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YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF ORAL AND MAXILLOFACIAL SURGERY

THE EFFECTS OF NEUROINFLAMMATION ON NEUROPATHIC PAIN IN RATS

DOCTOR OF PHILOSOPHY THESIS

BEGÜM VAROL, DDS

SUPERVISIOR

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İSTANBUL-2021

THESIS APPROVAL FORM

Institute : Yeditepe University Institute of Health Sciences
Programme : Oral and Maxillofacial Surgery
Title of the Thesis : The Effects of Neuroinflammation on Neuropathic Pain in Rats
Owner of the Thesis : Begüm VAROL
Examination Date : 08.07.2021

This study have approved as a Doctorate Thesis in regard to content and quality by the Jury.

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APPROVAL
This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

09/07/2021

Begüm VAROL

ACKNOWLEDGEMENTS

Doktora eğitimim ve tez yazım sürecim boyunca, cerrahi deneyimlerini ve teorik bilgisini paylaşmaktan çekinmeyen, hastalarına yaklaşımı ve mesleki başarılarını daima örnek aldığım, bu çalışmanın tamamlanmasında özel zamanını esirgemeyen ve kadın cerrah olmanın gücünü kendisinden aldığım, tez danışmanım, sayın hocam, Ağız, Diş ve Çene Cerrahisi Anabilim Dalı Başkan'ı Sayın **Prof. Dr. Nurhan GÜLER'e,**

Bu çalışmanın nöropatolojik değerlendirmelerinde birlikte çalışma onuruna eriştiğim, çok kıymetli zamanını ayıran Sayın **Prof. Dr. Aydın SAV'a,**

Bu çalışmanın gerçekleşebilmesi için gerekli tüm şartları sağlayan, teorik bilgisini ve enstitü imkanlarını esirgemeyen Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü başkanı Sayın **Prof. Dr. Bayram YILMAZ'a,**

Etik kurul onayından deneyin son aşamasına kadar yardımlarını esirgemeyen, deney sürecinde en kilit rolleri üstlenen başta **Dr. Vet. Engin SÜMER** olmak üzere bütün **Yeditepe Üniversitesi Deneysel Araştırma Merkezi çalışanlarına,**

Gerek lisans gerek doktora akademik eğitimlerim boyunca çok kıymetli bilgilerini aktaran değerli bölüm hocalarım **Prof. Dr. Ahmet H. ARSLAN,** ve **Doç. Dr. Ediz DENİZ'e** daima yaslanacak bir omuz olan canım hocam **Prof. Dr. Ceyda ÖZÇAKIR TOMRUK**'a.

Doktora hayatım süresince desteklerini esirgemeyen **Doç. Dr. Fatih CABBAR'a,** iş ahlakı ve cerrahi tekniklerini daima örnek aldığım canım hocam **Dr. Öğr. Üyesi M. Çağrı BURDURLU'ya,** asistanlığımın ilk gününden son gününe tüm vakalarıma sabırla yardımcı olan, sorularımı yanıtsız bırakmayan hocam **Dr. Öğr. Üyesi Volkan Çağrı DAĞAŞAN'a,**

Zorlu ameliyatlarda hayatımızı kolaylaştıran, göz göze gelerek bile anlaşmayı başardığımız başta **Semra TİK** olmak üzere tüm ameliyathane hemşirelerine, klinik işleyişinde keyifle çalışmamızı sağlayan başta **Elif ÖZGÜN TATAR** olmak üzere tüm doktora-master kliniği çalışanlarına,

Doktora eğitimimin ilk gününden itibaren klinikte elim ayağım olan ve her an yardımıma koşan **Dr. Hakkıcan TEKİN, Dr. N. Sezer IŞIKSAÇAN, Dr. Hanzade ALADAĞ** ve tüm asistan arkadaşlarıma,

Hayatın zorluklarına birlikte göğüs gerdiğim, yaşamımı neşeli ve anlamlı hale getiren, İstanbul'daki ailem, kardeşlerim olan çok değerli dostlarım **Dt. Simay YILMAZ, Dt. Dila ÖZBOZDOĞANLI, Dt. Yağmur YILDIRIM, Dt. Deniz FINDIK BALCI, Dt. Dilek DÜZENLİ, Dt. Buğra AYDEMİR,** ve **Dt. Kerim AKTAŞ'a,**

Ömrüm boyunca hem en yakın arkadaşlarım hem de ikinci anne babam olan, varlıkları en büyük şansım dediğim, canım teyzem ve eniştem **Hülya ŞAT, Cüneyt ŞAT** ve kuzenim **EMRE ŞAT'a**,

Hayatımın en değerlileri, doğduğum ilk günden bu güne yaşamımın her alanında arkamda hissettiğim, sevgilerini benden asla esirgemeyen, bu güne sayelerinde ulaştığım en büyük destekçilerim annem **Derya BANK** ve babam **Cengiz BANK**'a

Son olarak, artık mesafeleri aşarak kavuşmayı umduğum, hayatımın neşe kaynağı canım eşim **Barış VAROL'a** sonsuz teşekkürler.

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LIST OF SYMBOLS AND ABBREVIATIONS

IASP:	International Association for the Study of Pain
NP:	Neuropathic Pain
PPTTN:	Painful Post-Traumatic Trigeminal Neuropathy
DHN:	Dorsal Horn Neurons
CNS:	Central Nervous System
TLRs:	Toll-like receptors
GABA:	Gamma-Aminobutyric Acid
DAMPs:	Damage-Associated Molecular Patterns
PAMPs:	Pathogen-Associated Molecular Patterns
LPS:	Lipopolysaccharide
SDH:	Spinal Dorsal Horn
CCI:	Chronic Construction Injury
TN:	Trigeminal neuralgia
OMFS:	Oral and Maxillofacial Surgeons
HZ:	Herpes Zoster
AFP:	Atypical Facial Pain
PNI:	Peripheral Nerve Injury
IHS:	International Headache Society
QST:	Quantitative Sensory Testing
NOFP:	Neuropathic Orofacial Pain
NMDAr:	N-methyl-D-aspartate receptors
CN V:	Cranial Nerve V
TNF-α:	Tumor Necrosis Factor Alpha
IL-1β:	Interleukin-1β
IL-6:	Interleukin-6
MHC:	Major Histocompatibility Complex
IL-4:	Interleukin 4
IL-13:	Interleukin 13
pH:	Power of hydrogen
Th1:	T Helper Type 1

Th2:	T Helper Type 2
IoN:	Infraorbital Nerve
IoN-CCI:	Chronic Constriction Injury of the Rat's Infraorbital Nerve
VFF:	Von Frey Filament
OPAD:	Orofacial Pain Assessment Device
FG:	Face Grooming
iFG:	Isolated Face Grooming
Vhcl:	Vehicule
MSA:	Mechanical Static Allodynia
PFA:	Paraformaldehyde
Pr5:	Principal Sensory Trigeminal Nucleus
MIG:	Monokine Induced by Gamma Interferon
YÜDETAM:	Yeditepe University Faculty of Medicine Experimental Research Center
µm:	micrometer
mm:	milimeter
cm:	centimeter
kg:	kilogram
mg:	miligram
min:	minute
µL:	microliter
µM:	micromolar
W:	Watt
%:	percent
°C:	Celcius degree
<:	less than
>:	more than

ABSTRACT

VAROL, B. (2021). The Effects of Neuroinflammation on Neuropathic Pain in Rats, Yeditepe University, Institute of Health Sciences, Department of Oral and Maxillofacial Surgery, PhD Thesis, Istanbul.

The aim of this experimental study is to investigate whether there is any association between the chronic neuroinflammation induced by Lipopolysaccharide (LPS) on neuropathic pain (NP) produced by applying the infraorbital nerve chronic constriction injury (IoN-CCI) and the alterations on microglia and astrocytes in CNS in rats. 24 male, 14-16 weeks adult, Sprague-Dawley rats with the weight of 200–250 g were used. Two experimental groups were randomly made according to the perineural injection they received Vehicule (Vhcl-saline) and LPS groups depend on the receiving 50 µL Vhcl or 50 µL of LPS in NaCl 0.5 % for each perineural injection. Each group was included 12 rats. Two different parameters (i) the evaluation of spontaneous pain by quantifying the isolated facial grooming (iFG) episodes and (ii) evaluation of mechanical static allodynia (MSA) by a graded series of Von Frey filament (VFF) testing were performed for behavioral assessments on day -1, 1, 8, 15, 22, 27, 31, 35, 39. CCI-IoN was performed on day 23. The detection of microglia and astrocytes alterations on spinal trigeminal nucleus caudalis (Sp5c) were performed immunohistochemically by using CD68 and GFAP antibodies, respectively. A significant increase was found in iFG responses of Vhcl and LPS groups ($p<0.01$). iFG (the spontaneous pain) in LPS was achieved on day 8, 22, and the increase rate of iFG in LPS group was more significant ($p=0.001$). There were significant differences in the mean VFF scores (MSA) of 10 gr between Vhcl and LPS groups on days 1 ($p=0.045$), while there were significant differences in the mean VFF scores (MSA) of 6 gr between Vhcl and LPS groups on days 8 ($p=0.004$). Repeated perineural LPS injections produced a significant increase in spontaneous pain as compared to Vhcl on days -1, 8, 22 ($P < 0.001$). A statistically significant increase was found in VFF scores (MSA) of 2,4,6,8,10 grs in Vhcl and LPS groups by time ($p=0.001$, $p<0.01$). Repeated perineural LPS injections produced a significant increase in VFF testing scores between day 1 and day 22 following the first injection, showing increased MSA, as compared to the Vhcl group. There were no statistical significances found in between groups and, the mean CD68 and GFAP-positivity according to both sides of Sp5c ($p>0.05$). The mean CD68 and GFAP-positivity in both sides in Vhcl and LPS groups were statistically significant ($p<0.01$). The increased GFAP-positivities both sides were

found in each group. A negative correlation was found between CD68-positivities on both sides of Sp5c and VFF 4 gr in the LPS group (r=-0,828 and r=-0,828, p<0,05, respectively) while no correlation was found between iFG and CD68 and GFAP-positivities on both sides of Sp5c in Vhcl group (p>0,05). We concluded that (i) IoN-CCI induced NP connected with central sensitization, showed the reactive astrocytes and reflect mirror image MSA (ii) it supports the hypothesis that microglia can be significant for initiation, while astrocytes are important for the maintenance of NP.

Keywords: Lipopolysaccharide, Chronic Construction Injury to Infraorbital Nerve, Mechanic Static Allodynia, Spontaneous Pain, Von Frey Filament, Neuropathic Pain, Neuroinflammation, Microglia, Astrocyte

ÖZET

VAROL, B. (2021), Sıçanlarda Nöroinflamasyonun Nöropatik Ağrı Üzerine Etkileri, Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Ağız, Diş ve Çene Cerrahisi Anabilim Dalı, Doktora Tezi, İstanbul.

Bu deneysel çalışmanın amacı, sıçanlarda Lipopolisakkarit (LPS) ile oluşturulan kronik nöroenflamasyonun, infraorbital sinire uygulanan kronik konstriktif hasar (IoN-CCI) modeli ile üretilen nöropatik ağrı (NP) ve santral sinir sistemindeki mikroglia ve astrositlerdeki farklılaşmalar üzerinde etkisi olup olmadığını araştırmaktır. Çalışmada 24 adet, erkek, 200-250 gr, Spraque- Dawley sıçan kullanıldı. Sıçanlar uygulanan perinöral enjeksiyonun 50 µL Vehicule (Vhcl-saline) ya da 50 µL LPS (%0,5'lik serum fizyolojik içerisinde) olmasına göre randomize olarak iki gruba ayrıldı. Her grupta 12 sıçan yer aldı. Davranışsal değerlendirmeler için -1., 1., 8., 15., 22., 27., 31., 35., 39. günlerde iki farklı parametre (i) spontan ağrının tespit edilmesi için izole yüz ovalama hareketinin kaydedilmesi (iFG) (ii) mekanin static alladoninin tepit edilmesi için kademeli Von Frey Filament (VFF) testinin uygulanması kullanıldı. 23. Günde IoN-CCI yapıldı. Spinal trigeminal nükleus kaudalis (Sp5C) üzerindeki mikroglia ve astrositlerdeki değişimlerin varlığı sırasıyla CD68 ve GFAP antikorları kullanılarak immünohistokimyasal olarak yapıldı. Vhcl ve LPS gruplarının iFG yanıtlarında anlamlı artış saptandı ($p<0.01$) ve LPS'de spontan ağrının şiddetini tanımlayan iFG'ye 8., 22. günlerde ulaşıldı ve LPS grubunda iFG artış oranı daha anlamlı bulundu. ($p=0,001$). 1. günde Vhcl ve LPS grupları arasında 10 gr için ortalama VFF skorlarında (MSA) anlamlı fark bulunurken ($p=0.045$), 6 gr için ortalama VFF skorlarında (MSA) anlamlı farklılıklar 8. günde tespit edildi. ($p=0.004$). Tekrarlayan perinöral LPS enjeksiyonlarının, -1, 8, 22. günlerde Vhcl grubuna kıyasla spontan ağrıda anlamlı artış gösterdiği kaydedildi. ($P < 0.001$). Vhcl ve LPS gruplarında 2,4,6,8,10 gr'lık VFF skorlarında (MSA) zamana göre istatistiksel olarak anlamlı bir artış bulundu ($p=0,001$, $p<0,01$). Tekrarlayan perinöral LPS enjeksiyonları, ilk enjeksiyonu takiben 1. ve 22. günler arasında VFF testi skorlarunda Vhcl grubuna kıyasla, artan MSA'nin göstergesi olarak, istatistiksel olarak anlamlı bir artış bulundu. Gruplar arasında ortalama CD68 ve GFAP pozitifliklerinin karşılaştırılmasında Vhcl ve LPS gruplarında her iki tarafta ortalama CD68 ve GFAP pozitifliği istatistiksel olarak anlamlı farklılık bulunmadı ($p>0.05$). Vhcl ve LPS gruplarında her iki taraftaki Sp5c'de ortalama CD68 ve GFAP pozitifliği istatistiksel olarak anlamlı bulundu ($p<0.01$). Her iki grupta da sol ve sağ tarafta artmış GFAP pozitifliği bulundu. LPS grubunda sol ve sağ Sp5c hücrelerde CD68 pozitifliği ile VFF 4 gr arasında negatif korelasyon saptanırken

(sırasıyla r=-0,828 ve r=-0,828, p<0,05) Vhcl grubunda sol ve sağ Sp5c hücrelerde CD68 pozitifliği ile VFF gramları arasında korelasyon bulunmadı (p>0,05). Çalışmadan elde edilen sonuçlar, (i) reaktif astrositlerin, santral sensitizasyon ile bağlantılı CCI-IoN ile indüklenen NP'nin göstergesi olduğu ve MSA'nın ayna görüntüsünü yansıttığı (ii) NP'nin başangıcı için mikroliaların, sürdürülmesi için ise astrositlerin önemli olabileceği hipotezini desteklenmektedir.

Anahtar kelimeler: Lipopolisakkarit, İnfraorbital Sinirde Kronik Konstrüktif Hasar Modeli, Mekanik Statik Allodini, Spontan Ağrı, Von Frey Filamenti, Nöropatik Ağrı, Nöroinflamasyon, Mikroglia, Astrosit

1.INTRODUCTION and PURPOSE

The most prevalent cause for an individual's visit to the dentist is pain in the face region. Orofacial pain is a form of discomfort that affects the head, face, and oral cavity's soft and hard parts. The phrase "neuropathic orofacial pain" refers to a collection of symptoms described in the International Headache Society's most recent categorization system. Trigeminal post-traumatic neuropathic pain, sometimes called painful post-traumatic trigeminal neuropathy (or PPTTN for short), is among the most prevalent types of NP in the orofacial area. Just as other forms of pain, PPTTN similarly causes high distress and affects the quality of life. A major cranial trauma, oral trauma, and minor dental interventions such as dental implant placements, impacted mandibular third molar extractions, endodontic-material extraction into mandibular, even application of local anesthetic solution with a needle can induce PPTTN. In the clinical phenotype of PPTTN, positive signs including allodynia and hyperalgesia and negative signs including hypoesthesia and anesthesia can be observed.

Neuroinflammation is described as the inflammation of nervous tissue. It is started by responding to several endogenous and exogenous causes, including neural injury, attacking pathogens, and toxic compounds. The importance of inflammation following nerve damage in the introduction of neuropathic pain (NP) has been established. The inflammatory response is induced following acute tissue injury, which is critical for the development of NP. Ectopic activities and sudden pain may be induced by the development of inflammation in a person. In addition, neuropeptide expression changes in the trigeminal ganglion, signaling a functional shift. Continuous activity from primary afferents transmitted to dorsal horn neurons (DHN) in the central nervous system causes systemic changes. The central nervous system (CNS) becomes progressively sensitized when initial nociceptive afferent input is replicated, resulting in increased reactions, a process known as "wind up." Clinical hypersensitivity and/or allodynia are symptoms of DHN hypersensitivity. Secondary hyperalgesia can be detected in undamaged areas in the vicinity of the injury by clinical examination. In individuals with severe face pain, this condition is known as central sensitization, and it is the cause of increased pain and spread to neighboring regions.

In the CNS, glial cells, expressing receptors and carrier proteins for many neurotransmitters, are essential for starting and maintaining NP. Neuroinflammation process can be identified by glial cell activation, inflammatory molecules release, increased permeability of the blood-brain barrier, and immune cell recruitment into the brain. Microglial cells are tissue-resident macrophages inside the CNS, accounting for 5%–10% of total cells therein a mature human's CNS (1). Under steady-state conditions, microglia are sustained in a resting state. Microglia are immediately activated by presence of insult or infection. Their ramified morphology transforms into an amoeboid shape, allowing the cells to be phagocytic and mobile.

Trigeminal nerve injury creates a separate time window of glial activation in the trigeminal spinal subnucleus caudalis (Vc), connected with the initiation and maintenance stages of orofacial NP. It is assumed that microglia and astrocytes have a major role in the different NP stages (2).

Activation of microglia and astrocyte has been noticed in central NP models like spinal cord infection, spinal cord injury models, and peripheral NP models like peripheral nerve injury (PNI). Additionally, astrocytes have a close association with chronic pain behavior and synapse following nerve damage and are found more persistent than the reaction of microglia. Through the pain development, microglia responses are characteristically early and transiently; on the other hand, astrocyte activation occurs later and continues longer. Liddelow et al. theorize that A1 reactive astrocytes might be neurotoxic, while A2 reactive astrocytes might be neuroprotective. These outcomes may explain precisely the dual effects of reactive astrocytes in injury and disease in CNS (3,4).

There are 40 microglia-selective molecules that are elevated after PNI in mice have already been identified, suggesting that microglia is among the major cell types involved in the pathogenesis of NP. To explain how microglia induce pain hypersensitivity and how upregulated chemicals play a role. In rodents, behavioral traits indicating decreased pain have been reported. And although their functions are unknown, it is probable that they effectively link and create one or more recognizable signaling pathways required for PNI-induced changes in synaptic function and structure in the SDH. Furthermore, under normal circumstances, activation of microglia causes pain hypersensitivity, which leads to discomfort. These discoveries gave rise to the notion of

“microgliopathic pain”, which is defined as “dysfunctional-microglia-derived pain in the absence of primary nerve injury”. Microglia also are active in the brain in NP, leading to the sensory and non-sensory (memory, reward, and emotion) aspects of the scenario, according to recent research (5). Many researches have proved that reactive astrocytes provide chronic pain development. Nevertheless, the guarding effect of reactive astrocytes about pain is still not entirely understood, and it is unclear if they have a bilateral function in chronic pain or not (6).

There are several preclinical animal models designed for a precise understanding of the mechanisms of NP, providing a detailed study for researchers. Since NP has multiple etiologies, different animal models have been designed. Nerve injury models, drug-induced NP models, disease-induced neuropathy models, and miscellaneous models are the four types of NP animal models. Of nerve injury models, chronic construction injury (CCI) is one of the most accepted animal models of NP, developed by Bennett and Xie. This model makes possible to observe mechanical static allodynia and spontaneous pain as an indicator of NP in the orofacial area by applying CCI to the infraorbital nerve (IoN).

The aim of this experimental study is to investigate whether there is any association between the chronic neuroinflammation induced by LPS on NP produced by applying the CCI to IoN and the alterations on microglia and astrocytes in CNS in rats.

2. LITERATURE REVIEW

2.1. Neuropathic Orofacial Pain

Trigeminal neuralgia, or TN for short, can be described as a short-lasting, intense, and unilateral orofacial painful sensation. The pain is most often defined as electrical, shooting, stabbing, or piercing. There are trigger areas, and innocuous stimuli in these zones may drive pain. TN outbursts are typically unprovoked, and causes aren't always known or accessible. Due to the large number of TN patients and widespread and incorrect dental treatments, there should be a lack of understanding among dentists regarding the features of TN. Individuals experiencing inexplicable discomfort that might be TN are usually referred to oral and maxillofacial surgeons (OMFS). Those with TN discomfort, however, might be sent to OMFS for extractions. It is essential to emphasize that any invasive dental treatment should not be performed unless clinical indications and complaints indicate that it is necessary. OMFS may be able to assist elderly individuals with TN who are not candidates for central therapy methods. They have access to a variety of peripheral therapy choices that provide brief relief (7-9). Acute herpes zoster (HZ) is defined as the reactivation of the dormant HZ virus in the dorsal root ganglion, resulting in dermatomal vesicular eruption. Headache, discomfort, lethargy, and itching are all symptoms of an acute HZ eruption. There is constant discomfort, which is typically accompanied by lancinating aches. Mechanical allodynia, stimulus-dependent pain, and sensory thresholds that have often increased bilaterally and into neighboring dermatomes are further signs of HZ. Emergency rooms and OMFS departments in hospitals are common places for HZ cases to appear. Effective management of HZ patients, as well as the fast implementation of antiviral and adjuvant therapies, can significantly minimize long-term morbidity. This demonstrates the significance of oral and maxillofacial viewpoints (10).

A potential condition of painful traumatic trigeminal neuropathy, i.e. chronic idiopathic face pain, attracts attention in the traumatic orofacial neuropathies (earlier named as atypical facial pain). Atypical facial pain (AFP) has enough data to suggest that it is a chronic neuropathic disease, and some individuals with AFP have some degree of sensory impairment. The emergence of everyday discomfort that is initially limited but

may spread, according to the International Headache Society (IHS) defining criteria for chronic idiopathic face pain. Pain and any other physiological condition are not linked to sensory loss. AFP is usually characterized as stabbing, throbbing, and burning. A high number of individuals suffering from this mention a long-lasting and chronic painful sensation for several years, yet there are some periods that are reported to be pain-free (11,12).

Atypical odontalgia is another possible symptom of painful traumatic trigeminal neuropathy and is described as “a severe, throbbing tooth pain, with no major pathology” by the International Association for the Study of Pain. Nevertheless, the IHS does not include the atypical odontalgia into the classification and suggests that it can be a subentity of persistent idiopathic facial pain. It is controversial that whether the atypical odontalgia is a neuropathic or neurovascular syndrome (13,14). Complex regional pain syndrome is another potential syndrome causing the painful traumatic trigeminal neuropathy. Previously, other classifications have been used as causalgia, sympathetic dystrophy, or maintained pain. The pain is usually pricking or scorching, and it is felt deep in the afflicted limb's farthest portion. Most individuals complain of discomfort while they are at rest, although movement and joint pressure can elicit or exacerbate the pain. Mechanical or thermal allodynia are the other sensory abnormalities that complex regional pain syndrome patients may experience. And, hyperalgesia is not limited to the nerve territories (15,16).

Even though most of the oral surgical procedures heal without any remaining problems, a modest percentage of patients can experience residual pain. In the OMFS perspective for traumatic neuropathies, there are a few points that should be considered. A cautious surgical technique to avoid large tissue injury and direct neuronal damage is vital. In order to prevent chronic pain, a sufficient amount of local anesthesia and an appropriate technique selection is important. Patients who had microtrauma and fractures in the orofacial area are usually taking care of by OMFS. It is clear that the early management strategies focus on life-saving involvements and repairing form and fiction. On the other hand, strict attention should be given to avoid pain and nerve injury. Effective and early diagnosis for trauma-related pain is thought to improve prognosis, and clinically distinguishing between sensory impairment caused by nerve damage and

operational edema is difficult. Quantitative sensory testing (QST) can be vital in these cases (8).

It is very beneficial to define commonly used pain describing terms. Allodynia is discomfort induced by a stimulation which ordinarily doesn't always cause discomfort. Analgesia refers to the absence of pain in the face of ordinarily painful stimuli. Anesthesia dolorosa refers to discomfort in an anesthetic region. Dysesthesia is an uncomfortable anomalous feeling that can be induced or occurs spontaneously. Hypoalgesia is a term that describes the sensation of pain being decreased in response to a typically painful stimulus. Hypoesthesia refers to a loss of sensitivity to sensory stimuli that does not include the special senses. An aberrant feeling, either induced or random, is referred to as paresthesia. Some of these sensory complaints, particularly mechanical and thermal allodynia, have been linked to NP.

Myelinated and unmyelinated nerve fiber hyposensitivity, clinically characterized by increased detection thresholds to physiological and electrical stimuli and heat, indicates extensive mechanical nerve injury. Hyposensitivity or hypersensitivity may result from partial damage. Other nociceptive mechanisms, on the other hand, may have distinct sensory characteristics. Cold hypersensitivity in large myelinated A-beta nerve fibers is defined by a reduced detection limit in perineural inflammation, particularly in the early stages. Furthermore, in order to identify centrally mediated pain disorders, a meaningful reduction in the time between detecting and pain thresholds has been demonstrated. As a result, results from Quantitative Sensory Testing (QST) might provide crucial information for treatment decisions, such as whether to employ centrally acting medicines and when to undertake microsurgical repairs (17).

NP currently described as "pain arising as a direct consequence of any lesion or disease affecting the somatosensory system" (18). Chronic pain may result from a peripheral nerve, ganglion, dorsal root, or central nervous system trauma to the somatosensory system. The phrase neuropathic orofacial pain (NOFP), also known as neuropathic craniofacial pain, refers to a collection of conditions described in the most current IHS classification (7). Trigeminal post-traumatic neuropathic pain, commonly known as PPTTN, is one of the most prevalent types of NP in the orofacial area (19, 20).

NOFP is classified as two types based on their symptomatology as episodic and continuous. Episodic neuropathies are characterized by brief, acute, electrical, or paroxysmal pain, such as trigeminal neuralgia. The pain that is continuous with a burning sensation can be characterized by inflammation in nerve structures named as neuritis, or posttraumatic neuropathy. There is another classification as peripheral or central, according to the location of the priming event. In the clinical presentation of PPTTN, there are several syndromes, including trigeminal neuralgia, glossopharyngeal neuralgia, acute herpes zoster, postherpetic trigeminal neuralgia, traumatic neuropathies, and mental nerve neuropathy, often synonymously referred to as "numb chin syndrome." These syndromes should also be apprehended and taken into consideration (8, 21).

The prognosis of PPTTN is poor. Only one-third of the patients developed improvement according to long-term follow-ups. A significant improvement might be seen only in 10% to 20%. Most of the patients still undergo some sort of pain even 13 years after onset, on average (22).

Major discomfort and substantial changes in quality of life are common side effects of NP. PPTTN is a critical issue in the orofacial area, both in terms of theoretical understanding and therapeutic treatment. This could be brought on by significant craniofacial injury, mouth injuries, and minor dental procedures like root canal. Atypical odontalgia, also known as AFP, phantom tooth pain, anesthesia dolorosa, and orofacial complex regional pain syndrome, are all names for this disease (23, 24).

PPTTN is, in many aspects, essentially contradictory. Total denervation of a body region gives numbness, known as a "negative" symptom of neuropathy. However, nerve injuries and nerve diseases generally result in "positive" symptoms, including paresthesia (e.g., pins-and-needles), dysesthesias (unpleasant paresthesia) (25).

NP after a trigeminal nerve injury has been inadequately described. Multiple related terms exist including phantom tooth pain (26), atypical odontalgia (27), anesthesia dolorosa (28), deafferentation pain (29), and persistent idiopathic facial pain (7, 11). More definitive terms such as post-traumatic neuropathy have been defined by Graff-Radford et al. Woda and Benoliel et al. have proposed criteria for PPTTNs, as shown on Table

2.1.1 Nowadays, these criteria are leading discussions for establishing internationally accepted criteria (25).

Table 2. 1. Diagnostic Criteria for painful peripheral traumatic trigeminal neuropathies

Diagnostic Criteria		
A	Stimulus dependent pain or spontaneous pain predominantly influencing the receptive field of one or more branches of the trigeminal nerve	
B	Pain that forms within 3 months after an identifiable traumatic event to the painful region or related innervation, lasting more than 3 months	
C	At least one of these clinical neurological dysfunctions: Positive signs: Allodynia, Hyperalgesia, Swelling or flushing and/or: Negative signs: Hypoesthesia, Anesthesia	
D	Neurophysiology or imaging showing a neurological lesion and its location	
E	Not associated with another disorder	
Diagnostic Level	Fulfills criteria A, B and E	**Possible NP**
	Fulfills criteria A, B, C or D, and E	**Probable NP**
	Fulfills criteria A, B, C and D, and E	**Definite NP**

PPTTN diagnosis is essentially based on signs and symptoms that are occasionally linked with proof of disease, structural injury, or damage. One of the regional neuralgias and PPTTN can be confused. Particularly, extraoral PPTTN should diagnose relatively straightforward when there is a reported trauma and signs of it like a visible scar. PPTTN has been documented after intrusive operations in the face area, such as maxillary sinus surgery. Because there are no outward indicators to depend on, the diagnosis becomes more difficult in this scenario. Intraorally, the phase can be much more complicated, with

odontogenic pain, or discomfort from adjacent anatomical structures like the masticatory muscles or the maxillary sinus, as clinical findings (30, 31).

NOFP is assumed to be common. Yet, there is a shortage of reliable data to prove these considerations, partially due to inconsistency in the definitions and clinical criteria. The prevalence of NP in primary medical care units is among 2% and 11% (32); in more extended population studies, it has been in 6–8% (33). In various cohorts of PPTTN, patients are usually female, and onset is typically during the age of 45–50. PPTTN patients with more intensive pain express increased levels of depression and pain catastrophizing, as well as subsequently lowered coping mechanisms and quality of lifes (34, 35). It is important to note that severe damage to the trigeminal nerve usually results in no residual deficit or nonpainful neuropathy. Painful neuropathy affects just a tiny percentage of people. The development of PPTTN and its characteristics vary among individuals with the same trauma. A mixture of psychosocial, environmental, and genetic factors possibly produces such variability (36, 37).

The frequency of PPTTNs is either unusually low or mainly undetected when it comes to the number of surgical operations executed by dentists. Nonetheless, some individuals develop persistent pain as a result of minor nerve injuries, such as root canal treatment, or major nerve bundle injuries, such as face bone fractures.[1] Moderate hypoesthesia of the infraorbital nerve is typical in the event of zygomatic complicated fracturing (39).

Mandibular third molar extractions are frequently correlated with transient hypoesthesia (40), distrusting sensation in 0.3% to 1% of cases. Sensory changes might continue in the inferior alveolar nerve or the lingual nerve area for ranging episodes. Inferior alveolar nerve damages are more frequent than lingual nerve injuries but might generally come from particular extraction techniques including nerve retraction and may rise to 4% (36, 41, 42).

Dental implants are the most common procedures in dentistry associated with PPTTN. A general neural complication after implant placement is adjacent nerve injury, altered sensation, and neuropathic pain occurrence (43). The incidence of nonpainful neurosensory disturbance provides a wide range from 0.6% to 36% submitting that both

permanent and transient changes are covered. The incidence of postimplant PPTTN is not clear, yet some studies suggest about 8% (44-46). The damage might directly happen during osteotomy and ⁄ or implant placement, indirectly due to bleeding and pressure build-up in the vicinity of the nerve or a perineural inflammatory response (25).

Non-painful sensory differentiations associated with endodontic treatments are common but underreported. The reasons for these nerve injuries can be an apical infection or inflammation, accidentally hypochlorite injection, and extrusion of root canal filling materials resulting in chemical injury additionally to the physical insult (47-49). Persistent pain after successful endodontic treatments have seen in 3–13% of the cases (26). Factors connected with persistent pain were: previous chronic pain problems, extended preoperative pain duration, or a patient's history with a painful treatment in the orofacial area, and female gender (23).

All of the invasive dental procedures are the possible risk factors for nerve injuries due to the local anesthetic or physical trauma by the needle. They are seemingly generated by anatomic characteristics. According to recent studies, injuries in the lingual nerve stand more permanent than the injuries in the inferior alveolar nerves. It can be commonly seen when the procedure was noted as painful or after repeated injections (50). The probability of diverse local anesthetic solution usage (e.g., lidocaine, articaine, mepivacaine) on producing different grades of nerve toxicity was a topic of discussion. However, the data found in the recent studies had been equivocal (51).

The features of PPTTN range from patient to patient after identical injuries. Genetic, psychosocial and environmental factor plays a key role for these verifications. An additional concern is that the trigeminal nerve can have complex differences in the pathophysiological aspects causing the pain, relatively to spinal nerves (36, 37).

PPTTN might show a clinical phenotype including mixtures of evoked and spontaneous pain and of positive symptoms such as dysesthesia and negative symptoms such as numbness. Sensory changes like hyperalgesia can be found in extratrigeminal areas submitting more extensive alterations in the central somatosensory mechanism. Thermal modalities are normally conserved. There are some other patient complaints of flushing, swelling, local redness, a foreign body, heat or cold (22, 52).

The pain is present in the injury area, or at the distal dermatome of an injured nerve. Demonstrable sensory dysfunction accompanies the pain, especially when there is an injury in a major nerve branch. The pain occurs unilaterally. It may be accurately settled to the dermatome of the involved nerve and might be diffuse by spreading over dermatomes. Crossing the midline is unusual. The pain score is varying from moderate intensity (VAS 5) to severe intensity (VAS 8) and it is in form of the shooting or burning sensation (53). The pain is commonly continuous, remaining most of the day and on most days. Paroxysmal pain can be induced by touch or function or can be spontaneous. PPTTN clinical cases with a distinct “triggering-like” mechanism have been observed, but they are rare. A latent or refractory period does not follow them. Severe allodynia or a positive Tinel sign can be seen more frequently (8).

PPTTN is usually affiliated with solid psychosocial suffering, and patients with more severe pain describe high levels of pain catastrophizing and depression and considerably diminished quality of life and coping mechanisms (54).

Patients with intraoral PPTTN often have a history with too many repeated consultations with several specialists and have taken multiple treatments, including repeated invasive procedures carrying a big risk for symptom aggravation (55). They usually have different treatment procedures for pain elimination, including occlusal adjustments, surgeries, and pharmacotherapy (22). Remarkably, these patients are at risk for going through unnecessary dental treatments and oral surgeries. Unfortunately, the reason for that is most of dentists rarely encounter PPTTN cases. As a result of that, they might suspect it delayed in the progression of the condition (52).

Nerve injury can be properly confirmed using brainstem reflexes and sophisticated neurophysiologic testing. However, they aren't easily obtainable in dental offices, thus evaluations must rely on QST. QST offers dependable and repeatable data that is critical for research, patient evaluation, and therapy, particularly in the case of traumatic neuropathies. In the absence of a QST equipment, sensory modalities can be examined using "gross" testing using a blunt object, heated and cooled instruments, cotton balls, or a basic pin. Using dental instruments might be another option for testing gross changes (56). For instance, a dental probe can be bused for testing pin-prick sensation. Areas affected should be carefully marked, mapped, and photographed in order to use in

the patient’s documentation, evaluation, and follow-up. Besides all of these, orofacial structures should be carefully examined and appropriate radiological imaging should be done to determine the degree of injury from a dental implant or to locate a foreign body or sequestra, to detect a fracture or other injuries (25).

A beneficial grading system for NP in general, has been published. This grading system includes a list of necessities to be able to express a pain as definite, possible, or probable NP. This grading method requires the evidence of sensorial alterations in the painful region. In the definition of orofacial somatosensory function, a variety of quantitative and qualitative psychophysical techniques have been proposed and used. Because the most definite and accurate procedures require numerous repetitions of inputs, selecting a particular technique entails time concerns, among other factors. Standardization of both thorough psychophysical testing and testing is recommended as a main approach to improve diagnostic confidence and increase awareness of somatosensory alterations and neural processes in diverse orofacial pain disorders, as well as for therapeutic strategies (56, 57). Nevertheless, multiple factors may differ the psychophysical output response, for instance, patients' mood, attention, drowsiness, ongoing litigation, cognitive function, psychological distress, cooperation, age, and gender. All of these examinations must be done by a multidisciplinary group of experts (56, 58, 59).

2.2. Physiology of Orofacial Pain

Normal pain pathways include activation of a nociceptor as an answer to a painful stimulus. With the sodium flows in through voltage-gated sodium channels and potassium flows out, a depolarization wave is transferred to the first-order neurons. Neurons are divided into three groups. The peripheral sends signals to the spinal cord through 1st-order neurons. The thalamus sends signals to the primary sensory cortex via 2nd-order neurons, while the thalamus sends signals to the primary sensory cortex via 3rd-order neurons. The trigeminal nucleus's first-order neurons terminate in the dorsal horn of the spinal cord or the brain stem. Voltage-gated calcium channels in the pre-synaptic axon terminal open in there and let the calcium rush in. Calcium enables glutamate release into the synaptic space as an excitatory neurotransmitter. Glutamate binds to N-methyl-D-aspartate receptors (NMDAr) on the second-order neurons, and depolarization proceeds.

Next, the second-order neurons cross over in the spinal cord and continue to the thalamus to synapse with third-order neurons. There is also an inhibitory pathway that blocks the transmission of the pain signal in the dorsal horn. The synapse between the first-order neurons and the second-order neurons is regulated by interneurons with the help of gamma-aminobutyric acid (GABA) release as an inhibitory neurotransmitter resulting in pain suspension. So, the underlying mechanism of pain enhancement may be the effect of suppression of inhibitory synaptic connections (40, 60, 61).

The trigeminal nerve, also known as cranial nerve V (CN V), is a sensory nerve that supplies sensation to the orofacial area. Fig. 2.2.1 depicts the trigeminal pain pathway in the orofacial region.

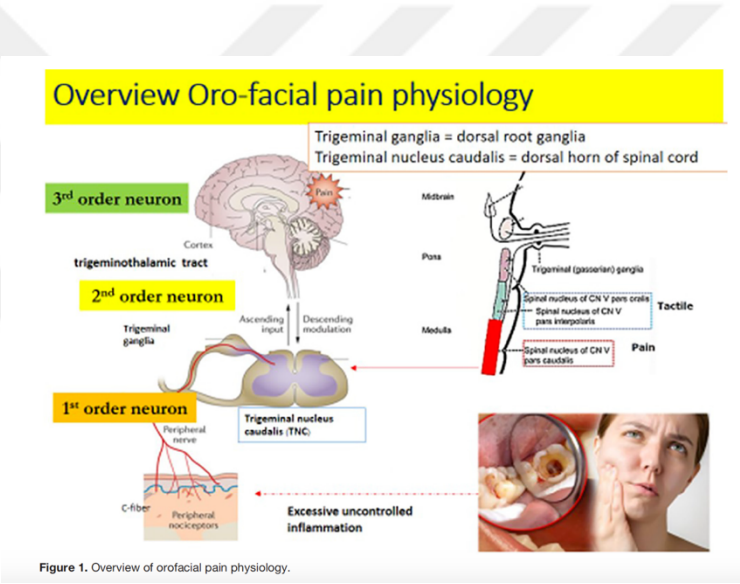


Figure 2.1. The trigeminal pain pathway of the orofacial area (62)

The 1st-order neurons in the trigeminal nerve generate increased pain signals towards the trigeminal ganglia in response to persistent inflammation or repetitive painful stimuli at peripheral nociceptors in the orofacial region. Pain signals are subsequently transmitted to 2nd-order neurons in the brainstem's trigeminal nucleus caudalis. The 2nd-order neurons are stored in the caudal trigeminal nucleus. Following that, messages are transmitted to the thalamic 3rd-order neurons through the ventral trigeminothalamic tract (63, 64).

Pain signal transformation is the mission of nociceptive fibers containing free nerve endings that makes dense networks with multiple branches. These branches are the

sensory receptors for pain termed as nociceptors. There are different types of nerve fibers, also known as axons. All of the peripheral organs connect via these fibers to the spinal cord. The variety of these fibers depends on both in thickness and diameter of the myelin sheath surrounds them, resulting in speed variations. The larger the diameter of the fiber, the thicker the myelin sheath, and the faster the fiber conducts nerve impulses. Aβ fibers are typically the largest, myelinated axons of sensory neurons providing innervation. They are known as low threshold mechanoreceptors. While low thresholds can create action potentials in response to very weak stimulation, high thresholds are demanding strong stimulation to create an action potential. C fibers are the unmyelinated axon of a sensory neuron with their thin diameter, less than 1.5 mm, considered as high-threshold nociceptors. However, depending on the types of stimuli, whether mechanical, thermal, or chemical, different types of C fibers might have a high threshold or low threshold responses (65).

2.3. Pathophysiology of Neuropathic Orofacial Pain

PPTTN has a pathophysiology that involves a series of events in the neurological system. Neuronal plasticity refers to the changes in physical, functional, and metabolic properties that occur as a result of these occurrences. The majority of what we know about this issue is based on pain models based on the spinal nerves, and the trigeminal system differs from these models in a number of ways. Tissue damage and inflammation; nerve damage, inflammation, and ectopic action; phenotypic neuronal alterations; sympathetic nervous system (SNS) engagement; CNS alterations and the functions of central glial cells and peripheral satellite glia are only a few of the many diverse aspects of these occurrences that need to be addressed (66-68).

The inflammatory liquid is a mixture of bioactive chemicals produced in response to a variety of stimuli and muscle damage that can either trigger or sensitize nociceptors explicitly or implicitly. Surface receptors on neurons regulate these alterations, which are reversible. As a result, nociceptors exhibit abnormal neurophysiological activity, which could be provoked or spontaneous. ***Peripheral sensitization*** is the name for this rapid change in the sensitivity of receptors. Percussion sensation in a tooth, for example, is an example of allodynia in periapical periodontitis. In permanent pulpitis, pain arises

spontaneously, and the application of ice, which is typically mildly unpleasant, now causes severe agony known as hyperalgesia (69-71).

The importance of inflammation following a nerve damage in the introduction of NP has been established. Structures of the trigeminal system are typically enclosed areas, such as the inferior alveolar canal, and pressure caused by inflammation might result in open nerve injury. As a result, intensive treatment of inflammation affecting the trigeminal nerve would be encouraged (69-71).

An inflammatory response is induced after acute tissue injury, which is critical for the start of NP. Even when there is no axonal nerve damage in the nerve trunk, perineural inflammation promotes spontaneous activity that is typically observed after a nerve damage and induces mechanosensitivity in myelinated axons. As a result, the presence of inflammation in a single person might cause ectopic activity and discomfort (36, 72).

In case of a critically injured neural tissue appearance resulting from a trauma such as transection, cell death can happen. But, if the proximal stump manages to survive, healing process includes disorganized sprouting of nerve fibers that develops a neuroma (36).

Production of neuroma is usually dependent on the degree of nerve damage and always formed when there is a cut in the perineurium. The lighter injuries, such as nerve compression or constriction, might be a reason for focal demyelination and neuroma formation. These areas can be identified with ectopic discharge, partly due to the upregulation of specific sodium and calcium channels and the downregulation of potassium channels. The cell bodies of injured nerves in the dorsal root or trigeminal ganglia may also show ectopic activity. These phenomena slightly demonstrate spontaneous NP. In addition, ectopic activity in neuromas is intensified by chemical and mechanical stimulation; so, the pain is felt when injury sides and neuromas are touched (36, 73).

"Peripheral neuritis" is a term for a definition of generalized neuropathies associated with autoimmunity, chemical poisoning, alcohol, or insufficient nutrition with an inflammatory factor. Neuritis is currently used to define localized nerve pathologies

subsequent inflammation. Wherever through the nerve, an inflammation may be a reservoir for pain felt in the organ innervated by the nerve. There are two different ways for inflammation to affect the nerve. One is effecting directly by their mediator secretion, mostly cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (74). The second way is effecting indirectly by the concomitant edema. Both mechanisms may be a reason for nerve damage in case of persisting long. The accompanying symptoms for this condition have been described in recent studies, and tactile allodynia has been reported to play an important role in myelinated nerve fibers. Dental or other invasive procedures in the orofacial area may produce temporary perineural inflammation, yet this generally gives no symptoms. However, some other conditions like paranasal sinusitis, pathologies in the temporomandibular joint, or early malignancies may cause symptomatic perineural inflammation and pain (17, 75).

Perineural inflammation also begins nerve trunk mechanical sensitivity and spontaneous neurophysiological activity, which might persist for several days. This abnormality is known as leading the way for initiating and maintaining the NP (72, 76). Perineural inflammation on a nerve trunk without clearly axonal damage is adequate to induce pain in the organ innervated by the involved nerve. After an inflammatory process takes place in the nerve, hypersensitivity starts immediately and may continue for several days. Unfortunately, pain is not restricted to the affected site. There is a phenomenon called "mirror image NP" where pain rises on the contralateral unaffected due to the potency of the inflammatory or immune response (40, 77, 78).

Neuropeptide expression in the trigeminal ganglion changes after a nerve damage, indicating a physiological shift. Aß fibers, for example, generally transmit benign impulses. When they are catalyzed by infection or trauma, however, a phenotypic change occurs with the production of substance P, and Aß fibers gain the capacity to generate pain signals in response to peripheral stimulation, which may be the cause of allodynia (75).

Individuals with anxiousness or who are under stress may have greater pain, which is indicated by heightened sympathetic activation. An increase of a-adrenoreceptors in the dorsal root ganglion and the trauma area, which causes responsiveness to the circulating catecholamines, might be the cause. Furthermore, sympathetic fibers grow in

a basket-like pattern around the neurons of the dorsal root ganglia, increasing sensory–sympathetic exchanges. Nonetheless, this phenomenon has not been well documented in the trigeminal ganglion, which might explain why sympathetically sustained craniofacial discomfort is rather infrequent (37, 67).

Continuous activity from primary afferents transmitted to DHN in the CNS causes central alterations. The CNS becomes progressively sensitized when initial nociceptive afferent input is repeated, resulting in increased reactions, a process known as "wind up." Extended DHN depolarization leads to the sensitivity of a calcium channel called NMDAr, which is typically inhibited by magnesium ions. The magnesium ion plug is removed when NMDAr is activated, and calcium ions are released into the DHN, priming a variety of intracellular processes. Although NMDAr activation does not cause nociception, it increases neuronal excitability and is thought to be important in the establishment of central sensitization. Other calcium channels, as well as NMDAr, are triggered by recurrent pain stimuli, indicating that intracellular calcium and DHN sensitivities are increased. Clinical hypersensitivity and/or allodynia are symptoms of DHN hypersensitivity (79).

Hypersensitivity might cause neighboring DHNs to activate both directly (through neurotransmitter diffusion) and indirectly (via the amplification of quiet inter-DHN connections). This widens the field that is receptive, leading to discomfort in areas that aren't typically affected by the peripheral nerve. Secondary hyperalgesia can be detected in undamaged areas in the vicinity of the injury by clinical examination. In individuals with severe face pain, this condition is known as ***central sensitization***, and it is the cause of increased pain and spread to neighboring regions. Because the first changes in neuronal excitability are activity-dependent, they can be controlled by modulating peripheral nociceptive input. Long-term changes in the dorsal root ganglion and DHNs, on the other hand, begin with continued stimulation; such modifications include changed gene transcription and expression, as well as downregulation of repressor mechanisms, resulting in increased excitability (25).

Within a few months of nerve damage, neural death can be detected, especially in C fibers. The removal of damaged C fibers (from lamina I and lamina II) may allow Aß fibers to sprout from the deeper lamina, increasing pain produced by mild contact,

however Aß sprouting has yet to be explored. Nerve damage is thought to produce excitotoxic cell death, which depletes inhibitory interneurons and increases discomfort. However, it is unnecessary for persistent pain development. One of the fundamental parts of balanced nociception is a supraspinal modification of peripheral signals. In inflammatory or NP states, reduced central inhibition that enhanced facilitation can be indicated (80, 81).

2.4. Microglia and Astrocyte Activation in Neuropathic Orofacial Pain

Microglia, astrocytes, and oligodendrocytes are the three primary types of glial cells in the CNS. Glial cells have been implicated in the development and management of NP in latest research. Many neurotransmitters have receptors and carrier proteins in glial cells. Thus, these become rather implemented in terms of pain modulation. In response to neural impulses, glial cells can release excitatory chemicals. Prostaglandins, pro-inflammatory cytokines, nitric oxide, and glutamate are examples of excitatory chemicals that can enhance neurotransmitter release from primary afferents and DHN hyperexcitability. The effectiveness of opioid analgesia has been shown to be harmed by glial cells. Glial cells are often stimulated by bacteria or viruses. It's possible that this is the underlying cause of pain and allodynia in certain systemic illnesses. The role of glial cells in the onset and progression of chronic NP has been investigated. Glial cells, with the exception of immediate nociceptive responses, are implicated in the process of pathological discomfort, making them a promising therapeutic goal (82-84).

Neuroinflammation is described as the inflammation of nervous tissue. It is started by responding to several endogenous and exogenous causes, including neural injury, attacking pathogens, and toxic compounds. This process can be identified by glial cell activation, inflammatory molecules release, increased permeability of the blood-brain barrier, and immune cell recruitment into the brain. Neuroinflammation is started by microglia, known as the resident immune cells of the central nervous system. In the normal, healthy CNS, microglia are typically in a resting state, distinguished by a ramified morphology, through interactions with the cell surface and soluble agents from circumambient cells. In this steady-state of microglia, its morphology has ramified processes with a very small cell body. These ramified microglia contribute to brain homeostasis by supporting myelin turnover, removing or remodeling synapses,

controlling neural firing, and cleaning the circumferential environment for pathogens or tissue damage actively. Interactions with membrane-bound and soluble factors from neighboring cells provide maintenance for microglia. There are microglia receptors carrying immunoreceptor tyrosine-based inhibitory subjects in their intracellular domain. When these receptors phosphorylated, they suppress downstream immune signaling. In addition, there are also ligand-receptor interactions that control microglial activation, including CD47, CD22, and HSP60 on neurons binding to CD45, SIRP alpha and, TREM-2, sequentially, on microglia. Neuropathic factors like beta-NGF and BDNF also release from neurons by the inhibition of major histocompatibility complex (MHC) class II and co-stimulatory molecule expression; these factors contribute to the immune-suppressed state of microglia. Additionally, numerous neuropeptides and neurotransmitters like dopamine and GABA suppress the production of the pro-inflammatory cytokine by bind to their corresponding receptors on microglia. After the loss of these inhibitory signals, microglial activation starts (85).

Microglia are immediately activated by insult or infection. Their ramified morphology transforms into an amoeboid shape, allowing the cells to be phagocytic and mobile. They also modify the cell surface proteins and enzyme expression. The secretion of immune response molecule expression is also started by activated microglia. Even though a full understanding is not achieved yet, it is assumed that microglia activation is started by both elimination of the inhibitory neuronal signaling pathway and pattern-recognition receptor activation, for instance RAGE and tool like receptors (TLRs), by either endogenous DAMPs or exogenous PAMPs. There is a wide list of stimuli activating microglia, including LPS, disease-specific proteins like Amyloid-beta and alpha-Synuclein, pesticides and, molecules generated from neural injury, involving nucleotides, extracellular matrix substrates, and glutamate.

Concurrently with microglia stimulation, it could produce a variety of phenotypes with distinct cell surface and intracellular indicators, secreting different substances, and performing various tasks. Furthermore, during an inflammatory response, the cells have the ability to switch between morphologies. M1 microglia are the first to respond to an insult. TNF-alpha and IFN-gamma are released by astrocytes and Th1 cells. Microglia polarized to the M1 phenotype produce proinflammatory cytokines, chemokines, and redox signaling molecules in response to these cytokines and bacterial-derived products

including LPS and trauma-induced cellular debris. On their cellular membrane, they also have MHC class II, co-stimulatory molecules, and scavenger receptors. M1 microglia were able to destroy and phagocytize foreign and cellular debris after these changes, as well as transport and differentiate T cells to initiate an autoimmune reaction. With the help of M2 microglia, the inflammatory reaction becomes increasingly anti-inflammatory over time. After stimulation using interleukin 4 (IL-4) or interleukin 13 (IL-13) released by Th2 cells, microglia polarize to the M2 phenotype. M2 microglia secrete anti-inflammatory cytokines and growth factors to aid in the reduction of the inflammatory reaction and the healing of injured tissue (Fig 2.4.1) (85).

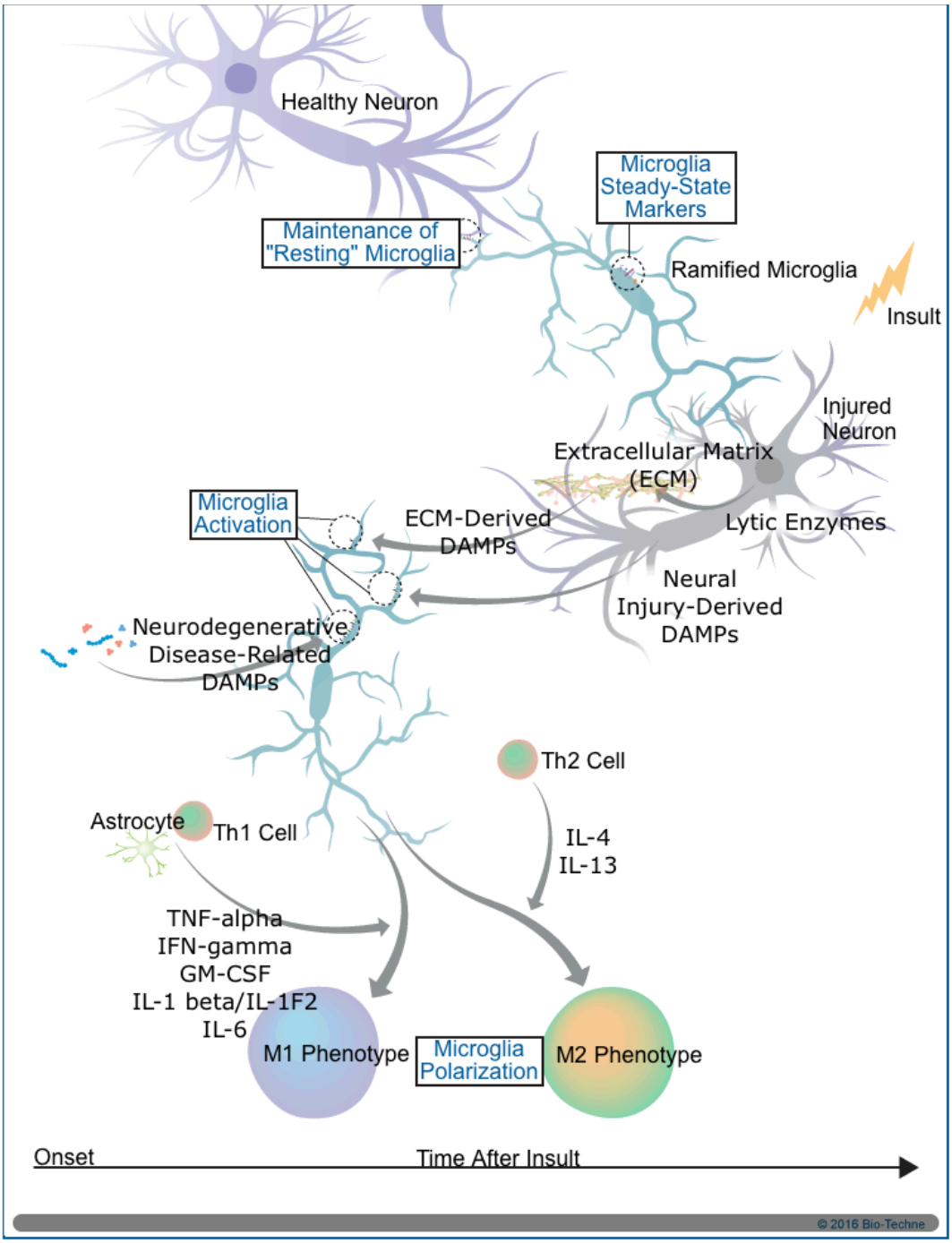


Figure 2.2. Microglia activation during neuroinflammation (85)

Astrocytes, the most abundant cell type in CNS, perform vital roles in maintaining CNS homeostasis. However, after noxious stimulation and nerve injury, the phenotype, functions, and gene expression of astrocytes may endure a significant difference, known as reactive astrogliosis. Through this process, astrocytes modify into several subsets, such as reactive astrocytes and scar-forming astrocytes. Reactive astrocytes are classified into toxic A1 astrocytes, which provoke rapid neuronal death and oligodendrocytes, and

neuroprotective A2 astrocytes, which support survival of neurons and tissue repair. Reactive astrogliosis are able to enhance neuroprotection and nutritional support for injured neurons. Activation of astrocytes can promote the development and maintenance of chronic pain by delivering signaling molecules. Additionally, current studies have revealed that activated astrocytes in brain regions linked to emotion regulation are associated with emotional dysfunction under chronic pain conditions (86, 87).

Astrocytes display numerous morphological and functional modifications after noxious stimulation and injuries, including morphological variations, proliferation, gene expression differences, significant molecular alterations, and functional modifications. The alteration of astrocytes from normal to reactive phenotypes include several intercellular and intracellular signaling pathways. Signaling molecules activating astrocytes could be released from various cell types, which are neurons and glial cells including microglia, oligodendrocytes, astrocytes, and inflammatory cells. The signaling molecules are not just participated in the astrocytes activation but further in chronic pain development; nevertheless, it is unclear if their role in chronic pain development interferes with the regulation of astrocyte activation (86, 87).

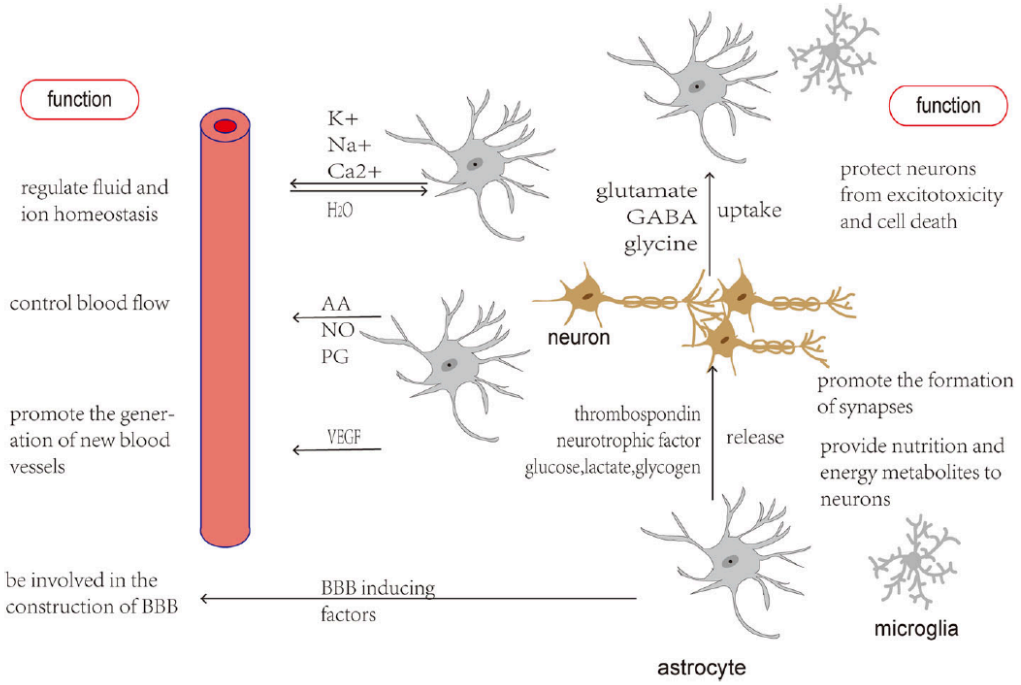


Figure 2. 3. Astrocyte function in the CNS (6)

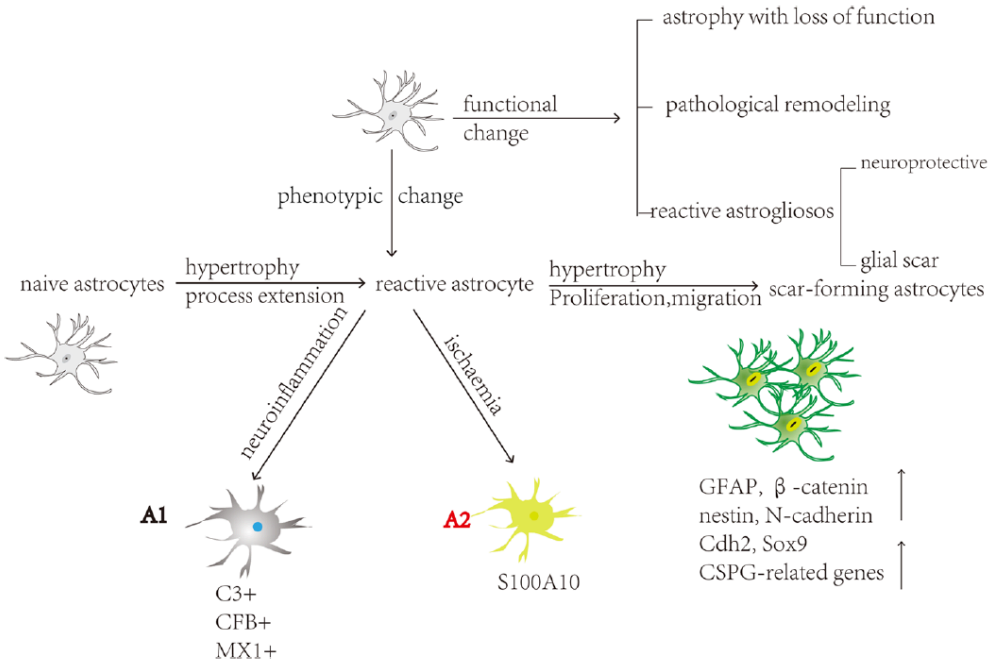


Figure 2. 4. Morphological and functional changes after noxious stimulation and an injury to the nerve (6)

2.5. Animal Models for Neuropathic Pain

Standardized and readily repeatable animal models of neuropathic pain are essential for expanding our understanding of the processes involved with NP and evaluating the analgesic potential of the new pharmacotherapies for NP therapy. The good examples should be able to produce sensory impairments such random pain, allodynia, and hyperalgesia over a long period of time (88).

In the aim of a precise understanding of the mechanical bases of NP, preclinical animal models have been present numerous advantages. First of all, they provide a detailed study of NP at the cellular and molecular levels. Secondly, they promote the description, design, and testing of pain-relieving candidates. And, finally, they give us details about the safety of these new compounds prior to testing and using them in the human population (88).

There are various animal models developed because NP has many etiologies. Four of these models are: 1. Nerve injury, 2. Drug-induced, 3. Disease-induced neuropathy, and 4. Miscellaneous (89).

Nerve injury models are divided into two categories: central and peripheral. Peripheral nerve injury models can be listed as; axotomy model (complete sciatic nerve transection, neuroma model), chronic constriction injury (CCI), partial sciatic nerve ligation (Seltzer Model), spinal nerve ligation, spared nerve injury, tibial and sural nerve transection, ligation of common peroneal nerve, sciatic cryoneurolysis, caudal trunk resection, sciatic inflammatory neuritis, cuffing-induced sciatic nerve injury, photochemical-induced sciatic nerve injury, laser-induced sciatic nerve injury. Weight loss or contusive spinal cord damage, excitotoxic spinal cord injury, photochemical spinal cord injury, and spinal hemisection are all examples of central nerve injury models. Anti-cancer drugs (vincristine, cisplatin, oxaliplatin, paclitaxel) and anti-HIV medicines (2,3-dideoxycytidine, didanosine) can be classified as **drug-induced animal models** (88).

Disease-induced animal models can be categorized as; diabetes-induced neuropathy, bone cancer pain models, HIV-induced neuropathy, post-herpetic neuralgia.

Chronic ethanol consumption or withdrawal, pyridoxine-induced, TN OFP, and acrylamide-induced models are the subgroups for **miscellaneous animal models** (88).

Principles of injuries in all of these animal models and the species using in them precisely have been summarized on Table 2.5.1.

Table 2.2. Principles of injuries in animal models and the species

Axotomy model (complete sciatic nerve transection; neuroma model)	Complete transection of sciatic nerve	Rats
Chronic constriction injury	Four loose ligatures around sciatic nerve	Rats, Mice
Partial sciatic nerve ligation (Seltzer Model)	Tight ligation of one-third to half of sciatic nerve	Rats, Mice
Spinal nerve ligation	(i) Tight ligation of L5, L6 spinal nerves (ii) tight ligation of L7 spinal nerve	Rats, Macaa
Spared nerve injury	Axotomy of tibial and common peroneal nerves	Rats, Mice
Tibial and sural nerve transection	Axotomy of tibial and sural nerves	Rats
Ligation of common peroneal nerve	Ligation of common peroneal nerve	Mice
Sciatic cryoneurolysis	Freezing of the sciatic nerve	Rats
Cuffing-induced sciatic nerve injury	Implantation of polyethylene cuff around sciatic nerve	Rats, Mice
Photochemical-induced sciatic nerve injury	Thrombosis in small vessels supplying sciatic nerve by photosensitizing dye and laser	Rats, Mice
Laser-induced sciatic nerve injury	Radiation mediated reduction in blood supply to sciatic nerve	Rats
Weight drop or contusive spinal cord injury	Dropping a weight over the exposed spinal cord	Rats, Mice
Excitotoxic spinal cord injury	Intraspinal injections of excitatory amino acids	Rats, Mice

Photochemical spinal cord injury	Thrombosis in blood vessels supplying the spinal cord by photosensitizing dye and laser	Rats
Spinal hemisection	Laminectomy of T11–T12 segments.	Rats
Drugs-induced		
Anti-cancer agents (vincristine, cisplatin, oxaliplatin, paclitaxel)	Direct injury of drugs to the nerves of peripheral nervous system	Rats, Mice, Guineapigs
Anti-HIV agents (2,3-dideoxycytidine, didanosine)	Direct injury of drugs to the nerves of peripheral nervous system	Rabbits, Rats
Diabetes-induced neuropathy		
Streptozotocin-induced	Persistent hyperglycemia-induced changes in the nerves	Rats, Mice
Genetic models	Persistent hyperglycemia-induced changes in the nerves	Rats, Mice
Bone cancer pain models		
Femur, calcaneus,tibial, humerus bone cancer pain	Inoculation of cancerous cells into respective bones	Rats, Mice
Neuropathic cancer pain	Growing a tumor in vicinity of sciatic nerve	Mice
Skin cancer pain	Injection of melanoma cells in plantar region of hind paw	Mice
HIV-induced neuropathy	Delivery of HIV-1 protein gp120 to sciatic nerve	Rats
Post-herpetic neuralgia		
Varicella Zoster virus	Injection of viral infected cells in the footpad	Rats, Mice
Herpes simplex virus	Depletion of capsaicin-sensitive	
Non-viral mode	Afferents with resiniferotoxin	Rats
Chronic ethanol consumption/withdrawal	Administration of ethanol over extended period (around 70 days)	Rats
Pyridoxine-induced	Administration of high dose pyridoxine for long period	Dogs, Rats

Trigeminal Neuralgia	Compression of trigeminal ganglion chronic constriction injury to infra-orbital nerve	Rats
Orofacial pain	Injection of formalin, carragenan into temporomandibular joints and maxilla	Rats, Mice
Acrylamide-induced	Administration of acrylamide for prolonged period	Rats

2.5.1. Chronic Constriction Injury of the Rat's Infraorbital Nerve (IoN-CCI)

One of the most accepted animal models of NP is a peripheral mononeuropathy model in rats by CCI to the sciatic nerve, developed by Bennett and Xie. [2] Based on the main model, Desure et al have been recently summarized the chronic constriction injury of the Rat's IoN (Fig 2.5.1) (91).

The IoN-CCI model's major goals are to conduct pharmacological trials of current or newly developed analgesic medications, as well as to investigate the pathophysiological processes underlying the creation and maintenance of trigeminal neuropathic pain (TNP) and to create novel treatment methods (91).

The objective of IoN-CCI is to surgically induce an infraorbital nerve (IoN) insult in a rodent that leads the formation of both induced and spontaneous nocifensive activity, which may be utilized to explore the causes of TNP and its therapy (90). After IoN-CCI, rats brush their faces more often and asymmetrically, with face wash strokes mostly directed towards the nerve-injured IoN area. Solitary face grooming is the only way to measure spontaneous NP in rats. Rats demonstrate changes in reaction to tactile stimulation and spontaneous behavior after IoN-CCI, which are symptoms of mechanical allodynia and chronic discomfort. The non-painful sensory disturbances such as anesthetic IoN blockade and unilateral vibrissae clipping do not or only very shortly make an important change in face grooming behavior. On the other hand, unpleasant facial treatment, such as formalin injection, does. Following IoN-CCI, aberrant face grooming response was recorded as a behavioral indicator of random, highly unpleasant feelings in

the damaged nerve's region. This response in rats gives remarkable similarity with spontaneous pain experienced by patients (93, 94). The other changes seen in rats after IoN-CCI is that they give an altered response to tactile stimulation, which is indicative of mechanical allodynia. In the first weeks following IoN-CCI, mild or moderate tactile stimuli have no longer responded by rats. This hyporesponsiveness is then suddenly shifted into extreme hyperresponsiveness. Nocifensive behavior can be elicited by even the tiniest stimuli. Although the start of hyperresponsiveness varies amongst rodents, most of them become hyperresponsive 3 to 4 weeks post-op (92).

The IoN-CCI requires placing two loosely tightened ligatures to IoN. For this purpose, there a few steps must be done. In order to having access to IoN, there are three approaches to practice: intra-orbital, intra-oral and peripheral approach. The peripheral approach the easiest one can be made by an incision into the hairy skin caudal to the vibrissal pad. However, the intra-orbital approach has a distinct advantage that it leaves the fine musculature that controls vibrissal movements intact. Moreover, it eliminates an incision wound in the territory of the injured nerve that can extremely hamper sensory testing in that area (91, 95). In order to IoN with the infra-orbital technique, a midline scalp incision should be made approximately 25 mm, placed equally distanced both anteriorly and posteriorly from the center of the eyes. The skull and nasal bone should be exposed with the help of this incision. A surgical loop or an operation microscope is a necessity for this procedure. A cotton swap and Dumont forceps can be used to dissection the edge of the orbit free, made by the frontal, lacrimal, zygomatic, and maxillary bones. The orbital contents should be smoothly deflected without overstretching the anterior ethmoidal nerve crossing superiorly to the IoN. Two ligatures must be done 2 mm apart from each other to create the targeted results. Additionally, any analgesia should not be applied to eliminate post-surgical pain due to avoiding pre-emptive analgesic effects on NP development (91).

The majority of NP animal research that use spinal nerve models emphasize nocifensive responses including withdrawal reactions and vocalization, as well as sensitivity to heat or physical stimuli. However, persistent pain, not allodynia or hyperalgesia, is the most common clinical symptom among patients (94, 95). The isolated face grooming assessment for spontaneous pain, Von Frey filament (VFF) applications to test mechanical allodynia, thermal hyperalgesia assessment, and cold allodynia

assessment are all NP assessments after IoN-CCI. Meal duration as an assessment for orofacial nociceptive responses and a lately improved apparatus named as the Orofacial Pain Assessment Device (OPAD) are the other two options for orofacial pain evaluations. There can be a considerably high variability between the amount of face grooming behavior performed by rats. In recent studies, it is reflected that observing rats more frequently can be a solution and help to reduce the variability (98-100).

Within a week, behavioral abnormalities emerge, with peak pain-related reactions and postural asymmetries occurring in the second week after surgery. These changes have been shown to last for at least 7 weeks after surgery (90, 101). Some histological investigations suggest that myelinated axons are more severely damaged than non-myelinated axons, and some electrophysiological research report a decrease in nerve conduction velocity. It has recently been proposed that C-fiber sensitization might be the cause of the behavioral alterations observed following damage in this paradigm. However, it has recently been discovered that partial nerve injury leads to sensitization of both A- and C-fibers, which play an important role in the initiation and maintenance of pain behavior (102, 103).

The ligature material may affect the outcome of the observed sensory abnormalities. An appropriate material of ligature for CCI in rats was studied earlier by Maves et al. Their study shows that neither plain nor silk gut ligatures that loosely tied around the sciatic nerve create any behavioral indication of NP. With no difference in physical withdrawal limit, chromic gut ligatures with an increasing diameter cause a dose-dependent decrease in thermo withdrawal latency to von Frey stimulation. Heat withdrawal delay decreases with the thickness of the ligature: 25% for 4-0 chromic gut sutures, 39% for 3-0 chromic gut sutures, and 41% for 2-0 chromic gut sutures. Also, loose ligation of the sciatic nerve with 4-0 chromic gut sutures was proceeded by placing 4-0 chromic gut sutures via the sciatic nerve terminating with the very same behavioral signs as NP (104).

The use of both plain and chromic gut sutures in surgery was shown to cause a local tissue inflammatory reaction in clinical settings. It was also discovered that using chromic gut sutures resulted in scarring on the sciatic nerve as well as a thick encapsulation of the ligatures. Silk sutures, on the other hand, elicited very mild

responses. Swelling and strangling of the nerve underneath the ligature have been described as other important consequences of a constricting ligation with 4-0 chromic gut sutures in previous research studying the pathophysiology of NP using the Bennett and Xie (1988) model. Such events result in the formation of transparent ridges on the nerve and, eventually, the constriction of the nerve beneath the ligatures, which is associated with a decrease in the number of big, myelinated axons (90, 105, 106).

Robinson et al. found that using a catgut ligature provides more stable symptoms of hyperalgesia and allodynia than using a synthetic silk suture. Preference of catgut ligature usage has been strongly recommended when long-term behavioral deficits are assured. Oppositely, van der Wal et al. investigated the best ligature material for CCI in mice, and they found silk more effective than catgut for NP investigation (107).

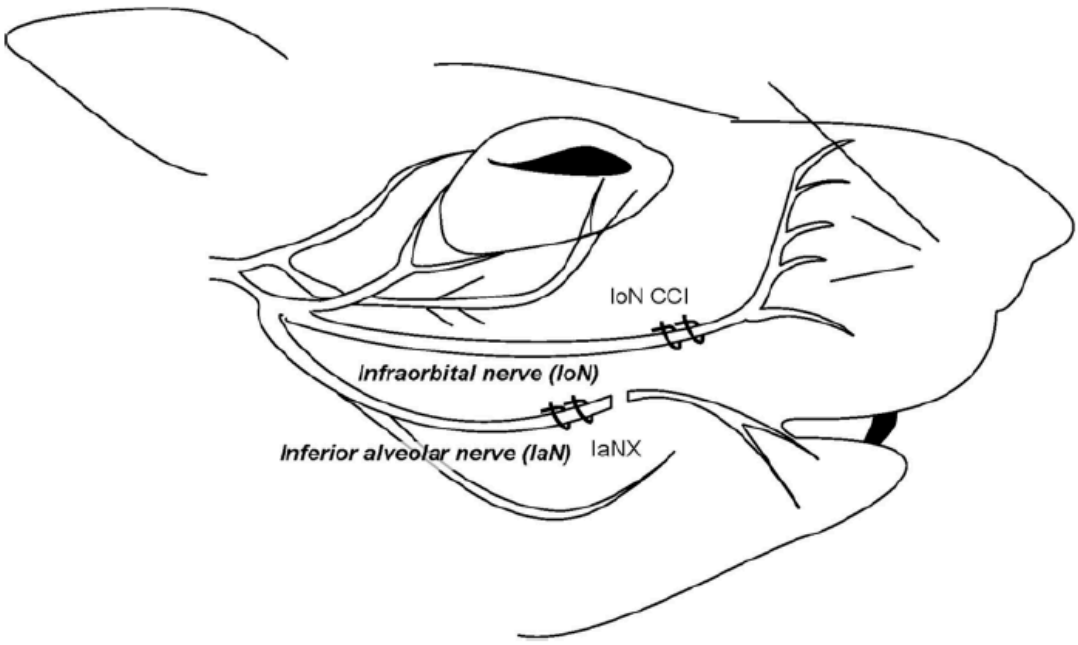


Figure 2.5. The chronic constriction injury of the Rat's Infraorbital Nerve

2.6.1. Face Grooming Behavior

Grooming is defined by etiologists as "a key activity for an animal's survival that includes all types of care and attention to the surface of the body". Grooming motions are

used by animals to remove surface material from sensory organs and protect the integument from biological elements such as microorganisms. Grooming behavior in rodents is also vital for spreading pheromones for social signaling and thermoregulation. The role of Grooming behavior as a response to painful stimuli has a critical role for experimental pain research among the diversity of functions (93, 108).

Grooming activities elicited by painful stimuli or regional discomfort have a distinct mechanism than grooming activities connected to social signaling, arousal reduction, pelage, or thermoregulation, according to etiological research. Grooming in rodents that does not cause a painful irritation involves lengthy periods of attention and care for the pelage and seems to be well-organized and completed. There are also short episodes of grooming actions particularly focused on the painful or irritated body region, for instance; in the intense irritation of a specific body area such as spreading mineral oil into the fur, in a painful irritation produced by biological factors such as ectoparasites and in physical objects damaging the skin such as splinters. Several animal models of chronic pain used the severity and length of spontaneously occurring events of focused grooming, that is licking, biting, or stroking of the affected region, as a behavioral measure of spontaneous pain (109-111).

Face grooming (FG) behavior has been reported in the territory of the injured nerve after IoN-CCI, and there is a substantial difference between FG that is not preceded or followed by body grooming, referred to as "isolated face grooming," (iFG), and FG that is a part of a broader body grooming, referred to as "face grooming during body grooming." Furthermore, the distinction between grooming elicited by regionalized discomfort, which includes short periods of grooming specifically directed to the agitated or painful area of the body, and grooming elicited by non-painful irritation, which includes prolonged and organized episodes starting with FG and ending with body licking, is properly defined. These results indicate that solitary face grooming following PNI in the trigeminal system can produce a significant amount of non-evoked facial discomfort (111, 112).

The reliability and validity of iFG as non-evoked facial pain measurement has been examined previously. iFG versus FG during body grooming, the difference between small and large observation cage and, the differences between unilateral and bilateral IoN-

CCI surgery are the three main topics discussed. It has been shown that unilateral IoN-CCI surgery increases the amount of time spent on iFG behavior while having no effect on the amount of time spent on FG during body grooming, which varies substantially depending on the observation cage size. IoN-CCI, on the other hand, produces iFG as a particular outcome. Pre-operatively, it is almost non-existent; that is, just a few rats engage in iFG, and only to a limited amount. Almost all rats continue to generate iFG episodes directed at the damaged nerve area after surgery (113).

The FG activity was more apparent after bilateral IoN-CCI surgery, although it was not better persistent. Nonetheless, while being observed in the tiny monitoring enclosure, FG behavior became more prominent and consistent. Dessure et al. found that bilaterally ligated rodents displayed substantially higher iFG behavior than unilaterally ligated animals pooled in their research. This result may represent alterations in core neuronal processing following IoN-CCI (114).

2.6.2. Von Frey Filament Testing

Because rats cannot be directly measured for pain, many approaches for measuring nociception or pain-like responses were devised. Depending as to whether or not an external stimulus is utilized to elicit a pullout reaction, these behavioral evaluations can be categorized as stimulus-evoked or non-stimulus evoked (spontaneous) nociception. Stimulus-evoked methods, which are manual and electronic VFF tests, the Hargreaves and Randall- Selitto test, were initially generated and continued widespread use. On the other hand, non-stimulus evoked methods including burrowing, gait analysis, grimace scales and weight bearing, have developed as a result of the concerns about clinical translatability of stimulus-evoked nociception (115).

The following are some of the methods for assessing pain-like behavioral responses in rodents: impulse evoked pain like actions with mechanical stimuli, such as manual and electronic Von Frey filament tests, and Randall-Selitto test; heat stimuli tail-flick test, hot plate test, Hargreaves test, thermal probe test; cold stimuli cold plate test, acetone evaporation test, cold plantar assay; tetanus toxin toxin toxin toxin Grimace scales, burrowing, weight bearing, and gait analysis, as well as automated behavioral analysis, are all examples of non-stimulus induced nociception (115).

Maximillian Von Frey invented a therapeutic technique known as VFF or Von Frey hairs in 1898. This apparatus is designed to test touch sensitivity by pushing on the epidermis with a thorn attached to the end of a strand until the strand begins to bow out. This approach was used to determine the pressure sensitivity of nerve fibers in the skin (116).

Von Frey used a balance to calibrate the fibers, varying stiffness by altering the length or using various hardness fibers. The pressure sensibility was measured by noting if pressing a filament on the skin caused any feeling. Von Frey improved on this approach of assessing pressure sensibility in subsequent experiments, but his approach stayed intact (116).

The working principle of VFF is applying a calibrated and graduated force to a sensory field, the measured force being that which is reached at the point when VFF buckles. VFF were integrated for sensory testing where the endpoint was subjective reported by the patient during the sensation (117). The manual VFF test is used for evaluating mechanical allodynia in rats and mice. Despite the development of electronic VFF test, manual Von Frey remains excepted as a gold standard for stating mechanical thresholds in rats and mice (115).

In people, the intensity of discomfort is usually determined by a verbal or other subjective assessment of suffering. QST, on the other hand, has the potential to become the gold standard in both medical practice and clinical research (31, 118). VFF are used to test mechanical responsiveness in animals by applying a controlled stress to a part of the subject's body. The use of these filaments has been used to evaluate mechanosensitivity with precision. Many distinct tests are used to study protective behaviour in animal studies. For instance, hind paw withdrawal, tail-flick, and jump-flinch are all painful stimulation reactions that may be sensitized by high-intensity laser beams, formalin injection, hot plates, or spinal nerve ligation surgery. The strength of a noxious stimulus that causes the cutoff response or the delay to react to a stimulus are used to quantify these responses (119-121).

Astrocytes organize neuronal development by secreting synaptogenic molecule secretion and excess synapses purification. They sustain homeostasis of CNS and support

neuronal survival by shuttling metabolites, secreting trophic factors with the blood flow regulation. They also react to a CNS disease and injury with a process termed reactive astrogliosis, which is activated glia state cells that contribute to both inflammation and the resolution of it. There are many roles of astrocytes discovered, yet countless of them still need to be made. New and discovered devices to culture astrocytes in vitro and handle them in vivo have advanced research into the function of astrocytes. Common astrocyte markers and reagents are shown in Table 2. 3. Many tools utilized in microglia studies do not specify between microglia and CNS macrophages, and this restriction leads to innovation in new tools to investigate microglia in vivo and in vitro. Common microglia markers and reagents are shown in table 2.4. (122-126).

Table 2.3. Common astrocyte markers and reagents

Gene (protein)	Labeled Cells (CNS)	Genetic Lines	Antibodies	Notes
Gfap (GFAP)	Astrocytes + NPCs	Fluorescent reporter, Cre, CreERT2	Y	Upregulated in some reactive astrocytes
Aldh1l1 (ALDH1L1)	Astrocytes	Fluorescent reporter, CreERT2 (new)	Y	
Slc1a3 (GLA ST)	Astrocytes + NPCs	Fluorescent reporter, CreERT	Y	Developed by Jeremy Nathans (Mouse Genome Informatics)
Slc1a2 (GLT1)	Astrocytes + NPCs	Fluorescent reporter	Y	
S100b (S100B)	Astrocytes + OL lineage	Fluorescent reporter, CreERT2	Y	
Gjb6 (CX30)	Astrocytes + NPCs	CreERT2	Y	
Slc6a11 (GAT3)	Astrocytes + NPCs	CreERT2	Y	
Nes (NESTIN)	Astrocytes + NPCs	Fluorescent reporter, Cre, CreERT2	Y	Upregulated in some reactive astrocytes
Vim (VIMENTIN)	Astrocytes + NPCs	Fluorescent reporter, LacZ	Y	Upregulated in some reactive astrocytes
C3	Astrocytes + NPCs	Fluorescent reporter (not finalized)	Y (human)	Upregulated in A1 reactive astrocytes; in situ hybridization required for murine tissue

Table 2.4. Common microglia markers and reagents

Gene (protein)	Labeled Cells (CNS)	Genetic Lines	Antibodies	Notes
Cx3cr1 (CX3CR1)	Microglia and other myeloid lineage cells	Fluor. reporter, Cre, CreERT	Y	Quadruple-colored PrismPlus lines available
Aif1 (IBA1)	Microglia and other myeloid lineage cells	Fluor. reporter	Y	Increase in IBA1 staining often used to suggest activation
Ptprc (CD45)	Microglia and other myeloid lineage cells	Fluor. reporter, Cre	Y	
Itgam (CD11b)	Microglia and other myeloid lineage cells	DTR/GFP line, Cre	Y	
Tmem119 (TMEM119)	Microglia	N	Y	Protein does not label young microglia
Sall1 (SALL1)	Microglia	CreERT	Y	
Fcrls (FCR LS)	Microglia	N	Y	
P2ry12 (P2RY12)	Microglia	N	Y	
Adgre1 (F4/80)	Microglia and other myeloid lineage cells	Cre	Y	
Cd68 (CD68)	Microglia and other myeloid lineage cells	Fluor. reporter, rtTA, CreERT2	Y	Often used as a marker of microglial activation
Cd40 (CD40)	Microglia and other myeloid lineage cells	N	Y	
Csf1r (CSF1R)	Microglia and other myeloid lineage cells	Fluor. reporter, Cre		Required for microglial survival

3. MATERIALS and METHODS

3.1. Experimental Animals

The study was conducted at Yeditepe University Experimental Animal Research Center. All experimental procedures were approved by Yeditepe University Animal Experiments Local Ethics Committee (YÜDHEK) (2020/11-7). Protocols for maintenance and care of experimental animals complied with the guidelines of the International Association for The Study of Pain (127).

24 male, 14-16 weeks adult, Sprague-Dawley rats with the weight of 200–250 g were used. The sample size of the study was determined according to the results of the power analysis. The total sample size was found to be n=24, using the G-POWER program with an effect size of 0.5, a power of 80%, and a margin of error of 0.05. All of the animals were kept under standard laboratory conditions with a partially reversed 12-h light, 12-h dark cycle in a T=22 ± 1 °C room with a humidity of 40-60% and given access to ad libitum food and water (Fig 3.1).

Figure 3. 1. The place where the animal Housing

3.2. Experimental Design

Animals initially presented to a 3-day adaptive period in the experimental environment. To do that, rats were put in an individual cage for regular application of Von Frey Filaments on their face by the researcher for 30 minutes. Any scoring was not made for behavioral responses during this adaptation process. This procedure was applied for each and every experimental animal in this study. The schematic presentation of the experimental design is shown on table 3.1.

After animals are adopted enough for 3 days, two experimental group was randomly made according to the perineural injection they received Vehicule (Vhcl) (NaCl, 0.5 %) and LPS groups. Each group was included 12 rats.

On day -1, the experiment started with the behavioral assessments. Two different parameters (i) the evaluation of spontaneous pain by iFG assessment and (ii) evaluation of MSA by VFF testing were performed for behavioral assessments.

3.2.1. Evaluation of Spontaneous Pain by Isolated Face Grooming Assessment

Behavioral testing session was including observation of free behavior. A darkened testing room was lightened by a 60 W red light bulb hung 1 m above the center of the test area. Besides the center, the circumferences were leaving dusk, and the rest of the room as dark. White noise (50 dB) was provided as a background noise for decreasing the intervention from unexpected auditory stimulation. For the observation process, rats were individually placed in a transparent plastic cage without bedding (l x w x h: 24 cm x 14 cm x 17 cm) with a mirrored back. A video camera was positioned 1 m in front of the cage. Spontaneous pain-like behavior was videotaped for 10 minutes while ensuring the experimenter was not presenting in the test room (Fig 3.2) (91, 93, 114).

Deseure et al. originally explained measuring the iFG activities on groups (n=6) as a method of assessing spontaneous pain-like activity (101). An observer who was blinded to the rat's treatment group examined videotaped behaviors offline. The emphasis of the video study was on random iFG activity that was neither preceded nor followed by body grooming (114). Using a timer to assess the amount of time spent on iFG activities

throughout the evaluation period, the sum of time spent on iFG episodes was computed (92).

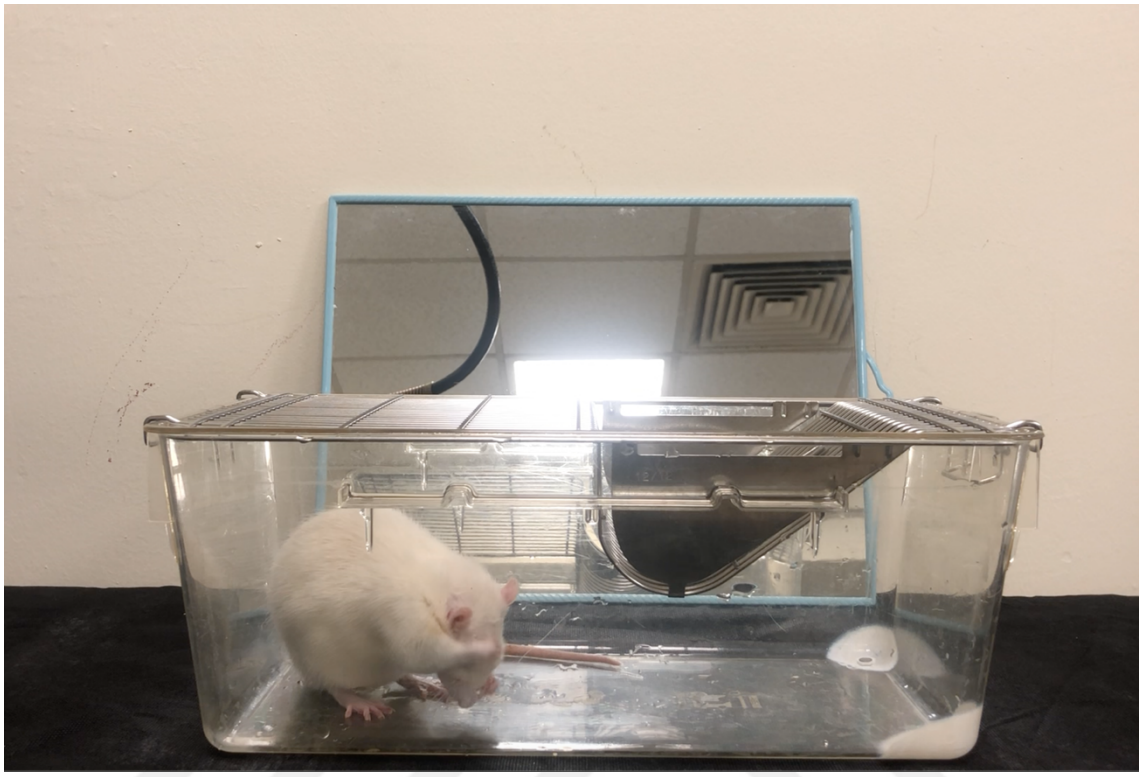


Figure 3. 2. Recording of the face grooming episodes

Table 3. 1. The schematic presentation of the experimental design

	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
VFF Testing																																									
Isolated Face Grooming Evaluation																																									
Perineural injections																																									
IoN-CCI																																									

3.2.2. Evaluation of Mechanical Static Allodynia by Von Frey Filament Testing

A graded series of VFF (Bioseb, Chaville, France) were used for mechanical stimulation. These filaments were ranging from 1 to 10 g bending force producing by plastic monofilaments of equal length and varying diameters depending on the force required to bend (Fig 3.3) (92). MSA was assessed by a blinded investigator to the experimental group. Rats were individually placed in transparent plastic cages with bedding (l x w x h: 24 cm x 14 cm x 17 cm) with a folding lid so that it can be simply opened and closed (91). For the record of the observation of responses to mechanical stimuli, application of VFF was videotaped with a camera located at the eye level of the experimenter, who sat quietly on the opposite side. A 60 W red bulb hung 1 m above the center of the test area as the same in iFG evaluation procedure. Mechanical stimulation was given to the right infraorbital facial area on hairy skin within the IoN territory by slowly reaching into the cage (90). Rats were habituated to the observation cage and reaching movements for 10 minutes before the actual VFF application session starts. The adaptation process includes the experimenter opening the lid and smoothly touching the walls of the cage with a rod similar to VFF 30 seconds (Fig 3.4).

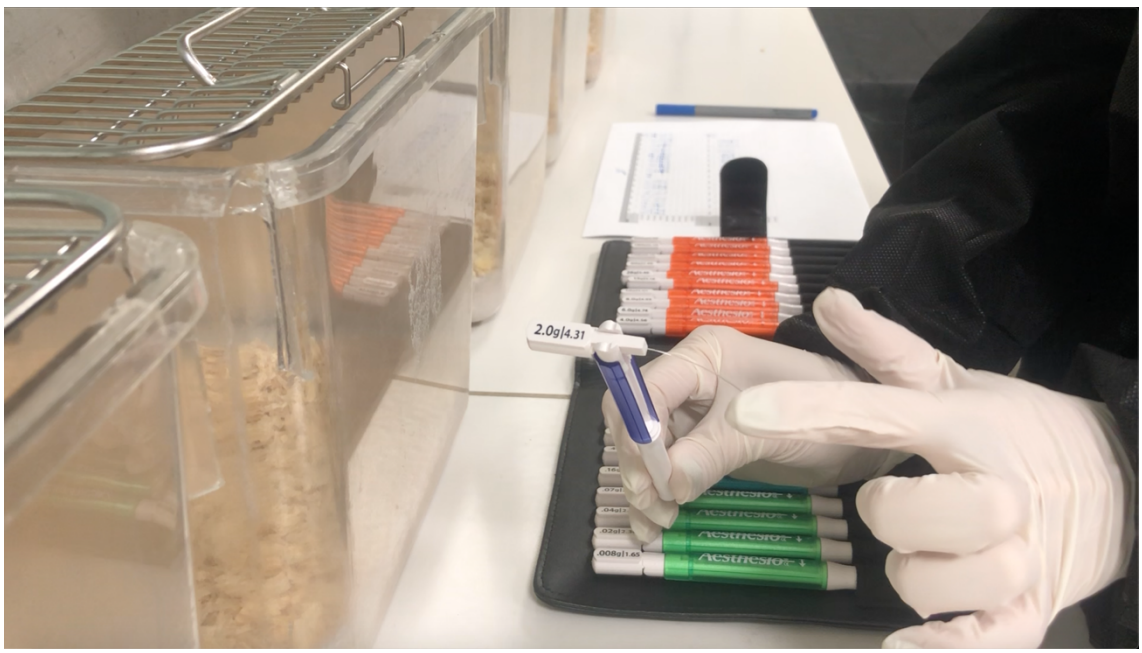


Figure 3. 3. Von Frey Filaments

Figure 3. 4. Application of Von Frey Filaments

The actual stimulation session was begun when the rat was in a sniffing/no locomotion state. In this state, rats should be neither moving nor freezing but exhibiting sniffing behavior with four paws placed on the ground only when continuing the position. Each VFF was applied individually 5 times, 3 seconds apart from each other in ascending order of intensity (128).

The behavioral response was assessed utilizing the response scoring system previously described by Vos et al (93). For the precise examination of the responses, videotaped scenes were played in slow motion. Table 3.2 is showing the response scoring system; which are 0 was given for no response, 1 was given for non-aversive response by just detection of the filament, 2 was given for mild aversive response by detecting the filament and withdrawal from stimulation, 3 was given for strong aversive response by detecting the filament, withdrawal and escaping or attacking, 4 was given for prolonged aversive behavior by detecting, withdrawal, escaping or attacking and asymmetric face grooming (92). The mean of all response scores was described as the final response (Fig 3.5).

Table 3. 2. Scoring for the responses to the Von Frey Filaments

	Response Elements Observed				
Category of Response	**Detection**	**Withdrawal**	**Escape or Attack**	**Face Grooming**	**Score**
No Response	0	0	0	0	0
Nonaversive Response	1	0	0	0	1
Mild Aversive Response	1	1	0	0	2
Strong Aversive Response	1	1	1	0	3
Prolonged Aversive Response	1	1	1	1	4

Figure 3. 5. Scoring the Responses to Von Frey Filaments

VFF indications were measured between 12:30 and 5:30 P.M. and iFG recordings were done between 11:00 A.M. and 3:00 P.M. on Days −1, 1, 8, 15, 22, 27 (4 days after IoN-CCI), 31 (8 days after IoN-CCI), 35 (12 days after IoN-CCI), and 39 (16 days after IoN-CCI) (128).

On day 0 perineural injections were started in groups depending on the injections they receive. Vhcl or LPS injections were made at 1:30 P.M. on days 0, 3, 5, 11, 13, 17, and 20.

3.3. Perineural Injections

A 1 mL-micro-syringe with a needle (29Gx12,7 mm) was used for perineural injections. Injections were made by locating the needle 4-5 mm posteriorly to the right maxillary incisor for performing a parallel insertion to the maxillary bone surface in the intraoral mucosa. With this technique, the perineural injection was conducted nearby IoN emergence from the infraorbital foramen. 50 µL Vhcl or 50 µL of LPS (300 µM, Santa Cruz, Fig 3.6) in saline (0.9% NaCl) was prepared for each perineural injections (Fig 3.7). 129 On day 23., IoN-CCI surgery was performed for all the experimental animals.

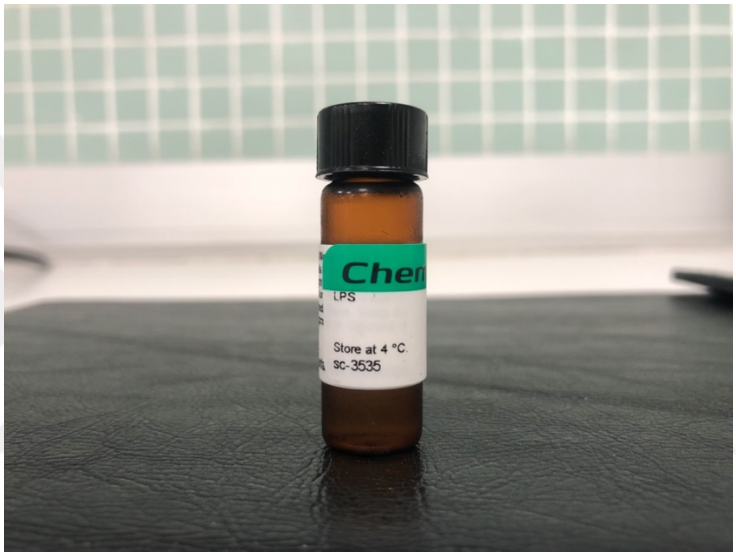


Figure 3. 6. LPS for perineural injections

Figure 3. 7. LPS injection into the vicinity of IoN

3.4. CCI-Surgery

Rats were intensely anesthetized with a combination of Ketamine and Xylazine (90 mg/kg + 5 mg/kg) and treated with atropine (0.1 mg/kg). The anesthesia level was controlled by stretching one leg and pinching the skin between the toes to secure sufficient pressure is applied. A heated pad was used to control the body temperature, and a stereotaxic frame was applied to fixate the rat's head. All the operation was performed under a Zeiss operation microscope (10-25 x) (92).

As a first step of the surgery, a mid-line scalp incision was made with the purpose of exposing the skull and nasal bone. The edges of the orbit, created by frontal, lacrimal, maxillary, and zygomatic bone, were dissected with the help of a forceps and a cotton swab, which was also helpful for removing the excessive blood in the view area. Surgery was proceeded with tenderly diverging the orbital contents from the surrounding tissues. In order to avoid the overstretching the anterior ethmoidal nerve, which crosses superiorly to the IoN. After giving access to the IoN, it was dissected free from the surrounding connective tissue with radial motion. Two chromic catgut ligatures (5-0; Ethican, Johnson & Johnson, Belgium) separated by 2 mm interval were loosely tied around the IoN. [4]The ligations were able to delay the circulation through the superficial vasculature without occlusion and decreased the diameter of the IoN by a remarkable amount, as described in the previous studies about producing the desired amount of constriction.[2] In the final part of the surgery, the mid-line scalp incision was sewed up with 5-0 Pronele sutures. In the Vhcl group, the same procedure was used for the IoN exposure without any ligature or direct contact. Analgesia for post-surgical pain was not given in order to eliminate pre-emptive analgesic effects on the development of NP (91, 92).

The post-operative measurements of spontaneous pain like behaviors were done on days 27 (4 days after IoN-CCI), 31 (8 days after IoN-CCI), 35 (12 days after IoN-CCI), and 39 (or 16 days after IoN-CCI) (128).

All animals were euthanized via isoflurance overdose on the 40th day and their brain and brain stem samples were retrieved (Fig 3.8). Tissue samples were immediately stored in 4% paraformaldehyde (PFA).

Figure 3. 8. Rat Brain and Brain Stem

3.5. Immunohistochemistry & Hematoxylin and Eosin staining

Mouse brains were put in 4% PFA overnight at 4° celcius, then transferred to 30% sucrose in phosphate buffer for at least 18 hours of cryoprotection at 4° celcius. The samples were dehydrated with graded ethanol and embedded in paraffin after being converted to 70% ethanol. On a sliding freezing microtome, brain samples were sliced transversely into 30-m-thick slices, collected in an anti-freeze solution [0.05M sodium phosphate buffer (pH 7.3) containing 30% ethylene glycol and 20% glycerol], and kept at -18 C until use. The counterstain used in the experiment was hematoxylin and eosin. For H&E staining, hydrated slides were washed in xylene for five minutes 3 times and in alcohol for five minutes 3 times, then rinsed in tap water for two minutes, immersed in 0.1 percent hematoxylin for 3.5 minutes, washed with alcohol for two minutes, immersed in 0.1 percent eosin for 7 minutes, and dehydrated with graded alcohol. The coverslips were then placed on the segments.

The immunohistochemical staining procedure was performed by following steps: Formalin-fixed paraffin-embedded TMA tissue blocks were cut into 3-m-thick slices and left overnight in an oven to dry at 45° celcius. According to the pre-published procedure, the sections were put in a BOND-MAX Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany). To begin, samples were deparaffinized and pre-treated for 20 minutes at 100 degreed celcius with Epitope Retrieval Solution 2 (EDTA-buffer pH8.8). After the washing processes, the Bond Polymer Refine Detection Kit DS9800 was used to apply peroxidase as a blocking agent for ten minutes (Leica Microsystems GmbH). Samples were washed and incubated with the main antibody for 30 minutes in succession. The following were the primary antibody concentrations: rabbit anti-GFAP (1:3000, Leica, Germany), goat anti-CD68 (1:100, Leica, Germany). Tissues were then treated in polymer for 10 minutes before being developed with DAB-Chromogen for ten minutes.

Images captured by using an Olympus BX51 microscope (Tokyo, Japan). IHC reactivations were analyzed using Aperio (Leica, Germany) under 4x magnification. Cell counting of both GFAP and CD68-positive cells was performed on digitized images by Sectra (Cologne, Germany) program. On digitized Sp5C slides, the appropriate "hot spots" were point-of-interest. The hematoxylin-stained nucleus associated with each cytoplasmic antibody, reactivity was considered to be the stereotypical phenotype. The cell count for each hot spot was repeatedly applied on six selected sections for each animal in the study, by 400x magnification, that consisted of 500 cells. Two different quantification methods were performed to evaluate the activated microglia and reactive astrogliosis, i.e., semiquantitative scoring (score 0-4) and the digital morphometric analysis on digitized visuals, respectively.

3.6. Statistical Analysis

In this study, statistical analysis was performed with SPSS 24.0 program. Descriptive statistical methods (mean, standard deviation, median, frequency, ratio, interquartile range) were utilized to evaluate the distribution of variables. All data were expressed as mean ± SD. The Mann-Whitney U test was applied to compare the quantitative data between two groups that did not show normal distribution, to find comparison of the mean iFG episodes describing spontaneous pain, and the mean VFF

testing scores of pain scores of MSA of applied grams in Vhcl and LPS groups according to the observation days. Friedman Halton test was used in comparisons of three or more periods, finding the mean iFG episodes describing spontaneous pain, and the mean VFF testing scores for MSA of applied grams in Vhcl and LPS groups according to the observation days. Wilcoxon test was used to determine the difference between two periods. Correlation of the mean iFG episodes and VFF testing scores on day 39, and CD68 and GFAP-positivity on cells according to the left and right sides of Sp5c for both groups were done by using Spearman's Correlation. The results were evaluated at $p<0.01$ and $p<0.05$ for level of significance.

4.RESULTS

All animals are survived in the time of perineural Vhcl and LPS injection, surgery, and the observation days.

The mean iFG episodes of Vhcl and LPS groups according to the observation days are shown on Table 4.1. While a significant increase was found in iFG responses of Vhcl and LPS groups ($p<0.01$), the increase rate of iFG describing the severity of the spontaneous pain in LPS group was more significant ($p=0.001$) (Table 4.1)

Table 4. 1. The mean iFG episodes of Vhcl and LPS groups according to the observation days.

Groups	Observation Days	N	Mean±SD	[b]*p*
Vhcl	Day -1	6	18.02±3.15	***0.001****
	Day 1	6	19.00±2.00	
	Day 8	6	18.00±2.62	
	Day 22	6	21.72±3.08	
	Day 27	6	47.16±4.62	
	Day 31	6	45.09±5.76	
	Day 35	6	45.60±5.76	
	Day 39	6	48.10±4.85	
LPS	Day -1	6	19.05±2.00	***0.001****
	Day 1	6	20.99±1.54	
	Day 8	6	30.66±5.81	
	Day 22	6	39.82±4.41	
	Day 27	6	64.31±3.98	
	Day 31	6	68.41±7.50	
	Day 35	6	69.46±3.04	
	Day 39	6	75.21±6.55	

*[b]Friedman Halton Test **p<0.01*

Comparison of the mean iFG episodes of Vhcl and LPS groups according to the observation days are shown on Table 4.2. While in LPS group, the mean iFG episodes showing severe spontaneous pain were higher than Vhcl group on days 8, 22, 27, 31, 35, 39. (p=0.006, p=0.004, p=0.004, p=0.004, p=0.002, p=0.002, respectively), there was no significant difference on the mean iFG episodes in both groups on day -1 and day 1. Statistically significant difference was found between groups according to observation days (p<0.01) (Table 4.2).

Table 4. 2. Comparison of the mean iFG episodes of Vhcl and LPS groups according to the observation days

Observation days	Groups	N	Mean ± SD	[a]*p*
Day -1	Vhcl	6	18.02±3.15	0.423
	LPS	6	19.05±2.00	
Day 1	Vhcl	6	19.00±2.00	0.109
	LPS	6	20.99±1.54	
Day 8	Vhcl	6	18.00±2.62	***0.006****
	LPS	6	30.66±5.81	
Day 22	Vhcl	6	21.72±3.08	***0.004****
	LPS	6	39.82±4.41	
Day 27	Vhcl	6	47.16±4.62	***0.004****
	LPS	6	64.31±3.98	
Day 31	Vhcl	6	45.09±5.76	***0.004****
	LPS	6	68.41±7.50	
Day 35	Vhcl	6	45.60±5.76	***0.002****
	LPS	6	69.46±3.04	
Day 39	Vhcl	6	48.10±4.85	***0.002****
	LPS	6	75.21±6.55	

[a]Mann Whitney U Test **p<0.01

The mean VFF testing scores of applied grams (2,4,6,8,10) according to observation days in both groups are shown on Table 4.3. Statistically significant increase was found in VFF testing scores describing pain scores of MSA of 2,4,6,8,10 grs in Vhcl and LPS groups by time (p=0.001, p<0.01)

Table 4. 3. The mean VFF testing scores of applied grams in Vhcl and LPS groups according to the observation days.

Groups	Observation days	N	Mean ± SD					[b]p
			2 gr	*4 gr*	*6 gr*	*8 gr*	*10 gr*	
Vhcl	Day -1	12	1.08±0.29	1.17±0.39	1.42±0.51	1.25±0.45	1.25±0.45	***0.001*** **
	Day 1	12	1.00±0.00	1.17±0.39	1.83±0.39	1.25±0.45	1.25±0.45	
	Day 8	12	1.08±0.29	1.17±0.39	1.17±0.39	1.42±0.51	1.42±0.51	
	Day 15	12	1.08±0.29	1.17±0.39	1.83±0.39	1.50±0.52	1.67±0.49	
	Day 22	12	1.17±0.39	1.17±0.39	1.42±0.51	1.50±0.52	1.67±0.49	
	Day 27	12	1.08±0.29	1.17±0.39	2.83±0.39	1.33±0.49	1.42±0.51	
	Day 31	12	1.33±0.49	1.42±0.51	2.25±0.45	1.67±0.49	1.67±0.49	
	Day 35	12	2.00±0.00	1.08±0.29	3.00±0.00	2.25±0.45	2.5±0.52	
	Day 39	12	2.00±0.00	1.08±0.29	2.50±0.52	2.50±0.52	3.00±0.00	
LPS	Day -1	12	1.00±0.00	1.08±0.29	1.17±0.39	1.25±0.45	1.25±0.45	***0.001*** **
	Day 1	12	1.00±0.00	1.08±0.29	1.08±0.29	1.58±0.51	1.67±0.49	
	Day 8	12	1.33±0.49	1.50±0.52	1.42±0.51	1.67±0.49	1.83±0.39	
	Day 15	12	1.42±0.51	1.58±0.51	1.08±0.29	1.83±0.39	1.83±0.39	
	Day 22	12	1.42±0.51	1.58±0.51	1.67±0.49	2.00±0.60	2.08±0.67	
	Day 27	12	1.33±0.49	1.50±0.52	1.08±0.29	1.83±0.39	2.00±0.60	
	Day 31	12	1.50±0.52	1.67±0.49	1.83±0.39	2.17±0.39	2.50±0.52	
	Day 35	12	2.08±0.67	2.58±0.51	1.42±0.51	2.92±0.29	3.25±0.45	
	Day 39	12	2.25±0.45	2.83±0.39	1.92±0.51	3.42±0.51	3.42±0.51	

[b]Friedman Halton Test **p<0.01

Comparisons of the mean VFF testing scores describing pain scores of MSA of applied grams in both groups according to the observation days are shown on Table 4.4.

The mean VFF scores of 2 gr between Vhcl and LPS groups were not statistically significant in all observation days ($p>0.05$). No pain scores of MSA were obtained from all days.

While there were significant differences in the mean VFF testing scores describing pain scores of MSA of 4 gr between Vhcl and LPS groups on days 15 and 22 ($p=0.039$, $p=0.039$, respectively) and in these days increased pain scores of MSA were obtained from the applied 4 gr. No significant differences were found on days -1, 1, 8, 27, 31, 35, 39 ($p>0.05$).

There were significant differences in the mean VFF testing scores describing pain scores of MSA of 6 gr between Vhcl and LPS groups on days 8, 15, 22, 27, 31, 35, 39 ($p=0.004$, $p=0.039$, $p=0.030$, $p=0.001$, $p=0.039$, $p=0.005$, $p=0.006$, respectively) and in these days increased pain scores of MSA were obtained from the applied 6 gr. No significant difference was found between Vhcl and LPS groups on days -1 and 1 ($p>0.05$).

While there were significant differences in the mean VFF testing scores describing pain scores of MSA of 8 gr between Vhcl and LPS groups on days 22, 27, 31, 35, 39 ($p=0.046$, $p=0.015$, $p=0.015$, $p=0.001$, $p=0.001$, respectively) and in these days increased pain scores of MSA were obtained from the applied 8 gr. No significant differences were found between Vhcl and LPS groups on days -1, 1, 8, 15 ($p>0.05$).

There were significant differences in the mean VFF testing scores describing pain scores of MSA of 10 gr between Vhcl and LPS groups on days 1, 8, 27, 31, 35, 39 ($p=0.045$, $p=0.039$, $p=0.023$, $p=0.002$, $p=0.003$, $p=0.014$, respectively) and in these days increased pain scores of MSA were obtained from the applied 10 gr. No significant difference was found between Vhcl and LPS groups on days -1, 15, 22 ($p>0.05$). (Table 4.4)

Table 4. 4. Comparison of the mean VFF testing scores of applied grams in Vhcl and LPS groups according to the observation days

			2 gr		4 gr		6 gr		8 gr		10 gr	
	Groups	**N**	**Mean ± SD**	**[a]p**	**Mean ± SD**	**[a]p**	**Mean ± SD**	**[a]p**	**Mean ± SD**	**[a]p**	**Mean ± SD**	***[a]p***
Day -1	**Vhcl**	12	1.00±0.00		1.17±0.39		1.08±0.29		1.25±0.45		1.25±0.45	***0.990***
	LPS	12	1.00±0.00	***0.317***	1.08±0.29	***0.546***	1.17±0.39	***0.546***	1.25±0.45	***0.999***	1.25±0.45	
Day 1	**Vhcl**	12	1.08±0.29		1.17±0.39		1.08±0.29		1.25±0.45		1.25±0.45	***0.045*****
	LPS	12	1.33±0.49	***0.985***	1.08±0.29	***0.546***	1.42±0.51	***0.065***	1.58±0.51	***0.105***	1.67±0.49	
Day 8	**Vhcl**	12	1.08±0.29		1.17±0.39		1.08±0.29		1.42±0.51		1.42±0.51	***0.039****
	LPS	12	1.42±0.51	***0.140***	1.50±0.52	***0.090***	1.67±0.49	***0.004*****	1.67±0.49	***0.229***	1.83±0.39	
Day 15	**Vhcl**	12	1.17±0.39		1.17±0.39		1.42±0.51		1.50±0.52		1.67±0.49	***0.356***
	LPS	12	1.42±0.51	***0.065***	1.58±0.51	***0.039****	1.83±0.39	***0.039****	1.83±0.39	***0.090***	1.83±0.39	
Day 22	**Vhcl**	12	1.08±0.29		1.17±0.39		1.42±0.51		1.50±0.52		1.67±0.49	***0.107***
	LPS	12	1.33±0.49	***0.187***	1.58±0.51	***0.039****	1.92±0.51	***0.030****	2.00±0.60	***0.046****	2.08±0.67	
Day 27	**Vhcl**	12	1.33±0.49		1.17±0.39		1.17±0.39		1.33±0.49		1.42±0.51	***0.023****
	LPS	12	1.5±0.52	***0.140***	1.5±0.52	***0.090***	1.83±0.39	***0.001*****	1.83±0.39	***0.015****	2.00±0.60	
Day 31	**Vhcl**	12	2.00±0.00		1.42±0.51		1.42±0.51		1.67±0.49		1.67±0.49	***0.002*****
	LPS	12	2.08±0.67	***0.418***	1.67±0.49	***0.229***	1.83±0.39	***0.039****	2.17±0.39	***0.015****	2.50±0.52	
Day 35	**Vhcl**	12	2.00±0.00		2.17±0.58		2.25±0.45		2.25±0.45		2.50±0.52	***0.003*****
	LPS	12	2.25±0.45	***0.625***	2.58±0.51	***0.081***	2.83±0.39	***0.005*****	2.92±0.29	***0.001*****	3.25±0.45	
Day 39	**Vhcl**	12	1.00±0.00		2.50±0.52		2.50±0.52		2.50±0.52		3.00±0.00	***0.014****
	LPS	12	1.00±0.00	***0.070***	2.83±0.39	***0.09***	3.00±0.00	***0.006*****	3.42±0.51	***0.001*****	3.42±0.51	

[a]Mann Whitney U Test *****p<0.01*** ****p<0.05***

The evaluations of spontaneous pain by iFG episodes before and after IoN-CCI are shown in Fig.4.1 and Fig 4.2, respectively. Repeated perineural LPS injections in the vicinity of IoN produced a significant increase in spontaneous pain as compared to Vhcl on days -1, 8, 22 ($P < 0.001$). After IoN-CCI, iFG episodes showing spontaneous pain were increased in LPS group as compared to Vhcl on days 27, 31, 35, 39 ($P < 0.001$).

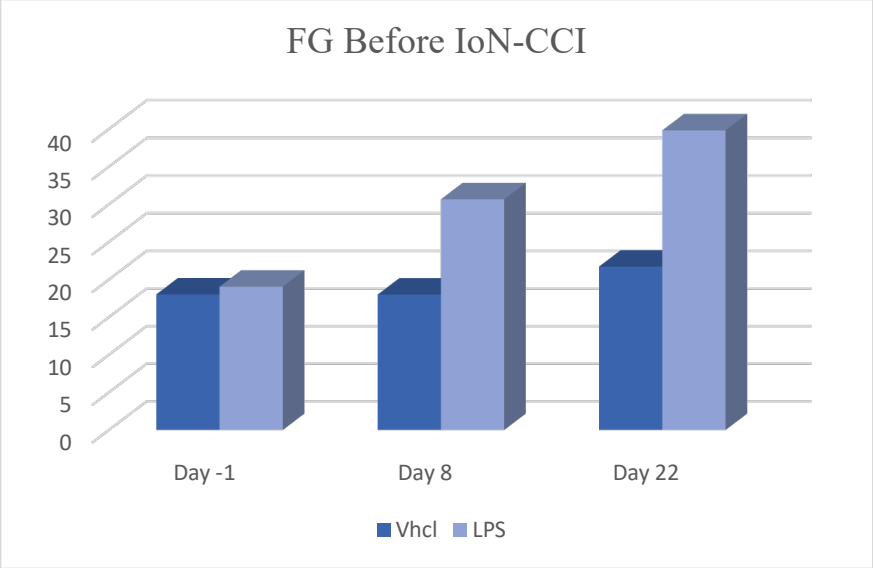


Figure 4. 1. The evaluations of spontaneous pain by iFG episodes before IoN-CCI

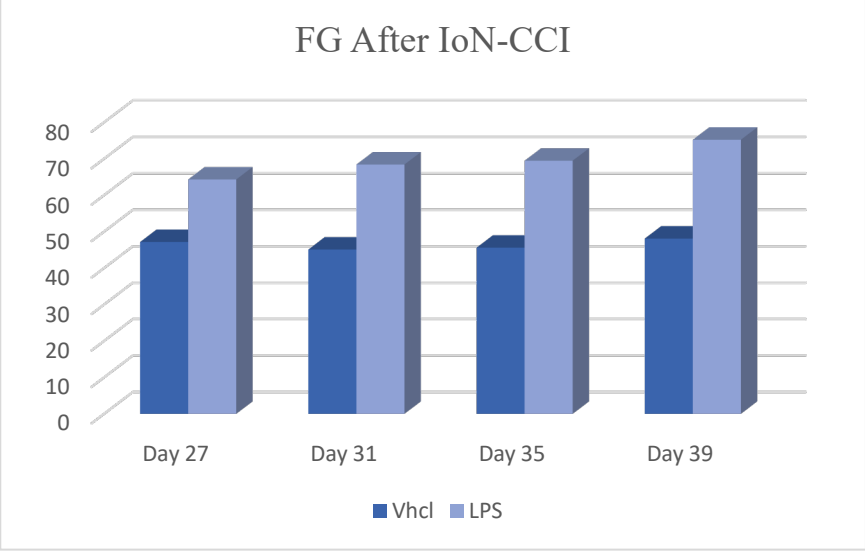
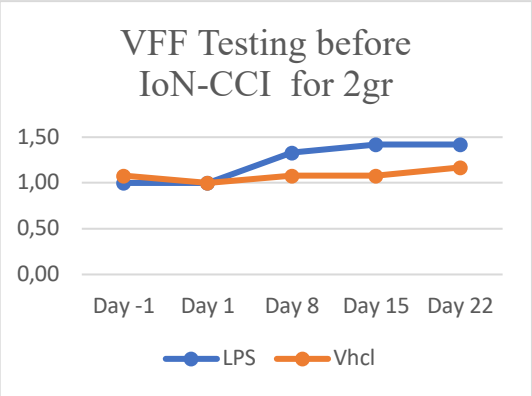


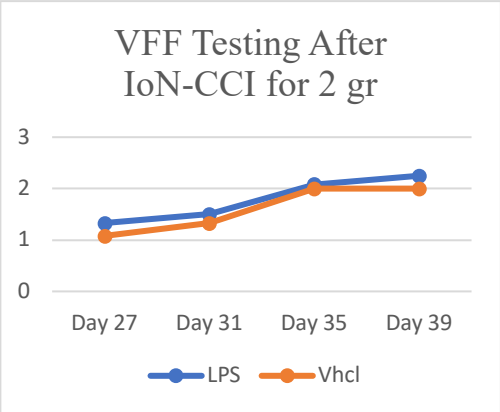
Figure 4. 2. The evaluations of spontaneous pain by iFG episodes after IoN-CCI

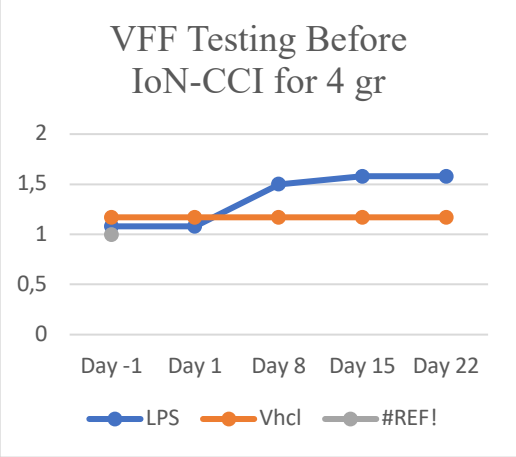
The evaluations of mechanic static allodynia by VFF testing before and after IoN-CCI are shown in Fig.4.3 and Fig 4.4, respectively.

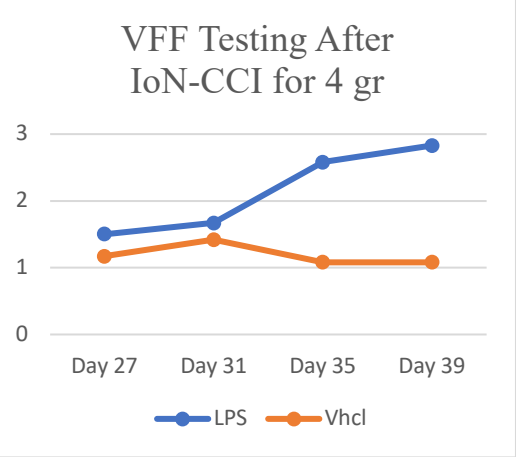
Repeated LPS injections induce pain scores of MSA in rats. Repeated perineural LPS injections in the vicinity of the IoN produced a significant increase in VFF testing scores between day 1 and day 22 following the first injection, showing increased MSA, as compared to Vhcl group. On day 1, a significant difference in VFF testing scores between Vhcl and LPS groups was observed only for mechanical stimulations of 10 gr ($p < 0.01$), whereas at day 22, increased pain scores were observed in VFF testing scores between Vhcl and LPS groups for mechanical stimulations of 4, 6, 8 gr's ($p < 0.05$) as an indicator of preexisting neuroinflammation resulting in increased MSA in rats.

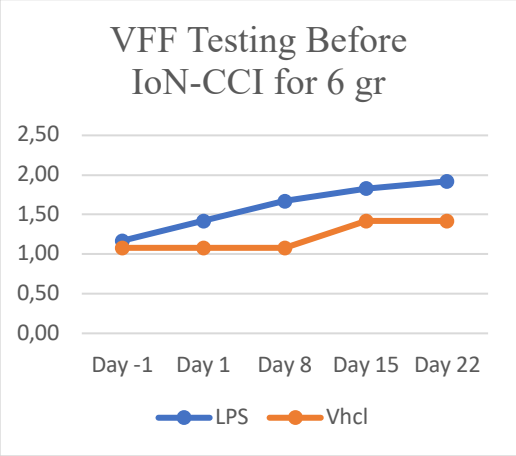
Repeated LPS injections stimulate MSA development and increase its severity after IoN-CCI. After Ion-CCI on day 27, a significant difference in VFF testing scores between Vhcl and LPS groups was observed for mechanical stimulations of 6, 8, 10 gr's ($p < 0.01$, $p<0.05$, $p<0.05$, respectively). On days 31, 35, 39 a significant difference in VFF testing scores between Vhcl and LPS groups was also observed for mechanical stimulations of 6, 8, 10 gr's ($p < 0.01$, $p < 0.01$, $p<0.05$, respectively).

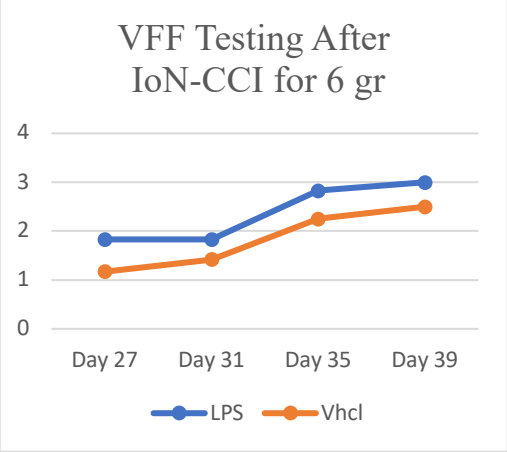

VFF Testing before IoN-CCI for 2gr
1,50
1,00
0,50
0,00
Day -1
Day 1
Day 8
Day 15
Day 22
LPS
Vhcl


VFF Testing After IoN-CCI for 2 gr
3
2
1
0
Day 27
Day 31
Day 35
Day 39
LPS
Vhcl


VFF Testing Before IoN-CCI for 4 gr
2
1,5
1
0,5
0
Day -1
Day 1
Day 8
Day 15
Day 22
LPS
Vhcl
#REF!


VFF Testing After IoN-CCI for 4 gr
3
2
1
0
Day 27
Day 31
Day 35
Day 39
LPS
Vhcl


VFF Testing Before IoN-CCI for 6 gr
2,50
2,00
1,50
1,00
0,50
0,00
Day -1
Day 1
Day 8
Day 15
Day 22
LPS
Vhcl


VFF Testing After IoN-CCI for 6 gr
4
3
2
1
0
Day 27
Day 31
Day 35
Day 39
LPS
Vhcl

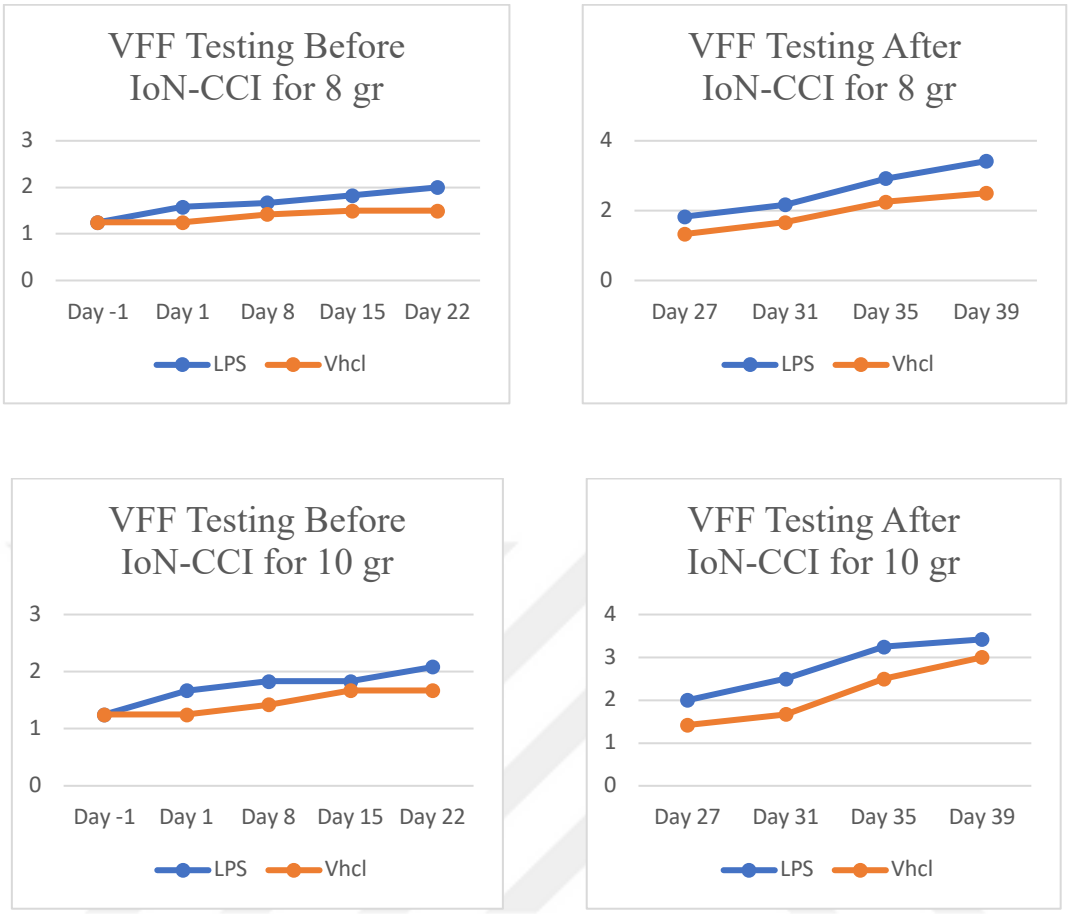


Figure 4. 3. VFF Testing scores for mechanic static alladony for 2, 4, 6, 8, 10 grs before and after IoN-CCI

H&E staining of brain, as a counterstain, on Sp5c in Vhcl group are shown on fig. 4.4a, 4.4b, 4.4c, 4.4d. The appropriate "hot spots" were point-of-interest on figures described Sp5c.

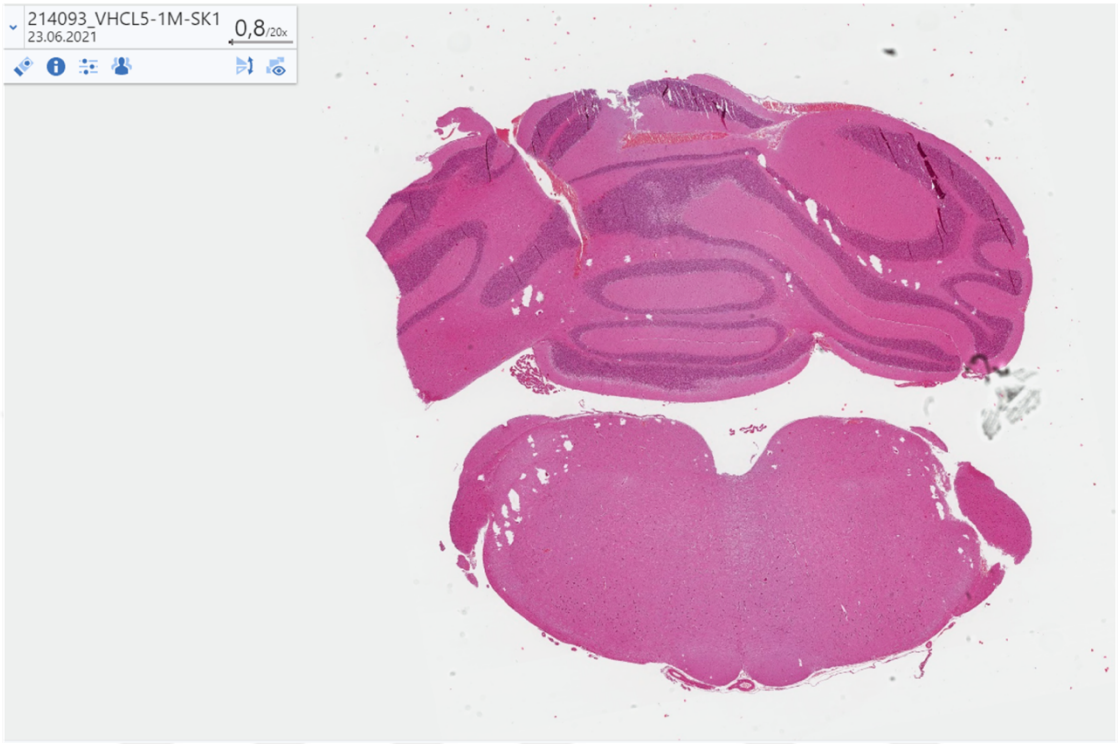


Figure 4. 4a. Detail of bilateral Sp5c, in Vhcl group (H&E, 16X)

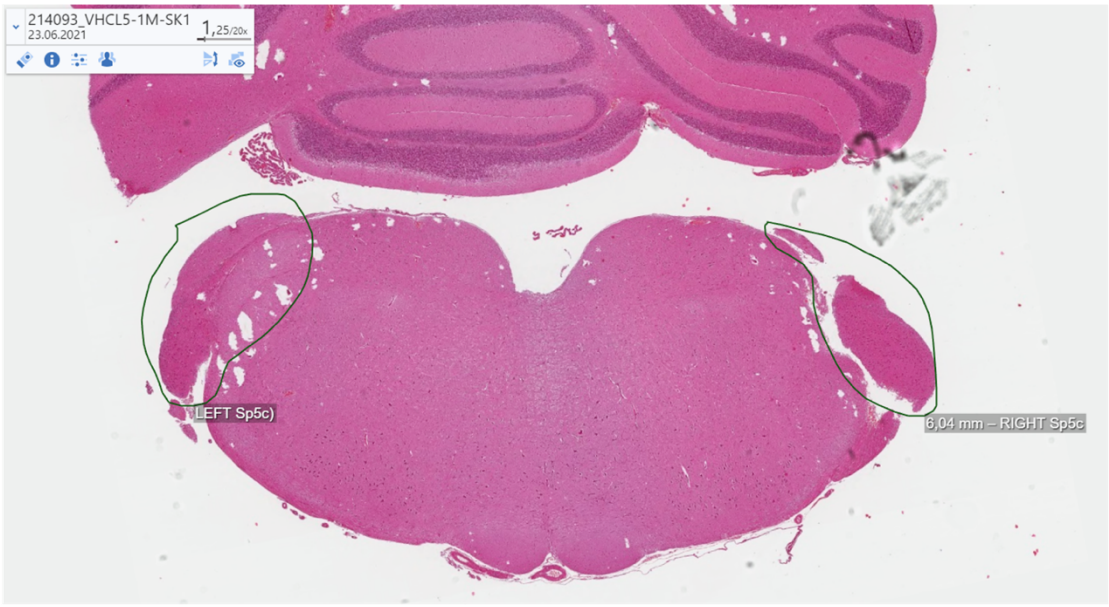


Figure 4. 4b. Detail of bilateral Sp5c, in Vhcl group (H&E, 25X)

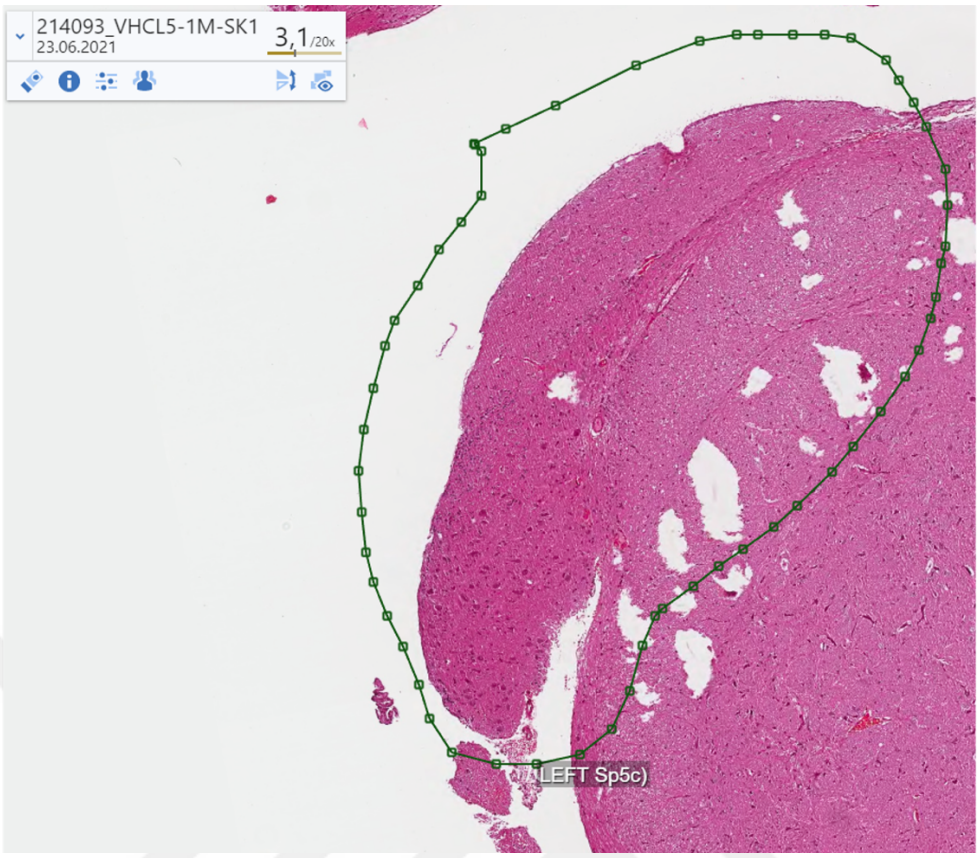


Figure 4. 4c. Detail of left Sp5c, in Vhcl group (H&E, 62X)

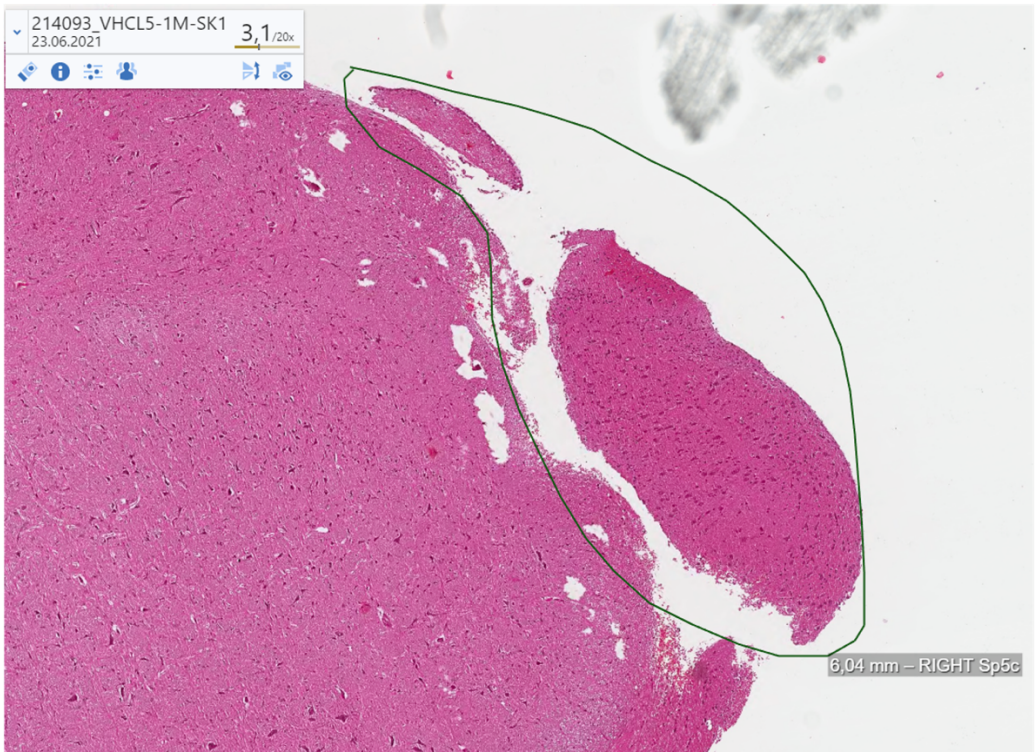


Figure 4. 4d. Detail of right Sp5c, in Vhcl group (H&E, 62X)

H&E staining of brain, as a counterstain, on Sp5c in LPS group are shown on fig. 4.5a, 4.5b, 4.5c, 4.5d. The appropriate "hot spots" were point-of-interest on figures described Sp5c.

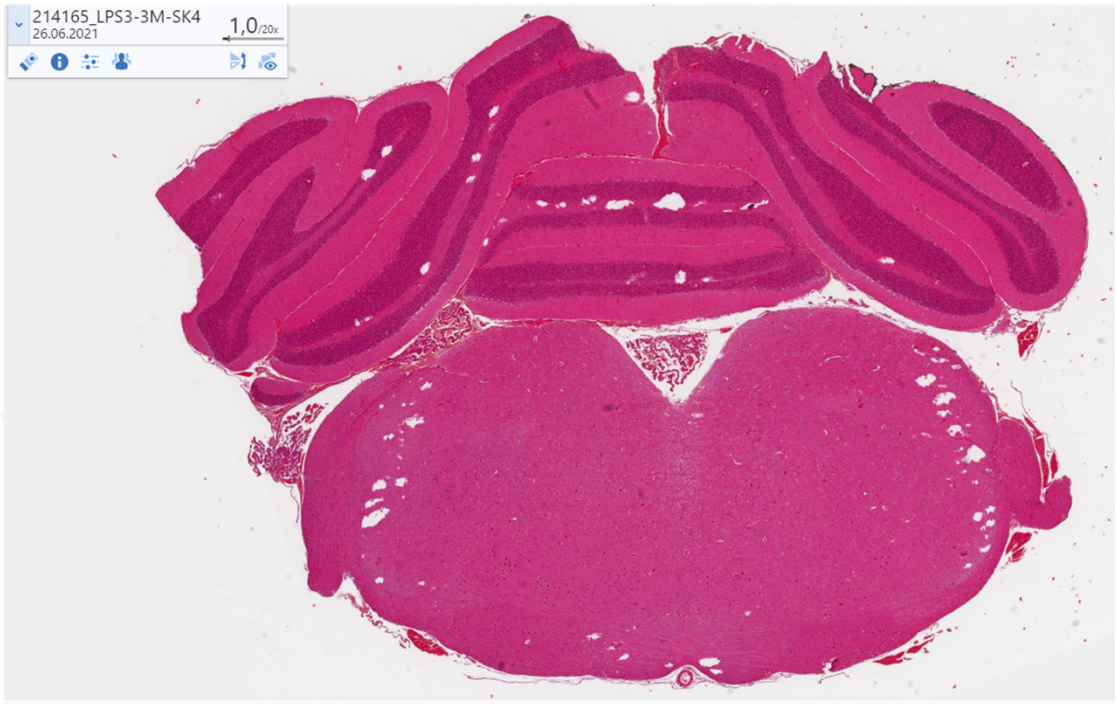


Figure 4. 5a. Detail of bilateral Sp5c, in LPS group (H&E, 20X)

Figure 4. 5b. Detail of bilateral Sp5c, in LPS group (H&E, 20X)

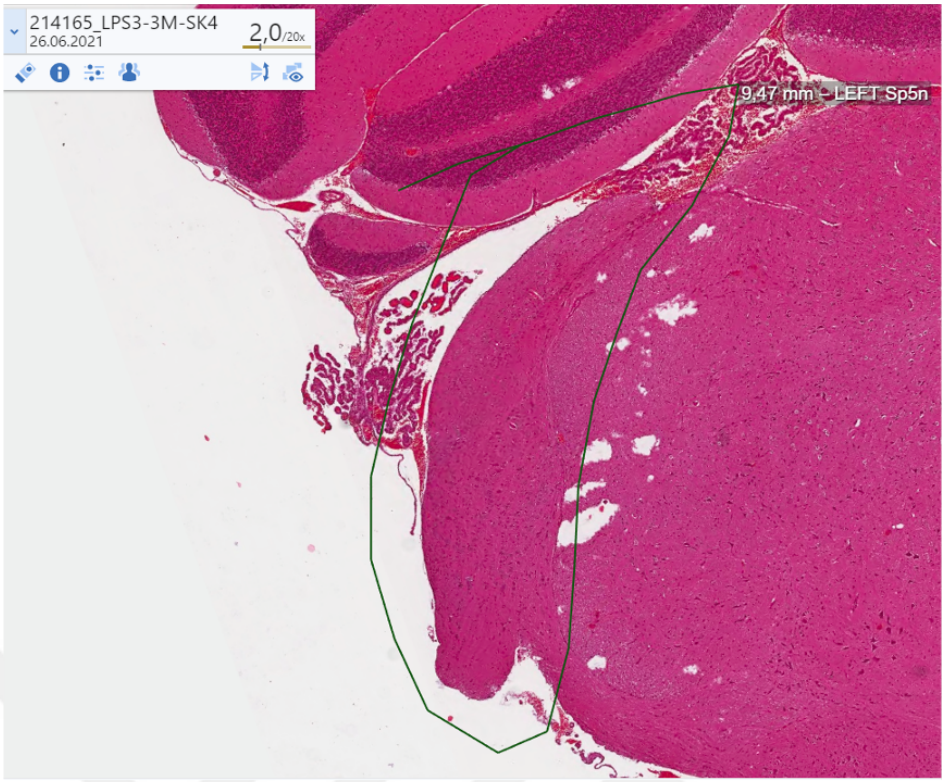


Figure 4. 5c. Detail of left Sp5c, in LPS group (H&E, 40X)

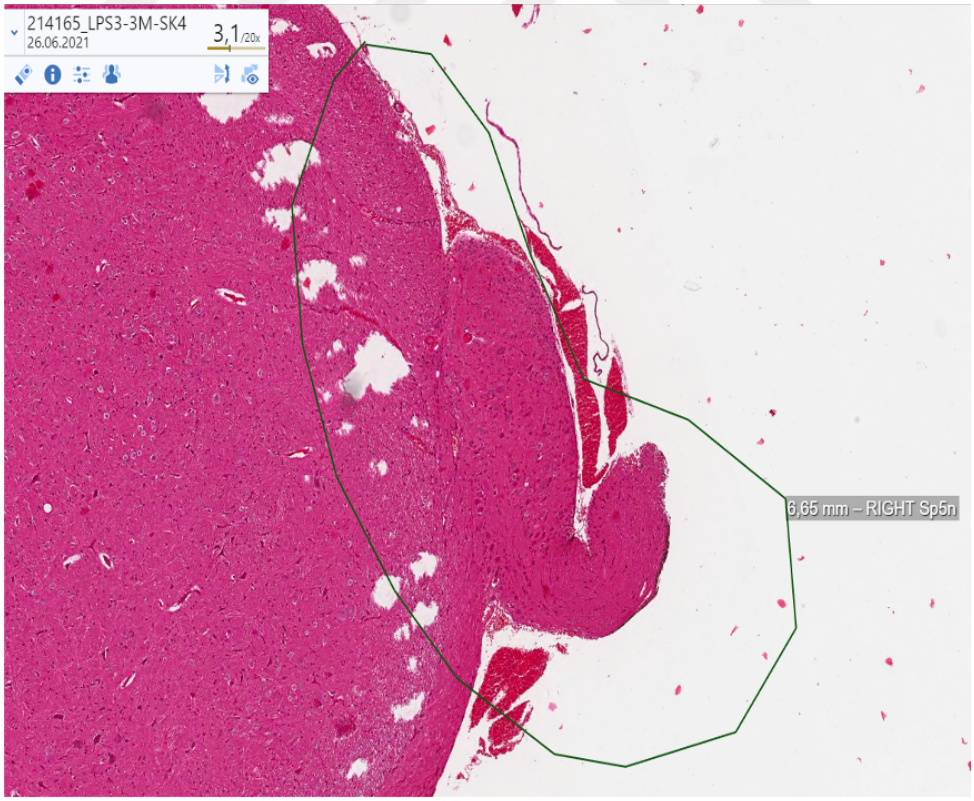


Figure 4. 5d. Detail of right Sp5c, in LPS group (H&E, 62X)

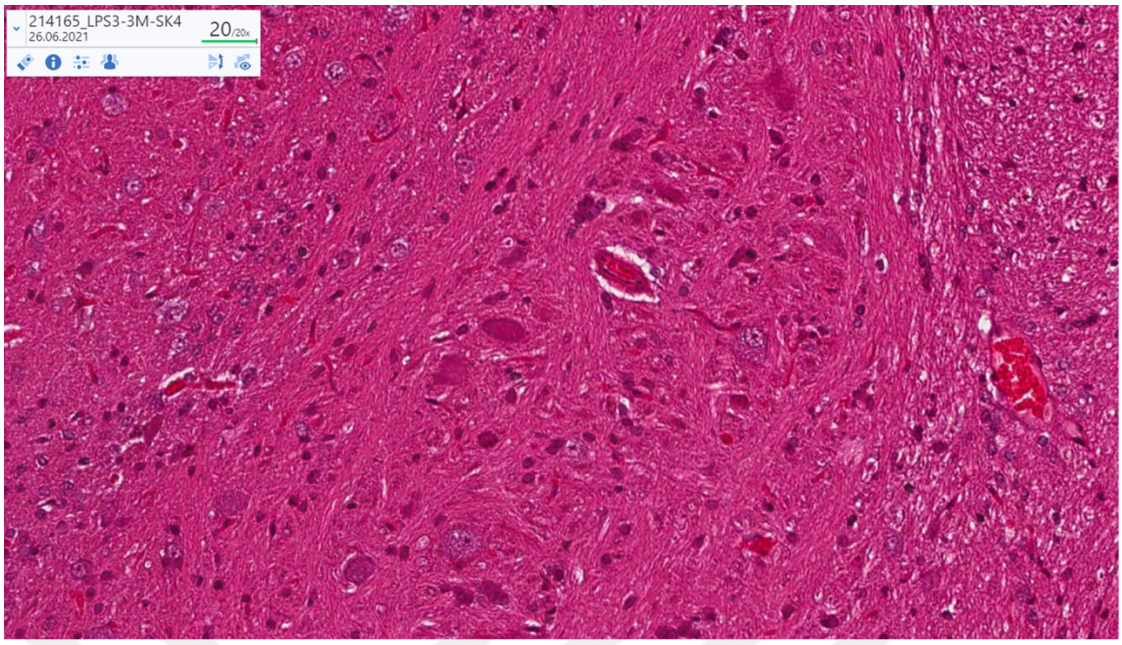


Figure 4. 5e. Detail of left Sp5c, in LPS group (H&E, 400X)

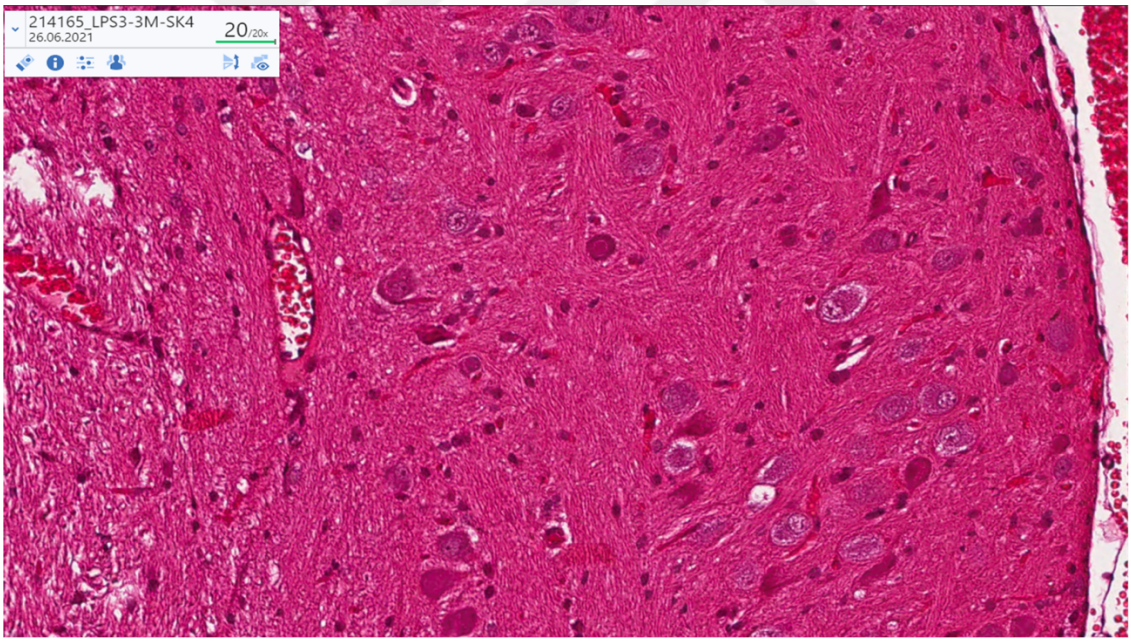


Figure 4. 5f. Detail of right Sp5c, in LPS group (H&E, 400X)

CD68 and GFAP-positivities were detected neither left nor right sides of Sp5c in 5 animals in each groups.

The comparison of the mean CD68-positive microglia in both groups are shown on Table 4.5. There were no statistical significancy found between Vhcl and LPS groups and, the mean CD68-positivity according to the left and right side of Sp5c were not significant. ($p>0.05$).

Table 4. 5. Comparison of the mean CD68-positivity in Vhcl and LPS groups according to side of Sp5c.

Sp5c	*Groups*	*N*	*Mean ± SD*	*[a]p*
Left	***VHCL***	7	52.86±21.06	***0.848***
	LPS	7	47.50±30.23	
Right	***VHCL***	7	41.21±30.27	***0.337***
	LPS	7	38.50±37.92	

[a]Mann Whitney U Test

Immunohistochemistry with CD68 on Sp5c in Vhcl and LPS groups are shown on fig. 4.6a, 4.6b, 4.6c, 4.6d. and fig. 4.7a, 4.7b, 4.7c, 4.7d, 4.7e, 4.7f. The appropriate "hot spots" were point-of-interest on figures described Sp5c.

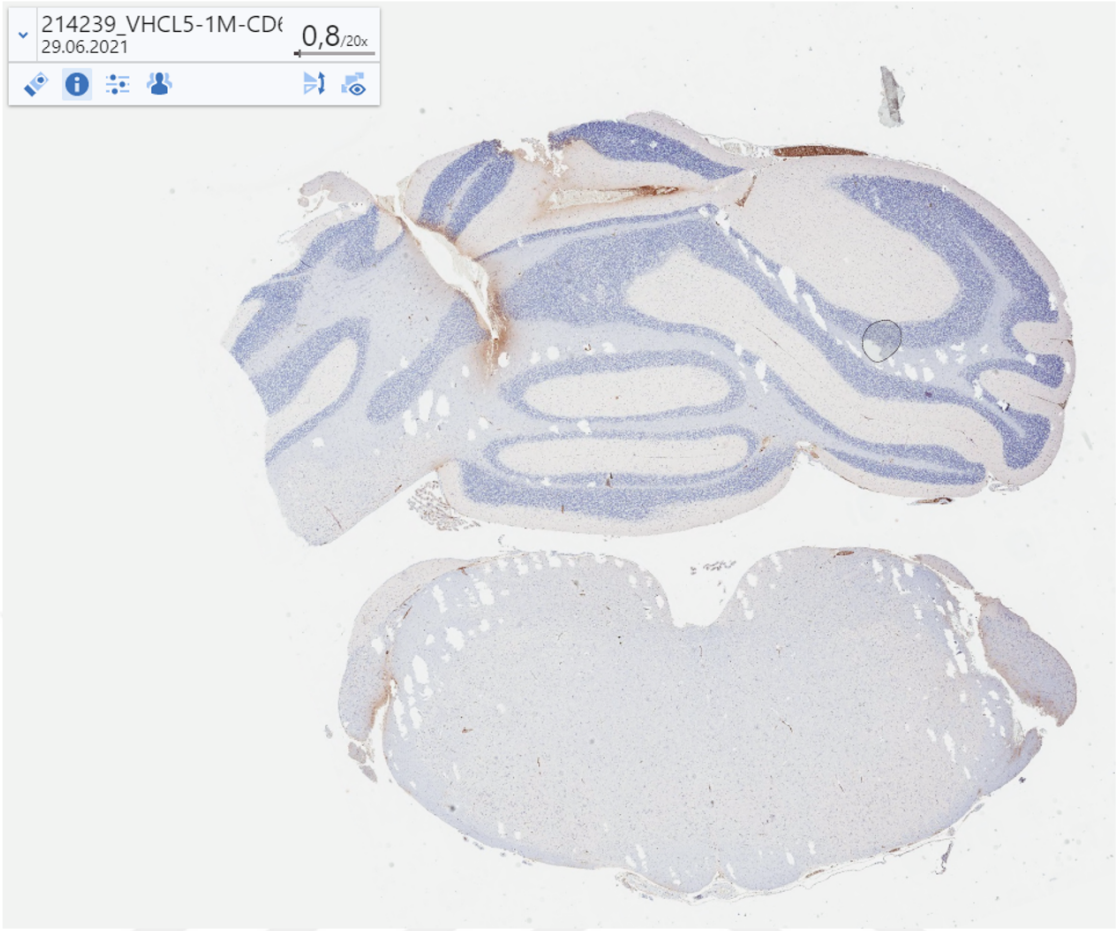


Figure 4. 6a. CD68-positive microglia in Sp5c of Vhcl group (Anti-CD68, 16X)

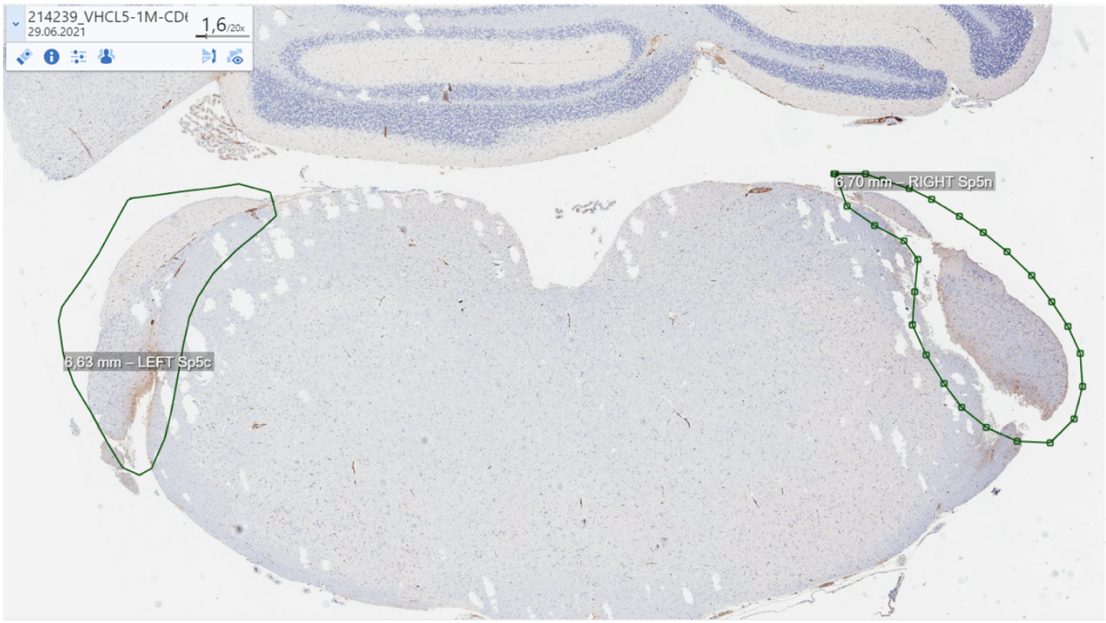


Figure 4. 6b. CD68-positive microglia in Sp5c of Vhcl group (Anti-CD68, 32X)

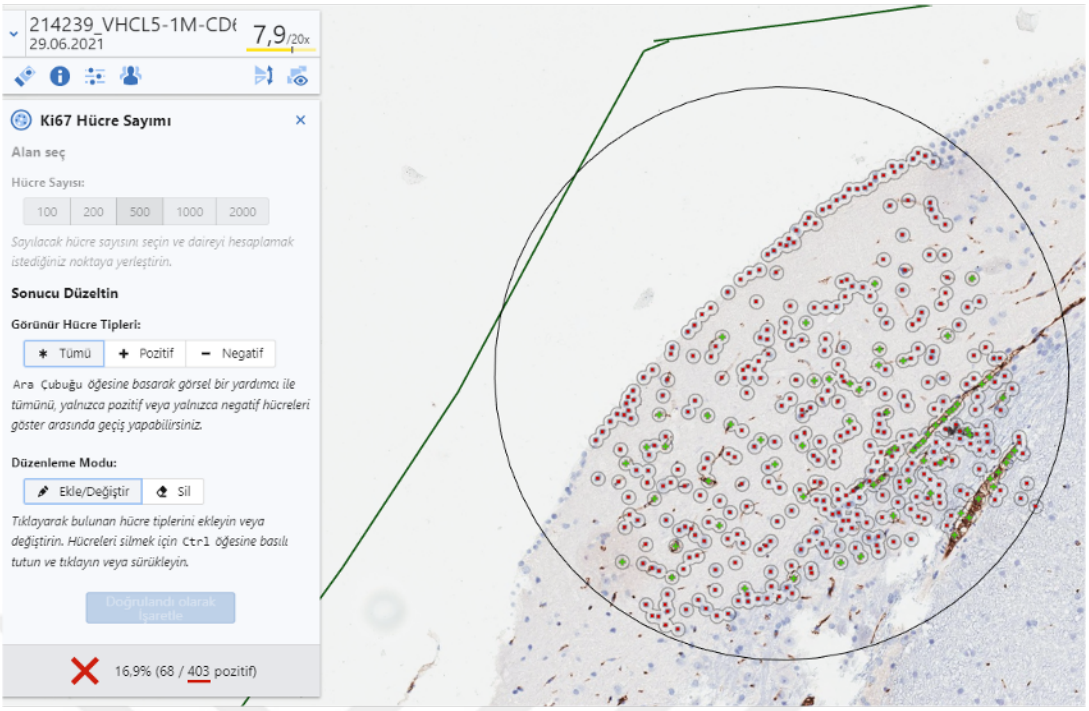


Figure 4. 6c. CD68-positive microglia in left Sp5c of Vhcl group (Anti-CD68, 158X)

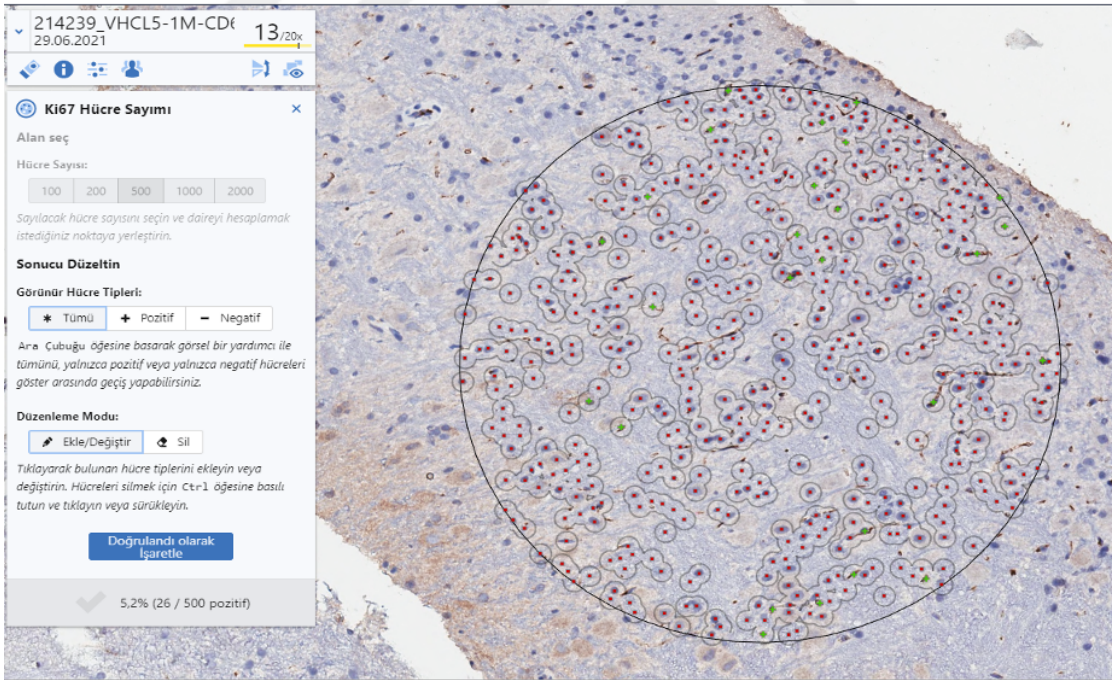


Figure 4. 6d. CD68-positive microglia in right Sp5c of Vhcl group (Anti-CD68, 260X)

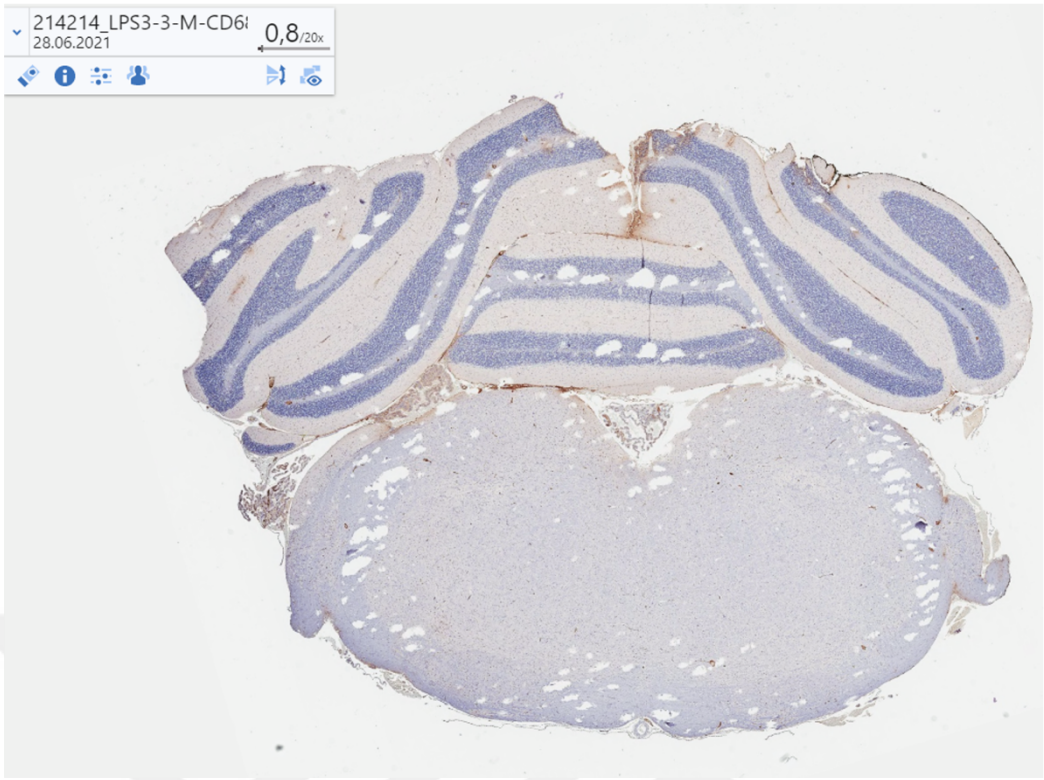


Figure 4. 7a. CD68-positive microglia in Sp5c of LPS group (Anti-CD68, 16X)

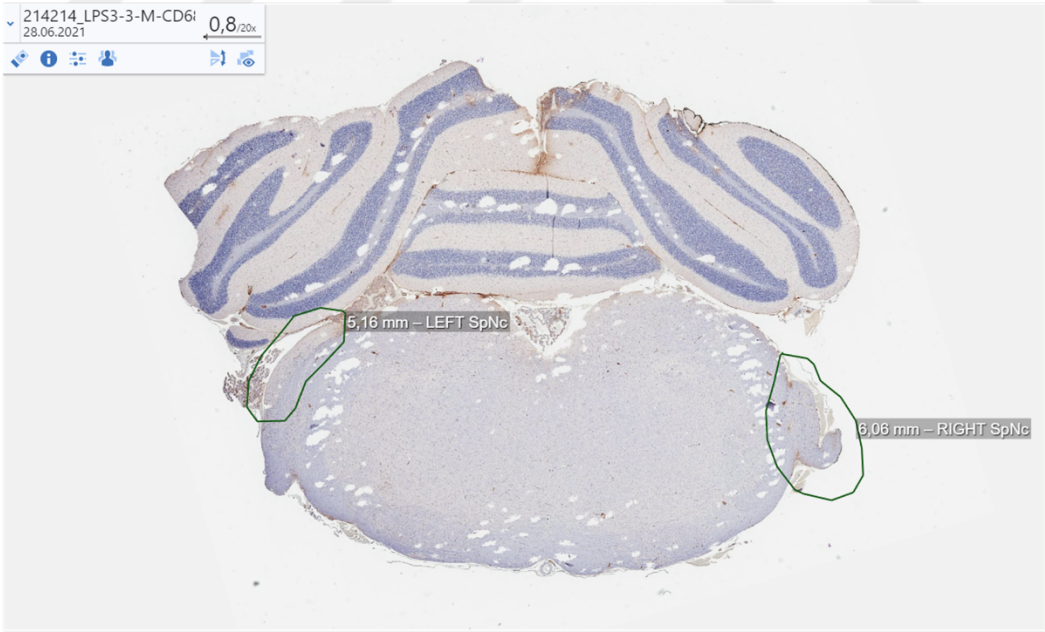


Figure 4. 7b. CD68-positive microglia in Sp5c of LPS group (Anti-CD68, 16X)

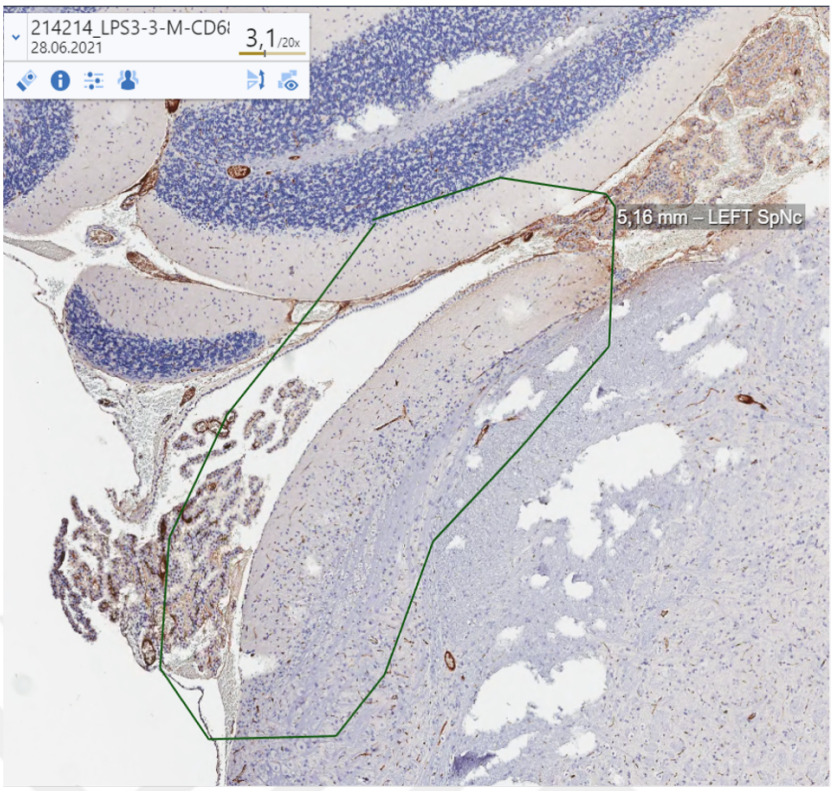


Figure 4. 7c. CD68-positive microglia in left Sp5c of LPS group (Anti-CD68, 62X)

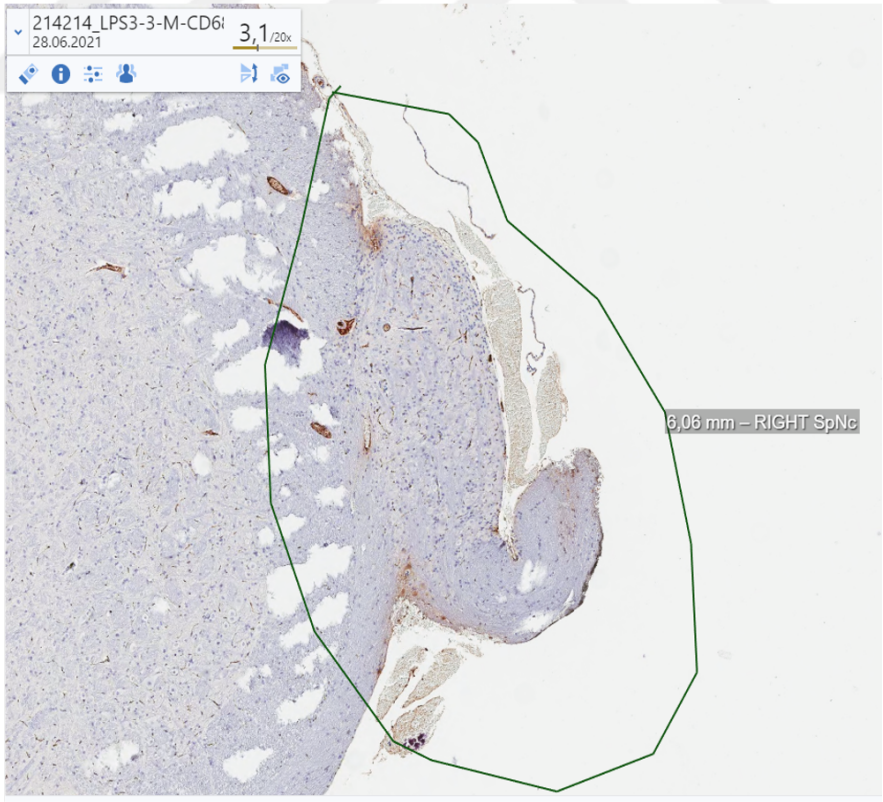


Figure 4. 7d. CD68-positive microglia in right Sp5c of LPS group (Anti-CD68, 62X)

Figure 4. 7e. CD68-positive microglia in left Sp5c of LPS group (Anti-CD68, 260X)

Figure 4. 7f. CD68-positive microglia in right Sp5c of LPS group (Anti-CD68, 260X)

The comparison of the mean GFAP-positive astrocyte in both groups are shown on Table 4.6. There were no statistical significancy found between Vhcl and LPS groups and, the mean GFAP-positivity according to the left and right side of Sp5c were not significant. (p>0.05).

Table 4. 6. Comparison of the mean GFAP-positivity in Vhcl and LPS groups according to side of Sp5c.

Sp5c		***N***	***Mean ± Ss***	***[a]p***
Left	***VHCL***	7	416.00±45.90	***0.302***
	LPS	7	436.92±28.53	
Right	***VHCL***	7	448.06±28.35	***0.439***
	LPS	7	452.67±53.94	

[a]Mann Whitney U Test

Immunohistochemistry with GFAP-positivity on Sp5c in Vhcl and LPS groups are shown on fig. 4.8a, 4.8b, 4.8c, 4.8d. and fig. 4.9a, 4.9b, 4.9c, 4.9d, 4.9e, respectively. The appropriate "hot spots" were point-of-interest on figures described Sp5c.

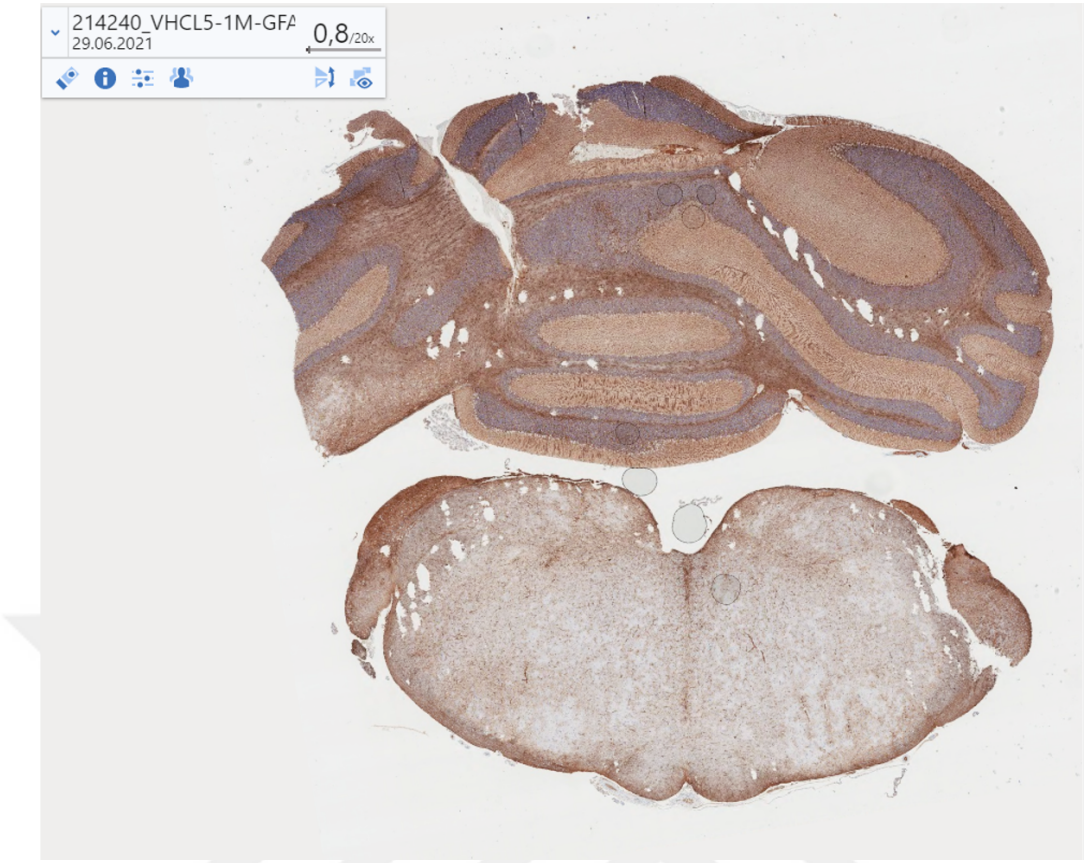


Figure 4. 8a. GFAP-positive astrocytes in Sp5c of Vhcl group (Anti-GFAP, 16X)

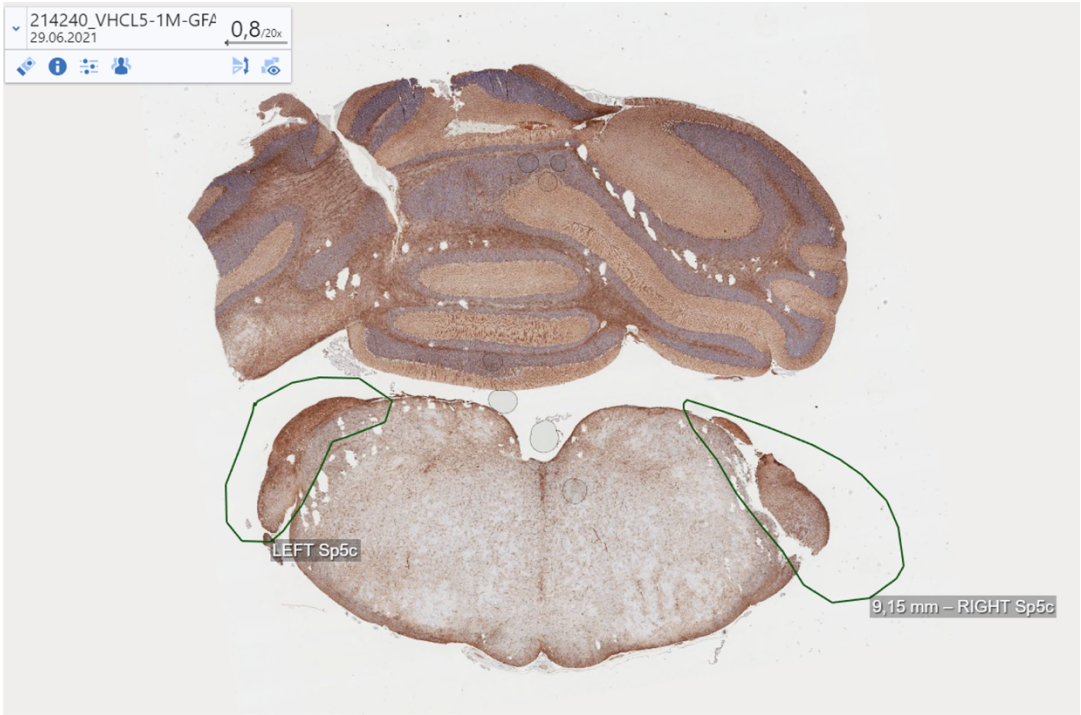


Figure 4. 8b. GFAP-positive astrocytes in Sp5c of Vhcl group (Anti-GFAP, 16X)

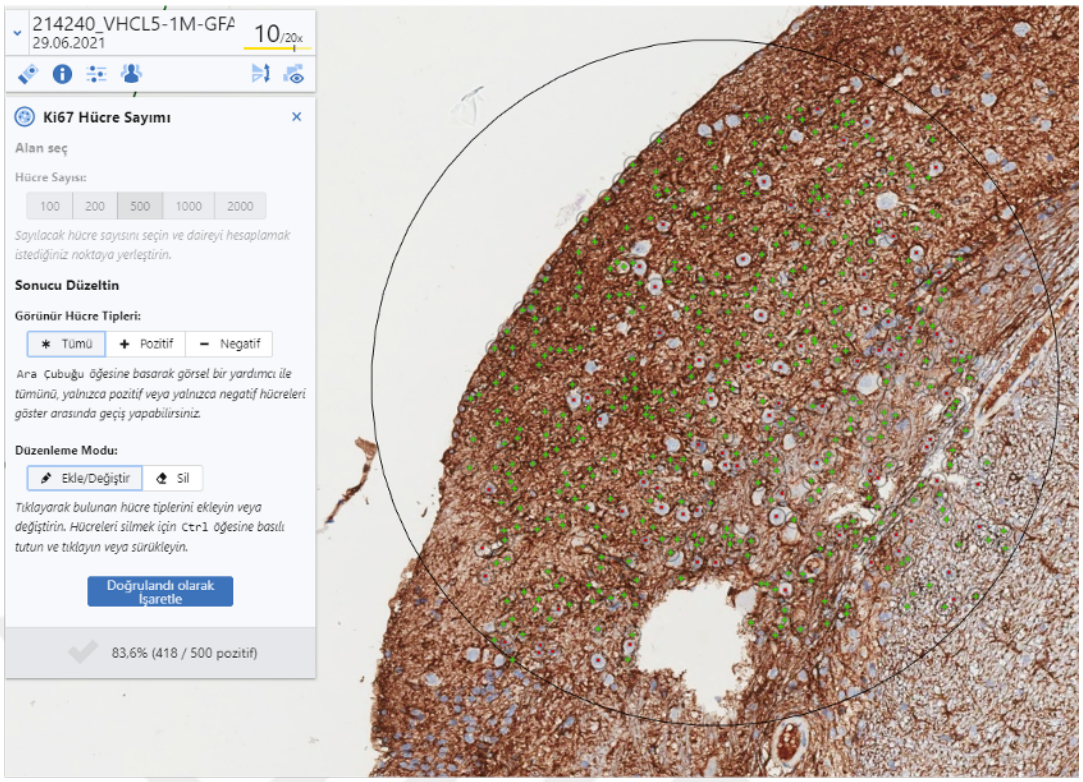


Figure 4. 8c. GFAP-positive astrocytes in left Sp5c of Vhcl group (Anti-GFAP, 200X)

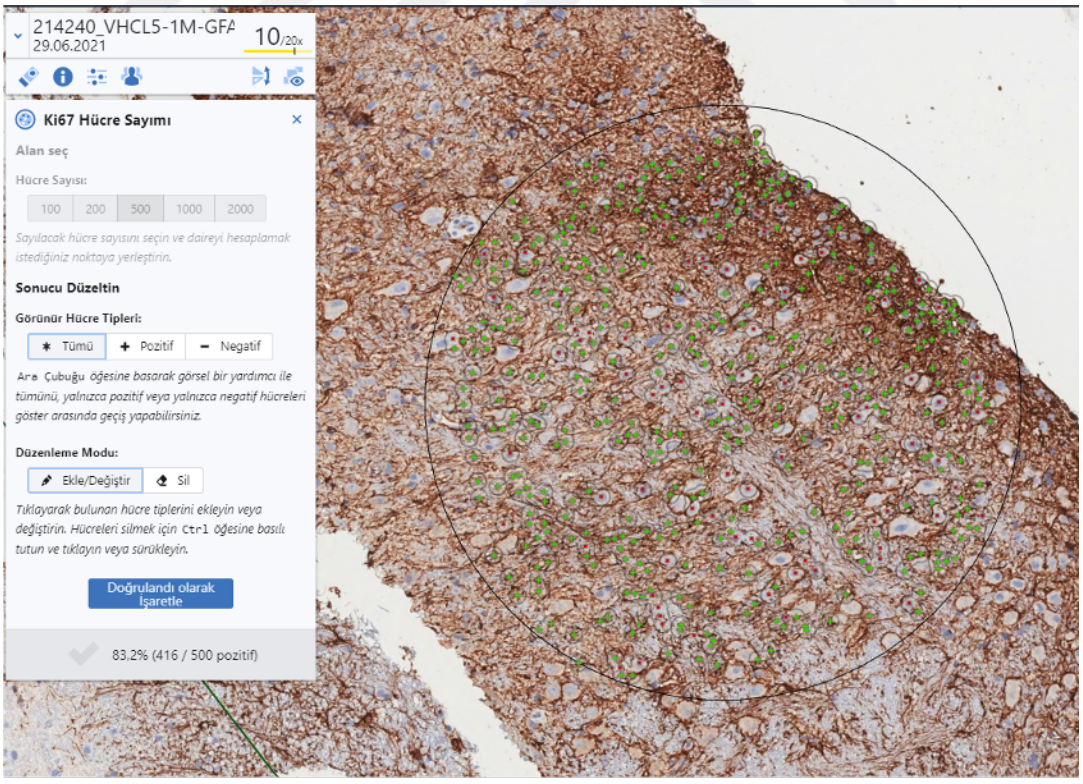


Figure 4. 8d. GFAP-positive astrocytes in right Sp5c of Vhcl group (Anti-GFAP, 200X)

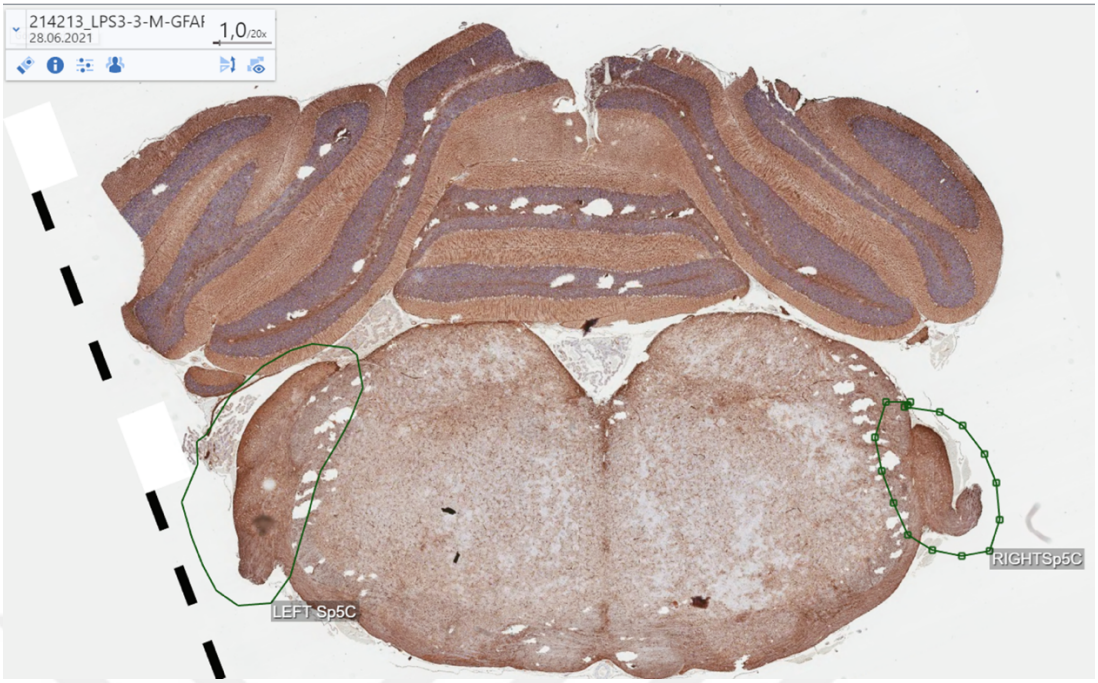


Figure 4. 9a. GFAP-positive astrocytes in Sp5c of LPS group (Anti-GFAP, 20X)

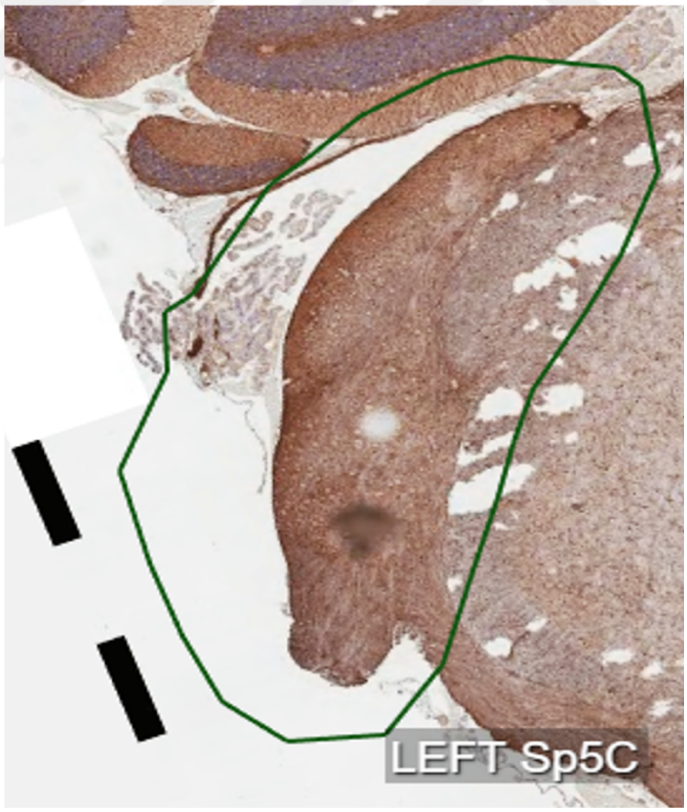


Figure 4. 9b. GFAP-positive astrocytes in left Sp5c of LPS group (Anti-GFAP)

Figure 4. 9c. GFAP-positive astrocytes in right Sp5c of LPS group (Anti-GFAP)

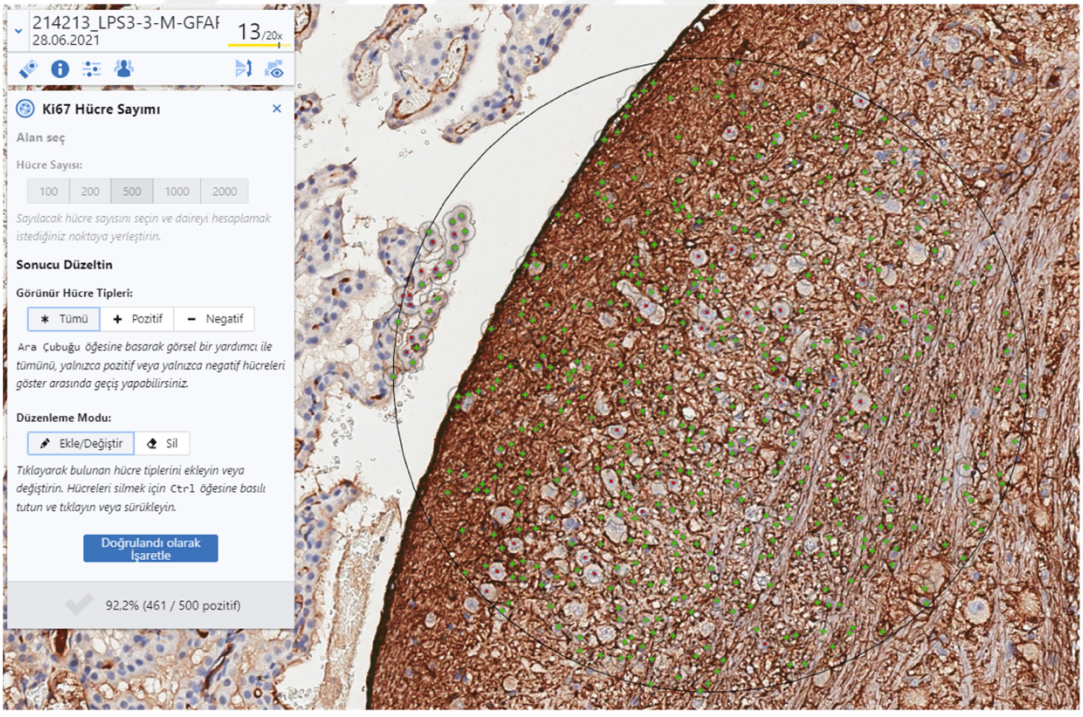


Figure 4. 9d. GFAP-positive astrocytes in left Sp5c of LPS group (Anti-GFAP, 260X)

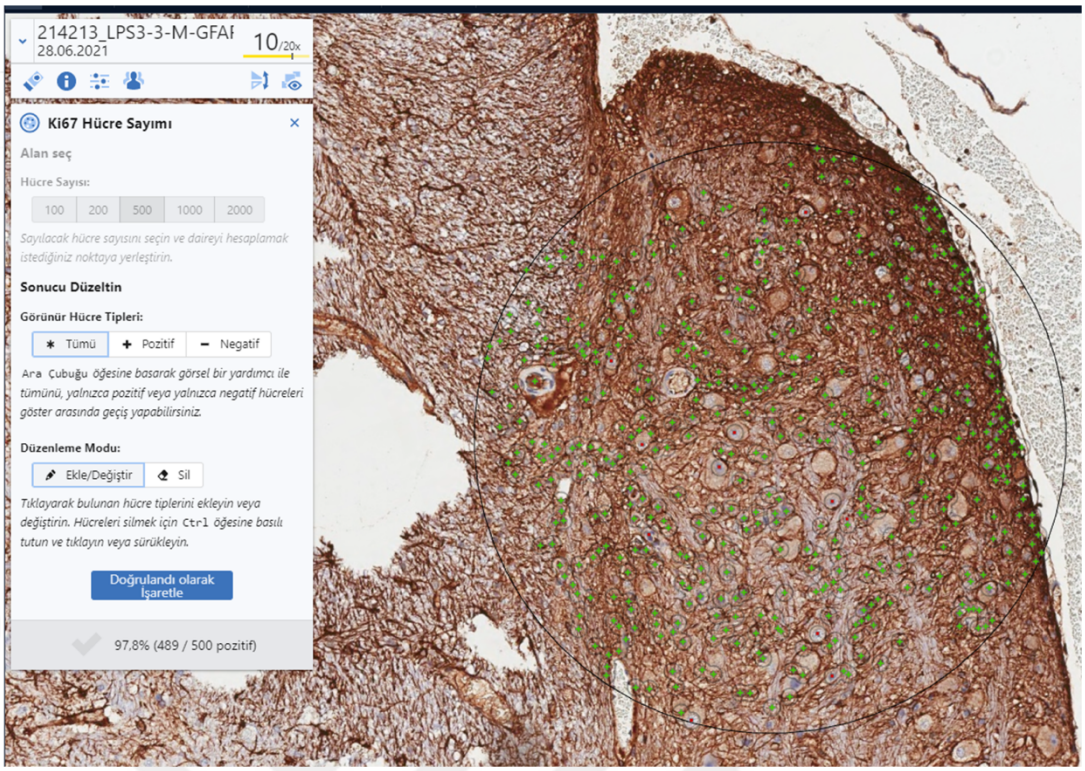


Figure 4. 9e. GFAP-positive astrocytes in right Sp5c of LPS group (Anti-GFAP, 200X)

Comparisons of the mean CD68 and GFAP-positivities between left and right Sp5c according to groups are shown in Table 4.7. The mean CD68 and GFAP-positivities in left and right sides in Vhcl and LPS groups were statistically significant (p=0.001; p<0.01, p=0.003; p<0.01, respectively). The increased GFAP-positivity on left and right sides were found in Vhcl and LPS groups.

Table 4.7. Comparisons of the mean CD68 and GFAP-positivities between left and right Sp5c according to groups.

		N	*Mean ± Ss*	*[a]p*
VHCL Left	***GFAP***	7	416.00±45.90	***0.001*****
	CD68	7	52.86±21.06	
VHCL Right	***GFAP***	7	448.06±28.35	***0.001*****
	CD68	7	41.21±30.27	
LPS Left	***GFAP***	7	436.92±28.53	***0.003*****
	CD68	7	47.50±30.23	
LPS Right	***GFAP***	7	452.67±53.94	***0.003*****
	CD68	7	38.50±37.92	

[a]Mann Whitney U Test *****p<0.01***

Correlation of the mean iFG, showing spontaneous pain, and 2, 4, 6, 8, 10 grs of VFF testing scores, describing mechanic static allodynia on day 39, and CD68 and GFAP-

positivities on cells according to the left and right sides of Sp5c for both groups are shown on Table 4.8.

No correlation was found between iFG and CD68 and GFAP-positivity on cells of both sides of Sp5c ($p>0.05$) in Vhcl group. Negative correlation was found between CD68-positivity on cells in left and right Sp5c and VFF 4 gr in LPS group ($r=-0.828$; $p=0.042$; $p<0.05$, $r=-0.828$; $p=0.042$; $p<0.05$, respectively) .

Table 4. 8. Correlation of the mean iFG, showing spontaneous pain, and 2, 4, 6, 8, 10 grs of VFF testing scores, describing MSA on day 39, and CD68 and GFAP-positivities on cells according to the left and right sides of Sp5c for both groups

	LPS (n=6)								***VHCL (n=6)***							
	GFAP Left		***GFAP Right***		***CD68 Left***		***CD68 Right***		***GFAP Left***		***GFAP Right***		***CD68 Left***		***CD68 Right***	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Face Grooming Episodes	*-0.086*	***0.872***	*0.371*	***0.468***	*0.771*	***0.072***	*0.314*	***0.544***	*-0.085*	***0.871***	***0.714***	***0.111***	*-0.600*	***0.208***	*-0.486*	***0.329***
VFF 2.gr	*-0.131*	***0.805***	-0.655	***0.158***	-0.393	***0.441***	0.131	***0.805***	0.000	***1.000***	0.207	***0.694***	0.000	***1.000***	0.000	***1.000***
VFF 4.gr	0.000	1.000	*0.005*	***0.999***	-0.828	***0.042****	-0.828	***0.042****	0.000	***1.000***	0.207	***0.694***	0.000	***1.000***	0.000	***1.000***
VFF 6.gr	0.000	1.000	*0.005*	***0.999***	0.000	***1.000***	0.000	***1.000***	0.000	***1.000***	0.207	***0.694***	0.000	***1.000***	0.000	***1.000***
VFF 8.gr	*-0.131*	***0.805***	-0.655	***0.158***	-0.393	***0.441***	0.131	***0.805***	0.000	***1.000***	0.207	***0.694***	0.000	***1.000***	0.000	***1.000***
VFF 10.gr	*-0.621*	***0.188***	0.000	***1.000***	-0.207	***0.694***	-0.207	***0.694***	0.000	***1.000***	0.207	***0.694***	0.000	***1.000***	0.000	***1.000***

r=Spearman's Correlation *p<0.05

5. DISCUSSION

Because of pathologically modified mechanisms in CNS, pain cannot be described simply as a transient continuation of intense peripheral nociceptive impulses. It is assumed that abnormal brain reactions through pathways of pain transmission, that start at dorsal root ganglion and go to spinal cord, thalamus, and cortex, create chronic pain. Persistent orofacial pain is commonly seen after trigeminal nerve damage which is able to generate NP connected with central sensitization at level of Sp5c (130-132). Recently, it has been stated that not only neurons are involving in chronic pain states; but also, glial cells, including microglia and astrocytes, can be potential additional members in initiating and maintaining NP. There is a close interaction between these glial cells and neurons, so transmission of the modulate pain, especially under pathological conditions (133,134).

A 5% – 10% of a human adult's CNS is made up of microglia, macrophages that live in the tissues. When the human is healthy, microglia persist through lifetime as they are self-renewing cells. Since microglia demonstrate a novel cellular mark in comparison to immune cells like macrophages, circulating monocytes, and other myeloid cells, the development of microglia is a distinct gene expression program (135, 136).

Microglia and astrocytes perform as potential modulators of NP by releasing several cytokines and chemokines. They have some overlapping activities in regulating CNS's innate immunological response, despite their distinct roles in brain activity. In NP, both microglia and astrocytes become activated, resulting in proinflammatory responses with severe consequences like as persistent inflammation and neuronal hyperexcitability. Now, the major cellular mechanism for SDH microgliosis following PNI is thought to be local growth of resident microglia through proliferation (134, 137).

Microglial stimulations in the SDH have been documented in 3 distinct ways during NP conditions: (i) Microglia shape changes from thinner and branching pathways in the "resting" state to hypertrophy with thicker and retracted branches in the "activated" state. Proliferation or microgliosis is also linked with morphological changes (ii) the active microglia display changes in surface markers and proteins that are membrane-bound or embedded, including CD44, CD11b, P2X4 receptors, TRL4, and MHC II (iii)

the activation of spinal microglia in NP is characterized by phosphorylation of mitogen-activated protein kinases, including the p38 and Src-family kinases (138, 140).

Spinal microglia are activated through early NP, which occurs before astrogliosis. This supports a recent argument suggesting that microglia are significant in starting and the astrocytes during the continuation of NP. The importance of the no projection of glial cells (or neuroglia) for brain might be explained by through ascending neuronal transmission on glial changes (141).

In CNS, the response of microglia and astrocytes after peripheral nerve damage happens by a neural pathway, in the area where the ending of the fibers of the injured nerves are located. Lee et al. found that, especially in the area where nociceptive primary afferents end (which is sp5c) and where the synapse of larger A-beta afferent fibers (which is principal sensory trigeminal nucleus (Pr5)) take place, the ligation of mental nerves provoked the activation of microglia and astrocytes (142).

Activated microglia can be identified by their specific morphology. Additionally, microglia proliferate and enhance the expression of receptors or cell surface markers. Also, a change in the functional activities, including phagocytosis, migration to the injured area, and pro-inflammatory substances production and release, can be seen in the activated microglia (143).

There are two possibilities considered as the mechanisms for SDH microgliosis induced by peripheral nerve injury. One potential cause is the colony stimulating factor 1 expression involving IL-1β of the surrounding glia, while the 2nd possible cause is the influx of the circulating monocytes (derived from bone-marrow) into the parenchyma of the SDH, which, in turn differentiates into cells that are microglia-like (135, 144, 145).

NP models have been used to investigate pathological changes and their molecular and cellular processes. As an example, there are models investigating peripheral nerve injury using rodents. Alterations in neurons were considered to be the cause of these changes for a long time, however, evidence during the last two decades shows that non-neuronal cells, monocytes, macrophages, T cells, and glial cells play important roles in the nervous system (5, 146).

To explain the potential contribution of glial cells in NP, and to provide a precise comprehension for the multi-dimensional profiles of microglial activation in reply to different insults, the aim of the present experimental study was to investigate whether there is any association between the chronic neuroinflammation on NP produced by applying nerve injury (IoN-CCI) and inflammation (LPS), and the alterations in microglia and astrocytes in CNS in rats.

In the trigeminal spinal subnucleus caudalis (Vc), TN creates a separate glial activation time frame, connected with the initiation and maintenance stages of orofacial NP. Microglia and astrocytes are thought to have a major part in the different stages of NP (2).

Activation of microglia and astrocyte has been noticed in central NP models like spinal cord infection, spinal cord injury models, and peripheral NP models like PNI. Additionally, astrocytes have a close association with chronic pain behavior and synapse following nerve damage and are found more persistent than the reaction of microglia. Through the pain development, microglia responses are characteristically early and transiently; on the other hand, astrocyte activation occurs later and continues longer. Liddelow et al. theorize that A1 reactive astrocytes might be neurotoxic, while A2 reactive astrocytes might be neuroprotective. These outcomes may explain precisely the dual effects of reactive astrocytes in injury and disease in CNS (3, 4).

Astrocyte reactivity is a well-known characteristic of multiple diseases, including spinal cord injuries, chronic pain, and neurodegenerative diseases. Recently, many researchers have proved that reactive astrocytes provide chronic pain development. Nonetheless, reactive astrocytes' guarding role for pain and whether they have more that one function regarding chronic pain is still not entirely clear (6).

Cytokines are significant mediators between microglia and astrocytes. To verify which cytokines microglia use to signal A1 induction, Liddelow et al purified microglia by immunopanning and determined which cytokines were secreted by resting and LPS-activated microglia. They have revealed that activated microglia provoke the transformation of astrocytes toward A1 astrocytes by releasing Il-1α, TNF, and complement component 1q (C1q). Xu et al. have shown that MFG-E8 regulates A1/A2

astrocytic conversion by upregulation of PI3K-Akt pathways and downregulation of NF-κB pathways in vitro. Hence, distinct inhibition of these signaling molecules might ease chronic pain (3).

A1 astrocytes secrete a neurotoxin that induces rapid death of neurons and oligodendrocytes. A1 astrocytes have lost many characteristic astrocytic functions, including the ability to promote neuronal survival and outgrowth, promote synapse formation and function, and to phagocytose synapses and myelin debris (121). At present, it is known that reactive astrocytes have A1 and A2 subtypes and activated microglia have M1 and M2 subtypes. Yet, there may be other glial cell subtypes having various missions in the disease development process (6).

Crosstalk among astrocytes and microglia are partly maintained via secreted mediators such as growth factors, neurotransmitters and gliotransmitters, cytokines, chemokines, mitogenic factors, innate immunity mediators and tissue-degeneration molecules, ROS (reactive oxygen species), and metabolic mediators (e.g. glutamate) that can be used for cell metabolism and can further mediate changes in tissue. Purinergic signaling via P2Y receptors, which are found in astrocytes and microglia, can play a pivotal part in microglial-astrocyte interaction during the inflammatory response (147).

Microglia-derived interleukin (IL)-18 stimulates astrocytes in the spinal cord, resulting in tactile allodynia, according to the research. This research indicates that communication between microglia and astrocytes is required for NP formation in the spinal cord (148).

Liu et al. have described that the CXCL12/CXCR4-mediated crosstalk among astrocytes and neurons preserves NP by a central sensitization mechanism. They also suggest that cytokines have a significant role in the crosstalk among glial cells and neurons. Hence, further studies must be done to evaluate the molecular mechanism of this crosstalk in the spinal cord and brain in the context of chronic pain (149).

Lee et al. discovered evidence of the existence of a unique phenotype of activated microglia as a reaction to nerve injury, inflammation, and infection in their recent in vivo research. When compared to the phenotype of M1 macrophages, LPS-induced activation

of microglia exhibits a characteristic inflammatory profile. As a result, morphological changes in microglia reflect altered activation states influenced by various inputs such as circulating immune mediators or injured neurons (142).

In present study, we preferred LPS, which it is a strong inducer of A1 astrocytes (149) and is an activator of TLR4 signaling, a receptor expressed specifically by microglial cells in the rodent CNS (151-153). In all rats, we produced inflammation by LPS and NP by IoN-CCI in the right side of rats. Our finding could not explain whether the activated microglia (the mean CD68-possitivity was 38,50±37,92) and astrocytes (the mean GFAP- possitivity was 452,67±53,94) were caused by LPS or IoN-CCI. Since all immunohistochemical staining were performed on day 39 (the day of animal sacrification). Future studies are needed to make this clarification.

Forty microglia-selective molecules that are elevated after PNI in mice have already been identified, suggesting that microglia is among the major types of cells involved in the pathogenesis of NP. Behavioral phenotypes suggestive of decreased pain are found in rats helping understand how microglia generate pain hypersensitivity and what role upregulated chemicals play. Even though their roles are unknown, it is probable that they functionally link and create one or more recognizable signaling pathways required for PNI-induced changes in synaptic structure and function in the SDH. Furthermore, normally, activation of microglia causes pain hypersensitivity, which leads to discomfort. These findings gave rise to the idea of microgliopathic pain (pain caused by malfunctioning microglia in the absence of acute nerve injury). Microglia are also active in the brain in NP, contributing to the sensory and non-sensory (i.e. reward, emotion, and memory) components of the scenario, according to recent research (5).

Three different groups of researchers indicated to microglia participation in NP post PNI, demonstrating the specific function of microglia in pain. According to Jin et al., spinal nerve ligation causes phosphorylation of the p38 MAP kinase only in CD11b (OX-42)-expressing spinal microglia but not in NeuN-expressing neurons or GFAP-expressing astrocytes. Furthermore, intrathecal injection of a p38 inhibitor reduced mechanical allodynia, a key feature of NP after nerve injury. After transecting the spinal nerve, Raghavendra et al. found that spinal minocycline injection, a non-specific inhibitor of microglia, reduced allodynia and mechanical hyperalgesia in the initial but not the later

phases. This prevention of nerve damage in the early stages was connected to a reduction in microglial activation in the spinal cord. Tsuda et al. found that injecting ATP-activated microglia into the spinal cord was enough to cause mechanical allodynia within about 60 minutes of treatment and that; (i) Nerve injury causes activation of P2X4 (ATP receptor subtype), particularly in spinal cord microglia, and (ii) Spinal inhibition of P2X4 reduced mechanical allodynia. They confirmed the role of p38 in spinal microglia in the formation of mechanical allodynia after nerve damage. Coull et al. demonstrated in their 2005 study that, to stimulate NP, spinal microglia produce brain-derived neurotrophic factor (154, 157).

Models created using mice imply that microglia are the initial defence, exhibiting activation and rapid migration to the injury site in order to phagocytoze dead tissue and particles. The activation of astrocytes, which releases inflammatory mediators that signal to microglia and can attract monokine induced by gamma interferon (MIG) invading hematogenous cells, particularly monocyte-derived macrophages, follows the microglial response. GFAP is upregulated in reactive astrocytes, and their morphology changes. This suggests that formation glial scars might limit damage within the involved site (150).

Standard markers for identifying changes on microglia and astrocytes are Iba1, CD11b, CD68 for microglia, GFAP, AQP4, ALDH1 for astrocytes. A high level of CD68 is commonly regarded for activated microglia; even if for separate microglia states, it will become complicated to get satisfying markers. GFAP has been given to be extremely particular, and it has also been recommended a glutamate transporter such as GLAST.It has been well defined that CD68-positive microglia and macrophages present several inflammatory markers. The presence of intracellular iron has been linked with microglia polarization and macrophages towards a proinflammatory state (158). Extreme expression of GFAP resulting with morphological remodelings like hypertrophic processes is a distinctive characteristic of reactive astrocytes. Reactive astrocytes promote neuronal activity (123).

Xu et al. (159) were used CD11b to evaluate GFAP and microglia changes for caudal medulla astrocytes. They observed that, in the sham and the contralateral side of partial IoN ligated rat groups, intensity of CD11b was average and it was regularly distributed within the caudal medulla. Microglia were found to be CD11b positive in the

ipsilateral side of partially IoN ligated rats on 1-day post-op and seemed to be activated with enlarged soma and thicker processes compared to same rats' contralateral side. There was activated microglia expression following partial ION ligation in the medial part of superficial lamina, compared to the activation of microglia that found normal in the sham group. GFAP expression in the caudal medulla was found modest and homogeneous everywhere in the superficial lamina on the contralateral side of the partially IoN ligated group and on both sides in the sham group. But, the ipsilateral side had strong GFAP staining with long processes and hypertrophic cell bodies on day 8 after surgery. In the medial part of the superficial lamina, the most prominent astrocytic activation was marked in the ipsilateral side. A significant increase was observed in GFAP staining in the ipsilateral caudal medulla comparing to the contralateral side in the sham group on day 8 after surgery. In present study, we observed CD68 and GFAP-positivities at level of Sp5c in rat brain.

It is previously shown by Watkins et al. (160) that the activation of spinal glial could be a conducive agent for the spinal level's pain hypersensitivity. Induced in partial IoN ligation, Xu et al. observed glial activation in their study. In the same study, the most prominent glial activation was observed in the caudal medulla. Notably, microglia were found earlier activated than astrocytes in partial IoN ligation, giving the idea that temporal changes in the activated microglia are not the same in astrocytes. This is compatible with the findings of Kreutzberg et al. pointing that microglia are the first responders in CNS to inflammation, ischemia, trauma, and tumors (161).

Asano at al. have reported that the number of c-Fos-IR cells and the fluorescence intensity of Iba1, GFAP, and C1q were significantly increased in the Vc following infraorbital nerve injury (IoNI). the enhancement in the excitability of Vc nociceptive neurons is produced by astrocytic activation via the signaling of C1q released from activated microglia in the Vc following IONI, resulting in persistent orofacial neuropathic pain. In conclusion, trigeminal nerve injury aspects microglia and astrocyte activation, which causes strong activation of Vc nociceptive neurons. Activated microglia induce C1q after trigeminal nerve injury, and C1q released from microglia is thought to be involved in astrocytic activation in the Vc, which causes hyperactivity in Vc nociceptive neurons, resulting in orofacial neuropathic pain (162).

In present study, we preferred to use CD68 and GFAP in order to define alteration on microglia and astrocytes in LPS and IoN-CCI induced neuroinflammation in Sp5 of rat brain. In findings of result, the mean CD68-positivity in Vhcl and LPS was (52.86±21.06, 47.50±30.23, respectively) in left and (41.21±30.27, 38.50±37.92, respectively) in right Sp5c. The mean GFAP-positivity in Vhcl and LPS was (416.00±45.90, 436.92±28.53, respectively) in left and (448.06±28.35, 452.67±53.94, respectively) in right Sp5c. CD68 and GFAP-positivities were detected neither left nor right sides of Sp5c in 5 animals in each group. There were no statistical significances found in between groups and, the mean CD68 and GFAP-positivity according to the left and right side of Sp5c were not significant ($p>0.05$). In comparisons of the mean CD68 and GFAP-positivities between left and right Sp5c according to groups, the mean CD68 and GFAP-positivity in left and right sides in Vhcl and LPS groups were statistically significant ($p=0,001$; $p<0,01$, $p=0,003$; $p<0,01$, respectively). The increased GFAP-positivity on left and right sides were found in both groups. This could be explained by that IoN-CCI induced NP showed of reactive astrocytes (5). Furthermore, LPS activated microglia-conditioned medium strongly induced A1 astrocytes found that neuroinflammation and ischemia induced two different types of reactive astrocytes (121). In the literature it is unclear if reactive astrocytes have a bilateral role in chronic pain or not. It hypothesizes that various subtypes of reactive astrocytes may perform dual roles in chronic pain connected with an injury in CNS (120). In present study, the finding of bilateral GFAP activities on Sp5c might be evidence that dual roles of reactive astrocytes could be seen in chronic pain.

There are three animal models of NP used in the literature, including: 1. The NP model following a nerve injury to sciatic, IoN and mental nerves; 2. the local infection-like pathology following a bacterial toxin (LPS) injection; 3. The local sterile inflammation, following Complete Freund's Adjuvant, or CFA (142).

As Gram-negative bacteria derivated endotoxin, LPS is a well-established agent to mimic the endogenous effect during infection. LPS application is associated with producing hyperalgesic priming through direct and/or indirect action on trigeminal nociceptors emerging in peripheral neuroinflammation. To evaluate the local perineural inflammation, in our study we also use LPS as it fundamentally can be found in the oral

cavity produced by gram-negative bacteria such as Porphyromonas gingivalis causing periodontal diseases (142, 163).

In a retrospective study done by Dieb et al., it is found that 73% of patients diagnosed with NOP reported tooth or gingiva pain before a nerve lesion associated with dental treatment and and 95% received privileged dental treatment, in line with an inflammatory-disease diagnosis. The study also found that LPS was presented for the trigeminal-neuron sensitization in the neurovascular bundle (as known as dental pulp, DP), through TLR4 activation, giving an understanding about DP-inflammation guided neuronal phenotype alterations. They concluded that these findings were explained by 2 possible suggestions (i) Inflammation caused by microorganisms can cause neuronal phenotypic changes that lead to the development of NP, with an increased risk following a subsequent iatrogenic lesion (ii) Prior to the dental deafferentation, the pain could already be neuropathic was not of inflammatory origin although not traumatic, like resulting from a metabolic condition, an undiagnosed viral infection, hormonal imbalance or impairment of descending controls (164, 165).

The function of intraperitoneal or peripheral injection of LPS in neuroinflammation has been mentioned in a significant number of animal experiments. The results of the behavioral and biochemical experiments show that LPS given intraperitoneally does not trigger a neuroinflammatory process. According to Tiwari et al., intraperitoneal LPS administration, whether with a short-term or long-term exposure, could not be able to cause neuroinflammatory reactions (166-171).

Systemic LPS injection causes septic shock and fever, and it relates to cytokine (e.g. TNF-a, IL-1b, and IL-6) elevations, in brain and circulation, resembling the ones encountered during natural infections. Nevertheless, a local LPS injection's influence on the CNS as well as its correlation with pain behavior triggered by locally applied LPS was not yet explained. Regarding that, we preferred local application of LPS into the vicinity of IoN to eliminate the septic shock and fever and to evaluate the results of peripheral neuroinflammation on CNS (164, 173, 174). Based on the suggestions in literature, we prefer to use local administration of LPS by a perineural 50 μL LPS injection (300 μM in saline) (0.9 percent NaCl) conducted nearby IoN emergence from the infraorbital foramen on days 0, 3, 5, 11, 13, 17, and 20 (129).

There are a few studies examining the behavioral effects of LPS pretreatment in the orofacial area. iFG scores describing spontaneous pain and VFF testing scores showing MSA which are the type of pain-like behavior are the proof for the effects of preexisting neuroinflammation inducing NP (128).

In present study, a significant increase was found in iFG responses of Vhcl and LPS groups ($p<0.01$), and the increase rate of iFG describing the severity of the spontaneous pain in LPS group was more significant ($p=0.001$). Repeated perineural LPS injections in the vicinity of IoN produced a significant increase in spontaneous pain as compared to Vhcl on days -1, 8, 22 ($P < 0.001$). Statistically significant increase was found in VFF testing scores describing pain scores of MSA of 2,4,6,8,10 grs in Vhcl and LPS groups by time ($p=0.001$, $p<0.01$). Repeated perineural LPS injections in the vicinity of the IoN produced a significant increase in VFF testing scores between day 1 and day 22 following the first injection, showing increased MSA, as compared to Vhcl group. In present study, on correlation of the mean iFG, showing spontaneous pain, and 2, 4, 6, 8, 10 grs of VFF testing scores, describing MSA on day 39, and CD68 and GFAP-positivities on cells according to the left and right sides of Sp5c for both groups; no correlation was found between iFG and CD68 and GFAP-positivity on cells of both sides of Sp5c ($p>0,05$) in Vhcl group. Negative correlation was found between CD68-positivity on cells in left and right Sp5c and VFF 4 gr in LPS group ($r=-0,828$; $p=0,042$; $p<0,05$, $r=-0,828$; $p=0,042$; $p<0,05$, respectively). The finding of negative correlation on MSA and CD68 activity on both Sp5c were supported to the study of Han et al (174). They used by a selective inhibitor of microglial cell activation, on mechanical allodynia and mirror-image mechanical allodynia in rats with compression of the trigeminal ganglion, as measured on postoperative day 14 by suggesting this mirror-image mechanical allodynia was apparently not caused by the sham surgery, because control rats did not develop contralateral mechanical sensitivity until 40 days postoperatively. Several studies have already reported the development of mirror-image pain in NP animal models. Milligan et al. provided evidence that mirror-image inflammatory NP is created through glial and proinflammatory cytokine activities. Also, our finding supported a current hypothesis that microglia can be significant for initiation, while astrocytes are important for the maintenance of NP (78, 92).

According to Boucher et al., one day following LPS injection, VFF scoring to 8 gr produces spontaneous pain-like behavior representing mechanical statical allodynia.

However, on day 8, increased pain-like behaviors were observed as low stimulations as 1 gr in the LPS group comparing to the Vhcl group. They observed spontaneous pain-like behavior on days 1,8 and 22. Our results were similar like that of Boucher et al. suggesting the amount of LPS injections show being proof for the effects of preexisting neuroinflammation inducing NP (128) and in adult rats, the nociceptive response in the intra-plantar formalin test increased by the administration of LPS (175).

In present study, LPS was applied the same dose and use the same time line for its administration data obtained from present study showed that iFG (spontaneous pain like behavior) was achieved on day 8, 22 the increase rate of iFG describing the severity of the spontaneous pain in LPS group was more significant ($p=0.001$), there were significant differences in the mean VFF testing scores describing pain scores of MSA of 10 gr between Vhcl and LPS groups on days 1 ($p=0.045$) while There were significant differences in the mean VFF testing scores describing pain scores of MSA of 6 gr between Vhcl and LPS groups on days 8 ($p=0.004$). Our findings were not like that of Boucher et al.

In the study of Ma et al., MSA provoked in their animal model is a consequence of central sensitization generated by the persistent immune reaction and sensory dysfunction produced by the physical compression of the IoN (188).

Early histological investigations show that significant degradation of myelinated axons distal to the ligation site is the main pathology in CCI, with less apparent harm to nonmyelinated axons. There are little or no pathological changes at the ligation's proximal location (102, 176). CCI prevented the response of both myelinated and nonmyelinated axons. Gabay et al. (103) have investigated the responsible axons for the behavioral modifications that occur following CCI. They also point to an equivalent reduction in A and C axons numbers located distant to the lesion in the CCI model, conserving a fixed balance between the two fiber types. The primary afferents in charge of pain behavior can be classified into two groups as injured and uninjured peripheral neighboring axons which shift hypersensitized. Both nerve fiber types may induct, as well as continue the pain behavior, after a damage to the nerves (177-179). Earlier Tal and Eliav et al. (180) proved myelinated A-fibers' ectopic activity to have a principal electrophysiological characteristic. Recently, Baba et al. found that ectopic activity was reliable for

spontaneous pain behavior and was promoting to central sensitization, as well. Certainly, changes in behavior after CCI regularly mediated by myelinated A-fibers. Under unusual circumstances such as injury and inflammation, it was suggested that peripheral and central sensitization were the mechanisms responsible for mechanical allodynia (181, 182). Based on the conclusion of these studies, we did not examined histologically IoN in both groups by assuming this evidence.

In the literature, partial trigeminal nerve injury studies have essentially worked IoN-CCI with loose ligatures in rats (92, 93). There are also other animal models such as chronic tooth infection (183), peripheral inflammation (184, 185), and nerve transection (186, 187). The recurrent asymmetric FG episodes directed to the injured nerve territory after IoN-CCI have been interpreted as behavioral signs of spontaneous NP. According to Xu et al., partial IoN ligation provoked persistent behavioral and anatomical variations, including mechanical allodynia enduring over 3 weeks. These periods vary from 2 to 10 weeks. In present study, after IoN-CCI, iFG episodes showing spontaneous pain were increased in LPS group as compared to Vhcl on days 27, 31, 35, 39 ($P < 0.001$) since surgery was performed on day 23 showing postoperatively day 4, 8, 12 and 16 respectively. These findings supported to the result of studies reporting that iFG episodes showed a significant increase in LPS group compared to Vhcl group on day 4 and 8, and highly significant increase by day 16 (91, 129). iFG episodes were observed 3 days after surgery based on timeline of iFG scores observation days by showing difference result of Xu et al which they showed that the partial ION ligated group presented significantly more FG episodes than the sham operated group on day 1 after nerve injury indicating the increased FG episodes in mice are specific for the acute phase following surgery. We also evaluated in our study that iFG episodes did not go back to the beginning scores before IoN-CCI during 2 weeks post-surgery. On the contrary, there is a study showing that IoN-CCI created an increased FG episodes remaining for 130 days (92).

In a study conducted by Xu et al. (159), mice exhibited significant allodynia following the partial ION ligation starting from day 1 and lasting for three weeks. Similarly, our assessment of the mechanical allodynia process lasted for 2 weeks for rats. They also observe a significant increase in FG episodes in mice one day after partial ION ligation compared to the sham mice group. Yet, FG episodes were back to normal within 3 days post-surgery. In our findings, FG episodes never went back to the beginning

scores. That might be a result of the LPS caused preexisting neuroinflammation. Additionally, they measure the body weights of mice and found them increased 2 weeks later partial IoN ligation above the preoperation measurements. We did not evaluate the body weights of rats since there is no reliable data about the effect of NP on body weight.

Microglial activation designated by upregulation of OX42 is widely applied as a biomarker as a neuropathic injury detector either in the spinal cord or the spinal trigeminal nucleus. The elevated OX42 positive immunoreactivity is apparent in the spinal trigeminal nucleus for 10 weeks showing chronic neuroinflammation and the activation of the microglial. The spinal trigeminal nucleus is the region of synaptic connection among the trigeminal nerve endings and the second-order neurons in the sensory transmission pathway, carrying the messages concerning pain to the higher center of the brain. Microglial activation for a long term has not yet been published in any other mouse and rat NP models. Our study is also a short-term study. In this study, sufficient histological evidence was obtained by combining mild nerve compression and inflammation in their model besides central activation of microglia at the level of the second-order spinal trigeminal relay neurons (155).

In the study done by Ma et al., as a marker for activated macrophage, CD68 was used, similar to our study. Ten weeks post-op, CD68 antibody in the nerve trunk, a marker for active macrophages, revealed a substantial immune cell infiltration at the chromic gut suture attachment site. In the experimental group, they also discovered a substantial reduction in mechanical threshold, which continued more than 10 weeks, explaining that chromic gut suture produces NP-related behaviors persistent for over 10 weeks in mice. Histology results of the study of Ma et al. showed that there exists neuroinflammation where the chromic gut suture was placed. Simultaneously with edema in axon-bundles, in the areas between the axons on the serial IoN segments was CD68-positive (188).

Boucher et al. found that, in the LPS group, the activation of the microglia confirmed by Iba-1 staining was highly noticeable. Yet, only qualitative evidence was used to get those findings which is the main limitation of the study. Realizing that, we conducted our study to eliminate that limitation by counting the activated microglia in Sp5c (128).

PNI causes not just NP, that is diagnosed by mechanical allodynia, or pain triggered by ordinarily benign stimuli, but also significant microgliosis in the spinal cord. Gliosis is a general reactive alteration of glial cells in response to traumas and injuries that typically entails glial cell hypertrophy or proliferation. Microglia undergo morphological changes in microgliosis, transforming from ramified to amoeboid types with larger specialized cells and shorter processes. Gilmore et al. (1975) revealed that after sciatic nerve transection, non-neuronal cells (i.e. neuroglia) proliferated in the spinal cords of irradiated, young mice. By using Nissl and H&E staining, Gilmore and Skinner et al. discovered that sciatic nerve damage in adult mice resulted in non-neuronal cell growth in the spinal cord (189, 190). Eriksson et al. (1993) used immunostaining with OX-42 (an antibody that recognizes receptor CD11b) to demonstrate remarkable microgliosis in the pain-modulating spinal cord and brain stem post nerve damage, with others demonstrating a significant microglia proliferation, visible two days post nerve damage (191-193).

In present study, the alterations on microglia and astrocytes by using CD68 and GFAF antibodies were evaluated on day 39. Even if iFG describing the severity of the spontaneous pain and 4 gr of VFF testing scores describing pain scores of MSA were observed on days 8, we did not have any rat brain to show CD68 and GFAF-positivities on day 8. Further studies are needed (i) to clarify alterations on microglia and astrocytes according to the first observation days of spontaneous pain and MSA. (ii) to use cytokines and specific markers to show activated microglia and astrocytes on NP.

6. CONCLUSION

1. Neuroinflammation was produced by the administration of LPS into the vicinity of IoN, and NP was produced by IoN-CCI on 24 male rats without complications, infections, or deaths during all stage of study.

2. iFG describing the severity of the spontaneous pain in LPS was achieved on day 8, 22 and the increase rate of iFG in LPS group was more significant. This finding supports that local administration of LPS evoke increased spontaneous pain as a pain like behavior.

3. There were significant differences in the mean VFF scores (MSA) of 10 gr between Vhcl and LPS groups on days 1 while there were significant differences in the mean VFF scores (MSA) of 6 gr between Vhcl and LPS groups on days 8. Repeated perineural LPS injections produced a significant increase in iFG scores as compared to Vhcl on days -1, 8, 22. Statistically significant increase found in VFF scores (MSA) of 2,4,6,8,10 grs in Vhcl and LPS groups by time. Repeated perineural LPS injections produced a significant increase in VFF testing scores between day 1 and 22 following the first injection, showing increased MSA, as compared to Vhcl group. These findings also showed that local administration of LPS provoke increased iFG and MSA as a pain like behavior which it was the proof for the effects of preexisting neuroinflammation inducing NP.

4. There were no statistical significances found in between groups, and the mean CD68 and GFAP-positivity according to both sides of Sp5c. In comparisons of the mean CD68 and GFAP-positivities according to groups, the mean CD68 and GFAP-positivity in both sides in Vhcl and LPS groups were statistically significant. This could be explained by that CCI-IoN induced NP showed the reactive astrocytes.

5. The increased GFAP-positivity on left and right sides were found in both groups. This finding could be explained by that IoN-CCI induced NP showed of reactive

astrocytes, and the finding of bilateral GFAP activities on Sp5c might be evidence that dual roles of reactive astrocytes could be seen in chronic pain.

6. Negative correlation was found between CD68-positivity on cells in left and right Sp5c and VFF 4 gr in LPS group while no correlation was found between iFG and CD68 and GFAP-positivities on cells of both sides of Sp5c in Vhcl group, supporting the development of mirror-image pain in NP animal models.

7. Our finding could not explain whether the activated microglia (the mean CD68-possitivity was 38,50±37,92) and astrocytes (the mean GFAP-positivity was 452,67±53,94) were caused by LPS or CCI-IoN. Since all immunohistochemical staining were performed on day 39 (the day of animal sacrification) future studies are needed to make this clarification

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