AN INVESTIGATION ON MELATONIN ADMINISTRATION IN PAINFUL DIABETIC NEUROPATHY: CONTRIBUTION OF CATION CHANNELS AND ADRENERGIC RELATED EFFECTS

Doctoral Thesis

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Eskişehir 2023

AN INVESTIGATION ON MELATONIN ADMINISTRATION IN PAINFUL DIABETIC NEUROPATHY: CONTRIBUTION OF CATION CHANNELS AND ADRENERGIC RELATED EFFECTS

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Thesis submitted for the degree of
Doctor of Philosophy
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Eskişehir
Anadolu University
Graduate School of Health Sciences
January 2023

FINAL APPROVAL FOR THESIS

This thesis titled “An Investigation on Melatonin Administration In Painful Diabetic Neuropathy: Contribution of Cation Channels and Adrenergic Related Effects” has been prepared and submitted by Ilhem DALLALI in partial fulfillment of the requirements in “Anadolu University Directive on Graduate Education and Examination” for the Degree of Doctor of Philosophy (PhD)/Proficiency in Pharmacology Department has been examined and approved on 03/01/2023.

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ÖZET

AĞRILI DİYABETİK NÖROPATİDE MELATONİN UYGULAMASI ÜZERİNE BİR ARAŞTIRMA: KATYON KANALLARININ KATKISI VE ADRENERJİK İLİŞKİLİ ETKİLER

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Anadolu Üniversitesi, Sağlık Bilimleri Enstitüsü, Ocak 2023

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Diyabetik nöropatik ağrı, henüz radikal tedavisi bulunamamış oldukça karmaşık bir sendromdur ve hastanın yaşam kalitesini belirgin şekilde etkiler. Melatonin, geniş bir etki yelpazesi ile bu alanda gelecek vaat eden bir ilaçtır. Bu çalışmada, melatoninin diyabetik nöropatik ağrı üzerindeki etkileri ve etki mekanizması in vivo davranışsal ve in vitro elektrofizyolojik deneylerle incelenmiştir. STZ kaynaklı diyabetik modelde, 14 gün boyunca 50 mg/kg melatonin subakut uygulaması, lokomotor aktiviteyi bozmaksızın sırasıyla Hargreave ve e-Von Frey testlerinde diyabet kaynaklı hiperaljezi ve allodini tepkilerini referans ilaç gabapentin ile karşılaştırılabilir düzeyde azaltmıştır. Melatoninin antihiperaljezik ve antiallodinik etkilerinin α1, α2 ve β-adrenoseptör antagonist uygulamaları ile tersine çevrildiği bulguları, bu etkilerin noradrenerjik sistemin katılımıyla gerçekleştiğine işaret etmiştir. Ayrıca, melatoninin K^+ akımları ve aksiyon potansiyeli ile ilgili parametreler üzerindeki etkilerinin in vitro olarak patch clamp deneyleri ile analiz edilmesiyle, melatoninin K^+ kanallarını açmakla birlikte I_A akımını arttırdığı ve böylelikle nöronal aktivite ve uyarılabilirliğini azalttığı tespit edilmiştir. Sonuç olarak melatonin, antihiperaljezik ve antiallodinik etkilerini nöropatik ağrının giderilmesinde temel bir yolak olan K^+ kanal ve noradrenerjik sistem eksenini kullanarak göstermiştir. Dolayısıyla, melatoninin diyabetik nöropatik ağrı tedavisinde güvenli ve etkin bir şekilde kullanılabileceği ve klinikte umut verici bir yaklaşım sağlayabileceği düşünülmektedir.

Anahtar Sözcükler: Melatonin, Diyabetik nöropatik ağrı, Dorsal kök ganglionu, K^+ kanalları, Patch clamp.

ABSTRACT

AN INVESTIGATION ON MELATONIN ADMINISTRATION IN PAINFUL DIABETIC NEUROPATHY: CONTRIBUTION OF CATION CHANNELS AND ADRENERGIC RELATED EFFECTS

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Anadolu University, Graduate School of Health Sciences, January 2023

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Diabetic neuropathic pain is a highly complex syndrome whose radical treatment has not been discovered yet and it affects quality of life of the patient evidently. Melatonin is a promising drug in scope of this area with a wide spectrum of effects. In this study, the effects and mechanism of action of melatonin on diabetic neuropathic pain have been investigated through in vivo behavioral and in vitro electrophysiological experiments. In the STZ-induced diabetic model, subacute administration of 50 mg/kg, melatonin for 14 days reduced diabetes-induced hyperalgesia and allodynia responses comparable to the reference drug gabapentin by regulating locomotor activity using the Hargreave's and e-Von Frey tests, respectively. The antihyperalgesic and antiallodynic effects of melatonin were reversed with α1, α2 and β-adrenoceptor antagonist administrations findings pointed out that these effects were mediated by the participation of noradrenergic system. On the other hand, through in vitro analyzing effects of melatonin on parameters related to action potential, together with its effects on K^+ currents, performed via patch clamp experiments, that exerts a reducing effect on neuronal activity and excitability via opening of K^+ channels by increasing the amplitude of I_A current, which is one of the systems that play a primary role in pain relief. In conclusion, melatonin shows its antihyperalgesic and antiallodynic effects by using the K^+ channel and the noradrenergic system axis. Thus, melatonin provides a promising clinical approach of diabetic neuropathic pain with safety and efficiently.

Keywords: Melatonin, Diabetic neuropathic pain, Dorsal root ganglion, K^+ channels, Patch clamp.

ACKNOWLEDGEMENTS

January 2023

I would like to express my gratitude to Prof. Dr. Yusuf ÖZTÜRK for being my mentor and leading my journey in my PHD studies, for the time he devoted to providing me with the tools essential to the conduct of this research and for giving me the opportunity to be a part of the first team that worked in the electrophysiology laboratory which is the first one among pharmacology departments of pharmacy faculties in Turkey.

I am extremely thankful to Doç. Dr. Nurcan BEKTAŞ TÜRKMEN for being there at the entire thesis process, offering invaluable assistance, the priceless advices and insights, guidance and limitless help she offered, to let me surpass an important part of the difficulties that I faced in through this years.

My sincere thanks go to Dr. Feyza ALYU ALTINOK, for all the valuable contributions, the invaluable assistance, to the support she offered in the hard days.

To Prof. Dr. Rana ARSLAN for her limitless guidance and help that she offered, for all the important research work that included me in, for which I am greatly thankful.

I express my limitless thanks to my lab colleagues Ahmed and Raouf for everything they did to help to accomplish this work and all the thing we have been through together.

Most importantly, I am grateful for my family's unconditional, unequivocal support. To my father, the most person that encouraged me to get to this stage, his endless love, and prayers gave me strength at all moments. I express my deepest thanks to my brother and sisters who keep me grounded, remind me of what is important in life, and is always supportive of my adventures.

This thesis dedicated to the soul of My Mother, who I think is proud of her daughter, who is obtaining the highest academic degree. I wish that you were here.

Last but not least, I would like to express appreciation and gratitude to my priceless friends Adham, Alaa and Zeineb. Your love, support and patience during my thesis was very much appreciated. Thank you for your presence in my life and for all the good and difficult days we lived together.

I would like to acknowledge Yurtdışı Türkler ve Akraba Topluluklar Başkanlığı 'YTB' for providing me with doctorate Türkiye Scholarship, I would also like to thank the financial supporter of this research, Scientific Research Projects 'BAP', Anadolu University, Project no:2203S022.

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

Here I declare that this thesis carried out by myself is an original work; all the processes including preparation, data collecting, data analysis and presentation of the observed results and regarding information has been done in compliance with ethical rules and standards; all the literature utilized for the discussion of the results obtained within this work have been specified in references section this document to be evaluated through scientific plagiarism detection program used by Anadolu University and no plagiarism detected. I notify that if any inconsistency appears concerning my work, I consent all moral and legal consequences**.**

Ilhem DALLALI

03.01.2023

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LIST OF SYMBOLS AND ABBREVIATIONS

4-AP : 4-AminoPyridine

4P-PDOT : 4-Phenyl-2-PropionamiDotetralin

5-HT : Serotonin

ADP : After Depolarization

AHP : After Hyper Polarization

AP : Action Potential

ATP : Adenosine Triphosphate

Aβ-LTMs : Low-Threshold Mechanoreceptors

BBZDR/Wor : Inbred Bio-Breeding Zucker Diabetic Rat

CaV : Voltage-gated Ca^{2+} channels

cGMP : Cyclic guanosine monophosphate

Cm : Membrane Capacitance

CNS : Central Nervous System

DM : Diabetes Mellitus

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : Dimethyl Sulfoxide

DN : Diabetic Neuropathy

DNP : Diabetic Neuropathic Pain

DRG : Dorsal Root Ganglia

EGTA : Ethylene Glycol TetraAcetic Acid

F : Faraday Constant

FBS : Fetal Bovine Serum

G : Conductance

GΩ : Gigaohm

GABA : Gamma-Aminobutyric Acid

GBP : Gabapentin

gf : gram force

GLUT2 : Glucose transporter 2

HCN : Hyperpolarization-activated cyclic nucleotide-gated cation channels

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HVACC : High-Voltage Activated Calcium Channels

I_A : Transient fast-inactivating K^+ current type A

IASP : International Association for the Study of Pain

I_D : Transient slowly inactivating K^+ current

I_h : Hyperpolarization- activated inward current K^+ current

I_K : Sustained delayed rectifying K^+ current

I_M : Non-inactivating K^+ current Type M

IPA : Integrated Patch Amplifier

i.p. : Intraperitoneal

IR : Immunoreactivity

i.v. : Intravenous

I-V : Current-Voltage

K^+ : Potassium Channel

K2p : Two-pore domain leak channels

K-ATP : ATP-sensitive Potassium Channel

KCa : Calcium-activated K^+ Channel

KCNQ : Potassium Voltage-Gated Channel Subfamily Q

KIR6.2 : Inward-Rectifier Potassium Ion Channel

Kv : Voltage-Gated K^+ Channel

LANSS : Leeds Assessment of Neuropathic Symptoms

MΩ : Megaohm

MAPK : Mitogen-Activated Protein Kinase

%MPE : Maximum possible effect

mRNA : Messenger RNA

MT : Melatonin

NADPH : Nicotinamide adenine dinucleotide phosphate

NaV : Voltage-Gated Na^+ Channels

NF-Kβ : Nuclear Factor-Kappa B

NGF : Nerve growth factor

NMDA : N-methyl-D-aspartate

NO : Nitric Oxide

ns : Not Significant

p : p-value

pA : Pico Ampere

PAG : Periaqueductal gray

PAI1 : Plasminogen Activator Inhibitor-1

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

PNS : Peripheral Nervous System

PNP : Peripheral Neuropathic Pain

PROP : Propranolol

PZ : Prazosin

Rm : Membrane Resistance

RMP : Resting Membrane Potential

ROS : Reactive Oxygen Species

RVM : Rostral Ventromedial Medulla

SA : Spontaneous Activity

SCI : Spinal Cord Injury

SD : Sprague-Dawley

S.E.M. : Standard Error of the Mean

siRNA : Small Interfering RNA

SNL : Spinal Nerve Ligation

SNRIs : Serotonin and Noradrenaline Reuptake Inhibitors

SSRIs : Selective Serotonin Reuptake Inhibitors

STZ : Streptozotocin

TCA : Tricyclic antidepressants

TEA : Tetraethylammonium

TNF-α : Tumor necrosis factor-alpha

TRPA1 : Transient Receptor Potential Ankyrin-like 1

TRPC6 : Transient Receptor Potential Canonical 6

TRPM : Transient Receptor Potential Melastatin

TRPV1 : Transient Receptor Potential Vanilloid 1

TTX : Tetrodotoxin

TTX-R : TTX-resistant

TTX-S : TTX-sensitive

WHO : World Health Organization

YOH : Yohimbine

1. INTRODUCTION AND PURPOSE

Since neuropathic pain is a type of pain that greatly reduces the quality of life, and radical treatment is still not possible, and in most cases, adequate analgesia cannot be provided [1]. Diabetic neuropathy (DN), one of the most common chronic complications of diabetes mellitus (DM), causes neuropathic pain as a result of nerve damage and is a major cause of morbidity and mortality in the diabetic population [2]. Diabetic neuropathic pain (DNP) pathophysiology is still unknown. Several mechanisms for its pathogenesis have been proposed, including structural changes in the peripheral nervous system[3, 4], and functional changes such as abnormal hyperexcitability of the dorsal root ganglion (DRG) [5]. However, the relative contributions of various functional subtypes are largely unknown. Despite its widespread prevalence and clinical significance, diabetic neuropathy is poorly controlled by analgesics, and successful therapy remains a challenge due to the limited efficacy and significant adverse side effects of currently available drugs.

Several clinical trials have been carried out over the last 20 years to investigate the therapeutic utility of melatonin, a neurohormone that has been shown to have many physiological effects in various fields of medicine, including diabetes and pain management. As a prescription drug or as a non-prescription supplement product, it is an agent of widespread use worldwide for many indications. Due to its widespread use, it is considered as a promising pharmacological agent in scientific studies. Furthermore, most of the studies documented melatonin's very low toxicity over a wide range of doses. Alike, more clinical and preclinical studies are required to specify whether melatonin can be utilized in pharmacotherapy [6]. Melatonin has attracted significant interest in the recent years. All evidence points to its antinociceptive properties in several animal model studies, potential beneficial effects after its proven use to be very safe and efficient in the management of various chronic pain paradigms, particularly neuropathic pain. This led to its clinical use for a variety of pathological conditions, however, the underlying mechanisms of the pharmacodynamic processes are still unknown [7–9].

With its effects observed in pain-related in several studies, melatonin provides a very promising approach in neuropathic pain syndrome [7, 10–14]. In this research, a comprehensive examination has been made by in vivo and in vitro methods, a detailed preclinical study on the use of melatonin in diabetic neuropathic pain. The effects of melatonin and its related potential mechanisms were studied in experimental animals for

which a DNP model was developed. Whether or not the model is formed will be tested by measuring thermal hyperalgesia and mechanical allodynia in experimental animals [15]. A mechanistic approach was adopted in the in vivo pain experiments. It is aimed to add to the examination of the adrenergic system-related involvement in the effects of melatonin by pre-administration of alpha 1 and alpha 2-adrenoceptor antagonists and non-selective beta-adrenoceptor antagonists. The fact that the involvement of processes at the adrenoreceptor level in the antinociceptive effects of the melatonin receptor agonist agomelatine has been demonstrated, reveals the necessity of examining the involvement of these receptors in the antihyperalgesic and antiallodynic effects of melatonin in diabetic neuropathic pain [16].

The literature demonstrates a link between pain and melatonin administration. There is no study on diabetic neuropathy and including electrophysiological methods with high validity among the studies showing the positive effects of melatonin on pain. With this study, which includes in vivo and in vitro methods, a detailed study related to the effects of melatonin, a test substance that has attracted attention recently, will be brought to the literature. The findings can be used to guide novel drug development studies toward melatonin that will reinforce the clinical use of this active molecule.

Primary dorsal root ganglion neurons are frequently used in studies on evaluating peripheral neuropathy and are biologic materials that can be reflected to the clinical conditions in electrophysiological experimental design. The purpose of this research is to investigate the effects of melatonin on electrophysiological parameters in DRG neurons obtained from rats and to carry out an effective and mechanistic preclinical research. DRG neurons are the sensory pathway's initial neurons and they have been used frequently in the studies investigating nociception related mechanisms of pharmacological agents [17–19]. Neurons in the dorsal root ganglia (DRG) contain nociceptors, which generate pain perception via ion channels. These ganglia may be novel therapeutic targets in neuropathic pain [20]. Pain threshold and the pain transmission are affected by changes in these neurons. There are studies in the neuropathic pain model that look at substances that affect currents like potassium (K^+) or action potential (AP) currents in DRG neurons using electrophysiological methods. Changes in current-related parameters in DRG neurons are associated with analgesic effects [21]. In the light of these data, in this study, it is aimed to investigate the effects of melatonin on AP parameters and the changes in K^+ current. Changes in the I-V curve indicate which currents/channels the test substance

acts on, from the ranges in which they occur. There have been few studies that show melatonin has a positive effect on pain-related HVACC, TRP channels, and some electrophysiological parameters (indicating excitability) in DRG neurons [22–25]. Among the studies showing the positive effects of melatonin on pain, there is no study examining the melatonin effects of DRG neurons obtained from experimental animals with DNP models including electrophysiological methods with high validity.

Finally, with the current study which includes in vivo and in vitro methods, a detailed study related to the effects of melatonin, a test substance that has attracted attention recently, founded on the hypothesis that melatonin has antihyperalgesic and antiallodynic effects associated with the noradrenergic system and exerts a reducing effect on neuronal activity and excitability via opening of potassium channels by acting on DRG neurons, which is one of the systems that plays a primary role in pain relief. The discovery of this compound's mechanisms and pathways may lead to a better knowledge about pathologies of chronic pain and the development of novel medications that will be brought to the literature. This will enable the sharing of results that will reinforce the clinical use of this active molecule.

2. REVIEW OF THE LITERATURE

2.1. Pain

The International Association for the Study of Pain (IASP) asserts that pain considered as "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [26]. Pain plays a crucial role of human experience [27]. Pain was long thought to be only a symptom of an underlying disorder or illness. However, it is now considered as a disease in itself [28, 29].

In the absence of an objective measure, pain is a subjective and highly individualized complex experience varying degrees influenced by biological, psychological, and social factors, with the painful stimulus being physical or mental in nature [29–31]. Pain can impair a person's quality of life and overall important functioning [32]. Only the patient knows if the pain is present and how the experience feels, because one person to another may respond to pain differently, this individual pain experience is determined by several factors, including each person's unique genetic characteristics as well as emotional, motivational, psychological state, and of course cognitive. Pain response was also influenced by environmental factors such as their gender, previous pain memories and experiences, general health condition, and cultural and social influences [33].

The pain epidemiology is difficult to determine because symptoms are subjective and there is no consensus on the definitions and diagnoses of some conditions. According to a WHO study conducted within the scope of primary health care services in its centers around the world, chronic pain affects 21.5% of the global population, with a higher prevalence in the elderly 33% [34, 35]. This indicates that approximately one out of every five people has a complaint of chronic pain, and among those who have chronic pain, 10.4-14.3% have moderate to severe disabling chronic pain [36]. And 10.1% of all drugs that are prescribed to adults are analgesics, and it is estimated that the annual cost of pain is between 560-635 billion dollars in the USA according to 2010 data [37].

2.1.1. Classification of pain

Pain is classified into two types based on its duration: acute pain and chronic pain (persistent pain), or based on the underlying pathology (for example, neuropathy or cancer) [38].

Acute pain is a type that lasts less than 30 days, according to some, while others claim that acute pain can refer to any pain that resolves before 3 or 6 months. This is a type of sharp and severe pain that has a specific cause, such as tissue damage, starts suddenly, and lasts for a short period of time. In terms of location, time, and severity, there is a strong connection between pain and the lesion that causes pain. The pain felt in cases such as burns, cuts, bone fractures, surgery and infection is this type of pain and usually goes away when the underlying cause is treated. It is regarded as a valuable survival mechanism that provides both protection and healing [39, 40].

In contrast to acute pain, According to the IASP, chronic pain is “pain that has persisted beyond normal tissue healing time”, it is lasting more than 3-6 months; sometimes of an unknown cause, Pathological processes such as inflammation, trauma, AIDS, diabetes, and cancer are the primary causes of chronic pain, which is identified as continuous or persistent pain in terms of time and severity [35]. In the transmission pathways of painful stimulation It is challenging to localize the source of chronic pain due to the multisynaptic connections found [41]. It is known that in chronic pain cases, pain may be accompanied by some psychosocial and behavioral disorders [42, 43]. Chronic pain could be divided into neuroanatomically and neurophysiologically; nociceptive pain, neuropathic pain, deafferentation pain is classified as reactive pain and psychosomatic pain [42].

2.1.2. Physiology of pain

Nociception is the whole of electrochemical events that occur between the formation of damage of tissue and the feeling of pain [44]. It involves the process of encoding and transmitting the pain signal along the path from the painful stimulation point in the periphery to the upper centers in the CNS, including the cerebral cortex, where awareness of pain occurrence occurs [45]. The first step in detecting harmful environmental stimuli and causing pain sensation is "nociceptors", a stimulation of primary sensory neurons. Nociceptors play a crucial role in preserving the body's integrity, as they perceive these harmful stimuli and create the necessary response to avoid them. For this reason, they are activated only after they reach a certain threshold and form an action potential (AP). This harmful stimulation, which activates the receptors, provides the release of various compounds (e.g., serotonin, bradykinin, histamine, K^+, substance P, prostaglandins) [46]. The physiological process of pain,

which begins with the stimulation of nociceptors, follows the stages of transduction, conduction, transmission and perception occurs.

Transduction; it describes the process of converting chemical (endogenous or exogenous), thermal (hot or cold) or mechanical (incision, swelling, pressure, tumor growth) harmful stimulus into nociceptor-level electrical activity [45, 47, 48]. Nociceptors, also called pain receptors, are specialized neurons located at the free ends of unmyelinated C-fibers and partially myelinated Aδ-fibers, which can be modified for specific stimuli [49, 50]. Nociceptors distinguish between harmful and harmless stimuli; they are sensitive to chemical, thermal or mechanical stimuli and are classified according to these specific stimuli, when exposed to noxious stimuli for an extended period of time, the nociceptors may develop sensitivity and pain hypersensitivity occurs whither the thresholds are decreased, so that stimuli would normally not produce immediately pain (allodynia), or an increase in responsiveness occurs, so that the noxious stimuli produce prolonged and exaggerated pain (hyperalgesia). Sensitization can be central or peripheral [46, 48]. Nociceptors can be activated by exogenous or endogenous substances. These endogenous substances include inflammatory mediators (bradykinin, prostaglandins, arachidonic acid derivatives), neurotransmitters (noradrenaline, serotonin, neurokinins, excitatory amino acids, histamine) and growth factors (nerve growth factor, NGF) [51]. When a sensory depolarization threshold occurs, the activation of voltage-gated sodium (NaV) and calcium channels (CaV), resulting in an action potential. The membrane repolarizes when voltage-gated potassium channels (KV) open, inactivating NaV channels and bringing the neuron back to rest. The AP then transmitted in a process along the distal axon known as transduction [52]. Thus, AP is initiated and various peptides and mediators such as cholecystokinin, substance P, bradykinin, histamine and serotonin are discharged at and around the injury site. And through these, nociceptor sensitivity and vascular permeability increase [53]. This initiated stimulus is transmitted to the dorsal horn via myelinated and unmyelinated fibers from peripheral nociceptors.

Conduction velocity, degree of myelination, and diameter of nociceptive nerve fibers are used to classify them. In the peripheral nervous system, there are 3 main types of sensory fibers: C-fibers and A-fibers (Aβ-fibers, Aδ-fibers), and all three have various properties that allow them to perceive and carry different sensory information. Myelinated Aδ-fiber axons allow APs to circulate through the central nervous system (CNS) at high conductance rates ranging from 5 to 30 m/s [54]. The other type is

unmyelinated C-fiber axons whether or not they are myelinated, they conduct slower with conduction rate directly related to sensory neuron axons' diameter. Many nociceptors with speed transmit of 0.4-1.4 m/sm, have unmyelinated axons (C-fibers) with small diameter and surrounded by Schwann cells [49]. Among them, C-fibers constitute the smallest and slowest conduction unmyelinated fiber type of primary afferents. C-fibers have a higher activation threshold than others and selectively perceive nociceptive and painful stimuli [55]. The Distinct sensations, precisely localized, and sharp sent by A fibers that define pain intensity and pinpoint its source, whereas the C fibers related to a badly localized stimuli, producing a longer-lasting burning sensation [47, 54, 56].

As an example, consider touching accidentally a hot oven, "first pain" directly transmitted by Aδ-fibers which is a sudden well-localized, and short-term sharp pain, while C-fibers transmit "secondary pain." [52]. Secondary pain describes a pain that is felt as burning and stinging with a delay and usually lasts for a long time, worsening in some cases. These various nociceptors' characteristics and pain types are illustrated in (Figure 2.1.).

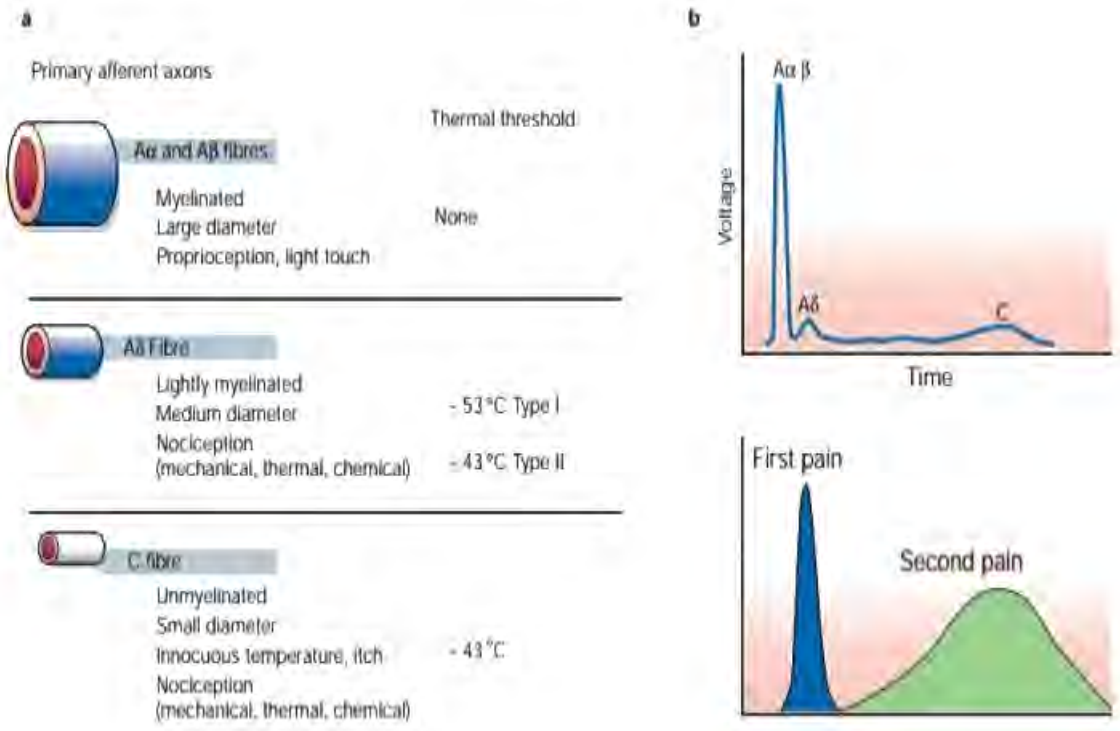


Figure 2.1. *Different nociceptive fibers and characteristics and their detection of different types of pain. (Fields, 1987).*

Their cell bodies are located in the dorsal root or trigeminal ganglia, just like all primary sensory neurons in the somatosensory system, and branch from a single axon to peripheral branches to innervate peripheral targets and enter the CNS to connect with second-order nociceptive neurons. It divides into the central axon[50]. Primary nociceptors in the periphery transmit harmful stimuli to cell bodies in the dorsal horn through a process known as transmission [52]. In other words, transmission; It is the stage in which impulses spread throughout the sensory nervous system [42]. With second-order neurons, primary afferents synapse and bring excitatory signals to the dorsal horn. These second-order neurons are located in the laminating regions of the dorsal horn, and each lamina consists of different cellular structures. These layers, called rexed laminae, are classified into 10 types according to their functions and their anatomical structures in the spinal cord cross-section are summarized in (Figure 2.2.). Of these, lamina I cells generally respond to harmful and thermal stimuli, and lamina II plays a role in perceiving sensory input as "painful", while lamina V neurons receive information from the axons of C-fibers, Aβ and Aδ that are involved in projecting information to the thalamus and brain stem via the spinothalamic pathway [48, 57]. While the lamina I-V provides projection to the thalamus via the spinothalamic pathway and to subcortical structures such as the parabrachial nucleus, periaqueductal gray (PAG)-rostral ventromedial medulla (RVM) via the spinobulbar pathway; Lamina II contains inhibitory interneurons that regulate opioids such as GABA, glycine and enkephalins, and exerts a modulatory effect on spinothalamic-spinobulbar projection neurons [58]. Apart from these pathways, the spinothalamic pathway, spinoreticular pathway, spinomesencephalic pathway and spinocervical pathway are among the ascending pathways that are associated with nociception [48].

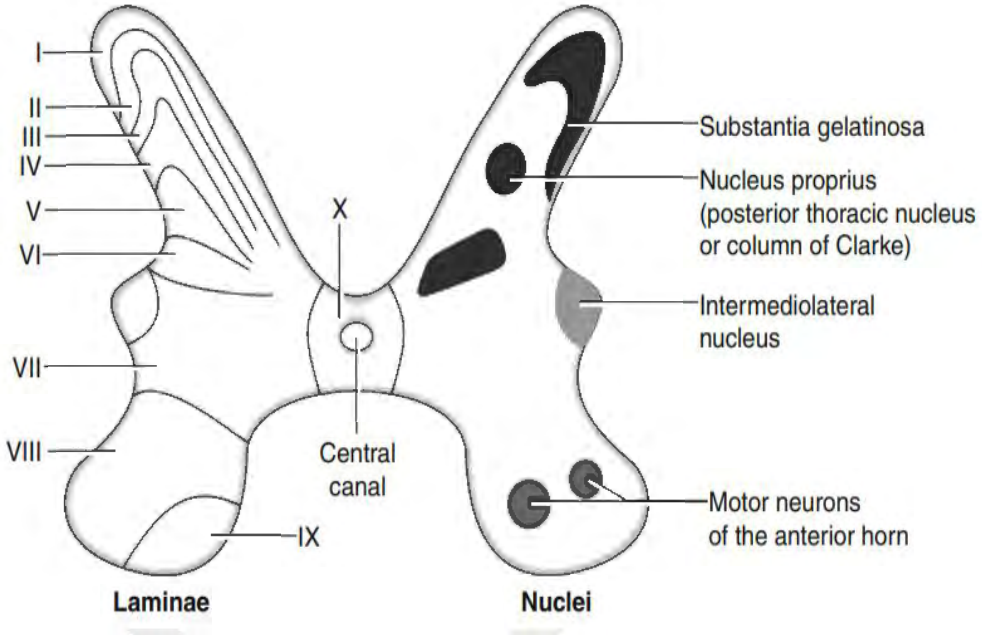


Figure 2.2. *Schematic representation of the various sensory endings in the dorsal horn of the spinal cord (Xing et al., 2011).*

The signals of the spinothalamic pathway, which is one of the most important nociceptive ascending pathways, go through the medulla of the spinal cord and synapse with thalamic neurons. Here, signals are sent to various pain-perceiving regions of the somatosensory cortex via nerves in the thalamus [52]. Perception, which is seen as the result of neuronal activity associated with the transmission of information that begins with harmful stimuli, can be characterized as the consciously aware of pain. When the information transmission generated by the noxious stimulus reaches a threshold for detection, it creates an information network between cortical and subcortical gray matter [46].

Pain experience occurs when signals reach the cortical structures of the brain [52]. Although the physiology behind perception is complicated and poorly understood, but it is known that behavioral and cognitive processes can affect how pain is perceived. Relaxation, meditation, positive mental thinking, and distraction may significantly impact how pain is perceived and can reduce pain, or similarly, conditions such as anxiety or depression often increase the feeling of pain [59]. The modulation of the afferent input generated against the noxious stimulus can occur at any level, from the periphery to the cortex. Between the central and peripheral nervous systems, the dorsal horn serves as a connection that makes it remarkable for physiological modulation.

In the dorsal horn, four mechanisms provide modulation: (i) endogenous opioids provide presynaptic inhibition between primary afferents and second order neurons, (ii) segmental (local) inhibition; local inhibition mediated by inhibitory neurotransmitters (GABA, glycine) in the dorsal horn independent of descending pathways, (iii) gate-control theory; through an inhibitory interneuron that acts as a physiological “gate”, C- and A-δ fibers' nociceptive signals are inhibited when non-nociceptive Aβ-fibers are activated, (iv) descending inhibition: mediated by neurotransmitters such as 5-HT, noradrenaline, dopamine in inhibitory pathways initiated from the midbrain pain modulation mechanisms have been described [48, 60, 61]. Ascending and descending pain pathways are displayed in (Figure 2.3.). Descending inhibitory pathways; it is initiated from various brain regions such as the rostral anterior cingulate cortex, amygdala, and hypothalamus. It is transmitted to the PAG and from there it is projected to the spinal dorsal horns via the RVM [62]. In this way, nociceptive traffic is directly or indirectly affected and the perception of pain changes.

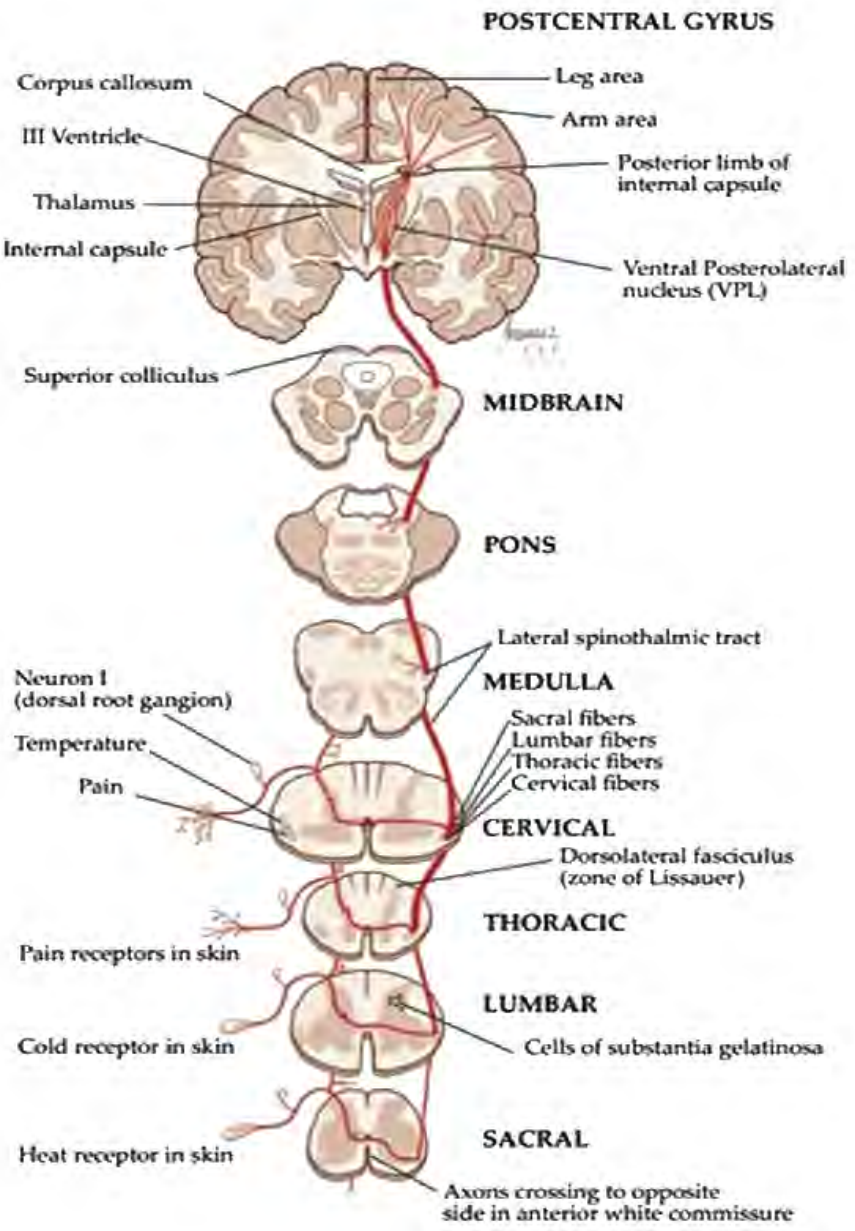


Figure 2.3. *Ascending and descending pain pathways (Bourne et al., 2014).*

2.2. Neuropathic pain

IASP defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system" [38]. Neuropathic pain (NP) is complex and severe pain syndrome, caused by a dysfunction or lesion in the CNS, PNS, or both and defined by a variety of symptoms and signs [63–65], This could interfere with normal nerve function and pathology, resulting in incorrect signal transmission [66].

Neuropathic pain can be a persistent or present as recurrent painful episodes. There are central and peripheral mechanisms in the maintenance and initiation of NP, and it has been demonstrated that immune and inflammatory reactions are also involved in its

pathophysiology [65, 67]. Although; neuropathic pain can occur due to ischemic conditions such as neurodegenerative, diabetic neuropathy, autoimmune or vascular conditions, exposure to toxins, infection, trauma, tumor or hereditary diseases, also, it can potentially occur with neurological diseases of unknown causes such as idiopathic neuropathies (entrapped neuropathies, fibromyalgia) [68]. While there are examples such as trigeminal neuralgia, postherpetic neuralgia, pain due to peripheral nerve damage, post-amputation pain, and painful polyneuropathies peripherally, among the types of neuropathic pain, there are various types of pain syndromes, including pain due to multiple sclerosis in central cases, spinal cord damage, and post-stroke pain [69].

Because of the scarcity of comprehensive epidemiological studies, the prevalence of neuropathic pain reportedly varies between 6.9% and 10% of the general population [70]. An investigation into over 12000 patients with neuropathic pain and nociceptive found that NP symptoms were present in 40% of all patients [71]. According to reports, advanced age as well as the existence of predisposing diseases such as DM, multiple sclerosis, stroke and the increased survival rate of cancer patients after chemotherapy cause an increase in this frequency [69, 72–74].

2.2.1. Symptoms of neuropathic pain

Neuropathic pain may begin spontaneously and be felt in a continuous manner or in ways that begin with a stimulus. The pain felt independent of the stimulus usually starts as paresthesia and continues as dysesthesia and is felt in the form of burning, stinging, pounding and throbbing in patients, or it can occur with symptoms similar to electric shock; Loss of touch, vibration and temperature perceptions can also manifest as numbness and tingling [65].

Pain on stimulus refers to an increased reaction to painful stimuli (hyperalgesia), which can be mechanical, thermal, or chemical, or pain reaction to a normally painless stimulus (allodynia) [75]. While hyperalgesia gives a clinical symptom of increased afferent activity against stimuli due to nociceptors' sensitization, allodynia is mostly linked with changes in the CNS [55, 76]. It is observed that negative (sensory reduction/loss) and positive (hyperalgesia, allodynia) sensory symptoms occur together in neuropathic pain.

2.2.2. Pathogenesis of neuropathic pain

Some peripheral and central mechanisms participate in the formation of NP. These central mechanisms include, spinal reorganization, cortical reorganization and disinhibition, central sensitization (wind-up phenomenon); Among the peripheral mechanisms, sensitization of nociceptor, primary afferent neuron collateral sprouting, ectopic and spontaneous discharges, ephaptic conduction, changes in ion channel expressions, sympathetic neuron sprouting in the DRG can be counted [77–79].

2.2.3. Types of neuropathic pain

Table 2.1. lists the most common types of neuropathic pain:

Table 2.1. *Types of neuropathic pain (Dworkin et al., 2003)*

Peripheral neuropathic pain	Central neuropathic pain
Chronic inflammatory demyelinating polyneuropathy	Multiple sclerosis-related pain
Painful diabetic neuropathy	Parkinson disease-related pain
Nerve compression or infiltration by tumor	HIV myelopathy
Trigeminal neuralgia (tic douloureux)	Poststroke pain
Chemotherapy-induced polyneuropathy	Postradiation myelopathy
Toxic exposure-related neuropathies	Syringomyelia
HIV sensory neuropathy	Postischemic myelopathy
Complex regional pain syndrome	Compressive myelopathy from spinal stenosis
Iatrogenic neuralgias	Posttraumatic spinal cord injury pain
Posttraumatic neuralgias	
Alcoholic polyneuropathy	
Postherpetic neuralgia	
Idiopathic sensory neuropathy	
Entrapment neuropathies	
Postradiation plexopathy	
Radiculopathy	
Phantom limb pain	
Nutritionaldeficiency-related neuropathies	

2.3. Diabetic neuropathic pain

Diabetic neuropathic pain (DNP) is the most common serious complication of DM [80, 81] and its progression has an adverse effect on the patient's quality of life [82]. This persistent and incapacitating complication has been estimated that 34–50% of DPN patients experience some degree of peripheral neuropathic pain (PNP) [83, 84]. Diabetic

neuropathy, which affects sensory, motor and autonomic nerves in a wide variety of ways, is one of the primary causes of increased mortality and morbidity in diabetics [2, 85, 86].

2.3.1. Epidemiology of diabetic neuropathy

According to the International Diabetes Federation, diabetes affects more than 425 million people worldwide today; this number could reach 628 million in 2045 [87]. The prevalence of neuropathy, one of the most important complications of DM, is expected to increase with this disease. It is known that diabetic neuropathy affects approximately 50% of DM patients [88]. Diabetic neuropathy is found in 66% of Type 1 diabetes patients and 49% of Type 2 diabetes patients [89]. While diabetic neuropathy was found to be present in 30% of hospital screenings and 10-20% of community screenings; the annual incidence of the syndrome has been reported to be around 2% [90]. A study conducted in 2009, reported that approximately 16% of adult diabetic patients in our country suffer from neuropathic pain [90].

2.3.2. Types of diabetic neuropathy

Autonomic neuropathy, proximal asymmetric mononeuropathy (diabetic amyotrophy), chronic inflammatory demyelinating polyradiculopathy, distal symmetrical polyneuropathy, nerve compression syndromes, truncal radiculopathy, and cranial mononeuropathy are the seven types of diabetic neuropathy [91, 92].

2.3.3. Symptoms of diabetic neuropathy

People with diabetic neuropathic pain (DNP) often develop symptoms that are dependent on the duration of the neuropathy, and initially affect the feet and progress proximally. These symptoms are mostly sensory in nature and can be classified as positive or negative. Unpleasant positive symptoms, which manifest initially, include dysesthesias (burning pain or electric shock), paresthesias (tingling), spontaneous pain and pain hypersensitivity to mechanical and thermal stimuli, termed as allodynia and hyperalgesia formed in Aδ and C-type fine fibers. In addition to negative symptoms such as heat hypoalgesia [93].

The pain, which can be moderate or severe in diabetic neuropathy, usually worsens at night and causes sleep disturbances. Diabetic neuropathy's prevalence of painful symptoms is difficult to estimate, as the disease can be associated with negative symptoms and cause both loss of sensation and pain in the same patient [80, 94]. Numerous sensory problems, such as loss of sensation in the extremities, which usually

results in foot ulcers and Charcot neuroarthropathy, which can lead to limb amputations, can be experienced by DNP patients [95, 96].

In diabetic neuropathy the felted pain may be permanent and may be accompanied by cutaneous allodynia; This has a significant impact on patients' quality of life, as well as their capacity to carry out daily and regular activities and their mood [97, 98]. Long-term hyperglycemia and pain could be related to sleep disruption, loss of mobility, daytime tiredness, anxiety, depression, and independence [99–101].

2.3.4. Stages of diabetic neuropathy

The staging system is proposed by Dr. Peter Kynaston Thomas as follows [102],

- Stage without neuropathy: There are no symptoms and only two abnormalities in tests, including autonomics.
- Asymptomatic neuropathy: There are no symptoms, but there are two or more abnormalities in functional tests.
- Symptomatic neuropathy: Two or more functional abnormalities with mild symptoms.
- Disabling neuropathy: Symptoms are associated with disability and there are two or more functional abnormalities.

2.3.5. Diagnosis of diabetic neuropathy

In diabetic neuropathy, firstly, after taking the detailed history of the patient; it is graded through visual analog scales on which simple clinical tests are applied, in which senses such as pain, touch, and vibration are evaluated. Pain scales such as the Leeds Assessment of Neuropathic Symptoms (LANSS) [103] and the Neuropathic Pain Scale [104], which were created to evaluate neuropathic pain, have clinical applications in the diagnosis of pain in diabetic patients [105].

More sensitive tests, for example “quantitative sensory tests” and “sensory and motor nerve conduction velocity measurement”, are also used to diagnose subclinical neuropathy in diabetic neuropathy and track disease advancement [106, 107]. In addition, corneal confocal microscopy or skin biopsy are among the highly sensitive methods used to visualize fine nerve fiber damage [108].

Differential diagnosis of conditions such as drug or heavy metal-induced neurotoxicity, osteoarthritis, alcohol-related neuropathy, post-herpetic neuralgia, thyroid and electrolyte balance disorders, inflammatory or congenital causes, which may mimic

neuropathic pain symptoms, in patients who are thought to have neuropathic pain from diabetes is also important [109].

2.3.6. Risk factors of diabetic neuropathy

Major risk factors that may trigger or exacerbate neuropathy in diabetic patients include long-term exposure to diabetes, failure to control hyperglycemia, age of the patient, hypertension, hypoinsulinemia and hyperinsulinemia. It is known that genetic predisposition, obesity, hyperlipidemia, tobacco, alcohol use, cardiovascular disease and albuminuria which are called independent risk factors, also pose a risk for neuropathy. Among all the factors, the most determinant for the development of neuropathy is the fasting plasma glucose level [110–112].

2.3.7. Diabetic neuropathy pathogenesis

Despite its widespread clinical and prevalence significance, the pathophysiology of diabetic neuropathy remains a mystery, and molecular and ionic mechanisms underlying diabetic neuropathy are not well understood. Various mechanisms can sensitize (become hyperexcitable) pain-sensing sensory neurons, or nociceptors, in response to diabetes-related pathological conditions or peripheral tissue injury [113]. However, it is known that metabolic, vascular, neurotrophic, genetic and immune factors are involved in the pathophysiology of diabetic neuropathy [114, 115]. Various modifications such as protein kinase C activation, increase in advanced glycosylation products, decrease in neurotrophic factors, increase in oxidative stress, alterations in ion channel expression and function activated as a result of abnormal hyperexcitability of nociceptive dorsal root ganglion (DRG) and central nervous system (CNS) neurons [5]. Several mechanisms, however, have been charged in its pathogenesis to take part in the development process of DN [113, 116–118].

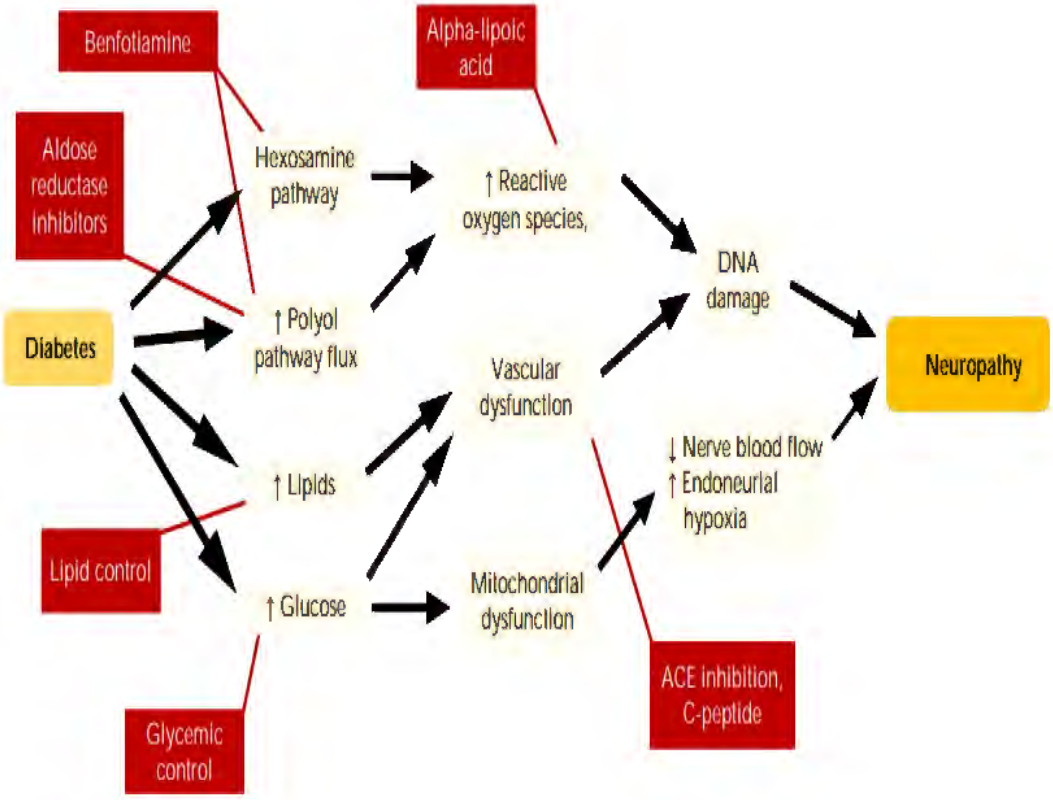


Figure 2.4. *Etiopathogenesis of diabetic neuropathy (Javed, Petropoulos, et al., 2015)*

The main mechanisms underlying the etiopathogenesis are summarized below:

2.3.7.1. *Polyol pathway mechanism*

When the polyol pathway is activated, as a result of chronic hyperglycemia, excessive sorbitol accumulation occurs in the cell; myoinositol level decreases and as a result Na^+/K^+ ATPase activity decreases [116, 119, 120]. NADPH depletion resulting from increased polyol pathway activation; It reduces nitric oxide and glutathione production, resulting in decreased vasodilation, increased oxidative stress, and deterioration of endothelial cell functions [120, 121].

2.3.7.2. *Protein kinase C mechanism*

Hyperglycemia causes protein kinase C to be activated; This leads to MAPK activation, changing gene expression, triggering stress genes to produce heat shock proteins, etc. leads to increased protein and apoptosis. Protein kinase C activation also causes changes in blood flow. It raises vascular permeability and causes abnormal angiogenesis. In addition, nuclear factor-kappa B (NF-Kβ) activation increases in

endothelial cells due to hyperglycemia, which increases the formation of reactive oxygen derivatives (ROS) [122, 123].

2.3.7.3. *Hexosamine pathway mechanism*

N-acetyl-D-glucosamine is a fructose-6-phosphate substrate; it binds to the transcription factor, leading to abnormal modifications in gene expression. It impairs the functions of pancreatic β-cells by increasing oxidative stress. This molecule also causes overexpression of plasminogen activator inhibitor-1 (PAI1) and vascular smooth muscle cells undergo mitosis, resulting in atherosclerosis [124].

2.3.7.4. *Mechanism of the advanced glycosylation products pathway*

In DM, some advanced glycolysis products and oxidative stress occur in parallel with the increased glycolysis events [125]. Precursors of advanced glycosylation products; It binds to its specific receptors in endothelial cells, macrophages, and microglia, leads to modification of plasma proteins and initiates production of ROS. Because of pro-inflammatory gene expression, cytokines, and growth factors such as interleukin, TNF-α, insulin-like growth factor-1 are expressed. Procoagulant and pro-inflammatory substances are expressed by endothelial cells. NF-Kβ is activated and apoptosis is triggered [126].

2.3.7.5. *Reactive oxygen derivatives formation mechanism*

ROS levels rise in Type 1 and Type 2 diabetes. Advanced glycosylation products, polyol, protein kinase C and hexosamine pathways; it directly affects the redox capacity of cells by causing ROS production in diabetic neuropathy. Neuronal damage and microvascular complications happen as a result of oxidative stress resulting from the autoxidation of glucose [127, 128]. In diabetes-related distal polyneuropathies, thin myelinated and unmyelinated fibers are lost, and axonal regeneration is increased; this causes abnormal impulses and neuropathic pain. In distal polyneuropathies that are not accompanied by pain, thick fibers are primarily affected, and myelinated fiber losses are accompanied by acute axonal degeneration and vasculopathy [129].

2.3.7.6. *Microvascular changes*

While metabolic factors are at the forefront at the beginning of diabetic neuropathy, it has been stated that vascular-ischemic disorder becomes involved as the duration of diabetes increases [130]. Ischemic neuronal damage as a result of vascular thickening, hyalinization, and decreased oxygenation, as well as inflammation, are factors

that aggravate neuropathy [117]. C peptide deficiency, a protein consisting of 31 amino acids that plays a role in insulin and/or insulin folding [120]; It is well known that neuronal degeneration and axonal dysfunction caused by chronic hyperglycemia, as well as microangiopathy and microvascular reactivity due to nitric oxide deficiency, worsen the neuropathy picture [131].

2.3.7.7. *Channels sprouting*

However, various mechanisms, including structural changes in the PNS, have been embroiled in its pathogenesis [3, 4], The abnormal hyperexcitability of dorsal root ganglion (DRG) neurons is thought to contribute to diabetic neuropathic pain, but the relative contributions of these specific functional subtypes are largely unknown [5, 132].

According to the most widely accepted hypothesis that unsettled damaged nerve endings can generate action potentials, which the central nervous system (CNS) interprets as pain [133, 134]. Ongoing pain and/or the hypersensitivity connected with DNP are resulted because of modifications in primary afferent excitability neurons, that conclude an abnormal spontaneous activity (SA) exhibited from both A- and C-fiber DRG neurons, that considered as a key feature of neuronal hyperexcitability [135–138].

In STZ-induced rats, several electrophysiological alterations, including an elevated incidence of SA in Aβ-LTMs (low-threshold mechanoreceptors) and C and A-β-nociceptors that are likely to participate in the pathophysiology of DNP, which can result in paresthesias /dysesthesias related with diabetic neuropathy, as well as a reduced electrical threshold, faster AP and after-hyperpolarization (AHP) kinetics [132, 139]. Indeed, alterations in the ion channel's expression in these peripheral fibers are a direct outcome of nerve damage, leading to hyperexcitability, which is strongly related to NP [140].

2.3.8. Synaptic control

Many neurotransmitters or neuromodulators take part in pain formation and create antinociceptive or pronociceptive responses [141]. When nociceptive transmission reaches the upper cortical centers, it activates many synaptic events that will provide pain control by activating inhibitory neurons in order to reduce pain [142]. While many neurochemicals such as GABA, glycine, serotonin, endoopioids, acetylcholine, dopamine, noradrenaline, endocannabinoids, glutamate, and the activation or inhibition of different receptor types of these neurochemicals are implicated in the transmission of

pain and control, only the information related to the noradrenergic system, which is considered within the scope of the thesis, is detailed. According to reports, the development of mechanical allodynia and thermal hyperalgesia, which causes NP, is associated with weakening in the functions of descending antinociceptive pathways that mediated by endogenous opioids monoamines such as serotonin and noradrenaline [143–145]. Pain stimuli transmitted to the brain stem through ascending spinal cord pain pathways cause the formation of inhibitory descending messages reaching the dorsal horn by using noradrenergic, serotonergic and opioidergic pathways [146].

2.3.8.1. ***Role of the noradrenergic system***

Noradrenaline's effects on pain regulation are significantly mediated by α-adrenoceptors,, however, It is possible that adrenaline-induced regulation of pain is primarily mediated by β-adrenoceptors [147]. In supraspinal regions, both α 2- and α 1-adrenoceptors are broadly distributed, which is consistent with ascending noradrenergic pathways' broad distribution [147]. All the subtypes of the α1-adrenoceptor have been detected in the DRG, but the most strongly expressed is α1A subtype [148, 149]. Also, α2-adrenoceptors visibly expressed in dorsal root ganglion neurons, α2C subtype is the most abundantly expressed and associated to pain, whereas α2B is rare in the DRG [150].

Of the noradrenaline-specific adrenoceptors, α1- and β-receptors show more facilitator activity, while α2 adrenoceptors show inhibitory activity [151]. It is well known that 2-adrenoreceptors' inhibitory effects on noradrenergic descending inhibitory pathways reduce the transmission of pain [146, 152]. α2-adrenoceptors; it reduces the effect of intracellular adenylcyclase by directly modifying the activity of Gi-mediated or ion channels such as K^+, Ca^{2+} or Na^+/H^+ antiport channels [146]. Noradrenaline exerts a strong antinociceptive effect in living things thanks to spinal α2-adrenoceptors [153]. Owing to the α2-adrenoceptor, inhibitory interneurons actively suppress pain with post-synaptic inhibition [154]. α2-adrenoceptor agonists also have a very strong analgesic effect in pain caused by nerve damage in rodents and humans [155]. It is also reported that α2-adrenergic agonists and opioid agonists exert synergistic effects [156]. Experiments have shown that the α2-adrenoceptor agonist, clonidine, not only shows good analgesia, but also potentiates the analgesic effect of opioids [157]. Stimulation of the α2-adrenoceptor by noradrenaline provides peripheral analgesia by causing enkephalin release and indirectly μ1 receptor activation [158]. Although studies on noradrenergic receptor activation tend to have agonistic effects on α2-adrenoceptors,

there are many studies showing that α1- and β-adrenoceptor agonism also participate in pain control [158, 159]. In a study, it was shown that noradrenaline facilitates GABAergic and glycinergic inhibitory synaptic transmission in the dorsal horn of the spinal cord, but not excitatory glutamatergic transmission. It has been shown to do this by triggering depolarization via α2- and β-receptors as well as α1-adrenoceptors, and then triggering action potentials that will stimulate synaptic terminals and cause the release of glycine and GABA [151]. In addition, stimulation of β2-adrenoceptors is necessary and essential for antidepressants to exert their antiallodynic effect against neuropathic pain [160].

2.3.9. Treatment of diabetic neuropathic pain

Today, radical treatment of diabetes-induced neuropathy is not possible [161]. Therefore, the basic medical approach in the treatment of DN can be expressed as preventing the occurrence of the disease and controlling the minor and/or major complications that occur with the disease.

4 basic treatment strategies are adopted for the treatment of patients with DN. These are "glycemic control", "therapeutic approaches for pathogenic mechanism", "handling risk factors and complications (lifestyle changes)" and "symptomatic treatment" [162, 163]. Among these approaches, besides pharmacotherapy, there are physical therapy and rehabilitation, cognitive and behavioral treatment and interventional treatment applications [164].

2.3.9.1. *Glycemic control*

Preventing hyperglycemia and reducing the patient's blood glucose to normoglycemic limits are the primary goals expected to be achieved in diabetic patients, regardless of the type and stage of neuropathy. With pharmacotherapy and lifestyle changes provided glycemic control can delay or even prevent neuropathy; it is known that it also significantly slows down the progression of existing neuropathy [165]. In order to achieve glycemic control in diabetic patients, sulfonylureas, metformin, glitazones and incretinmimetics (GLP-1 analogs and dipeptidyl-peptidase IV inhibitors) and α-glucosidase inhibitors are used alone or in combination. In cases where these antidiabetics are not successful in the management of diabetes type II, insulin administration is started [166].

2.3.9.2. *Lifestyle changes*

Lifestyle changes should be made to reduce cardiovascular risk factors such as high blood pressure and hyperlipidemia in the prevention and control of diabetes-induced neuropathy. Alcohol consumption and smoking should be avoided, and attention should be paid to body weight. It is known that exercise and diet regulation have a great contribution to the treatment of diabetes-induced neuropathic pain due to their positive effects on metabolic events in the body [94, 162].

2.3.9.3. *Treatment approaches for pathogenetic mechanisms*

Approaches that target pathogenic mechanisms, which is another strategy in the treatment of DN, focus on the processes that cause the emergence of the disease rather than the symptoms. Some examples of drugs used in this type of treatment or under development are as follows [120, 167]:

- Drugs such as sorbinil, tolrestat, ponalrestat, fidarestat, epalrestat, zenrestat and ranirestat, which are aldose reductase inhibitors that act on the polyol pathway
- Protein kinase C inhibitor drugs such as ruboxistaurin
- Agents such as benfothiamine that act on the hexosamine pathway
- Agents such as aminoguanidine and n-phenylacylthiazolium that act on the advanced glucolysis pathway
- Inhibitors such as α-lipoic acid, nicotinamide, resveratrol, rutin, taurine and trigonelline, which prevent the formation of reactive oxygen species

2.3.9.4. *Symptomatic treatment*

Symptomatic treatment, which is another strategy in the treatment of DN, is treatment aimed at eliminating only the symptoms of neuropathy, unlike the treatment approach for pathogenic mechanisms [168, 169]. The drug groups that are frequently used for symptomatic treatment in DNP, which is a type of pain that is difficult to cope with, are presented in table 2.4

Tricyclic antidepressants (TCA) are the first preferred drug group in the treatment of all neuropathic pains, not only in DN, but also in trigeminal neuralgia. It has been reported that those with tertiary amine structure are more effective than those with secondary amine structure. The negative side of drugs in this group is that their side-effect profiles are higher than other drugs [164]. In cases where TCA use is risky in terms of side effects, selective serotonin reuptake inhibitors (SSRIs) can be used as an alternative.

Serotonin and noradrenaline reuptake inhibitors (SNRIs), which inhibit both serotonin and noradrenaline reuptake, have become more preferred due to the weak efficacy of SSRIs [120]. It has been reported that amitriptyline from TCAs and venlafaxine and duloxetine from SNRIs are most frequently preferred drugs in order to treat DN [170–172]. It has been reported that antidepressant drug therapy is beneficial in terms of reducing DN-related hyperexcitability and hyperalgesia, as well as in the treatment of depressive symptoms due to pain and chronic hyperglycemia [173, 174].

Anticonvulsants are drugs that are generally used when the desired benefit cannot be obtained from the first and second choice drugs in the DN treatment guidelines [175]. In addition, it has been reported that anticonvulsants are more effective in the neuropathy treatment such as driving or electric shock. According to certain suggestions, the effects of antiepileptic drugs are probably related to the GABAergic system and stabilization of neuronal membranes acting on Na^+ current [129, 176]. Although pregabalin, carbamazepine, gabapentin, sodium valproate, lamotrigine and topiramate are frequently used drugs for the treatment of DN, it is noteworthy that there are different preferences in various treatment guidelines. The American Neurological Association recommends sodium valproate and carbamazepine as the second choice after pregabalin along with gabapentin, on the other hand, the European Federation of Neurological Societies does not recommend the use of these drugs [177]. While gabapentin and pregabalin, a drug with a similar mechanism of action, are more preferable options with low side-effect profiles; drugs such as carbamazepine, phenytoin, and lamotrigine are drugs with a wide side effect profile that require blood levels to be monitored [178]. It is known that from time to time, the use of drug combinations is necessary when a single drug is not effective [179].

Tramadol, a weak opioid analgesic, is the most effective drug in its group against diabetic neuropathic pain. Dextromethorphan and morphine are less effective than tramadol. The controlled release form of oxycodone has also been observed to significantly reduce pain. Side effects and addiction risks of opiates such as rebound headache, nausea, constipation, and sedation limit their use. The use of opioids is only evaluated when other treatment options fail [177, 180].

In topical use, the pain complaints of patients can be reduced by the use of agents such as capsaicin, isosorbide dinitrate, lidocaine, ketamine and clonidine [166, 181]. In addition, there are some agents that act by slowing down the development of neuropathy.

Table 2.2. *Drug groups used in diabetic neuropathic pain (Civelek & Kuşkonmaz, 2015).*

Drug Group	Drug	Dose (mg)	Side Effect
Tricyclic Antidepressants (TCA)	Amitriptyline	50-100	Dry mouth, visual disturbance, somnolence, drowsiness, tachycardia, urinary retention, constipation
	Desipramine	25-150	Dry mouth, visual disturbance, somnolence, drowsiness, tachycardia, urinary retention, constipation
	Imipramine	25-150	Confusion, dry mouth, visual disturbance, somnolence, drowsiness, tachycardia, urinary retention, constipation
SNRI	Venlafaxine	37.5-150 (once a day)	Cardiac conduction disturbance, fatigue, drowsiness, vomiting
	Duloxetine	60 (once a day)	Anorexia, somnolence, vomiting, drowsiness
SSRI	Paroxetine	40 (once a day)	Anorexia, somnolence, vomiting, drowsiness
	Citaloprom	40 (once a day)	Diarrhea, tremor, impotence
Anticonvulsants	Pregabalin	50-150 (three times a day)	Edema, Somnolence, confusion weight gain
	Gabapentin	300-1200 (three times a day)	somnolence, ataxia, drowsiness, confusion
	Carbamazepine/ Oxcarbazepine	up to 200 (four times a day)	Leukopenia, somnolence, drowsiness, vomiting

opioids	Tramadol	50-100 (twice a day)	somnolence, constipation, vomiting
	Oxycodone	10-30 (twice a day)	somnolence, constipation, vomiting
Topical Agents	Capsaicin	0.075% (four times a day)	Irritation in the application area
	Lidocaine	0.04% (once a day)	Irritation in the application area
	isosorbide dinitrate	Spray application up to 30 frames (four times a day)	Irritation in the application area

2.3.10. Experimental models of diabetic neuropathy

Most information on the pathogenesis of diabetes-induced neuropathic pain has been obtained through diabetic rodent studies. Commonly used experimental models include streptozotocin (STZ)-treated mice and rats, high-fat diet-fed rodents, food- and chemical-combination-induced models; Spontaneous or genetically engineered models include Zucker diabetic obese mice, Type 1 insulinopenic BB/Wor and Type 2 hyperinsulinemic diabetic BBZDR/Wor rats, non-obese diabetic mice, Akita mice, and leptin and leptin receptor deficient mice [182, 183].

In order to investigate the mechanisms of diabetic neuropathic pain and evaluate prospective treatments, the STZ-induced diabetic rat has become a more popular and frequently used model of type 1 diabetes, as an experimental paradigm than other models of DNP because of its relative lack of extrapancreatic toxicity, greater stability, rapid induction and of course low cost [182–184]. It's worth noting that hyperglycemia and hypoinsulinemia caused by streptozotocin that indirectly activates an apoptotic program that demolishes all the cells expressing the GLUT2 transporter and especially pancreatic β-cells [80, 139, 185], leading to diabetes mellitus type1. The toxicity of STZ is due to the nitrosoamide moiety. [182]. The STZ-model is well-known display long-term behavioral signs of diabetic neuropathy including heat hypersensitivity and mechanical [132].

2.4. Primary Afferent Neurons

2.4.1. DRG neurons

The cell bodies of somatosensory PANs are clustered together in ganglions called DRG positioned outside the central nervous system (CNS), along the dorsal root of each spinal nerve [186]. DRG neurons are types of pseudounipolar, in which the short axon splits into two divisions; the first branch is peripherally terminated, which constitutes dendrites and carry stimuli from the body to the spinal ganglion cell, and the second branch is centrally terminated, which constitutes the cell axon and carry the stimuli from the spinal ganglion cell to the spinal cord [187, 188]. (Figure 5.1).

APs are generated in DRG's cell body in response to a primary sensory neuron located in the cell body; originates from the peripheral receptive field stimulated externally noxious stimuli (thermal, mechanical, chemical, etc.) [189]; it extends to reach the spinal cord particularly the dorsal horn, and then transferred to the thalamus and associated areas of the brain. The axonal extensions of these neurons emerge together from the trunk; then they diverge in opposite directions at a structural junction in the shape of the letter "T". The electrical signals are being spread from the periphery while reaching the dorsal horn by helping of T region; it can act as a barrier or a reducing filter for action potential (AP) transmission [190, 191]. The firing pattern produced by DRGs provides information about the stimuli intensity, duration and location [192].

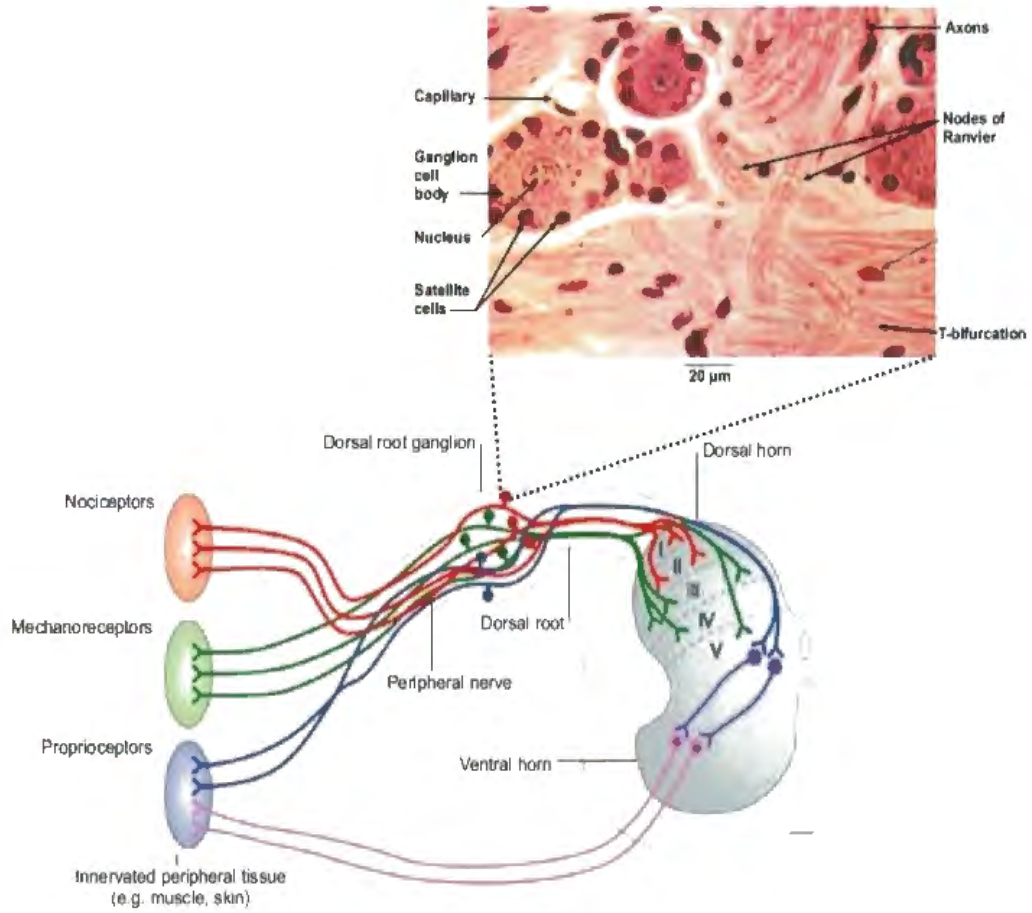


Figure 2.5. *DRG neuron anatomy as a component of the reflex arc (Caspary & Anderson, 2003).*

DRGs are known to have heterogeneous neuronal groups and there are different classifications of these groups [193–195]. Each sensory modality is displayed by a distinct DRG neuron, so the DRGs represent afferent neurons with various actions which are formed by the DRGs [196], However, when classifying DRGs, this diversity is generally ignored, I place of that they have used conduction velocity (calculated in m/s: slow < 1; medium: 1-8; fast: 9-60) and size of the cell (measured by diameter or capacitance: Small :10-30 µm or < 70 pF; Medium: 31-40 µm or 71-90 pF; Large: 41-60 µm or > 90 pF) [132, 186]. Other classification attempts include; immunoreactivity (IR) based [196] as well as the other is AP's shape based [186] have been proposed into two types that implicates the first derivative (dV/dt) of the action potential's repolarizing phase. One type, show a shift through the descendant phase of AP, distinctly

distinguishable in the dV/dt as well as AP duration become longer. The other type, lacks this inflection and has a shorter duration [193–195].

DRG neurons are the first pathway of sensory neurons and they have been used frequently in the studies investigating nociception related mechanisms of pharmacological agents [17–19].

2.4.2. Ion Channels expressed in DRG Neurons

Ion channels are formed from complex proteins that form pores in cell membranes to allow ions to flow across the hydrophobic core. Electrical signals result from temporary local changes that drive membrane potential away from its resting value, which underlie cognition, muscle contraction/ relaxation, neurotransmitter release, neuronal signal transmission, sensory transduction and preserving electrolyte balance are propagated throughout a neuron or nervous fiber. Ion channels mediate those alterations [51, 197, 198]. DRG neurons contain various types of channels that are usually classified depending on structure and function.

Multiple researches have exposed the crucial role of persistent raises in excitability of primary afferent and persistent spontaneous activity in the initiation and maintenance of peripherally induced neuropathic pain [199].

There are several channels that are reasonable as the logics for DRGs to be taken into consideration as pain transmitters, K^+, Na^+, Ca^{2+}, TRP and HCN channels are relatively present in DRG and involved in neuropathic pain.

2.4.2.1. *Sodium channels*

Voltage-gated Na^+ channels (Nav) are essential in DRG neurons' pain-conducting C-fibers, as well as in the AP's initiation and propagation, because they are in charge of the first membrane depolarization phase. They have been discovered in neurons, particularly DRG and cells that are electrically excitable, as well as in non-excitable cells at lower levels [200]. The Nav channels are divided into two categories based on the tetrodotoxin sensitivity: the first is tetrodotoxin resistant (TTX-R) and the second is tetrodotoxin sensitive (TTX-S). These channels are also critical in the development of pain signals in humans, with Nav1.7 channel in the DRG is loss-of-function mutations causing total pain insensibility [201]. Nav 1.7 null mutations cause the dystrophy of large sensory fibers and, as a result, the inability to feel pain [202]. The mutation of Nav1.9 channel in the DRG may also result in pain perception loss [203].

2.4.2.2. *Calcium channels*

Calcium voltage-gated channels (Cav), especially the T-type channels, have been linked to chronic pain in the DRG. T-type channels are abundant in cell bodies, and their activation may contribute to spontaneous pain by lowering the APs threshold [204, 205]. Injection of T-Type antagonists intraperitoneally have demonstrated to mitigate chronic and acute pain behaviors [206]. Furthermore, N-type channels considered the vast Cav majority expressed at DRG neurons presynaptic terminals and are implicated in neurotransmitter release. DRG neurons have been found to express three different types of Cav channels; Cav2.1–2.3, Cav3.1–3.3, and Cav1.2–1.3 [207].

2.4.2.3. *TRPV channels*

One of the fact that DRGs express transient receptor potential vanilloid 1 (TRPV1) channels suggests that they are pain transmitters, a TRP ion channel superfamily member, which respond to capsaicin and heat activated (>43 °C) [208]. Ca^{2+} influx in the DRG neurons results in depolarization and an induced series of APs; Capsaicin sensitizes DRG neurons, which is used to demonstrate the presence of TRPV1 [209]. Therefore TRPV1 is a thermo-nociception channel, however, it is not the only one in charge of this task, as long as neuropathic thermal hyperalgesia persists properly complete in mice with the TRPV1 gene knocked out [210], TRPA1 and TRPC6 are two other TRP family members found in DRG neurons [211].

2.4.2.4. *HCN channels*

Hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels are largely expressed in the nervous system, including sensory neurons. They have an essential function following AP in the repolarization phase by inducing a current depolarizing with high activity, which facilitates the creation of a new AP [212, 213]. I_h has been observed in all DRG neurons, especially with an abundant expression of HCN1 and HCN2 subunits in large diameter neurons, these neurons display much larger I_h amplitude, while small diameter neurons expresses predominantly HCN3 [214]. Indeed I_h has found to raise in DRG neurons with large diameter following spinal nerve ligation (SNL - a neuropathic pain model) in rat [215]. Another study discovered a significant increase in I_h after DRG chronic compression in rats [216]. Furthermore peripheral block of HCN channels with ZD7288 has been illustrate to reduce mechanical allodynia induced by sciatic nerve injury [217] and inhibit ectopic spikes in Aβ fiber and not in Aδ fiber of

DRG rats [215]. These observations make I_h a relevant target of study in neuropathic DRG neurons [218].

2.4.3. Potassium channels

K^+ channels are the largest and the most packed, broadly dispersed, with a variety range of neuronal ion channels, ruled by over 100 genes encoding the pore-forming α-subunits of K^+ channels [219, 220]. K^+ channels are a group of ion channels that control the intrinsic neuronal electrical properties [221]. The exact position of K^+ channels straight with the dendro-somatoaxonic surface of the neurons is a crucial factor in defining its functional significance [222, 223].

K^+ channels act as a significant function in neuronal repolarization following Na^+ induced depolarization. Upon activation, K^+ channels opening is voltage dependent and allow for extremely fast transmembrane K^+ efflux, which influences AP threshold, waveform, and frequency (restoring the resting potential of the axonal membrane) [224, 225]. Depending on their level of activation, deactivation and inactivation characteristics, expression, conduction properties, and electrochemical ionic gradient across the cell membrane [226, 227]. All K^+ channel types functions are diminished by the processes plurality giving raise to altered expression and/or alterations of post-translational, resulting in excitability augmentation of DRG cell bodies and nociceptive free nerve endings, modifies the conduction of the axon and increases the secretion of neurotransmitters from spinal dorsal horn primary afferent terminals [224, 228] (Figure 1).

K^+ channels expressed in DRG are divided into four distinct categories include; voltage-gated channels (Kv1-Kv12), calcium-activated K^+ channels (KCa1.1, 2.1, 2.2, 2.3, and 3.1) [229, 230], inward rectifying (KATP and KIR6.2) [231], and two-pore domain leak channels (K2p; TWIK related channels) [232].

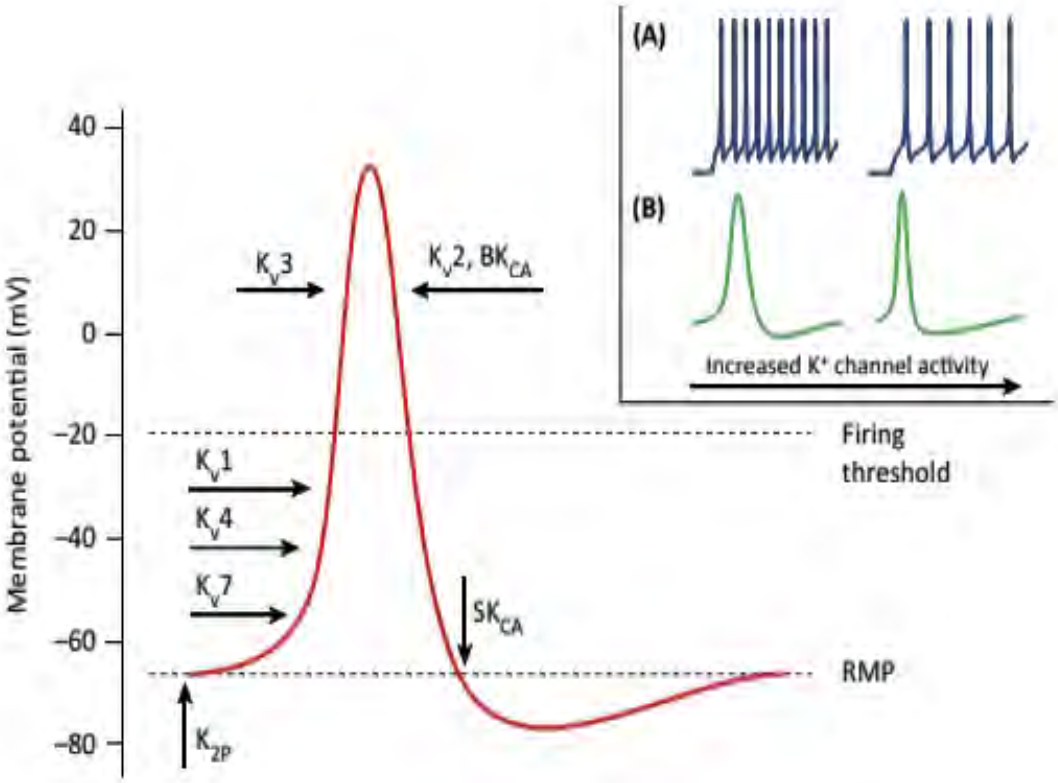


Figure 2.6. *Different potassium channel activation during action potential (AP) firing in sensory neurons (Tsantoulas & McMahon, 2014).*

2.4.3.1. *Voltage-gated K^+ channels in DRG neurons*

Voltage-gated K^+ channels (Kv) are the most abundant superfamily among K^+ channels, in humans, consisting of 40 genes [225, 233]. They represent the most diverse group by 12 families (Kv1-Kv12) [233, 234]. Kv channels are tetramers of α subunits containing 6 transmembrane helixes S1 to S6, capable of forming homo or heterotetramers [235–237].

Kv plays a role in pain perception. These potassium-selective transmembrane ionic pores exhibit diverse clustering mechanisms and, importantly, regulate the RMP and spike interval to enhance neuronal excitability [238, 239]. When at rest, the Kv channels reduce neuronal hyperexcitability by closing the membrane potential to -90mV, which is the K^+ equilibrium potential (-50 to -60mV in spinal ganglion sensory neurons) [234]. They have a crucial role in regulating the RMP, duration of AP, resistance of the membrane, and the release of neurotransmitters [240].

As their names suggest, K_V currents are classified as transient fast-inactivating (I_A), transient slowly inactivating (I_D), sustained delayed rectifying (I_K), and non-inactivating (I_M). Even though this classification is oversimplified, it is useful as a

beginning for investigating the various Kv composition s in physiological systems. These currents are also found in neurons of the dorsal root and trigeminal ganglia [241–244].

According to their inactivation kinetics and sensitivities to tetraethylammonium (TEA), and 4-aminopyridine (4-AP), Kv channels are classified into two major types: rapidly activated and inactivated 'transient' A-type currents (I_A) and rapidly activated and slowly inactivated 'delayed' currents (I_K) and [242, 245, 246]. The A-type currents are particularly important in controlling spike onset, AP firing threshold, and frequency of firing [245]. Besides, in most excitable cells, outward rectifying potassium currents (I_K) have been observed [247]. They are thought to identify the threshold of the AP firing, to regulate the rate of repolarization and after-hyperpolarization phases of action potentials, furthermore primary sensory neurons' resting potential [248–250]. Time-dependent inactivation is slow or non-existent.

The majority of Kv channels expressed in DRGs are; Kv1.1-1.6, Kv2.2, Kv4.2, Kv4.3, Kv3.4 [251, 252]. Among the A-type Kv channels, Kv1 channel family, the most amply expressed channels in DRG neurons are Kv1.1, Kv1.2 and Kv1.4 [245, 253, 254]. Kv1.4 (I_A) appears to be the most expressed Kv isoform in DRG neurons as most small diameter DRG Kv1.4-positive neurons do not detectably express other Kv1 subunits [255]. They are specifically inhibited by 4-AP [245, 256] with fast inactivation [257]. While, Kv3.4 (I_A) channel are high voltage activated with fast inactivation, slow recovery from inactivation and hypersensitive to TEA and 4-AP [252, 258]. Besides, the A-type Kv4s are found in small and large nociceptors in addition to the dorsal horn of the spinal cord with predominance expression of Kv4.3 and Kv4.2 in small sized cells [251, 259–261]. Kv4s and Kv1.4 are low voltage channels, with the former recovering slowly and the latter recovering quickly, and they are accountable for the hyperpolarizing voltage in steady-state. Multiple studies have linked the dysfunction of A-type Kv channels in DRG neurons to persistent pain sensitization, emphasizing their importance [252].

The KCNQ/M (Kv7) channels are a family of five voltage-gated K^+ channel subunits M-type Kv channels (Kv7.1-7.5) [262, 263] encoded with the KCNQ1-5 genes [264]; among those, KCNQ3and KCNQ2are abundantly exclusively expressed in the peripheral nervous system (PNS) especially in the DRG neurons [265–267]. Kv7 channels are low-threshold, slowly activating and slowly deactivating channels. The Kv7 channels are known as delayed rectifiers because of their slow activation and proclivity to drive outward current [268]. This current is a non-inactivating K^+ current (the M

current), which, when combined with an inactivation protocol, can be applied to distinguish, and isolate Kv7 currents from other currents produced in the cell. Pharmacological agents can also be used to detect currents generated by these channels that modulate the action of Kv7 channels, such as retigabine or the less powerful flupirtine, or Kv7 current inhibitors, XE991 antagonist [268, 269]. The suppression of M currents raises neuronal excitability [244, 270, 271], while their improvement has a deafening effect [272–275]. Their essential role is regulating the firing of action potentials (APs) and neuronal excitability [244, 264], also their usefulness in direct research into the membrane excitability mechanism and treatment of related disorders [269, 276].

Mutations in Kv channel genes can cause a variety of severe hereditary disorders [277, 278], and importantly to this work, they are involved in the development of the chronic pain syndrome [279].

2.4.3.2. *Involvement of DRG Kv channels in chronic pain*

The underlying mechanism of neurological problems such as epilepsy and chronic pain is defined as neuronal hyperexcitability [280]. The DRG neurons hyperexcitability is linked to neuropathic pain. DRG neurons may exhibit abnormal firing properties or neuronal cross-excitation by neighbors following a nerve injury [219, 251], producing inappropriate impulse activity that may underlie an abnormal sensations.

In chronic pain states, several data has noted that inflammation or nerve injury changes K^+ channel activity in pain pathway neurons, having a prominent role on DRG hyperexcitability, thus starting to reveal its potential as a novel therapeutic target [224, 254, 264, 281]. Controlling membrane excitability and treating chronic pain could be mediated by kv channels as a target of this problem [282]. In fact, DRG neurons excitability reduced by applying K^+ channel openers to the cell body or terminals, while the neurons firing increase by applying the K^+ channel blockers [220, 269, 283, 284]. Furthermore, the existing data indicate so far that a number of antinociceptive drugs act as direct openers of K^+ channels in the spinal cord [220].

Several studies have illustrated that downregulation of expression of K^+ channels in pain animal models. For instance, Kv1.1 decreases mechanosensitivity (the mechanical sensitivity threshold gets higher) at the C-mechanonociceptor terminals [285]. Loss-of-function of Kv1.1 results in lower firing thresholds, reduced heat and mechanical pain, as well as improved sensitivity in both phases of the formalin test [286, 287]. By contrast, Kv1.2 channel downregulation in DRG neurons in response to nerve injury contributes

to cold and mechanical neuropathic pain by depolarizing the resting membrane potential (RMP), lowering the threshold current, and increasing firing rates in myelinated neurons [254, 288–290].

It has also been evidenced that Kv 1.4 subunit expression is decreased in DRG neurons (Yang et al. 2004) after spinal cord injury (SCI) chronic pain model. Also a reduction of firing threshold and fast activation of Kv1.4 observed after 4-AP application [255, 256].

Kv4.3 channel knockdown causes hypersensitivity, Kv4 channels are being implicated more directly in the chronic pain development. After the onset of STZ-induced diabetic neuropathy, there are significant declines in putative nociceptors' A-type Kv currents and Kv4 expression [291–293]. The expression of Kv4.2 and Kv4.3 mRNAs in streptozotocin-induced diabetes DRG neurons is decreased by 50%, in addition to a significant decrease in A-type Kv currents [291, 292]. Also, in a chronic pain bone cancer model of, Kv4.3 siRNA injection in the lumbar spinal cord prevents diclofenac from reversing the mechanical allodynia phenotype while having no effect on thermal hyperalgesia [294], in addition to reduced DRG excitability. These various studies associate Kv channels as modulators of inflammatory and neuropathic pain signaling in afferent neurons. Specifically, they suggest a reduction of A-type K^+ currents through the downregulation of Kv channels [254].

In dorsal root ganglion neurons from diabetic rats, KCNQ2/3/5 channel mRNA and protein levels were significantly reduced, which was followed by a decline in I_M density and raises in neuronal excitability [262]. The hyperexcitability decreased after retigabine was used to activate the KCNQ channels, and KCNQ channels inhibition with XE991 increased the hyperexcitability. Also, retigabine treatment decrease both mechanical allodynia and thermal hyperalgesia, in contrast, rats with diabetic neuropathic pain, the antagonist XE991 increased both mechanical allodynia and thermal hyperalgesia [139, 268, 295].

2.5. Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) (figure 7.1) is a neurohormone that is primarily synthesized during the dark period in the pineal gland and released into the circulatory system according to a circadian rhythm [296–298]. These extrapineal sources would contribute little to the plasma concentration of melatonin, however, they would have considerable importance for the paracrine and/or autocrine action of this hormone

[299]. Melatonin cannot be stored at the synthesis site and, therefore, is secreted directly into the cerebrospinal fluid and vascular circulation [300]. Aaron Lerner discovered and isolated it from bovine pineal in 1958 [301]. Ever since melatonin has been researched in a variety of tissues and conditions [302].

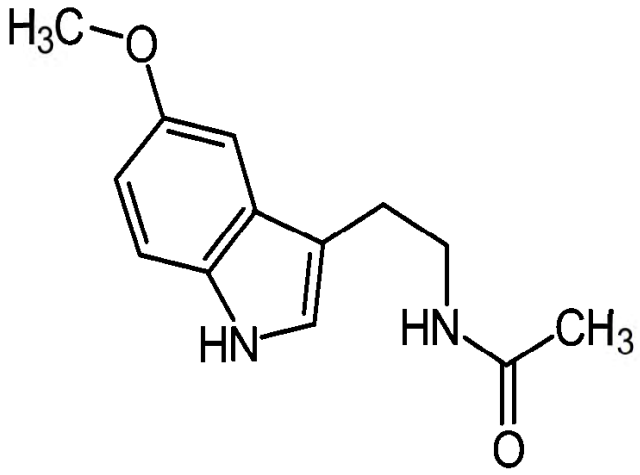


Figure 2.7. *Melatonin Chemical structure (N-acetyl-5-methoxytryptamine) (Tordjman et al., 2017).*

Its main biological function includes the controlling of the circadian rhythm (waking and sleep phase), the improvement of sleep quality [303], seasonal reproduction and the function of the retina [304]. Melatonin also has a number of pharmacological properties, that make it a therapy management of various pathological conditions [305], including anti-inflammatory, anti-apoptotic, antioxidant, anxiolytic, and locomotor activity-regulating [303, 306–308]. Furthermore, a several studies have shown melatonin's effectiveness in treating various pain syndromes [7], because of its antinociceptive [309–312] and antihyperalgesic and analgesic effects [7, 8, 313–315]. Also it has been reported to be neuroprotective against diabetic neuropathy [316, 317].

The study of the relationships between glucose metabolism, diabetes, and the effects of melatonin is a subject of great interest [318]. It has been suggested that treatments with antioxidants may be an remarkable preventive therapeutic option of diabetes-related vascular complications [319]. Melatonin is one of nature's most potent antioxidants that prevents macromolecular oxidative damage, such as proteins, lipids, and nucleic acids [320]. The antioxidant protection of melatonin has been demonstrated both in vivo and in vitro at the levels of the cell membrane, mitochondria, and nucleus [321, 322]. Aside from its role as a free radical scavenger, also superoxide dismutase,

glutathione peroxidase, and glutathione reductase are all antioxidant enzymes that are stimulated, which further promotes its ability to decrease free radical toxicity and their associated reagents [323]. Research has shown that melatonin could restore impaired antioxidant status in streptozotocin-induced diabetic rats [324]. Melatonin has been suggested to be possible effective treatment for hyperglycemia-related symptoms. Likewise, Decreased melatonin synthesis has been reported in several animal models of DM [316, 325], its long-term use reduced hyperlipidemia and hyperinsulinemia while restoring the polyunsaturated fatty acid ratio in diabetic rats' serum and tissues [326] by improving insulin sensitivity and promoting better glycemic control in white adipose tissue [327–329].

2.5.1. Antinociceptive effects of melatonin

Melatonin has complicated effects on spinal nociception that are primarily inhibitory which has been illustrated in behavioral and electrophysiological studies [330, 331]. Evidence suggests that the melatonin receptors on the membrane (MT1 and MT2) found in structures of the nervous system are taking part in nociceptive transmission [310], such as spinal cord's dorsal horn, trigeminal nucleus, trigeminal tract, and the thalamus [332, 333]. In nociceptive, inflammatory, and neuropathic pain models, these receptors are engaged in a critical role in antinociception induced by melatonin [334–337].

Research has shown that melatonin have potent neuroprotective effects in a streptozotocin-induced diabetic neuropathic pain model, according to most recent proof from in vitro and in vivo studies [316, 317]. Melatonin's anti-neuroinflammatory properties make it a powerful modulator in an extensive neurological disorders' variety [307]. Melatonin has been illustrated in preclinical and clinical studies to have anti-allodynia effects in the controlling of neuropathic pain and inflammatory [309–312]. Moreover, there are studies showing the melatonin’s effect on chemotherapy-induced pain. Also, according to some reports, melatonin may reduce opioid-induced hyperalgesia, as well as prevent astrocytic activation in the spinal dorsal horn [313, 314]. Many researchers have recently reported the effectiveness of melatonin in the treatment of NP in which co-administration of melatonin with 4P-PDOT, the MT-2 selective antagonist suppressed the antihyperalgesic and antiallodynic effects of MT in a rat model of capsaicin-induced neuropathic pain [338]. Another study, found that melatonin administration also has antihyperalgesic and antiallodynic effects in cuff-implanted mice

in neuropathic pain behavior by looking at the DRG in a sciatic nerve cuffing, upregulation of MT2 expression in DRG, revealed by western blot showing that DRG MT2 receptors are important in mediating the sensory component of neuropathic pain [339]. Also, in spinal nerve ligation (SNL) model, melatonin administration can significantly reduce tactile allodynia in SNL rats, whereas MT2 and opioid receptor antagonists blocked MT's antiallodynic effects [335].

Melatonin has sparked a lot of interest in recent years. All the evidence points out its antinociceptive properties in various animal models studies, potential beneficial effects after its proven uses to be extremely safe and effective in the treatment of various chronic pain paradigms, especially neuropathic pain. Which accelerate its use clinically for different pathological cases and in undergoing surgery's patients [7–9].

2.5.2. Possible pathways of melatonin’s antinociceptive action

Melatonin's antinociceptive properties are still unclear, although preclinical studies have demonstrated their efficacy in different pain models, various questions about the mechanism of action is still unanswered and not yet fully understood. Melatonin has the potential to interfere through a number of antinociceptive mechanisms, by acting directly through the most importantly MT1/MT2 melatoninergic receptors present in both the spinal cord and the CNS, and indirectly through interaction with a variety of neurotransmitter systems and receptor sites, including opioidergic (δ/κ/μ receptors), adrenergic (α1 and α2 receptors), glutamatergic (NMDA receptor), GABAergic system, dopaminergic (D2 receptor), muscarinic receptor, nicotinic receptor, and nitric oxide (NO)-arginine pathway, with alterations in K^+ ion channels [7, 8, 308, 310, 340–342]. Figure 7.2 showing melatonin's possible mechanisms of antinociception.

Melatonin is widely assumed to have a direct effect on antinociceptive responses via activation of the MT1 and MT2 receptors of melatonin, as evidenced by a number of experimental studies. The MT’s analgesic action in nociceptive pain are primarily selectively mediated by MT2 subtype but not MT1 type receptors [343], this has become possible by using the common melatonin receptor (MT1/MT2) non-selective antagonist luzindole that blocked melatonin's dose-dependent analgesic action [336, 344, 345]., and confirmed by using selective and affinity MT2 receptor antagonists 4P-PDOT [338].

Melatonin's effects are determined by the location and melatonin receptors type [346]. The activation of MT2 and MT1 receptors of melatonin reduces NO-cyclic AMP formation and nociception. Because of its various roles in nociceptive transmission,

melatonin act on NMDA receptor-dependent intracellular NO generating pathways is believed to be implicated for its effect [347]. Thus, one proposed mechanism of action is activation of the cGMP system. Melatonin may thus recapitulate the stimulation of α1-adrenergic, α2-adrenergic, nicotinic, and muscarinic receptors by increasing the NO-cyclic GMP pathway, resulting in antinociception [348]. Melatonin can also indirectly act on opioid receptors to activate them, inhibit the expression of 5-lipoxygenase and cyclooxygenase 2, and act as opener of various K^+ channels. The free radicals have been affected and melatonin acts as a scavenger and the pro-inflammatory cytokines production has been inhibited by modulating GABAA receptor function [310].

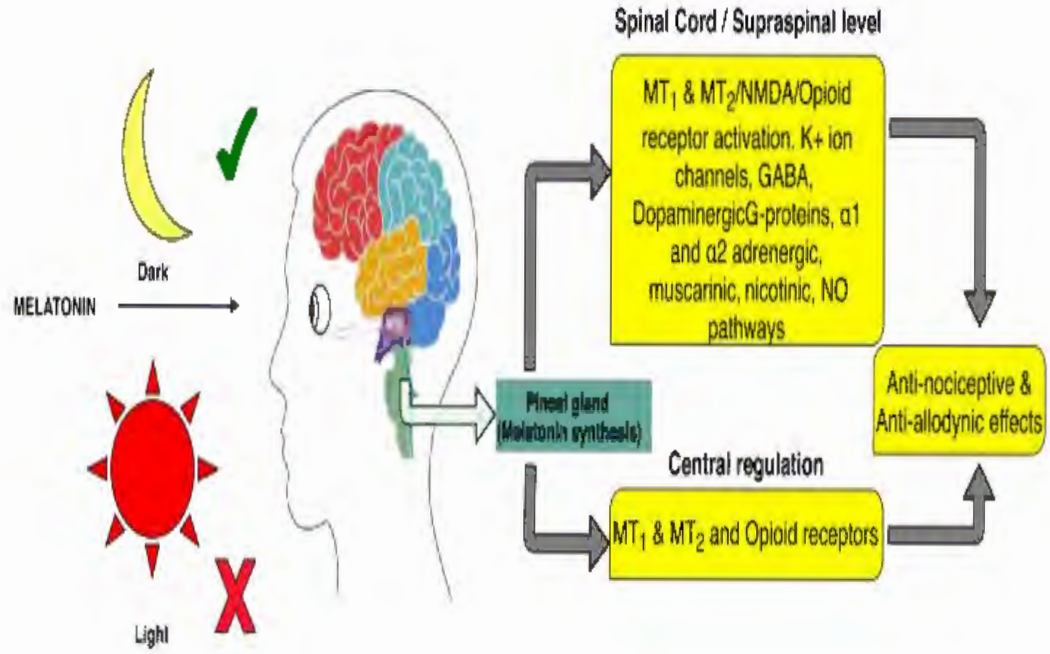


Figure 2.8. *The possible mechanisms through with melatonin promotes anti-allodynic and anti-nociceptive effects (Kuthati et al., 2019).*

2.5.3. Various electrophysiological characteristics of melatonin

Electrophysiological and behavioral investigations have demonstrated that melatonin has several effects which mainly have inhibitory features regarding spinal nociception [312, 330, 331]. Studies reporting that melatonin has a decreasing effect on the neuronal firing rate which suggests an inhibitory effect on excitability supporting that concept [349–351]. Recently, melatonin has been found to reduce excitability in DRG neurons with inflection on the repolarization phase of the AP in DRG neurons. In that

study it was suggested that melatonin has a greater pharmacological potency on N_{inf} neurons and considering the fact that this type of neurons is related to nociception, for the preclinical research concerning the therapeutic use of melatonin, this type of DRG neurons should be utilized. Authors suggested that melatonin may act on DRG on the modulation of specific type of pain sensation [23]. In another study, in DRG neurons of rats, applied melatonin (1.0–1000.0 nM) has been found to block the generation of APs (a marker for an inhibitory activity on the excitability of the neurons) in a concentration dependent manner. It has affected the passive properties such as RMP and the input resistance, causing a hyperpolarization and an increase, respectively. Also, in the same study, it was also shown that melatonin altered the active electrophysiological parameters of the AP, amplitude of the action potential was shown to be increased by melatonin. These effects were demonstrated to be blocked in the presence of luzindole, a non-selective melatonin receptor antagonist [352, 353], indicating hormonal effects of melatonin, and when it binds to MT_1 receptors the effect is induced, since from the data obtained via quantitative PCR, it was concluded that DRG expresses MT_1 receptors rather than MT_2 [327].

There are several reports regarding melatonin's effects on $Ca2^+$ currents. The effects of melatonin on high-voltage activated calcium channels (HVACC) in DRG neurons were evaluated using the whole-cell patch clamp technique. Extracellular application of melatonin has been demonstrated to inhibit HVACC in a dose dependent manner. Authors stated that even though the certain physiological importance of melatonin inhibitory actions on HVACC is not clear, it may have antinociceptive effects [22]. Melatonin supplementation has been found to decrease DRG neuron Ca^{2+} influx through TRPM2 and voltage gated calcium channels in wireless (2.45 GHz)-induced oxidative injury [24]. Melatonin has been shown also to modulate TRPM2 and TRPV1 channels in DRG neurons of diabetic rats. This modulation, by regulating the involvement of these channels to Ca^{2+} entry, was suggested to be the reason for melatonin to show neuroprotective activity [354].

K^+ currents in DRG neurons have also been studied in several research concerning pain. An inhibition on K^+ currents such as A-current, delayed rectifier and Ca^{2+}-sensitive K^+ currents were suggested to be linked to persistent increases in primary afferent excitability in neuropathic pain [229, 355, 356]. All mentioned studies are in parallel with the fact that an increase in general activity of the neuron in DRG is a marker for pain.

Several effects on K^+ currents of melatonin have been introduced. Melatonin has been shown to open several K^+ channels [310], by increasing the delayed rectifier outward K^+ channels in primary culture cerebellar granule cells without changing activation and inactivation properties [357]. Also it has been illustrated to activate an outward K^+ current and inhibit I_h, hyperpolarization activated inward cation current, in suprachiasmatic nucleus neurons [358]. Besides, 2-iodomelatonin, a high affinity melatonin agonist, has been demonstrated to inhibit A-type transient outward K^+ currents [359, 360].

Since effects of melatonin on electrophysiological properties of DRG and other neurons have been suggested to prone to be inhibitory in several studies, unveiling its effects on K^+ currents appear to be an important step for further investigations.

3. MATERIALS AND METHODS

3.1. Experimental Animals

All animal experimental protocol and care procedures were performed strictly in accordance with the Directive 2010/63/EU of the European Parliament and of the Council and approved (Decision No: 2021-35 and 2022-07) by the Local Ethics Committee of Anadolu University, Eskisehir, Turkey.

8-12 weeks-old male Sprague-Dawley (SD) rats weighing 250–300 g were used (n=8). Animals were housed maximum 5 per cage with free access to food (standart chow) and drinking water. Rats were acclimated for one week prior to behavioral experiments in a quiet animal breeding room with a 12-hour light/dark cycle (8 a.m. to 8 p.m.) under temperature control (23 ± 2 °C) and relative humidity (50 ± 10%). All in vivo experiments were conducted in a double-blind manner from 9:00 a.m. to 5:00 p.m.

3.2. Chemicals

Used chemicals are listed in (Table 3.1.).

Table 3.1. *List of used chemicals*

Name of Chemical	Supplier
Melatonin (MT)	Acros organics part of Thermo Fisher Scientific (Geel, Belgium)
Gabapentin (GBP)	ChemCruz, Santa Cruz Biotechnology Inc. (Dallas, TX, USA)
Yohimbine hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Prazosin hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Propranolol hydrochloride	Sigma-Aldrich (St. Louis, MO, USA)
Streptozotocin (STZ)	Sigma-Aldrich (St. Louis, MO, USA)
Ketamine	Richter Pharma ag, Austria / Interhas A.S. (Ankara, Turkey)
Xylazine	Bioveta a.s., Czech Republic / Intermed Ecza Deposu (Ankara, Turkey)
Citric acid monohydrate	Sigma-Aldrich (St. Louis, MO, USA)
Trisodium citrate dihydrate	Sigma-Aldrich (St. Louis, MO, USA)
Physiological saline solution (0.9% sodium chloride)	Polifarma (Istanbul, Turkey)
Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO, USA)
Phosphate buffered saline (PBS)	Sigma-Aldrich (St. Louis, MO, USA)
Dulbecco's Modified Eagle's Medium (DMEM**)**	Sigma-Aldrich (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	Sigma-Aldrich (St. Louis, MO, USA)
Penicillin- streptomycin	Sigma-Aldrich (St. Louis, MO, USA)
Trypsin 0.25%	Sigma-Aldrich (St. Louis, MO, USA)
Collagenase type IV	Sigma-Aldrich (St. Louis, MO, USA)
Potassium chloride (KCl)	Wisent (Quebec, Canada)
Sodium chloride (NaCl)	Wisent (Quebec, Canada)
Mg-ATP	Wisent (Quebec, Canada)
EGTA	Wisent (Quebec, Canada)
HEPES ACID	Wisent (Quebec, Canada)
D-glucose	Wisent (Quebec, Canada)
Potassium hydroxide (KOH)	Wisent (Quebec, Canada)
Magnesium chloride ($MgCl_2$)	Wisent (Quebec, Canada)
Calcium chloride ($CaCl_2$)	Wisent (Quebec, Canada)
Tetraethylammonium chloride (TEA)	Sigma-Aldrich (St. Louis, MO, USA)
Tetrodotoxin (TTX)	Sigma-Aldrich (St. Louis, MO, USA)

3.3. Apparatus

Used apparatus are listed in (Table 3.2).

Table 3.2. *List of used apparatus*

Name of the Apparatus	Brand and Model
Electronic Von Frey device	Ugo Basile, No: 38450 (Gemonio, VA, Italy)
Hargreave's thermal testing device	Ugo-Basile, No:37370 (Gemonio, VA, Italy)
Activity cage-locomotor activity apparatus	Ugo-Basile, No:7441 (Gemonio, VA, Italy)
Micropipette Puller	Sutter Instrument, Model: P-97 (Novato, CA,USA)
Patch Amplifier	Sutter Instrument, Model: IPA (Novato, CA,USA)
Anti-vibration table	Kinetic Systems, Inc , Model: 9100 S (Boston, MA,USA)
Glass pipettes	Sutter Instrument, Model: BF150-110-10 (Novato, CA,USA)
Microscope	Sutter Instrument, Model: Sutter BOB™ (Novato, CA,USA)
Motorized Micromanipulator	Sutter Instrument, Model: MP-285 (Novato, CA,USA)
Multi-Micromanipulator System	Sutter Instrument, Model: MPC-385 (Novato, CA,USA)
Perfusion system	Sutter Instrument, (Novato, CA,USA)
Analytic lab balance	Ohaus, No: E 12140 (Greifensee, ZRH, Switzerland)
Ultrasonic water bath	Bandelin Sonorex , No:3-4D-12207 (Berlin, Germany)
ACCU-CHEK Active glucometer device	Roche, No:5237 (Basel, Switzerland)

3.4. Experimental groups and drug administration

To assess the antiallodynic and antihyperalgesic effects of melatonin and its mechanism of action, rats were divided into 15 groups. (n = 8 in each). These groups are respectively;

1. (Healthy control) group: 4 weeks after the citrate buffer (*i.v.*) injection, rats were received vehicle (*i.p.*) for 14 days,

2. (Healthy+MT-50) group: 4 weeks after the citrate buffer (i.v.) injection, rats were received melatonin (50 mg/kg, *i.p.*) for 14 days.

3. (DM control) group: 4 weeks after STZ (*i.v.*) injection, rats were received vehicle (*i.p.*) for 14 days,

4. (DM+MT-50) group: 4 weeks after STZ (*i.v.*) injection, rats were received melatonin (50 mg/kg, *i.p.*) for 14 days,

5. (DM+GBP) positive control group: 4 weeks after STZ (*i.v.*) injection, rats were received gabapentin (50 mg/kg, *i.p.*) for 14 days,

6. (PROP+Vehicle) group: pretreatment of nonselective β-adrenergic antagonist (5 mg/kg, *i.p.*) propranolol, 30 min before the last vehicle administration,

7. (PROP+MT-50) group: pretreatment of nonselective β-adrenergic antagonist (5 mg/kg, *i.p.*) propranolol, 30 min before the last melatonin administration,

8. (YOH+Vehicle) group: pretreatment of α2-adrenergic antagonist (4 mg/kg, *i.p.*) yohimbine, 30 min before the last vehicle administration,

9. (YOH+MT-50) group: pretreatment of α2-adrenergic antagonist (4 mg/kg, *i.p.*) yohimbine, 30 min before the last melatonin administration,

10. (PZ+Vehicle) group: pretreatment of α1-adrenergic antagonist (10 mg/kg, *i.p.*) prazosin, 30 min before the last vehicle administration,

11. (PZ+MT-50) group: pretreatment of α1-adrenergic antagonist (10 mg/kg, *i.p.*) prazosin, 30 min before the last melatonin administration,

12. (Healthy control patch clamp+ MT-10 μM) group,

13. (Healthy control patch clamp+ MT-100 μM) group,

14. (DM control patch clamp+MT-10 μM) group,

15. (DM control patch clamp+MT-100 μM) group,

The mechanical and thermal thresholds of all animals included in the experimental groups were measured with the following devices and recorded as baseline values just before the STZ injection at week 0. Before the start of drug applications after STZ injection at week 4, behavioral tests again measured thermal and mechanical thresholds and evaluated the development of neuropathy.

4 weeks after diabetes induction, subacute melatonin, gabapentin, and vehicle treatments will be started and will continue for 14 days [10, 15, 361] and were administered every morning between 9:00 and 09:15 A.M. throughout the experimental

protocol. At the end of week 6, 24 h after administration of last dose, the behavioral experiments were performed again and the effect of the drug was evaluated.

For in vivo experiments, melatonin will only be administered at a dose of 50 mg/kg, which is often accepted as a possible effective dose in the literature and in current studies, first dissolved in ethanol and diluted to 1% (v/v) in physiological saline solution final concentration. Rats in the vehicle control groups daily received equivalent volumes of 1% ethanol in saline [11, 12, 362]. As a diabetic positive control group, gabapentin (50 mg/kg, *i.p.*) used as a reference drug for neuropathic pain assessment, since it has been demonstrated to induce antihyperalgesic, antiallodynic, and neuroprotective effects in NP [363, 364]. Gabapentin dissolved in saline [365, 366]. Yohimbine, prazosin, and propranolol were dissolved in distilled water [367, 368]. All drug applications were administered intraperitoneally (*i.p.*).

For electrophysiological experiments, melatonin was dissolved in absolute ethanol and daily stock solutions were prepared. The final ethanol concentration never exceeded 0.04% (v/v) [23]. Standard external solution was used to dilute the stock solution for patch clamp recordings to get 10 μM and 100 μM final concentrations of melatonin and was applied by perfusion [357]. Also TEA (5 mM) was dissolved in the bath solution [369].

3.5. Experimental Methods

3.5.1. Establishment of diabetes

Rats in the diabetic groups will be fasted overnight before the administration of a single (50 mg/kg, *i.v.*) dose of STZ (prepared in a 0.1 M citrate buffer, pH of 4.5) in the tail vein [370]. Following STZ injection, 5 mmol/L glucose solution will be provided in the waterers to reduce or prevent hypoglycemic shock and hyperinsulinemia caused by STZ is due to its cytotoxicity to pancreatic β cells [132, 371]. 72 h after injection, animals were fasted overnight and blood samples were obtained by nicking the lateral tail vein using a sterile surgical scissors and the blood sugar measurements will be done [363] with ACCU-CHEK® Active glucometer (Roche, No:5237, Basel, Switzerland). Animals with blood glucose levels over 300 mg/dl will be considered as diabetic. All healthy rats used as control of diabetic rats will be injected intravenously (*i.v.*) with the same volume of citrate buffer [15, 361].

Time for development of nociceptive perception deficits in diabetic rats has been suggested to be 4 weeks. Presence of neuropathic pain will be tested with e-Von Frey and Hargreave's tests in all experimental groups before starting subacute melatonin, gabapentin and vehicle administration (at 4th week following STZ injection) [15, 361] and taking the dorsal root ganglion (DRG) neurons for electrophysiological studies.

3.5.2. Neuropathic pain tests

3.5.2.1. *Evaluation of mechanical allodynia (e-Von Frey test)*

Pain thresholds of rats to mechanical stimulus were measured using an electronic Von Frey device (Ugo Basile, No: 38450, Gemonio, VA, Italy) (Figure 3.1.). As much as the electronic Von Frey device's capacity [1=1000 gram force (gf)] allows, continuous force is applied under user control and automatically records the animal's response. Mechanical allodynia was assessed by determining the rat's paw withdrawal threshold in response to mechanical stimulus developed using the electronic von Frey device. Before the mechanical allodynia assessment, rats were placed in special plastic cages placed on a perforated metal platform and acclimatized for 15-30 minutes. Mechanical stimulation was generated by rapidly increasing force under operator control by means of a rigid stainless steel filament applied perpendicularly to the mid-plantar surface of of the left hind paw. The force (gram=gf) causing the animal to pull its paw was recorded by the device. The average of 3-4 consecutive measurements taken at 3-minute intervals for each rat was evaluated as the animal's withdrawal threshold. In order to prevent tissue damage in the paw, the cut-off point was determined as 50 gf [372].

Figure 3.1. *E-von Frey apparatus*

3.5.2.2. *Evaluation of thermal hyperalgesia (Hargreave's test)*

Pain thresholds of rats to thermal stimulus were measured with the plantar test-Hargreaves apparatus (Ugo-Basile, No:37370, Gemonio, VA, Italy) (Figure 3.2.). Thermal hyperalgesia was evaluated over the rat's hind paw withdrawal latency in response to thermal stimulus. Before starting the test, rats were placed in their transparent plastic cages placed on a special glass platform and acclimated to the environment for 15-30 minutes. A high-intensity movable radiant heat source was placed under the glass platform coinciding with the mid-plantar surface of the hind paw of the rat, and it was monitored whether the animal was motionless during the measurement. The time from the onset of radiant heat until the rat pulls its paw is automatically recorded by the device. The average of 3-4 consecutive measurements taken at 3-minute intervals for each rat was evaluated as the animal's withdrawal threshold. Due to the infrared heat intensity of the plantar test device, it prevents damage to the paw tissue of the animal. The cut-off time of the experiment was determined as 30 seconds in order to pass the test [12].

Figure 3.2. *Hargreave's apparatus*

Percentage of maximum possible impact (%MPE), based on the thresholds obtained from electronic von Frey and plantar tests, was used for analyses and it was calculated using the following formula [373]:

%MPE = [(post-drug measured threshold – pre-drug measured threshold) / (cut off value – drug pre-measured threshold)] x 100 (3.1)

3.5.3. Evaluation of locomotor activity (*Activity cage test)*

The spontaneous locomotor activities of rats were evaluated by the activity cage-locomotor activity apparatus (Ugo-Basile, No:7441, Gemonio, VA, Italy) with a plexiglass walls and 40 × 40 × 31 cm dimensions (Figure 3.3.). Horizontal and vertical movements of the animals interrupted the infrared rays, detected by two sets of emitter/sensor arrays. These interruptions were automatically counted and recorded by the unit's internal memory allow the user to evaluate and analyze animal activity. Locomotor activities will be recorded for a period of 15 min before e-Von Frey and Hargreave's tests [374]. The locomotor activity studies were repeated for 3 times such behavioral tests (week 0, 4 and 6).

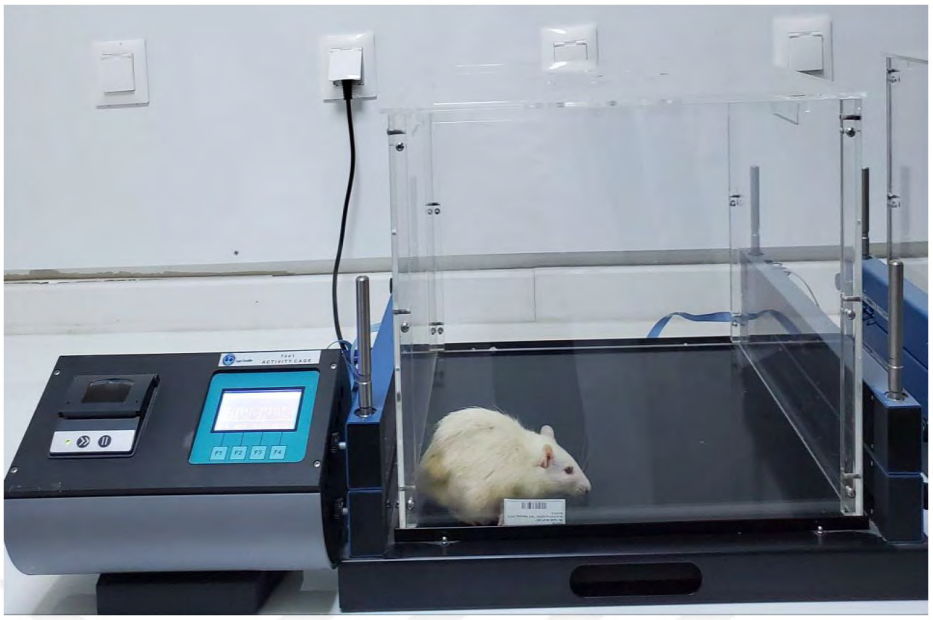


Figure 3.3. *Activity cage apparatus*

3.5.4. Investigation of adrenergic mechanism

In order to investigate the possible contribution of adrenergic receptors to the antiallodynic and antihyperalgesic effects of melatonin in diabetic neuropathic pain, following the 14 days melatonin administration, propranolol (a non-selective β-adrenergic receptor blocker, 5 mg/kg, *i.p.*) [375], yohimbin (α2-adrenergic receptor blocker, 4 mg/kg, *i.p.*) [367], and prazosin (α1-adrenergic receptor blocker, 10 mg/kg, *i.p.*) [376], were injected 30 minutes before the last melatonin or vehicle administrations. All of the behavioral tests were performed 30 min after these administrations [15, 363, 364].

3.6. Electrophysiological studies

3.6.1. DRG dissection and primary DRG cell culture preparation

For a full anesthesia with a duration of 5 to 8 minutes, the rat was anesthetized using a 90 mg/kg ketamine + 10 mg/kg xylazine mixture (1 ml/ kg, *i.p.*) administered [377]. After the animal was completely under anesthesia, the vertebral column was exposed through a back skin incision, then removed and transferred directly into a 50 ml falcon tube filled with ice cold PBS for 5 minutes before beginning DRG collection [378–380].

DRG extraction and mechanical digestion of the ganglia were completed in a laminar flow cabinet in order to avoid contamination and ensure working in sterility. At

4 C° the vertebral column is placed in a petri dish filled with DMEM solution; iris scissors were used to cut the vertebral column into two symmetrical pieces from the centerline (Figure 3.4.). After carefully removing the spinal cord, small-medium diameter DRGs were isolated using a surgical lab tweezer, individual ganglia were isolated and placed in a petri dish containing DMEM+Penicillin-Streptomycin [378, 380] (Figure 3.5.). To avoid debris or other types of cells in the obtained cell culture, DRGs were cleaned as thoroughly as possible with a surgical lancet and iris scissor [378, 380]. The enzymatic digestion began when clean ganglia were placed in an Eppendorf tube filled with solution of 2 mg of collagenase type IV dissolved in 1 ml of DMEM+Penicillin-Streptomycin and allowed to incubate for 45 minutes at 37 °C and 5% CO2. Every 10 minutes, the ganglia were resuspended [380, 381].

The supernatant was discarded after the first incubation period and three washing cycles with PBS (discard the supernatant, add 1 ml of PBS and centrifuge for 30 s) were carried out, washed ganglia were then placed in 1 ml of DMEM+Penicillin-Streptomycin containing 100 µl of 0.25% Trypsin and 6 minutes incubation, DRGs are resuspended after 3 minutes [378, 380, 381]. Another three DMEM washing cycles were performed (discard the supernatant, add 1 ml of DMEM and centrifuge for 45 to 60 s), then the trypsin digested DRGs were then mixed with 1 ml of DMEM in an Eppendorf tube before being transferred to a 15 ml falcon tube containing 1 ml of DMEM for a total volume of 2 ml of DMEM ganglia solution contained [378, 380, 381].

Mechanical digestion was performed sequentially by gentle pipetting at a rate of 4 times per minute for 5 minutes with a cut 1 ml blue tip, followed by 5 minutes with a non-cut 1 ml blue tip [378].

Finally, the DRGs cell suspension was transferred into 12.5 ml of DMEM+Penicillin-Streptomycin + FBS for 1 ml of cells [380, 381]. Before performing electrophysiological recordings, the cells were given a 2 to 3 hour rest period.

A significant proportion of the neurons obtained from each animal's DRGs will be formed in the diabetic control and healthy control groups, in which the test substance will be administered in the chamber [382, 383].

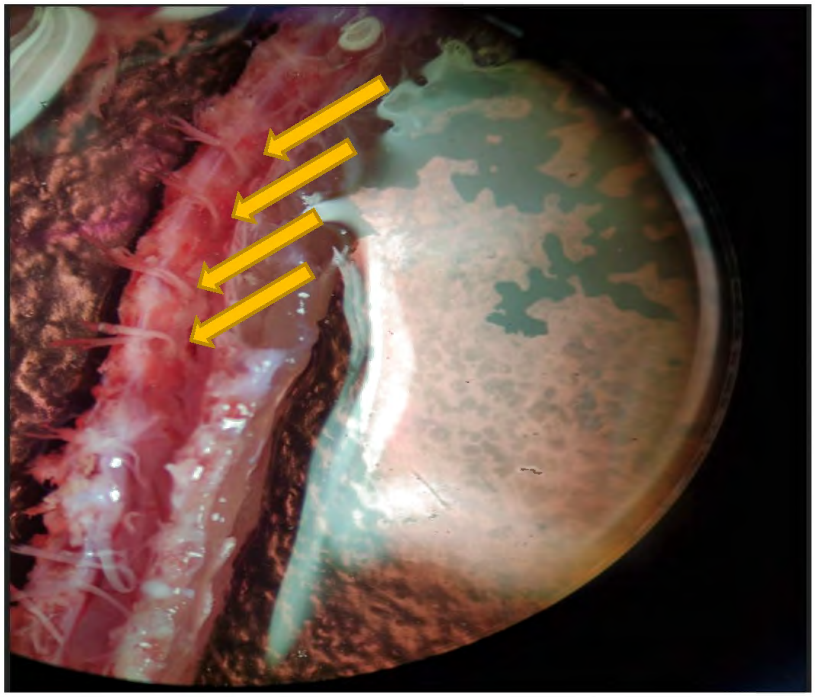


Figure 3.4. *DRG's in vertebral column under stereomicroscope*

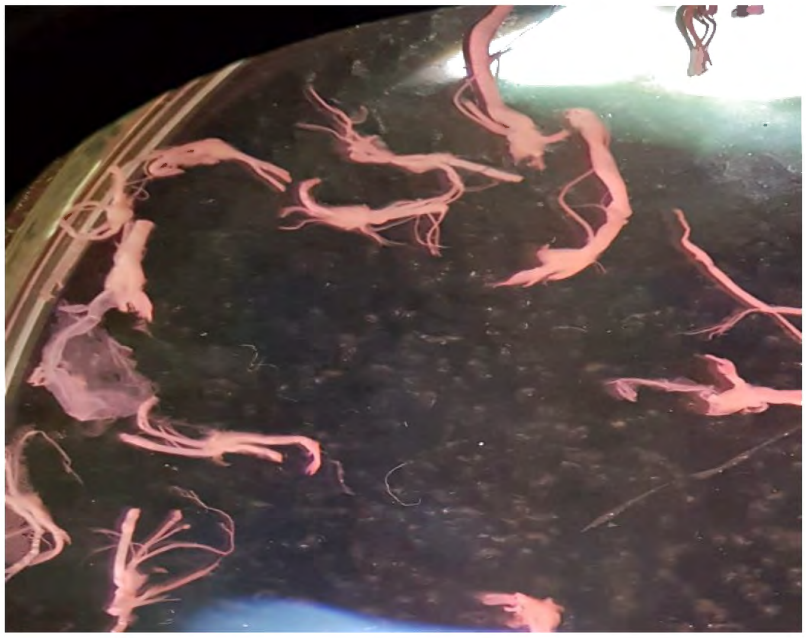


Figure 3.5. *Isolated whole DRG's*

3.6.1.1. *Current clamp recording solutions*

Table 3.3 shows the internal solution (pipette solution) used in current clamp mode for AP recordings, as well as the concentrations of its components, and the pH was adjusted to 7.3- 7.4 with KOH [384].

Table 3.3. *Pipette solution for AP recordings*

Internal solution components (313 mOsm)	**Concentration (mM)**
HEPES Acid	10
D-glucose	10
NaCl	10
EGTA	5
Mg-ATP	4
KCl	130

Table 3.4 shows the external solution (bath solution) used in current clamp mode for AP recordings, as well as the concentrations of its constituents, and the pH was adjusted to 7.4 with NaOH [384].

Table 3.4. *Bath solution for AP recordings*

External solution components (321 mOsm)	**Concentration (mM)**
HEPES Acid	10
D-glucose	10
KCl	5
$CaCl_2$	2.5
$MgCl_2$	1.2
NaCl	140

3.6.1.2. *Voltage clamp recordings*

Table 3.5 shows the internal solution (pipette solution) used in voltage clamp mode for current recordings, as well as the concentrations of its components, and the pH was adjusted to 7.2 with KOH [384].

Table 3.5. *Pipette solution for currents recordings*

Internal solution components (310 mOsm)	Concentration (mM)
KCl	140
NaCl	10
HEPES Acid	10
D-glucose	3
EGTA	1.1
$MgCl_2$	2
$CaCl_2$	0.1

Table 3.6 shows the external solution (bath solution) used in voltage clamp mode for current measurements, as well as the concentrations of its constituents, and the pH was adjusted to 7.3 with NaOH [357, 384].

Table 3.6. Bath solution for currents recordings

External solution components (320 mOsm)	Concentration (mM)
NaCl	140
KCl	3
HEPES Acid	10
D-glucose	10
$MgCl_2$	1
$CaCl_2$	1
TTX	0.001

3.6.2. Patch-clamp recordings

A pipette filled with conductive solution is placed on the ion channel-containing cell membrane. The pipette is linked to an amplifier, which both imposes currents and measures potentials (current clamp) or imposes potentials and measures currents (voltage clamp). Leaks must therefore be avoided [385, 386].

The patch-clamp technique was used to record the whole-cell currents in dissociated small-medium diameter DRG neurons cell culture after 2-3 hours of resting [387] at room temperature (23.6 ± 0.4°C) [369]. This technique is obtained through applying powerful suction to the membrane, causing it to rip. Measurements of global potentials and currents are possible because the pipette is in contact with the entire cell cytoplasm. Also this

method authorizes for the injection of substances into the cytoplasm for experimentation [385, 386, 388] (Figure 3.6.).

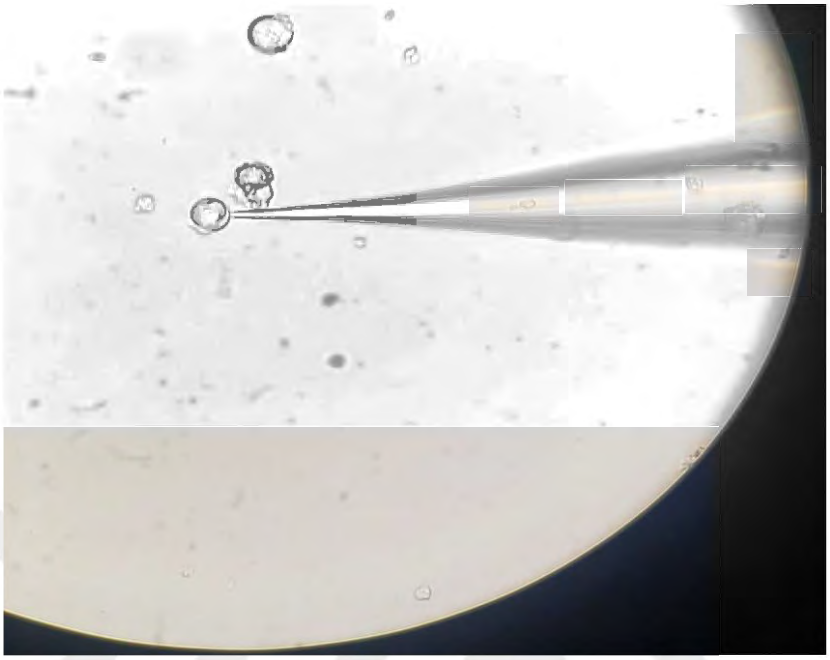


Figure 3.6. *Pipette in contact with DRG cell*

Melatonin's effect on the outward K^+ channel current was investigated; the standard pipette solution was used for the whole-cell voltage-clamp recording model. TTX (1 mM) was added to the bath solution to remove the voltage-gated inward sodium current [357]. And for only recording the transient outward K^+ current (A-type), to inhibit K-type K^+ current, TEA (5 mM) was added to the bath solution [369]. For a longer effect, 10 μM and 100 μM of melatonin was applied via perfusion. During the experiment, the bath solution was subjected to gravity perfusion to washout out after drug application [357]. As shown in (Figure 3.7.) the electrophysiological rig used in the patch clamp experiments.

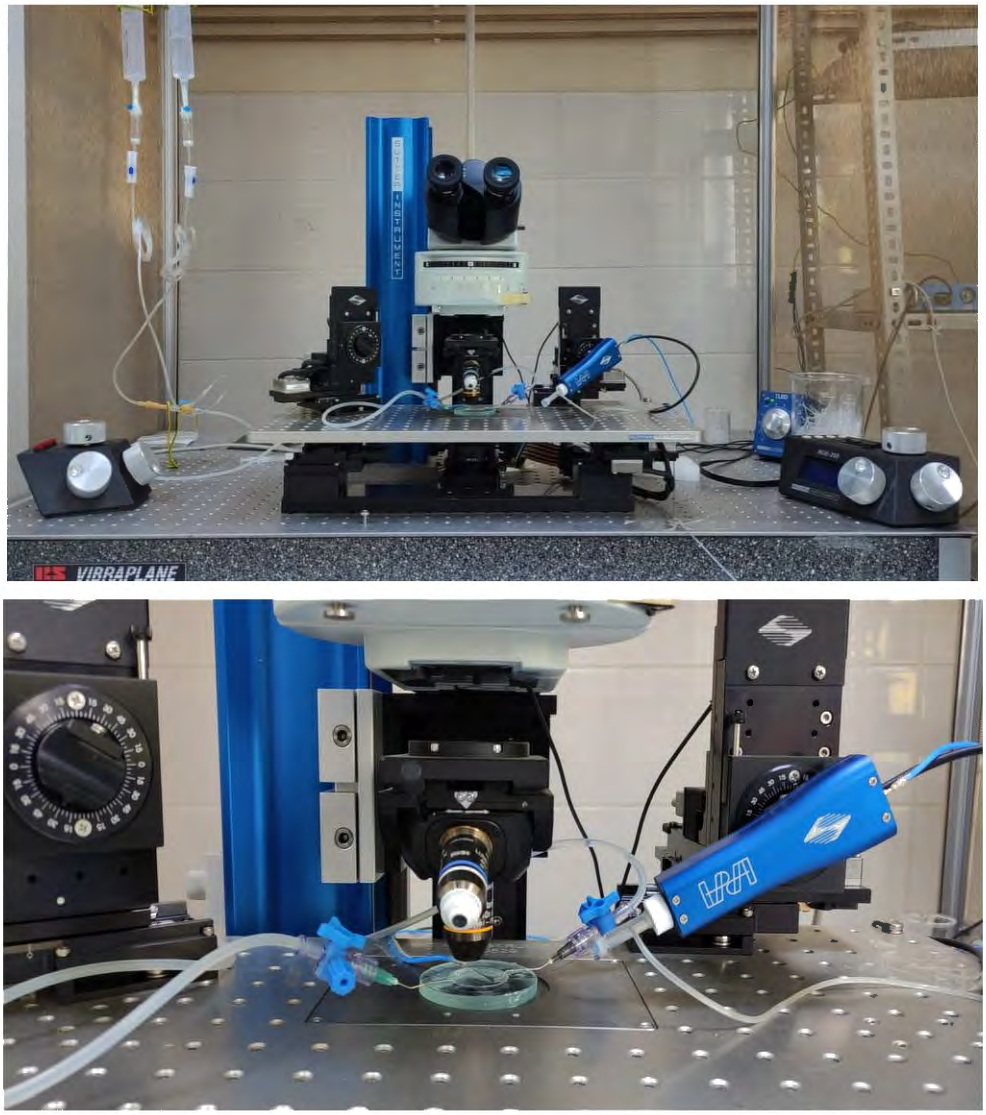


Figure 3.7. *Electrophysiology rig*

3.6.2.1. *Voltage-clamp recording technique*

Voltage-clamp ion current recordings from acutely dissociated DRG cells were made in whole-cell configuration. A thin wall glass pipette (Sutter Instrument BF150-110-10) has been used to form GΩ-seals and pulled using P-97 Micropipette Puller (Sutter Instrument). The pipette was filled with the internal solution for voltage calm recording and the pipette resistance was 2-5 MΩ.

By applying a negative pressure with a 1 ml syringe or through the mouth, the transition to whole-cell configuration was accomplished. By a significant decrease in resistance in series to around 10 MΩ and an increase in capacitance of the membrane, the passage to the entire cell is distinguished. Conduction of these recordings was at room temperature [388].

Following clamping the membrane potential to −60 mV, 300 ms depolarizing pulses to 0 mV were used. The current-voltage curve (I-V curve) depolarization is produced in 10 mV increments from −60 mV to +60 mV. Melatonin was perfused after obtaining a stable outward current in response to multiple depolarizing steps to 0 mV [388].

Currents were measured in voltage-clamp mode using a single headstage version of the Integrated Patch Amplifier (IPA) (Sutter Instrument). Data Acquisition and Analysis Software (SutterPatch 2.0.4) installed on Windows®10. Data were sampled at 25 kHz and filtered at 5 kHz using the IPA's built-in filter. Using the software's automatic compensation option, electrode compensation and series resistance compensation were applied automatically. The same software was used to analyze the data (SutterPatch 2.0.4).

3.6.2.2. *Current-clamp recording technique*

The voltage variation of acutely dissociated DRG cells was recorded using current-clamp recordings in whole cell configuration. GΩ-seals were created by filling Thin Wall glass filamented pipette (Sutter Instrument BF150-110-10) with the internal solution for current-clamp recordings and pulling them with a P-97 Micropipette Puller (Sutter Instrument). The final a pipette resistance was 4-6 MΩ. The moving to whole cell configuration was accomplished by applying negative pressure either by mouth or with a 1 ml syringe and was noticeable by a significant decrease in series resistance to around 10 MΩ and an increase in membrane capacitance. The recordings were made at room temperature.

Data was collected in current-clamp mode with a single headstage version of the Sutter Instrument Integrated Patch Amplifier (IPA), SutterPatch® Data Acquisition and Analysis Software (SutterPatch 2.0.4) installed on Windows®10. The acquisition sampling rate was 25 kHz, and the IPA's built-in filter was used to filter it at 5 kHz. During a few minutes of whole-cell recording, spontaneous firing activity was monitored. The AP threshold was determined by depolarizing current steps of 10 pA for 10 ms ranging from 0 pA to 300 pA, and the minimum injected current amplitude that elicited an AP was chosen [388].

The value obtained was used to elicit APs several times; once a consistent outcome is obtained, melatonin is perfused and changes in signal amplitude, half-width, fast AHP,

ADP, and medium AHP are monitored. The same software was used to analyze the data (SutterPatch 2.0.4).

3.7. Statistical Analysis

Statistical analysis of the in vivo data was performed with the Graphpad Prism (ver. 5.03) program. One-way ANOVA, followed by post-hoc Tukey HSD multiple comparison test have been used to evaluate the MPE% values of melatonin effects in e-Von Frey and plantar tests. Data from devolpment of NP, locomotor activity and mechanism studies were evaluated with two-way ANOVA followed by Bonferroni multiple comparison tests. Results are given as mean ± standard error of mean. A value of $p<0.05$ was considered significant.

OriginPro 2021 (64-bit) 9.8.0.200 (Learning Edition), copyright ©1991-2020 OriginLab corporation, and GraphPad prism (ver. 8.02) programs have been used in in vitro data. Electrophysiological data are given as mean ± standard error of the mean. Unpaired Student's t-test was used when comparing DM cells with healthy control cells to evaluate Ap and AP parameters, also paired student t-test has been used to compare the effect of the drug on the same cell. A value of $p <0.05$ was considered significant.

4. RESULTS

4.1. Behavioral results

4.1.1. Devolopment of neuropathic pain

The changes in the mechanical and thermal thresholds occurred by injection of citrate buffer or STZ after 4 weeks in the Figure (4.1.A) and (4.1.B), respectively. As expected, it has been observed that mechanical and thermal threshold were significantly decreased only in STZ injected groups at the 4^{th} week compared to self-baseline values at 0^{th} week ($^{***}P < 0.001$) and also significantly decreased compared to two healthy groups (healthy control and healthy+MT-50 groups) at the 4^{th} week ($^{+++}P< 0.001$ and $^{<<<}P< 0.001$, respectively). (Fig. 4.1.A.: Treatment: $F[10,77] = 14.00$; $P < 0.001$, Time factor: $F[1,77] = 69.61$; $P < 0.001$, Interaction: $F[10,77] = 15.56$; $P < 0.001$ and Fig. 4.1.B.: Treatment: $F[10,154] = 16.86$; $P < 0.001$, Time: $F[1,154] = 66.99$; $P < 0.001$, Interaction: $F[10,154] = 15.06$; $P < 0.001$, respectively).

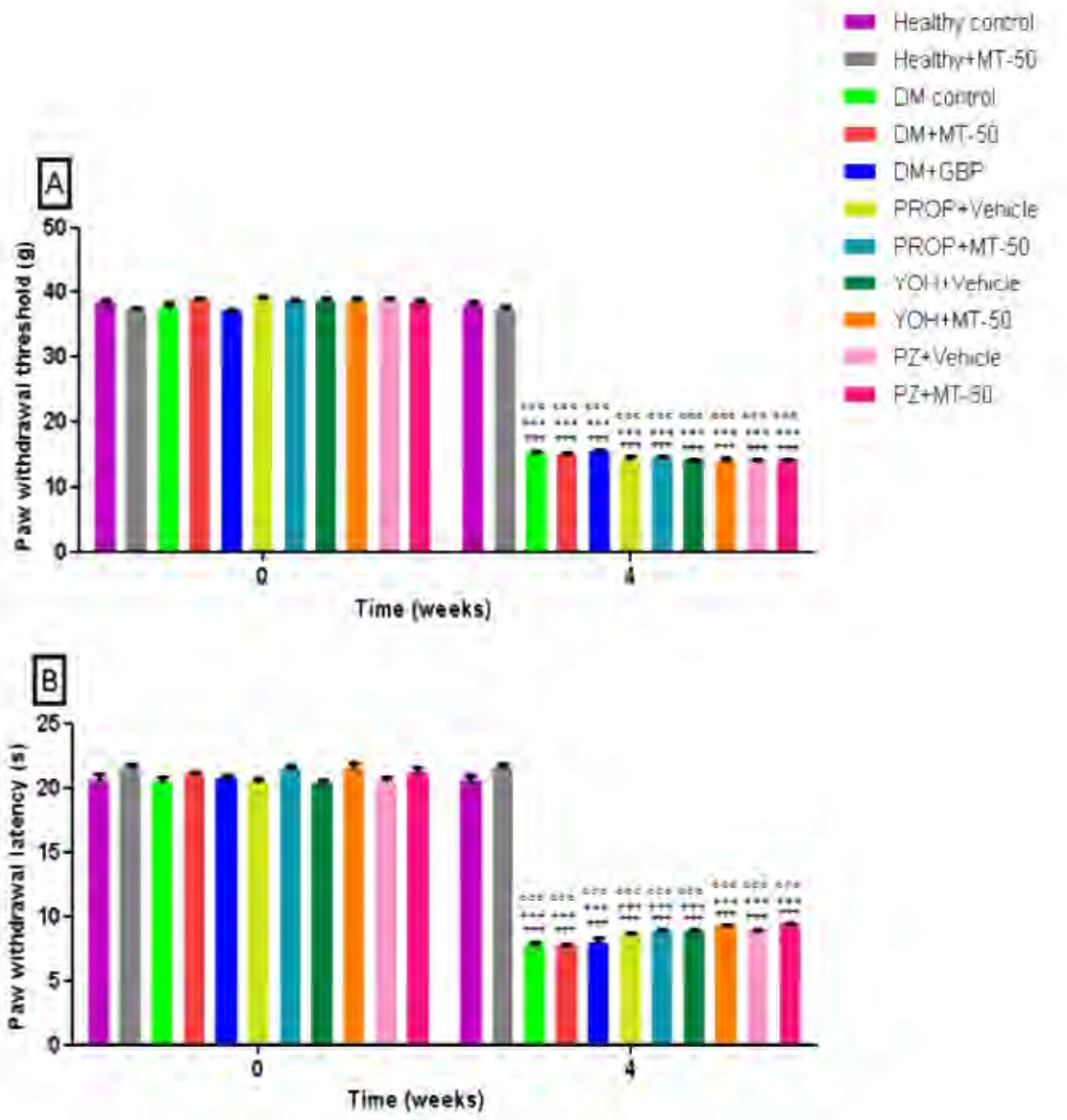


Figure 4.1. *The mechanical (A) and thermal (B) thresholds of the experimental groups at the 0th and 4th weeks. DM: Diabetes Mellitus; MT: Melatonin; GBP: Gabapentin; PROP; Propranolol; YOH; Yohimbine; PZ; Prazosin. ***P<0.001; significance of STZ injected groups compared to self-baseline values at the week 4. $^{+++}$P<0.001; significance compared to the healthy control group at week 4. $^{<<<}$P<0.001; significance compared to the (Healthy+MT-50) group at week 4. Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.2. Evaluation of antiallodynic activity

MPE % values at week 6 and the paw withdrawal threshold values measured at 4th and 6th week in healthy control and diabetic rats assessed in the e-Von Frey test following subacute administration of vehicle, melatonin (50 mg/kg) and gabapentin (50 mg/kg) are displayed in (Fig. 4.2 and 4.3).

In figure 4.2 the MPE % values calculated of Healthy+MT-50 group was significantly ($^{+++}P<0.001$) increased compared to healthy control group. Similarly, the MPE % values of both DM+MT-50 and DM+GBP were significantly ($^{***}P<0.001$) increased compared to the DM control group (Fig. 4.2: $F[4,35] = 186.4$; $P < 0.001$).

The figure 4.3 shows the effects of 14 days of subacute treatments on paw withdrawal thresholds. The significant ($^{***}P<0.001$) enhancements in paw withdrawal thresholds were demonstrated in Healthy+MT-50, DM+MT-50 and DM+GBP groups comparing between the 4th and 6th weeks. However, as expected paw withdrawal threshold was decreased significantly ($^{**}P<0.01$) in DM control group. At week 6 compared to Healthy control group, while the paw withdrawal threshold values of group Healthy+MT-50 was found to be significantly ($^{+++}P<0.001$) high, the threshold values of groups DM+MT-50 and DM+GBP groups were found to be significantly ($^{+++}P<0.001$) low. Additionally, the paw withdrawal thresholds of both DM+MT-50 and DM+GBP groups were significantly ($^{\&\&\&}P<0.001$) increased compared to DM control group (Fig. 4.3: Treatment: $F[4,35] = 82.88$; $P < 0.001$, Time: $F[1,35] = 7.96$; $P < 0.001$, Interaction: $F[4,35] = 8.50$; $P < 0.001$).

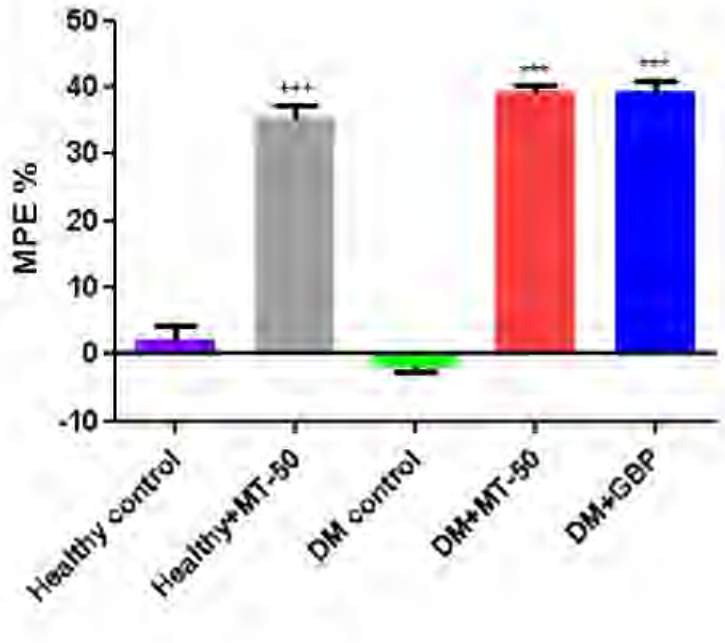


Figure 4.2. *MPE% values of healthy rats injected vehicle (Healthy control), and 50 mg/kg melatonin (Healthy+MT-50), diabetic rats injected vehicle (DM control), 50 mg/kg melatonin (DM+MT-50) and 50 mg/kg gabapentin (DM+GBP) in the E-von frey test.* $^{***}P<0.001$*; significance compared to DM control group,* $^{+++}P<0.001$*; significance compared to the healthy control group, One-way ANOVA, followed by post-hoc Tukey HSD multiple comparison test were applied using ± S.E.M. values (n=8).*

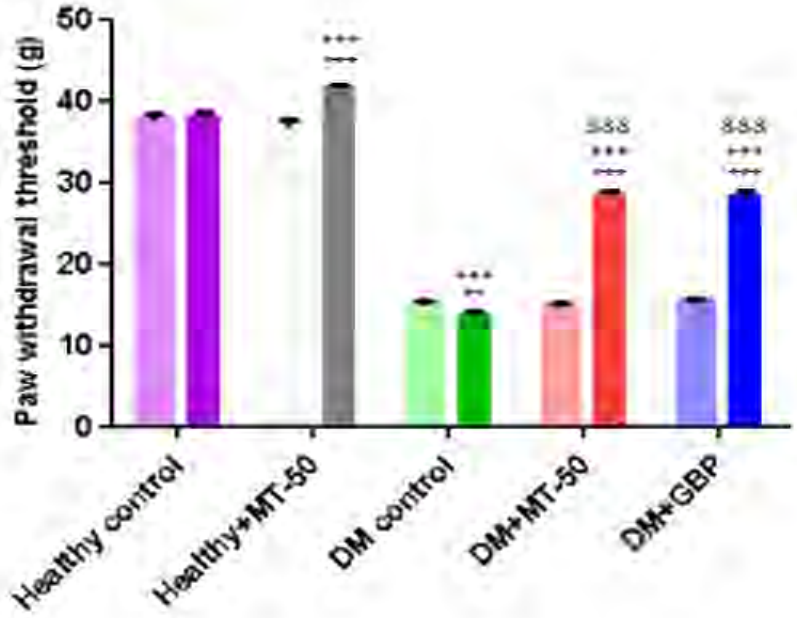


Figure 4.3. *Paw withdrawal thresholds (g) of healthy rats injected vehicle (Healthy control), and 50 mg/kg melatonin (Healthy+MT-50), diabetic rats injected vehicle (DM control), 50 mg/kg melatonin*

*(DM+MT-50) and 50 mg/kg gabapentin (DM+GBP) in the E-von frey test. ***P<0.001; significance between the 4th and 6th weeks of groups themselves, +++P<0.001; significance compared to the healthy control group at week 6, &&& P<0.001; significance compared to the DM control group at week 6, Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.3. Evaluation of antihyperalgesic activity

MPE % values at week 6 and the paw withdrawal threshold values measured at week 4 and 6 in the Hargreave's test (plantar test) of healthy control and diabetic rats following subacute administration of vehicle, melatonin (50 mg/kg) and gabapentin (50 mg/kg) are displayed in (Fig. 4.4 and 4.5).

In figure 4.4, the MPE % of Healthy+MT-50 group was significantly ($^{+++}P<0.001$) increased compared to healthy control group. Similarly, the MPE % of both DM+MT-50 and DM+GBP were significantly ($^{***}P<0.001$) increased compared to the DM control group. Besides, the significant ($^{\&\&\&}P<0.001$) enhancement was found in MPE% of DM+MT-50 group compared to Healthy+MT-50 group (Fig. 4.4: $F[4,35] = 128.1$; $P < 0.001$).

The figure 4.5 shows the effects of 14 days of treatments on paw withdrawal latencies. The significant ($^{***}P<0.001$) enhancements in paw withdrawal latencies were demonstrated in Healthy+MT-50, DM+MT-50 and DM+GBP groups comparing between the 4th and 6th weeks. However, as expected paw withdrawal latency was decreased significantly ($^{**}P<0.01$) in DM control group. At week 6 compared to Healthy control group, while the paw withdrawal latency values of group Healthy+MT-50 was found to be significantly ($^{+++}P<0.001$) high, the threshold values of groups DM+MT-50 and DM+GBP groups were found to be significantly ($^{+++}P<0.001$) low. Additionally, the paw withdrawal latencies of both DM+MT-50 and DM+GBP groups were significantly ($^{\&\&\&}P<0.001$) increased compared to DM control group (Fig. 4.5: Treatment: $F[4,35] = 80.70$; $P < 0.001$, Time: $F[1,35] = 8.10$; $P < 0.001$, Interaction: $F[4,35] = 9.75$; $P < 0.001$).

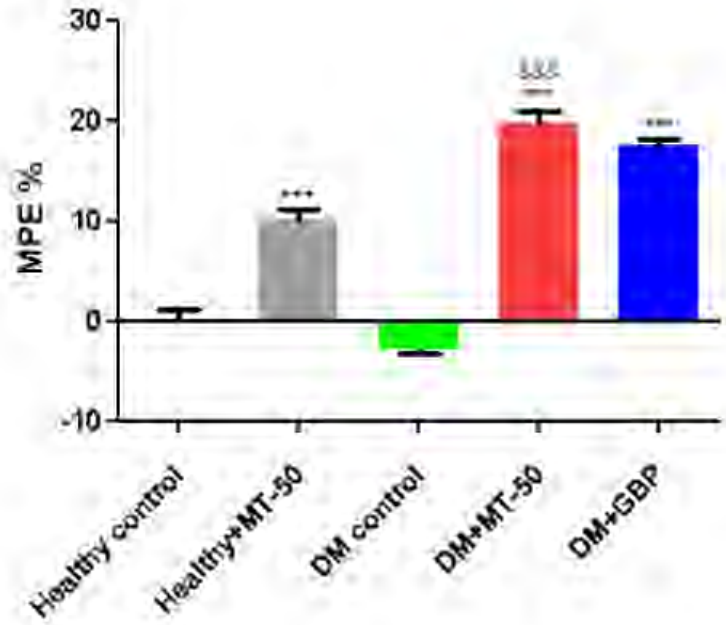


Figure 4.4. *MPE% values of healthy rats injected vehicle (Healthy control), and 50 mg/kg melatonin (Healthy+MT-50), diabetic rats injected vehicle (DM control), 50 mg/kg melatonin (DM+MT-50) and 50 mg/kg gabapentin (DM+GBP) in the Hargreave's test (plantar test).* $^{***}P<0.001$*; significance compared to DM control group,* $^{+++}P<0.001$*; significance compared to the healthy control group,* $^{\&\&\&}P<0.001$*; significance compared to the Healthy+MT-50 group. One-way ANOVA, followed by post-hoc Tukey HSD multiple comparison test were applied using ± S.E.M. values (n=8).*

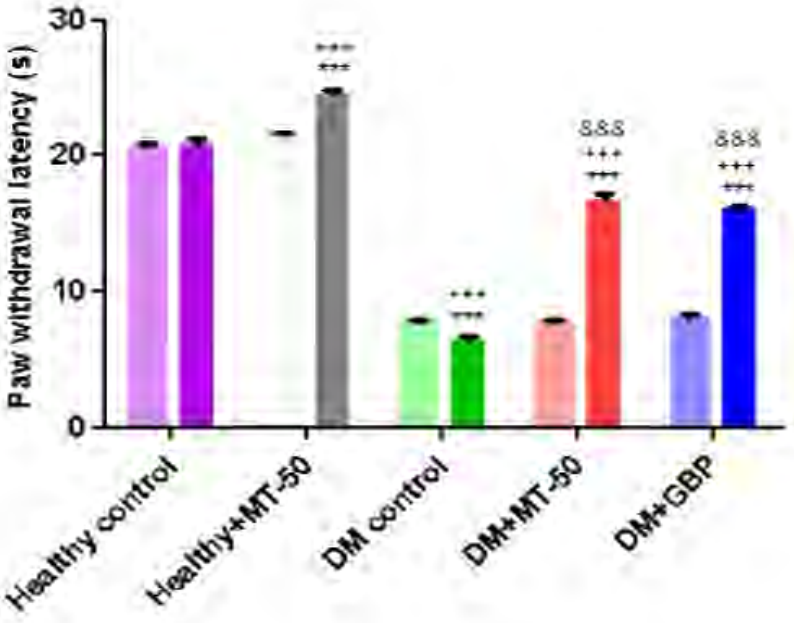


Figure 4.5. *Paw withdrawal latencies(s) of healthy rats injected vehicle (Healthy control), and 50 mg/kg melatonin (Healthy+MT-50), diabetic rats injected vehicle (DM control), 50 mg/kg melatonin (DM+MT-50) and 50 mg/kg gabapentin (DM+GBP) in the Hargreave's test (plantar test).* $^{***}P<0.001$*; significance between the 4th and 6th weeks of groups themselves,* $^{+++}P<0.001$*; significance compared to the healthy control group at week 6,* $^{\&\&\&}P<0.001$*; significance compared to the DM control group at week 6, Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.4. Locomotor activity

Figure 4.6 shows melatonin's effects (50 mg/kg) treatment on the horizontal (A) and vertical (B) locomotor activity counts of rats assessed in the activity cage tests. After 4 weeks, the spontaneous locomotor activities for all diabetic groups, were significantly lower compared to healthy control group at week 4 ($^{***}P<0.001$) and also week 6 ($^{\wedge\wedge\wedge}P<0.001$). Interestingly, at week 6, DM+MT-50 and DM+GBP groups did induce significant additional changes in the locomotor activity (horizontal: $^{+++}P< 0.001$ and $^{+++}P< 0.001$, respectively; vertical: $^{+++}P< 0.001$ and $^{+}P< 0.05$, respectively) compared to DM control group. Comparison between the 4th and 6th weeks of groups themselves, the significant enhancements was shown in DM+MT-50 and DM+GBP groups (horizontal: $^{\&\&\&}P< 0.001$ and $^{\&}P< 0.05$, respectively; vertical: $^{\&\&\&}P< 0.001$ and $^{\&\&}P< 0.01$, respectively) (Fig. 4.6.A.: Treatment: $F[4,35] = 44.24$; $P < 0.001$, Time: $F[2,70] = 22.96$; $P < 0.001$, Interaction: $F[8,70] = 21.82$; $P < 0.001$ and Fig. 4.6.B.: Treatment: $F[4,35] =$

44.75; P < 0.001, Time: F[2,70] = 22.71; P < 0.001, Interaction: F[8,70] = 19.59; P < 0.001, respectively).

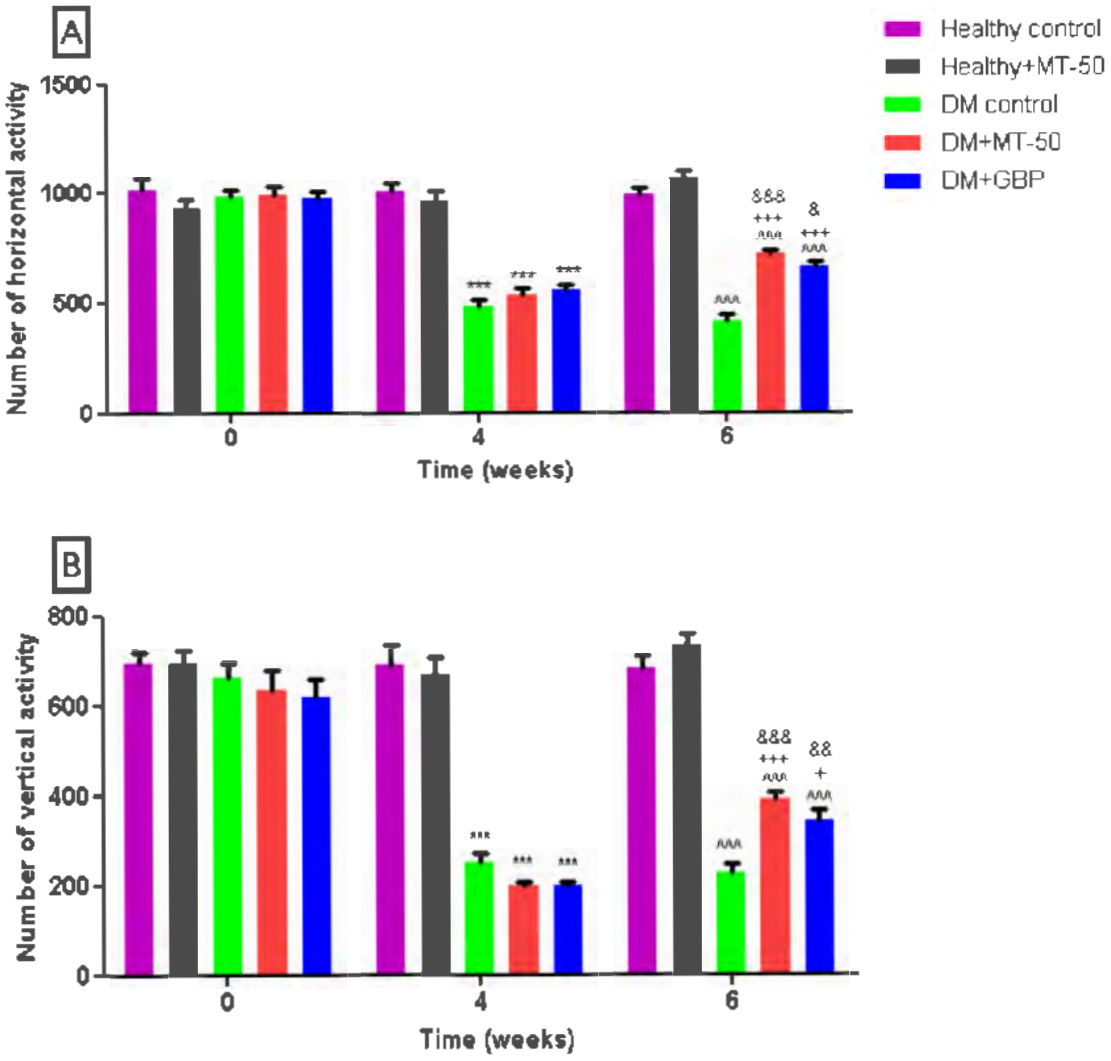


Figure 4.6. *Evaluation of melatonin effects on locomotor activities measured in the activity cage test. Number of horizontal (A) and vertical (B) activities for healthy rats injected vehicle (Healthy control), and 50 mg/kg melatonin (Healthy+MT-50), diabetic rats injected vehicle (DM control), 50 mg/kg melatonin (DM+MT-50) and 50 mg/kg gabapentin (DM+GBP) at 0^{th} , 4^{th} and 6^{th} weeks*** $P<0.001$; significance compared to the healthy control group at week 4, ^^^ $P<0.001$; significance compared to the healthy control group at week 6. + $P<0.05$, +++ $P<0.001$; significance compared to DM control group at week 6. & $P<0.05$, && $P<0.01$, &&& $P<0.001$; significance between the 4th and 6th weeks of groups themselves. Two way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.5. Adrenergic mechanism studies

4.1.5.1. *Effect of propranolol administration*

Figure 4.7. shows the effect of propranolol pretreatment on the antiallodynic response (A) and antihyperalgesic response (B) induced by the administration of 50 mg/kg melatonin (MT-50).

Antiallodynic and, also antihyperalgesic effects of alone MT-50 group showed significantly ($^{***}P<0.001$) increase compared to alone vehicle group. Propranolol pretreatment was antagonized the antiallodynic and antihyperalgesic effects of melatonin ($^{\&\&\&}P<0.001$) compared to alone MT-50. Moreover, the antiallodynic and antihyperalgesic effects of pretreated melatonin group was remained statistically significant ($^{+++}P<0.001$) compared to pretreated vehicle group (Fig. 4.7.A.: Treatment: $F[1, 28] = 76.93$; $P < 0.001$, Antagonist: $F[1, 28] = 11.51$; $P < 0.001$, Interaction: $F[1, 28] = 10.85$; $P < 0.001$ and Fig. 4.7.B.: Treatment: $F[1, 28] = 81.96$; $P < 0.001$, Antagonist: $F[1, 28] = 5.70$; $P < 0.001$, Interaction: $F[1, 28] = 7.97$; $P < 0.001$, respectively).

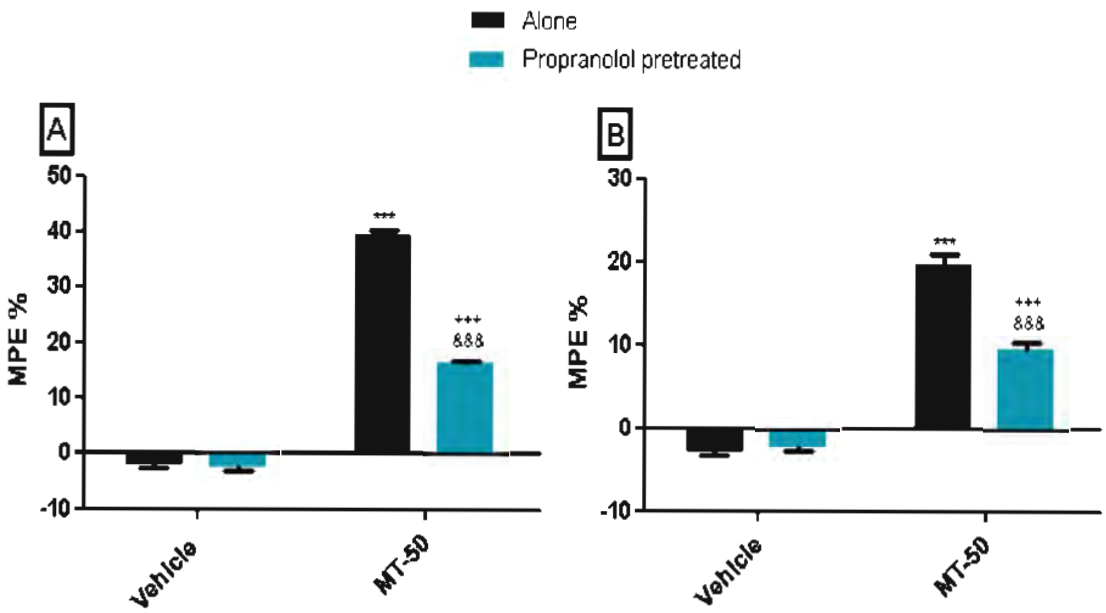


Figure 4.7. *Effect of propranolol treatment on the effect of melatonin (50 mg/kg, 14 d) in E-von frey test (A) and Hargreave's test (plantar test) (B). ***P < 0.001; significant differences based on comparisons to the alone vehicle group. &&&P < 0.001 significant differences based on comparisons to the alone MT-50 treated group. +++P < 0.001; significant differences based on comparisons to the propranolol pre-treated vehicle group. Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.5.2. *Effect of yohimbine administration*

Figure 4.8. exhibits the effect of yohimbine administration on the antiallodynic response (A) and antihyperalgesic response (B) induced by the administration of 50 mg/kg melatonin (MT-50).

Antiallodynic and, also antihyperalgesic effects of alone MT-50 group showed significantly ($^{***}P<0.001$) increase compared to alone vehicle group. Pretreatment with yohimbine antagonized the antiallodynic and antihyperalgesic effects of melatonin ($^{\&\&\&}P<0.001$) compared to alone MT-50. Moreover, the antiallodynic and antihyperalgesic effects of pretreated melatonin group was remained statistically significant ($^{+++}P<0.001$) compared to pretreated vehicle group (Fig. 4.8.A.: Treatment: $F[1,28] = 72.36$; $P < 0.001$, Antagonist: $F[1,28] = 11.62$; $P < 0.001$, Interaction: $F[1,28] = 14.15$; $P < 0.001$ and Fig. 4.8.B.: Treatment: $F[1,28] = 68.71$; $P < 0.001$, Antagonist: $F[1,28] = 11.89$; $P < 0.001$, Interaction: $F[1,28] = 15.30$; $P < 0.001$, respectively).

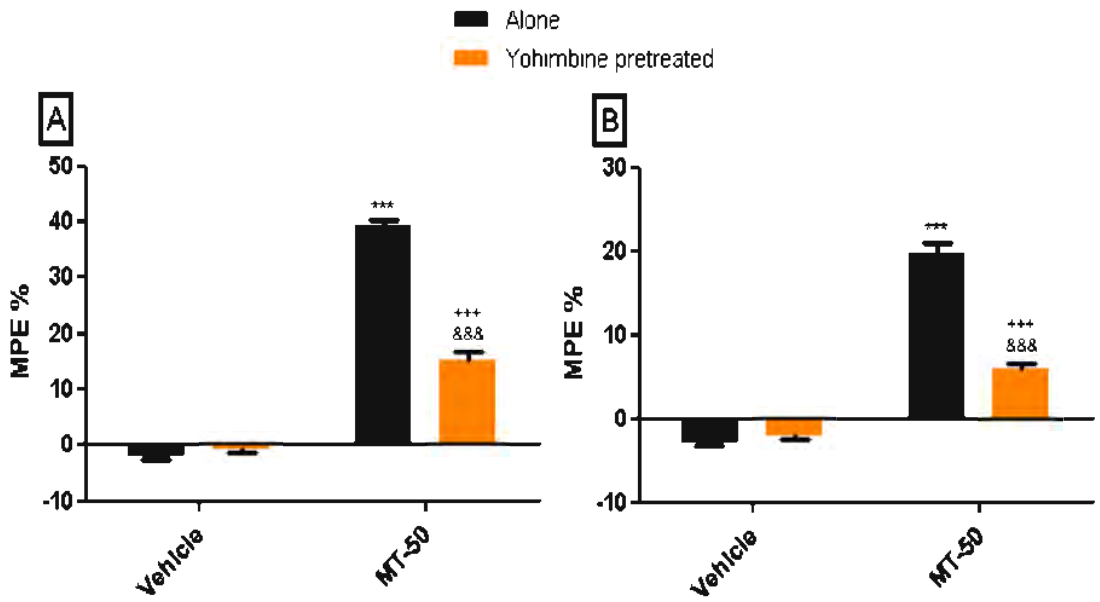


Figure 4.8. *Effect of yohimbine treatment on the effect of melatonin (50 mg/kg, 14 d) in E-von frey test (A) and Hargreave's test (plantar test) (B).* $^{***}P < 0.001$*; significant differences based on comparisons to the alone vehicle group.* $^{\&\&\&}P < 0.001$ *significant differences based on comparisons to the alone MT-50 treated group.* $^{+++}P < 0.001$*; significant differences based on comparisons to the yohimbine pre-treated vehicle group. Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.1.5.3. *Effect of prazosin administration*

Figure 4.9. demonstrates the effect of prazosin administration on the antiallodynic response (A) and antihyperalgesic response (B) induced by the administration of 50 mg/kg melatonin (MT-50).

Antiallodynic and, also antihyperalgesic effects of alone MT-50 group showed significantly ($^{***}P<0.001$) increase compared to alone vehicle group. Administration of prazosin reversed the antiallodynic and antihyperalgesic effects of melatonin ($^{\&\&\&}P<0.001$) compared to alone MT-50. Moreover, the antiallodynic and antihyperalgesic effects of pretreated melatonin group was remained statistically significant ($^{+++}P<0.001$) compared to pretreated vehicle group, even prazosin shows a significantly increase ($^{***}P<0.001$) in the prazosin pretreated vehicle group in the antiallodynic and antihyperalgesic effects (Fig. 4.9.A.: Treatment: $F[1,28] = 89.35$; $P < 0.001$, Antagonist: $F[1,28] = 1.08$; $P < 0.001$, Interaction: $F[1,28] = 8.49$; $P < 0.001$ and Fig. 4.9.B.: Treatment: $F[1,28] = 81.93$; $P < 0.001$, Antagonist: $F[1,28] = 1.52$; $P = 0.0029$, Interaction: $F[1,28] = 12.56$; $P < 0.001$, respectively).

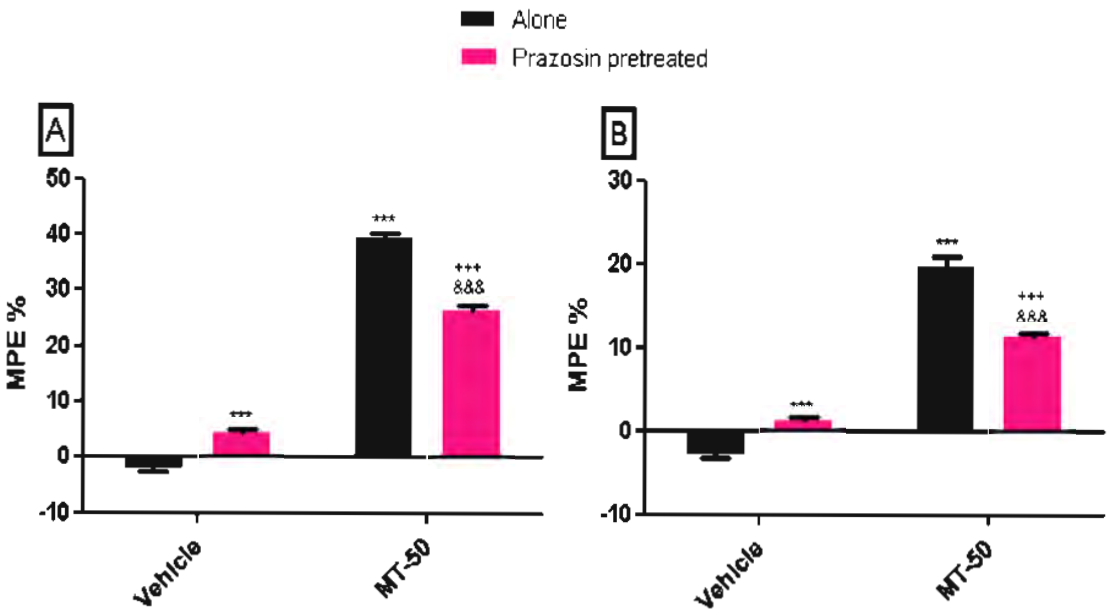


Figure 4.9. *Effect of prazosin treatment on the effect of melatonin (50 mg/kg, 14 d) in E-von frey test (A) and Hargreave's test (plantar test) (B).* $^{***}P < 0.001$*; significant differences based on comparisons to the alone vehicle group.* $^{\&\&\&}P < 0.001$ *significant differences based on comparisons to the alone MT-50 treated group.* $^{+++}P < 0.001$*; significant differences based on comparisons to the prazosin pre-treated vehicle group. Two-way ANOVA followed by Post hoc Bonferroni test were applied using ± S.E.M. values (n=8).*

4.2. Electrophysiological results

4.2.1. Potassium current recordings

4.2.1.1. *Effect of melatonin on K^+ current in DM DRG cells*

The whole-cell voltage-clamp recording technique was performed with the standard pipette solution to study the melatonin's effect on the outward K^+ currents. The external solution contained TTX (1 mM) to remove the voltage-gated inward sodium current. As two types of outward K^+ currents, a delayed rectifier K^+ current and a transient outward K^+ current, were expressed on the rat DRG neurons, TEA (5 mM) the specific blocker of delayed rectifier outward K^+ current was used in the bath solution. Under this condition, delayed rectifier K^+ current was inactivated and inhibited, while a fast activate and inactivate outward K^+ current 'transient' A-type currents (I_A) current was evoked by 300 ms depolarizing pulses from the holding potential of −60 mV to +60 mV at a 15 s interval. The effect of melatonin on K^+ current generally very rapidly occurred, reached its maximal effect within 50s, and then recovered almost to the control level after washout. The effect of melatonin on the K^+ current was dose-dependent (Fig. 4.10.F).

Melatonin application reversibly increased the current amplitude of transient K^+ I_A current in diabetic DRG cells tested (Fig. 4.10). The percentage of activation induced by 100 µM concentration melatonin significantly activated the potassium current by 36.2% (9.37 ± 0.28 pA, 14.70 ± 0.47 pA, $P<0.001$). Also, 10 µM significantly affected I_A density by 25.6% (9.37 ± 0.28 pA, 12.61 ± 0.30 pA, $P<0.001$).

Figure 4.11 displays the effects of 10 and 100 µM melatonin application on K^+ activation current traces in DM DRG cells, causing a significant activation of the K^+ peak current between DM control cells before and after application of melatonin in a dose-dependent manner ($^{***}P<0.001$).

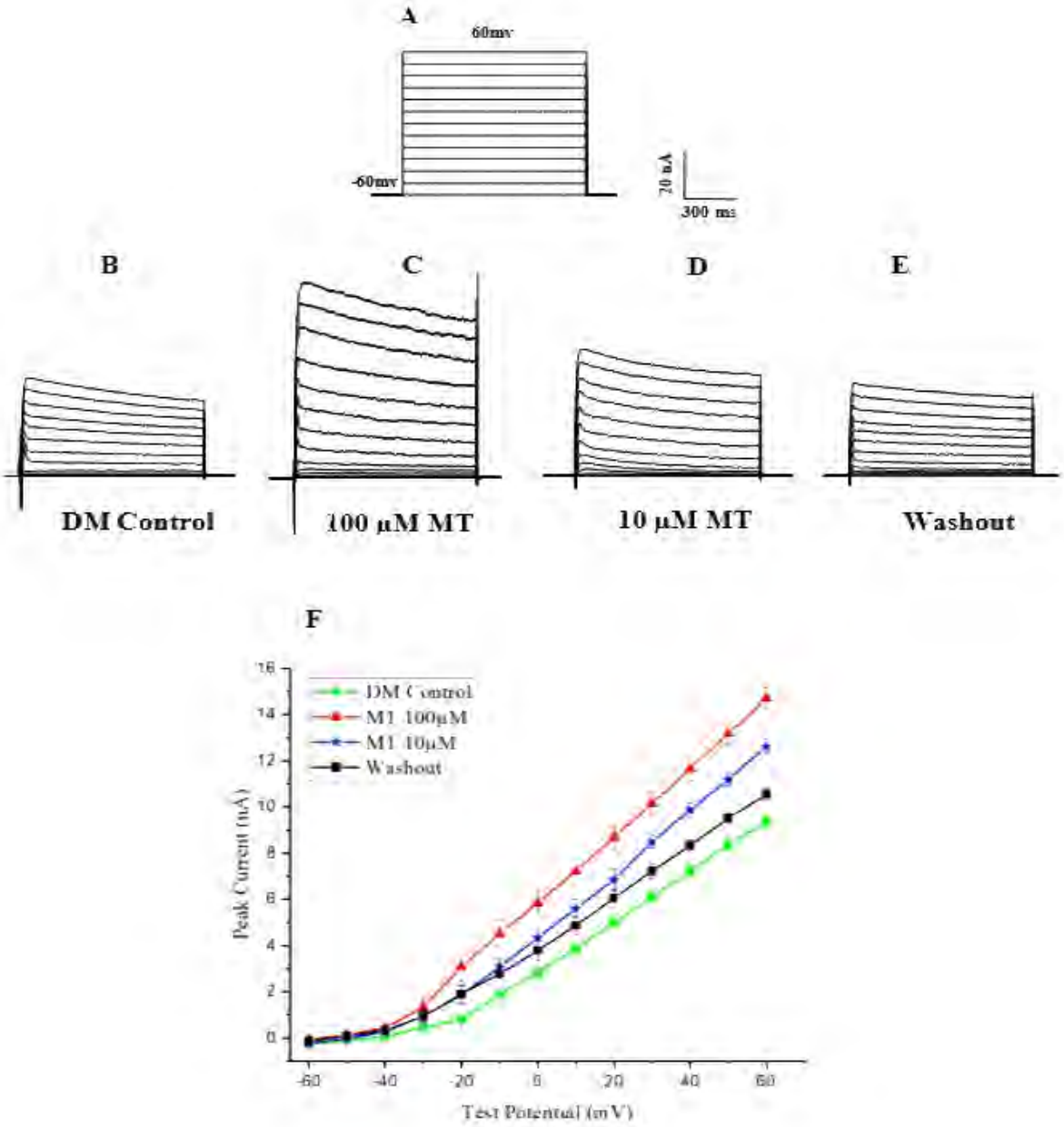


Figure 4.10. *Effect of 100, 10µM MT on the activation of I_A current in DM control DRG cells. (A) Potential steps to elicit the current, in which the cells were evoked by depolarizing pulses ranging from -60 to +60 mV in steps of 10 mV every 15s from a holding potential of -60 mV. (B)DM control DRG neurons, (C) DM+100 µM MT, (D) DM+10 µM MT, and (E) washout. (F) Summarized current-voltage (I-V) relationships of I_A currents before and after application of 10, 100 µM MT and washout. Paired Student's t-test were applied using ± S.E.M. values (n = 10 for 100 µM, n=8 for 10 µM).*

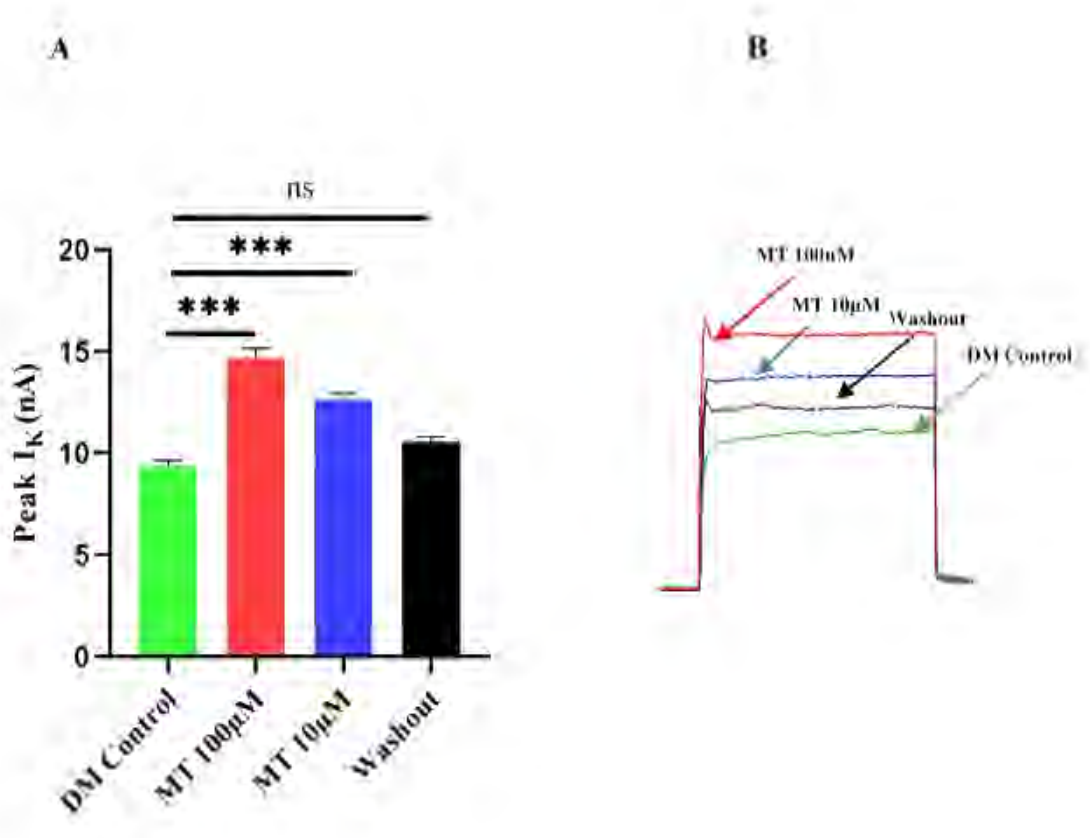


Figure 4.11. *Effect of MT on K^+ activation current traces. (A) The K^+ current activation traces obtained in the absence and presence of MT at the 100 µM and 10 µM in DM control cells. (B) Superimposed K^+ current evoked by 300 ms depolarization pulse from+60mV applied at holding potential -60 mV. ***P<0.001; significance between DM control before and after application of MT. Paired Student's t-test were applied using ± S.E.M. values (n = 10 for 100 µM, n=8 for 10 µM).*

4.2.1.2. *Effect of melatonin on K^+ current in healthy DRG cells*

In order to evaluate whether the K^+ current is affected by melatonin in healthy control neurons, we studied with the same melatonin concentrations in healthy control DRG. Figure 4.12 shows that treatment with melatonin 100 µM significantly reversed the changes in I_A K^+ current density of DRG neurons with a small effect on I_A currents in DRG neurons from healthy control rats which affected by 14.8% (11.78 ± 0.27, 13.83 ± 0.50 pA, P<0.001). While 10 µM melatonin shows an insignificant effect (11.78 ± 0.27 pA, 11.95 ± 0.40 pA).

The figure 4.13 demonstrate melatonin's effect on K^+ activation current traces acquired in the absence and presence of 10 and 100 µM concentrations of melatonin in

healthy control cells. 100 µM melatonin significantly affected I_A in healthy cells ($^{***}P<0.001$), but there was no significant effect of 10 µM melatonin on the healthy control DRG cells.

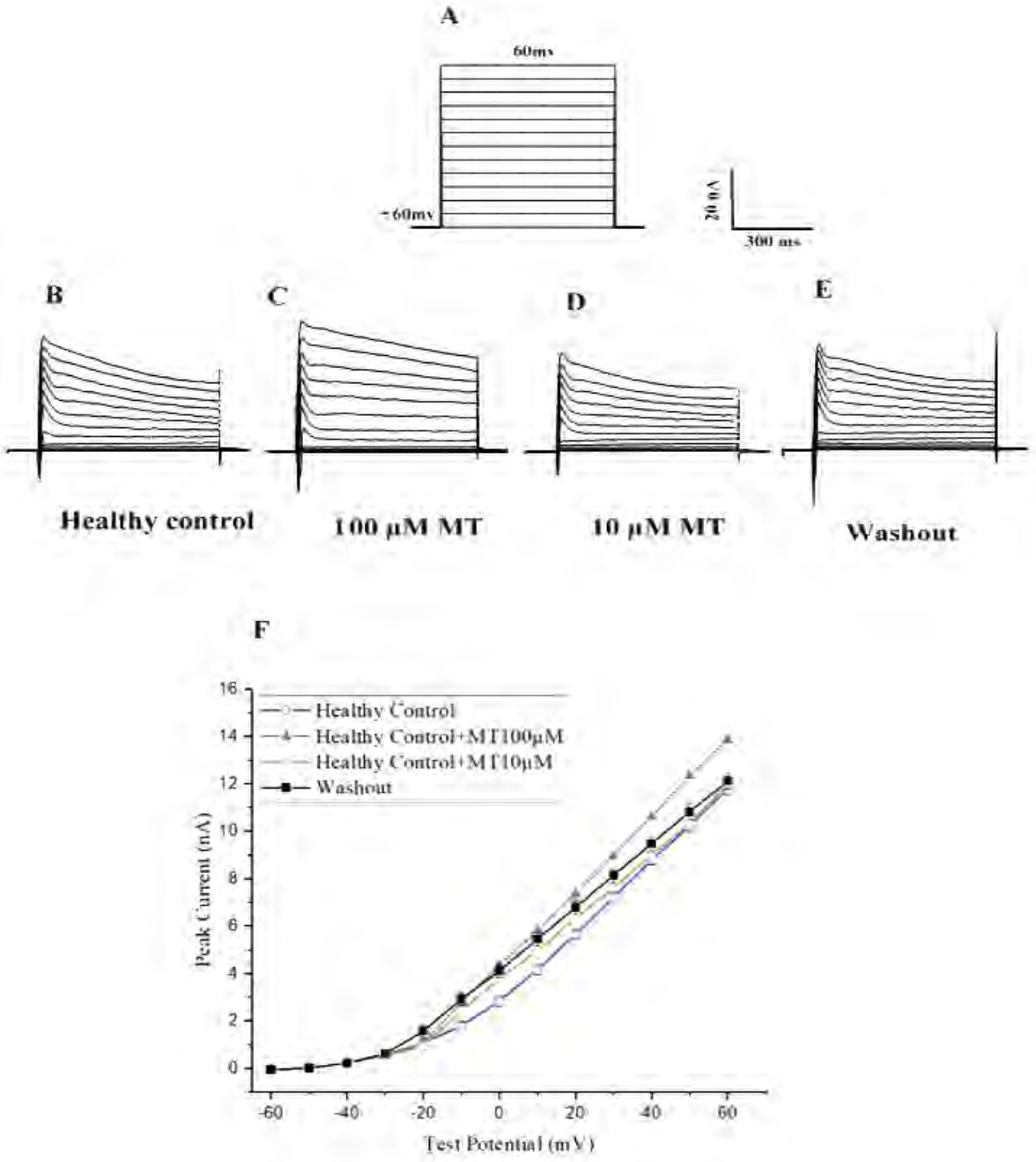


Figure 4.12. *Effect of 100, 10µM MT on the activation of I_A current in healthy control DRG cells. (A) Potential steps to elicit the current, in which the cells were evoked by depolarizing pulses ranging from -60 to +60 mV in steps of 10 mV every 15s from a holding potential of -60 mV. (B)Healthy control DRG neurons, (C) Healthy+ 100 µM MT, (D) Healthy+10 µM MT, and (E) washout. (F) Summarized current-voltage (I-V) relationships of IA currents before and after*

application of 10, 100 µM MT and washout. Paired Student's t-test were applied using ± S.E.M. values (n = 10 for 100 µM, n=8 for 10 µM).

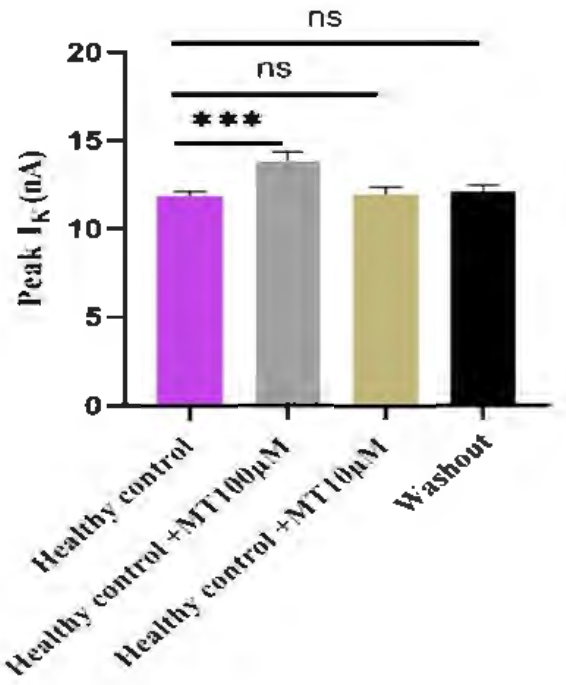


Figure 4.13. *Effect of MT on K+ activation current traces obtained in the absence and presence of MT at the 100 µM and 10 µM in healthy control cells. ***P<0.001; significance between healthy control before and after application of MT. Paired Student's t-test were applied using ± S.E.M. values (n = 10 for 100 µM, n=8 for 10 µM).*

4.2.1.3. K^+ current activation traces comparison between DM and healthy control cells

Comparing K^+ current activation traces in healthy control with DM control in Figure 4.14. A significant reduction in the I_A current density ($^{***}P<0.001$) observed in the diabetic rat's DRG neurons compared with the healthy control (9.37 ± 0.28 pA in DM group and 11.78 ± 0.27 pA in the healthy control group, P<0.001). All data are expressed as mean ± S.E.M.

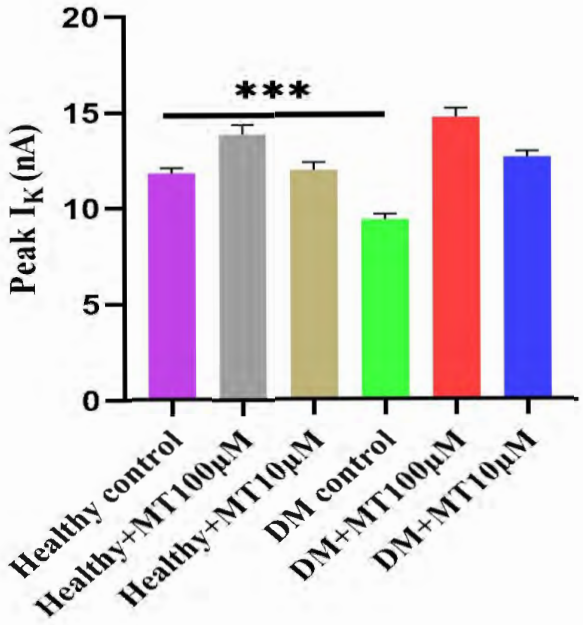


Figure 4.14. *Comparing K^+ current activation traces obtained in the absence and presence of MT at the 100 µM and 10 µM in healthy control with DM control cells. $^{***}P<0.001$; significance between DM and healthy control groups. Paired Student's t-test were applied using ± S.E.M. values (n = 10 for 100 µM, n=8 for 10 µM).*

4.2.2. Action potential parameters

4.2.2.1. *Effect of melatonin on the hyperexcitability of DRG neurons in healthy and diabetic rats*

To study the melatonin's effect on neuronal excitability of DRG cells in healthy and diabetic rats. Minimum injected depolarizing currents required to elicit AP that induce AP firing, and consecutive AP were elicited after applying 2x rheobase in DM and healthy control DRG cells.

In figure 4.15, DM cells were excited, and the number of AP spikes significantly increased compared to the healthy control cells ($^{\#\#\#}P<0.001$) (10.2 ± 0.81 for DM and 4.7 ± 0.73 for healthy control). After 100 µM melatonin application on the hyperexcitability of DRG neurons in diabetic and healthy DRG neurons. The number of AP spikes significantly decreased in DM cells after applying 100 µM melatonin ($^{***}P<0.001$) (10.2 ± 0.81 before and 2.8 ± 0.38 after MT). Also, 100 µM melatonin has a significant effect on the number of AP spikes ($^{**}P<0.001$) in the healthy control cells (4.7 ± 0.73 before and 4.4 ± 0.49 after MT). All data are expressed as mean ± S.E.M.

The minimum current required to elicit AP was higher in the healthy control cells.

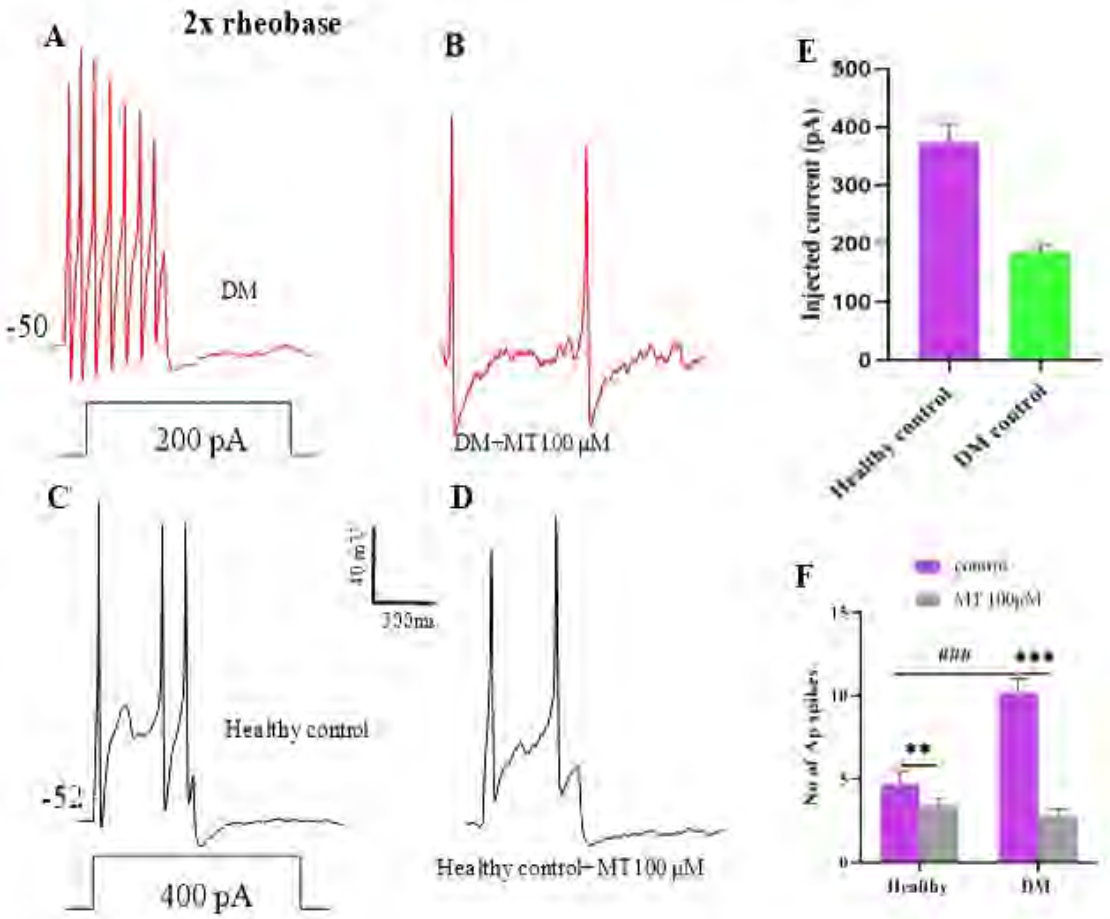


Figure 4.15. *The inhibitory effect of MT on the hyperexcitability of DRG neurons in diabetic and healthy DRG neurons. Representative traces illustrating the spike firings in response to a depolarizing current step from; (A) DM control, (B)DM+ 100 µM MT, (C) Healthy control, and (D) Healthy+ 100 µM MT. (E) Represent the rheobase required to elicit AP in healthy and DM cells. (F) Quantification of the effect of MT 100 µM on the number of AP spikes in diabetic and control cells. $^{###}P<0.001$; significance between DM and healthy control groups, $^{***}P<0.001$; significance between DM and (DM+100 µM MT) groups, $^{**}P<0.001$; significance between healthy control and (healthy+100 µM MT) groups. Paired Student's t-test were applied using ± S.E.M. values (n=10).*

4.2.2.2. *Effect of melatonin on AP parameters of DM and healthy DRG cells*

4.2.2.2.1. *Effect of MT on AP parameters of DM cells*

Figure 4.16 shows a statistically significant decrease in the threshold and amplitude, an increase in duration, and hyperpolarization of action potential after using 10 and 100 µM melatonin in DM cells ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) (n=10 for each group). All data are expressed as mean ± S.E.M.

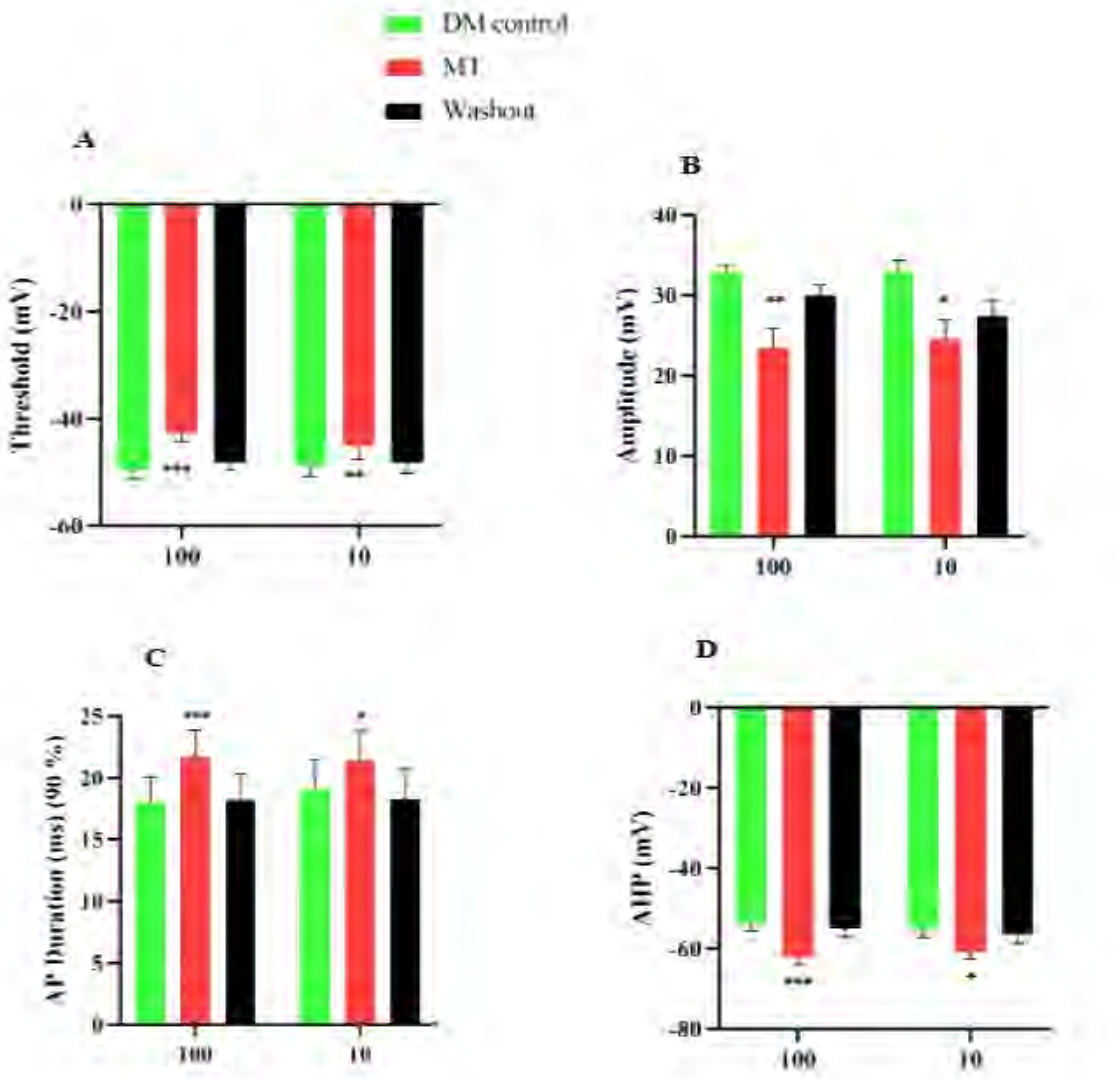


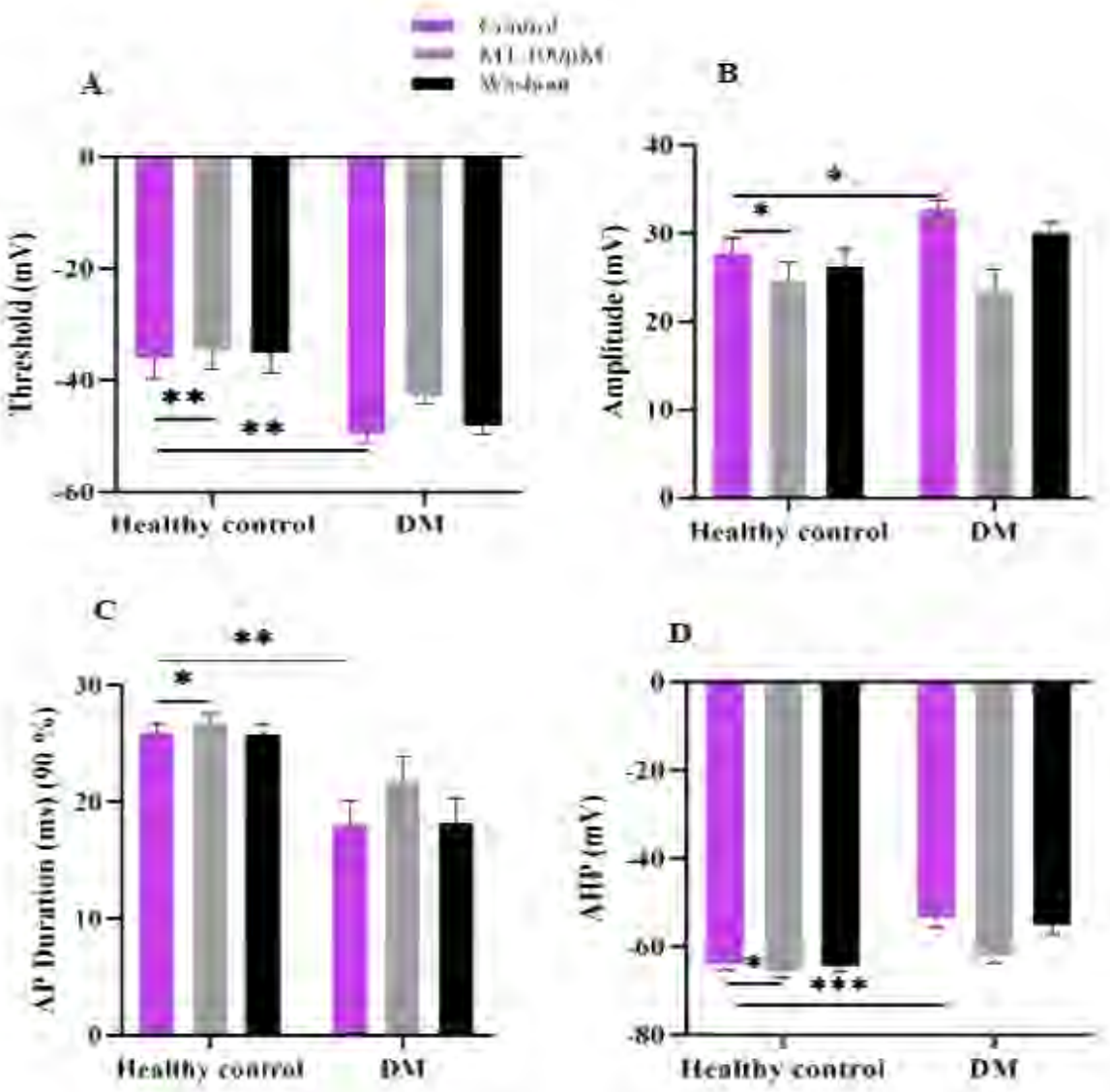
Figure 4.16. *MT effects on AP parameters of DM cells. Different aspects of AP parameters have been evaluated in DM cells before and after application of 10 and 100µM MT. (A) threshold, (B) amplitude, (C) APD (90%), and (D) AHP. $^{*}P<0.001$, $^{**}P<0.001$ $^{***}P<0.001$; significance compared to the DM control group. Paired Student's t-test were applied using ± S.E.M. values (n=10).*

4.2.2.2.2. *Effect of MT on AP parameters of healthy control cells comparing with DM cells*

To determine the melatonin's effect of on neuronal excitability of DRG cells in healthy and diabetic rats. A total of 40 cells were used. The electrophysiological parameters' control values of AP used in this investigation can be found in (Fig.4.17). Results showed that 100 µM melatonin causes a significant increase in the AHP to the negative side

(-53.52 ± 2.08 before, and -55.12 ± 2.14 mV after MT, P<0.001) for DM and (-62.18 ± 1.59 before, and -60.90 ± 1.73 after MT, P<0.05) for healthy control group.

Threshold has been changed significantly (-49.56 ± 1.65 before, and -48.77 ± 2.06 mV after MT, P<0.001) for DM and (-42.18 ± 1.53 before, and -45.02 ± 2.54 after MT, P<0.01) in healthy group. Also, melatonin causes a significant decrease in the amplitude and increase of duration of action potentials, in which they are among the parameters affecting neuronal excitability. For comparison, the following parameters of AP statistically and significantly differed between the DM group and the healthy group by paired Student's t-test. (n=10 for each group) ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). All data are expressed as mean ± S.E.M.


A
B
C
D
Threshold (mV)
Amplitude (mV)
AP Duration (ms) (90 %)
AHP (mV)
Healthy control
DM

5. DISCUSSION AND CONCLUSION

In this thesis study, it was demonstrated for the first time that the administration of the neurohormone melatonin in rats with diabetic neuropathy showed antiallodynic and antihyperalgesic effects, and this effect was associated with the noradrenergic system and exerts a reducing effect on neuronal activity and excitability via opening of K^+ channels by increasing the amplitude of I_A current, which is one of the systems that play a primary role in pain relief.

It is an undeniable fact that one of the most common symptoms of diabetes, which is common today, is neuropathic pain, and the living conditions of patients are limited by frequently complaining of pain [82]. Therefore, experimental studies are in the discovery of new drugs for the relief of neuropathic pain that develops with diabetes. In experimental studies, diabetes is induced to improve diabetic neuropathy, and pain thresholds against various stimuli decrease after a certain waiting period as a result of developing neuropathy [84, 361]. In this study, an agent named STZ was used to induce diabetes. STZ is a chemical that occurs naturally and is derived from *Streptomyces achromogenes* that is especially toxic to the pancreatic insulin-producing beta cells in mammals [389]. It is noteworthy that hyperglycemia and hypoinsulinemia caused by STZ that indirectly activates an apoptotic program that destroys pancreatic β-cells and all the cells expressing the GLUT2 transporter [80, 139, 185], leading to diabetes mellitus type1. As stated in the literature, it is known that the blood values measured after 72 hours increase to indicate the development of diabetes and animals with over 300 mg/dl are considered to have Type I diabetes [15, 132]. In the thesis study, except for the healthy control groups, as a result of measuring the blood sugars of the animals treated with STZ after 72 hours, it was observed that the blood sugar values were above 300 mg/dl and these animals were taken to a resting period of 4 weeks for the development of DN.

One of the most prominent symptoms of the development of diabetic neuropathy is pain. Observed pain is evaluated as peripheral neuropathic pain and markedly is characterized with developing allodynia and hyperalgesia [93]. In this study, pain thresholds against mechanical and thermal stimuli were measured before the animals were induced diabetes. After a 4-week rest period, pain thresholds were measured again before the drugs administrations because it is known that the thermal hyperalgesia and mechanical allodynia have been shown to occur during the first month following the initiation of hyperglycemia in this model [390, 391] and an accepted indicator of diabetic

neuropathy considered by 20% decrease in thresholds [392]. According to the findings, pain thresholds against mechanical and thermal stimuli were significantly lowered in diabetic animals except for healthy groups. In the experimental set, thresholds against mechanical stimuli were determined with the e-Von Frey device. With this device, the filament applied to the animal's claw is normally a non-noxious stimulus, but it is perceived as a painful stimulus in animals with neuropathy and their threshold for the stimulus decreases [372]. This decrease in the threshold is considered as the development of allodynia [393]. Therefore, it was determined that allodynia developed in animals with diabetes. Differently, the thermal stimulus is perceived as a painful noxious stimulus and the decrease in the threshold against the thermal stimulus is accepted as the development of hyperalgesia [394]. In the experimental set, the thresholds against thermal stimuli were determined by the Hargreaves method, and a significant decrease was observed in the thermal thresholds after 4 weeks in the animals determined to have developed diabetes [12]. Therefore, the development of both allodynia and hyperalgesia occurs in the animals to be tested in this study and are suggested to be an indicator of early PDN.

After the neuropathic pain was developed, a single daily dose of solvent was applied to healthy and diabetic control animals for 14 days, and melatonin was administered to other groups at a dose of 50 mg/kg and, gabapentin was administered at a dose of 50 mg/kg as a reference drug. At the end of the 14th day, pain thresholds against mechanical and thermal stimuli were measured again in order to determine how drug treatments applied to developing allodynia and hyperalgesia. Based on these measured thresholds, % MPEs were calculated, and these values were accepted as an indicator of the antiallodynic and antihyperalgesic effects of melatonin and gabapentin. It has been seen that; in animals with diabetic neuropathy, 14 weeks of melatonin treatment showed antiallodynic and antihyperalgesic effects by raising pain thresholds against both mechanical and thermal stimuli. These observed effects were at the same level as gabapentin, an anticonvulsant drug that is frequently prescribed in the clinic for diabetic neuropathic pain. Gabapentin is such an effective drug, along with its needed effects, gabapentin may cause some unwanted and serious side effects include somnolence, dizziness, diarrhea [395], myopathy [396, 397], anxiety, respiratory depression [398] , and increased suicide behavior are the rare but serious side effects [399–401], so some patients need different treatment approaches that can be an alternative to gabapentin. The fact that melatonin has similar efficacy with gabapentin suggested that melatonin is a

potential agent that can be evaluated. Moreover, melatonin has more advantages than gabapentin in term of safety, it is a neurohormone that has been shown to have many physiological effects. As a prescription drug or as a non-prescription supplement product, it is an agent of widespread use worldwide for many indications. Due to its widespread use, it is considered as a promising pharmacological agent in scientific studies. Several studies have shown its high efficacy and safety profile in the management of various chronic pain paradigms, especially neuropathic pain, especially with its very low toxicity over a wide range of doses. Therefore, it can be considered as a safe option in treatment [6–9].

In order to evaluate whether melatonin treatment shows variability in the presence of diabetes, a group of healthy animals was also administered melatonin for 14 days. It is known that the drug efficacy can show differences in the presence and absence of disease [7, 310, 312]. Looking at the results of the thesis study; while no difference was observed in melatonin's antiallodynic activity in healthy and diabetic animals, it was determined that its antihyperalgesic effect showed differences in the absence and presence of diabetes. The antihyperalgesic effect of melatonin in diabetic animals is significantly higher than the antihyperalgesic activity in healthy animals. To be reminded again, hyperalgesia is distinguished by an increased painful stimuli response and allodynia is an innocuous stimulus pain response [75]. The difference that melatonin shows on these two painful sensations this may be due to their different pathophysiological origins. As follows; while hyperalgesia gives a clinical symptom of increased afferent activity against stimuli as a result of sensitization of nociceptors, allodynia is mostly associated with changes in the central nervous system[55, 76]. Moreover, the fact that the antihyperalgesic effect is at different levels in healthy and diabetic animals may be due to the fact that the drug effect occurs in the direction of correcting a pathophysiological event that is impaired by diabetes.

In the thesis study, besides its antinociceptive effects, melatonin was also evaluated in terms of locomotor activity. It should be noted that false positive results in evoked and non-evoked pain measures can occur due to effects other than analgesia, such as motor side effects , a change in locomotor activity, sedation or drug-induced anxiety may appear as a side effect of the drug [402]. A decrease in locomotor activity can be considered as sedation, while an increase can be considered as a psychostimulant effect and locomotor activity-regulating[306, 308]. In this study, the variability in the horizontal and vertical

mobility of the animals was evaluated as the variability in the locomotor activity. As expected with diabetes, the decrease in locomotor activity occurred at the end of the 4th week. Diabetes causes a decrease in locomotor activity due to the damage various transmission of neurotransmitters such as dopamine and noradrenalin [403, 404]. In our study, we did not see the sedative effect of melatonin on healthy rats in this dose as they have mentioned in the literatures. Although 2 weeks of treatment with melatonin and, also gabapentin were not reach locomotor activity to the level of healthy animals, they increased locomotor activity in diabetic animals. In these conditions, this effect of melatonin was evaluated as an advantage as well as antiallodynic and antihyperalgesic effects in diabetic animals. Although this positive effect was observed at similar levels to gabapentin, its relative correction effect was slightly better than gabapentin. This is a finding that may provide an advantage to melatonin. It is known that the increase in locomotor activity mainly occurs as a result of stimulation via transmitters such as noradrenaline and dopamine and adenosine receptor blockade [402, 403]. The reason that melatonin increases the decreased locomotor activity in diabetic animals may be that it triggers any of these mechanisms, and it is already known that these neurotransmissions are impaired by diabetes and affect the locomotor deterioration.

After the action analysis studies of melatonin were completed, studies on the mechanisms by which the observed effects were revealed were started. Evaluation and determination of the mechanism of action of drugs with pharmacological activity is important in terms of mechanism of action-based treatment approaches, in terms of making or predicting the pharmacological profile of the drug. The formation of neuropathic pain and the relief of pain occur as a result of the organized work of many pathways in the body. However, according to recent findings, the noradrenergic system plays a prevailing role in neuropathic pain pharmacotherapy, while the other systems such as dopaminergic and serotonergic systems have only modulatory effects [405, 406]. This suggests that drugs affecting the noradrenergic system may have curative potential in neuropathic pain. It has been shown that melatonin exert antinociceptive process by improving noradrenaline transmission in the supraspinal level indirectly by a mechanism through one possible mechanism of action that proposed to be, is the activation of the cGMP system, and then by increasing NO-cyclic GMP pathway, it is possible that melatonin may mediate adrenergic receptor stimulation, thereby producing antinociception [7, 340, 348]. For these reasons, it was planned to investigate the

contribution of the noradrenergic system to its effect on neuropathic pain by *in vivo*. For this purpose, it was aimed to evaluate the contribution of alpha-1, alpha-2 and beta receptor stimulation, which play a role in noradrenergic pain modulation, to the effect of melatonin. Several studies on noradrenergic receptor activation tend to α-adrenoceptors play an important role in the pain-regulating effects of noradrenaline, however, β-adrenoceptors also contribute to pain control by mediating adrenaline-induced pain modulation [4, 147, 158]. Besides, it was shown that noradrenaline facilitates GABAergic and glycinergic inhibitory synaptic transmission in the spinal cord dorsal horn, but not excitatory glutamatergic transmission. It has been shown to do this by triggering depolarization via α2- and β-receptors as well as α1-adrenoceptors, and then triggering action potentials that will stimulate synaptic terminals and cause the release of GABA and glycine [151]. Of the noradrenaline-specific adrenoceptors, α1- and β-receptors show more facilitator activity, while α2 adrenoceptors show inhibitory activity [151].

The beta receptor antagonist propranolol has been used to evaluate the involvement of β-adrenergic receptor stimulation. Propranolol is an antagonist that closes all types of β-receptors [407]. Although stimulation of β2-adrenoceptors is necessary and essential for antidepressants to exert their antiallodynic effect against neuropathic pain [160]. Melatonin's effect was significantly antagonized by the pre-application of propranolol, its effectiveness was determined to be significant. From this point of view, beta receptor stimulation can be evaluated as the mechanism of action responsible for some of the antiallodynic and antihyperalgesic effects of melatonin.

For the evaluation of alpha receptors stimulation participation, the α-1 adrenergic-antagonist prazosin and the α-2-adrenergic antagonist yohimbine were included in the study, respectively. Results from these antagonists' pre-administration were observed similar to the results obtained from the pre-administration of propranolol; the non-selective β-adrenergic antagonist. Although α-receptor antagonists antagonize the effect of melatonin, it was observed that the effect of melatonin continued. It seems that prazosin produces a statistically significant but also negligible effect when solvent pre-treated. Prazosin is a ligand with an inverse agonistic effect on α1-receptors [408]. Therefore, this significant effect observed under these experimental conditions may be due to its inverse agonistic effect. However, in this experiment, prazosin was used to take advantage of its antagonistic effect. It is already known that inverse agonists show antagonistic effects in the presence of agonists.

It is well established that α-adrenergic receptors stimulation plays a pivotal role in pain relief with generally known that noradrenergic descending inhibitory pathways reduce pain transmission and nociception through the inhibitory action of α2-adrenoreceptors by reducing the effect of intracellular adenylcyclase by directly modifying the activity of Gi-mediated or ion channels such as Na^+/H^+ antiport, Ca^{2+} or K^+ channels [146, 147, 152, 409]. Noradrenaline exerts a strong antinociceptive effect thanks to spinal α2-adrenoceptors that inhibitory interneurons actively suppress pain with post-synaptic inhibition [153, 154]. Although studies on noradrenergic receptor activation focus heavily on agonistic effects on α2-adrenoceptors, there are many studies showing that α1-adrenoceptor agonism also participates in pain control [4, 158]. Activation of postsynaptic α1 adrenergic receptor participates in antinociception by increasing the release of GABA or glycine by local inhibitory neurons [58, 410].

The fact that the pharmacological effect is not fully antagonized by the antagonists used in the mechanism of action studies indicates that the evaluated noradrenergic system does not undertake the pharmacological effect of melatonin alone. As a matter of fact, considering that pain relief is a complex process involving many pathways, the results are not surprising.

Another physiological proof in which the aim of this *in vitro* study was investigated melatonin's effects on electrophysiological parameters in primary DRG neurons obtained from rats and to carry out an effective and mechanistic preclinical research. Whether, dorsal root ganglion neurons that frequently used in studies on evaluating peripheral neuropathy, DRG neurons are biologic materials that can be reflected to the clinical conditions in electrophysiological experimental design investigating nociception related mechanisms of pharmacological agents [17–19]. Potassium channels are deemed important therapeutic targets for controlling membrane excitability and managing chronic pain [282].

Kv current density was noticeably decreased in the DRG neurons in diabetic rats [411]. We find that there was a significant reduction in the Kv outward I_A current density in the diabetic rats DRG neurons, as observed in other studies that shown a robust decreases in DRG neurons' A-type Kv currents of STZ-induced diabetic neuropathy model [291–293]. Specifically, they suggest a reduction of I_A K^+ currents through the downregulation of Kv channels [254]. Our study findings revealed that treatment with 100 μM melatonin significantly caused the opening of K^+ channels by reversing the

changes and increasing the amplitude of the outward A-type Kv current (I_A) in both diabetic and healthy cells, but the effect was more obvious in diabetic than healthy control. While 10 µM melatonin also produced a significant increase in I_A amplitude in the diabetic cells group but the effect was statistically insignificant in the healthy control cells group. This effect may be due to the direct action of melatonin on potassium channels. We can explain the fact that the effect of melatonin on potassium currents in diabetic animals is more pronounced than in healthy animals especially with higher dose, by the fact that melatonin prevents the deteriorating effect of diabetes on potassium flow, in other words, it has a neuroprotective effect in streptozotocin-induced rat model of diabetic neuropathy as shown in the study conducted by Negi et al., [412]. As a matter of fact, the improvement in this direction that can be observed in potassium current is an indicator of the neuroprotective effect.

On the level of the action potential, our study showed that melatonin in a concentration of 100 and 10 µM exerts reducing effects on neuronal activity and excitability. The ability to inhibit repetitive neuronal firing represents a crucial treatment strategy for chronic pain [413]. The spontaneous action potential disappeared in the presence of melatonin and the potential threshold increased significantly. This inhibition to action potential might be because of regulation ion channels [414].

In our study melatonin decreased the firing frequency after applying 2x rheobase in both diabetic and control cells, but the effect was profound and more significant on the diabetic cells than healthy control. Therefore, the changes applied in neuronal excitability by melatonin can be caused by its effect on the function of ion channels. The study by Olivera-Abreu et al. showed that melatonin reduces neuronal excitability by increasing the rheobase, which is the minimum current intensity required to produce an action potential, which is dependent on the activity of voltage-dependent sodium channels [327]. Basically, most of neuronal excitability is dependent on the function of ion channels. Ion channels, including the group that are voltage dependent, are widely distributed in the peripheral nervous system of vertebrates and invertebrates and are responsible for generating and modulating neuronal excitability, through regulating the shape, firing pattern, duration of the action potential membrane resistance and the release of neurotransmitters [240, 415], they play a very key role in the pathogenesis of neurological diseases such as chronic pain and epilepsy [280].

It has been shown that melatonin increased the amplitude of the after-hyperpolarization potential (AHP) in DRG neurons. The potential following hyperpolarization plays an important role in regulating the frequency of occurrence of action potential and cell excitability [416]. In our study melatonin significantly affect AHP in both diabetic and healthy cells causing a significant increase in the AHP to the negative side. Increasing the conductivity of the membrane to potassium making the membrane potential more negative during the AHP phase prevents the occurrence of action potentials and prevents excessive excitability of the cell [417]. On the other hand, since the negative after-potential is affected by potassium currents, perhaps melatonin has increased the AHP amplitude by increasing potassium conductance and thereby reducing the frequency of electrical activity of neurons. Other parameters of action potential have been changed in our study; the threshold changed to a less negative value which means that the cell will need higher stimulation to action potential firing. Also, MT causes a significant increase in the duration of action potentials and decrease in the amplitude, which they are among the parameters affecting neuronal excitability.

Finally, due to the lipophilic nature of its structure, melatonin can pass through the cell membrane and intracellular organelles and directly affect intracellular receptors and targets, including intracellular reserves [23]. The results of our study showed that melatonin exerts an effect on neuronal activity and excitability via opening of K^+ channels by increasing the amplitude of the outward A-type Kv current, showing that I_A plays a key role in regulating repetitive discharges and the excitability of action potential [418]. The effect of melatonin on the general increase of potassium currents has been associated with membrane hyperpolarization [419]. Also, melatonin can reduce excitability by activating the outward potassium flow [420]. Totally based on our in vitro electrophysiological studies, we can say that melatonin has an antinociceptive effect also, after turning potassium current and affecting the neuronal excitability in action potential in diabetic DRG conditions in favor of relief of neuropathic pain, by the existing data that indicate so far as a variety of antinociceptive drugs act by directly opening spinal K^+ channels [220].

In conclusion, if we evaluate the in vivo and in vitro data of the study together, it can be said that melatonin shows its antihyperalgesic and antiallodynic effects by using the potassium channel and the noradrenergic system axis. It is known that mechanisms

and pathways where potassium channels and noradrenergic system work together to pain relief.

Radical treatment is not yet possible in diabetic neuropathy with a high prevalence. For this reason, it is necessary to evaluate active substances that are different from current treatment approaches. Seeing the positive effects of melatonin, which has gained popularity recently in diabetic neuropathy will attract clinical attention and provide approaches to be developed. The data obtained by behavioral test and patch clamp methods enable the design of further studies (changes in the current-voltage curve give an idea about different pathways) but leads us to infer that melatonin exerts its antinociceptive action via new pathway. Further research is needed to determine the precise molecular mechanisms of melatonin's actions in this type of pain, and then to combine the new antinociceptive therapeutic strategies. Due to insufficient evidence to support melatonin's impact on neuropathic pain. This data will be obtained to emphasize the importance of melatonin and its derivatives in new drug development studies.

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APPENDICE I

APPENDICE II