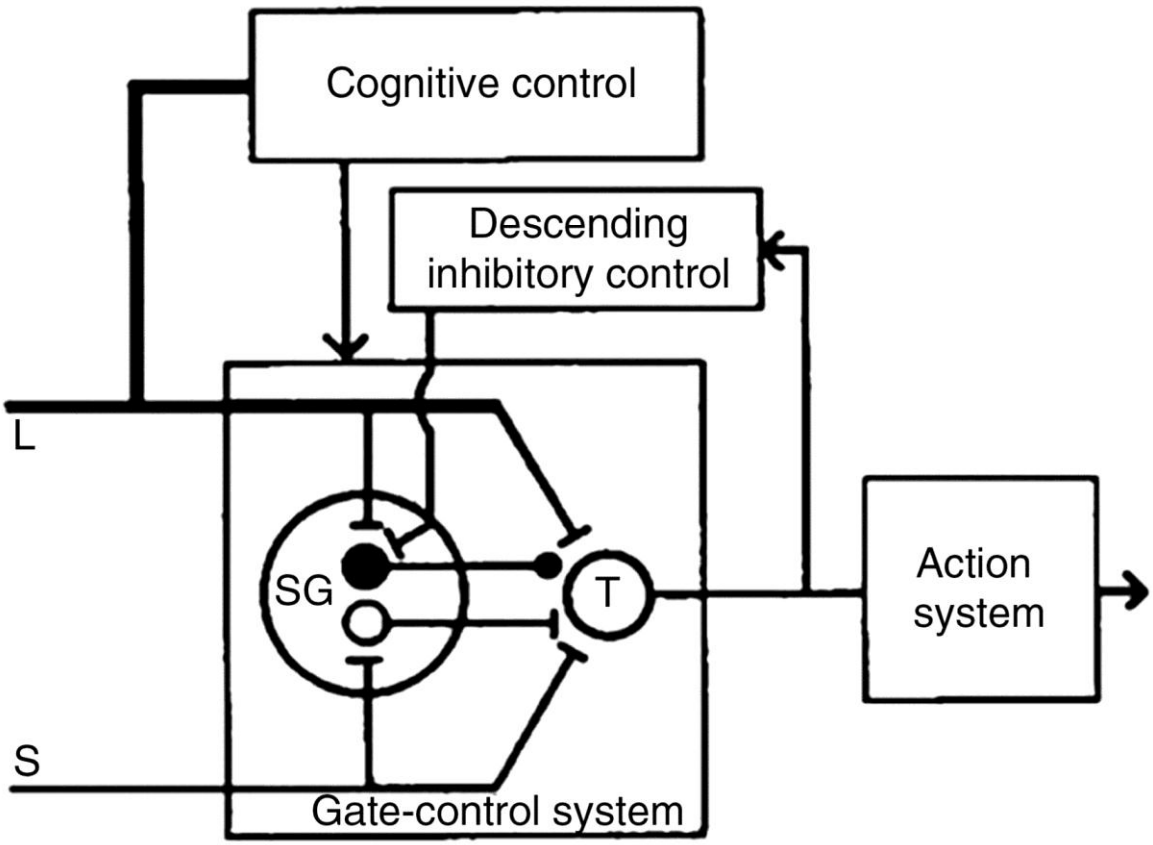

Figure 1: The gate control theory of pain model.

A presynaptic gate in the substantia gelatinosa (SG) of the spinal dorsal horn between primary afferent and projection neurons is controlled by the balance of activity between the large diameter (L) and small diameter (S) fibres. When the L-fibre input outweighs that of the S-fibres, the gate opens, permitting activation of transmission neurons (T). Central nervous system mechanisms (descending control) are postulated to modulate the gate.

2.4.2 Dorsal Horn Neurochemicals

In the superficial dorsal horn, a large variety of receptor classes and neurotransmitters are found. Peripheral noxious stimuli lead to nociceptor activation followed by release of neurotransmitters in the dorsal horn. The most important neurotransmitter classes for nociceptive transmission are excitatory amino acids and neuropeptides (D'Mello and Dickenson, 2008).

Glutamate is the principal excitatory neurotransmitter at the synapse between primary afferent nociceptors and the dorsal horn (Goudet *et al*., 2009). The activity of this amino acid is mediated by ionotropic or metabotropic glutamate receptors (Basbaum *et al*., 2009). The ionotropic receptors can be divided into three subcategories; N-methyl-D-aspartate (NMDA), alpha-amino-hydroxy-5-methyl-4-isoxazole- proprionic acid (AMPA), and kainate receptors. Activation of NMDA receptor results in a large influx of sodium and calcium ions into the neurons leading to depolarization (Basbaum *et al.,* 2009). The NMDA receptor complex is a multimeric channel permeable to sodium and calcium ions, and is both ligand and voltage gated. At normal resting potential, magnesium ions blocks the ionophore of the NMDA receptor, and relief of this blockade only occurs after membrane depolarization. This depolarization occurs after prolonged activation of AMPA receptors by glutamate leading to activation of NMDA receptors, which causes large prolonged depolarization assosiated with calcium ions influx (Schaible and Richter, 2004; Goudet *et al*., 2009).

This process underlies the medium to long term changes that occur in chronic pain states, including changes in peripheral receptive fields, induction of gene transcription and long term potentiation (LTP) (Giordano, 2005). The NMDA receptor is also hypothesized to be implicated in thermal and mechanical hyperalgesia (Zhou *et al*.,1996; Sandkühler, 2009). The metabotropic glutamate receptors (mGluRs) comprise of three groups (I-III) and at least 8 subtypes, (mGluR1 – mGluR8), of which at least two are present in the spinal cord (Fürst,

1999; Riedel and Neeck 2001; Goudet *et al*., 2009). Group I mGluRs may play a modulatory role in nociceptive processing, central sensitization and pain behaviour (Budai and Larson, 1998). The role of mGluRII and III is less clear (Budai and Larson, 1998; Ji *et al*., 2003; Goudet *et al*., 2009).

High intensity or prolonged C-fiber activity causes the release of the tachykinin and substance-P (SP) (Schaible and Richter, 2004; Andersson *et al*., 2008). Post-synaptically, SP initially binds to neurokinin-2 (NK-2) receptors, and subsequently to high affinity neurokinin (NK-1) receptors that are sensitive to lower SP concentrations (Andersson *et al*., 2008; Basbaum *et al*., 2009). SP acts at NK-1 receptors to induce translational and post-translational protein products that further alter the function and synaptic microstructure of cells receiving and transmitting nociceptive input (Luo *et al*., 2001; Basbaum *et al.*, 2009).

Sensitized C-fiber afferents retrogradely release SP which acts on NK-1 receptors in mast cells to induce pro-inflammatory chemicals mainly histamine and serotonin (5-HT). 5-HT may inhibit or facilitate nociceptive transmission depending on the experimental procedures used to study it or the species under study, amongst other factors (Schaible and Richter, 2004). C-afferents also releases other peptides such as cholecysyokinin (CCK), somatostatin, neurokinin A (NKA) and calcitonin gene-related peptide (CGRP). CCK acts nociceptively mainly via an indirect action by inhibiting antinociceptive effects of opioids (Riedel and Neeck, 2001). CGRP activates the induced isoenzyme nitric oxide synthase (iNOS) to enhance production of nitric oxide (NO) and increases peripheral vasodilation (Riedel and Neeck, 2001). NO acts as a non-adrenergic, non-cholinergic neurotransmitter and has been proposed to act in conjunction with NMDA receptors to initiate presynaptic glutamate release and thereby enhance nociception (Fürst, 1999). This is supported by findings showing that LN-nitroarginine methyl ester (L-NAME), a NO synthase inhibitor, enhances the antinociceptive effect of oxotremorine (Machelska *et al*., 1999).

The effects of histamine, 5-HT, SP, and CGRP are synergistic. In peripheral tissue, free 5-HT acts at 5-HT3 receptors on C-fiber terminals to directly induce a fast Na^+-dependent depolarization, sensitizes NK-1 receptors to SP, and evokes co-release of CGRP to increase iNOS production, thereby perpetuating the cycle of C-fiber-mediated neurogenic inflammation and pain (Cao *et al*., 1998).

2.4.3 Ascending spinal cord pathways

Nociceptive information from the peripheral system ascends in the white matter of the spinal cord through second order neurons. These transmit the nociceptive impulses to structures of the brain stem and diencephalon including the thalamus, periaqueductal substance, parabrachial region, reticular formation of the medulla, amygdaloid complex, septal nucleus, and hypothalamus (Willis and Westlund, 1997; Millan, 1999). There are five main ascending systems that are implicated in nociception. These include; the spinothalamic, the spinoreticular, the dorsal column, the spinomesencephalic and the spinocervical systems.

The **spinothalamic tract** (STT) is located in the anterolateral quadrant of the spinal cord and is the most prominent nociceptive pathway in the spinal cord. The STT mainly originates from cells in Rexed's lamina I, IV and V (Millan, 1999; Almeida *et al*., 2004). The pathways terminate in the contralateral thalamus via two projections. In the lateral projection, they terminate in the ventral posterior lateral nucleus and the ventral posterior inferior part of the lateral thalamus. These neurons are believed to play a role in the sensory and discriminative aspects of pain. In the medial projection, axons originate from deeper parts of the dorsal horn, and ventral horn, and terminate in the central lateral locus of the thalamus (Almeida *et al*., 2004). These axons reflect input from larger and more diverse receptive fields and are implicated in the affective-motivational dimension of pain (Price, 1999; Almeida *et al*.,

2004). Spinothalamic tract is very important in transmission of signals assosiated with pain and temperature sensation.

Spinoreticular tract originates from the deep layers of the gray matter (laminae VI and VII) and ascends through the anterolateral quadrant to terminate in reticular formation of the brainstem. One part of the tract terminates in several nuclei in pons and medulla such as nucleus paragigantocellularis, nuclei reticularis, pontis caudalis and oralis; nucleus gigantocellularis and nucleus subcoeruleus. Another major termination is in the parabrachial region, including the locus coeruleus and parabrachial nuclei (Willis and Westlund, 1997). This tract is involved in the motivational-affective characteristics of pain, and in the activation of brain stem structures responsible for descending suppression of pain (Price *et al*., 2000; Almeida *et al*., 2004).

The **spinomesencephalic tract** includes projections to different areas in the midbrain. Most axons project from layers I, II, IV, V and VI of the spinal cord. The tract primarily terminates in the superior colliculus and the periaquiductal grey (PAG) (Almeida *et al*., 2004). Projections to the PAG mainly activate the descending pain control networks, and are also involved in autonomic and somatomotor aspects of defence reaction (Almeida *et al*., 2004). Nociceptive activity in the superior colliculus is thought to be involved in multisensory integration, behavioural reactions and orientation to pain (Basbaum *et al*., 2002).

The **spinocervical tract** is predominantly assosiated with transmision of tactile stimuli but may also act as a minor pathway for noxious information. Its neurons originate in layers III and IV of dorsal horn and ascend in the postero-lateral cord and end in the lateral cervical nucleus where most of its neurons cross over and project to the thalamus (Almeida *et al*., 2004).

The **dorsal column pathway** mainly originates from lamina III and V and project to the dorsal column nuclei. Although the vast majority of these fibres are non nociceptive, some

nociceptive neurons project through this tract. The tract is organaized into two distinct pathways. One pathway is close to the midline of the spinal cord, originates from the lumbo-sacral region, while the other is at the junction of the gracile and cuneiform bundles, and originates from the thoracic column (Almeida *et al*., 2004). The pathway is involved in visceral pain transmission (Willis and Westlund, 2001; Palecek, *et al*., 2002).

The **spino-parabrachial-amygdala** originates in lamina I and V of the dorsal horn and ascends in the dorsolateral funiculus. It projects to the parabrachial area of the pons and from there to the amygdala. This system may normally be involved in fear and memory of pain, as well as in behavioural and autonomic reactions to noxious events such as vocalization, fighting, freezing, pupil dilation and cardiorespiratory responses (Millan, 1999; Almeida *et al*., 2004).

Several other nociceptive pathways have been described such as the **spinolimbic tracts** which consists of the **spinoreticulothalamic**, the **spinoamygdalar** and the **spinohypothalamic pathways** (Burstein *et al*., 1990). Neurons of the spinohypothalamic tract originate from similar areas as those of STT and terminate in the hypothalamus. This tract contributes to activation of the motivational component of pain and in initiating neuroendocrine and autonomic responses assosiated with painful stimulation (Almeida *et al*., 2004).

2.5 Supraspinal pain-regulating centres

Nociceptive neurons have been identified in portions of the medulla, pons, mesencephalon (midbrain), diencephalon (thalamus and hypothalamus), and cerebral cortex. The brainstem structures (medulla, pons, midbrain) contribute to nociceptive function through their contributions to the reticular system and the periaqueductal gray matter (PAG).

The reticular formation is a core of isodendritic neurons sending collaterals to the spinal cord, reticular neurons, various sensory and motor nuclei of the brainstem, the diencephalon, and to the cerebral cortex (Thurmon *et al*., 1996). The reticular system is critical to integration of the pain experience, as nociceptive input generates a profound effect on reticular neuronal activity. Ascending reticular neurons mediate the affective and motivational aspects of pain through their projections to the medial thalamus and limbic system (Giordano, 2005).

The periaqueductal gray (PAG) of the midbrain is a major locus of integration for homeostatic control. Although noted for its importance in the descending modulation of nociceptive information, it also extends ascending projections to the thalamus and hypothalamus, thereby providing an indirect alternative pathway for nociceptive sensory activity to reach diencephalic structures (Behbehani, 1995; Willard, 2008).

The thalamus serves as the relay point for sensory information en route to the cerebral cortex and is composed of numerous complex nuclei, several of which play key roles in nociception (Cross, 1994). Neurons from the lateral thalamic nuclei project to primary somatosensory cortex, where a conscious localization and characterization of the pain occurs. Neurons from the medial nuclei are projected to the anterior cingulate gyrus, which has been suggested to be involved in perception of suffering and emotional reactions to pain (Willard, 2008). The limbic system, also called the paleocortex, consists of the amygdala, hippocampus, septal nuclei, preoptic region, hypothalamus, and certain thalamic components. Limbic structures mediate aversive drive and thus influence the motivational component of pain and determine purposeful behavior (Millan, 1999).

Impulse transmission to the cerebral cortex is believed to play a vital role in integrating pain perception. Imaging studies in human beings indicate that several discrete regions of cortex are activated by noxious stimulation: the primary and secondary somatosensory cortices, the anterior insular cortex, and the anterior cingulate (a component of the limbic-associated

cortex), providing convincing evidence that cortical regions are in fact targets for noxious input (Talbot *et al*., 1991). Although the functional and structural species differences occurring at this level are undoubtedly more significant than at any other point along the nociceptive pathway, it seems clear that the cortex is able to modulate both the cognitive and aversive affective aspects of pain sensation and to mediate increasingly complex behavior patterns (Thurmon *et al*., 1996; Millan, 1999).

Several brainstem regions are involved in the modulation of the nociceptive transmission through the descending inhibitory regions of the spinal cord (Giordano, 2005). Connections from the brainstem to the spinal cord can modify information that is coming from the peripheral system to the brain.

The descending pathways include: the corticospinal, the raphe spinal and the reticulospinal (Millan, 1999; Giordano, 2005). The cortical spinal cells terminate in the LIII–LVI or even in LVII in the cat and are absent in LI and LII. The influence of the cortical spinal pathway upon dorsal horn interneurons includes a prominent inhibition in LV and excitation in LVI but no effect in LIV (Millan, 1999, Giordano, 2005). The raphe spinal system arises from the midline raphe magnus of the brain stem and consists of bilateral pathways descending in the dorsal lateral funiculli and terminates in LI, LII, LV and medial parts of LVI and LVII. The parts of the dorsal horn, which receives input from the raphe magnus, are those parts concerned with nociceptors and which give rise to spinothalamic and spinoreticular tracts (Millan, 1999, Giordano, 2005).

2.6 Role of descending modulatory systems in nociception

Descending pain inhibitory pathways have an important role in regulating pain by providing negative feedback control of nociceptive signals at the spinal cord level (Fields and Basbaum, 1999). The activation of descending inhibitory controls by a painful stimulus may not only

serve reduction of excessive pain by negative feedback loops, but it may also help in sharpening up of the contrast between the stimulus site and adjacent areas (Le Bars *et al*., 1979). Higher central nervous system activity controlling behavior provides another physiological way to recruit descending pain modulatory pathways, as shown by the modulation of responses of nociceptive spinal neurons by behavioral context and attention (Dubner, 1985). Similarly, mood and emotions may modulate pain through action on descending pain modulatory pathways (Suzuki *et al*., 2004). Analgesia induced by some centrally acting drugs involves activation of descending pain inhibitory pathways (Pertovaara and Almeida, 2006).

The major descending systems that modulate pain at the spinal level include opioidergic, GABAergic, cholinergic, noradrenergic and serotonergic systems.

2.6.1 Opioidergic system

It is important in supraspinal and spinal antinociceptive mechanisms. Opioidergic mechanisms are mediated by four types of opioid receptors (μ-, κ-, δ- and nociceptin opioid peptide). The μ-receptor is generally considered the most essential in antinociceptive actions, but κ and δ have also been shown to mediate antinociception. The endogenous ligands for opioid receptors can be divided into three different families of opioid peptides; endorphins, enkephalins and dynorphins (Fürst, 1999). Activation of opioid receptors can inhibit Ca^{2+} channels specifically on afferent C-fibres and thereby inhibit their spinal activity. Opioid receptors are also present on interneurons and cell bodies of second order neurons, where the nociceptive information can be blocked (Taddese *et al*., 1995; Ossipov *et al*., 2004). The various types of opioid receptors have high affinity for naloxone (Yaksh, 1987a). Opioid receptors in the spinal cord are found throughout the spinal gray matter, with a higher density in the dorsal horn (Fields *et al*., 1980; Slater and Patel, 1983; Morris and Herz, 1987). Opioid

receptors are also found pre and post synaptic to small afferent teminals (LaMotte *et al*., 1976; Gamse *et al*., 1979; Fields *et al*., 1980; Yaksh, 1987b). The interaction of opioidergic system with NMDA receptors possibly contributes to its antinociceptive actions, development of tolerance and opioid dependance (Mollereau *et al*., 2005). The opioids may act by modulating the NMDA receptor–mediated electrophysiological events or by interacting at an intracellular level (Mao, 1999).

2.6.2 GABAergic system

Studies have shown that gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the central nervous system (CNS) of vertabrates (Schmidt, 1973; Roberts, 1984). GABAergic interneurons are involved in tonic inhibition of nociceptive input. GABA normally plays an inhibitory role in dopaminergic cells (Giordano, 2005). Opioids and endogenous opioid neurotransmitters activate the presynaptic opioid receptors on GABAergic neurons. This inhibits the release of GABA in the ventral tegmental area. Inhibition of GABA allows the doperminergic neurons to fire more vigorously causing the release of extra dopamine in the nucleus accumbens. The two types of GABA receptors; the ligand gated Cl^- channel (GABA-A) and the GTP-binding protein coupled receptor (GABA-B) are important in spinal antinociception (Enna and McCarson, 2005). Activation of GABAergic interneurons also reduces the release of excitatory neurotransmitters such as glutamate, SP and CGRP from primary nociceptive afferents (Furst, 1999; Giordano, 2005). The inhibitory effects of GABA-A are preferentially through postsynaptic mechanisms, while those of GABA-B are presynaptic through the suppression of the effects of excitatory amino acids from the primary nociceptive terminals (Giordano, 2005).

2.6.3 Cholinergic system

The cholinergic system is diffuse and innervates most regions of the CNS (Nicoll *et al*., 1990). The cholinergic receptors are divided into two main groups: muscarinic (mAChRs) and nicotinic (nAChRs) receptors. Muscarinic actions can be excitatory or inhibitory while nicotinic actions are usually excitatory (Pert, 1987). Muscarinic receptors are G-protein coupled receptors with seven trans-membrane domains. The muscarinic receptors were initially defined as receptors activated by muscarine and blocked by atropine (Dale, 1914). This definition was accepted until 1980, when pharmacological studies demonstrated that the effects mediated by mAChRs could not be due to one receptor type, but that there had to be two subtypes (Hammer *et al*., 1980; Caulfield, 1993). Further pharmacological investigations showed that at least three (Birdsall *et al*., 1983; Caulfield and Straughan, 1983) and later four (Waelbroeck *et al*., 1990) muscarinic subtypes existed. These pharmacologically defined subtypes were termed M_1, M_2, M_3 and M_4. Molecular biology techniques have made cloning of genes coding for muscarinic receptors possible and demonstrated the presence of five muscarinic receptor subtypes. The cloned subtypes are termed M_1, M_2, M_3, M_4 and M_5 (Caulfield and Birdsall, 1998). The different subtypes differ in function with regard to their specific G-protein coupling and second messenger activation. Subtypes M_1, M_3 and M_5 couple to the Gq protein, which activates the inositol polyphosphate generation, which in turn leads to a stimulated effect of the cell (Eglen, 2005; Jones and Dunlop, 2007). Subtypes M_2 and M_4, on the other hand, couple to the Gi protein and thereby inhibit cAMP generation, which in turn inhibits the function of the cell (Caulfield, 1993; Lambert, 1993; Eglen, 2005). Muscarinic receptors have been found in the spinal cord grey matter, including the superficial laminae in several species such as humans and rats (Gillberg *et al*. 1989; Höglund and Baghdoyan, 1997).

Nicotinic receptors are pentameric transmembrane proteins belonging to the family of ligand-gated ion channels. Like the muscarinic receptors, they were defined by Sir Henry Dale, as receptors that were activated by nicotine and blocked by curare (Dale, 1914). The receptor consists of five subunits arranged symmetrically in the cell membrane to form a central pore. Several types of subunits have been described, which can be divided into two main categories: The alpha (α) subunits (α1–9), that possess adjacent cysteines for acetylcholine binding, and the non-α subunits (β1–4, δ, ε and $_{\gamma}$) that lack the cysteines (Dani and Bertrand, 2007). About 15 mammalian subunits have been cloned (Corringer *et al*., 2000; Le Novere and Changeux, 2001). Various nicotinic receptor subtypes are present in the spinal cord in the superficial laminae. However, the nicotinic receptors appear to exist in lower quantities than muscarinic receptors in the spinal cord of rats (Gillberg *et al*., 1989; Khan *et al*., 1994, 1997).

Several studies have been undertaken to investigate the involvement of the cholinergic receptor system in antinociception at both the supraspinal and the spinal level. Systemic as well as intrathecal administration of muscarinic agonists produce potent antinociception in several species (Yaksh *et al*., 1985; Gower, 1987; Gillberg *et al*., 1989; Zhuo and Gebhart, 1991; Iwamoto and Marion, 1993; Abram and O'Connor, 1995; Lambert and Appadu, 1995). The different pharmacological studies that have attempted to determine the spinal muscarinic subtypes relevant for the antinociceptive effect, suggested involvement of the M_1, M_2, M_3 and M_4 subtypes (Bartolini *et al*., 1992; Naguib and Yaksh, 1997; Ellis *et al*., 1999; Duttaroy *et al*., 2002; Lograsso *et al*., 2002).

Neuronal nicotinic receptors are considered a promising target in pain treatment (Flores and Hargreaves, 1998). An involvement of nicotinic receptors in antinociception has been known for several decades. In 1932, antinociception of nicotine was reported (Davis *et al*., 1932), an effect that has been verified by other studies (Sahley and Berntson, 1979; Iwamoto, 1991). Other nicotinic agonists that produce antinociception after supraspinal or systemic

administration are epibatidine (Qian *et al*., 1993; Curzon *et al*., 1998; Lawand *et al*., 1999; Abelson and Höglund, 2002; Dulu *et al*., 2014), A85380 (Curzon *et al*., 1998) and ABT-594 (Bannon *et al*., 1998; Bitner *et al*., 1998). The antinociceptive effects of nicotinic agonists administered into the spinal cord are somewhat controversial, since both nociceptive and antinociceptive effects have been observed (Khan *et al*., 1998).

The cholinergic receptor system has been found to interact with most other receptor systems in the spinal cord. Muscarinic receptors have been shown to be involved in spinal antinociceptive mechanisms mediated by the GABAergic (Baba *et al*., 1998; Chen and Pan, 2003), opioid (Harris *et al*., 1969; Pert, 1975; Chen and Pan, 2001; Dulu *et al*., 2014), and adrenergic (Detweiler *et al*., 1993; Klimscha *et al*., 1997; Pan *et al*., 1999; Honda *et al*., 2002, 2003) receptor systems. Nicotinic receptors are also involved in modulation of nociceptive information by other receptor systems. Interactions with particularly the serotonergic and adrenergic systems have been demonstrated (Iwamoto and Marion, 1993; Bitner *et al*., 1998; Cordero-Erausquin and Changeux, 2001; Li and Eisenach, 2002). In addition, both muscarinic and nicotinic receptors have been suggested to play an important role in the antinociceptive mechanism of NO in the spinal cord (Xu *et al*., 1996, 2000).

Based on these findings, there should be little doubt that the cholinergic receptor system is an important component in antinociceptive mechanisms. However, the underlying mechanisms responsible for the cholinergic contribution to spinal antinociception are far from fully understood. One conceivable explanation could be that stimulation of muscarinic or nicotinic receptors results in a release of acetylcholine in the spinal cord, and that acetylcholine in turn inhibits the nociceptive transmission. In 1945, a study showed that subcutaneous injection of the acetylcholine esterase inhibitor, neostigmine (prostigmine), significantly increased the antinociceptive effect of morphine in humans (Flodmark and Wramner, 1945). Intrathecal administration of neostigmine has revealed that part of its antinociceptive effect is mediated

at the spinal cord level in both humans and animals (Bouaziz *et al*., 1995; Hood *et al*., 1995; Hwang *et al*., 1999). Since neostigmine prevents degradation of acetylcholine in the synaptic cleft, the amount of acetylcholine increases. This strengthened the theory that endogenous acetylcholine contributes to the inhibition of nociceptive information at the spinal cord level. Little has been reported from the few studies that have been performed to evaluate this theory (Bouaziz *et al*., 1996; Eisenach *et al*., 1996).

2.6.4 Noradrenergic system

The noradrenergic system is one of the diffusely organaized systems in the central nervous system which is assosiated most with the locus coeruleus (Gebhart, 2004). The locus coeruleus is located in the rostral pons in the floor of the rostral part of the fourth ventricle

Antinociception by activation of descending noradrenergic fibres has partially been attributed to the direct inhibition of nociceptive spinal neurons (Gassner *et al*., 2009). Noradrenaline is known to have a significant antinociceptive influence through action on spinal α2-adrenoceptors (Yaksh *et al*., 1985). The source of spinal noradrenaline is descending axons originating in the noradrenergic neuronal cell groups of the brainstem (Proudfit, 1987; Jones *et al*., 1991), particularly the locus coeruleus (A6) but also noradrenergic cell groups A5 and subcoeruleus (A7) (Kwiat and Basbaum, 1992). The A5, A6 and A7 cell groups are connected with other pain control centers and all of them receive projections from the PAG (Bajic and Proudfit, 1999).

Additionally, the locus coeruleus receives projections from the central nucleus of the amygdala, preoptic area, paraventricular nucleus of the hypothalamus and lateral hypothalamus (Cedarbaum and Aghajanian, 1978). The descending noradrenergic systems terminates in the marginal layer LII, IV,VI and the ventral horn. Of the nuclei projecting to noradrenergic cell groups of the brainstem, the parabrachial nucleus is noteworthy since it is

an important relay for nociceptive signals from the superficial laminae of the spinal cord to the amygdala and hypothalamus, structures involved in control of emotional responses and stress, respectively (Bernard *et al*., 1996; Gauriau and Bernard, 2002). Due to their anatomical connections to multiple forebrain areas, the descending noradrenergic systems provide a putative subcortical relay for descending antinociceptive actions from some forebrain areas (Jasmin *et al*., 2004). Moreover, the descending analgesic influence triggered by PAG stimulation is partially mediated by recruitment of the descending noradrenergic system (Peng *et al*., 1996), through projections of the PAG and rostral ventral medulla (RVM) to noradrenergic cell groups of the brainstem (Sim and Joseph, 1992; Bajic and Proudfit, 1999). Noradrenaline can act on four distinct receptor subtypes, alpha-1 (α1), alpha-2 (α2), beta-1 (β1) and beta-2 (β2) (Furst, 1999). Noradrenergic descending inhibition directly excites GABAergic spinal lamina II but not lamina III neurons which facilitate GABA release in the dorsal horn via the activation of α1-adrenoceptors in addition to its direct inhibitory action on excitatory spinal dorsal horn neurons (Gassner *et al*., 2009).

The descending noradrenergic systems have a low tonic nociceptive activity, since α2-adrenoceptor antagonists (Pertovaara, 1993) or knockouts of various subtypes of α2-adrenoceptors (Malmberg *et al*., 2001) have not consistently produced increases in pain-related responses to brief noxious stimuli in animals without sustained pain. Reports from studies involving knockout of the dopamine, β-hydroxylase gene have shown the absence of noradrenaline and only minor and submodality selective effects on pain sensitivity were expressed (Jasmin *et al*., 2004), supporting the concept that noradrenergic systems have little influence on baseline pain sensitivity. During persistent pain, however, noradrenergic systems have a more important role. This is shown by the findings that a lesion of the noradrenergic locus coeruleus (Tsuruoka and Willis, 1996) or a knockout of α2A-adrenoceptors (Mansikka *et al*., 2004) significantly increased pain-related reflex responses in animals with

inflammatory pain, indicating an involvement of the noradrenergic feedback inhibition in the regulation of sustained pain.

2.6.5 Serotonergic system

Most of the afferents utilizing serotonin (5-HT) originate from the nuclei of median raphe. Diffuse afferents from these nuclei innervate virtually all levels of the central nervous system from the sacral spinal cord up through the telencephlon (Nicoll *et al*., 1990). This wide distribution of 5-HT provides the basis for an influence on numerous central nervous system functions such as endocrine activity, appetite, sleep mechanisms, sexual behaviour, temperature regulation, motor activity, and some cognitive functions including learning and memory (Slater and Blundell, 1980; Cox *et al*., 1981; Steinbusch, 1981; Nicoll *et al*., 1990)

There are seven subtypes of serotonergic receptors, $5\text{-}HT_{1\text{-}7}$ (Nelson, 2004). With the exception of 5-HT3 receptor which is a ligand gated ion channel, all other receptors are G protein coupled seven transmembrane receptors that activate an intracellular second messenger cascade (Nelson, 2004; Nichols and Nichols, 2008).

Serotonergic fibres terminate on enkephalinergic interneurons of the immediate gray of the spinal cord (Basbaum, 1999). They project to the presynaptic terminals of primary afferents, which mediate pain and utilize substance P. The serotonergic input is excitatory to the enkaphalinergic interneurons, which in turn are inhibitory on the primary afferents. Thus raphe neurons can inhibit the flow of pain information from the periphery. Serotonergic fibres also terminate on parasympathetic neurons in the intermediolateral cell column of the spinal cord (Furst, 1999). The raphe is inhibitory to these neurons.

Stimulation of the raphe nuclei produces a powerful analgesia and thus blocks pain transmission. Depletion of 5-HT by P-chlorophenylamine reduces stimulation-produced analgesia which is reversible by administration of 5-hydroxytryptophan, a 5-HT precursor

(Giordano, 2005). Serotonin may not be directly involved in the inhibition of pain transmission since serotonergic agonists do not have significant analgesic effects (Furst, 1999).

2.7 Models of nociception

Models of nociception involve the application of various noxious or innocuous stimuli. These models are essential for uncovering the mechanisms of nociception in vertebrates for the development of analgesics for use in both animal and human pain. More potent and selective therapeutics may be developed as further progress is made. Nociceptive tests use electrical, thermal, mechanical, or chemical stimuli (Le Bars *et al*., 2001). Some of them rely on the latency of appearance of an avoidance behavior, usually a withdrawal reflex of the paw or the tail. In this case the stimulus may be considered as fixed. The tests that use thermal stimulation include the tail flick test, the hot- or cold-plate tests, and the radiant heat paw-withdrawal test. Nociceptive tests can also rely on the stimulus threshold necessary to elicit an avoidance behavior. In this case, the stimulus is either variable, with increasing value, or the test may use successive incremental stimuli at a fixed value. These tests involve mechanical stimulation and include the von Frey filaments, the Randall–Selitto analgesimeter, and recent tests based on strain gauges held by forceps or fingers. The development of dynamic hot and/or cold plates has allowed the assessment of thermal thresholds in awake rodents. Electrical thresholds are also studied, particularly as a control for other behavioral experiments. Some nociceptive tests can rely on the observation and scoring of specific behaviors. This is the case for assessing cold allodynia with acetone or for tests using inflammatory or irritating chemical stimuli (Sandkühler, 2009). The results obtained in most nociceptive tests show a relatively low interindividual variability compared with what is observed in other fields of behavioral studies, such as mood disorder-related studies or

operant behavior studies. As a consequence, experiments on nociceptive responses can often be conducted with fewer animals than what would be necessary for these other studies (Barrot, 2012). The choice of test is a critical step. Indeed, different nociceptive modalities are at least partially processed through different molecular transducers and fibers (Delmas, 2008; Scherrer *et al*., 2009). Moreover, genetic or pharmacological manipulation may dissociate these various modalities (Scherrer *et al*., 2009).

2.7.1 Tail flick test

The tail flick is one of the oldest nociceptive tests (D'Amour and Smith, 1941). The measured parameter is the latency, in seconds, for tail flick reflex following tail exposure to a heat stimulus. The stimulus may be applied by dipping the tail tip into a bath at a controlled temperature, exposing the tail to a controlled infrared heat beam or by heating the tip of the tail by a light bulb shining through a small aperture using a specialised analgesimeter, and the withdrawal response is detected and timed by a photocell circuit (Le Bars *et al*., 2001). The tail flick is a spinal reflex, but it is subject to supraspinal influences that can affect this reflex (Yaksh and Rudy, 1978; Millan, 2002). This test is highly sensitive to opiates (Le Bars *et al*., 2001). Because it has mostly been used to study the response to analgesic drugs, the heat intensity is usually set up for fast withdrawal latencies (around 2–4 s), but it can be adjusted when pain models are studied. A lengthening of the reaction time is interpreted as an analgesic action. Stronger intensities create a "floor" effect that makes it difficult to detect hyperalgesia. Weaker intensities (longer latencies) make it difficult to detect analgesia, and increase the probability of a non reflexive tail movement (Barrot, 2012). Tail flick test is relatively easily done in rats with habituation to manipulation. The advantages of this method are its simplicity and the small inter-animal variability in reaction time measurements under a given set of controlled conditions. A potential difficulty of the test is to maintain the animal in a correct posture without inducing unwanted stress (Barrot, 2012). Another pitfall may be

related to the role of the tail in thermoregulation of rodents. A decrease in tail temperature will produce an increase in the tail-flick response (Berge *et al*., 1988). Monitoring the tail temperature may therefore be important to avoid false conclusions about the antinociceptive effect of an agent (Le Bars *et al*., 2001). The tail-flick test has been applied to many animal models such as rats (Cecchi *et al*., 2008), mice (Tseng *et al*., 2011; Wu *et al*., 2011), naked mole rats ((Dulu *et al*., 2014), and currently to the *Tachyoryctes splendens*.

2.7.2 The formalin test

The test was first introduced by Dubuisson and Dennis (Dubuisson and Dennis, 1977) in cats and rats and adapted for use in mice (Hunskaar *et al*., 1985; Murray *et al*., 1988; Shibata *et al*., 1989) and other animals such as primates and naked mole rats (Dulu *et al*., 2014). It involves subcutaneous injection of a dilute solution of formalin into the dorsal or ventral surface of the hind paw, inducing a variety of recuperative behaviours that last approximately for 1 hour. In comparison with various algogens known to cause inflammation and/or hyperalgesia, including yeast, carrageenan, serotonin, kaolin, platelet-activating factor and mustard oil, only formalin and acetic acid produce obvious evidence of spontaneous nociception in the rat (Wheeler-Aceto *et al*., 1990) and other animal species.

A unique characteristic of the formalin test is its biphasic nature in a large number of species including rats, mice and naked mole rats. Subcutaneous formalin produces two distinct phases of behavioral responses and firing of Aδ and C dorsal horn convergent neurons (Dickenson and Sullivan, 1987, Heapy *et al*., 1987; Puig and Sorkin, 1996). The early or acute phase (0 to 5 minutes post injection) is thought to reflect direct activation of nociceptors, whereas the late tonic phase (aproximately 15 to 60 minutes) has been attributed to central sensitization (Coderre *et al*., 1990;Vaccarino and Chorney, 1994). There is consensus that the late tonic phase behaviors are driven in part by the central sensitization of spinal cord circuits

secondary to the barrage of input that occurs during phase I (Dubuisson and Dennis, 1977; Tjolsen *et al*., 1992). There is evidence of an important contribution of ongoing afferent firing during phase II (Taylor *et al*., 1995). In fact, electrophysiological studies demonstrated that Aδ- and C-fiber nociceptors exhibit sustained firing during both phases of the formalin test, and even that presumably non-nociceptive Aβ-fibers are activated during phase I (Puig and Sorkin, 1996; Mccall *et al*., 1996). The interphase period (5 to 15 minutes) has been thought to be due to inhibition at the supraspinal (Franklin and Abbott, 1993) or spinal level (Henry *et al*., 1999). The late phase is sensitive to a number of drugs including non steroidal anti-inflammatory drugs (NSAIDs), κ-opioid agonists, and gabapectin that are less effective or ineffective against the early phase and other acute models of nociception (Le bars *et al*., 2001). The intensity of the behavioural response is independent from the extent of the inflammation (Brown *et al*., 1968; Wheeler-Aceto *et al*., 1990; Wheeler-Aceto and Cowan, 1991). The formalin test has been used to investigate analgesic effects of oxotremorine, the muscarinic receptor agonist (Yaksh *et al*., 1985; Capone *et al*., 1999; Abelson and Hoglund, 2002; Dulu *et al*., 2014), epibatidine (the nicotinic receptor agonist) in rats, mice, naked mole rats (Qian *et al*., 1993; Curzon *et al*., 1998; Boyce *et al*., 2000; Dulu *et al*., 2014), morphine, nefopam and paracetamol (Kanui *et al*., 1993), codeine, naproxen and dexamethasone (Karim *et al*., 1993) and opioid peptides (Towett *et al*., 2009). Besides being simple to perform, the formalin test is reported to have a fair degree of objectivity, validity, reproducibility and quantifiability (Alreja *et al*., 1984). Prior to this study the formalin test had not been applied to the *Tachyoryctes splendens.*

2.7.3 Writhing test

This is a nociceptive test used to study visceral pain and cutaneous pain. It has also been used as a "standard" pharmaceutical screening tool since its initial description in the 1950s (Vander

Wende and Margolin 1956; Siegmund *et al*., 1957; Carroll and Lim 1958; Koster *et al*., 1959). The writhing test involves intraperitoneal injection of a chemical irritant to cause a behavioural response in the experimental animal. After intraperitoneal injection of the noxious agent, the rat and the mouse show a response consisting of a wave of constriction and elongation passing caudally along the abdominal wall, sometimes accompanied by twisting of the trunk and followed by extension of the hind limbs which can be quantified (Le Bars *et al*., 2001). This response has been variously called "writhing" (Vander Wende and Margolin, 1956), "stretching" (Koster, *et al*., 1959), "cramping" (Murray and Miller, 1960) and "squirming" (Whittle, 1964). Because of the emotional implications of these terms, it was later called the "abdominal constriction response" (Collier *et al*., 1964).

The writhing test has variations that have been described in primates, cats, dogs, and guinea pigs but predominantly in rats and mice (Ness, 1999). Methodology has varied with the use of endothelin, bradykinin, adenosine 5'-triphosphate, acetylcholine, magnesium sulfate, hypertonic saline, and iodinated radio-contrast agents as intraperitoneal irritants (Gyires and Torma, 1984). However, the most commonly employed agents for the writhing test have been phenylquinone and acetic acid. The writhing test is typically carried out in unanesthetized rodents using an intraperitoneal injection of either a fixed dose or weight adjusted dose of dilute acetic acid (0.6 to 9% V/V) or phenylquinone (0.1 to 0.3%) solutions. Responses have been quantified as all-or-none responses, but more commonly the number of writhes is counted in 5-min intervals for 30 to 60 min.

This model of visceral pain has proven predictive value as a screening tool for analgesic actions (Porreca *et al*., 1987), but methodological and ethical concerns have presented significant constraints to the use of the model. Multiple nonanalgesics such as atropine and naloxone have been demonstrated to have profound inhibitory effects in the writhing test (Hendershot and Forsaith, 1959; Chernov *et al*., 1967; Taber *et al*., 1969; Le Bars *et al*.,

2001), and so the specificity of the model has been questioned. The reliability of the response has also been problematic in that less than 8% of animals may demonstrate no evidence of any writhing response (Hendershot and Forsaith, 1959). Within-animal reproducibility of responses has not been demonstrated because animals are typically sacrificed at the end of experiments.

2.7.4 Hot plate test

This assay was originally described by Woolfe and MacDonald (1944) although the version most often used today was modified by Eddy and Leimbach (1953). In this test, the animal is confined by Plexiglass walls to a metal or porcelain surface heated to a specified temperature commonly (50 to 56 ºC), and the latency to the performance of an endpoint response considered indicative of nociception. A 52 or 55 °C set up allows observing baseline latencies between 5 and 10 seconds for paw licking, depending on the material of the plate and the animal species. The plate material may influence heat conduction and explain small differences in latency values between the available brands of hot plates. These temperatures are 10–15 °C higher than the response threshold of heat nociceptors (Yeomans and Proudfit, 1996), which reflects the time required for skin temperature to increase until detection of the nociceptive stimulus, and the delay to elicit the withdrawal response. Higher temperatures are commonly not used because of the risk of burns. Responses in the hot-plate test are supraspinal (Le Bars *et al*., 2001). Mice placed on a hot plate will exhibit one or more of the following: freezing, exploring, forepaw licking, grooming, hind paw lifting/guarding, hind paw licking, hind paw fluttering (shaking or stamping) and vertical jumps (Hunskaar *et al*., 1986). The behaviors observed in the rat are more complex when compared to the mouse (Le Bars *et al*., 2001). Some studies are specifically based on jump latency, particularly in mice. This parameter should be used with caution as it results in longer latencies, which may

sometimes raise ethical issues, and may also lead to a learning/anticipation process limiting the possibility to repeat measures on the same animal (Le Bars *et al*., 2001). Small differences in the plate temperature can result in important differences in response latency. Using reliable plates specifically designed for behavioral tests, with fast adjustment of temperature changes and with a 0.1 °C precision in the temperature control, is thus important.

As far as analgesic substances are concerned, the paw-licking behavior is affected only by opioids in mice (Neelakantan and Walker, 2012). On the other hand, the jumping reaction time is increased equally by less powerful analgesics such as acetylsalicylic acid or paracetamol, especially when the temperature of the plate is 50 °C or less (Ankier, 1974) or if the temperature is increased in a progressive and linear fashion, e.g., from 43 to 52 °C at 2.5 °C/min (Hunskaar *et al*., 1985). Temperatures of 60 ºC caused licking and shaking of both fore and hind limbs in the East African root rat (Towett and Kanui, 1995). The specificity and sensitivity of the test can be increased by measuring the reaction time of the first evoked behavior regardless of whether it is paw-licking or jumping (Carter, 1991), or by lowering the temperature (Plone *et al*., 1996). Like other nociceptive tests, the hot plate test has been applied to a wide range of animal models such as mice (Sabbithi *et al*., 2013), rats (Gunn *et al*., 2011), the naked mole rat (Kanui and Hole, 1990), *Tachyoryctes splendens* (Towett and Kanui, 1995).

2.7.5 Radiant heat paw withdrawal test

The radiant heat paw-withdrawal test was described by Hargreaves *et al*. (1988). The test differentiates the left and right hind paw responses to heat in freely moving rodents. It has also been referred to as the Hargreaves Method or by the brand name, "Plantar" (Barrot, 2012). In this test animals are placed in clear boxes on a glass surface. A controlled heat beam system is placed below the glass and is moved under a hind paw. A timer is activated at the

onset of the stimulus, whereas the paw withdrawal automatically stops the timer. This test takes longer to complete than the hot-plate test. It requires a period of habituation to the box before each testing procedure. Moreover, each paw is tested independently, often with alternative measures of left and right hind paw withdrawal that are done repeatedly to average the results. However, with adequate set ups, a few animals can be tested in parallel and this test has the advantage to allow differentiating the response of both hind paws. It is valuable when working with unilateral models of pain with injections in the paw or the knee or with manipulations of the sciatic nerve. It is also useful for tests requiring topical application of a substance, either proalgic or analgesic. The contralateral paw can be used to provide an internal control for the experiments.

2.7.6 Yeast or Carrageenin induced hyperalgesia

This test involves injection of an irritant material such as yeast, croton oil, or carrageenin into the hind paw of the animal (Vinegar *et al*., 1987; Morris, 2003). This causes inflammatory changes and hyperalgesia. Pain is then quantified by applying pressure on the swollen paw by means of a metal cylinder and the pressure (mmHg) at which the animal begins to vocalize or struggle is recorded. The contralateral paw is used as the positive control. This test has been used to distinguish between drugs acting in the CNS and locally at the site of inflammation. It is also sensitive to narcotic as well as non narcotic analgesics (Morris, 2003).

2.7.7 Adjuvant induced arthritis

This test provides a chronic pain model for studying the effects of anlgesics. Sodium urate crystals and Mycobacterium butyricum with Freud's adjuvant (Pircio *et al*., 1975) have been used to induce polyarthritis in rats. Oedema of the paw and vocalization following manipulation of the joint are two of the responses that have been used to study the effects of analgesics in this test.

2.7.8 Didanosine-induced peripheral neuropathy

Didanosine is a nucleotide reverse transcriptase inhibitor (NRTI) that is commonly used in therapy of Human Immunodeficiency Virus patients to reduce the effects of the virus. Peripheral neuropathy is among the most frequent side effects with some of the dideoxynucleosides, mainly zalcitabine, didanosine and stavudine (LeLacheur and Simon, 1990; Cui *et al*., 1997). To elucidate the mechanisms underlying this peripheral neuropathy, a new model of enhanced nociception using the NRTI was developed and adopted for use in rats (Joseph *et al*., 2004). Oral or intravenous administration of these drugs has been observed to induce hyeralgesia and allodynia in rats (Joseph *et al*., 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Animal samples

The animals used in these experiments were captured alive from Lower Kabete area in Nairobi county, Kenya. A total of 120 adult male and female *Tachyoryctes Splendens* weighing 160 to 280g (Kingdon, 1974; Nowak, 1999) were selected on capture and put in well aerated buckets that were used in transportation by road to a laboratory in Chiromo campus, University of Nairobi.

Modifications of one of the capture methods described for capturing naked mole rats in the fields (Jarvis, 1978) was used to trap the East African root rats. The method is based on the fact that this rodent just like the naked mole rat has a habit of investigating and blocking up opened sections of their burrow system. Fresh mole-hills were identified and each was marked using a coloured paper stuck to a tall stick for ease of location of make-shift traps placed within the coffee plantations. The entrance to the burrow was exposed by removing the soil cover and a careful investigation to establish their likely location within the burrow was carried out. A trap made of a wire noose was placed along the circumference of the burrow from the direction the root rat was suspected to be. The wire was tied onto a long stick anchored to the ground, a string was tied onto the stick to exert tension and anchored to the ground at the entry of the burrow. Some cut mexican marigold (*Tagetes minula*) was placed in front of the string at the entrance of the burrow as bait. The burrow was then covered back with soil and a period of silence was observed to allow the rodents to access the bait. The rodent would be attracted by the smell of the bait when it came to investigate the burrow and it would then be captured by the wire noose on its girth once it chews off the string blocking its way to access the bait. The traps were inspected every fifteen to twenty minutes and

captured animals were removed from the wire noose and kept singly in well aerated buckets. The traps were removed after every trapping excercise.

In the laboratory, the animals were kept singly in strong wire mesh and plastic glass cages (30×35×30 cm) containing untreated wood sawdust and some grass for nesting. The bedding was changed twice a week to ensure the cages were clean and damp-free. Animal house temperatures were maintained at 21^{o}-25 ºC and light was maintained at 12/12 dark/light cycle. The animals used in the study were fed fresh sweet potatoes, carrots and freshly cut grass *ad libtum*. No water was provided since these rodents have not been observed to drink any when provided in earlier studies. It is likely that they get water from the feeds provided.

The animals were habituated to the laboratory conditions for at least 21 days before they were used for the experiments. Before any experiment, the animals were examined for fitness by checking on their touch reflex, locomotion and response to sounds and vibrations. Each animal was used once and sacrificed after the experiment using an overdose of halothane.

All the methods and techniques used were approved by the faculty board on Ethics and Animal use committee.

3.2 The Formalin test

A transparent Perspex chamber (measuring 30×30×30 cm) was used to contain and observe the animals. The root rats were acclimatized to the observation chamber for 60 minutes daily during the 21-day acclimation period. Prior to the formalin test, each animal was again acclimatized to the Perspex chamber for 30 minutes. The root rat was restrained and using a 100 µl (U-100 insulin) syringe with a 30-gauge needle, 20 microlitres of 8% formalin in 0.9% sodium chloride solution was injected intradermally into the dorsal side of the right hind paw (**Plate 2)**. The volume and concentration of formalin used was based on preliminary studies. The animal was returned to the observation chamber immediately after the formalin injection

and the observation period of one hour started. A mirror was placed behind the observation chamber to allow for unobstructed view of the animal (**Plate 3**). Pain behavior was quantified by scoring the total amount of time (in seconds) the animal spent licking/biting the injected paw. The time spent licking or biting the injected paw following the injection of formalin was recorded in blocks of 5 minutes for 60 minutes.

The experiments were done in a room with very minimal noises and vibrations. The experiments were always performed between 8 a.m. and 2 p.m.

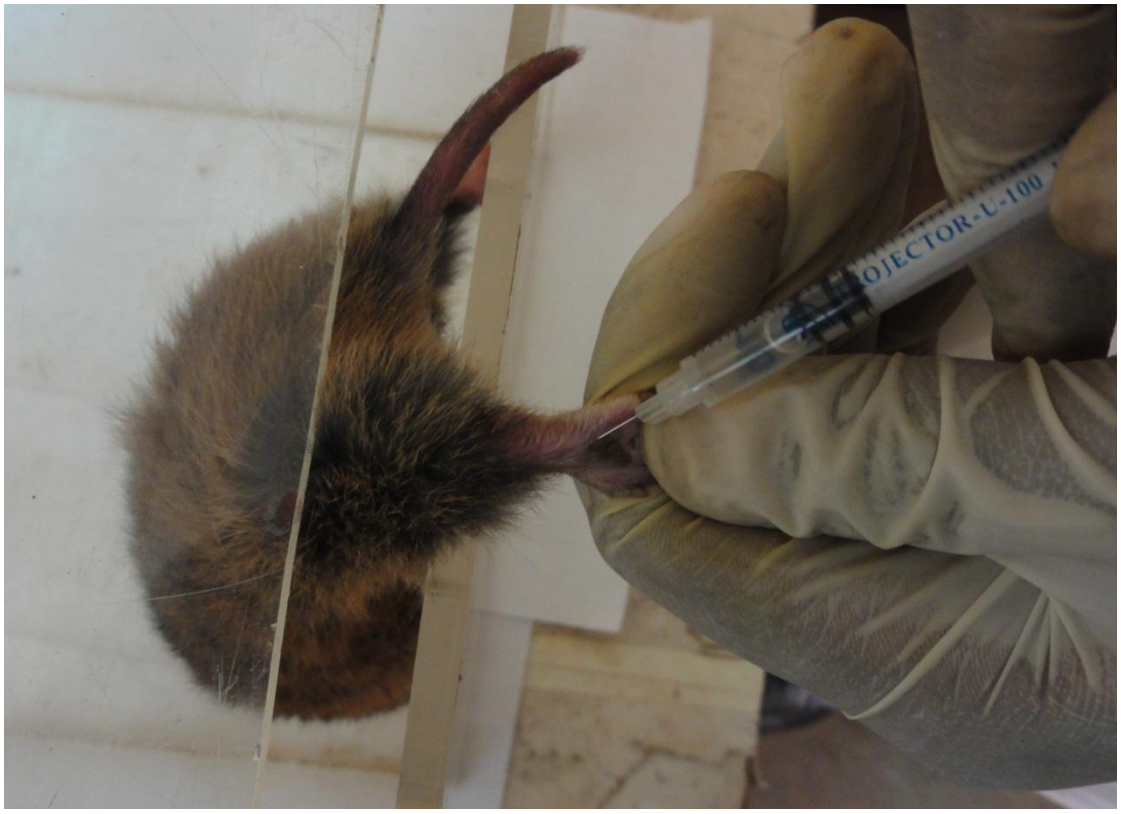

Plate 2: Intradermal injection of 20 µl of 8% formalin using a 100 µl syringe.

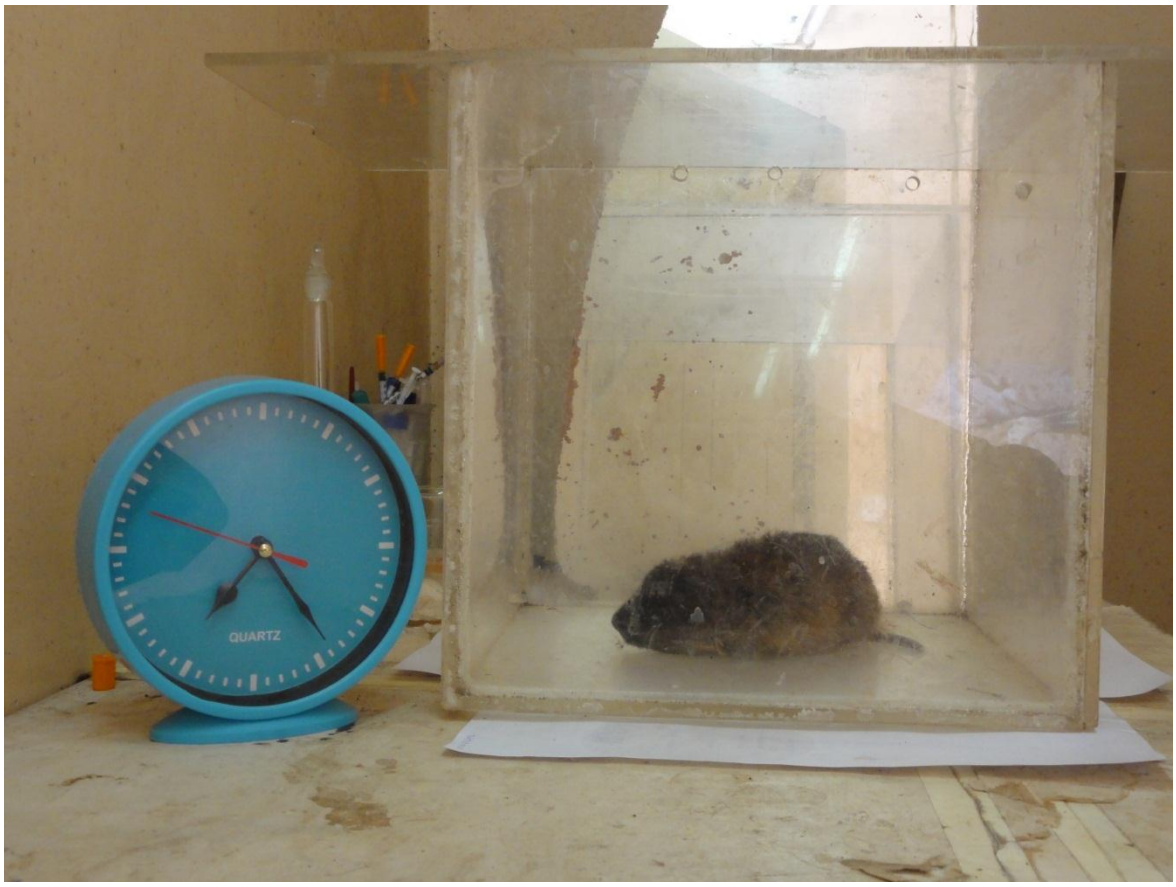

Plate 3: Observation of the pain behaviour with a mirror behind the Perspex observation chamber.

3.3 The Tail flick test

The tail-flick test was performed using an IITC model 33D analgesimeter (IITC Inc., Woodland Hills, CA, USA) with a sensitivity setting of 10 and beam at 8. Before the start of the experiments, the root rats were acclimated to the restrainer for 30 minutes per day for a period of 21 days. Before the experiments, the root rats were again acclimated to handling and the restrainer for thirty minutes. To establish the base line latencies, the rodent was placed gently in the restrainer and a radiant beam focused on the dorsal surface of the tail, at a mid-point to the tip (**Plate 4)**. In order to protect the tail from tissue damage, cut-off was initially set at 10 seconds and in the absence of a tail-flick, the cut-off time was extended to

20 seconds. The latency from the application of heat until the animal flicked its tail or reached the cut-off was recorded.

Plate 4: The East African root rat placed inside a makeshift restrainer during the tail-flick test.

3.4 Acetic acid writhing test

In the pilot studies, the acetic acid induced writhing test was carried out in the East African root rats using different concentrations and volumes of acetic acid, one of which was 50 µl of 8% acetic acid in 0.9% sodium chloride solution. The acid was administered intraperitoneally (**Plate 5**). The root rat was put into a clear Perspex glass chamber for observation of abdominal writhes for 30 minutes. Control experiments were carried out using saline, administered in the same way.

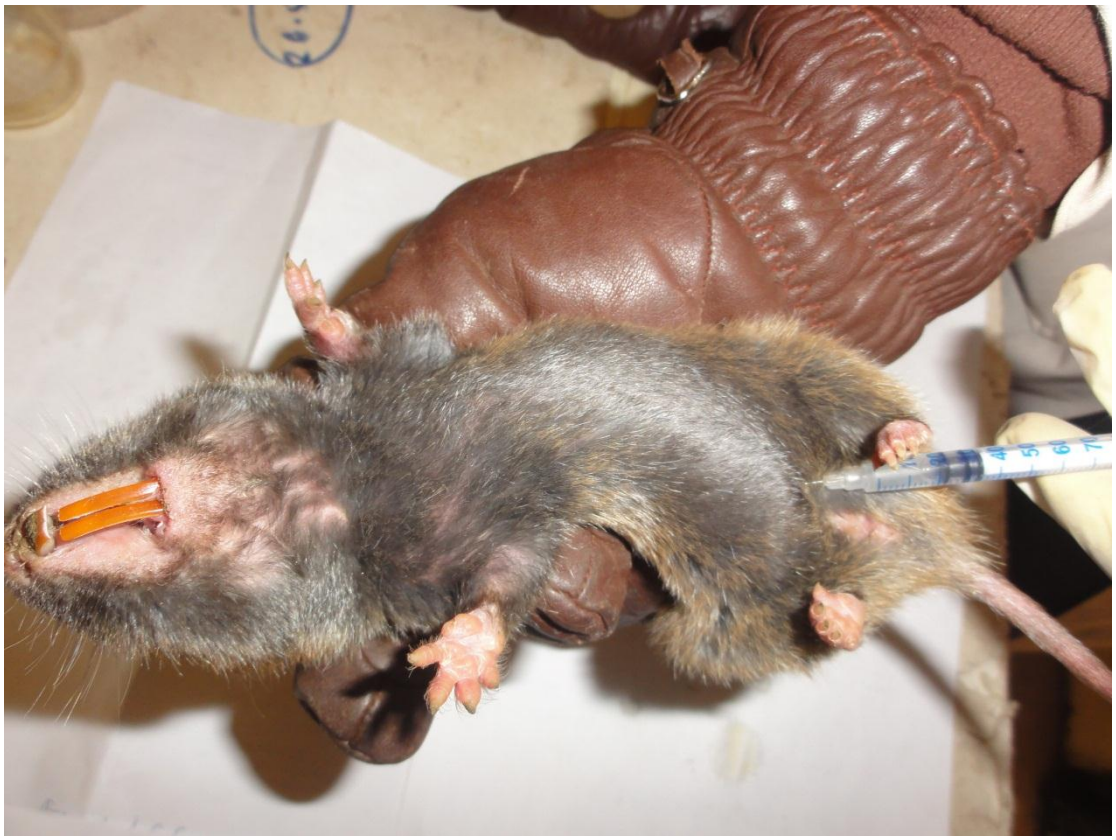

Plate 5: Intraperitoneal administration of acetic acid in the writhing test.

3.5 Drugs

Oxotremorine sesquifumarate salt, (±)-epibatidine dihydrochloride, morphine sulphate (Sigma-Aldrich, Stockholm, Sweden) naloxone, atropine sulphate salt and mecamylamine hydrochloride (Tocris Bioscience, Copenhagen, Denmark) were used in the study. All drugs were weighed and dissolved in 0.9% saline and stored as a stock solution at a temperature of 2-4°C. Fresh preparations were made daily. All the precautions regarding handling and stability of the drugs as recommended by the manufacturer were followed strictly.

3.6 Drug administration

All the drugs except formalin were injected intraperitoneally, 30 minutes prior to testing. The injections were given in a volume of 50 microlitres, using a U-100 insulin syringe with a 30-gauge needle. The doses were based on data from preliminary studies, as well as from doses used in related studies (Dulu *et al*., 2014). The mAChR agonist oxotremorine, was administered in doses of 20, 30 and 60 µg/kg body weight. Oxotremorine, 30 µg/kg body weight was also co-administered with the mAChR antagonist atropine (3 mg/kg body weight). The nAChR agonist epibatidine was administered in doses of 1, 3 and 10 µg/kg body weight. Epibatidine at 3 µg/kg body weight was also co-administrered with the nAChR antagonist mecamylamine (50 µg/kg body weight). Morphine (1, 3 and 6 mg/kg dose levels) was administerd. Morphine (3 mg/kg) was also co-administered with naloxone (2.5 mg/kg). Control animals were injected with 50µl, 0.9% physiological saline. Each treatment group had 6 animals and all treatments were administered in a randomized order. Co-administration of drugs was performed in accordance with previous studies, where the same chemicals were tested (Dulu *et al*., 2014).

3.7 Data analysis

In the formalin test, the mean of each 5 minute data for a group of root rats was calculated to give the mean time (seconds) spent licking/biting the injected paw. All values were expressed as mean ± standard error of mean (SEM). To determine significant differences among the experimental groups, the data was analyzed with SPSS version 12.0.1. The two tailed unpaired Student's test was used when comparing two experimental groups. The one-way analysis of variance (ANOVA) and a Tukey's post hoc test were also performed. P-value equals to or lower than 0.05 ($P \leq 0.05$) was considered significant.

In the evaluation of the analgesic median effective dose (MED_{50}) values, the Litchfield–Wilcoxon II method was employed (Litchfield and Wilcoxon, 1949). The analgesic data were expressed as a percentage of the maximum possible inhibition effect of a given drug.

The percentage inhibition was calculated as follows;

% Inhibition = (Post drug inhibition – Pre-drug inhibition)/ (Pre-drug inhibition) *100.

CHAPTER FOUR

4.0 RESULTS

4.1 The Formalin test

The intradermal injection of formalin (8%) elicited behavioural responses that included favouring, licking and biting of the injected paw. A mean licking/biting response of 80.1 ± 10.83 seconds was recorded in control animals and when this value was compared with that of saline injected animals (8.4 ± 0.98 seconds) was statistically significant ($P \leq 0.001$; **Fig. 2**) in the first block of 5 minutes (early phase). There was no statistically significant difference in pain behaviour in the rest of the intervals, commonly referred to as late phase, and for this reason the experiments were only restricted to the early phase.

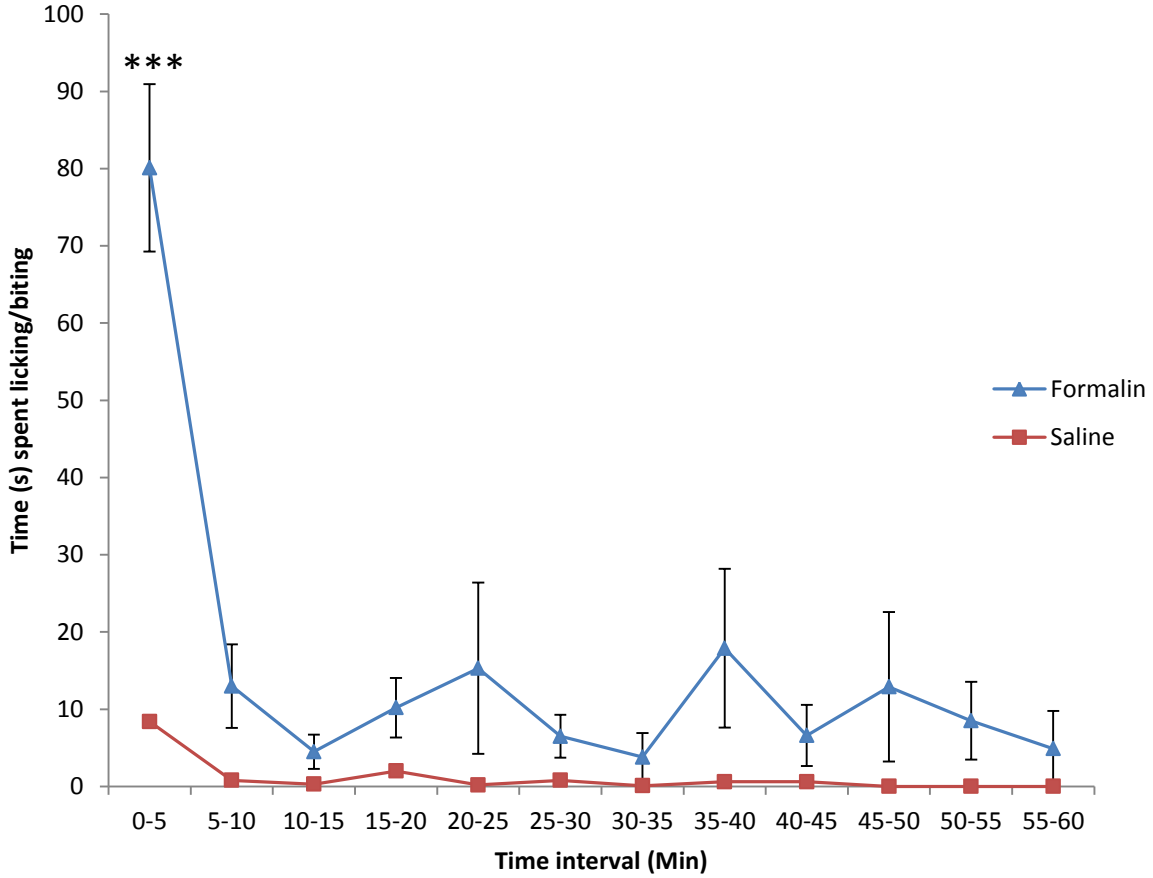

Figure 2: Time-course of pain behaviour after intradermal injection of 20 µl of 8% formalin or 20 µl of physiological saline into the dorsal right hind paw of *Tachyoryctes splendens*. Values are means ± S.E.M., n = 6 in each group. Treatment means were compared using Students t-test subsequent to ANOVA, the level of significance was set at $P \leq 0.05$ and *** denotes $P \leq 0.001$.

4.1.2 Effects of oxotremorine and atropine

The effects of oxotremorine on the mean licking/biting response in the formalin test were investigated. In the early phase, the mean times spent licking/biting the injected paw after intraperitoneal injection of 20, 30 and 60 µg/kg were 62.9 ± 14.54, 3.5 ± 0.45 and 4.7 ± 0.64 seconds respectively, while that for controls was 76.9 ± 3.22 seconds. Statistical evaluation of the data using ANOVA and the Tukey post hoc test showed that the effect of oxotremorine (30 or 60 µg/kg) was statistically significant ($P \leq 0.05$; **Fig. 3a**). There was also a statistically significant difference ($P \leq 0.05$) between oxotremorine (30 or 60 µg/kg) and oxotremorine (20µg/kg) in the early phase of the test.

The coadministration of oxotremorine (30 µg/kg) and atropine (3 mg/kg) caused a mean licking/biting time of 64.4 ± 6.87 seconds. On comparing this mean licking/biting time (64.4 ± 6.87 seconds) with that of oxotremorine (30 µg/kg) alone a statistically significant difference was noted ($P \leq 0.001$; **Fig. 3b**). There was no statistical difference ($P \geq 0.05$) between the saline, atropine and the oxotrmorine-atropine treated groups. The median effective dose (MED_{50}) of oxotremorine was approximately 21 µg/kg **(Fig. 3c).**

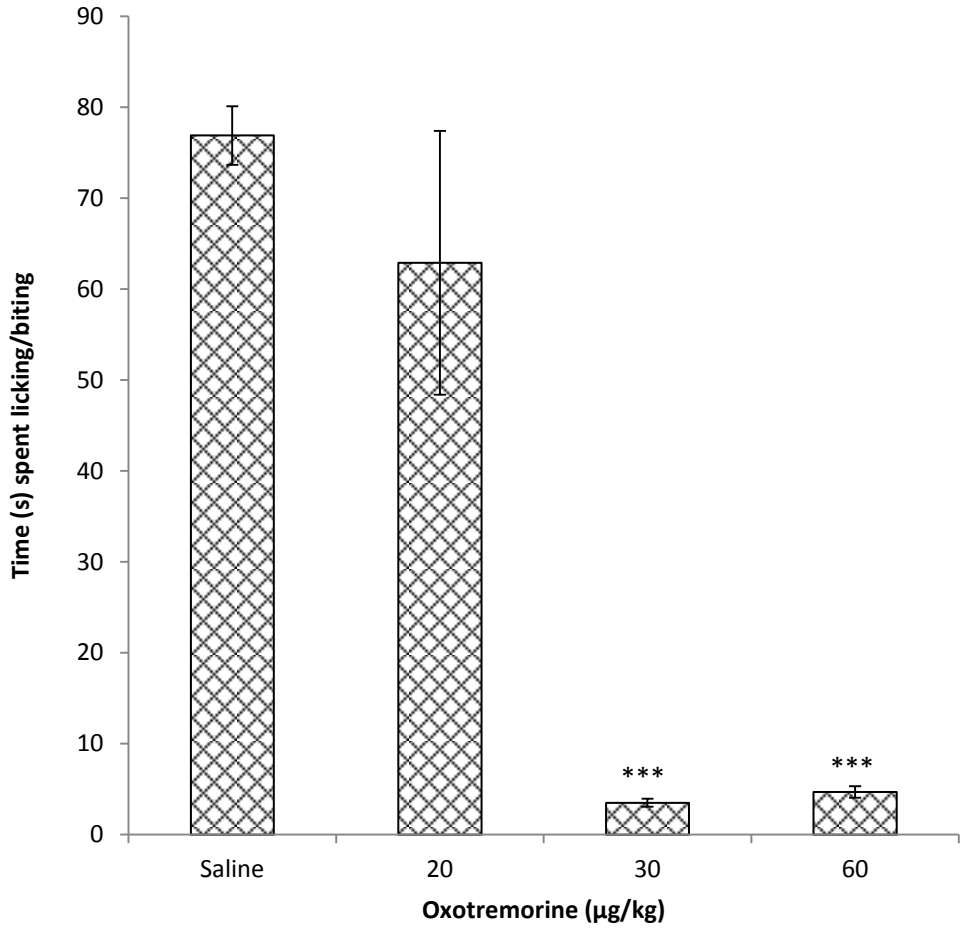

Figure 3a: Effects of intraperitoneal administration of saline or oxotremorine (20, 30 or 60 µg/kg) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. *** denotes $P \leq 0.001$.

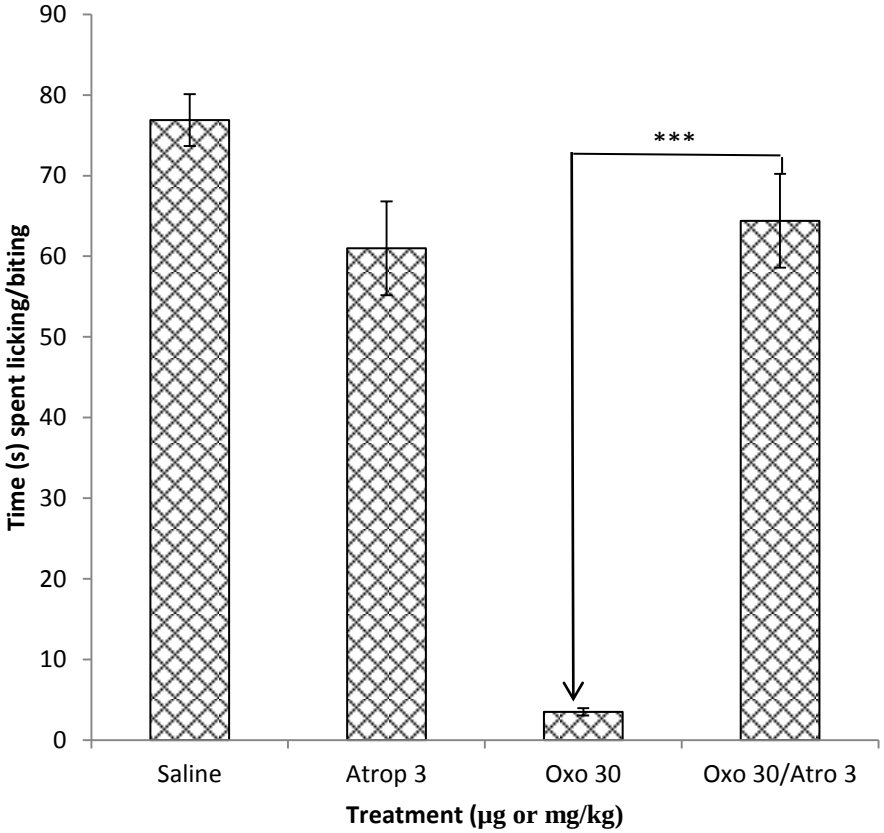

Figure 3b: Effects of intraperitoneal administration of saline, 3 mg/kg atropine (Atrop 3), 30 µg/kg oxotremorine (Oxo 30) or a combination of 30 µg/kg oxotremorine and 3mg/kg atropine (Oxo 30/Atro 3) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. *** indicates significant difference ($P \leq 0.001$) between the oxotremorine (30µg/kg) group and that given the combined treatment.

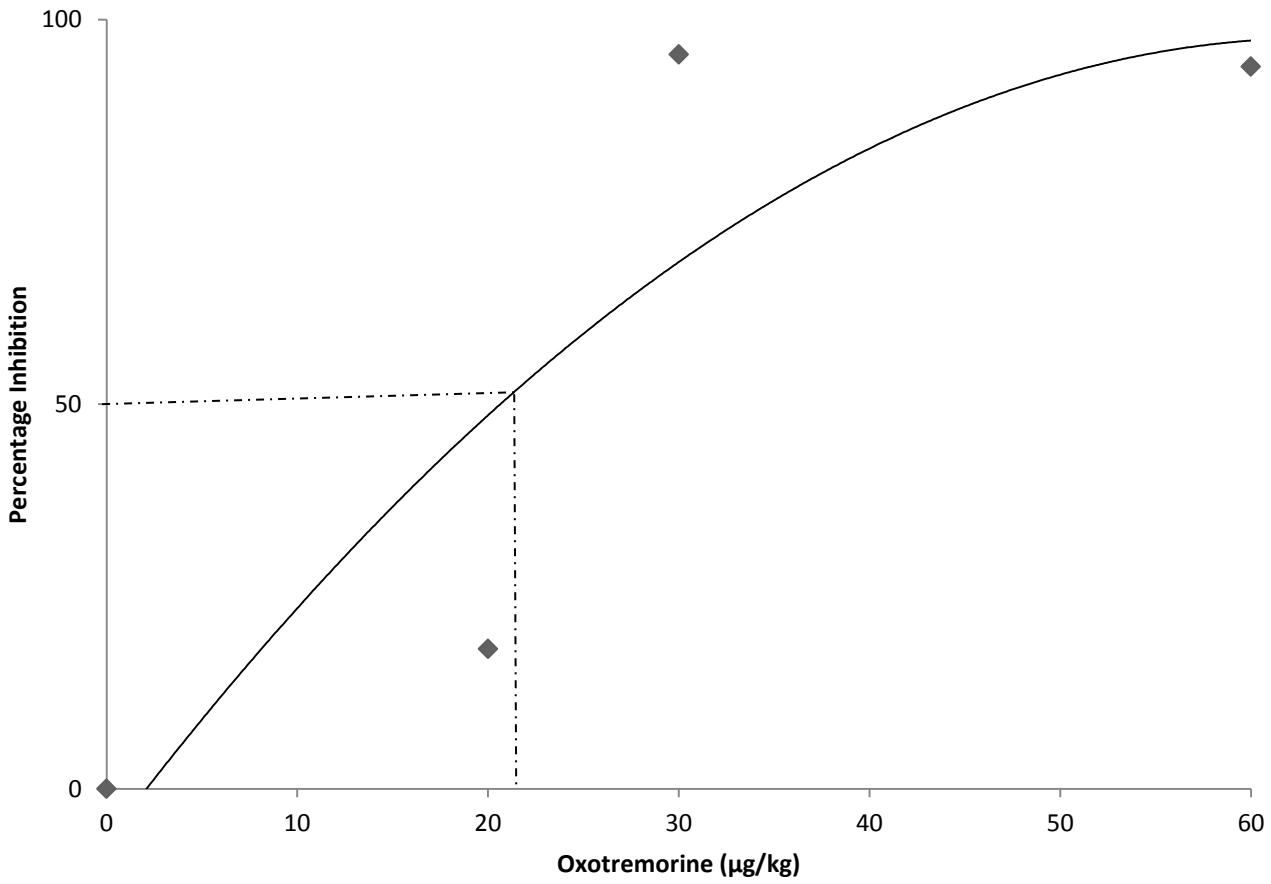

Figure 3c: The median effective dose (MED_{50}) response curve of oxotremorine following intraperitoneal administration in the formalin test in the *Tachyoryctes splendens*. Approximately 21 µg/kg of oxotremorine inhibited the licking/biting response by 50%.

4.1.3 Effects of epibatidine and mecamylamine

The effects of epibatidine on the mean licking/biting response in the formalin test were investigated. In the early phase, the mean times spent licking/biting the injected paw after intraperitoneal injection of 1, 3 and 10 µg/kg were 61 ± 6.76, 49 ± 8.19 and 5 ± 2.96 seconds resspectively, while that of controls was 76.9 ± 3.22 seconds. Statistical evaluation of the data using ANOVA and the Tukey's post hoc test showed that the effect of epibatidine (3 or 10 µg/kg) was statistically significant ($P \leq 0.05$; **Fig. 4a**). There was also a statistical significant difference between epibatidine 10µg/kg and epibatidine (1 or 3 µg/kg).

The coadministration of epibatidine (10 µg/kg) and mecamylamine (50 µg/kg) caused a mean licking/biting time of 57 ± 8.57 seconds. On comparing this mean licking/biting time with that of epibatidine (10 µg/kg), a statistically significant difference ($P \leq 0.001$; **Fig. 4b**) was noted. Multiple comparison test showed a statistically insignificant difference ($P \geq 0.05$) between saline- or mecamylamine- (mec 50) and the epibatidine–mecamylamine-treated groups. The MED_{50} of epibatidine was approximately 4.5 µg/kg **(Fig. 4c).**

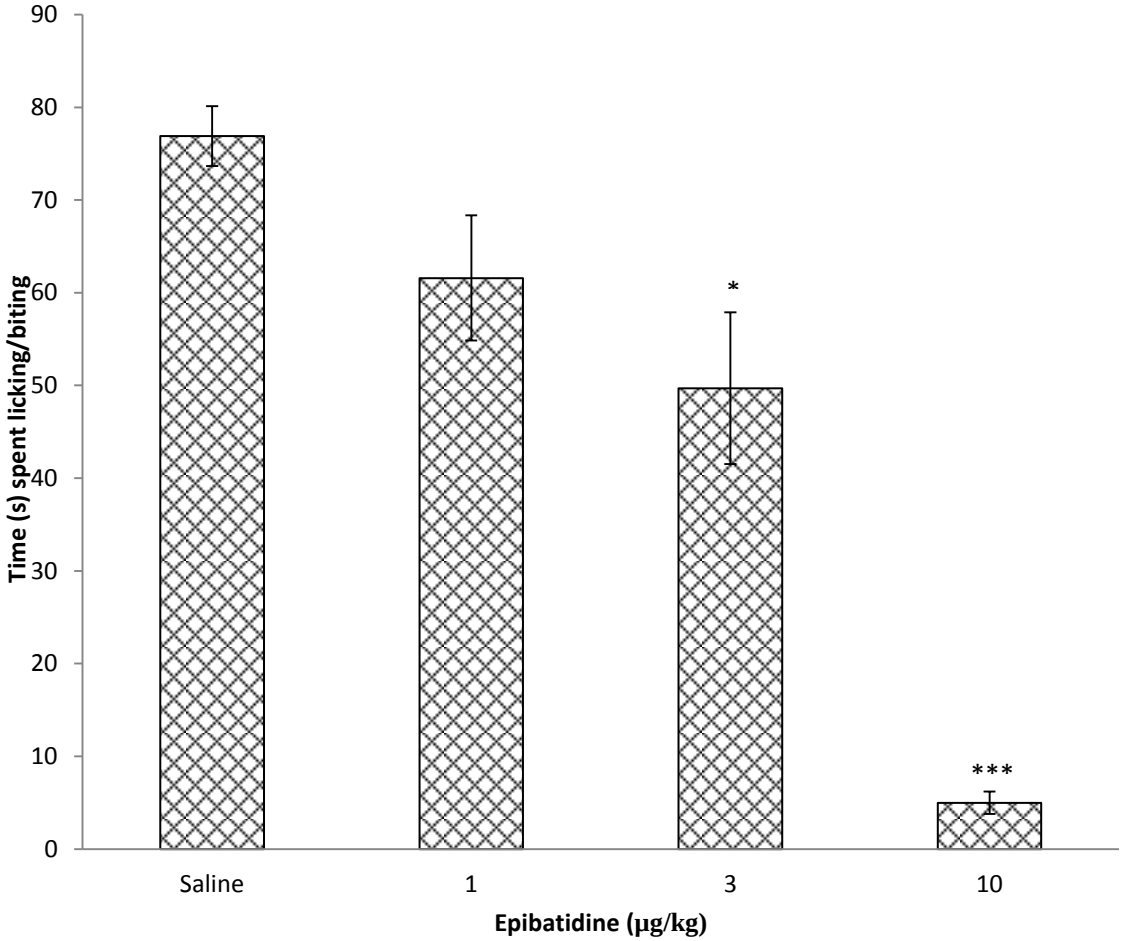

Figure 4a: Effects of intraperitoneal administration of saline, or epibatidine (1, 3 or 10 µg/kg) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. * and ***denote $P \leq 0.05$ and $P \leq 0.001$ respectively.

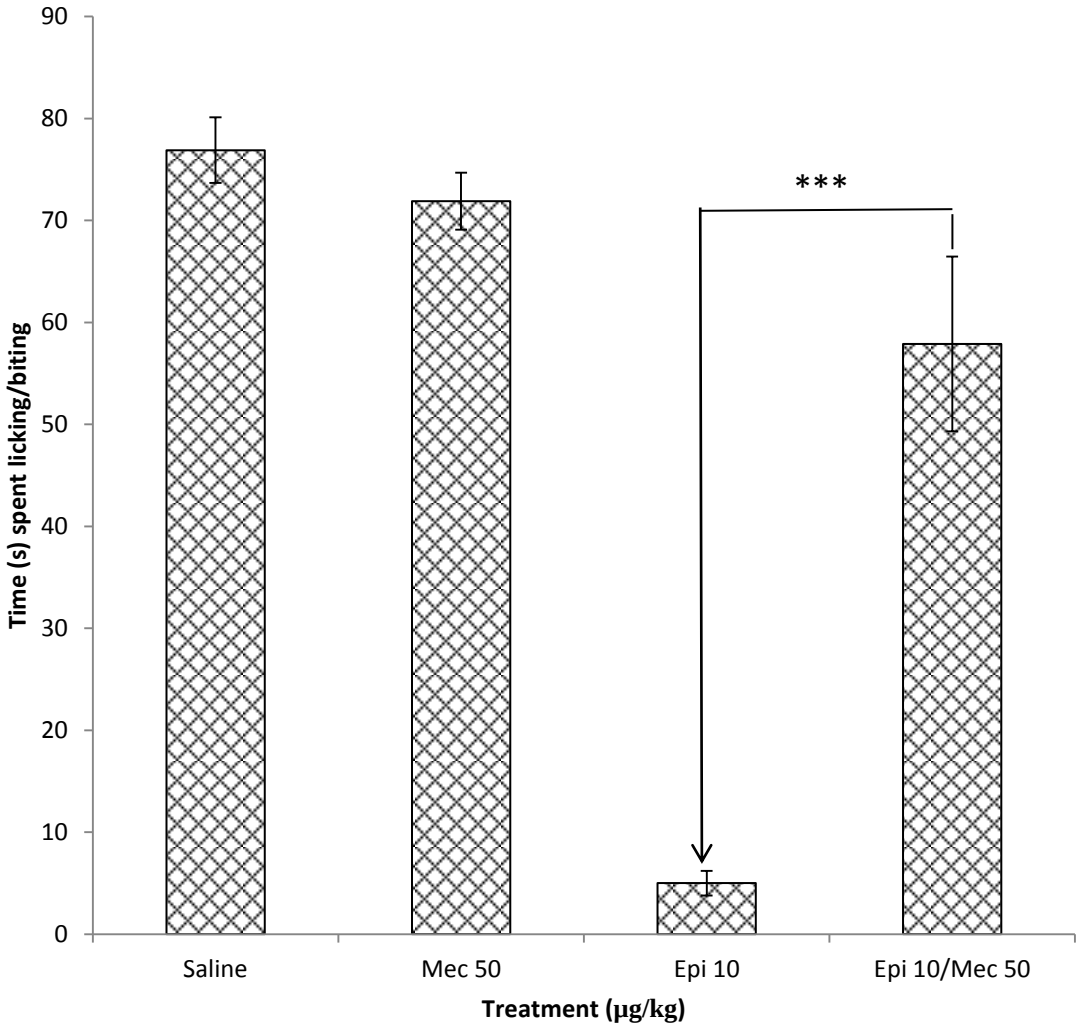

Figure 4b: Effects of intraperitoneal administration of saline, 50 µg/kg mecamylamine (Mec 50), 10 µg/kg epibatidine (Epi 10) or a combination of 10 µg/kg epibatidine and 50 µg/kg mecamylamine (Epi10/Mec 50) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. *** indicates significant difference ($P \leq 0.001$) between epibatidine (10µg/kg) and the group given the combined treatment.

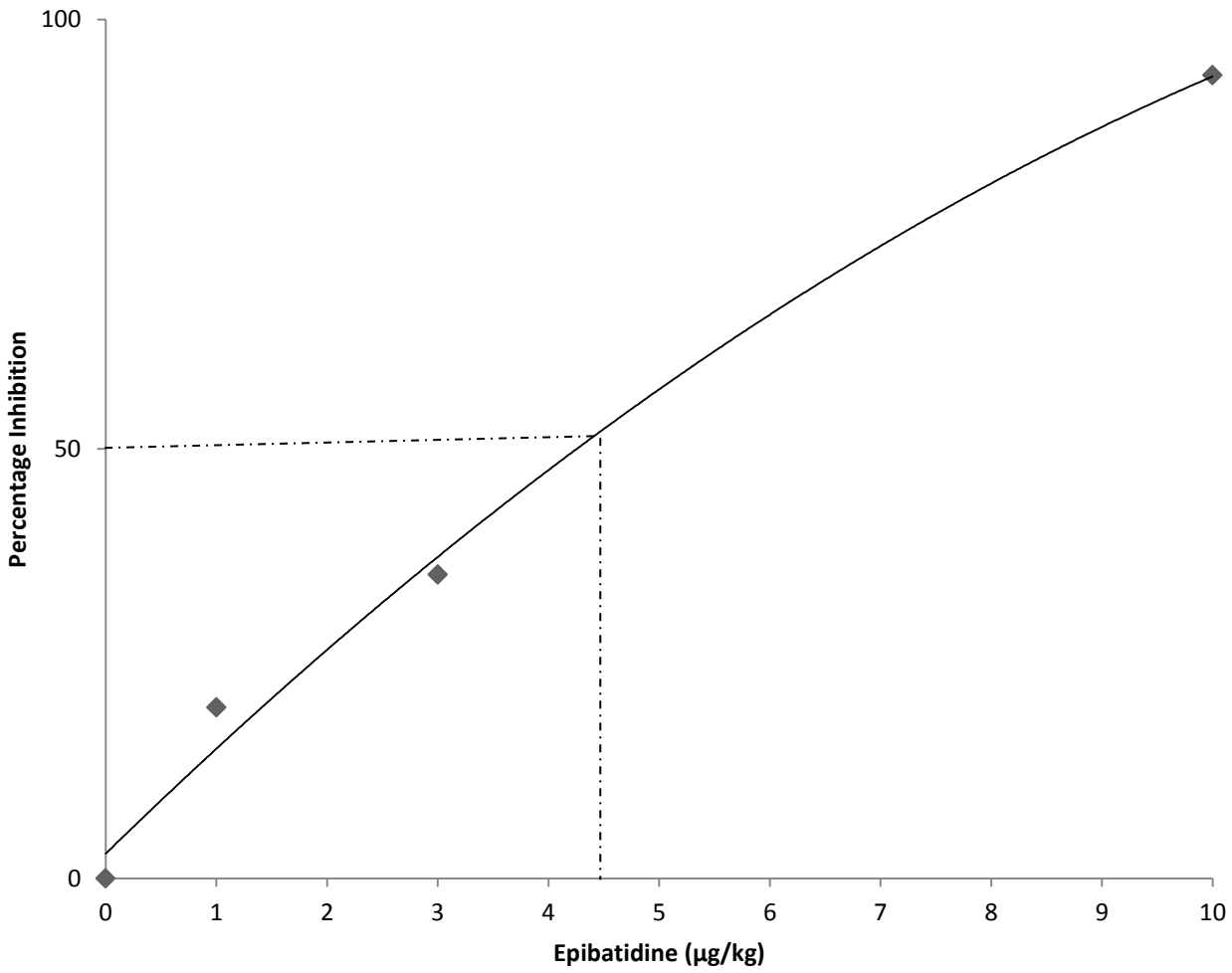

Figure 4c: The median effective dose (MED_{50}) response curve of epibatidine following intraperitoneal administration in the formalin test in the *Tachyoryctes splendens.* Approximately 4.5 µg/kg of epibatidine inhibited the licking/biting response by 50%.

4.1.4 Effect of morphine and naloxone

The effects of morphine (1, 3 and 6 mg/kg) on the mean licking/biting response in the formalin test were investigated. In the early phase, the mean times spent licking/biting the injected paw after intraperitoneal injection of 1, 3 and 6 µg/kg were 48 ± 7.16, 4.7 ± 1.03 and 3.7 ± 1.76 seconds resspectively, while that of controls was 76.9 ± 3.22 seconds. Statistical evaluation of the data using ANOVA and the Tukey's post hoc test showed that the effect of morphine (3 and 6 µg/kg) was statistically significant ($P \leq 0.05$; **Fig 5a**). There was a statistical significant difference ($P \leq 0.001$) between morphine (3 or 6 mg/kg) and morphine (1 mg/kg). There was no statistically significant difference ($P \geq 0.05$) between morphine 3 mg/kg and morphine 6 mg/kg.

The coadministration of morphine (3 mg/kg) and naloxone (2.5 mg/kg) caused a mean licking/biting time of 57 ± 7.91 seconds. On comparing this mean licking/biting time (57 ± 7.91 seconds) with that of morphine (3 mg/kg) alone, a statistically significant difference ($P \leq 0.001$ **Fig. 5b**) was noted. There was no statistical difference ($P \geq 0.05$) between the saline- or naloxone- and the morphine / naloxone-treated groups. The MED_{50} of morphine was approximately 1.4 mg/kg **(Fig. 5c).**

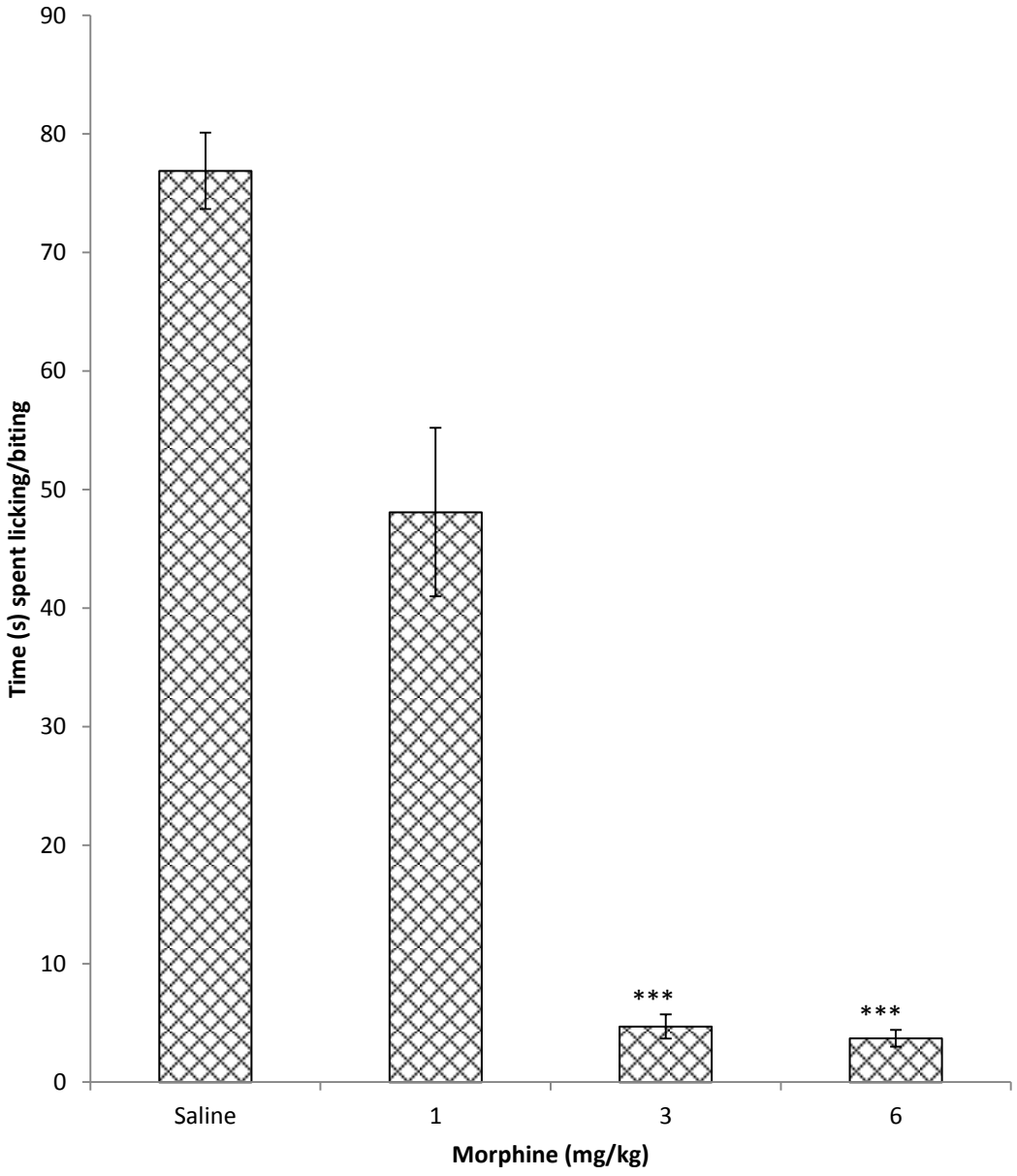

Figure 5a: Effects of intraperitoneal administration of saline or morphine (1, 3 or 6 mg/kg) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. *** denotes $P \leq 0.001$.

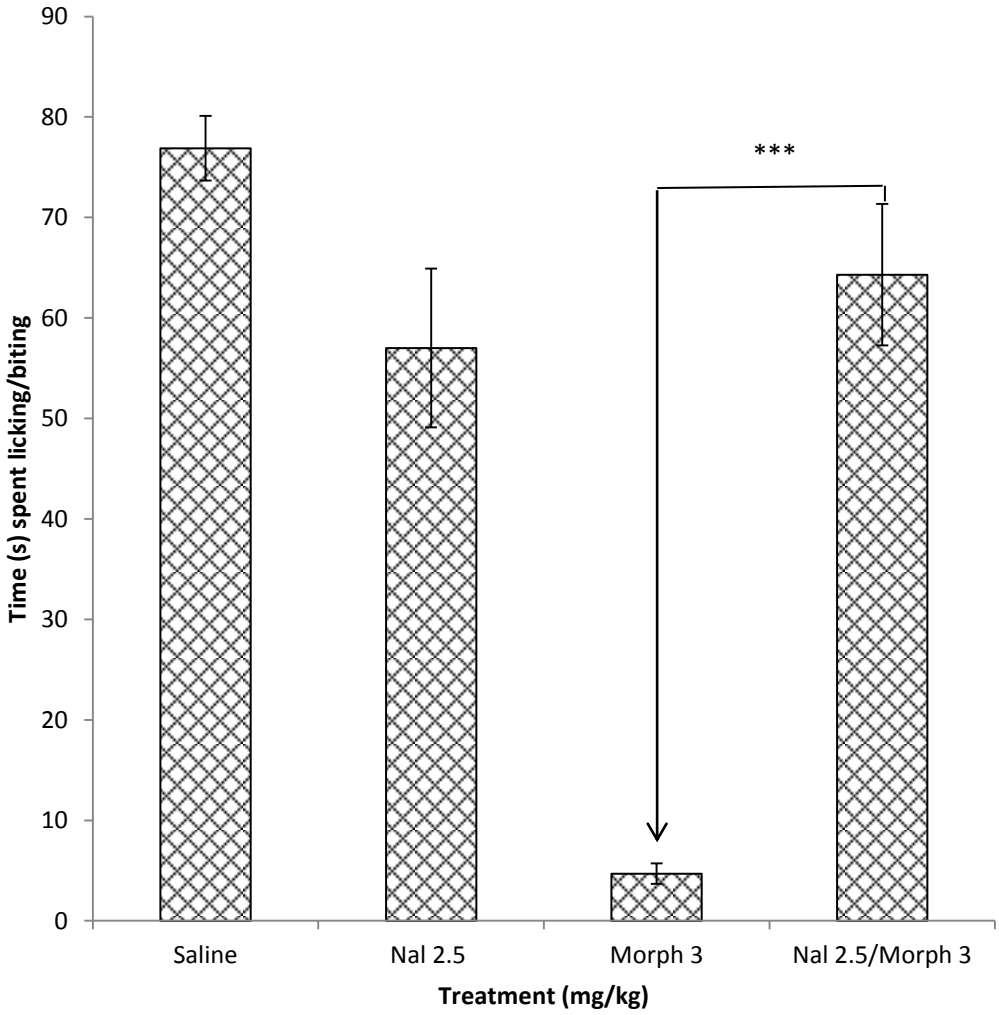

Figure 5b: Effects of intraperitoneal administration of saline, 2.5 mg/kg naloxone (Nal 2.5), 3 mg/kg morphine (Morph 3) or a combination of 2.5 mg/kg naloxone and 3 mg/kg morphine (Nal 2.5/Morph 3) on the mean licking/biting response in the formalin test (8%) in the *Tachyoryctes splendens*. Values are means ± S.E.M. and n = 6 in each group. Treatment means were compared using Tukey post hoc test subsequent to ANOVA and the level of significance was set at $P \leq 0.05$. *** denotes a significant difference ($P \leq 0.001$).

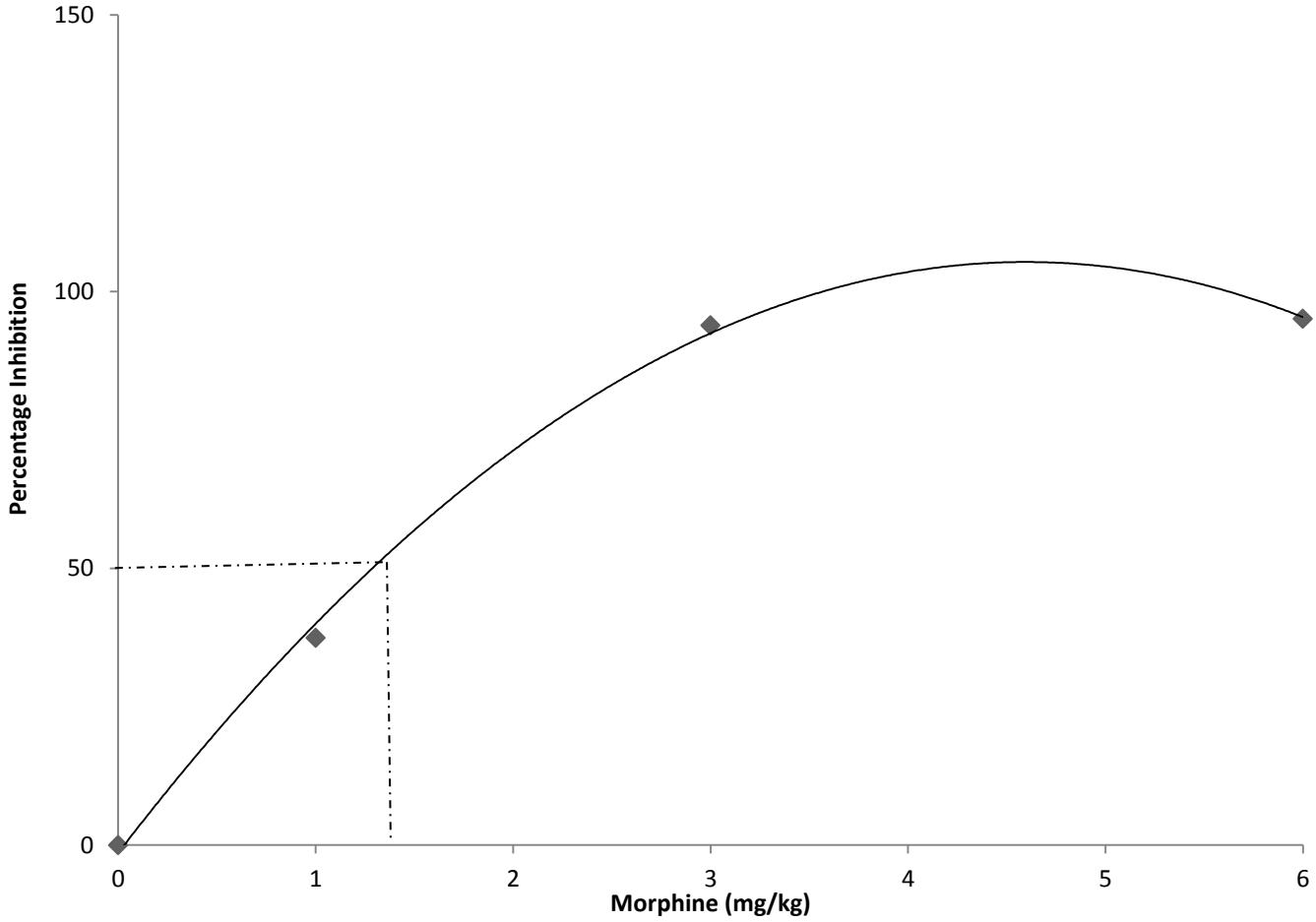

Figure 5c: The median effective dose (MED_{50}) response curve of morphine following intraperitoneal administration in the *Tachyoryctes splendens.* 1.4 mg/kg of morphine inhibited the licking/biting response by 50%.

4.2 The Tail flick test

All the animals tested did not respond within the 10 seconds cut off point on the tail flick analgesimeter and were assigned a score of zero. When the cut off point was increased to 20 seconds, the animals still did not respond to thermal pain and were also assigned a score of zero (**Table 1**).

4.3 Acetic acid writhing test

The animals tested using this nociceptive test remained calm with very little movement in the observation chamber. The typical "writhe" was not observed in the 30 minute observation period in these animals (**Table 2**). The grooming behaviour observed was similar to that observed in untested animals. Higher concentrations of acetic acid (10%) also caused no change in behaviour.

Sex	Weight (g)	Cut off time (s)	
		10	**20**
M	163.4	0	0
M	179.9	0	0
F	187.1	0	0
M	175.7	0	0
M	191.2	0	0
F	186.3	0	0

Table 1: The scores of the tail flick test during the cut off times of 10 and 20 seconds in the tail flick analgesimeter in the *Tachyoryctes splendens*.

Sex	Weight	0-5	5-10	10-15	15-20	20-25	25-30
F	169.9	0	0	0	0	0	0
F	172.8	0	0	0	0	0	0
M	201.3	0	0	0	0	0	0
F	181.1	0	0	0	0	0	0
F	171.7	0	0	0	0	0	0
M	169.2	0	0	0	0	0	0

Table 2: The number of writhes observed during the 5 minutes observation intervals for 30 minutes following intraperitoneal injection of 50 µl of 8% acetic acid in the *Tachyoryctes splendens*.

CHAPTER 5

5.0 DISCUSSION CONCLUSIONS AND RECOMMENDATIONS

5.1 The formalin test

The injection of formalin 8% caused licking/biting and favoring of the injected paw.The licking/biting of the hind paw was however the most common pain related behavior in this rodent. The concentration of formalin used in this study was based on the pilot studies whereby lower concentrations did not produce clearly quantifiable behaviour. This is in agreement with earlier reports that intensities of pain behaviors are dependent on the concentration of administered formalin (Rosland *et al*., 1990; Aloisi *et al*., 1995).

The formalin-induced behaviour was monophasic and lasted 5 minutes (early phase). This was very unique and contrary to what is reported in rats (Dubuisson and Dennis, 1977; Curzon *et al*., 1998; Capone *et al*., 1999), primates (Alreja *et al*., 1984) and mice (Hunskaar *et al*., 1985; Hunskaar and Hole, 1987) and in the naked mole rats (Kanui *et al*., 1993; Karim *et al*., 1993; Towett *et al*., 2009, Dulu *et al*., 2014) which display a characteristic biphasic behaviour with distinct early and late phases. The early phase response in the formalin test is attributed to direct peripheral nociceptor activation by formalin (Le Bars *et al*., 2001). Aδ-fibres are responsible for the early phase of the formalin test (Shibata *et al*., 1989), and being a vertebrate the *Tachyoryctes splendens* is not an exception.

The current data uniquely showed minimal, insignificant response to formalin-induced pain after 5 minutes. A similar observation has been reported in frogs (Oyadeyi *et al*., 2007) and in the speke's hinged tortoise (Wambugu *et al*., 2010; Dahlin *et al*., 2012). Some researchers have attributed the late tonic phase to central sensitization of spinal cord circuits secondary to the barrage of input that occurs during phase I (Dubuisson and Dennis, 1977; Coderre *et al*., 1990; Tjolsen *et al*., 1992; Vaccarino and Chorney, 1994). The late phase response is thought

to reflect an inflammatory reaction in the injected tissue (Hunskaar *et al*., 1986), signaled by peripheral mechanically insensitive afferents (MIAs) and C- fibres (Puig and Sorkin, 1995). The absence of the late phase in the *Tachyoryctes splendens* cannot be explained at the moment but a number of speculations can be made. It could be due to; the inflammatory phase coinciding with the acute phase, the inflammation not being sufficiently severe to cause pain, or that the inflammatory phase actually does not exist (Dahlin *et al*., 2012). It is also possible that the lack of nociceptive behaviours in the late phase could be due to a prolonged or potent inhibition at the spinal or supraspinal level in this species after the acute phase of nociceptor activation. Being an aggressive rodent, it is possible that the root rat pays more attention protecting itself than to the pain in the injected paw

In the present study, oxotremorine administered intraperitoneally, reduced the time spent licking/biting the injected paw in the early phase of the test in a dose-dependant manner. This finding is similar to what has been reported in Sprague-Dawley rats (Yaksh *et al*., 1985; Capone *et al*., 1999; Machelska *et al*., 1999; Abelson and Höglund, 2000; Abelson *et al*., 2004), mice (Wang *et al*., 2004) and in the naked mole rat (Dulu *et al.,* 2014). The intraperitoneal administration of atropine (3 mg/kg) alone did not have any significant effect on the time spent in pain behaviour in *Tachyoryctes splendens,* but on co-administration with oxotremorine it reversed its effects. Other researches have reported analgesia following administration of low doses of atropine (Ishii and Kurachi, 2006; Langmead *et al*., 2008) or increased pain sensitivity in both high and low doses of atropine in rats (Abelson and Höglund, 2002). This may be due to species-differences or difference in methodology.

Intraperitoneal administration of epibatidine caused a decrease in pain behaviour in the early phase of the formalin test in the *Tachyoryctes splendens*. The antinociceptive effect of epibatidine was dose-dependent and was reversed by mecamylamine. This agrees with previous reports in mice (Qian *et al*., 1993), rats (Curzon *et al*., 1998; Boyce *et al*., 2000;

Kommalage and Höglund, 2004), and in the naked mole rat (Dulu *et al*., 2014). The effect of mecamylamine on epibatidine induced antinociception suggests that the antinociceptive effect of the agonist was mediated by nicotinic acetylcholine receptors. Similar observation has been noted in rats (Badio and Daly, 1994) and in the naked mole rats (Dulu *et al*., 2014). This data further provides evidence of the existence of cholinergic system in this solitary subterranean rodent.

Intraperitoneal administration of morphine caused a decrease in the pain behavior induced by the formalin test in the *Tachyoryctes splendens*. This effect was dose-dependent and was reversed by naloxone. This agrees with previously published studies in mice (Rosland *et al.*, 1990), rats and cats (Dubuisson and Dennis, 1978) and in the naked mole rats (Kanui *et al.*, 1993). Administration of naloxone alone had no effects on formalin induced pain in this species, as also reported in the naked mole rat (Dulu *et al*., 2014). The effect of naloxone on morphine-induced analgesia suggests that the antinociceptive effect of morphine is mediated by μ-opioid receptors suggesting an involvement of the opioidergic system in the regulation of pain in the *Tachyoryctes splendens*. This is contrary to the findings in an earlier study (Towett and Kanui, 1995), where intraperitoneal administration of morphine increased pain sensitivity in the hot plate test. This suggests the existence of different mechanisms for thermal and chemical nociception in this rodent.

Lower doses of epibatidine compared to those of oxotremorine and morphine were effective in reducing the licking/biting behaviour in the East African root rat, while morphine was the least sensitive. This may be due to the pharmacological binding properties, or the distribution and concentration of the receptors of these drugs in the East African mole-rat which is an area that warrants further investigation.

5.2 Tail flick test

In this study, the tail flick test did not induce any observable nociceptive behaviour in this species. It was therefore not possible to establish baseline responses to determine the effect of the reference compounds. This finding is contrary to what has been observed in mice (Yael *et al*., 2000), rats (Hai-chun *et al*., 2001; Illes *et al*., 2006) and in the naked mole rats (Dulu *et al*., 2014). The lack of response by the root rat in the tail flick test was unexpected since many of the experimental animals subjected to this test, have shown the tail flick response.

The tail has been reported to be the most important thermoregulatory organ of the rat and the heat loss is regulated by an on–off regulation of blood flowing in the tail, which leads to rapid variations in skin temperature (Milne and Gamble 1989, Tjølsen and Hole, 1992). The temperature of the tail therefore acts as a confounder in this test since a decrease in tail temperature produces an increase in the tail flick response. When animals are lightly stressed and activated due to experimental procedures, a considerable increase in tail skin temperature is regularly observed (Tjølsen *et al*., 1989). Rats restrained in tubes for a short time may show a considerable increase in the temperature of the tail (Tjølsen and Hole, 1992), probably due to vasodilation. This vasodilation could have contributed to the lack of an observable nocifensive behaviour in the *Tachyoryctes splendens* indicating that despite it being a subterranean rodent, it may have a similar thermoregulatory mechanism as the terrestrial rat. The tail of the *Tachyoryctes splendens* just like the rat is a complex structure, the movement of which is effected by between 8 and 14 muscles (Brink and Pfaff, 1980), and the conical form of which could influence how much of it and what types of receptors are affected by thermal stimulation. It is possible that its peripheral nociceptors in the tail are not sensitive to the thermal stimulus and hence it would not respond to the noxious heat applied to its tail. Since this rodent has a poorly developed sight system, it is very likely that other senses may

be very well developed that even minimal disturbances such as restrainment during the test could cause no response to the heat stimulus applied. As the tail-flick test is considered to mainly reflect a spinal reflex, part of this mediation is suggested to occur directly or indirectly at the spinal cord level. However, it should be pointed out that the anatomical structure of descending mechanisms in the *Tachyoryctes splendens* is unknown; hence the spinal portion of the antinociception in this species needs further investigation.

5.3 Acetic acid-induced writhing test

In the current study, the acetic acid-induced writhing test did not produce any observable and quantifiable nociceptive behaviour in the *Tachyoryctes splendens*. Even with increased concentrations and volumes of the acetic acid, the typical writhes observed on other rodents, were absent in this species. Though it could not be established why, a possible reason for this would be that the visceral nociceptors in this rodent are not sensitive to acetic acid. Since this root rat is primitive in terms of physiology and anatomy as compared to the laboratory rodents, perhaps the visceral receptors that mediate visceral pain are poorly developed. A problem with the sensitivity of the method could also be possible since it has been reported that the number of writhes in the writhing test are subject to a great deal of variability and that the reliability of the response is problematic in that less than 8% of animals may demonstrate no evidence of any writhing response (Hendershot and Forsaith, 1959), There is need to investigate visceral pain mechanisms in this species.

5.4 CONCLUSIONS

It has to be noted however, that this was the first study to be conducted in the East African root rat, a primitive fossorial rodent. These results, therefore, indicate that the cholinergic and opioidergic receptor systems are essential mediators of antinociception in this species The present study demonstrates that the formalin test is a good nociceptive test for studying

behavioural responses to acute pain and also a useful method for evaluating the antinociceptive effects of various drugs in the *Tachyoryctes splendens*

The tail-flick test and the acetic acid-induced writhing test appeared ineffective in producing quantifible pain behaviour in the *Tachyoryctes splendens*. More research needs to be done to provide additional information on the latter two nociceptive tests in this species. Further studies are also needed to establish the presence and distribution of muscarinic, nicotinic and opioid receptors, as well as the pharmacological binding properties of various ligands to these receptors so as to elucidate their involvement in antinociception in this species.

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APPENDICES

Appendix 1: Independent samples T-test for the formalin vs saline injected root rats

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Time in seconds	Equal variances assumed	4.738	.055	6.594	10	.000	71.70000	10.87391	47.47141	95.92859
	Equal variances not assumed			6.594	5.082	.001	71.70000	10.87391	43.88258	99.51742

Appendix 2: Post hoc analysis of oxotremorine treated East African root rats

Multiple Comparisons

Dependent Variable: Time in seconds
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	20micrograms/kg Oxo	14.00000	9.88012	.623	-15.0166	43.0166
	30micrograms/kg Oxo	73.40000*	9.88012	.000	44.3834	102.4166
	60micrograms/kg Oxo	72.20000*	9.88012	.000	43.1834	101.2166
	30micrograms + 3mg/kg Atropine	59.80000*	9.88012	.000	30.7834	88.8166
20micrograms/kg Oxo	Control	-14.00000	9.88012	.623	-43.0166	15.0166
	30micrograms/kg Oxo	59.40000*	9.88012	.000	30.3834	88.4166
	60micrograms/kg Oxo	58.20000*	9.88012	.000	29.1834	87.2166
	30micrograms + 3mg/kg Atropine	45.80000*	9.88012	.001	16.7834	74.8166
30micrograms/kg Oxo	Control	-73.40000*	9.88012	.000	-102.4166	-44.3834
	20micrograms/kg Oxo	-59.40000*	9.88012	.000	-88.4166	-30.3834
	60micrograms/kg Oxo	-1.20000	9.88012	1.000	-30.2166	27.8166
	30micrograms + 3mg/kg Atropine	-13.60000	9.88012	.648	-42.6166	15.4166
60micrograms/kg Oxo	Control	-72.20000*	9.88012	.000	-101.2166	-43.1834
	20micrograms/kg Oxo	-58.20000*	9.88012	.000	-87.2166	-29.1834
	30micrograms/kg Oxo	1.20000	9.88012	1.000	-27.8166	30.2166
	30micrograms + 3mg/kg Atropine	-12.40000	9.88012	.720	-41.4166	16.6166
30micrograms + 3mg/kg Atropine	Control	-59.80000*	9.88012	.000	-88.8166	-30.7834
	20micrograms/kg Oxo	-45.80000*	9.88012	.001	-74.8166	-16.7834
	30micrograms/kg Oxo	13.60000	9.88012	.648	-15.4166	42.6166
	60micrograms/kg Oxo	12.40000	9.88012	.720	-16.6166	41.4166

*. The mean difference is significant at the 0.05 level.

Appendix 3: Post hoc analysis of the epibatidine treated East African root rats

Multiple Comparisons

Dependent Variable: Time in seconds
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	1microgram/kg epi	15.30000	8.90047	.441	-10.8395	41.4395
	3microgram/kg epi	27.20000*	8.90047	.039	1.0605	53.3395
	10microgram/kg epi	71.90000*	8.90047	.000	45.7605	98.0395
	3mg/kg epi & meca	19.00000	8.90047	.237	-7.1395	45.1395
1microgram/kg epi	Control	-15.30000	8.90047	.441	-41.4395	10.8395
	3microgram/kg epi	11.90000	8.90047	.672	-14.2395	38.0395
	10microgram/kg epi	56.60000*	8.90047	.000	30.4605	82.7395
	3mg/kg epi & meca	3.70000	8.90047	.993	-22.4395	29.8395
3microgram/kg epi	Control	-27.20000*	8.90047	.039	-53.3395	-1.0605
	1microgram/kg epi	-11.90000	8.90047	.672	-38.0395	14.2395
	10microgram/kg epi	44.70000*	8.90047	.000	18.5605	70.8395
	3mg/kg epi & meca	-8.20000	8.90047	.886	-34.3395	17.9395
10microgram/kg epi	Control	-71.90000*	8.90047	.000	-98.0395	-45.7605
	1microgram/kg epi	-56.60000*	8.90047	.000	-82.7395	-30.4605
	3microgram/kg epi	-44.70000*	8.90047	.000	-70.8395	-18.5605
	3mg/kg epi & meca	-52.90000*	8.90047	.000	-79.0395	-26.7605
3mg/kg epi & meca	Control	-19.00000	8.90047	.237	-45.1395	7.1395
	1microgram/kg epi	-3.70000	8.90047	.993	-29.8395	22.4395
	3microgram/kg epi	8.20000	8.90047	.886	-17.9395	34.3395
	10microgram/kg epi	52.90000*	8.90047	.000	26.7605	79.0395

*. The mean difference is significant at the 0.05 level.

Appendix 4: Post-hoc analysis for morphine treated East African root rats

Multiple Comparisons

Dependent Variable: Time_in_sec
Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
controls	1mg/kg morph	28.80000*	7.62946	.008	5.5943	52.0057
	3mg/kg morph	72.20000*	7.62946	.000	48.9943	95.4057
	6mg/kg morph	73.20000*	7.62946	.000	49.9943	96.4057
	3mg/kg morph + 2.5 mg/kg nal	19.90000	7.62946	.126	-3.3057	43.1057
	2.5mg/kg nal	12.60000	7.62946	.573	-10.6057	35.8057
1mg/kg morph	controls	-28.80000*	7.62946	.008	-52.0057	-5.5943
	3mg/kg morph	43.40000*	7.62946	.000	20.1943	66.6057
	6mg/kg morph	44.40000*	7.62946	.000	21.1943	67.6057
	3mg/kg morph + 2.5 mg/kg nal	-8.90000	7.62946	.849	-32.1057	14.3057
	2.5mg/kg nal	-16.20000	7.62946	.303	-39.4057	7.0057
3mg/kg morph	controls	-72.20000*	7.62946	.000	-95.4057	-48.9943
	1mg/kg morph	-43.40000*	7.62946	.000	-66.6057	-20.1943
	6mg/kg morph	1.00000	7.62946	1.000	-22.2057	24.2057
	3mg/kg morph + 2.5 mg/kg nal	-52.30000*	7.62946	.000	-75.5057	-29.0943
	2.5mg/kg nal	-59.60000*	7.62946	.000	-82.8057	-36.3943
6mg/kg morph	controls	-73.20000*	7.62946	.000	-96.4057	-49.9943
	1mg/kg morph	-44.40000*	7.62946	.000	-67.6057	-21.1943
	3mg/kg morph	-1.00000	7.62946	1.000	-24.2057	22.2057
	3mg/kg morph + 2.5 mg/kg nal	-53.30000*	7.62946	.000	-76.5057	-30.0943
	2.5mg/kg nal	-60.60000*	7.62946	.000	-83.8057	-37.3943
3mg/kg morph + 2.5 mg/kg nal	controls	-19.90000	7.62946	.126	-43.1057	3.3057
	1mg/kg morph	8.90000	7.62946	.849	-14.3057	32.1057
	3mg/kg morph	52.30000*	7.62946	.000	29.0943	75.5057
	6mg/kg morph	53.30000*	7.62946	.000	30.0943	76.5057
	2.5mg/kg nal	-7.30000	7.62946	.928	-30.5057	15.9057
2.5mg/kg nal	controls	-12.60000	7.62946	.573	-35.8057	10.6057
	1mg/kg morph	16.20000	7.62946	.303	-7.0057	39.4057
	3mg/kg morph	59.60000*	7.62946	.000	36.3943	82.8057
	6mg/kg morph	60.60000*	7.62946	.000	37.3943	83.8057
	3mg/kg morph + 2.5 mg/kg nal	7.30000	7.62946	.928	-15.9057	30.5057

*. The mean difference is significant at the 0.05 level.