

**INVESTIGATING THE ANTIHYPERALGESIC EFFECTS OF
LEVETIRACETAM AND ITS INVOLVED MECHANISMS AT SUPRASPINAL
LEVEL IN A MODEL OF NEUROPATHIC PAIN**

Doctoral Thesis

Feyza ALYU TEKES

Eskişehir 2019

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DOCTORAL THESIS

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September 2019

JÜRİ VE ENSTİTÜ ONAYI

Feyza ALYU TEKES'in "Nöropatik Ağrı Modelinde Levetirasetam'ın Antihiperaljezik Etkilerinin ve Bu Etkilere Katılan Mekanizmaların Supraspinal Düzeyde İncelenmesi" "Investigating the Antihyperalgesic Effects of Levetiracetam and Its Involved Mechanisms at Supraspinal Level in a Model of Neuropathic Pain" başlıklı tezi 27.09.2019 tarihinde aşağıdaki jüri tarafından değerlendirilerek "Anadolu Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği"nin ilgili maddeleri uyarınca, Farmakoloji Anabilim Dalı Doktora Yeterlik Tezi olarak kabul edilmiştir.

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ÖZET

NÖROPATİK AĞRI MODELİNDE LEVETİRASETAM'IN ANTİHİPERALJEZİK ETKİLERİNİN VE BU ETKİLERE KATILAN MEKANİZMALARIN SUPRASPİNAL DÜZEYDE İNCELENMESİ

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Nöropatik ağrı sendromları, hastalarda yaşam kalitesini önemli derecede düşürmektedir. Ağrı yönetimi için daha etkili tedavilere ve ayrıntılı araştırmalara belirgin bir ihtiyaç vardır. Bu çalışmada levetirasetam'ın antihiperalezik etki mekanizmaları iyon kanallarının, nörotensinerjik ve kannabinoiderjik sistemlerin katılımı açısından supraspinal düzeyde incelenmiştir. İlaç uygulama bölgesi olarak ağrı süreçlerine belirgin ölçüdeki katılımı gösterilmiş olan ventral posterolateral talamus seçilmiştir. *In vivo* deneyler, mekanik allodini değerlendirilmesinde e-Von Frey, termal hiperalezi değerlendirmesinde ise plantar cihazı kullanılarak gerçekleştirilmiştir. Kronik konstrüksiyon hasarı modelinde levetirasetam'ın zamana ve doza bağlı belirgin antihiperalezik etkiler gösterdiği gözlemlenmiştir. Bu etkilere nörotensinerjik ve kannabinoiderjik katılım, antagonistlerin ön uygulaması ile araştırılmış ve her iki sistem için de önemli bir katılım tespit edilmiştir. Elektrofizyolojik çalışmalar, levetirasetam'ın yalnızca -130 ile -90 mV aralığındaki hiperpolarize membran potansiyellerinde aktif olan bir veya daha fazla akımı tetiklediğini göstermiştir. Bu bulgular HCN, K_{ir} veya GIRK kanallarının olası katılımına işaret etmektedir. Levetirasetam ve türevleri nöropatik ağrının farmakoterapisinde alternatif seçenekler olarak değerlendirilmelidir. Elde edilen sonuçlar, nöropatik ağrının tedavisi için levetirasetam türevlerine yönelik yeni ilaç geliştirme çalışmalarına öncülük edecek niteliktedir.

Anahtar Sözcükler: Levetirasetam, Nöropatik ağrı, Hiperalezi, Elektrofizyoloji, Talamus

ABSTRACT

INVESTIGATING THE ANTIHYPERALGESIC EFFECTS OF LEVETIRACETAM AND ITS INVOLVED MECHANISMS AT SUPRASPINAL LEVEL IN A MODEL OF NEUROPATHIC PAIN

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Neuropathic pain states disrupt quality of life. There is an evident need for more effective treatments and detailed research for its management. In this study, antihyperalgesic mechanisms of levetiracetam have been investigated at supraspinal level regarding ion channel activities, neurotensinergic and cannabinoidergic systems. Ventral posterolateral nucleus of thalamus was chosen as the site of administration since it functionally participates in pain processing to a great extent. *In vivo* experiments were performed by e-Von Frey and plantar apparatus for assessing mechanical allodynia and thermal hyperalgesia, respectively. Significant effects of levetiracetam were observed in a dose and time-dependent manner in chronic constriction injury model. Neurotensinergic and cannabinoidergic involvement was investigated by pre-treatment of antagonists and significant reversal was demonstrated for both systems. Electrophysiological studies showed that levetiracetam has a tendency to open one or more conductances which are only active at hyperpolarized membrane potentials ranging from -130 to -90 mV, indicating the possible involvement of HCN, K_{ir} or GIRK channels. Utilization of LEV and its derivatives can be assessed as alternative choices in pharmacotherapy of neuropathic pain. Obtained results can guide novel drug development studies towards levetiracetam derivatives for management of neuropathic pain.

Keywords: Levetiracetam, Neuropathic pain, Hyperalgesia, Electrophysiology, Thalamus

ACKNOWLEDGEMENTS

September, 2019

I would like to thank to Prof. Dr. Yusuf Ozturk, for being my mentor and guiding me in the way to become a scientist who appreciates her own vision, always attempts her dreams and has positivity. As being an example for initior work for pharmacology departments within Turkey, this thesis originated from his pasion about novelty and included patch clamp technique. This is one of the outputs of the electropysiology laboratory we set up under the leadership of him and is the first one among pharmacology departments of pharmacy faculties in Turkey. Within this work, the electrophysiological study performed at Camerino University was a harvest of the whole process.

Many thanks to Prof. Roberto Ciccocioppo for providing me the opportunity to work at his laboratory and supporting me at any time through the work schedule. My visit to Neuropsychopharmacology and Neuroscience Laboratory of the University of Camerino has been a higly efficient experience and provided me a better scientific vision. His support for the application process for European Molecular Biology Organisation Short-term Fellowship Program actualized that visit.

I would also like to thank to Assoc. Prof. Dr. Alessio Masi and Michele Petrella for guiding me in the field of electrophsiology, showing me how to think in this field of science.

Lastly, but not the least, I would like to thank all my family members and friends for supporting me through all this time.

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.

Feyza ALYU TEKES

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LIST OF SYMBOLS AND ABBREVIATIONS

ACC	: Anterior Cingulate Cortex
ACSF	: Artificial Cerebrospinal Fluid
AMPA	: Alpha-amino-3-hydroxy-5-methyl-4-isoxazole Propionic Acid
ANOVA	: Analysis of Variance
CB	: Cannabinoid
CB ₁	: Cannabinoid Receptor Type 1
CB ₂	: Cannabinoid Receptor Type 2
CCI	: Chronic Construction Injury
cGMP	: Cyclic Guanosine Monophosphate
CNS	: Central Nervous System
DMSO	: Dimethyl Sulfoxide
DRt	: Dorsal Reticular Nucleus
E _m	: Membrane Potential
EPSCs	: Excitatory Postsynaptic Currents
E _{res}	: Resting Membrane Potential
GABA	: g-aminobutyric Acid
GBP	: Gabapentin
GIRK	: G protein-gated Inwardly Rectifying K ⁺
HCN	: Hyperpolarization-activated Cyclic Nucleotide-gated
HEPES	: Hydroxyethyl Piperazineethanesulfonic Acid
HVA	: High Voltage Activated
I _h	: Hyperpolarization Activated Current
<i>i.p.</i>	: Intraperitonally
IPSCs	: Inhibitory Postsynaptic Currents
I-V	: Current- Voltage
K _{ir}	: Inwardly Rectifying K ⁺
LEV	: Levetiracetam
mGluR5	: Metabolic Glutamate Receptor 5
n	: Number of individuals
NaV1.3	: Tetrodotoxin-sensitive Channels
NMDA	: N-Methyl D-Aspartic Acid

NMDG	: N-Methyl-D-glucamine
NO	: Nitric Oxide
NOS	: Nitric Oxide Synthase
NS	: Nociceptive Specific
NT	: Neurotensin
NTS1	: Neurotensin Receptor Subtype 1
NTS2	: Neurotensin Receptor Subtype 2
p	: Probability Value
PAG	: Periaqueductal Gray
PBS	: Phosphate Buffered Saline
PE	: Polyethylene
PKC	: Protein Kinase C
PLA2	: Phospholipase A2
R_i	: Input Resistance
R_s	: Series Resistance
RVM	: Rostral Ventromedial Medulla
S.E.M.	: Standart Error of the Mean
Sham	: Sham operated / Placebo surgery
SNL	: Spinal Nerve Ligation
STT	: Spinothalamic Tract
STZ	: Streptozotocin
SV2A	: Synaptic Vesicle Protein 2A
VP	: Ventral Posterior Thalamus
VPL	: Ventral Posterolateral Nucleus
WDR	: Wide Dynamic Range
5-HT	: Serotonin
5-HT3	: 5-Hydroxytryptamine type 3

1. INTRODUCTION

Within the scope of this doctoral thesis, antihyperalgesic effects of levetiracetam (LEV), a novel generation antiepileptic drug [1] have been investigated at supraspinal level by *in vivo* behavioral and *in vitro* electrophysiological experiments.

1.1. Statement of Problem

Quality of life becomes severely disrupted in patients experiencing neuropathic pain because of the increase in drug prescriptions, visits to hospital and the morbidity from the pain itself plus the causative disease [2, 3]. Symptoms such as pain resulting from non-painful stimulations, burning and electrical-like sensations persist and have a trend to become chronic and non-responsive to therapy. Several mood disorders like anxiety, depression and sleep disturbances accompany the syndrome. Impairment of the quality of life has been shown to be more significant in patients suffering from chronic neuropathic pain compared to those with chronic non-neuropathic pain [2, 4, 5]. There is an evident need for more effective treatments and detailed research.

There are several central nervous system (CNS) inhibitory or facilitatory mechanisms that have been linked to enhanced or reduced pain sensation and that involves several brain areas and circuits [6]. Comprehension of which particular CNS areas are participating in these modulatory systems could have remarkable significance and may help to expand our concept of neuropathic pain and forming better approaches for pharmacotherapy that have basis in mechanisms for acute and also chronic kinds of pain.

Alteration of nociception at any level may modulate pain, an approach introducing another complexity. This is in compliance with the observations demonstrating that pharmacological agents can have different effects at different regions within the nervous system.

Clarifying the mechanisms of agents which have different mechanisms than classical treatments and focusing on specific CNS areas will open new horizons towards new treatments for neuropathic pain syndromes.

1.2. Purpose of the Study and Objective

Levetiracetam (LEV) belongs to a relatively new class of antiepileptics, has an excellent pharmacokinetic profile and is characterized by a distinct mechanism of action which makes this drug particularly attractive for the treatment of a number of CNS and peripheral disorders beyond seizures [7, 8]. LEV is a promising agent for several

indications. For its utilization in clinics, first there is a need for more clarification about its effects and related mechanisms. This work has focused on its antihyperalgesic effects and related mechanisms in neuropathic pain.

The diversity of neuropathic pain models provides researchers the opportunity to evaluate the different pathologies of these syndromes. Neuropathic pain models carry different pathophysiologies [9]. The purpose of this study has been investigating the effects of LEV in the chronic construction injury (CCI) model with a neuroanatomical manner so that to provide a mechanistic approach to elucidate the antihyperalgesic effects of this drug. Studies related to neurotensinergic and cannabinoidergic pathways are aimed to elaborate the mechanistic approach. By electrophysiological experiments carried out within this work, it has also been intended to investigate the mechanisms regarding ionic currents at a supraspinal level.

In the event of providing sufficient data about antihyperalgesic effects of LEV, utilization of it and its derivatives can be considered as alternative choices in pharmacotherapy of neuropathic pain. Obtained results can guide novel drug development studies towards LEV derivatives for management of pain.

1.3. Statement of Research Hypothesis and Rationale for Hypothesis

Understanding the functions of components of pain pathways within CNS is important to relieve mechanisms of agents. Ventral posterolateral nucleus (VPL) of thalamus has been shown to be highly related with pain in several studies. With regards to algnesia, this region has been linked to participate, mostly via activation [10-18].

Systemically administered LEV has been shown to demonstrate antihyperalgesic effects in neuropathic pain models, such as diabetic neuropathy and CCI [9]. Nonetheless, a study investigating its effects at a supraspinal level has not been performed so far. The VPL is one of the main supraspinal relay site for nociceptive input and appears to be a promising area that may be involved in mediating the effects of LEV. Since LEV has a wide range of mechanisms [7, 8], high possibility of observing an alteration by the drug on the pathological processes within VPL was predicted. For instance, involvement of hyperexcitability within VPL to neuropathic pain has been shown [13, 15, 18-21] which is also a process that might be affected by several mechanisms of LEV [7, 22-41].

Considering these facts, it has been hypothesized that the antihyperalgesic effects of LEV may involve mechanisms related to its effects on VPL.

Cannabinoidergic involvement to neuropathic pain within VPL has been shown [42]. In addition, cannabinoidergic pathways within VPL has been demonstrated to attenuate spontaneous activity and evoked responses, which also might be linked to one or more possible mechanisms of LEV [7, 22-41]. Thereby, in this work it was hypothesized that if LEV displays antihyperalgesic effects when administered directly to the VPL, those effects can be regulated, at least partly, by cannabinoidergic system.

Similar to the hypothesis regarding cannabinoidergic system and considering the evident participation of neurotensinergic system to the pain processing [43-50] and with regards to the finding that neurotensin (NT) has several effects [49, 51-56] resembling to effects of LEV, it is suggested that neurotensinergic pathway can be one of the mechanisms that LEV has with regards to antihyperalgesia it represents.

Blockage of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels within VPL has been shown to provide hyperalgesia [57], introducing a clue for the need for electrophysiological investigations. Since regulation on ion currents is one of the major pathways for effects of pharmacological agents [58], it was hypothesized that if intra-VPL injected LEV could display antihyperalgesic effects, those may can emerge from its modulation on electrophysiological parameters of VPL neurons, since changes of electrophysiological properties of these neurons have been linked to relief of hyperalgesia [18, 21, 59].

In conclusion, the present work stem from the hypothesis that LEV may have antihyperalgesic effects by acting on the VPL. Elucidation of the mechanisms of this compound can provide a deeper understanding of chronic pain pathologies and may help the development of innovative medications

2. REVIEW OF THE LITERATURE

Mechanisms underlying neuropathic pain and new insights to its management are poorly understood and thus, novel approaches to its pharmacotherapy has been limited and remains a high priority [60].

2.1. Neuropathic Pain

Pain plays a crucial role for sustaining survival, but it must be taken into attention that there are various types of pain and they should be discriminated carefully. A sensation of noxious stimuli, termed as nociceptive pain, functions as early-warning system and can be activated only by intense stimuli. After tissue damage inflammatory pain emerges through the activation of immune system to support healing by forming a state for averting movement or physical contact. Misfunctioning of the nervous system may result in a maladaptive but not protective type of pain, called pathological pain. When pathological pain arises from damage to nervous system it is termed as neuropathic pain. The other type of pathological pain, called dysfunctional pain, occurs in conditions such as irritable bowel syndrome, fibromyalgia or tension type headache, without any peripheral inflammation or noxious stimulus, rather than a damage or inflammation. Pain management should be targeted at the distinct mechanisms among mentioned varieties of pain [61, 62].

Neuropathic pain is irreversible and frequently characterized by persistent pain that is independent from the noxious stimulus. Another characteristic scenario for neuropathic pain is abnormal sensory perceptions such as the one emerged from innocuous tactile stimuli or exaggerated one that caused by mildly noxious stimuli, termed as allodynia and hyperalgesia, respectively. Imbalances within inhibitory and excitatory signaling, changes in ion channel functions and alterations regarding how the nociceptive signaling is modulated in the CNS have been involved in neuropathic pain [5]. The underlying mechanisms continue to be fully clarified and the development of agents to interrupt pain signaling is a major therapeutic direction for research [63, 64].

In symptomatic management of neuropathic pain, three main drug classes are commonly used: antidepressants, such as tricyclics; anticonvulsants, such as gabapentin (GBP) and carbamazepine; and opioids [29, 65-67]. However, using traditional agents has been suggested to be effective solely to a minimal extend for many patients and to be accompanied by an unacceptable level of adverse events [68, 69].

Considering the efficacy of opioids for neuropathic pain, data so far introduced controversy [70, 71]. First line therapy mostly includes the antidepressants [72], acting to elevate monoamine levels through a plurality of processes such as transporter reuptake reduction and/or receptor blockade. Even though tricyclic antidepressants are specifically effective in this regard, complete elimination has been shown to be rare [73-76].

Considering the mechanisms of antiepileptics for neuropathic pain treatment, three main processes have been suggested which all result in dampening neuronal hyperexcitability within the CNS: inhibition regarding glutamate related excitatory transmission, potentialization of γ -aminobutyric acid (GABA)-mediated transmission together with voltage activated ion channel blockage [77].

Research has been focused on discovering newer, less toxic drugs for management of neuropathic pain [68, 78]. A novel treatment approach has recently been suggested as a treatment strategy, based on targeting directly particular pain processes rather than the underlying disease per se [79-82].

2.1.1. Neuroanatomical basis of pain

An extensively distributed brain network has been suggested to participate in nociceptive processing since pain is a multifactorial and complex experience, as represented in Figure 2.1. To establish a more plausible framework for pain therapy, better understanding is needed regarding the way sensory information is processed while it moves from the sense organ to the cerebral cortex. In the scope of this thesis work, thalamus has been the focal point of interest since that region has been accepted as the principal relay site for nociceptive inputs to subcortical and cortical areas [83].

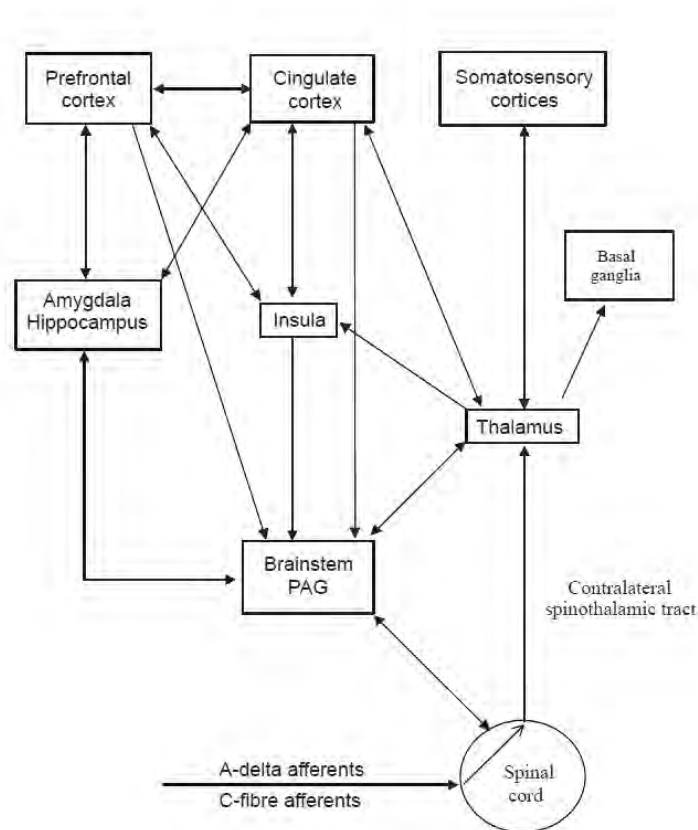


Figure 2.1. Schema of a number of the principal anatomical components for brain network in pain [84].

Nociceptive input travels from afferent fibers which synapse onto transmission neurons in the spinal cord. Following this, ascending fibers thru the contralateral spinothalamic tract carry the information. As the gateway for sensory dimension of pain, these ascending projections terminate within the ventrobasal and ventroposterior thalamus [77, 85-87]. Neurons within mentioned nuclei project to the somatosensory cortex, the pain response quickly get discriminated in cortex regarding its temporal encoding properties, providing the intensity and location of pain to be sensed [5, 77, 88].

Being the principal relay site for nociceptive stimuli to subcortical and cortical areas, thalamus carries a specific importance for pain signaling pathway. Within thalamus, anatomical and functional divisions have been determined depending on their connections to specific spinal cord laminae [89, 90]. Rostral projections from thalamus reach to the areas within, for instance, the amygdala and cortical sites. Nociceptive data coming from brainstem/the spinal cord reach to the central nucleus of the amygdala. Moreover, inputs from the cortex and also thalamus enter to the amygdala. This region

sends outputs to the thalamus and cortical sites, leading to integration of conscious and cognitive perceptions of pain [87].

Brainstem has a central role in mediating alterations regarding pain perception. Nociceptive input reaches to the brainstem and medulla through spinoreticular and spinomesencephalic tracts [83]. The dorsal column pathway together with the spinoreticular projections reach to the nucleus gracilis and cuneate nucleus [87]. Limbic projections relay in the parabrachial nucleus [91] prior to get in contact with the amygdala and hypothalamus, where central autonomic function, anxiety and fear are altered [4, 5]. Targeted mesencephalic nuclei include the midbrain periaqueductal gray (PAG) region, the dorsal reticular nucleus (DRt) in addition to rostral ventromedial medulla (RVM) [87].

There are descending projections from the DRt which have been shown to be critical components of the diffuse noxious inhibitory control pathway [87]. The descending pain modulatory framework represents a highly characterised anatomical system which enables the modulation of pain signaling by the organism (mainly at the dorsal horn level) to produce either inhibition (antinociception) or facilitation (pronociception) [92, 93]. Regulation of the descending pain modulation occurs via projections to the PAG, an area receiving data from diverse areas and communicates with the RVM together with other medullary nuclei sending descending projections to the spinal dorsal horn via the dorsolateral funiculus [87]. Moreover, PAG integrates data from within all the limbic system namely amygdala, frontal cortical structures and hypothalamus, with ascending transmission from the dorsal horn [92]. PAG has a regulatory role on processing the nociception which is mediated through descending noradrenergic and serotonergic processes originating from the brain stem, also acting within the dorsal horn by a multiplicity of receptor subtypes [92, 94]. Following chronic injury, the net action appears to shift toward a facilitation of nociceptive transmission even though the effect of descending modulation is subject to the equilibrium among facilitatory and inhibitory input to the spinal cord [95].

Among pain related supraspinal areas, the noradrenergic locus coeruleus has been shown to receive data from the PAG, communicate with the RVM and send descending inhibitory projections (noradrenergic) to the spinal cord. Pronociceptive and antinociceptive projections from the RVM negatively or positively arrange nociceptive data and maintain the regulations within endogenous pain system [83, 87]. In

neuropathic conditions, lacking of this noradrenergic control and through 5-HT₃ receptors, an increase in serotonin descending excitation have been demonstrated [5, 96].

Sustained activation of the pain transmission facilitatory descending pathways have been pronounced to be a reason for some states of chronic pain [97-99]. Direct effect of descending inputs originating from supraspinal level on spinal cord excitability has been shown, resulting in either inhibition or facilitation [95, 98, 100]. A variety of brain regions such as amygdala, frontal lobe, hypothalamus, insula, anterior cingulate cortex (ACC) and nucleus cuneiformis, RVM and PAG have been shown to take a part in this descending modulation. Insula and ACC, together with thalamus, have been termed as classic pain-processing brain regions. Increased lateral prefrontal cortex activation has been demonstrated to be linked to attenuated pain effect, probably via suppressing the functional connectivity among midbrain and medial thalamus, in this way driving endogenous pain-inhibitory mechanisms [83].

In chronic pain, alterations throughout the descending pain modulatory process among patients having either an activated and enhanced descending facilitatory system or a dysfunctional descending inhibitory system, have clearly been implicated so far [101-103].

As being the principal relay site for nociceptive inputs to subcortical and cortical areas, among the pathways mentioned above, thalamus is of great importance in pathophysiology of pain.

2.1.2. Difference between mechanical and thermal hyperalgesia

There have been pronounced to be diverse excitatory signal transduction systems and receptor subtypes among hyperalgesia and allodynia. To explore these differences at cellular level several electrophysiological and molecular studies performed so far with also the support of behavioral studies providing opinions about hyperalgesia in intact experimental animals. In a study carried out with awake animals at spinal cord level to investigate the diversity of systems for thermal hyperalgesia and mechanical allodynia, researchers found differences in signal transduction systems and receptor subtypes. Attention should be given to the fact that the CNS is a dynamic entity which process the information at many different levels and assemble systemically and differences in mechanisms should be expected at any level [104].

After an injury to the nerve, significant amount of C-fiber discharges has been proposed to release neurokinins, glutamate and some other substances from primary afferent terminals. Mentioned release results in fast synaptic potentials induced by actions in non-NMDA (N-Methyl D-Aspartic Acid) receptors and slow synaptic potentials generated from constant depolarization and the discharge of peptides (e.g., calcitonin gene-related peptide; substance P). Consequently, a voltage-dependent magnesium block depart from NMDA receptors and this process allows an influx of calcium (Ca^{2+}). Influx of Ca^{2+} induces a cascade of intracellular events, for instance nitric oxide synthase (NOS) activation and soluble guanylate cyclase, alteration of protein kinase C (PKC). Together with specific genes activation causing the development and maintenance of hyperalgesia, those processes are referred to be crucial central components for hyperalgesia [104].

The suggested differences in intracellular cascades between thermal hyperalgesia and mechanical allodynia at spinal level are as follows [104-106]: Thermal hyperalgesia is mainly linked to spinal NMDA receptor activation, PKC translocation and nitric oxide (NO) plus cyclic guanosine monophosphate (cGMP) production. Conversely, mechanical version occurs principally owing to the coactivation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and spinal metabotropic glutamate, phospholipase A2 (PLA2) activation together with cyclooxygenase products production. This complex system of neuronal formation provides flexibility for nervous system [104].

Considering the fact that in the spinal cord there is also diversity about responding to thermal and mechanical stimuli among neurons [107, 108], further consideration should be given to nociceptive information transmission. While 75% of neurons within spinal cord found to respond to NMDA, only 35% has been shown to be affected by glutamate [109] introducing the knowledge about presence of glutamate insensitive neurons which can be activated by an endogenous substrate other than glutamate; endogenous metabotropic glutamate and NMDA receptor agonists are released [110, 111], there is a difference within neurons concerning glutamate receptors [112, 113], distribution of metabotropic glutamate receptors and NMDA, AMPA are not even, but they are generally in the dorsal horn; [114-119]. NOS (+) cells shown to be not ubiquitously dispersed, however present mainly around the central canal plus dorsal horn [120, 121], NMDA and non-NMDA receptors are present at synaptic sites as an

evenly distributed manner [112, 122, 123]; a single neuron may have a condensed localization of a particular receptor subtype at diverse locations along the neuron [112, 113], synapse has been shown to have a greater concentration of intracellular Ca^{2+} than in other regions within the same neuron [124]; different glutamate receptor agonists cause activation of different intracellular enzymes [125, 126], which is a fact that can be linked to the mentioned Ca^{2+} microdomains; activation of intracellular enzymes are related to selective modulation of different glutamate receptors [127, 128], diverse glutamate receptor agonists induce gene expression via diverse ways for Ca^{2+} entry [129], when activated at high-frequency, C-fibers causes metabotropic glutamate receptor activation in CNS [130-132]. The possibility for the afferents to terminate on neurons which have solely NMDA receptors or only AMPA and metabotropic receptors, as well as the neurons containing all of the receptor subtypes, but having regional differences such as an evident overage of a single receptor subtype should also be considered. This complex system of neuronal formation provides flexibility for nervous system [133].

Collectively, differences mentioned above triggers consecutive processes in mostly separate pathways, supporting the event that there are differences between mechanical and thermal hyperalgesia. When hyperalgesia develops, the sensitivity of the neurons in the pathway for pain processing would rely on location and density of receptor and intracellular activity. It can be speculated that if major type of receptors a neuron bearing are the ones crucial for mechanical allodynia (AMPA and metabotropic receptors), when activated, that neuron would lead to mechanical sensitivity (or vice versa for thermal stimuli and NMDA) [112, 113].

Also, $\text{A}\delta$ mechanoreceptors and C-thermoreceptors have been suggested to synapse and signal on different regions or different neurons of nervous system, as there are evident differences among neurons in terms of location, subtype and density of receptors. There has also been another assumption regarding a feed-forward mechanism. If a neuron has numerous NMDA or activated more via NMDA, then following processes may not solely generate thermal hyperalgesia, but also suppress the processes generating mechanical allodynia. Consequently, thermal hyperalgesia and mechanical allodynia depend on activation of two diverse intracellular processes [104].

For thermal hyperalgesia formed by neuropathic pain, while the development has been linked to activation of both NMDA and non-NMDA receptors, maintenance has

been linked solely to activation of NMDA receptors [134]. Activation of metabotropic and AMPA receptors have been found to be non-related with thermal hyperalgesia [135-139].

NO has been proposed to be another component of the persistent NMDA-regulated thermal hyperalgesia in peripheral inflammation models [140, 141] or following acute administration of NMDA [136, 138, 140, 142]. As being one of the main targets of neuronal NO, soluble guanylate cyclase [143] has also been suggested to be involved in thermal hyperalgesia [138, 141, 144, 145].

AMPA, NMDA or substance P, when applied in high doses, have been shown to produce thermal hyperalgesia which was found to be related to prostaglandins [146]. In terms of PKC, a relationship between NMDA and PKC have been found in terms of thermal hyperalgesia [139, 147].

From the perspective of electrophysiology, statement of NMDA-mediated thermal hyperalgesia is as follows: the voltage-gated NMDA receptor has been shown to be not active at resting membrane potential (E_{res}) however, following high-frequency stimulation it becomes active, a situation that has been shown in the situation of hypersensitivity [104, 148, 149].

In mechanical allodynia, coactivation of AMPA and metabotropic receptors have been pronounced to be involved while lack of sufficient effects of NMDA for acute mechanical allodynia has been pronounced [108, 139, 140, 147, 150-154]. Even so, NMDA receptor activation has been demonstrated to increase the consequences of metabotropic glutamate and AMPA receptor coactivation in producing mechanical allodynia [147, 155]. Metabotropic glutamate receptor activation stimulates Ca^{2+} -permeable AMPA receptor has been suggested to induce Ca^{2+} influx and start a cascade of intracellular actions leading mechanical allodynia [108].

cGMP, NO and activation of PKC or phospholipase C (PLC) have been shown not to be take a part in mechanical allodynia [104, 140, 147, 156] while coactivation of glutamate and AMPA receptors have been shown to be capable of generate AA through stimulation of PLA2, leading to production of eicosanoids [147, 156, 157].

Both leukotrienes and prostaglandins have been suggested to have pivotal role in hyperalgesia [146, 147, 156, 158-162] and evidence supporting the fact that prostaglandins have particularly pivotal roles for the processes that underlie both persistent and acute mechanical allodynia have been shown [156, 159], while the

possibility of them not taking any significant part in thermal hyperalgesia was also pronounced [147, 158].

Another point of difference about processing of pain information has been suggested as follows: vast majority of A-fiber nociceptors in mice (A-fiber mechanonociceptors) has been pronounced to be sensitive to mechanical stimuli, while only a small proportion of them being responsible for both mechanical and thermal stimuli [163-166].

In inflammatory pain, mechanical allodynia was shown to be modulated by peripheral activation of NMDA, but not by non-NMDA receptors [167]. Nevertheless, in carrageenan-induced inflammation thermal hyperalgesia was shown to be linked to activation of peripheral non-NMDA as well as NMDA receptors [168].

Neurokinin 1 (NK-1) and Substance P receptors were shown to be important mediators of noxious mechanical stimuli [169-171], while they were found to be not related to thermal hyperalgesia [172].

There also has been found a correlation between glycinergic transmission and the difference between mechanical and thermal hyperalgesia [173].

2.1.3. Ventral posterolateral nucleus of thalamus and pain

The thalamus integrates nociceptive information and were suggested to be the main final area for the spinothalamic tract (STT) afferents. The ventral posterior (VP) thalamus is one of the nuclei within the lateral pathway and they mostly projects to the somatosensory cortex (S1 and S2), showing associations with pain regarding sensory-discriminatory features. Concerning motivational and effective components of pain, medial nuclei project to the insula and ACC [13].

Ventral caudal region neurons have been shown to be able to regulate the intensity of stimulus which was applied peripherally in clinical studies. In addition, elevation of electrical stimulation within the thalamus has been pronounced to be positively correlated with intensity of sensation and to lead pain, thermal (both warm and cold) and mechanical sensations and nonpainful paresthesia [10-13].

The localization of VPL is illustrated in Figure 2.2.

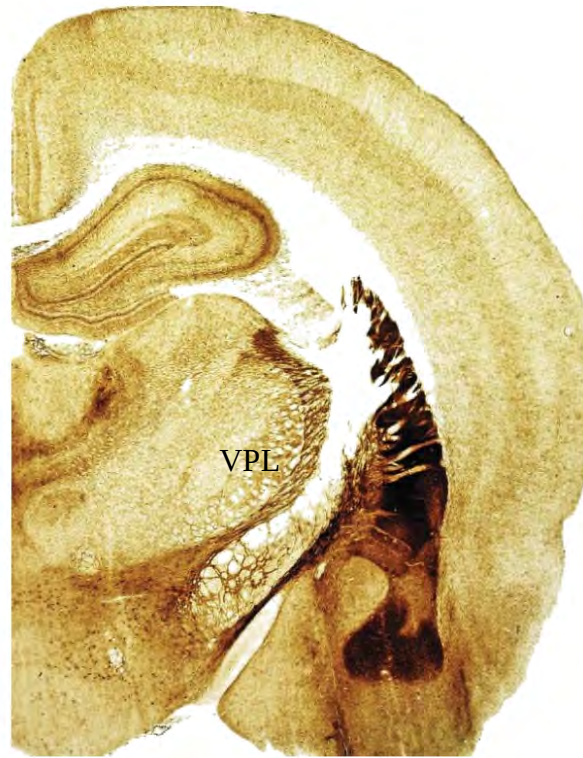


Figure 2.2. *Localization of VPL nucleus of thalamus [174]*

Neurons of STT–VP–S1–S2 pathway have been pronounced to be predominantly wide dynamic range (WDR) and dorsal horn has been shown to have projections to the VPL [13, 175]. Having nociceptive roles, WDR neurons distinguish between even little diversity in the intensity of stimulus [13, 176, 177], and this fact implies to a similar extent between animal and human studies [178].

For pain research, of particular interest is the VPL nucleus. It receives inputs coming from spinal sensory neurons, while also taking a part in sensory-discriminative aspects of pain [14, 15].

In studies mostly related to VP WDR neurons and mechanical stimuli and conducted via neuropathic pain models like sciatic nerve ligation, spinal cord injury, diabetic neuropathy and rheumatoid arthritis, researchers detected increased spontaneous and evoked activity in electrophysiological recordings [13, 18, 19, 179-182].

In neuropathic animals, gabapentinoids have been demonstrated to eliminate behavioral hypersensitivity evaluated via alterations in the withdrawal threshold [183] and diminish neuronal response at spinal level at stimulation having higher intensities [184]. Nevertheless, the mechanisms of gabapentinoids at neuronal level are poorly

understood, and too little has been demonstrated concerning their actions related to sensory processing within the brain. In a study aimed to examine the effects of pregabalin on central mechanisms, the drug was found to inhibit mechanical and heat induced electrophysiological responses in VP WDR neurons of neuropathic rats but not cold-evoked activity. The increase in the spontaneous WDR activity shown in neuropathic conditions was observed to be unaffected by pregabalin in means of inhibition, introducing the result that pregabalin shows modality-selective inhibition of evoked hypersensitivity however, not aberrant spontaneous activity [13].

Through the investigation of the mechanisms regarding the process leading to spinal hyperexcitability and nonspinal mechanisms, such as the dorsal column pathway which primarily innervates the VPL without many spinal terminations [179, 185] may converge onto thalamic relays, researchers have found out novel characteristics of thalamic neuronal hyperexcitability in neuropathic pain. Via *in vivo* electrophysiological experiments at 14 to 18 days postsurgery of CCI, significant increases in excitability and sensitivity were found [13].

There is a parallelism between the extensive changes in induced hypersensitivity among modalities, intensities and postsynaptic changes. GABAergic inhibition has been shown to be diminished in the dorsal horn after peripheral nerve injury [186]; nevertheless, that process does not lead to form a sensitivity to stimuli with low-threshold for prominent amount of thalamic nociceptive-specific (NS) neurons; reduction in GABAergic inhibition of spinal NS neurons was pronounced to be majorly diminished to gating of high-threshold input into the superficial dorsal horn. Eventually, VP NS neurons keep their ability regarding to process noxious input in neuropathy, with increased firing frequencies. Contradictory, significance in the absence of density-dependent changes in firing at the spinal level, an increase of neuronal receptive sizes and the enrollement of elevated numbers of neurons could combine onto thalamic relays and highlight the observed raised thalamic neuronal reactions [185, 187, 188]. This process has been suggested to reflect disinhibition at the spinal level, causing the accessing of polysynaptic interneuronal pathways from deeper to more superficial laminae [189].

Loss of nerve supply to the thalamus, named denervation commonly caused by the injury or other circumstances, can induce increased spontaneous activity [190], and

this ongoing activity has been suggested to not reflect spinal neuronal activity mostly [18, 19, 179].

Elevated spontaneous activity of spinal neurons was shown after sciatic and spinal nerve injuries, usually with a feature of firing in infrequent patterns [191, 192]. VP neurons have been shown to express rapidly repriming tetrodotoxin-sensitive channels (Nav1.3) following injury, participating to spinal knockdown of Nav1.3 and an elevation in basal neuronal excitability has been suggested to be the responsible process that attenuates these thalamic changes [19].

Increased spontaneous activity changes the coupling between thalamic and cortical structures and dynamics within thalamocortical networks [193]. This dysrhythmia at the thalamocortical level has been suggested to result in ongoing pain [194, 195].

When deep brain stimulation was applied to VPL, a reduced activity within that region accompanied by an attenuation in neuropathic pain observed together with a decrease in mechanical allodynia [16, 17]. Moreover, traumatic nerve injury induces hyperexcitability amongst primary afferents, dorsal horn and pain-signalling neurons of the VPL [15, 18].

2.1.3.1. Pain-related ion channel activities in VPL nucleus

In streptozotocin (STZ) induced diabetic neuropathy accompanied by mechanical allodynia, neurons within VPL have been shown to get into a hyperexcitability state with augmented reactions to pinch, press stimuli applied to peripheral receptive fields and phasic brush. Also, VPL neurons of diabetic rats have been shown to present amplified spontaneous activity, which is independent from ascending afferent barrage, and expanded receptive areas. These findings indicate hyper-responsiveness of neurons within VPL and an abnormal level of spontaneous activity contribute to diabetic neuropathic pain [18].

Together with the feature of underlying changes in burst firing effects of VPL neurons, elevated spontaneous and provoked firing within that region were linked to Nav1.3. This effect of Nav1.3 dysregulation on spinal and thalamic generation and augmentation of pain also contributes to post spinal cord injury chronic pain. Being similar in humans, this system has been suggested to be an efficient target for management of clinical pain [59].

Peripheral nerve injury has been shown to increase the expression of promptly-repriming Nav1.3 sodium (Na^+) channel amongst second-order dorsal horn nociceptive and first-order dorsal root ganglion neurons, withal showing modifier effects on the expression of Na^+ channel together with the neurons in higher-order VPL neurons. Researchers proposed that if misexpression of the Nav1.3 Na^+ channel occurs, it leads to an increase in the excitability of VPL neurons, and this event contributes to neuropathic pain [15].

Peripheral neuropathic pain was reported to be aligned with evident alterations in the firing of VPL neurons within the receptive areas in the contralateral injured hind paw. An increase in the rate of spontaneous firing and elevated afterdischarge rates together with provoked firing in response to non-noxious and noxious mechanical stimuli besides significant changes in bursting and rhythmic oscillation firing have been demonstrated. Nonetheless, the electrophysiologic characteristics of neurons within the regions other than the injured hind paw have been shown to be unchanged, except only for elevated reactions to brush stimuli [21].

10 days after CCI, an amplified spontaneous firing and elevated activity provoked by brush, pinch stimuli, and pressure in the VPL has been shown [15]. Even 7 days post-CCI, researchers found increased afterdischarge besides numerous diverse abnormal burst firing patterns not previously reported in neuropathic pain [19-21].

Changes such as spontaneous neuronal discharge in VPL have been suggested to be not affected by the input coming below the thoracic level since several data represented the fact that those changes induced by neuropathy endures to be high even following complete transection of the spinal cord. Only rhythmic oscillations were found to be abolished immediately after spinal transection [21].

As being an important component for neuronal coding and an effective regulator for impact postsynaptic neurons [196], burst firing effects downstreaming from the VPL in the somatosensory cortex. Temporal prevalence content of incoming signals can control bursting, that has been pronounced to be the case in neuropathic pain as they get significantly modified at peripheral and spinal cord levels [197]. The relative timing of burst spikes [196] and the duration of bursts [198] have been shown to be able to encode stimulus aspects specifically [199]. In terms of ventral thalamus, bursting was reported in awake rats, suggested to have a role in priming the thalamocortical loop for augmented signal detection [200, 201]. For neuronal projections such as reticular one to

thalamocortical neurons, low-threshold spike bursts were shown to be involved in sleep disturbance, epilepsy and chronic pain [202]. In addition, alterations in the patterns of burst firing have been suggested to be an evident marker for pain-induced neuroplasticity [203]. Electrophysiological changes within VPL has been pronounced to clearly take a part in sensitization of VPL neurons together with other parts of the nervous system, for instance; the somatosensory cortex and dorsal horn neurons, which visualized to the VPL [204].

A discharge of somatosensory cortex neurons regarding central pain has also been demonstrated to change in ways identical to those defined for VPL neurons [205]. Sensory disruption like thermal hyperalgesia and tactile allodynia have been linked to dysfunctional thalamocortical communication [21, 206].

VPL stimulation inhibits spinothalamic neurons [21, 207]. The possibility of the result namely activation of spinothalamic tract neurons protruding to the medial brain stem, as well as nucleus raphe magnus and the PAG after VPL stimulation has been pronounced [208-210]. Mentioned neuroanatomical regions have been shown to liaise descending antinociceptive control unto lumbar dorsal horn neurons [208, 211]. Another mechanism reported for the actions after VPL stimulation is the adequate activation progress within the somatosensory cortex to turn on corticofugal inhibitory pathways, thalamocortical-corticofugal projections restraining spinal cord nociceptive neurons [207]. Corollary within possible events, VPL stimuli has also been linked to local inhibition of neurons within that area through stoppage of membrane ion channels like voltage-gated currents [212], induction of early genes, synaptic exhaustion and depolarization blockade [213, 214] and following this the transmission of nociceptive signals to cortical areas.

2.1.4. CCI model of neuropathic pain

Usage of animal subjects in the field of pain research holds an especially important place because of similarities between human and animal neurophathic pain sensation. Neurophathic pain described as pain provoked by an injury at one or more parts in the nervous system. There have been a couple of models which are used to represent neurophathic pain. An example for the most widely used and highly validated models on peripheral neuropathy is the sciatic nerve CCI. Reason for this is because CCI's reputation on being a well-established, trustworthy, easy to achieve model that is consistent on showing neurophathic pain symptoms. Additionally, the CCI model has

further inflammatory mechanisms which in turn reproduce mixed etiology of neuropathic symptoms. All things considered, CCI has been suggested to be the closest model in research to mimic the actual conditions in human neuropathic pain cases [215-221]. The CCI-model is similar to clinical conditions of chronic nerve compression in humans that can occur after metabolic disorders, lumbar disk herniation or nerve entrapment, anoxia and heavy metal poisoning [222, 223].

Rat model unilateral mononeuropathy was reported in 1988. Briefly, the common sciatic nerve of an anesthetized rat is exposed by blunt dissection through biceps femoris in the middle of the thigh. Proximal to the sciatic's trifurcation, adhering tissue around 7 mm of freed nerve and 4 tied ligatures (4.0 chromic gut) are loosely around with a space about 1 mm [224, 225] which leads to nerve inflammation associated with extensive deafferentation distal to the placement of the ligatures [226-228]. Ligations are tightened enough to minimize the diameter of the nerve while causing no interruption to the circulation around the nerve [221, 229]. It is suggested that this model also involves an inflammatory component in the development of neuropathic pain since anti-inflammatory treatment of CCI-rats results in attenuation of hyperalgesia [230]. To prevent sepsis, post-surgical care involves administration of antibiotics. Allodynia and hyperalgesia develop from day 3 after surgery and persist for 7 months [228, 231, 232].

Hypersensitivity observed in neuropathic pain arises also due to the activation of immune system. Immune cells, mast cells and macrophages get activated as a response to nerve damage, a process triggering production of proinflammatory cytokines mostly tumor necrosis factor alpha, interleukin 1 beta and interleukin 6. Post-CCI process has the elevation of the levels of these cytokines as a principal contributor to its pathology [219, 233-236]. The activation of glial cells induced by CCI [237, 238]. Glial cells take a part in pain, for instance the immunomodulation via inducing anti-inflammatory cytokines like interleukin 10 and transforming growth factor- β [239].

An inflammatory reaction triggered by nerve injury is a section of the pathophysiology of neuropathic pain [64]. Inflammation as being the body's natural reaction in case of injury, it is essential for initiation of the repair operation[233]. The inflammatory response results in the injured nerve to sustain degeneration, as a result disrupting the normal pain transmission operation. Consequently, exaggerated and incommensurate pain impulses are transmitted. This process results in neuropathic pain

indications like allodynia and hyperalgesia. Within the pain transmission, neurons involving to the process have been shown to be activated specifically in regions very related with pain perception [64]. For instance, noticing the c-fos expressions in the brain by this process that might be analyzed by.

As being a useful marker for nociception [240], a dramatic increase of c-fos expression induced by an inflammation or tissue injury was detected. After activation of postsynaptic neurons by neurotransmitters or neuromodulators, like substance P, glutamate or brain-derived growth factor, sensitization appears subsequent inhibition of potassium (K^+) channel activities, elevated AMPA and NMDA receptors. This results in phosphorylation of extracellular signal-regulated kinase and eventually transcriptional adjustment of target genes like c-fos initiates [241]. Inflammation induced release of mediators cause sensitization of the primary afferent neurons. As a result, threshold for multiplication of action potential gets low and the firing assess of action potential rises. Consequently, hyperalgesia and allodynia, two features of neuropathic pain, occur [222].

There is a major mechanism suggested for neuropathic pain is as follows: defeat of nerve-blood barrier in sustaining the construction of the inner peripheral nerve and disrupted modulatory effects on the concentration and infiltration of ions leading to local inflammation and leakage [242].

Allodynia and hyperalgesia were shown to be more constant on post-surgery day 14 showing that the CCI model is qualified to be used at this time point. Moreover, day 14 was also the point time when peak responses were noticed [243]. Day (14) has been suggested to be a perfect time period for testing behavioral parameters since inflammation because of the surgery procedure supposed to disappeared by this exactly time, making the obtained pain reaction represent neuropathy wholly and solely [244].

c-fos expression in brain regions concerned in the nociceptive pathway and at spinal cord grade has been shown for an increase into CCI [221, 245-248]. Keeping in mind that higher c-fos expression is an indicative of higher neuronal activity [221, 245], it is noteworthy to pay attention to the finding that elevated number of c-fos 14 days post-CCI was evident at the laminae I–II to the deeper laminae of the spinal cord mostly of the endings of the primary afferents are exist at these regions [249, 250]. Surprisingly, 20 days after CCI c-fos levels have been suggested to return to levels similar to sham [251].

2.2. Patch Clamp Technique in Brain Slices: General Parameters and Principles

The technique of patch-clamp recording in brain slices is operative to a great variety of cell types in slices from almost all areas of the CNS [252-254]. The technique has been successfully applied in pharmacological studies for decades. Pioneering studies provided the option to utilize brain slices as a cellular physiology and an integral part of synaptic [255-257]. *In vitro* brain-slice preparation and use have allowed scientists to examine several aspects of the nervous system in an isolated preparation while sustaining significant extent of the brain's complement of neuronal connections.

An example of the patch clamp software screen showing a brain slice in contact with a pipette is showed in Figure 2.3.

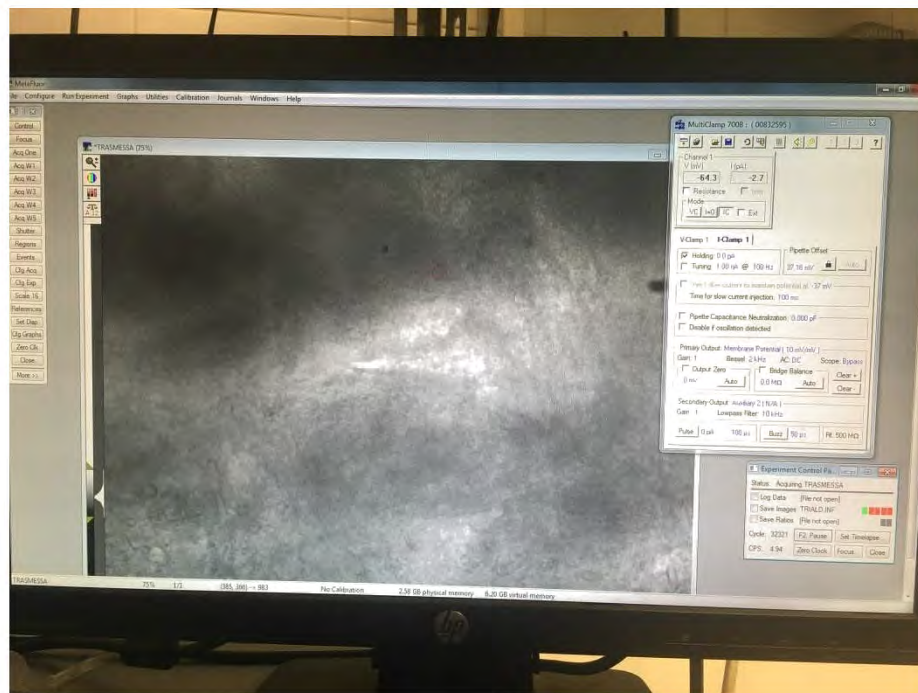


Figure 2.3. An example of patch clamp software screen

Whole-cell patch-clamp recording is an electrophysiological method studying the electrical characteristic of a substantial part of the neuron. In whole-cell arrangement, the micropipette gets in serried contact with the cell membrane, preventing current escape and in this way providing more accurate ionic current measuring than the intracellular sharp electrode recording technic previously used. It is possible to use whole-cell recording on neurons in diverse types of preparations, comprise neurons in brain slices,

cell culture models, dissociated neurons and in intact anesthetized or awake animals [258].

Active and passive biophysical properties of excitable cells can be enlightened by this method [259-261]. This technique is highly advantageous since it provides information concerning how specific manipulations such as pharmacological ones may change specific neuronal channels or functions in real-time. Specifically, when mixed with appropriate pharmacology, this method is a strong implement providing the ability to identify exact neuroadaptations that produced following any type of experiences. When applied on the neurons present in brain slices, whole-cell recording introduces benefits of recording in a relatively well-preserved brain circuits, for instance; in a physiologically pertinent context. It provides quantifying *ex vivo* preparation long-lasting changes in neuronal functions that progressed in animals intact and awake. This method also provides high regional specificity since brain regions can be identified visually.

Access to the intracellular area is provided in this technique, through giving access to the plasma membrane [262]. Thereby it becomes possible to study molecular targets or cellular mechanisms within different situations via arranging the ingredients or density of specific ions formulating the internal solution of the pipette.

Intrinsic cellular excitability (such as axosomato-dendritic ion channels: K^+ ; Na^+ ; Ca^{2+}) and neuronal synaptic factors (such as glutamate transmission) interact in a way that effects brain circuit activity and via whole-cell patch-clamp electrophysiological methods, changes in signals coming from alterations in intrinsic vs. synaptic excitability can be separated. Generally, assessment of synaptic excitability can be done by whole-cell voltage-clamp methods. Voltage-clamp recording mode permit the assessment of ion currents (mediated by e.g.; NMDA and AMPA receptors) through the plasma membrane while holding the membrane potential at a pre-set voltage. In studies using micropipette solutions with the content of a broad blocker of K^+ channels (key intrinsic excitability factors), cesium (Cs^+), prevention concerning the influence of intrinsic excitability factors on other quantifications can be provided. With pharmacological tools, such as GABA receptor antagonists or blockers of glutamate receptors in the extracellular solution, this method permit the measurement of glutamate receptor- and GABA mediated currents successively [263, 264].

Current-clamp recording mode is the way to assess intrinsic excitability. Contrary to voltage-clamp technique, this mode permit the quantification of changes of membrane potentials provoked via ion currents flowing through the membrane. Assessment can be done via measuring alterations in the neuronal capability for producing APS, a process that requires both Na^+ and K^+ channels. This also represents the reason to fill micropipettes with an internal solution that includes K^+ in place of Cs^+ for current-clamp recordings. An example for assesment of the contribution regarding intrinsic agents (such as K^+ channels) to neuronal firing without being infected by potential changes in synaptic excitability factors is a work design with pharmacological factors that block glutamate and A type GABA receptor-mediated currents disbanded in the artificial cerebrospinal fluid (ACSF) [265, 266].

Some basic parameters are as follows; post-synaptic currents (excitatory postsynaptic currents – EPSCs or inhibitory postsynaptic currents IPSCs) used to assess synaptic strength (via slope), the firing pattern such as latency to the 1st point, point frequency, waveform of action potential and number, which are molded through a coordinated and timed opening/closing of particular voltage-gated ion channels (Na^+ , Ca^{2+} , and K^+). Series resistance (R_s) is a parameter that can be controlled by the experimenter in a way as providing an efficient opening for recording while in transition from seal state to whole-cell configuration, since it is a mark of membrane opening. During recording R_s slowly increases, a process induced by several uncontrollable events such as membrane re-closing or debris blocking the pipette tip during the registration. In practice, by applying a slight suction, membrane can be re-opened although this might compromise the patch, and as a result a stable R_s can be maintained. Patch clamp experimenters throwing away the data when changes in R_s exceed 15%, however some laboratories accept the ratio of change as 20% [266-268].

Similar to R_s , input resistance (R_i) is also another parameter that should be monitored during experiments. 10% changes for R_i has been accepted to be sufficient to bias data. When R_i decreases, capability of neurons to produce spikes also reduces. When analyzing the data from the recording, experimenter should keep in mind that observed changes can be related to a change in R_s or R_i instead of the experimental manipulations such as pre- against post-effects of application of the drug to the bath [266].

2.3. Levetiracetam

LEV is a broad-spectrum, anticonvulsant well-tolerated with an action in the mechanism which have been pronounced to possibly be interesting regarding neuropathic pain management [269]. It is a second-generation anti-epileptic drug that belongs to the pyrrolidone family (chemical structure illustrated in Figure 2.4), a class of drugs with a wide spectrum of action. Having a distinct chemical structure than other antiepileptics, LEV possesses a unique pharmacological profile compared with the traditional anticonvulsants [7-9].

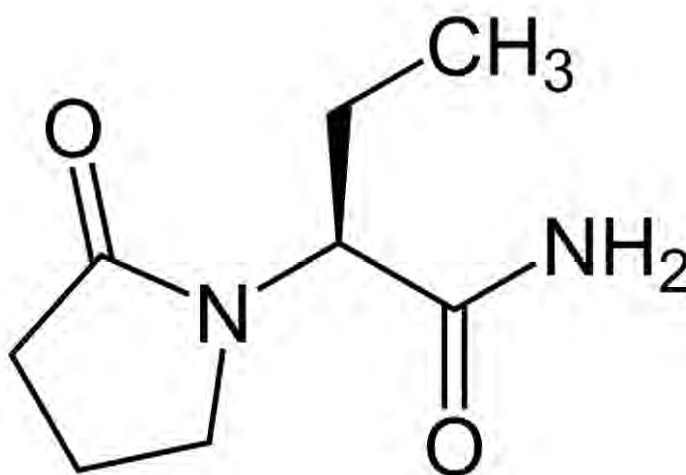


Figure 2.4. Chemical structure of LEV ((-)-S)- α -ethyl-2-oxo-1-pyrrolidine acetamide, $C_8H_{14}N_2O_2$)

LEV was synthesized in the early 1980s during a chemical follow up program intended to discover a second-generation of nootropic drug. In 1999 it was approved by the U.S. Food and Drug Administration, and also in Europe in 2000 [270] for myoclonic seizures, juvenile myoclonic epilepsy or primary generalized tonic-clonic seizures [271]. LEV displays an excellent pharmacokinetic profile and has been used in a wide range of clinically complex situations [270-273].

LEV misses anticonvulsant activity in acute animal seizure models such as pentylenetetrazol and maximal electroshock models, whereas in animal models of chronic epilepsy it provides a powerful seizure protection and antiepileptogenic activity [9, 274]. For a various forms of epilepsy, LEV has been proven to be effective within the prevention has been employed as monotherapy [275-278] and adjunctive treatment [279-283] to the prevention of early post-traumatic seizures [284-287]. Its efficacy and safety in neonatal seizures has been widely reported [288-290].

The effectiveness, low interaction with other drugs, favorable pharmacokinetic properties, high tolerability and several mechanisms of action proposed for LEV have drawn great interest and opened up novel avenues for clinical research [7].

2.3.1. Effects of LEV on hyperalgesia

There is evidence for LEV to possess beneficial properties in the hyperalgesia animal models and for being effective and safe in patients suffering from a diversity of painful central or peripheral neuropathic conditions [9, 68, 291-297]. Even if LEV showed to provide in inflammatory pain antihyperalgesic effects [22, 23, 298], chronic pain [299] and neuropathic pain [29, 295, 300, 301] models, the specific mechanism of action was not fully explained yet [7].

LEV has been appeared to be well tolerated and to display a clinically significant effect on multiple sclerosis related central neuropathic pain [276, 300-303] even though conflicting results concerning the effects of LEV have been found in central neuropathic pain induced by multiple sclerosis [295, 300].

The effectiveness of LEV in prophylactic or symptomatic migraine treatment has also showed in a several researches [301]. Moreover, LEV founded to be efficient in trigeminal neuralgia clinically [304, 305].

In STZ-induced diabetic neuropathy, LEV has been shown to display antihyperalgesic and antiallodynic activities with favorable effects on spinal cord and sciatic nerve which were accompanied by downregulation of microglia and astrocyte expression. Researchers declared that LEV has protective effects on siatic nerve and spinal cord. Altogether, utilization of LEV for alleviating neuropathic pain in diabetic patients was suggested, with also pointing the fact that most studies are still required to fully define the mechanisms [306].

Again, in the diabetic neuropathy, combinations of LEV with ibuprofen/aspirin/paracetamol were evaluated in means of thermal nociception. A synergism was reported [307]. However, in a persistent pain model LEV has ben shown to not have any significant analgesic effect [299].

In another study utilizing diabetic neuropathy, LEV has been shown to display a significant antihyperalgesic effect ($p < 0.001$) [29]. In inflammatory pain, antihyperalgesic effects of LEV has been shown to be, at least in part, linked to A type GABA, $\alpha 2$ -adrenergic receptors, opioid and serotonin (5-HT). Authors resumed that the activation of opioidergic system, in addition to that of the 5-HT and noradrenergic

systems, might indicate that the antihyperalgesia induced by LEV depends on the interaction of the descending inhibitory system [22].

Local peripheral antihyperalgesic and also anti-edematous effects of LEV have also been shown and been linked to 5-HT, adrenergic, opioid, adenosine and GABA receptors and their subtypes [23]. According to hyperalgesia in localized inflammation, the effects of the two-drug combinations of LEV with nonsteroidal analgesics (paracetamol, celecoxib and ibuprofen) and caffeine were evaluated. According to the results obtained, researchers suggested that the two-drug combination of LEV and nonsteroidal analgesics/caffeine might be functional in a treatment of inflammatory pain.

Clinical effectiveness of LEV regarding neuropathic pain was introduced in a study using electrical nerve stimulation. Another significant observation of the study was that the effects observed were seen most significantly after a while from reaching peak serum concentrations, which has been suggested to point to the possibility of LEV to be effective via its regulations in deep compartments of CNS [294].

Systemically administered LEV was found out to display antihyperalgesic effects in two neuropathic pain human models in a dose range comparable to the effective doses in epilepsy [274]. Even in healthy subjects, LEV was demonstrated to modify response to mechanical stimuli, whereas no effect on thermally evoked stimuli was observed. In that study LEV has been shown to display more efficient effects in diabetic neuropathy compared to CCI, a finding that points to the etiology-dependent antihyperalgesic effect of the drug. Authors suggested a therapeutical potential of LEV in neuropathic pain patients, in a way that might act preferably in specific neuropathic pain conditions [9]. In general, anticonvulsants have been accepted to show effects that are similar in terms of efficiency, and LEV seems to be different in this respect. With this feature, according to the type of neuropathic pain syndrome LEV has been pronounced to be the first antiepileptic drug with particular activity, a particularity probably emerging from the mechanism of action and original pharmacological profile [274, 308, 309]. The resemblance of the dose range between epileptic and neuropathic pain models propose that LEV can be considered for chronic pain at the dose used for antiepileptic treatment. Even with a highest dose used in that study was pronounced to be almost two times inferior compared to the median toxic dose value for deterioration of rotarod performance in rats [9, 274].

In another study assessing antihyperalgesic effects of LEV against thermal stimulus, results displayed a significant and reversible attenuation induced by the drug in a dose-dependent manner, only in neuropathic animals. Obtained results have been suggested to provide support to the validation of the promising therapeutic potential for LEV in neuropathic pain syndromes. Besides, in previous studies the effects of antinociceptive of LEV have also been inquired and shown in a rat model of peripheral neuropathy [9, 310, 311]. LEV has also been suggested to reduce anesthetic induced hyperalgesia in rats [291]. Also it refines nociceptive behaviors in rat models of neuropathic pain and persistent [312]. These facts obtained from previous data were the background for performing this work to evaluate mechanisms of LEV in neuropathic pain.

2.3.1.1. Mechanism of action demonstrated so far

Pharmacological mechanisms of LEV demonstrate diversity compared to other antiepileptic drugs. It was shown to not to regulate neuronal voltage-gated Na^+ and T-type Ca^{2+} currents, or show any significant induction on simplification of the GABAergic system [313]. On the other hand, LEV was founded to practice many atypical electrophysiological functions, which include removing of the suppressing effects of negative allosteric modulators of GABA- and glycine-gated currents, including β -carbolines and zinc [28] as well as the inhibition of high voltage activated Ca^{2+} currents [27]. It was suggested that its ability to hyperpolarize membrane potential via K^+ channels in dorsal root ganglions might be included in its possible mechanism to the analgesic effect [29], similar to retigabine which enhances K^+ channel activation [314].

The principal mechanism of action for LEV has been pronounced to be its inhibitory effect on presynaptic neurotransmitter release via fixing to the synaptic vesicle protein 2A (SV2A), which is integral membrane protein, at spinal and supraspinal level, which is accepted as a different mechanism from that of gabapentinoids [315-317]. Consequential modulation of neurotransmitter release has been claimed to display a similar net impact on signaling in the pain pathway with other antihyperalgesic drugs [295]. How LEV affects the SV2A still remains to be unknown. Nevertheless it is suggested that it is obligatory in the control of exocytosis and might prevent the exocytosis of glutamate, since a relation between potency and binding affinity in removing tonic seizures in audiogenic-sensitive rat has been shown [316].

In a review focused on studying utilization of LEV as a pharmacological agent for the treatment of epilepsy, neuronal damage and hyperalgesia, authors claimed that studies in the literature provide evidence of three major molecular targets: inhibition of Ca^{2+} N-type channels, SV2A protein and the neuromodulator action on 5HT, GABA, α 2-adrenergics, and μ -opioidergic pathways. Even so they agreed on the point that the pharmacodynamics of this drug has not been fully elucidated [7]

The unique binding site for LEV, the protein SV2A is particularly present in subcortical areas, like the thalamus [318]. It is noteworthy that the exact function of SV2A in synaptic vesicle cycling and neurotransmitter release remains uncertain. Nevertheless it is suggested that SV2A protein could alter the exocytosis of transmitter-containing synaptic vesicles and act as a transporter, modifying synaptic function. SV2A deficiency has been suggested to leading to an accelerated epileptogenesis and elevated seizure vulnerability [7, 319].

In general LEV has been claimed to not to affect normal brain physiology, indicating the possibility of its modulation occurs only when pathophysiological conditions. But a connection between elevated SV2A expression and changes in synaptic functioning has been shown, leading to the suggestion of the assumption that overly SV2 is as detrimental to neuronal function as too little of it [320]. On the other hand, no relation between the genetic variations of SV2 proteins and the response to LEV was found during evaluation of susceptibility to epilepsy [321].

Neuroprotective effects of LEV were also linked to its inhibitory effects on Ca^{2+} N-type channels, reversal of the inhibition by it on negative allosteric modulators like zinc, beta-carbolines of GABA, glycine-gated currents and inhibitory effects on Ca^{2+} release from intraneuronal stores [322]. Its ability to hyperpolarize membrane potential via modulating the activation of K^{+} channels and to inhibit Ca^{2+} entry into the cells also were pronounced to be possible mechanisms of action [24].

In the superior cervical ganglion neurons, LEV was claimed to attenuate Ca^{2+} current density [25]. Presynaptic inhibitory action of GABAergic neurons was suggested to be increased by LEV, while also it reduces the excitotoxic effect of glutaminergic neurons by blocking NMDA receptors. In addition LEV was reported to regulate AMPA receptor-mediated excitatory synaptic transmission in hippocampus via effecting presynaptic P/Q-type voltage-dependent Ca^{2+} channel, in this way leading a reduction in glutamate release and inhibition on the amplitude of excitatory postsynaptic

current [26]. Another mechanism for its neuroprotective effects was suggested to be its ability to upregulate the expression of glial glutamate transporters [323]

Micov et al. (2010) [22] studied the antihyperalgesic mechanisms of LEV regarding the contribution of opioidergic, adrenergic, GABAergic and 5-HTergic systems in a model of inflammatory pain. They reported that decreasing noradrenaline levels via its α_2 -adrenergic action, the indirect activation of central and/or peripheral GABA pathways by augmenting GABAergic neurotransmission, as well as by modulating 5-HT levels utilizing various 5-HT receptors were all involved. In addition, it was found that LEV also influences the μ -opioidergic receptors since they inhibit N-type Ca^{2+} channels in a voltage-dependent manner as well. Considering all, it is concluded that LEV has a broad spectrum of molecular targets, leading to the difficulty for clarification of its mechanisms of action.

New research avenues were being searched in content of determining the pharmacodynamics of LEV [7]. Neuroanatomical approach used in this thesis work provides a gateway in this manner, offering an opportunity to find ways about supporting the administration of this antiepileptic drug as an alternative treatment to neuronal damage and hyperalgesia.

2.3.2. Various electrophysiological characteristics of LEV

The actions of LEV on the neuronal high-voltage-activated (HVA) Ca^{2+} current on pyramidal neurones, identified visually in the CA1 area of rat hippocampal slices were shown to be inhibitory at a clinically relevant concentration [27]. In addition, the study investigating the effect of LEV on diverse HVA Ca^{2+} channels in newly-isolated CA1 hippocampal neurons of rats showed that LEV could not alter L-, P- or Q-type Ca^{2+} channel activity, even at the highest concentration used, whereas it specifically altered N-type Ca^{2+} channel activity [30]. LEV was also shown to reduce both extent and duration of paroxysmal depolarization shifts, as well as the concomitant elevation in $[\text{Ca}^{2+}]$ in neocortical slices (recordings were obtained from single pyramidal neurons within the slice) in a dose-dependent manner. Whole-cell patch-clamp recordings demonstrated that LEV decreased N-, and partially P/Q-type HVA Ca^{2+} currents evidently, whereas Na^+ currents remained unaffected [33]. LEV was found to be efficient and potent in inhibiting HVA Ca^{2+} currents in the cortical and periaqueductal grey neuronal populations, modulated only P- and L-type channels [8]. HVA Ca^{2+} currents were examined over patch-clamp recordings from rapidly-dissociated

pyramidal neurons, and LEV was found to reduce those currents [32]. It also lowers glutamate transmission via presynaptic P/Q-type Ca^{2+} channels on the the dentate gyrus granule cells and the amplitude of EPSC within that region [34]. LEV's inhibitory effects on the HVA L-type Ca^{2+} channels in hippocampal CA3 neurons in epilepsy were also shown by other researchers [35].

Effects of LEV on GABA- and glycine-gated currents were investigated by whole-cell patch-clamp methods administered on spinal and hippocampal neurons and cultured cerebellar granule and the results showed that observed reversal of the negative modulator activity on the two primary inhibitory ionotropic receptors might be relevant regarding the anti-epileptic mechanisms of LEV [28]. LEV was shown to strengthen GABA inhibition of neuronal circuits by blocking the GABA current down-stream (caused by repetitive administrations of GABA) in the cortex [36]. A decrease of excitatory synaptic transmission within hippocampal neurons by LEV was shown to involve zinc ion-dependent GABA type A receptor-mediated presynaptic modulation [37].

AMPA receptors have been shown to be altered by LEV since an evident reduction in amplitude, the frequency of mEPSCs and the kainate- and AMPA-induced currents was observed in cortical neurons in culture [38].

In a study which effects of LEV on AP formation and voltage-operated K^{+} currents were investigated in rapidly-isolated hippocampal CA1 neurons using whole-cell configuration, LEV was shown to reduce repetitive action potential formation, affect the single action potential, decrease the total amount of APs, reduce the total depolarisation region of repetitive action potentials, increase the duration of the first AP insignificantly, prolong the second AP, decrease the slope of rise, decrease the voltage-operated K^{+} current, and without effecting Na^{+} and A-type K^{+} currents reduced the delayed rectifier current [39]. The persistent Na^{+} current was shown to be not affected by LEV in CA1 hippocampal neurons [324].

A study investigating the possible neuroprotective effects of LEV on the electrophysiological changes on striatal neurons stimulated by *in vitro* ischemia, introduced that LEV induces a small reduction of excitatory postsynaptic potential amplitude (recorded after synaptic stimulation in corticostriatal slices via sharp intracellular microelectrodes), reduces HVA Ca^{2+} conductances and is ineffective on fast Na^{+} conductances (whole cell patch clamp recordings from rapidly-isolated rat

striatal neurons) [40]. The fact demonstrating lack of effects of LEV on Na^+ currents has been also shown by other researchers in rat hippocampal neurons [325].

LEV was reported to elevate the activity of the renal outer medullary K^+ channel (channels that maintain the resting membrane potential) [326]. The inhibitory effects of LEV in cell culture on slowly inactivating delayed rectifier K^+ current (KV3.1-encoded current) was pronounced to incorporate an underlying mechanisms through which it affects neuronal activity *in vivo* [327]. Also LEV was reported to restore altered astrocyte RMP through alterations in outward and inward rectifier currents, which points out stabilizing consequences for neuronal-glia interactions [328]. Depletion of Kv4.2, a dendritic K^+ channel crucial for synaptic plasticity and the regulation of dendritic excitability was shown to be blocked by LEV [329].

Recordings in rat brain slices from CA1 neurons to quantify LEV's effects on IPSCs showed that it reduces inhibitory currents in a frequency-dependent manner [330].

In a study which aimed to examine the actions of LEV on the Ca^{2+} signaling induced by membrane depolarization and excitability in sensory neurons via the whole-cell configuration and fura 2-based ratiometric Ca^{2+} -imaging methods, results showed that it significantly increases R_i and causes the membrane hyperpolarization from E_{res} in a dose-dependent manner. That study was pronounced to be the first evidence on the potential effects of LEV in the excitability of rat sensory neurons by an action that might involve K^+ channel activation and inhibition of Ca^{2+} entry [41].

Via targeting high-voltage, N-type Ca^{2+} channels as well as the SV2A, LEV has been suggested to impede impulse conduction across synapses [304].

2.4. HCN Channels

HCN channels have a regulatory role on neuronal excitability in both peripheral and central nervous systems since they are commonly expressed in both peripheric sensory neurons and CNS neurons [331, 332]. They generate an inward current (hyperpolarization activated current, I_h) at hyperpolarized membrane potentials, a mixed Na^+/K^+ current. Growing evidence shows that HCN channels are engaged within the development, progression and maintenance of chronic pain. But understanding the extent and mechanism of the impact they possess still requires further research [57].

In a study conducted to enlight the effects of the HCN channel activity in the thalamus on chronic pain, researchers injected a HCN channel blocker ZD7288 into the

VPL to the rats with monoarthritis or neuropathic pain. They observed a dose-dependent attenuation of mechanical allodynia and thermal hyperalgesia in addition to increased immunoreactivity of both HCN1 and HCN2 subunits within thalamus. They concluded with a suggestion such as the elevated HCN channel activity in the thalamus of the ascending nociceptive pathway plays an evident role in both inflammatory and chronic neuropathic pain conditions [57].

Anesthesia [333, 334], learning and memory [335, 336] and sleep and arousal [337] are some of the behavioral and physiological process which HCN activity takes a part in. Misregulations in their activity were demonstrated to lead to psychological and neurological disorders such as epilepsy [338], pain [339, 340], anxiety and addiction [341, 342].

Accumulating evidence indicates that deterioration of HCN channel activity is correlated with the development and maintenance of chronic pain, which is confirming the observation that inhibition of HCN channel activity introduces anti-nociceptive action [343-345]. In peripheral nerve injury, HCN protein accumulation along with up-regulation of I_h current was shown in dorsal root ganglion neurons [346] and the spinal cord [347]. Systemic or local application of a HCN channel blocker was demonstrated to reduce nociceptive behavior in animals [347].

At the supraspinal level, a correlation has been found between increased HCN activity and comorbidity or chronic pain. For instance, HCN1 expression level was found to be elevated in the amygdala of rats with CCI and inhibition of HCN channels was found to be anti-nociceptive [345]. In CCI model, enhanced I_h current and increased HCN protein expression level were also observed in the PAG region, a finding that shows parallelism with the observation that HCN channel blocker infusion to this area attenuated neuropathic pain [339, 348]. Microinfusion of the same HCN channel blocker into the cortex (medial prefrontal or anterior cingulate) was also demonstrated to produce an antinociceptive effect in CCI [340] or spared nerve injury [349]. These observations led to a conclusion that activation of HCN channels in ascending nociceptive pathways might be a crucial component in chronic pain conditions.

As receiving the nociceptive data from several ascending pain pathways and connecting to the limbic system and cortex, thalamus has a pivotal role in neuropathic pain. The findings obtained from human imaging studies showing that changes within

thalamus accompanies to neuropathic pain supports this statement [350]. Besides, the thalamic neuronal firing pattern has been shown to become irregular in patients within tractable pain as well [351, 352]. In the study investigating if the HCN protein expression within the thalamus gets altered in inflammatory and/or neuropathic pain conditions and if the HCN action in the thalamus can introduce any direct influence on nociceptive behavior, researchers found that chronic pain elevated HCN immunoreactivity and HCN activity inhibition in the thalamus attenuated thermal hyperalgesia and mechanical allodynia. In that study male Sprague Dawley rats and CCI model were utilized and test chemical was microinjected into the VPL [57], introducing significant similarity with the experimental methodology performed within this thesis.

In chronic pain that is induced by persistent inflammation, HCN channel activity was shown to be increased in both central and peripheral nervous systems, and VPL, being the final destination of the spinothalamic tract, has been suspected to be exposed to this increase [340, 353]. This suggestion was proved by Ding et al (2016) [57], who showed that microinfusion of ZD7288 into the VPL inhibited nociceptive behavior in rats with chronic pain induced by peripheral nerve injury (CCI) or inflammation, together with the finding that following nerve injury, HCN1 and HCN2 protein expression was elevated in the thalamus of the same rats.

Abnormalities in HCN channel activity were suggested to engage in the development and maintenance of chronic pain. I_h alters neuronal excitability by its influence on R_i and E_{res} in neurons [354]. Alterations in I_h induced by nerve injury was found to be related to the elevated sensory neuron firing [353, 355, 356].

Even though the presence of HCN channels were known, the study conducted by Ding et al. (2016) was the first study showing their contribution at VPL level to the neuropathic pain [57]. Another significant observation provided the perception of the specificity of HCN channels in neuropathic pain conditions since microinjection of the blocker did not alter thermal or mechanical nociceptive threshold in sham rats.

Results showing the participation of HCN channels to pain at VPL level are conforming to a recent finding introducing enhancer effect of activity of the HCN channels after nerve injury on synaptic transmission between the anterior cingulate cortex and thalamocortical projection [340]. Since the VPL nucleus is a main relay site of the spinothalamic track for temperature and pain sensation [357], it is not surprising to observe that in neuropathic rats, neurons of VPL nucleus exhibit higher spontaneous

firing together with evoked response [20]. Moreover, elevated HCN channel activity has been suggested to contribute to ectopic firing in chronic pain conditions [344, 355].

Intra-PAG or intrathecal application of HCN channel inhibitor was shown to decrease neuropathic pain behaviors [347, 348]. In addition to the report provided by Ding et al (2016) [57], this data also provides substantial support regarding an evident role of the supraspinal HCN channel activity in chronic pain.

HCN1 and HCN2 subunits amongst four HCN channel subunits (HCN1-4) were shown to be expressed in neurons through all the nervous system and pronounced to be highly relevant to pain modulation and processing [358-362]. HCN2 gene-knockout mice, which exhibited highly significant decrease in HCN2 protein expression in the brain areas such as thalamus and cortex, were shown to be conserved from thermal hyperalgesia and mechanical allodynia following inflammation [353], whereas HCN1 gene-knockout mice were shown to be partially conserved from generating cold allodynia [332]. Besides, elevated HCN1 and HCN2 protein expression was reported in the amygdala and PAG in chronic pain [345, 348].

In the study performed by Ding et al (2016) [57], it was demonstrated that HCN1 and HCN2 immunoreactivity was elevated in the thalamus of neuropathic rats. The exact mechanisms of HCN channel alterations in the thalamus effect chronic pain endures to be clarified, the data of the mentioned work suggest that inhibition of HCN1 and HCN2 activities might be a pharmacological approach in chronic pain.

2.5. Inwardly Rectifying K^+ Currents

Presence of inwardly rectifying K^+ (K_{ir}) currents in neurons have been shown in several studies [363-368]. Classical K_{ir} channels have been shown to be abundantly presence within brain, $K_{ir2.1}$ being expressed diffusely and inadequately in the entire brain, $K_{ir2.2}$ strongly in the cerebellum and moderately throughout the forebrain, $K_{ir2.4}$ in the cranial nerve motor nuclei of midbrain, medulla and pons, $K_{ir2.3}$ mainly in forebrain and olfactory bulb [369-373].

K_{ir} channels have been demonstrated to have contribution to the long-lasting action potential plateau and establishment of highly negative E_{res} , leading to hyperpolarization [374]. This hyperpolarization has been suggested to close voltage-gated Ca^{2+} [375]. The observed hyperpolarization of E_{res} is mainly related to an increase in K^+ conductance [376-381]. Excitability of the neuron has been shown to be highly affected by these channels since blockage has been demonstrated to cause

depolarization and initiation of action potential firing [382]. $I_{K_{ir}}$ within thalamocortical neurons has shown to represent an area of negative slope conductance in the current-voltage (I-V) relationship which generates K^+ currents activated by hyperpolarization, an effect mostly mediated by $K_{ir2.2}$ channels. Moreover, unblock of this current has been demonstrated to amplify hyperpolarization [383].

K_{ir} channels have been suggested to not solely regulate the active and passive electrical properties of cells, but also to participate in G protein-coupled receptor signaling as well. The possibility of their contribution to membrane excitability and cellular metabolic state was also proposed *in vivo* [384].

Extend of the K_{ir} conductance defines the value of E_{res} , in a way that if this conductance is high then the E_{res} becomes near to the equilibrium potential for K^+ . This results in absence of spontaneous electrical activity. This process and their essential voltage dependence provide these channels the ability to have a key role in the regulation of the action potential duration and maintenance of E_{res} in electrically excitable cells [385-387].

Inward rectification they induce has been shown to not to be an intrinsic function but a consequence of the block of outward K^+ flux by intracellular components like polyamines and Mg^{2+} . Induced inward rectification was shown to represent diversity among types of K_{ir} channels. For instance, $K_{ir2.x}$ and $K_{ir3.x}$ were pronounced to be strong, $K_{ir4.x}$ as intermediate and $K_{ir1.1}$ and $K_{ir6.x}$ as weak rectifiers [384].

To investigate the roles of these channels, Cs^+ and Ba^{2+} are often used. When applied, Ba^{2+} and Cs^+ have been shown to restrain K_{ir} currents in a voltage-dependent manner. The inhibition induced by these agents has been shown to be stronger at hyperpolarized potentials [388-391]. Moreover, at hyperpolarized membrane voltages, K_{ir} current has been shown to increase, at first time-independently and later time-dependently [392].

It has been shown that among dopaminergic neurons of substantia nigra pars compacta, NT induces an excitation via reducing $I_{K_{ir}}$ and increasing the cationic conductance [393].

2.5.1. G protein-gated inwardly rectifying K^+ channels

G protein-gated inwardly rectifying K^+ (GIRK) or K_{ir3} channels are components of a large family of inwardly rectifying K^+ channels (Kir1 – Kir7). Inward rectification

means an alteration in slope of the I-V relationship at the reversal potential (*i.e.* the zero current level) [394, 395].

GIRK channels hyperpolarize neurons in reaction to the activation of many G-protein coupled receptors and therefore regulate the excitability of neurons through, volume transmission slow synaptic potentials and GIRK-mediated self-inhibition [394].

Under physiological conditions E_{res} of a typical neuron has been shown to be positive to the equilibrium potential for K^+ . The little outward K^+ current via GIRK channels reduces the neuronal excitability. Neurotransmitters like dopamine, acetylcholine, serotonin, opioids, GABA, somatostatin and adenosine have been shown to activate these channels [396].

GIRK channel activation has been pronounced to modulate the neuronal network in several regions within brain at various levels. The basal activity of these channels has been demonstrated to contribute to the E_{res} , mainly altering the membrane voltage. This hyperpolarization of the E_{res} reduces electrical excitability. Additionally, receptor activation of GIRK channels has been shown to provide other levels of inhibition, to which three distinct alterations in signaling could be commonly described, namely neuron-to-neuron inhibition, neuronal self-inhibition, and network-level inhibition [394].

In mammals four GIRK channel subunits (GIRK1-4 or $K_{ir}3.1-3.4$) have been shown to be expressed. GIRK1-GIRK3 subunits are pronounced to be the main types present in brain, while GIRK4 expression was shown to be low and therefore not participating evidently to cerebral GIRK currents [397].

Involvement of GIRK channels to pain perception has been shown in animal models. When there appears an abnormality regarding their function, altered neuronal excitability and cell death occurs, which is the basis of the suggestion representing the possibility of their contribution to several disease states. Therefore, GIRK channels were suggested to be a valuable new therapeutic target [394].

The notion suggesting the implication of GIRK channels to pain perception is based on studies showing GIRK channel activation via endogenous pain modulators like endorphins and endocannabinoids together with analgesic drugs and studies conducted with mice with GIRK channels mutations [398-402].

GIRK channels are considered as new targets for the development of new therapeutic agents in neuropathic pain management. Still, the pharmacology of these

channels endures to be largely unidentified. Even though a number of drugs including antidepressants have been shown to block the GIRK channel, this inhibition requires relatively high drug concentrations and found to be not selective [403].

GIRK channels have been suggested to mediate a slower inhibitory postsynaptic potential after the fast inhibition mediated by ionotropic GABAA/glycine receptors [404].

Ligand bindings (such as hormones, neurotransmitters, drugs) to their G protein-coupled receptors activate GIRK channels. Afterwards, G protein stimulation, which consequently leads to activation of the GIRK channel, occurs. When this channel becomes open, it enables the migration of K^+ out of the cell, consequently leading to E_{res} become more negative, meaning that activation of GIRK channels reduces spontaneous AP generation and suppresses the releasing of excitatory neurotransmitters [405].

In rat thalamic neurons, a relation between GIRK and high voltage activated Ca^{2+} channels was demonstrated [406-408]. In sympathetic neurons of rats [409], GIRK channel overexpression has been shown to decrease basal Ca^{2+} channel facilitation and attenuate noradrenergic inhibition of Ca^{2+} channels.

Possibility of the modulation of NT on GIRK channel activity, probably via GABAergic and dopaminergic modulation, has been proposed. Authors suggested that NT may reduce both dopamine and GABA-mediated GIRK currents by causing direct modulation of GIRK channels. Further experiments are needed to test this hypothesis [410].

As we merely start to clarify the participation of GIRK channels with different subunit configurations, their respective expression pattern at supraspinal level will give more functional insight [394]. Further research is required to relate obtained cellular observations to behavior and function. The development of novel methods such as conditional and cell-specific knock-out mice lines will be crucial to further advance our clarification of GIRK function. These methods will be important for further research of GIRK channels in disease and may contribute to design specific drugs, selectively opening or closing GIRK channels consisting of different subunit compositions, in treatment. [394].

2.6. NT and Pain

NT is an endogenous neuropeptide with analgesic effects which are opioid-independent [43, 44, 49, 50]. These effects have been suggested to be mediated by both

NT receptor subtype 1 (NTS1) and NT receptor subtype 2 (NTS2) [44]. NT receptors were found widely in the CNS including some structures of pain circuits [44, 411-413]. Within the CNS, NT has been shown to be found exclusively in neuronal cell terminals, bodies, fibers [414] and in neuronal synaptic vesicles [415, 416], functioning as a neuromodulator or neurotransmitter [44].

According to the results obtained with radioimmunoassay method, presence of NT has been shown in the thalamus [417, 418], besides NTS1 has been shown to function within ventral thalamus [419]. Localization of the NT receptor NTS2 in brain was investigated and researchers found that lateral thalamus is a region where NTS2 is abundant [420]. NTS2 has a lower affinity for NT than NTS1, which is a high affinity receptor for NT [44, 421].

Administration of NT into pain related regions of nervous system, as PAG, RVM or spinal cord have been shown to induce a profound antinociception. Antinociception induced by NT has been shown not only in physiological pain but in chronic pain as well. So many evidence has provided the outcome that NT acts as a painkiller in different chronic pain models [44, 422]. Following nociceptive injury [46] or μ -opioid receptor agonist injection [47] or stress stimulation [48], NT has been shown to display potent analgesia within CNS [44].

Clinically it is not convenient to use NT as a painkiller since it is highly susceptible to degradation and cannot overpass the blood-brain barrier. So far excessive effort has been given to specify candidates that can mimic antinociceptive effects of NT [44].

As mentioned above, when administered in supraspinal level, such as intra-RVM or icv injection, NT and its analogues were reported to exert analgesic effects in visceral pain. The RVM is a substantial site involved in descending facilitation or inhibition of spinal nociceptive transmission [423] The dual influences from the RVM were reported to be mediated by anatomically distinct pathways, the descending facilitation or inhibition system [44, 422, 424-427]

The complex mechanism of NT within RVM has been suggested to involve a pathway such as; at low dose, descending facilitatory system activates via high-affinity NTS1 whereas high dose could also bind to the low-affinity NTS2 in RVM, which played an important role in descending inhibitory system [44, 45].

NTS1 was pronounced to be, not totally but mainly, the responsible subtype for the antinociceptive action of NT formed through RVM [44, 425, 428-430]. In neuropathic pain, especially at spinal level, endogenous NT was suggested to play a role in later stage rather than early stage [431, 432]. Analgesic role of NT at spinal level have been pronounced to be mediated by NTS1 [433]. In CCI model, intrathecal administration of NT was reported to alleviate thermal and mechanical hypersensitivities and this effect was linked to its interaction with mostly NTS1 [44].

Dose-dependent effects of NTS1 selective antagonist SR48692 injected to RVM were analyzed and researchers found out that endogenous NT induces facilitatory influences in the RVM. Results supporting the facilitatory effect of NT in visceral pain were obtained before as well [44, 429, 434-437].

Pronociceptive mechanism of endogenous NT emerges mainly in under normal condition, NTS2 likely being the predominate receptor [434, 438]. Data obtained from some pharmacological studies also showed that blockade of neurotensinergic signal by antiserum against NT or NTS1 specific antagonist SR 48692 has been shown to potentiate morphine-induced antinociception [44, 428, 439].

As being the first relay station for peripheral nociceptive input and sending the project fibers to supraspinal pain related structures, the spinal dorsal horn carries importance for pain signaling. NT has been shown to introduce alterations within and related to spinal dorsal horn, for instance intrathecal injection of NT or its analogues has been shown to exert analgesic effects [44, 433, 440-443]. An excitatory effect at spinal cord level was suggested to be the mechanism, similar to its effect in the PAG and RVM.

The mechanisms underlying NT induced antinociception in various pain related supraspinal structures like thalamus or ACC are far from being fully clarified [44].

NT has been suggested to inhibit both GABA and dopamine mediated inhibition within ventral tegmental area dopamine neurons [410]. It also was observed that NT enhances GABAergic activity in hippocampal CA1 region, via a modulatory effect on L-Type Ca^{2+} channels [53]. In the VPL nucleus, presence of GABAergic interneurons has been shown [444], and expression of NTS1 on GABAergic interneurons was detected, a finding supported by the blockage of the effects of NT by the specific NTS1 antagonist SR48692. In CA1 interneurons, NT was shown to increase rate of AP firing while decreasing the afterhyperpolarization amplitude. The way neurotensin alters

GABAergic neuronal activity within hippocampus was pronounced to be also by phospholipase C [53]. Several studies suggested NT mediated increase in release of GABA in brain areas like globus pallidus, hippocampus, prefrontal cortex and striatum [54, 445-449]. An involvement of NTS1 in this effect was supported by findings showing blockage of NT results in GABA release elevation by SR48692 [450].

In a study analyzing the effects of NT on levels of GABA and glutamate in the substantia nigra pars reticulata and on dialysate GABA levels in the ventral thalamus, it was shown that NT dose-dependently increased local dialysate glutamate levels while reducing both local and ventral thalamic GABA levels [451]. Neurons within substantia nigra were pronounced to inhibit the neurons in VPL nucleus via GABAergic synapses [452].

The functional interaction between NTS1 and opioid has been suggested to affect the alterations of morphine on supraspinally-mediated nociceptive processes [453] while also a synergistic effect at supraspinal level to decrease inflammatory pain between NTS2 and morphine was pronounced [44, 454].

Relation between neurotensinergic and cannabinoidergic pathways was shown in PAG level. Activation of NTS1 in PAG has been suggested to induce a metabolic glutamate receptor 5 (mGluR5) mediated release of endogenous cannabinoid, which in turn binds to cannabinoid (CB) receptor type 1 (CB₁) and decreases release of GABA, leading to a disinhibition [44, 455].

The PAG integrates inputs from higher brain regions and arranges nociception through a descending pathway through the RVM and ends in the spinal dorsal horn [100]. Microinjection of NT into PAG leads to reduced nociceptive responses [456], indicating the suggestion that PAG probably is one of the structures in which NT exerts its antinociceptive effect. Electrophysiological studies introduced the observation that NT have a predominant excitatory effect on PAG neurons, some projecting to RVM [457-459]. Receptor activation by NT leads to opening of nonselective cation channels [458]. Surprisingly, antagonist pre-application was found to be not enough to inhibit excitatory effects of NT on PAG-RVM neurons. In local neurons, NTS1 activation was shown to lead to glutamate release and action potential in PAG, while inhibiting GABAergic synaptic transmission which induces neuronal excitation and glutamate release, leading to mGluR5 mediated production of endocannabinoids. In turn, released endocannabinoids activates presynaptic CB₁ receptors and decrease GABA release in

the PAG [455]. NT relieves GABAergic inhibitory control on neurons projecting to RVM and by this way facilitates excitation in PAG [44].

For the ionic mechanisms leading to the NT mediated increase in GABA release, inhibition of resting K^+ channels resulting in depolarization of GABAergic interneurons, leading to an increase in GABA release and AP firing, or increasing AP firing frequency without affecting depolarization have been suggested. NT was also suggested to modulate some certain subtypes of Ca^{2+} channels. L-type Ca^{2+} channels have been suggested to take a part in NT mediated GABA release elevation [53]. Also, inhibition of N-type Ca^{2+} channels was shown [460] together with low-voltage-activated Ca^{2+} channels such as T-type Ca^{2+} channels and high-voltage-activated Ca^{2+} channels such as L-type or N-type Ca^{2+} channels [51-53]. The release of GABA induced by NT has been proposed to be mediated by NTS1 [53, 450]. Activation of NTS1 has been shown to be accompanied with PLC and as a consequence, activation of PKC. Mentioned processes together with intracellular Ca^{2+} release take part in the elevation of GABA release induced by NT [53]. The PKC activated by NT phosphorylates L type Ca^{2+} channels [53, 461].

NT has several roles for being a neurotransmitter, also being a modulator of other neurotransmitters consequently affecting serotonergic, dopaminergic, cholinergic, glutamatergic and GABAergic systems [49, 54-56]. NT has been studied regarding its interaction with the dopaminergic system widely, and its effects on other neurotransmitter systems have been demonstrated [462].

Studies investigating the effect of NT on cholinergic transmission introduced that cholinergic neurons are directly or indirectly modulated by NT. [463-466]. Data obtained from electrophysiological studies showed that NT produces depolarization and rhythmic bursting in cholinergic neurons [467-470]. Via a decrease in inwardly rectifying K^+ conductances among cholinergic neurons, NT has been shown to induce excitation [471].

Effect of NT on serotonergic neurotransmission has been studied mainly focusing on anatomical data, such as observation of neurotensinergic innervations to densely serotonergic areas [414, 472]. NTS1 also has been shown to be present on serotonergic neurons [473, 474]. The role of NT within raphe has been suggested to be related to nociceptive function of serotonergic system [429].

In terms of GABAergic neurotransmission, NT was demonstrated to increase GABAergic activity in hippocampus, striatum, prefrontal cortex and globus pallidus [54, 447, 450, 475], whereas it has a contrary effect by a dopaminergic system related mechanism in substantia nigra and the ventral thalamus, mainly mediated by NTS1 [451].

NT has been demonstrated to increase glutamate release in brain areas, like substantia nigra, the striatum, frontal cortex and globus pallidus [451, 476-478]. Since an excess of glutamate can induce excitotoxicity NT has been suggested to enhance glutamate excitotoxicity [479, 480]. Together with its enhancing effect on glutamate release, NT also proposed to modify the function of glutamate receptors and elevate apoptotic cell processes which is induced by glutamate exposure [480].

When released from intrinsic glutamatergic spinal cord neurons, NT through NTS1 receptors has been shown to hyperpolarize excitatory interneurons while depolarizing inhibitory interneurons [442]. This finding was pronounced to be in parallel with the observation that when administered intrathecally, NT introduces antinociceptive effects [481-483].

Activation of NTS1 has been shown to induce excitatory effects through G-proteins, leading to intracellular Ca^{2+} influx. Consequently, an increase in intracellular cAMP, cGMP, and inositol trisphosphate; and increased activities of Na^+/K^+ -ATPase, PLC and PKC occur [484-491]. NTS1 has been shown to have a close association with glutamatergic and dopaminergic processes promoting and reinforcing glutamate signaling in distinct brain areas via leading to an elevation of PKC activation and phosphorylation of the NMDA receptor [49].

2.7. Cannabinoidergic System within VPL

Supraspinal presence of CB receptor type 2 (CB_2) expression in the brainstem [492] and brain centers at higher levels, including the thalamus [493] and cerebellum [494] has been reported. Previously, a new contribution regarding CB_2 receptors at spinal level in altering nociception in neuropathic, but not sham-operated, rats [495] was shown, demonstrating their existence in the spinal cord of neuropathic rats [42, 496-498].

In a study conducted for determination of localization for CB_2 receptors at supraspinal level together with its localization on structures with well-accepted role in neuropathic pain processing such as the thalamus [182, 499], authors demonstrated that

within thalamus CB₂ receptors might take a part in the inhibitory effects of the ligands effective in neuropathic conditions. Von Frey filaments and *in vivo* electrophysiology techniques were utilized on male Sprague–Dawley rats. They reported an attenuation in evoked responses and spontaneous activity within VPL in neuropathic, but not sham-operated, rats by selective CB₂ agonist administered to thalamus directly. In other words CB₂ agonist attenuated responses of VPL neurons which are mechanically evoked. With SR144528, this effect was shown to be blocked whereas no blockage was demonstrated with AM251, the CB₁ receptor antagonist. That study was the first report introducing a functional effect of CB₂ receptors at supraspinal level regarding neuronal activity in neuropathic pain. Another observation of the study was the fact that neurons within VPL exhibit evidently higher burst and spontaneous activity in neuropathic rats, compared to sham-operated group. Besides VPL neurons response in a larger extent when noxious mechanical stimuli is applied to the neuropathic group compared to sham-operated rats, corroborating previous research in neuropathic rats [182] and a model of spinal cord injury [20, 42].

Presence of CB₂ receptors in thalamus was shown, even though it was at lower levels compared to CB₁ receptors [493], and authors suggested this receptor to modulate nociceptive processes. Absence of effects on the VPL neurons of sham-operated rats was pronounced to demonstrate the neuronal responses to be controlled less under nonpathological conditions at this level [42].

When administered directly to the thalamus, SR144528 alone shown to lack a modulatory effect on evoked or spontaneous responses, while increasing burst activity in neuropathic rats. Authors concluded CB₂ receptors within the thalamus might take a part in the alterations emerging from neuropathic pain states, since alteration of responses within VPL neurons in neuropathic, but not sham-operated, rats were observed [42].

CB₁ agonists have been shown to reduce pain behavior and evoke neuronal responses in neuropathic rats, but they were also pronounced to produce psychoactive side-effects [495, 500-503]. Microinjection of WIN55212, the mixed CB₁ and CB₂ receptor agonist, into the intralateral posterior thalamus has been shown to attenuate nociceptive behavioral processing in naive rats [504].

When administered systemically, selective CB₂ agonists were demonstrated to display analgesic effects in models of neuropathic [498, 502, 508, 509, 511] and inflammatory [498, 505-510] pain.

Even though absence of effects of JWH-133, a CB₂ receptor agonist, sham-operated group was shown, administration of this agent locally into the VPL has been demonstrated to attenuate the spontaneous activity significantly, whereas burst activity was found to be not affected. The relevance of mentioned differential effect of the CB₂ agonist on burst vs. spontaneous activity is unclear for now. The observation that the CB₂ receptor agonist can diminish the facilitated spontaneous activity of VPL neurons, together with the inhibition regarding both innocuous- and noxious-evoked responses, leads to the conclusion introducing possible role of the supraspinal CB₂ receptors in the inhibitory actions of systemically administered CB₂ receptor agonists in terms of mechanical allodynia [42, 498, 502, 508, 509, 511].

The contribution of CB₂ receptors at VPL level to pain in neuropathic rats might emerge from a consequence of elevated coupling of pre-existing receptors or increased receptor expression. In fact, after a damage to the peripheral nerve, protein expression specific for this receptor has been demonstrated to be elevated in the afferent terminals of sensory nerves in the superficial laminae of the spinal cord dorsal horn [496]. Besides, the assumed function of the endocannabinoids in altering thalamic neurons in neuropathic conditions was also investigated. Intrathalamic microinjection of SR144528 alone has been shown to not to change spontaneous or evoked responses significantly. Conversely, SR144528 has been shown to increase burst firing of VPL neurons in spinal nerve ligation model (SNL) rats. These results indicate a complex role of CB₂ receptors in the tonic control of activity of neurons in the thalamus. The importance for the absence of actions by CB₂ receptor antagonism on spontaneous activity compared to burst activity is not clear yet, however it has been suggested that CB₂ receptors might have an evident effect within VPL in tonic inhibition of neuronal activity. Also burst activity has been shown to transmit more input than single spikes, and there is more possibility of a burst than of single spikes generating a single postsynaptic spike [512]. Another suggestion was the one involving the data showing single spikes and burst firing to convey distinct information [513], in a way that some specific burst parameters convey specific information among neurons [514].

Data obtained from that study complement the work representing a new role for the CB₂ receptor at the spinal cord in neuropathic conditions, compared to sham-operated rats [495], and the study showing the presence of CB₂ receptor mRNA at the spinal cord level of neuropathic, but not sham-operated, rats [497]. Even though the exact roles of CB₂ receptors at supraspinal level in the control of nociceptive process is still unknown, CB₂ agonists have been pronounced to be antinociceptive. After nerve injury, the inhibitory effects of CB₂ receptor agonists have been suggested to be altered by thalamic as well as spinal [495, 497, 498] and peripheral [496, 515-517] sites of action.

3. MATERIALS AND METHODS

3.1. Experimental Animals

All animal care and experimental procedures were approved by the local ethics committee (Decision no: 2017-01 and 2019-01, Appendix I) and conducted in accordance with the standards of the European Community Guidelines, the Directive 2010/63/EU, on the care and use of laboratory animals.

10 to 12 weeks-old male drug or test naive Sprague Dawley rats weighing 200–250 g were used (number of individuals, n=7). The rats were kept in a storage room at a constant ambient temperature ($23 \pm 2^{\circ}\text{C}$) and relative humidity ($50 \pm 10\%$) under a 12 h light/dark cycle with free access to food (standart chow) and drinking water. Maximum number of rats per cage was 5.

3.2. Chemicals

Chemicals used and suppliers are listed in Table 3.1.

Table 3.1. *List of chemicals used*

Name of the Chemical	Supplier
LEV	Sigma Aldrich, USA
NT	Sigma Aldrich, USA
GBP	TCI Tokyo Chemical Industry, Japan
SR48692	Sigma Aldrich, USA
SR144528	Cayman Chemical, USA
ACSF	Tocris Bioscience, United Kingdom
Phosphate buffered saline (PBS)	Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, USA
Physiological serum	Polifarma İlaç San. Ve Tic. A.Ş., Tekirdag, Turkey
Ketamine	Richter Pharma ag, Austria / Interhas A.Ş. Ankara, Turkey
Xylazine	Bioveta a.s., Czech Republic / Intermed Ecza Deposu, Ankara, Turkey
Zinc polycarboxylate cement	Pentron, SpofaDental a.s., Czech Republic
Trypan blue solution	Sigma Aldrich, USA

3.3. Apparatus

Apparatus used are listed in Table 3.2, together with model and brand information.

Table 3.2. *List of apparatus utilized*

Name of the Apparatus	Brand and Model
Activity cage apparatus	Ugo Basile, Model no: 7441, 21036 Gemonio VA Italy
e-Von Frey	Ugo Basile, Model no: 38450, 21036 Gemonio VA Italy
Hargreaves apparatus	Ugo Basile, Model no: 37370, 21025 Gemonio VA Italy
Stereotaxic frame	World Precision Instruments (WPI), USA
Microinjection pump	Kent Scientific, Genie Touch, Torrington USA
Analytical balance	Ohaus, Switzerland
Vibratome	Leica VT1200S, Leica Biosystems Inc., IL, USA
Microscope	Olympus Corporation, MA, USA
Amplifier	Molecular Devices LLC, CA, USA

3.4. Administration of Drugs and Chemicals

GBP was chosen as positive control since it has been shown to induce antihyperalgesic, antiallodynic and neuroprotective effects in neuropathic pain [306].

Microinjection of the drugs to the awake and gently held animals performed at the following concentrations [518]; 3, 30 and 300 μg LEV dissolved in PBS [519], 100 μg GBP dissolved in saline [520, 521], 30 pmol and 10 nmol NT, as low and high doses considering the contrary effects, dissolved in 0.01 PBS in physiological serum, 3 fmol SR 48692 dissolved in 2% DMSO 0.01 PBS in physiological serum [428, 522], 48 ng SR144528 in 500 nL in ACSF [42].

Electrophysiological experiments were performed with 300 μg dose of LEV since most evident effects were observed at that dosage in *in vivo* experiments. For the mechanistic studies, 30 μg dose of LEV was chosen so that suppression of any possible reversal can be avoided.

Pretreatment and treatment of antagonists were performed 10 minutes before microinjection of LEV or its solvent, PBS, respectively [428].

3.4.1. Insertion of the cannula

At the same session with CCI surgery, cannula implantation was carried out. Rats were placed in the stereotaxic instrument. A guide cannula was implanted in the right VPL, since it is the contralateral site to the injury. Coordinates from the brain atlas were as follows [174]: 3.5 mm lateral to the midline, 3.4 mm posterior to the Bregma, distance to the skull surface 6.5 mm ventral. Fixation of the guide cannula to the skull was performed with jeweler's screws and dental acrylic. A 33-gauge stainless steel wire dummy cannula was used to reduce the incidence of occlusion by inserting into the guide cannula. Microinjection was performed through 33-gauge cannula which was extended 0.5 mm beyond the tip of the guide cannula. The 33-gauge cannula was attached to a Hamilton microsyringe through polyethylene-10 tubing (PE-10). Infusion pump was programmed to deliver a volume of 0.5 μ L over a period of 1 min. The needle was held for 1 more min before retraction to restrain any scattering [57].

Following the termination of each experiment, injection site was confirmed via visual examination of thalamus sections. Trypan blue was microinjected at the end of experiments and brains were collected. Coronal sections obtained from the injection site were used for visual examinations [57, 518]. An example is illustrated in Figure 3.1.

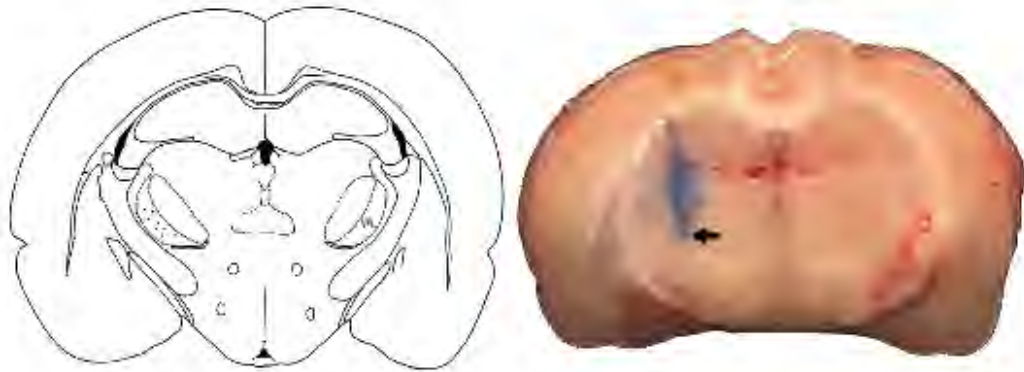


Figure 3.1. Representaton of the location for rat VPL [57].

3.5. Establishment of the Neuropathic Pain Model

The surgical procedure was performed aseptically. CCI model was done according to the method of Bennett and Xie [57, 229] with some modifications. Rats were anesthetized with 90 mg/kg ketamin (intraperitoneally, *i.p.*) and 10 mg/kg xylazine (*i.p.*) [523]. Afterwards, left sciatic nerve was exposed in the mid-thigh and using 4/0 silk sutures, four ligatures were loosely placed around the sciatic nerve, with a 1.0–1.5

mm interval. Skin incision was closed with hidden sutures. Rats in sham group received the same procedure but without nerve ligation.

Only the completely healed subjects were used for experiments. After surgical procedures, rats were housed in the cages individually for 7 days, since one week has been pronounced as the recovery time in several studies [312, 524, 525].

Considering the findings showing the most evident and stable effects of this model on post-operation day 14, assessments were started on that day [243, 244, 524].

The behavior and posture of the rats were carefully monitored on the first postoperative day and through the recovery from the anesthesia. The weight gain and general behavior of the rats were observed throughout the postoperative period. The development of mechanical allodynia and thermal hyperalgesia were assessed at the 13th day after surgery [42].

3.6. Assessment of Locomotor Activity

3.6.1. Activity cage apparatus

Horizontal and vertical locomotor activities of the rats were assessed by activity cage apparatus (Ugo Basile, Model no: 7441, 21036 Gemonio VA Italy) [526] for 20 minutes starting 15 minutes after microinjection on the post-operative day 18.

3.7. Assessment of Antihyperalgesic and Antiallodynic Responses

Experiments were performed at 15, 30, 45, 60 and 90 minutes after administration of the test chemical so that disappearance of the effect can be observed, supporting the observation for the effect to be drug related. Each test was performed at different days on the same animal, the interval being two days. Before test days, the subjects were habituated to test conditions in the test box via spending 1–2 h daily for 2 days [57, 518].

3.7.1. Hargreaves test

Hargreaves test was used for the assessment of thermal hyperalgesia since it is a commonly used and validated method. Hind paw withdrawal latency in response to radiant heat was measured using a plantar test apparatus using a modified Hargreaves thermal testing device (Ugo Basile, Model no: 37370, 21025 Gemonio VA Italy) and mainly according to the method described by Hargreaves et al. (1988) [528]. Rats were placed individually into a compartment enclosure on a glass surface. After a 15 min habituation -or until exploratory behavior was no longer observed- a mobile heat source

was positioned under the plantar surface of the right hind paw. Activated light beam of radiant heat was applied through the glass floor with a cut-off time of 30 sec [529]. Recording was done automatically by a digital timer as the response latency for paw withdrawal to the nearest 0.1 s. To prevent thermal sensitization the mean withdrawal latency (s) was determined from the average of two trials separated by a 5-min interval [173, 530].

3.7.2. E-Von Frey test

For the assessment of mechanical allodynia, e-Von Frey test was used since it is a validated method. Paw withdrawal thresholds to mechanical stimuli were measured using an electronic Von Frey device (Ugo Basile, Model No: 38450, Varese, Italy). Rats were placed in individual plastic cages on an elevated wire mesh platform and were allowed to habituate for at least 15 min or until exploratory behavior was no longer observed. A rigid tip was applied to the midplantar region of the left hind paw. Mechanical stimulation was terminated with the withdrawn move of the paw. Cut off was 50 g to avoid tissue damage. The mean of 2-3 consecutive measurements with 5 min intervals was used for the analysis [527].

3.7.3. Dynamic plantar test

For the animals in patch clamp groups, establishment of the neuropathy state was evaluated via mechanical allodynia assessment using the dynamic plantar aesthesiometer device (Ugo-basile, 37450, Verase, Italy).

Animals were placed in Plexiglas compartments individually, located on a perforated metal floor. Mechanical stimulus was applied by a metal rod (diameter, 0.5 mm) within the mobile device located under this metal floor. The rod applies increasing force to the paw via passing through the holes of the metal floor, and force stops automatically when the animal lifts his paw. Paw withdrawal thresholds of rats to the increasing force were measured with an accuracy of 0.1 g. Before measurements, 15 min habituation -or until exploratory behavior was no longer observed- was performed. [531, 532]. The average of 4-6 measurements obtained at 5-min intervals was calculated. Cut-off value was 50 g [531, 533].

3.8. Electrophysiological Studies

3.8.1. Preparation of the slices

For whole-cell electrophysiological recordings, 10 to 12 weeks-old sham and CCI operated male Sprague Dawley rats were used.

14 days after sham or CCI surgery, rats were deeply anesthetized with isofluorane and intracardially perfused with ice-cold N-Methyl-D-glucamine (NMDG)-based slicing solution containing (in mM): 2.5 KCl, 92 NMDG, 10 MgSO₄, 20 hydroxyethyl piperazineethanesulfonic acid (HEPES), 25 glucose, 1.2 NaH₂PO₄, 5 Na⁺ ascorbate, 30 NaHCO₃, 3 Na⁺ pyruvate, 2 thiourea and 0.5 CaCl₂. Brains were rapidly removed following decapitation and placed into an ice-cold NMDG solution. Acute coronal brain slices containing VPL, 200 µm thickness, were obtained using a vibratome (Leica VT1200S, Leica Biosystems Inc., IL, USA).

An image of brain slicing by vibratome is illustrated in Figure 3.2.



Figure 3.2. *Slice preparation – Vibratome sectioning*

Brain slices were transferred to a holding chamber filled with a solution containing (in mM): 92 NaCl, 2.5 KCl, 20 HEPES, 30 NaHCO₃, 1.2 NaH₂PO₄, 5 Na

ascorbate, 25 glucose, 3 Na⁺ pyruvate, 2 thiourea, 2 CaCl₂ and 1 MgSO₄. For all electrophysiological recordings, a single slice was transferred to the recording chamber and continuously perfused at a flow rate of 1.5 to 2.0 ml/min with ACSF, in mM: 125 NaCl, 2.5 KCl, 2.4 CaCl₂, , 1 MgCl₂, 26 NaHCO₃, 11 glucose, 1.25 NaH₂PO₄. All solutions were saturated with 95% oxygen and 5% carbon dioxide. Recordings were conducted at room temperature. Unless otherwise specified, all drugs were purchased from Sigma-Aldrich (Saint Louis, MO, USA) [534].

3.8.2. Patch clamp recordings

The image of electrophysiological rig used in this study is illustrated in Figure 3.3.

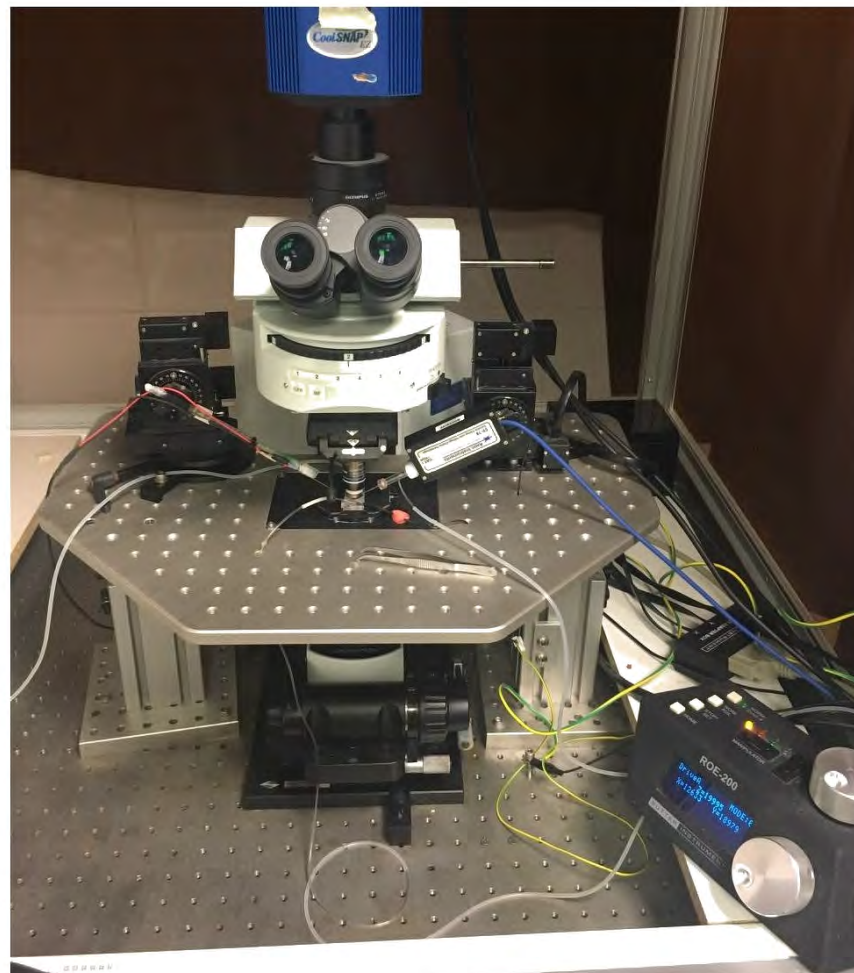


Figure 3.3. *Electrophysiology rig*

Neurons were visualized by an infrared differential interference contrast optics on an Olympus BX51WI microscope (Olympus Corporation, MA, USA). For whole-cell

recordings; patch pipettes (2.5–3.5 M Ω) containing (in mM): 120 K⁺ methane sulfonate, 15 KCl, 10 HEPES, 0.1 egtazic acid, 2 MgCl, 5 Na₂Phosphocreatine, 2 magnesium adenosine triphosphate, 0.3 guanosine 5'-triphosphate sodium (pH adjusted to 7.3 using KOH). Recordings were carried out using an Axon Multiclamp 700B amplifier (3 kHz low-pass filtered and 10 kHz digitized by a Digidata 1440A, Molecular Devices LLC, CA, USA) with Clampex software v10.6 (Molecular Devices LLC, CA, USA). Recorded neurons were held at -70 mV. Analysis of the recording was performed with Clampfit v10.6 (Molecular Devices LLC, CA, USA). R_s (10–20 M Ω) was monitored with a -10 mV hyperpolarizing pulse imposed every 60 s, and only recordings that remained stable over the period of data collection were used [534].

To examine the effects of CCI treatment on VPL neurons excitability, current-clamp input-output curves were obtained by injecting 500 ms-current steps with amplitude ranging from -300 to +1600 pA with 100 pA increments. I-V relationship were obtained by plotting the current response to voltage command steps of 2 seconds duration with 10 mV increments (-130 mV to -40 mV) starting from a holding potential of -70 mV. Membrane conductance was calculated as the slope of the linear regression obtained from the hyperpolarized part (from -130 mV to -70 mV) of the I-V relationship.

3.9. Statistical Analysis

GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, ABD) was used for statistical assessment and graph preparation. When multiple comparisons between groups were needed, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey honest significant difference multiple comparison test. Post-hoc Bonferroni multiple comparison test was applied following two way ANOVA when comparing all the groups with one particular group.

In patch clamp experiments, membrane conductance parameter was evaluated by unpaired t test, while I-V curves were analyzed by two way ANOVA following Sidak's multiple comparison test. For other parameters t test was used.

Results are expressed as mean $\pm$ standart error of the mean (S.E.M.). Probability value (p) was considered statistically significant when less than 0.05.

4. RESULTS

4.1. Effects on Locomotor Activity

As a marker for locomotor activity, effects of drugs or treatments on vertical and horizontal moves of the animals are shown in Figures 4.1-4.13. No significant difference has been observed for any group regarding drug, antagonist or solvent treatment. However, CCI establishment was found to decrease the number of vertical and horizontal moves in a statistically significant manner.

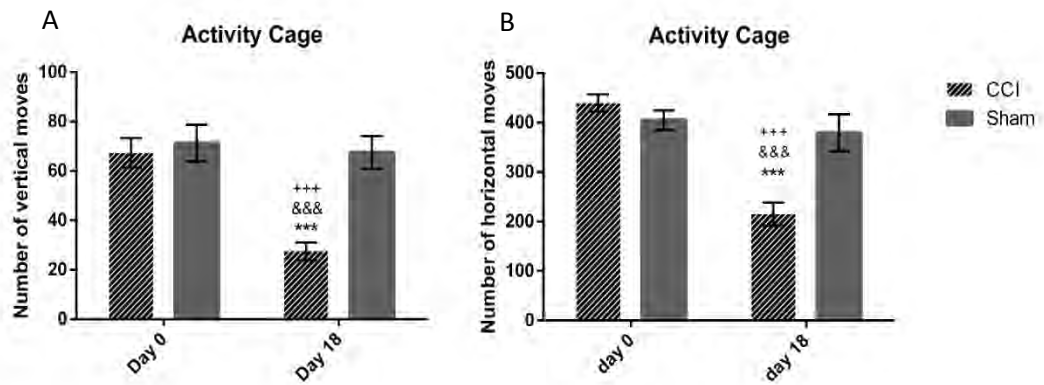


Figure 4.1. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and Sham groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; against day 0 Sham is $\&\&\&p < 0.001$ and against day 18 Sham is $+++p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

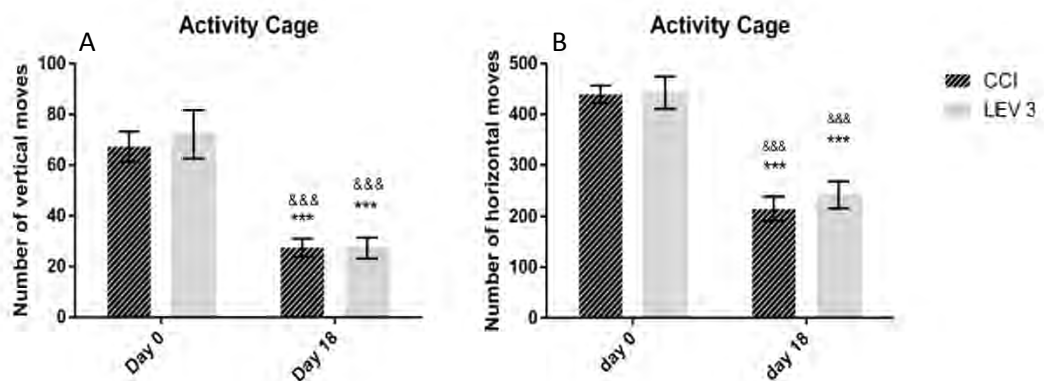


Figure 4.2. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and LEV 3 μg treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; and against day 0 LEV 3 μg treated group is $\&\&\&p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

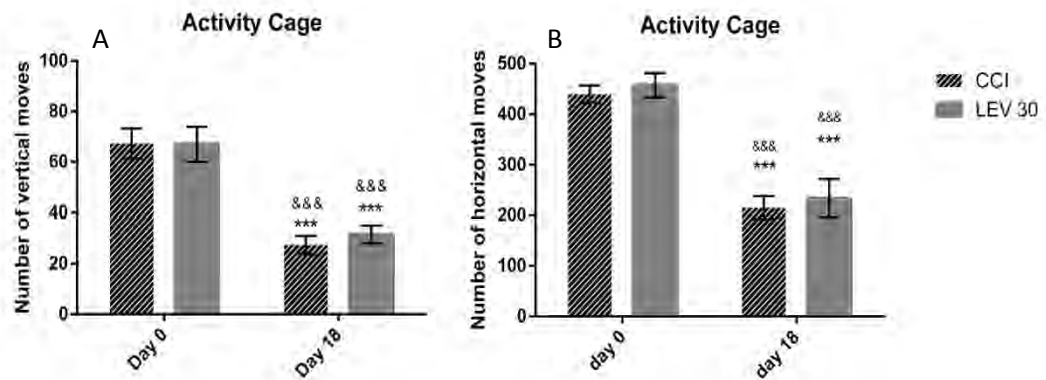


Figure 4.3. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and LEV 30 μg treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; and against day 0 LEV 30 μg treated group is $\&\&\&p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

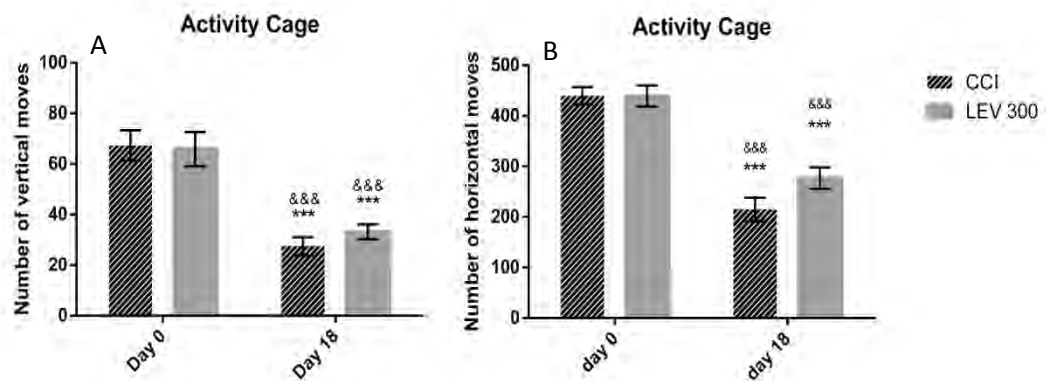


Figure 4.4. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and LEV 300 μg treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; and against day 0 LEV 300 μg treated group is $\&\&\&p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

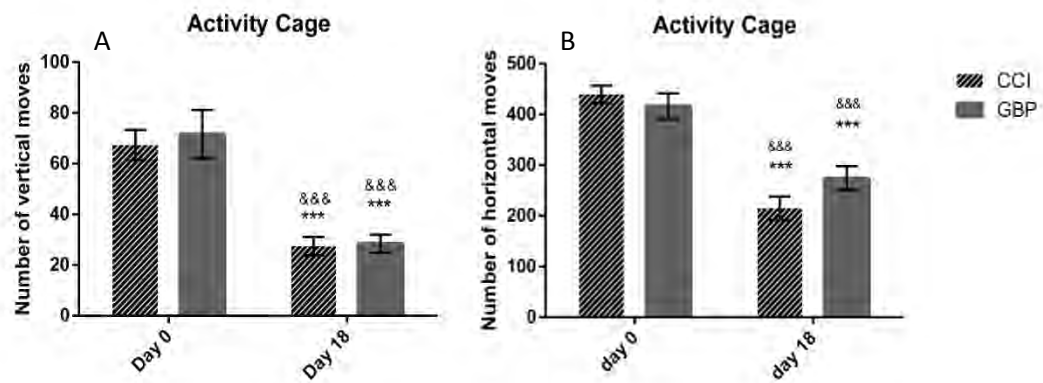


Figure 4.5. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and GBP treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** $p < 0.001$; and against day 0 GBP treated group is &&& $p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

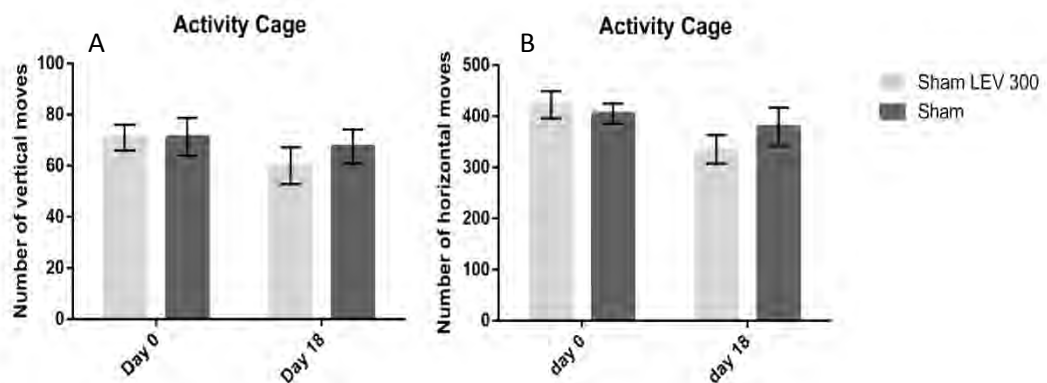


Figure 4.6. Number of vertical (A) and horizontal (B) locomotor activities of the rats in Sham and 300 μ g LEV treated Sham groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

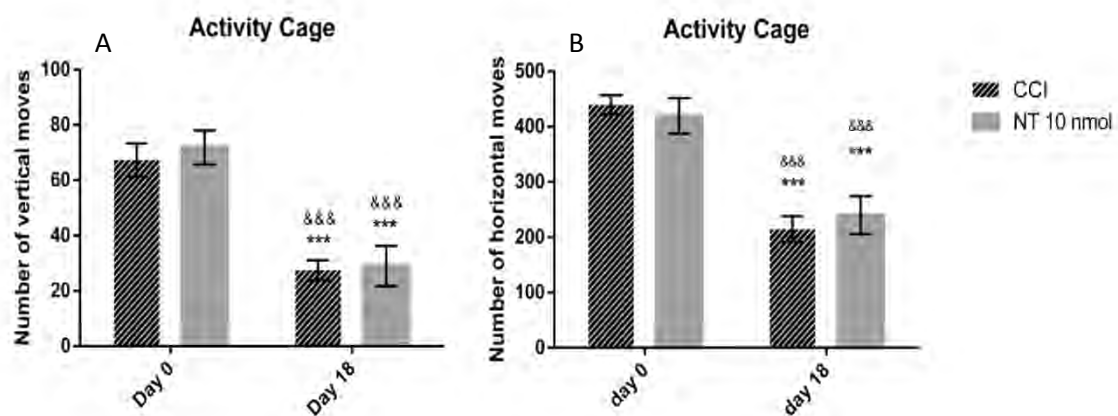


Figure 4.7. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and NT 10 nmol treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; and against day 0 NT 10 nmol treated group is $***p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

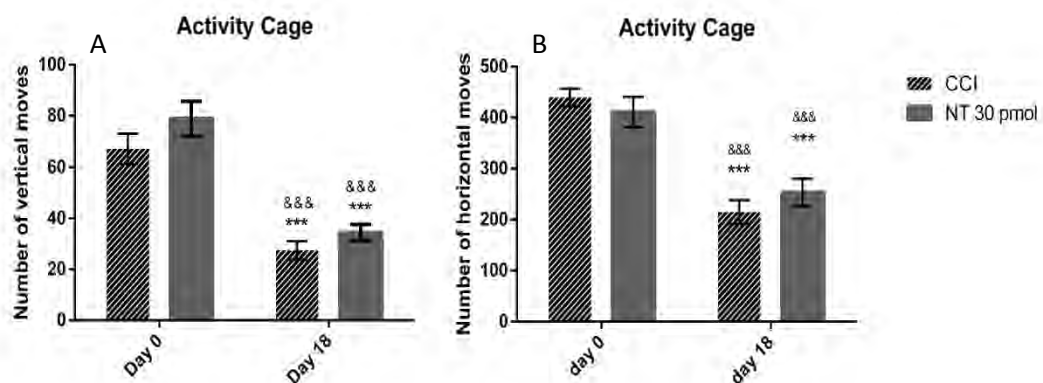


Figure 4.8. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and NT 30 pmol treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is $***p < 0.001$; and against day 0 NT 30 pmol treated group is $***p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

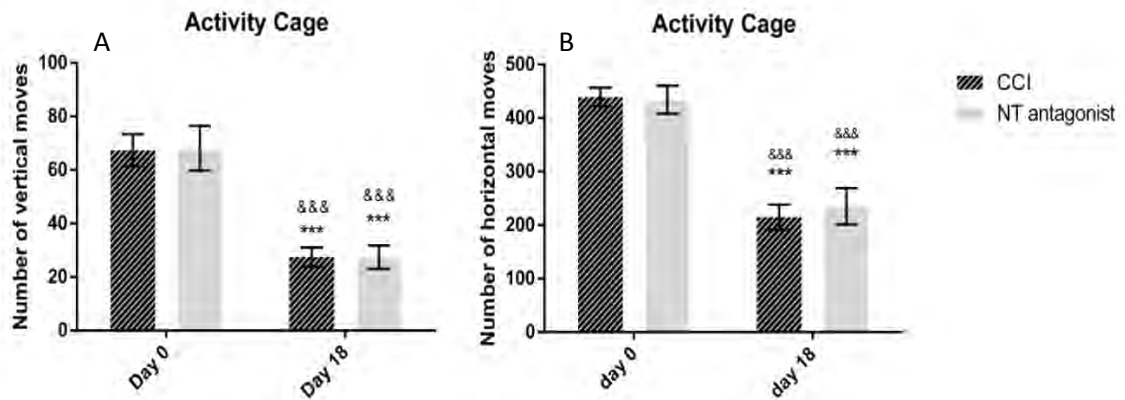


Figure 4.9. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and NT antagonist treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** $p < 0.001$; and against day 0 NT antagonist treated group is &&& $p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

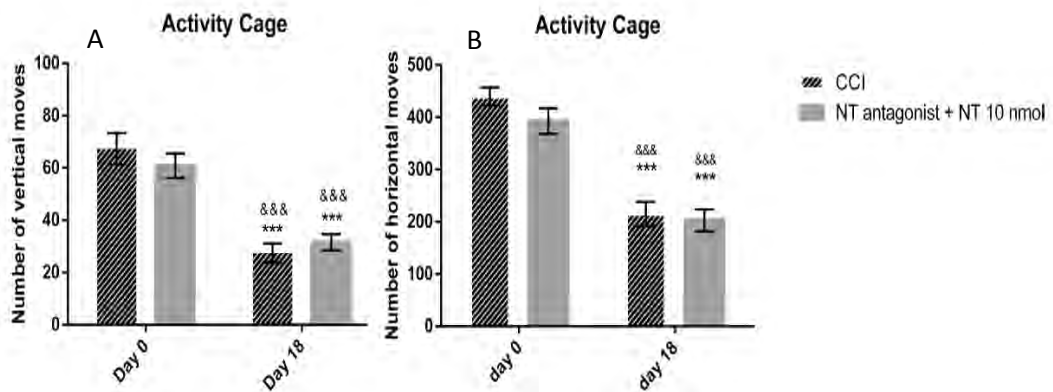


Figure 4.10. Number of vertical (A) and horizontal (B) locomotor activities of the rats in NT 10 nmol group pretreated with NT antagonist and CCI group in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** $p < 0.001$; and against day 0 NT 10 nmol group pretreated with NT antagonist is &&& $p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

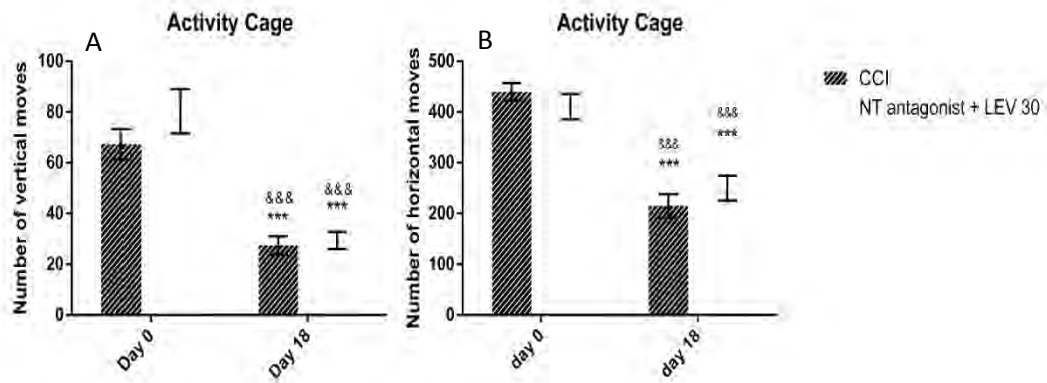


Figure 4.11. Number of vertical (A) and horizontal (B) locomotor activities of the rats in LEV 30 μ g group pretreated with NT antagonist and CCI group in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** $p < 0.001$; and against day 0 LEV 30 group pretreated with NT antagonist is &&& $p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

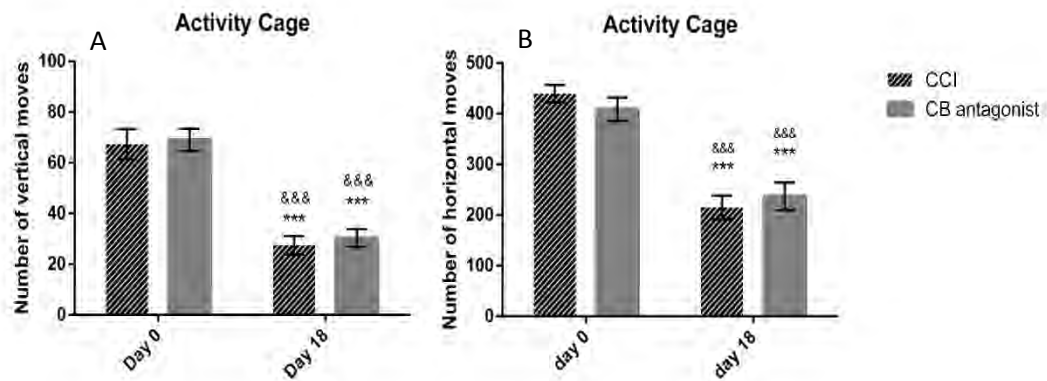


Figure 4.12. Number of vertical (A) and horizontal (B) locomotor activities of the rats in CCI and CB antagonist treated groups in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** $p < 0.001$; and against day 0 CB antagonist treated group is &&& $p < 0.001$. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

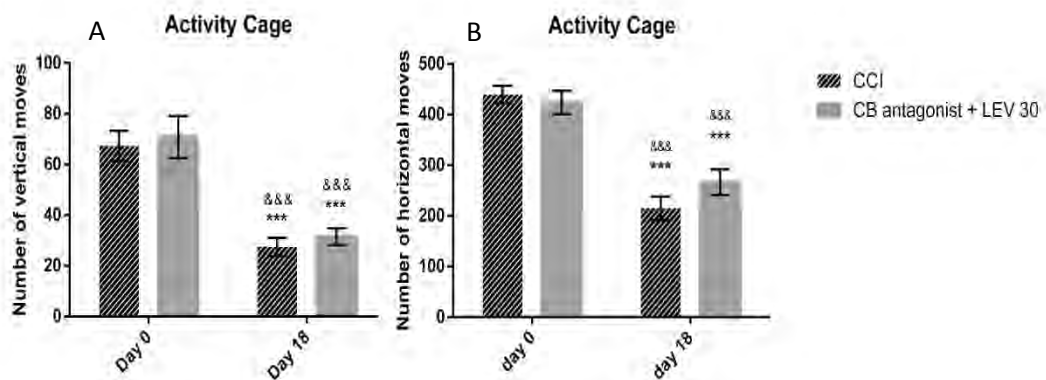


Figure 4.13. Number of vertical (A) and horizontal (B) locomotor activities of the rats in LEV 30 μ g group pretreated with CB antagonist and CCI group in the activity cage test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against Day 0 CCI is *** p < 0.001; and against day 0 LEV 30 group pretreated with CB antagonist is &&& p < 0.001. Two-way repeated ANOVA, posthoc Tukey test, $n=7$.

4.2. Effects on Hyperalgesia

Since allodynia and hyperalgesia after CCI were shown to be more stable on post-operative day 14 and this time point was pronounced to represent peak responses together with presenting the most ideal conditions regarding inflammation due to the surgery procedure, experiments were started at that time point within this work [243, 244].

4.2.1. Mechanical allodynia

A significant reduction in paw withdrawal threshold was observed between Sham and CCI group (Figure 4.14).

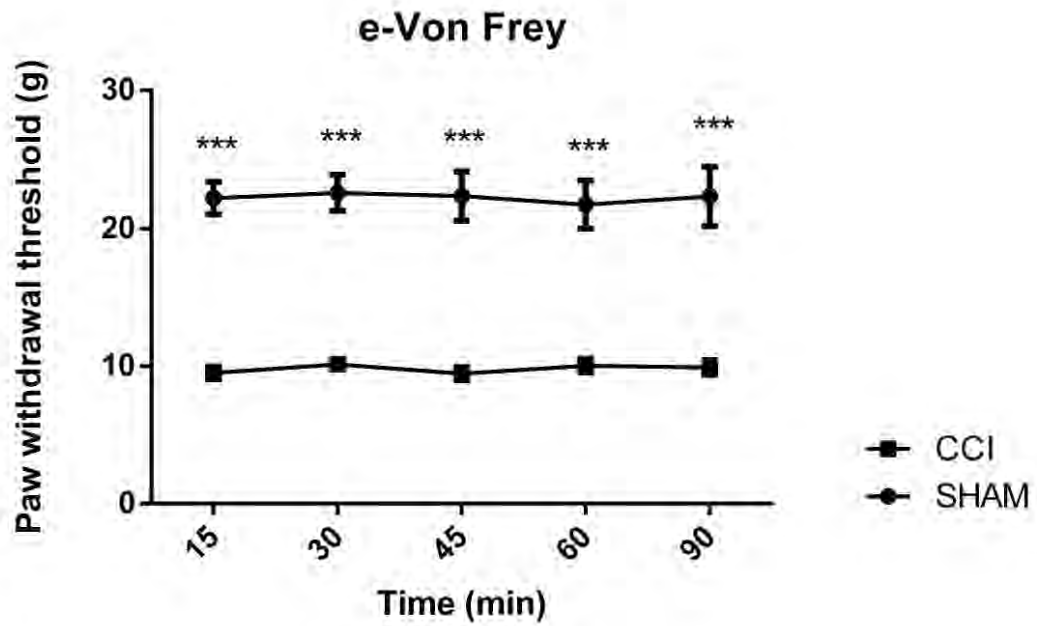


Figure 4.14. Paw withdrawal thresholds of rats in CCI and Sham groups in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Significant difference between groups is *** $p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

There was an antiallodynia induced by LEV 300 μ g observed on the 30th minute after microinjection in the sham-operated group (Figure 4.15). Dose and time-dependent antiallodynic effects of LEV are shown in Figure 4.16. Time-dependent antiallodynic effects of both LEV and GBP are illustrated in Figure 4.17, showing a more significant effect of GBP compared to LEV.

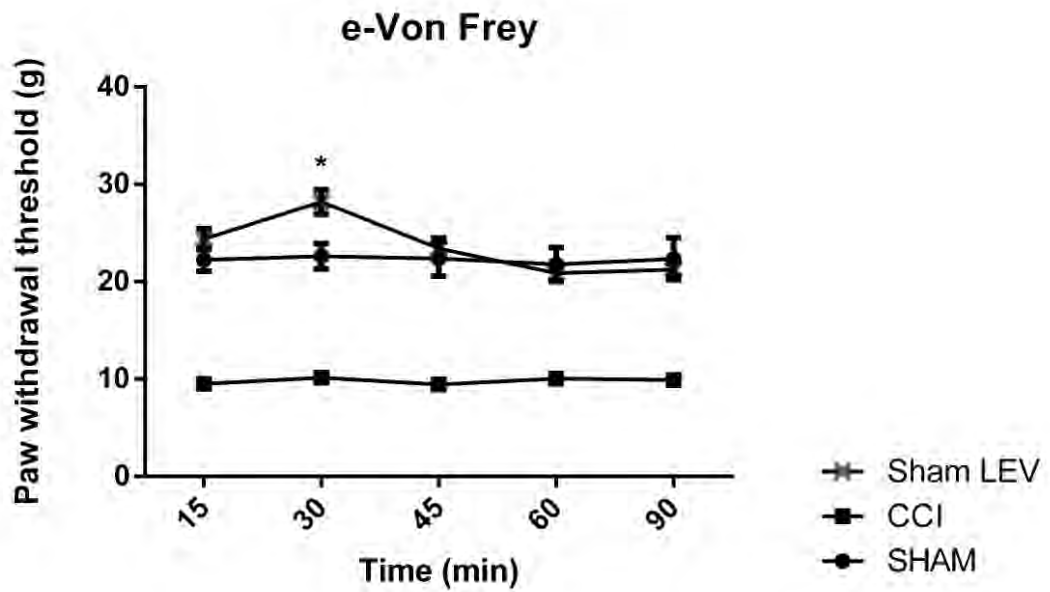


Figure 4.15. Paw withdrawal thresholds of rats in Sham group treated with 300 μ g LEV in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Significant difference against CCI group is * $p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

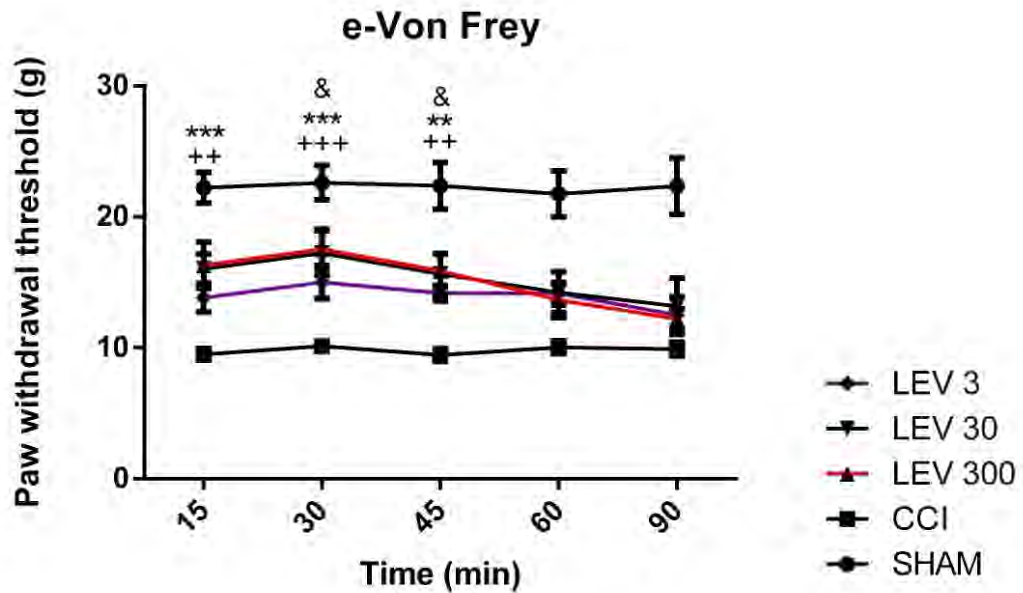


Figure 4.16. Paw withdrawal thresholds of rats in the groups treated with 3, 30 and 300 μ g LEV in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group are; for LEV 300 *** $p < 0.001$ and ** $p < 0.01$; for LEV 30 +++ $p < 0.001$ and ++ $p < 0.01$; for LEV 3 & $p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

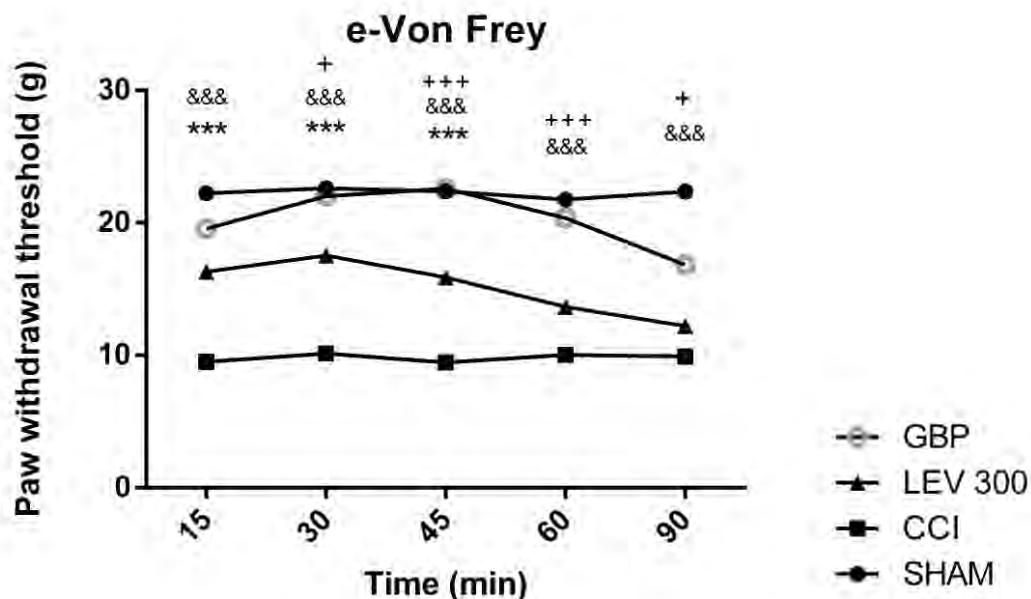


Figure 4.17. Paw withdrawal thresholds of rats in the CCI group and groups treated with GBP and 300 μ g LEV in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group for GBP group is $\&\&\&p < 0.001$, for LEV 300 is $***p < 0.001$; significant difference between GBP and LEV 300 groups are $+++p < 0.001$ and $+p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

4.2.2. Thermal hyperalgesia

As being a marker for hyperalgesic response against thermal stimuli, effects of test chemicals on paw withdrawal latency has been analyzed by Hargreaves test. A significant reduction was observed after CCI (Figure 4.18). No effect of LEV on this parameter was observed when microinjected to the sham group (Figure 4.19). Compared to LEV, GBP was found to be highly effective and its effects were time-dependent (Figure 4.20). A significant dose and time dependent effect for LEV microinjected to CCI rats was observed only at 15th minute after microinjection (Figure 4.21).

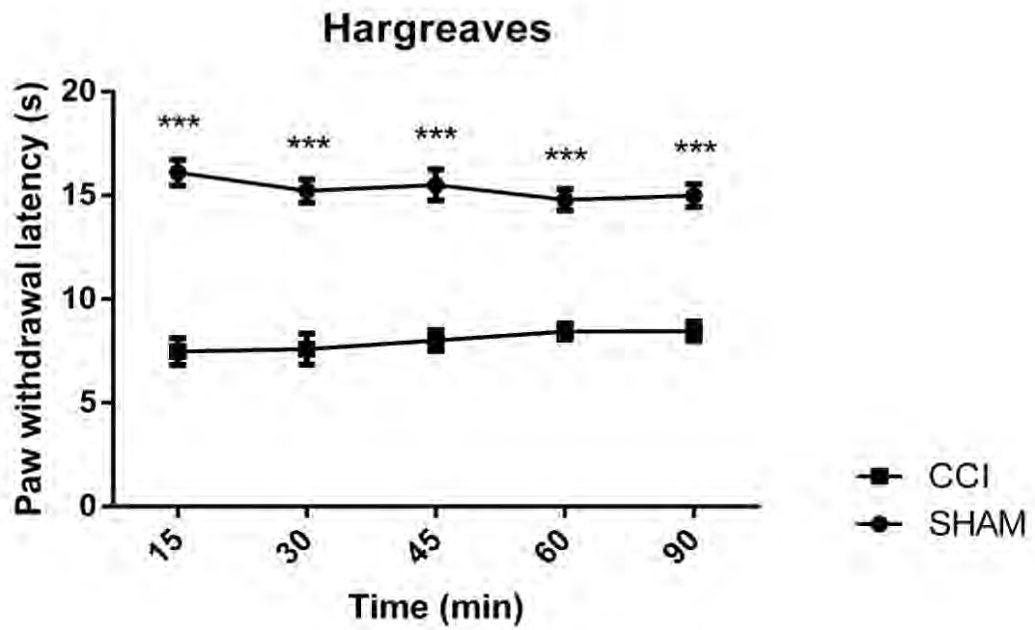


Figure 4.18. Paw withdrawal latency of rats in CCI and Sham groups in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Significant difference against CCI group for Sham group is *** $p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

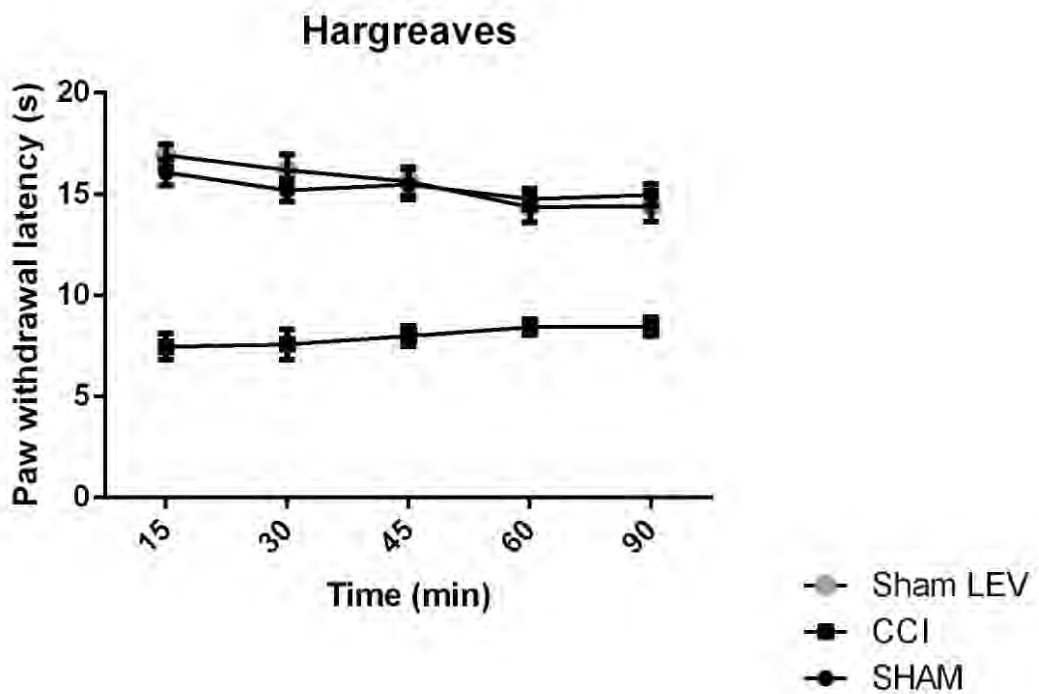


Figure 4.19. Paw withdrawal latency of rats in Sham group treated with 300 μ g LEV in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

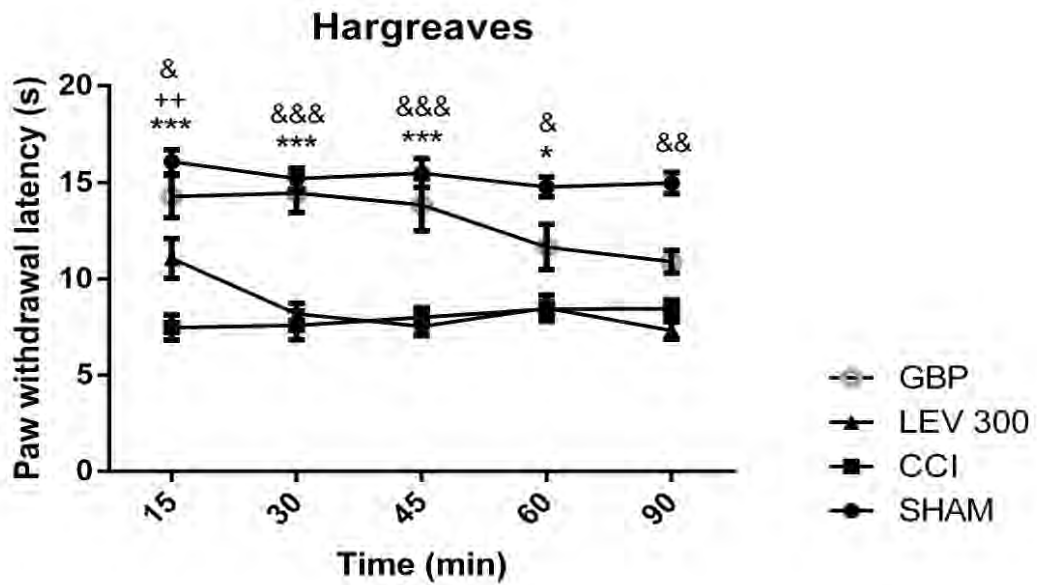


Figure 4.20. Paw withdrawal latency of rats in the CCI group and groups treated with GBP and 300 μ g LEV in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group are; for GBP group *** p < 0.001 and * p < 0.05, for LEV 300 is ++ p < 0.01; significant difference between GBP and LEV 300 groups are & p < 0.05, && p < 0.01 and &&& p < 0.001. Two-way ANOVA, post hoc Bonferroni test, n = 7.

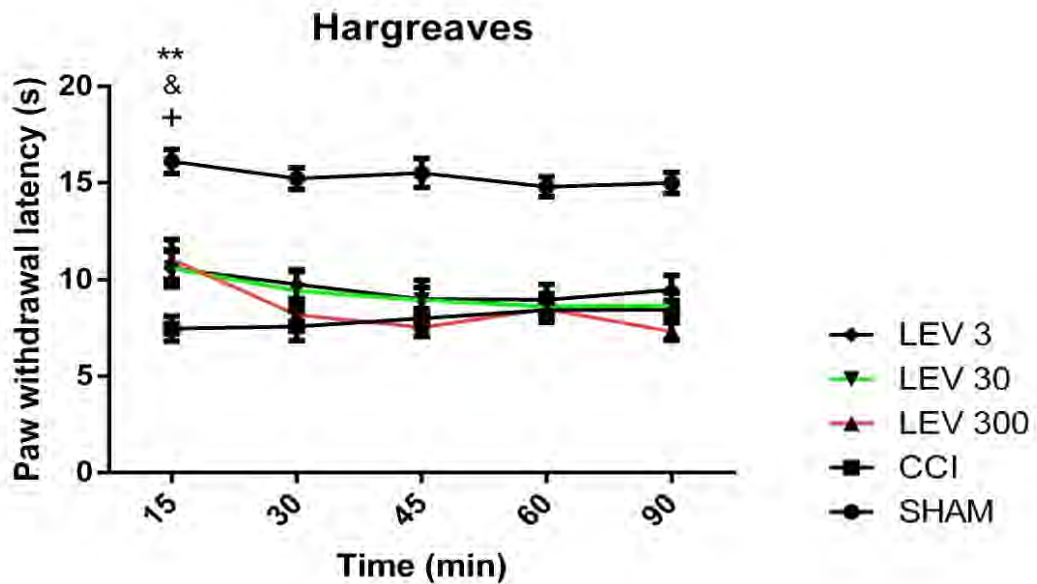


Figure 4.21. Paw withdrawal latency of rats in the groups treated with 3, 30 and 300 μ g LEV in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group are; for LEV 300 ** p < 0.01; for LEV 30 + p < 0.05 and for LEV 3 & p < 0.05. Two-way ANOVA, post hoc Bonferroni test, n = 7.

4.3. Neurotensinergic Contribution

4.3.1. Mechanical allodynia

In Figure 4.22, dose and time-dependent antiallodynia induced by NT microinjection is shown. NT antagonist SR 48692 was shown to have no effect on the parameter tested (Figure 4.23), but reversed the antiallodynia induced by NT (Figure 4.24). NT antagonism on antiallodynic effects of LEV is illustrated in Figure 4.25.

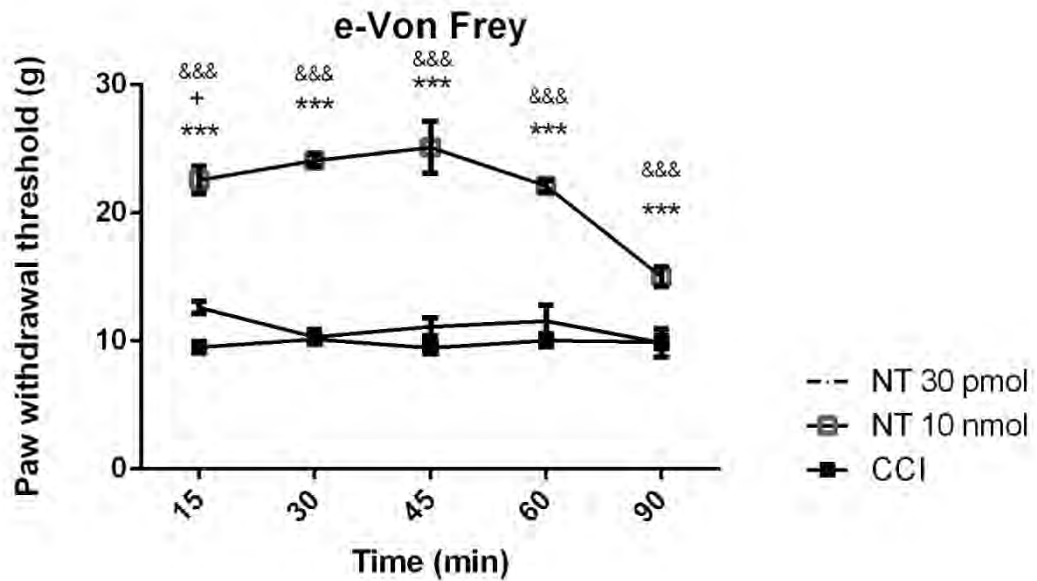


Figure 4.22. Dose-dependent antiallodynic effects of NT treatment in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group are; for NT 10 nmol group $***p < 0.001$ and for NT 30 pmol group $^+p < 0.05$; significant difference between NT 10 nmol and NT 30 pmol groups is $\&\&\&p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

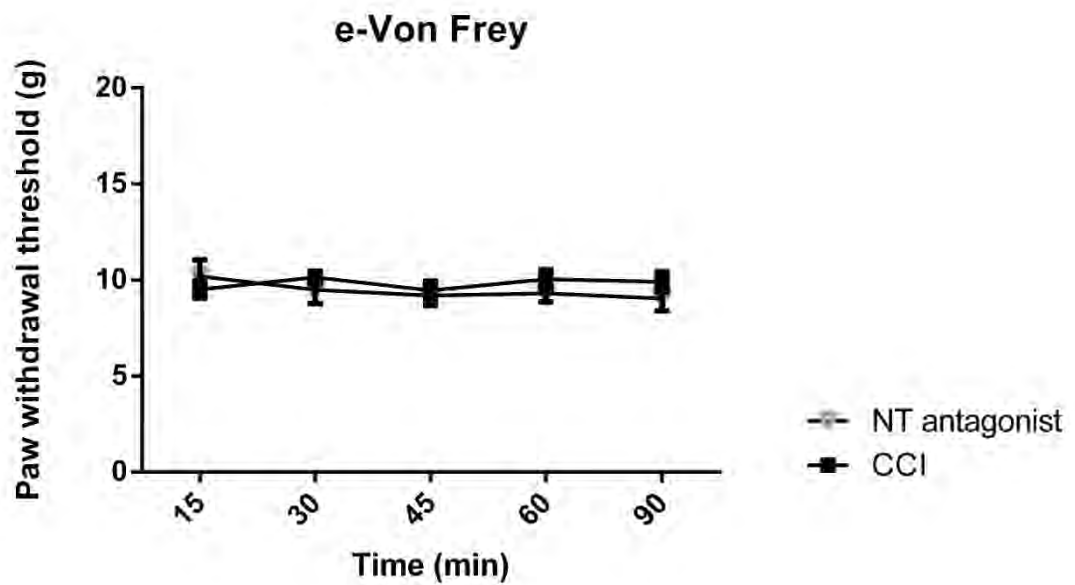


Figure 4.23. Paw withdrawal threshold values of rats in CCI group and NT antagonist treated group in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

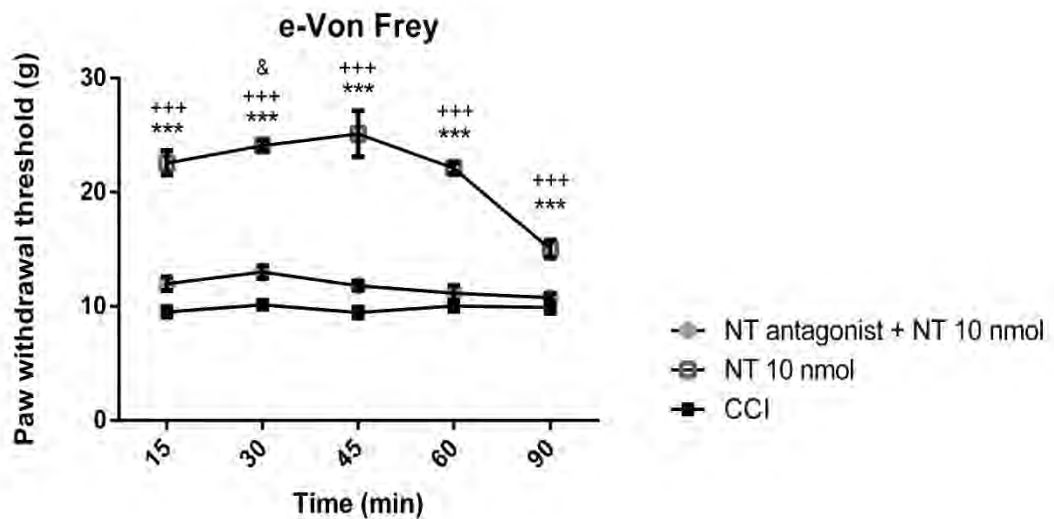


Figure 4.24. Effects of NT antagonism on the antiallodynia induced by 10 nmol NT regarding paw withdrawal threshold values in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group for NT 10 nmol group is $***p < 0.001$ and for NT antagonist pretreated NT 10 nmol group is $&p < 0.05$; significant difference between NT 10 nmol group and NT antagonist pretreated NT 10 nmol group is $+++p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

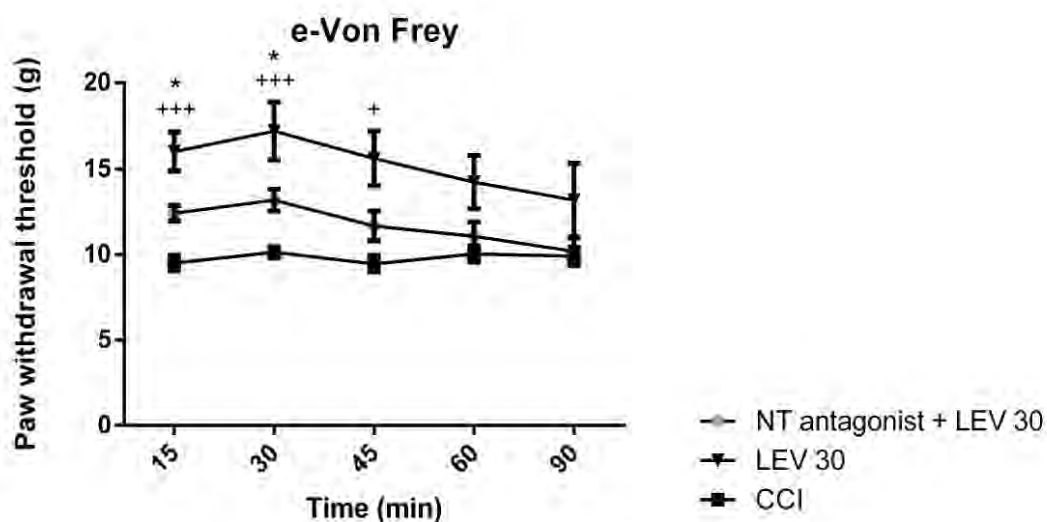


Figure 4.25. Reversal effect of NT antagonism on antiallodynic responses induced by 30 μ g LEV administration regarding paw withdrawal thresholds in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference between CCI and LEV 30 groups are $^{+++}p < 0.001$ and $^{+}p < 0.05$; significant difference between LEV 30 and NT antagonist pretreated LEV 30 is $^{*}p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

4.3.2. Thermal hyperalgesia

Dose and time dependent antihyperalgesic effects of NT are illustrated in Figure 4.26. SR 48692 was shown to have no effect on thermal hyperalgesia (Figure 4.27), but reversed the antihyperalgesia induced by NT (Figure 4.28) and LEV (Figure 4.29).

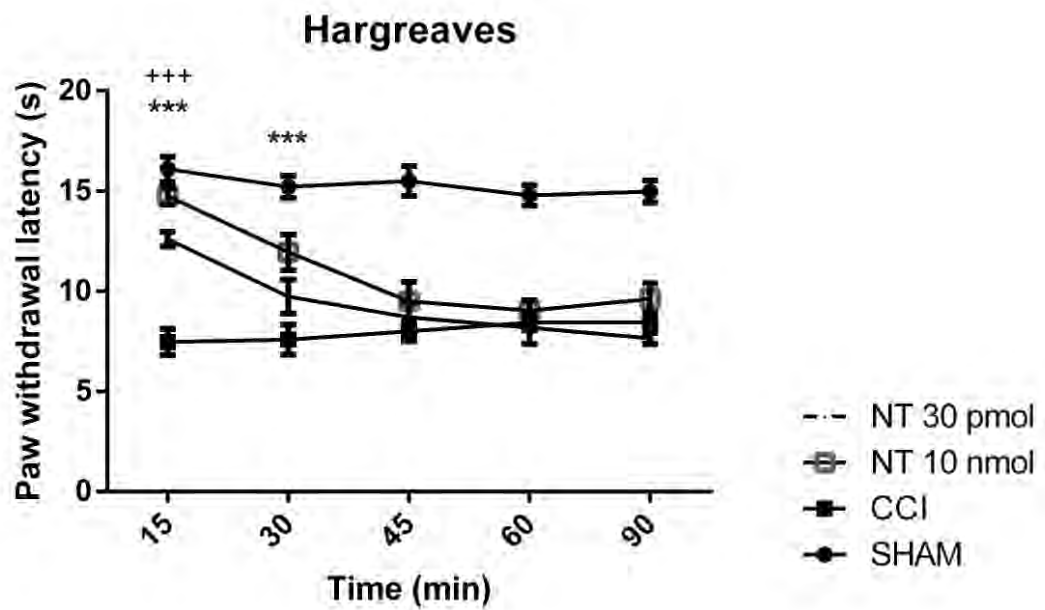


Figure 4.26. Dose and time-dependent antihyperalgesic effects of NT treatment in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group are; for NT 10 nmol group *** $p < 0.001$ and for NT 30 pmol group +++ $p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$

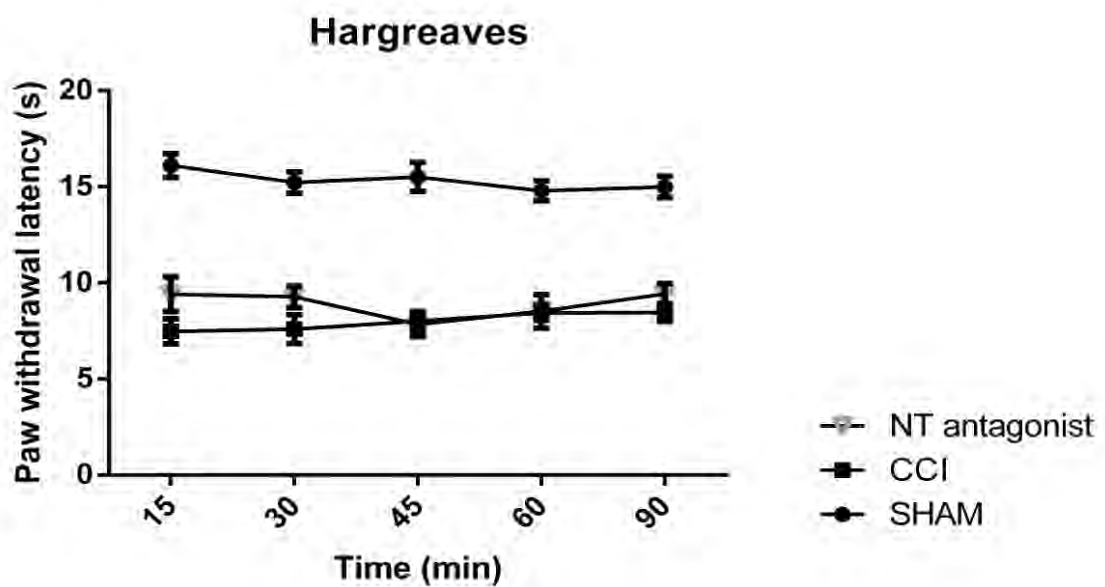


Figure 4.27. Paw withdrawal latency values of rats in CCI group and NT antagonist treated group in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

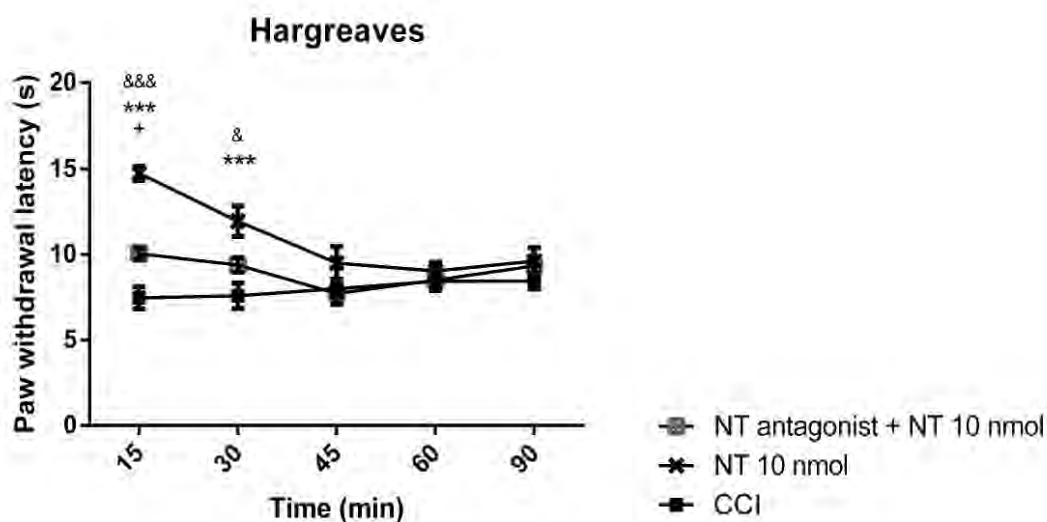


Figure 4.28. Effects of NT antagonism on the antihyperalgesia induced by 10 nmol NT regarding paw withdrawal latency values in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference against CCI group for NT 10 nmol group is $***p < 0.001$ and for NT antagonist pretreated NT 10 nmol group is $^+p < 0.05$; significant difference between NT 10 nmol group and NT antagonist pretreated NT 10 nmol group are $^&p < 0.001$ and $^&&&p < 0.001$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

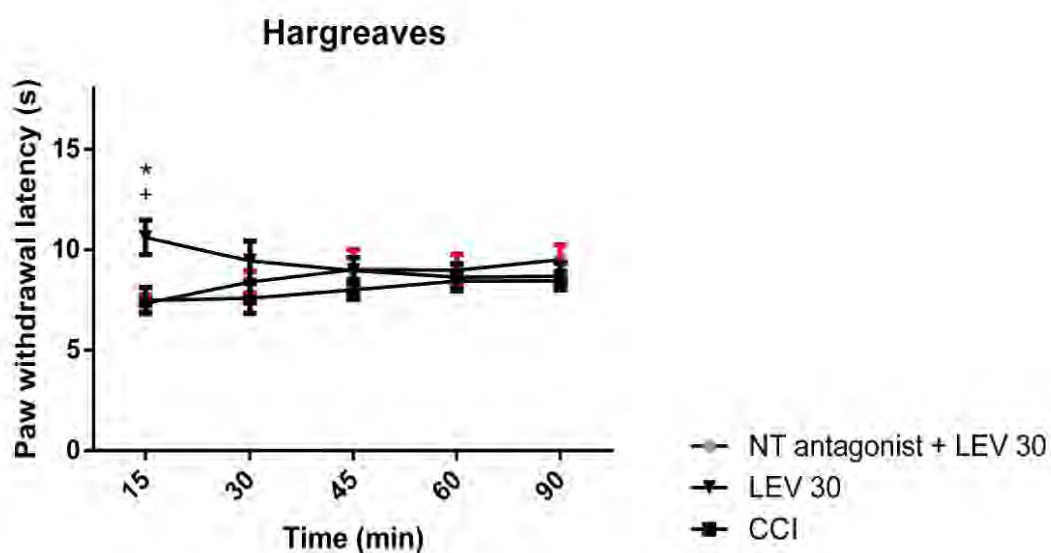


Figure 4.29. Reversal effect of NT antagonism on antihyperalgesic responses induced by 30 μ g LEV administration regarding paw withdrawal latency in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference between CCI and LEV 30 groups is $^+p < 0.05$; significant difference between LEV 30 and NT antagonist pretreated LEV 30 is $^*p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n=7$.

4.4. Cannabinoidergic Contribution

4.4.1. Mechanical allodynia

CB antagonist SR144528 was found to have no effects on mechanical allodynia (Figure 4.30).

Cannabinoidergic contribution is represented in the Figure 4.31 as the reversal effect of CB antagonist was found to be significant in 30 μ g LEV group pretreated with CB antagonist.

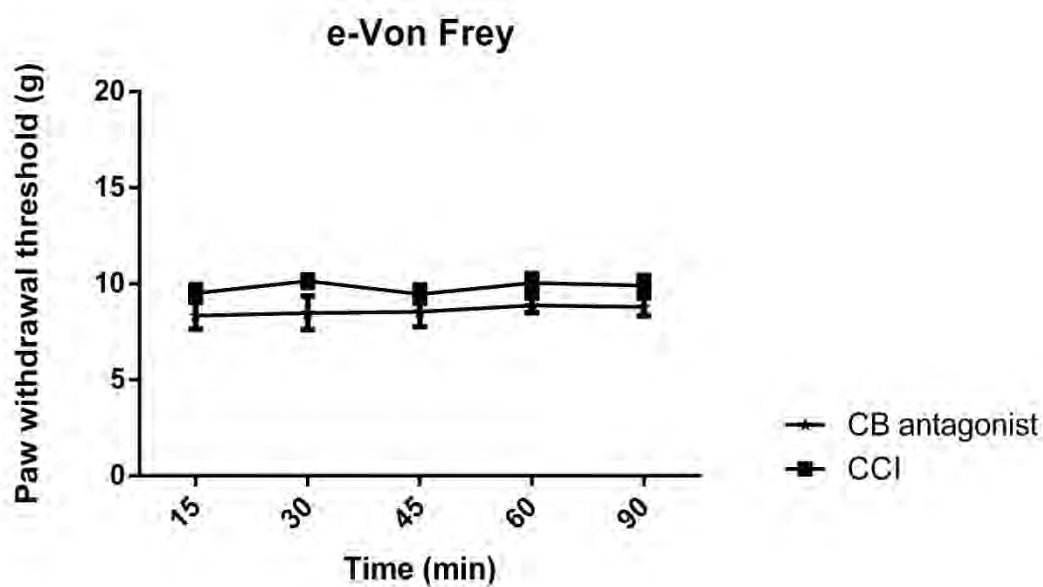


Figure 4.30. Paw withdrawal threshold values of rats in CCI group and CB antagonist treated group in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

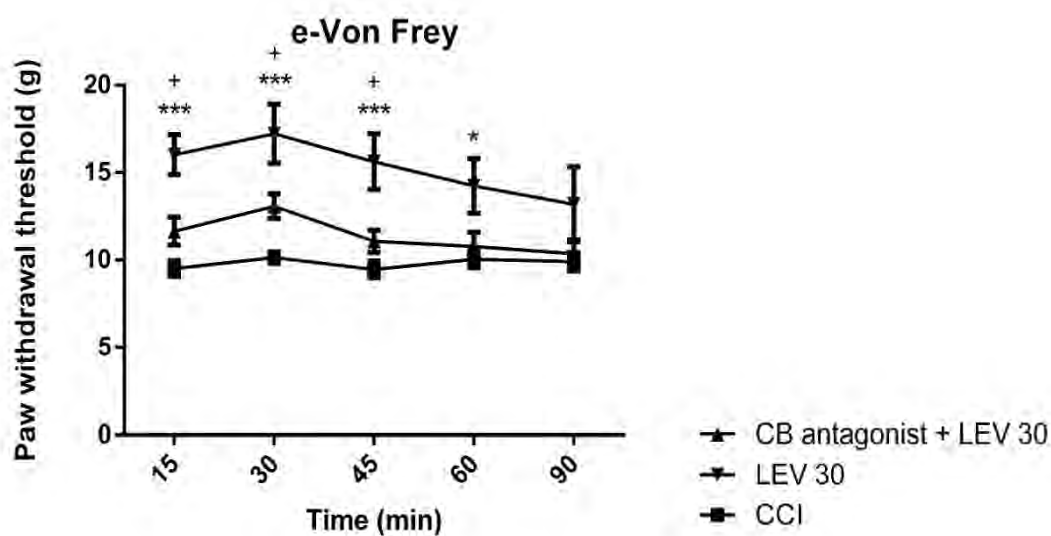


Figure 4.31. Reversal effect of CB antagonism on antiallodynic responses induced by 30 μ g LEV administration regarding paw withdrawal thresholds in the e-Von Frey test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference between CCI and LEV 30 groups are *** $p < 0.001$ and * $p < 0.05$; significant difference between LEV 30 and CB antagonist pretreated LEV 30 groups is ⁺ $p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

4.4.2. Thermal hyperalgesia

CB antagonist SR144528 was found to have no effects on thermal hyperalgesia (Figure 4.32), while it induced a reversal in the antihyperalgesic effects of 30 μ g LEV treatment (Figure 4.33).

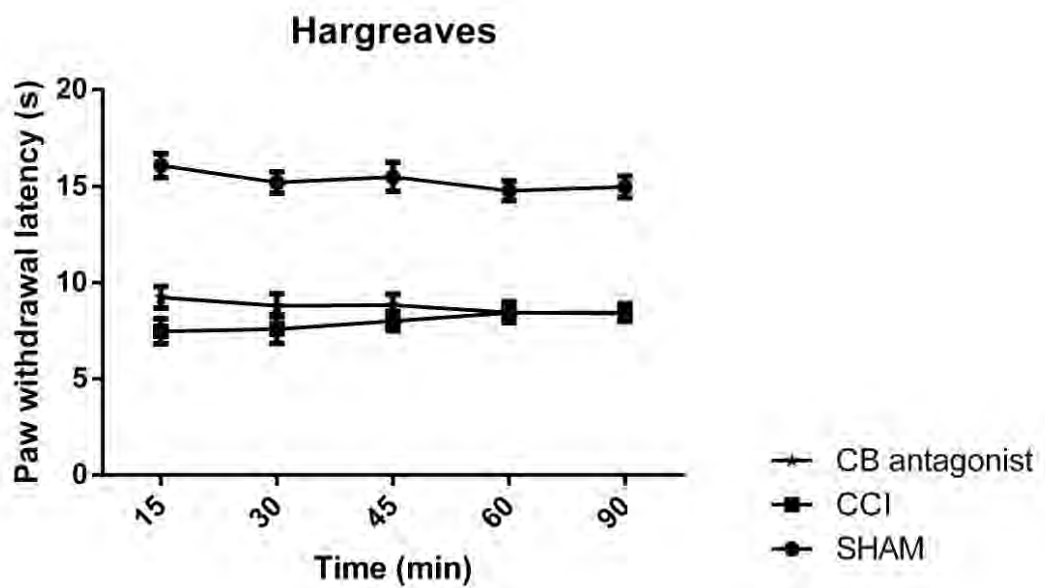


Figure 4.32. Paw withdrawal latency values of rats in CCI group and CB antagonist treated group in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

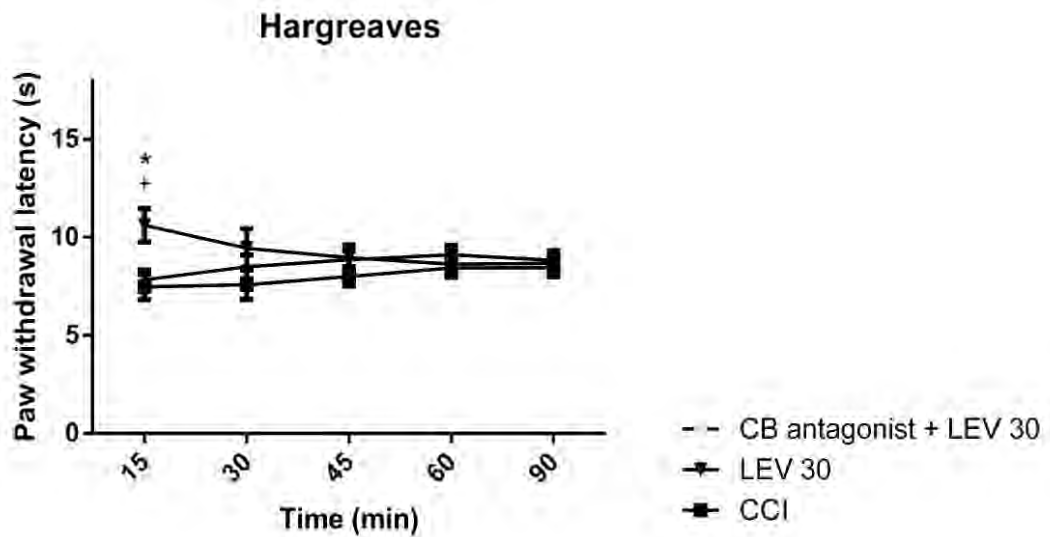


Figure 4.33. Reversal effect of CB antagonism on antihyperalgesic responses induced by 30 μ g LEV administration regarding paw withdrawal latency in the Hargreaves test. Values are given as mean $\pm$ S.E.M. Within the groups significant difference between CCI and LEV 30 groups is $*p < 0.05$; significant difference between LEV 30 and CB antagonist pretreated LEV 30 groups is $^+p < 0.05$. Two-way ANOVA, post hoc Bonferroni test, $n = 7$.

4.5. Electrophysiological Recordings

An evident significant difference was observed between sham-operated and CCI group utilized in patch clamp experiments (Figure 4.34).

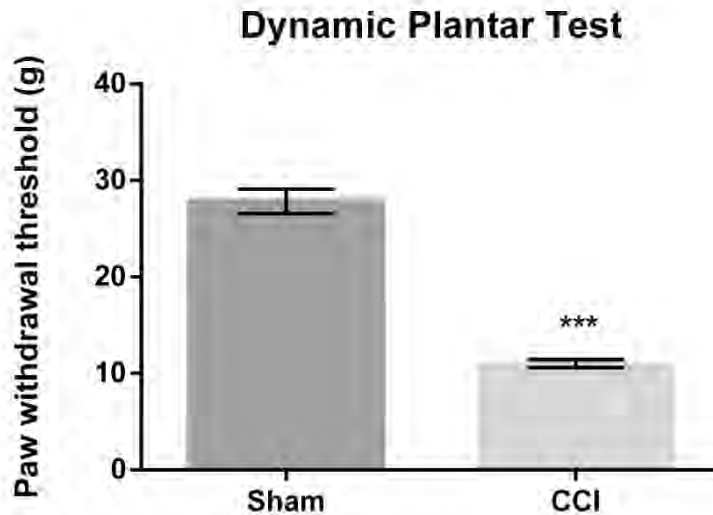


Figure 4.34. Paw withdrawal thresholds of rats in CCI and Sham patch clamp groups in the dynamic plantar test. Values are given as mean $\pm$ S.E.M. Significant difference between groups is *** $p < 0.001$. Unpaired student t test, $n = 10$.

4.5.1. Membrane passive properties

Effects of CCI establishment on passive membrane properties are represented in Figure 4.35. No significant difference was observed between groups regarding tested parameters.

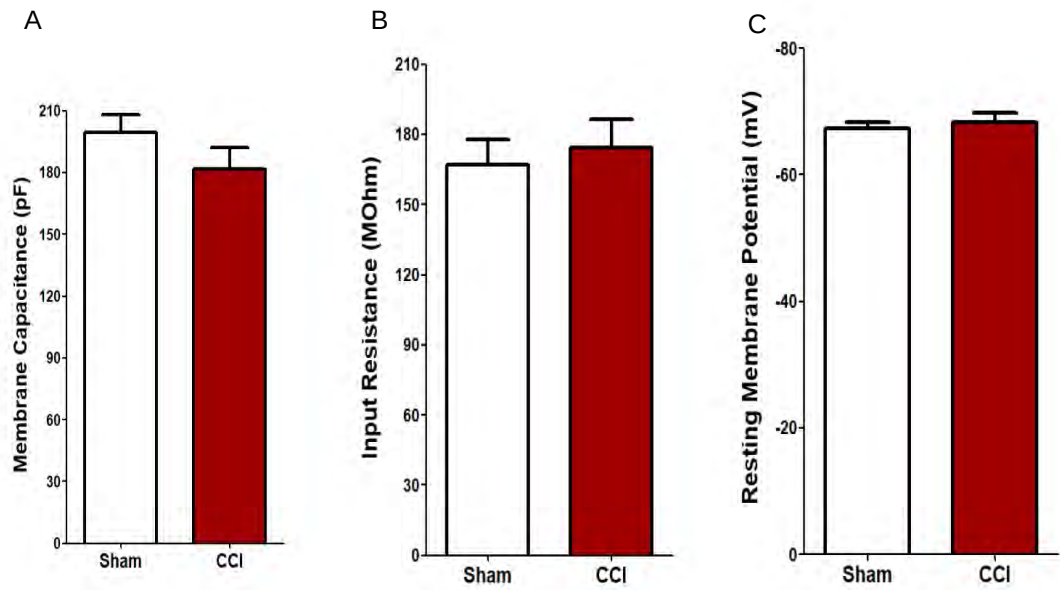


Figure 4.35. CCI treatment does not alter passive membrane properties of VPL neurons. (A) membrane capacitance (Sham: $n=24$; CCI: $n=21$; $p=0.1914$); (B) R_i (Sham: $n=24$; CCI: $n=21$; $p=0.6598$); (C) E_{res} (Sham: $n=24$; CCI: $n=18$; $p=0.5775$). Student's t -test revealed not significant differences between groups for all the parameter tested. Data represent mean values $\pm$ S.E.M..

4.5.2. Action potential frequency

Following CCI, significant difference regarding intrinsic neuronal excitability was observed at input currents 1400, 1500 and 1600 pA. (Figure 4.36).

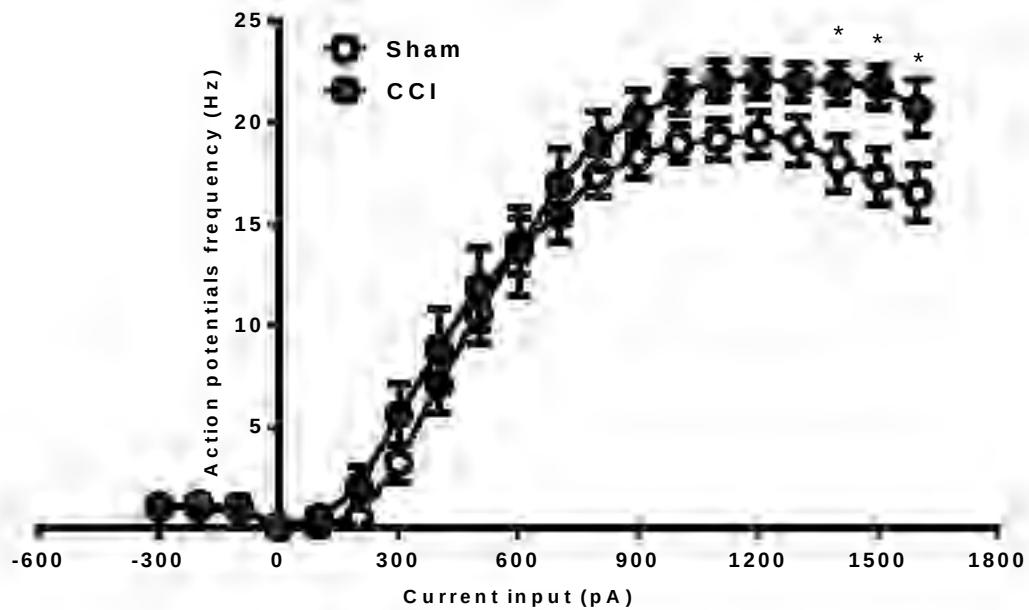
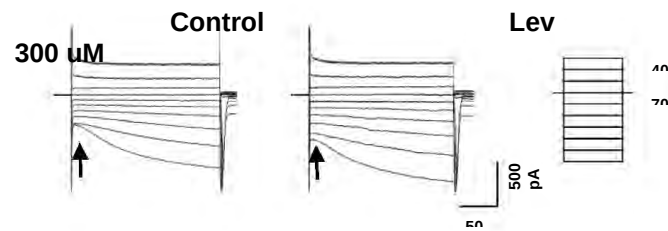


Figure 4.36. CCI treatment modifies VPL neurons intrinsic membrane excitability. Action potentials firing frequency plotted against each value of injected current (from -300 pA to +1600 pA). 2 way ANOVA revealed a significant effect of CCI treatment on intrinsic neuronal excitability at high input current levels. Data represent mean values $\pm$ SEM. Significant difference between groups is $*p < 0.05$.

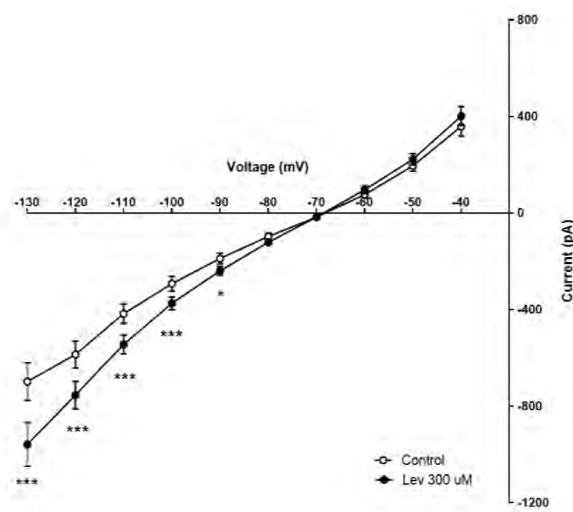
4.5.3. I-V curve and membrane conductance

Effects of LEV treatment on I-V relationship (B) and membrane conductance (C) are illustrated in Figure 4.37, together with protocol related representative traces (A).

A



B



C

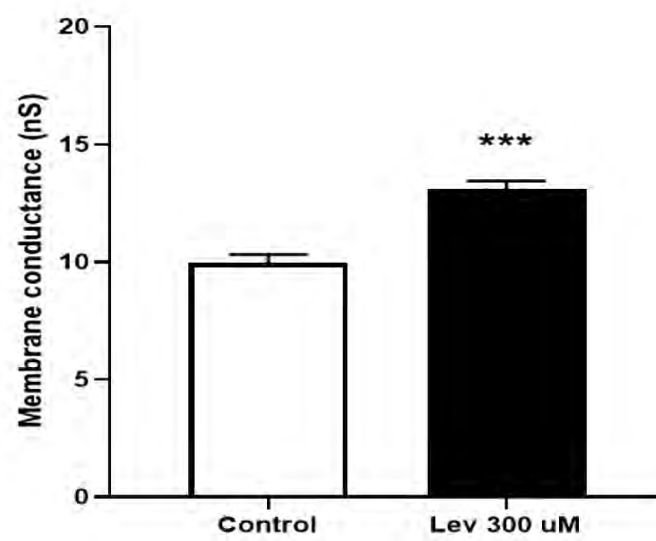


Figure 4.37. LEV increases membrane conductance at hyperpolarized potentials. (A) Representative traces of a current response to voltage command steps of 2 seconds duration made in 10

mV increments (-130 mV to -40 mV) from a holding potential of -70 mV. (B) I-V relationship in the absence (Control) and in presence of LEV (Lev 300 μ M, n=10) in neuropathic group. 2 way ANOVA revealed a significant effect of treatment with LEV ($p<0.0001$), command voltage ($p<0.0001$), and a significant treatment command voltage interaction ($p<0.0001$). Sidak post hoc analysis showed a significant effect of LEV at command voltage values from -130 mV to -90 mV. (C) Membrane conductance was measured as the slope of the I-V relationship between -130 mV and -70 mV. Student's *t* test revealed a significant increase of the membrane conductance in presence of LEV (Lev 300 μ M) compared to the control value ($p<0.001$). Data represent mean values $\pm$ S.E.M.; * $p<0.05$, *** $p<0.001$. Comparison of CCI and LEV (LEV) 30 μ groups.

5. GENERAL DISCUSSION, CONCLUSIONS, RECOMMENDATIONS

5.1. General Discussion

Within the scope of this work, it has been demonstrated that antihyperalgesic effects of LEV in neuropathic pain display mechanisms within VPL. Those mechanisms involve at least, but not the last, cannabinoidergic and neurotensinergic systems and possibly alterations on conductances which are activated at hyperpolarized potentials.

Understanding the correlational network of pain would be of great importance to translation of therapeutics, and in this thesis work LEV was examined within this context [13]. Although spinal mechanisms of processes concerning nerve injury resulting in the development of allodynia and hyperalgesia have been well investigated, supraspinal processes need further research. So far, a lack of effect has been shown with morphine administered at a spinal level, while a significant effect was observed when morphine was given systemically or supraspinally, especially for tactile allodynia [535-539].

As shown by several electrophysiological, radiological and anatomical evidence in humans and animals, VPL is takes an important part concerning perception of pain and the pathophysiology of chronic pain syndromes [89, 540-545].

Neurons in the human thalamic nuclei have been shown to induce excitatory responses to somatic sensory stimuli [546, 547]. Over a century ago, the proposition that elevated firing of thalamic neurons can cause hyperalgesia and allodynia was suggested for the first time [548]. Several recent observations have been found to subsequently support this theory, in such a way introducing that central pain syndromes can emerge from neuronal disinhibition, causing allodynia and spontaneous pain [18, 549, 550]. Stimulation of the primary ending area for spinothalamic tract, the ventral caudal region of the thalamus [551, 552], has been proposed to evoke a sensation of pain [10, 11, 553, 554].

Considering the fact that the ventrobasal complex of the mammalian thalamus nucleus has been shown to have nociceptive specific neurons, it is also obvious that VPL, as being a part of the mammalian thalamus nucleus, has nociceptive characteristics. [16, 555]. The VPL specifically receives thermal and tactile senses, sense of position and nociception from peripheral nervous system and transmits these information to the primary and secondary somesthetic areas of the cerebral cortex [17].

Neurons within VPL nucleus of neuropathic rats have been shown to exhibit elevated spontaneous firing as well as evoked response [20].

In neuropathic pain, pregabalin was shown to be ineffective on reducing aberrant spontaneous firing of VP WDR neurons [13] in contrast to its effects at spinal level [192], which shows consistency with the suggestion of excitability within thalamus not being fully dependent on spinal activity [13] also providing an insight to elucidation mainly focusing on supraspinal level. The fact that even after total transection of the spinal cord persistence of neuropathy induced changes within VPL draws attention to the high possibility that these changes are independent from ascending inputs [21].

In CCI it has been shown that VPL neurons sensitize to thermal as well as mechanical stimuli [15, 182]. In support to this statement, a significant difference between sham-operated and CCI group, both without LEV administration, with regards to this parameter was observed within this work (Figure 4.36) showing that CCI increases AP frequency to high current input within VPL.

As VPL represents a promising area to investigate antihyperalgesic effects of ligands and since LEV has a wide range of mechanisms, a high possibility of observing an alteration by the drug on the pathological processes within VPL was hypothesized. Moreover, in CCI, it has been shown that NMDA blockade can decrease nociceptive transmission within VPL [15, 499] and in another study LEV has been shown to reduce the excitotoxic effect of glutamatergic neurons by blocking NMDA receptors. [7, 34]. This resemblance supports the finding of this study demonstrating the antihyperalgesic effects of LEV at VPL level.

Recently a novel treatment approach has been suggested as a treatment strategy for neuropathic syndromes, focusing on targeting specific pain mechanisms rather than the underlying disease per se [79-82].

For many years the contribution of NT to pain processing has been investigated [556]. The antinociception induced by NT has been reported after its injection intracerebroventricularly [557], as well as into the regions rich in its innervation, areas like the thalamus, medial preoptic area, amygdala, nucleus raphe magnus and PAG [439]. The exact location of the microinjection site for NT has been suggested to be able to modify the intensity of the response, such as higher intensity reported for intra-amygdala injection than intracerebroventricular one [558]. In this work microinjection

of NT at a relatively high concentration was shown to induce significant antihyperalgesia.

At several different levels neurotensinergic activity modulate nociception [559]. It has been found to be even more potent than morphine [560, 561]. When administered centrally, NT has been shown to display an analgesic response in the acetic acid-induced writhing and the hot plate tests [50, 556, 562]. Following administration into RVM, NT has been demonstrated to induce a long-lasting antinociceptive effect in the tail-flick assay [435]. These findings support its effectiveness against nociception at diverse levels in supraspinal pain modulatory circuitry. Besides, NT also affects pain transmission directly in the spinal cord [563] in persistent [564] and of neuropathic pain [49]. The effect of NT on pain modulation has been proposed to be receptor-selective and dose-dependent [439]. Opposing and dose-dependent actions were suggested to be related to distinct and separate receptor subtype showing different affinity for the peptide as well as the involvement of diverse neuronal pathways that modulate pain [428]. Mounting evidence demonstrates that both NTS1 and NTS2 mediate the antinociceptive effects of NT, depending upon the species of rodent used and the antinociceptive test. Modulation of pain by NTS1 and NTS2 involves distinct spinal and/or supra-spinal neural circuits [49, 442]. In this study NTS1 was selected for further investigation.

NTS1 has been proposed to be the main receptor subtype regulating antinociceptive effects of NT [44, 433] while NTS2 was suggested to be the receptor subtype responsible for pronociceptive effects of NT under normal physiology [434, 438]. Accordingly, in this study NTS1 antagonist was used for the investigation of the involvement of neurotensinergic system to the antiallodynic and antihyperalgesic effects of LEV. Also, pretreatment of the NTS1 antagonist was performed before NT microinjection and a significant, but not full, reversal was observed, showing that NTS2 may also contribute to the antihyperalgesic and antiallodynic effects of NT at VPL level in CCI. Evidence showing that high NT dose also binds to NTS2 and introduce analgesia, and the finding showing synergism between NTS2 and morphine at supraspinal level supports this finding [44, 45, 453, 454].

Most of the studies demonstrating analgesic effects of NT have involved its high concentrations, since lower and probably more physiological concentrations induce hyperalgesia rather than analgesia [45, 425, 428, 439]. Moreover, administration of

SR48692, a relatively selective NTS1 antagonist which was also utilized in this study, into RVM or systemically, has been shown to facilitate analgesia, suggesting the high possibility of hyperalgesia function for endogenous NT [45, 428]. In this work, its administration has been found to exert no effect, while pre-administration leads to a reversal of antihyperalgesic effects of LEV and also NT. This may be related to its dosage and/or the possible neurotensinergic mechanisms within VPL not being the same as in other regions tested. Further investigation regarding its dose-dependent effects are necessary to understand the exact role of endogenous contribution of NT through NTS1 receptors among VPL to neuropathic pain.

In thermal analgesia, NTS1 has been shown to contribute to analgesic effects of NT [565, 566]. Opposite evidence has also been reported, such as the one showing the analgesic effects of NT not being antagonized by the NTS1-selective antagonist SR48692 [430], lack of correlation between the binding affinity of NT analogs to NTS1 and their analgesic effects [567] and lack of reduction of the NT-induced analgesia following of antisense oligonucleotides application targeted to NTS1 [43]. Nevertheless, in the case of formalin induced pain NTS1 has been found to be crucial in persistent pain pathways after systemic administration of morphine [49, 453]. And in this study, NTS1 was found to be the receptor subtype demonstrating to take a part in antihyperalgesia induced by LEV at the level of VPL.

NT was shown to lead to excitation within substantia nigra pars compacta dopaminergic neurons by reducing $I_{K_{ir}}$ and increasing the cationic conductance [393]. Moreover, NT was shown to decrease inwardly rectifying K^+ conductances among cholinergic neurons and induce excitation [471]. Within this study a possibility for LEV to strengthen K_{ir} currents was hypothesized. Also antihyperalgesic and antiallodynic effects of LEV was shown to be related to neurotensinergic system via antagonist pre-treatment. Nevertheless, the effect of NT antagonist was partial, therefore the possibility of several mechanisms including both neurotensinergic system and K_{ir} current alteration for LEV is still valid. Also, there is a possibility for NT to show diverse effects in different regions of the brain. Effects of NT on VPL neurons regarding K_{ir} channels with and without neuropathic pain state should be investigated with further studies.

Researchers speculated that NT may cause a direct effect on GIRK channel modulation, leading to an inhibition [410]. Similar to the suggestion above regarding the relationship between NT and K_{ir} channels, this speculation also should be tested at

VPL level with further studies since in this study the possibility of LEV to induce GIRK channel activation and have a mechanism related to NT has been suggested.

NT was shown to inhibit I_h in the rat [568]. In this study the possibility for LEV to inhibit the same current was pronounced, and effects of LEV was shown to be related to neurotensinergic system, supporting the hypothesis regarding I_h current inhibition.

Although NT has been shown to induce excitatory effects within PAG [456-459] it was also shown to depolarize GABAergic interneurons, alter L-type Ca^{2+} channels to increase GABA release [53], increase GABAergic activity in hippocampus, striatum, prefrontal cortex and globus pallidus [54, 447, 450, 475], inhibit N-type Ca^{2+} channels [460] and both low-voltage-activated Ca^{2+} channels such as T-type Ca^{2+} channels and high-voltage-activated Ca^{2+} channels such as L-type or N-type Ca^{2+} channels [51-53]. Its enhancing effect on the release of GABA has been proposed to be mediated by NTS1 [53, 450]. NT was also suggested to be a modulator for also the serotonergic, dopaminergic, cholinergic, glutamatergic and GABAergic systems [49, 54-56]. Besides its release from intrinsic glutamatergic spinal cord neurons acting on NTS1 receptors has been shown to hyperpolarize excitatory interneurons and depolarize inhibitory interneurons [442]. Showing a wide variety of mechanisms, effects and pathways of NT at VPL level in pain should be investigated. This study introduces the need for that research since an evident antihyperalgesic and antiallodynic effect of NT microinjected into VPL has been demonstrated *in vivo*.

NTS1-selective and non-selective NT agonists have been demonstrated to produce potent antiallodynic and antihyperalgesic effects in nerve injured rats [433]. Investigating the involvement of NTS2 the antihyperalgesic effects of LEV should be performed with further studies since the effects were not completely reversed with NTS1 antagonist pre-treatment. Sustained effects can be related to contribution of NTS2 subtype or other non-neurotensinergic mechanisms. In this aspect, it should be noted that significant reversal by cannabinoidergic antagonist pre-treatment was also observed. It can be assumed that sustained effects after NTS1 antagonist administration can be linked to involvement of CB_2 related mechanisms or neurotensinergic and cannabinoidergic involvement might be sharing same or similar pathways within VPL after LEV administration since a correlation between these two systems have been demonstrated before, in such a way that via activation of NTS1, an increase in the release of endogenous cannabinoid occurs [44, 455]. In the last case and also

considering its effect on hyperpolarization induced currents, sustained effects can be related to other mechanisms, for instance the pathways leading to its electrophysiological effects, or else which should be investigated with further studies.

Presence of CB₂ receptor expression in the thalamus [42, 493] has been reported, suggesting its modulatory effects on nociceptive neurotransmission. Authors reported a functional action of CB₂ receptors at supraspinal level on neuronal activity in neuropathic conditions [182, 498, 499, 502, 508, 509, 511]. Intrathalamic administration of a selective CB₂ agonist was shown to attenuate spontaneous activity and evoked responses of VPL neurons in neuropathic, but not sham-operated, rats, in other words attenuated mechanically evoked responses of VPL neurons [42]. Effects were shown to be antagonized by SR144528 but not by AM251 [182]. That finding was the underlying basis for investigating the contribution of this receptor subtype to the effects of LEV in this study. Moreover, some shared mechanisms have been shown for both CB₂ activation and LEV.

Effects of CB₂ receptor-mediated alterations within VPL were shown to be evident only under neuropathic conditions [42]. In this study, CB₂ antagonism was shown to attenuate antiallodynic and antihyperalgesic effects of LEV. A relation between neurotensinergic and cannabinoidergic systems was demonstrated [44, 455], and that finding supports the results observed from this study representing the contribution of both systems to the effects of LEV.

Even though administration of CB₁ agonists has been shown to inhibit pain behavior and evoke neuronal responses in neuropathic conditions, they were also pronounced to produce psychoactive side-effects [495, 500-503]. Considering this fact, CB₂ subtype was chosen for further utilization regarding the elucidation of antihyperalgesia induced by LEV.

The contribution of CB₂ receptors at VPL level has been reported to arise as a consequence of elevated coupling of pre-existing receptors to their signal transduction systems or increased receptor expression. It has been suggested that CB₂ receptors might act in tonic inhibition of neuronal activity within the VPL [42]. Having a wide range of mechanisms, in this study it is revealed that CB₂ related processes are contributing to one of the mechanisms of LEV.

The exact roles of supraspinal CB₂ receptors in nociception is still unknown [42]. Further investigation regarding this issue should be performed so that results to be

obtained could provide aspects for CB₂ related antihyperalgesia of LEV at VPL level to be enlightened in detail.

CB receptors are pronounced to be the pathway for GIRK channels in their role considering the inhibitory regulation of neuronal excitability in most brain regions. The function of GIRK channels has been shown to be involved in antinociception by cannabinoids [569]. These findings support the observations within this study in such a way that effects of LEV observed were shown to be related to cannabinoidergic system and a possibility of GIRK channel involvement were also predicted.

In the clinical study showing effectiveness of LEV on neuropathic pain, authors also suggested the possibility of LEV to be effective via its regulations in deep compartments of CNS since the effects observed were seen most significantly after a while from reaching peak serum concentrations [294]. Several studies introduced effectiveness of LEV in neuropathic conditions. Nonetheless the mechanisms involved for the observed effects are still not known completely [29].

When administered systemically, LEV has been shown to have antihyperalgesic effects in two models of human neuropathic pain [9, 274]. The similarity of the dose range between neuropathic pain and epileptic models suggests that the drug can be used in chronic pain patients at the dose used for the treatment of epilepsy in a safe manner.

Being different than the other anticonvulsants in terms of efficiency, LEV carries an etiology-dependent antihyperalgesic effect [9]. This specificity probably emerges from the original pharmacological profile and mechanism of action [274, 308, 309]. This observation leads to the conclusion that clinical utilization of LEV should be oriented according to the characteristics of the syndrome. This issue should be investigated in detail to form a better therapeutic approach.

In an *in vivo* study showing its antihyperalgesic effects, even the highest dose tested was shown to be significantly lower than the median toxic dose value for impairment of rotarod performance [9, 274], revealing a wide safety margin for LEV. In this study no significant alteration of locomotor activities was observed with LEV administration.

The presence of SV2A protein in thalamus [318] was another mainstay for this work with regards to the choice of a supraspinal microinjection site. Following the finding that LEV represents antiallodynic and antihyperalgesic effects, alterations regarding SV2A within VPL under neuropathic conditions should be investigated with

further studies. For instance, a correlation between alterations in synaptic functioning and increased SV2A expression has been shown before [320].

Modulations induced by LEV have been shown to occur only in the presence of pathophysiological conditions in general, leading to the suggestion that LEV may not affect normal brain physiology [320]. These suggestions were based on its effects through SV2A. Observation of its antiallodynic effects against mechanical stimuli in sham-operated rats within this study shows that LEV may affect at least some of the pathways involved in mechanical allodynia but not in thermal hyperalgesia since no significant effect was observed on thermal hyperalgesia in sham group treated with LEV. At VPL level, these differences should be investigated with molecular techniques. Also results showing that LEV is effective against mechanical but not thermal stimuli in sham group introduce a resemblance to the data obtained within this study [9].

In terms of mechanism of action, LEV differs from classical antiepileptic drugs [309]. Indeed, recent findings indicate reduced high voltage-activated Ca^{2+} currents, as a proposed mechanism for antihyperalgesic effects of LEV [27], particularly the N-type Ca^{2+} channels [30] since these channels have been suggested to be essential for the development of neuropathic pain [570] and N-type Ca^{2+} channel blockers have been demonstrated to reduce allodynia [571] and hyperalgesia [571, 572].

LEV was shown to hyperpolarize membrane potential via K^+ channels in dorsal root ganglions and this was suggested to be one of its possible mechanisms of action [29]. LEV hyperpolarizes the membrane from E_{res} , which means that LEV has effects regarding the neuronal excitability by an action that might involve activation of inhibition of entry of Ca^{2+} and K^+ channels [41].

Three major molecular targets have been suggested for LEV: inhibition of Ca^{2+} N-type channels, SV2A protein and the neuromodulatory action on 5HT, GABA, $\alpha 2$ -adrenergics, and μ -opioidergic pathways. Researchers agree on the fact that the pharmacodynamics of this drug has not been fully elucidated [7, 22]. With the results obtained from this study, cannabinoidergic and neurotensinergic pathways together with its effects on currents activated at hyperpolarized membrane potentials can be added to its pharmacodynamic features.

In cell culture LEV was shown to inhibit KV3.1 current [327]. In addition LEV has been demonstrated to restore altered astrocyte RMPs by modification of outward

and inward rectifier currents [328]. Depletion of Kv4.2 was shown to be blocked by LEV [329].

Its ability to hyperpolarize membrane potential via modulating the activation of K^+ channels and to inhibit Ca^{2+} entry into the cells were pronounced to be possible mechanisms of action [24]. Similarly, in electrophysiological studies carried out within this work, LEV has been found to increase membrane conductance of VPL neurons significantly only at hyperpolarized potentials (ranging from -130 to -90 mV). This might indicate that LEV alters a conductance which is active only at these potentials. Some of the possible conductances can be K_{ir} , GIRK and HCN channel current I_h .

Further pharmacological studies are necessary for the exact determination of which channels and subtypes are involved in the effects of LEV within these hyperpolarized potentials, which have been planned by the research team who carried out this work.

K_{ir} channels have been demonstrated to generate hyperpolarization [374]. Hyperpolarization of E_m induced by K_{ir} channels is mainly related to an increase in K^+ conductance [376-381]. Also unblock of this current has been demonstrated to amplify hyperpolarization [383]. In addition, its activation is supposed to reduce the excitability of the neuron since the opposite has been introduced showing that blockage of these channels induces depolarization and initiation of action potential firing [382]. The likelihood of the neuron to sustain an inactive state increases with the enhancement of this current [385-387]. $I_{K_{ir}}$ within neurons has shown to represent a region of negative slope conductance in the I-V relationship which generates K^+ currents activated by hyperpolarization, an effect mostly mediated by $K_{ir2.2}$ channels [383]. In this study a similar trend within I-V curve was observed following LEV administration.

At hyperpolarized membrane voltage levels, K_{ir} current has been shown to increase [392], making its involvement to the observed effects of LEV more likely only at hyperpolarized potentials. Since their inward rectification was shown to represent diversity among types, further studies are necessary to detect which subtype is involved [384].

An inhibitory effect of NT on I_{kir} and GIRK currents has been demonstrated [393, 410]. Findings of this study showed that LEV introduce antihyperalgesic effects, not totally but partially, through neurotensinergic system, and there is a high possibility of the drug to activate K_{ir} and GIRK currents. Three possible scenarios can be suggested if

LEV acts through these channels: The post-receptor signal transduction within neurotensinergic pathway induced by LEV can be different compared to the study showing the inhibitory effect of NT on these currents and/or the effect of LEV on these channels can be too evident that NT-regulated inhibition can not totally eliminate these effects. And lastly, neuropathic pain states can have modulatory effects on NT, such as alteration of the density and distribution of its receptors leading to different pathway activations or changing the endogenous concentrations of NT within the related area and resulting in dose-dependent diverse effects compared to physiological conditions.

GIRK channels are also inwardly rectifying K^+ channels that hyperpolarize neurons. Their hyperpolarizing effects occur as a result to the activation of several G-protein coupled receptors, controlling the neuronal excitability through GIRK-mediated self-inhibition [394]. Their presence within thalamus has been shown [573].

GIRK channel activation inhibits the release of excitatory neurotransmitters and spontaneous action potential formation [405]. Neurotransmitters like dopamine, opioids, acetylcholine, serotonin, GABA, somatostatin and adenosine have been shown to activate these channels [396].

GIRK channel activation has been pronounced to contribute to the RMP of neurons, mainly by membrane voltage shifting, decreasing electrical excitability. Since no effect of LEV has been observed with regards to Eres, possible contribution of these channels might involve other characteristic features. For instance, receptor activation of GIRK channels has been shown to provide other levels of inhibition, to which three different changes in signaling can be commonly described, namely neuron-to-neuron inhibition, neuronal self-inhibition and network-level inhibition [394].

Involvement of GIRK channels to pain perception has been shown in animal models, mainly through an abnormality regarding their function. GIRK channels have been demonstrated to be a valuable new therapeutic target for several diseases including neuropathic pain [394, 403].

With regards to the pain, the implication of GIRK channels has been suggested through observations linking these channels to pain modulators like endocannabinoids, analgesic drugs and endorphins [398-402].

Still, the pharmacology of these channels remains to be unexplored and more research is needed to link obtained cellular observations to behavior and function.

HCN channels have a regulatory role on neuronal excitability [331, 332]. They are specifically hyperactive in neuropathic conditions since microinjection of their blockers do not effect mechanical or thermal thresholds in sham rats [57].

I_h , an inward current HCN channels carry at hyperpolarized membrane potentials, is a mixed Na^+/K^+ current. Their activation has been demonstrated to contribute to the development and maintenance of chronic pain and enhanced activation of HCN channel after injury to the nerve on synaptic transmission, and antinociceptive effects of their antagonists have been shown [339, 340, 345-349]. Besides, under chronic pain conditions, increased activity of HCN channel has been suggested to be involved in ectopic firing [20,37]. Nerve injury induced alterations in I_h has been related to the elevated firing of sensory neurons [353, 355, 356]. Nevertheless, understanding the extent and mechanism of the impact they possess still requires further research.

The contribution of HCN channels to the neuropathic pain at VPL level has been demonstrated by evaluating thermal hyperalgesia and mechanical allodynia in the same model utilized within this thesis work and with same animal species. Authors concluded that the elevated activity of HCN channel in the VPL contributes to chronic neuropathic pain, but not in physiological conditions with regards to pain processing [57].

Observing no significant effect following LEV administration with regards to R_i and E_{res} strengthens the probability of this drug to inhibit HCN channel activity as a part of the mechanisms, since I_h alters neuronal excitability by its influence on R_i and E_{res} in neurons [354].

Compared to classical antiepileptics like carbamazepine and phenytoin together with novel agents such as lamotrigine and GBP, LEV has been shown to have a limited affinity for voltage dependent Na^+ channels, thus reducing the probability of disturbances in nerve conduction at demyelinating axons [574]. Conversely, a decreased neuronal excitability induced by LEV via several alternative mechanisms has been shown, which involve the facilitation of GABA and glycine inhibitory transmission, the inhibition of N-voltage-dependent Ca^{2+} channels, the reduction of intracellular Ca^{2+} release [575-577] and also via enhancing chloride currents in the A type GABA receptor [316, 578]. As known, the drug works mainly at the SV2A protein binding site at the synaptic vesicle and SV2A has been suggested to be crucial in the modulation of the exocytosis; stimulation of presynaptically located SV2A probably diminishes release of neurotransmitters [316]. Exocytosis of the excitatory neurotransmitter

glutamate has been shown to be inhibited by LEV [579]. Processes mentioned above might be crucial regarding antihyperalgesic actions of LEV in chronic neuropathic pain, which has been shown to involve sensitization of glutamatergic pathways and increased transmitter release [580, 581].

In this study it was observed that LEV displays more significant effects against mechanical stimuli compared to thermal stimuli. Evaluation of this finding requires the understanding of the possible differences between thermal and mechanical allodynia, which will also provide insight in the mechanism that LEV likely exhibits and/or difference of sensitivity of VPL for mechanical versus thermal stimuli.

There have been several reports regarding the diversity between mechanical allodynia and thermal hyperalgesia at spinal level. Considering the fact that spinal nociceptive input also includes ascending transmission and that process is under modulatory control from supraspinal levels, it is not surprising to observe differences about mechanical allodynia and thermal hyperalgesia [537-539].

Against thermal stimuli, LEV was shown to induce antihyperalgesia only in neuropathic rats [9, 310, 311]. The antihyperalgesic effects of LEV shown by Hargreaves method within this study may arise from its direct effects on VPL neurons under neuropathic conditions since no significant effect was observed in sham group treated with LEV. Particular attention should be drawn to the finding introducing the fact that in neuropathic rats, VP neurons elevate evoked responses to dynamic brushing, and punctate mechanical stimuli, but less so to heat stimulation. In addition, those neurons in SNL rats have been shown to exhibit higher rates of spontaneous firing [13].

More evident effect of LEV on mechanical allodynia observed in this study supports the assumption that LEV may alter mechanical allodynia related alterations rather than thermal hypersensitivity related ones to a greater extent in neuropathic conditions. Also, it was observed that only at 15th min after microinjection of LEV there was an antihyperalgesia effect against thermal stimuli while most evident antiallodynia against mechanical stimuli was observed around 30th min after microinjection. This difference may emerge from the diversity between pathways of thermal hyperalgesia and mechanical allodynia in terms of signal transduction from peripheral to supraspinal areas.

LEV has been shown to induce antihyperalgesia in healthy subjects against mechanical stimuli, whereas no effect on thermally evoked stimuli was observed [9].

That finding supports the result obtained within this work showing its antihyperalgesic effect on sham group only in mechanical allodynia. The resemblance between systemically and intra-VPL administration within this regard introduces the suggestion that mechanisms of LEV also has a link with mechanical hypersensitivity pathways in a greater extent compared to thermal processes.

While thermal hyperalgesia was suggested to include both spinal and supraspinal circuits, mechanical allodynia has been shown to include only a supraspinal loop. That difference was pronounced to be related to different fiber types affecting afferent inputs. Another indication was suggested for this process as thermal hyperalgesia was found to be possibly related to small-diameter opioid-sensitive primary afferent fibers, while mechanical allodynia was shown to be largely independent of small-fiber input in neuropathic pain [539, 582]. Diverse neuronal pathways were suggested to underlie the abnormal sensory responses to thermal and tactile stimuli [539]. Considering the fact introducing central pain to be also driven by the pathological activity of spinothalamic pathways, it can be suggested that diversity among mechanical allodynia and thermal hyperalgesia may also differ in signal process within supraspinal areas [583].

A greater extent of inhibition on responses of VP neurons of neuropathic rats, as normalizing the hypersensitivity, for mechanically evoked responses compared with heat shows high resemblance with the action of gabapentinoids on spinal lamina I and V neurons in neuropathic rats [584, 585]. Also there has been pronounced to be a correspondence between these findings and clinical research [586-590]. Together with the findings mentioned above, it can be suggested that VPL nucleus represents sensitivity to a greater extent to the mechanical stimuli compared to thermal stimuli. This suggestion is consistent with the results obtained in this study, representing a better effectiveness of LEV administered intra-VPL on mechanical allodynia.

The receptor location, density and intracellular activity of that neuron change the response against the thermal and mechanical stimuli. It can be concluded that within VPL the neurons receiving mechanical stimuli and can be affected by the mechanisms LEV introducing its effects are more abundant than the neurons receiving thermal stimuli, since more evident effects of LEV has been demonstrated for mechanical hyperalgesia. Based on the speculation showing that if a neuron had a greater proportion of receptors crucial for mechanical allodynia, namely AMPA and metabotropic receptors, than those linked to thermal hyperalgesia, namely NMDA receptors,

mechanical sensitivity might be the consequence for that neuron [112, 113, 123] and it can be hypothesized that within VPL LEV affects the neurons with higher proportion of AMPA and metabotropic receptors. This possible observation introduces a mechanism for the drug, such in a way that showing it may have effects on AMPA and metabotropic receptors more likely than NMDA receptors. Considering the effectiveness of the drug on thermal hyperalgesia, the finding showing that LEV reduces the excitotoxic effect of glutaminergic neurons by blocking NMDA receptors also supports the involvement of this pathway to its antihyperalgesic effects.

With regards to the fact that A δ mechanoreceptors and C-thermoreceptors have been suggested to synapse and signal on different regions or different neurons of nervous system, it can also be suggested that the input is processed through the neurons having more AMPA and metabotropic receptors within VPL, so LEV has been found to be more effective on mechanical allodynia when microinjected to VPL in this study [135-139, 158].

Even so, NMDA receptor activation has been purposed to increase the actions of coactivation of metabotropic glutamate and AMPA receptors in producing mechanical allodynia [147, 155]. It means that the hyperalgesia induced by LEV may also include mechanisms related to NMDA receptors evidently, even though its effects were found to be more significant for mechanical allodynia. Antihyperalgesia induced by LEV against thermal hyperalgesia supports this suggestion.

Considering the findings showing that substance P and NK-1 receptors have been shown to be important mediators of noxious mechanical stimuli [169-171], while they were found not to be related to thermal hyperalgesia [172], it can also be suggested that LEV has a more favorable effect on these pathways.

GBP was chosen as positive control since its effects has been demonstrated before [306]. In this study GBP represented more significant effects than LEV. The evident difference in efficiency between the actions of GBP and LEV may emerge from the distinct mechanisms of actions for these drugs: SV2A introduces different mechanisms from that of gabapentinoids [315-317]. Further research is promising regarding evaluation of its mechanisms so that the validity of gabapentinoids will increase for neuropathic pain treatment. An inhibitory effect of the gabapentinoid pregabalin on responses of VP neurons, as normalizing the hypersensitivity to heat and mechanical stimuli only in neuropathic rats introduces the suggestion that mechanism of action of

GBP becomes more effective in neuropathic pain state since the alterations in pathophysiology of neuropathic pain results in more sensitive conditions for those mechanisms [13]. GBP should also be studied in more detail to show its mechanisms within VPL, for instance, with patch clamp techniques. This investigation will be performed in forthcoming projects.

Caution is warranted while discussing the data, regarding the anatomical interpretation. Even though, further analysis of the diffusion of LEV into adjacent brain areas is necessary so that specificity of the effect in VPL can be detected. For certain confirmation electrophysiological studies in neuropathic SV2A gene-deleted mice at supraspinal level can be utilized, unfortunately this has yet to be applied [42].

According to altered responses linked to clinical neuropathic pain, mechanical allodynia was suggested to be the most appropriate behavioral measure, therefore mechanical allodynia was used for demonstration of the presence of neuropathic pain state among the patch clamp group [42].

5.2. Conclusions

In conclusion, LEV was shown to have antihyperalgesic effects in CCI model of neuropathic pain, which are modulated at the level of VPL by cannabinoidergic (via CB₂ receptor subtype) and neurotensinergic (via NTS1 receptor subtype) systems and possibly by alterations on conductances which are active at hyperpolarized membrane potentials. Another finding carrying importance in elucidating its mechanisms was the observation showing its more evident effect on mechanical allodynia rather than thermal hyperalgesia. Last mentioned difference might emerge from some possible diversity, regulation or alterations within VPL such as differences in receptor density, location, and intracellular activity. It can be concluded that within VPL the neurons receiving mechanical stimuli and can be affected by the mechanisms LEV introducing its effects are more abundant than the neurons receiving thermal stimuli. Considering the finding that AMPA and metabotropic receptors are far more involved than NMDA receptors to the mechanical allodynia [108, 139, 140, 147, 150-154], it also can be speculated that LEV may represent antihyperalgesic effects through AMPA and metabotropic receptors in a more favorable way compared to NMDA receptors.

Considering the fact that A δ mechanoreceptors and C-thermoreceptors have been suggested to synapse and signal on different regions or different neurons of the nervous system, it can also be suggested that the input is processed through the neurons having

more AMPA and metabotropic receptors within VPL, so LEV has been found to be more effective on mechanical allodynia when microinjected to VPL in this study [135-139, 158].

With regards to the findings showing that substance P and NK-1 receptors have been shown to be important mediators of noxious mechanical stimuli [169-171], while they were found to be not related to thermal hyperalgesia [172], it can also be suggested that LEV has a more favorable effect on these pathways.

Considering the effect of diversity among neuropathic pain models on altered observed efficacy of agents, this study provides another mechanistic insight. The mechanisms elucidated in this study was shown in the CCI model, a well established, trustworthy, easy to achieve animal model of chronic pain, having further inflammatory mechanisms which in turn reproduce mixed etiology of neurophatic symptoms and assumed to be the closest model in research to mimic the actual conditions in human neurophatic pain cases [215-221]. It produces a condition similar to clinical conditions of chronic nerve compression in humans that can occur after metabolic disorders, lumbar disk herniation or nerve entrapment, anoxia and heavy metal poisoning [222, 223]. From this aspect it can be concluded that LEV exerts its effects through the pathologies involved in these and similar conditions. Further research is necessary for better clarification.

Data obtained introduces VPL as a promising area for further pain research. Direct microinjection of LEV and antagonists and obtaining significant results provided findings that emphasize the importance and effective contribution of this brain region to neuropathic pain conditions.

The finding observed in this study and also other studies [306] showing a more significant effect of GBP compared to LEV introduces the conclusion that in clinics gabapentinoids may show better efficacy in neuropathic conditions.

Cannabinoidergic participation to the antihyperalgesia induced by LEV has been demonstrated in this study. Regarding the linkage between neurotensinergic and cannabinoidergic pathways [44, 455] it can be speculated that LEV may effect cannabinoidergic system through altering neurtensinergic system. In conclusion, contribution of both systems at VPL level to antihyperalgesic effects of the drug was shown.

5.3. Recommendations

While using LEV, it has been suggested that there is no need for routine blood tests, and with LEV the risk of drug interactions are smaller as compared to other drugs [591, 592]. These advantages support its utilization for further pain research. Antihyperalgesic effects of the drug represented in this study also supports the significance of further research so that LEV can be utilized in clinics more effectively.

Together with the antihyperalgesia observed in this study at VPL level, the clinical study suggesting that LEV represents its effects mainly through deep brain compartments [294] emphasize the need for investigation of mechanisms of LEV also within other supraspinal regions.

As being a main relay site for nociceptive input to supraspinal regions and having many regulatory roles in pain processing, VPL should be considered more in the field of pain research for investigating mechanism of drugs and candidates.

NT has been studied widely in the past, but recently the research about this neuropeptide lost momentum. Being effective within several neuroanatomical regions, neurotensinergic system should be further studied so that preclinical research can provide better understanding of the pathophysiology and pharmacology of pain.

Although the relation between neurotensinergic and cannabinoidergic systems was demonstrated at PAG level in a previous study [44, 455], this suggestion and the finding of this study showing that mechanism of action of LEV includes neurotensinergic and cannabinoidergic systems introduces the possibility of a relation between this systems at also VPL level. This possibility should be investigated with further research.

Since CB₁ is related to psychoactive side-effects [495, 500-503] pain research may focus on CB₂ for more reasonable therapies. Introduced effects of LEV within this work was shown to be partly mediated by CB₂, so derivatives of this drug can be evaluated mechanistically in studies involving the investigation of the participation for this receptor subtype.

Within pain research, data obtained from *in vivo* studies should be adapted to clinical approaches and pharmacotherapy should be designed according to the characteristics of the pain condition. This can be achieved by conducting studies with different neuropathic pain models to discover their pathophysiologic pathways and utilizing agents in those models. There are various types of mechanisms involved in

neuropathic pain, therefore effects of agents on these mechanisms should be determined in detail with different models to justify its effectiveness in clinical neuropathic pain conditions [294].

The experimental design used within this work can be considered when investigating derivatives of LEV for pain therapy and can be compared with LEV with regards to efficacy.

In clinics, LEV can be considered in pain conditions of chronic nerve compression in humans having resemblance with CCI, such as the ones occurring after metabolic disorders, lumbar disk herniation or nerve entrapment, anoxia and heavy metal poisoning [222, 223].

Having several functional connections with other brain areas, VPL has several regulatory roles other than the processes taking a part within its own structure. Electrophysiological studies regarding these connections and effects of LEV on these should and will be performed.

Pharmacological studies investigating the participation of the currents LEV was shown to possibly alter at hyperpolarized voltage levels in this study should and will be performed to clarify them specifically.

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APPENDICE I



T.C.
ANADOLU ÜNİVERSİTESİ

HAYVAN DENEYLERİ YEREL ETİK KURULU KARAR FORMU

Toplantı No: 1 Dosya Kayıt No: 19-01 BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	Nöropatik ağrı modelinde levatirasetam'ın antihiperalezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi.
	SORUMLU ARAŞTIRMACI ÜNVANI / ADI KURUMU	Prof. Dr. Yusuf ÖZTÜRK Anadolu Üniversitesi Eczacılık Fakültesi
	YARDIMCI ARAŞTIRMACILAR	Araş. Gör. Feyza ALYU TEKEŞ
	Hayvan Türü ve Sayısı	Sprague- Dawley/Erkek 30

DEĞERLENDİRİLEN BELGE

ARAŞTIRMA PROTOKOLÜ
ve EKLERİ

Var

KARAR BİLGİLERİ	Anadolu Üniversitesi Eczacılık Fakültesi Öğretim Üyesi Prof. Dr. Yusuf ÖZTÜRK'ün araştırma yürütücüsü olduğu 19-01 dosya kayıt numaralı "Nöropatik ağrı modelinde levatirasetam'ın antihiperalezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi" başlıklı başvuru; Deney Hayvanları Etik Kurulu Yönergesine uygun bulunarak onaylanmasına karar verilmiştir.	
	KARAR NO: 2019-01	KARAR TARİHİ: 08.01.2019

ETİK KURUL ÜYELERİ

ÜNVANI / ADI SOYADI ETİK KURUL GÖREVİ	BİRİMİ	TOPLANTIYA KATILMA	KARARA KATILMA İMZA
Doç. Dr. Bülent ERĞÜN BAŞKAN	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Özgür Devrim CAN ÜYE	Eczacılık Fakültesi Farmakoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Sinem ILGIN ÜYE	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Harun BÖCÜK ÜYE	Fen Fakültesi Biyoloji Bölümü	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Gökhan KUŞ ÜYE	Açıköğretim Fakültesi Sağlık Programları Bölümü	☐ Katıldı ☒ Katılmadı	
Doç. Dr. Gülşen AKALIN ÇİFTÇİ ÜYE	Eczacılık Fakültesi Biyokimya Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Yrd. Doç. Dr. Vet. Hek. Mustafa ESER ÜYE	Deney Hayvanları Arş. Ve Uyg. Birimi	☒ Katıldı ☐ Katılmadı	
Dr. Kürşat KARTAL ÜYE	Laboratuvar Hayvanları Bilimi Derneği Üyesi	☒ Katıldı ☐ Katılmadı	
Vet. Hek. Mustafa SAYIN ÜYE	Serbest	☐ Katıldı ☒ Katılmadı	



T.C.
ANADOLU ÜNİVERSİTESİ

HAYVAN DENEYLERİ YEREL ETİK KURULU KARAR FORMU

Toplantı No: 1 Dosya Kayıt No: 17-01 BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	Nöropatik ağrı modelinde levatirasetam'ın antihiperaljezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi.
	SORUMLU ARAŞTIRMACI ÜNVANI / ADI KURUMU	Prof. Dr. Yusuf ÖZTÜRK Anadolu Üniversitesi Eczacılık Fakültesi
	YARDIMCI ARAŞTIRMACILAR	Araş. Gör. Feyza ALYU TEKEŞ
	Hayvan Türü ve Sayısı	Sprague- Dawley 90

DEĞERLENDİRİLEN BELGE

ARAŞTIRMA PROTOKOLÜ
ve EKLERİ

Var

KARAR BİLGİLERİ

Anadolu Üniversitesi Eczacılık Fakültesi Farmakoloji Anabilim Dalı Öğretim Üyesi Prof. Dr. Yusuf ÖZTÜRK'ün araştırma yürütücüsü olduğu 17-01 dosya kayıt numaralı ve " Nöropatik ağrı modelinde levatirasetam'ın antihiperaljezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi " başlıklı başvuru; Deney Hayvanları Etik Kurulu Yönergesine uygun bulunarak onaylanmasına karar verilmiştir.

KARAR NO: 2017-01

KARAR TARİHİ: 15.12.2017

ETİK KURUL ÜYELERİ

ÜNVANI / ADI SOYADI ETİK KURUL GÖREVİ	BİRİMİ	TOPLANTIYA KATILMA	KARARA KATILMA İMZA
Doç. Dr. Bülent ERĞÜN BAŞKAN	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☐ Katıldı ☒ Katılmadı	
Doç. Dr. Özgür Devrim CAN ÜYE	Eczacılık Fakültesi Farmakoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç.Dr. Sinem ILGIN ÜYE	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Harun BÖCÜK ÜYE	Fen Fakültesi Biyoloji Bölümü	☐ Katıldı ☒ Katılmadı	
Doç. Dr. Gökhan KUŞ ÜYE	Açıköğretim Fakültesi Sağlık Programları Bölümü	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Gülşen AKALIN ÇİFTÇİ ÜYE	Eczacılık Fakültesi Biyokimya Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Yrd. Doç. Dr.Vet. Hek. Mustafa ESER ÜYE	Deney Hayvanları Arş. Ve Uyg. Birimi	☒ Katıldı ☐ Katılmadı	
Dr. Kürşat KARTAL ÜYE	Laboratuvar Hayvanları Bilimi Derneği Üyesi	☐ Katıldı ☒ Katılmadı	
Vet. Hek. Mustafa SAYIN ÜYE	Serbest	☒ Katıldı ☐ Katılmadı	



T.C.
ANADOLU ÜNİVERSİTESİ

HAYVAN DENEYLERİ YEREL ETİK KURULU KARAR FORMU

Toplantı No: 6 Dosya Kayıt No: 19-30 BAŞVURU BİLGİLERİ	ARAŞTIRMANIN ADI	Nöropatik ağrı modelinde levetirasetam'ın antihiperaljezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi.
	SORUMLU ARAŞTIRMACI ÜNVANI / ADI KURUMU	Prof. Dr. Yusuf ÖZTÜRK Anadolu Üniversitesi Eczacılık Fakültesi
	YARDIMCI ARAŞTIRMACILAR	Araş. Gör. Feyza ALYU TEKEŞ
	Hayvan Türü ve Sayısı	Sprague- Dawley/(Erkek) 8

DEĞERLENDİRİLEN BELGE

ARAŞTIRMA PROTOKOLÜ
ve EKLERİ

Var

KARAR BİLGİLERİ

Anadolu Üniversitesi Eczacılık Fakültesi Öğretim Üyesi Prof. Dr. Yusuf ÖZTÜRK'ün 15.12.2017 tarih ve 2017-01 karar no ile onaylanan, 08.01.2019 tarih ve 2019-01 karar no ile değiştirilen; 19-30 dosya kayıt no'lu " Nöropatik ağrı modelinde levetirasetam'ın antihiperaljezik etkilerinin ve bu etkilere katılan mekanizmaların supraspinal düzeyde incelenmesi" başlıklı projeye ait değişiklik başvurusu; Deney Hayvanları Etik Kurulu Yönergesine uygun bulunarak onaylanmasına karar verilmiştir.

KARAR NO: 2019-30

KARAR TARİHİ: 18.06.2019

ETİK KURUL ÜYELERİ

ÜNVANI / ADI SOYADI ETİK KURUL GÖREVİ	BİRİMİ	TOPLANTIYA KATILMA	KARARA KATILMA İMZA
Doç. Dr. Bülent ERĞÜN BAŞKAN	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Özgür Devrim CAN ÜYE	Eczacılık Fakültesi Farmakoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç.Dr. Sinem ILGIN ÜYE	Eczacılık Fakültesi Farmasötik Toksikoloji Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Harun BÖCÜK ÜYE	Fen Fakültesi Biyoloji Bölümü	☒ Katıldı ☐ Katılmadı	
Doç. Dr. Gökhan KUŞ ÜYE	Açıköğretim Fakültesi Sağlık Programları Bölümü	☐ Katıldı ☐ Katılmadı	
Doç. Dr. Gülşen AKALIN ÇİFTÇİ ÜYE	Eczacılık Fakültesi Biyokimya Anabilim Dalı	☒ Katıldı ☐ Katılmadı	
Yrd. Doç. Dr.Vet. Hek. Mustafa ESER ÜYE	Deney Hayvanları Arş. Ve Uyg. Birimi	☒ Katıldı ☐ Katılmadı	
Dr. Kürşat KARTAL ÜYE	Laboratuvar Hayvanları Bilimi Derneği Üyesi	☒ Katıldı ☐ Katılmadı	
Vet. Hek. Mustafa SAYIN ÜYE	Serbest	☐ Katıldı ☐ Katılmadı	

Anadolu Üniversitesi Hayvan Deneyleri Etik Kurulu Sekreterliği, Yunus Emre Kampüsü, 26470 ESKİŞEHİR

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APPENDICE II

Struttura di appartenenza Scuola del Farmaco e dei Prodotti della Salute , Università di Camerino,

Indirizzo della struttura_ Via Madonna delle Carceri 9, 62032 Camerino (MC)

Spett.^{le}

ORGANISMO PREPOSTO AL BENESSERE DEGLI ANIMALI

Università degli Studi di Camerino

oggetto: **Richiesta di Parere sul progetto di ricerca dal titolo:**

Ruolo del recettore PPAR γ nel dolore neuropatico: individuazione di nuove strategie farmacologiche.

redatto secondo l'Allegato VI del DLgs 26/2014

Distinti saluti

10/2016

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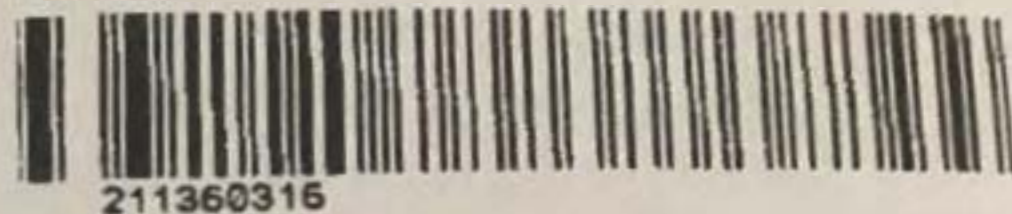


Ministero della Salute

Direzione Generale della Sanità Animale e dei Farmaci Veterinari
Ufficio 6

ANNA

0000705-P-13/01/2017



Università degli Studi di Camerino

pec:

c.a. Prof. Francesco AMENT A

e-mail:

e, per conoscenza

ASUR Camerino
Servizio Veterinario

OGGETTO: D.lgs. 26/2014 in materia di protezione degli animali utilizzati a fini scientifici.
Trasmissione autorizzazione ai sensi dell'art. 31.
Autorizzazione n° 1209/2016-PR (Risp. a prot. 1D580.5 del 16/11/2016)

Si trasmette l'autorizzazione n° 1209/2016-PR rilasciata in data 29/12/2016, ai sensi dell'art. 31 del D.lgs. 26/2014.

IL DIRETTORE DELL'UFFICIO 6
Dr. Vincenzo Ugo SANTARELLI

APPENDICE III



ESKİŞEHİR
OSMANGAZİ
ÜNİVERSİTESİ

ESKİŞEHİR OSMANGAZİ ÜNİVERSİTESİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
DENEY HAYVANLARI KULLANIM SERTİFİKASI

Belge No: 19-14

Sayın Feyza ALYÜ

24 Mart – 04 Nisan 2014 tarihleri arasında düzenlenen

“Deney Hayvanları Kullanımı İle İlgili B sınıfı Eğitim Programı” 80 saatlik teorik ve uygulamalarına katılarak tamamlamış ve yapılan sertifika sınavını başarıyla geçmiştir.

Prof. Dr. Hasan GÖNEN
Eskişehir Osmangazi Üniversitesi
Rektörü

