The Possibility Of Endolymphatic Hydrops In An Animal Migraine Model Created With Nitroglycerin And Analyses Of The Effects Of Migraine Treatment On The Cochlear And Vestibular System

by

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Koç University

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This is to certify that I have examined this copy of a doctoral dissertation by

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and have found that it is complete and satisfactory in all respects,

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Dedicated to my parents, who have always loved and supported me, and to my patients, who have trusted in me

ABSTRACT

The Possibility Of Endolymphatic Hydrops İn An Animal Migraine Model Created With Nitroglycerin And Analyses Of The Effects Of Migraine Treatment On The Cochlear And Vestibular System

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Background: VM is a different type of migraine with vestibular symptoms like vertigo and postural imbalance and also auditory symptoms like tinnitus, aural pressure, and muffled hearing. MD presents with vertigo, fluctuating hearing loss, tinnitus, and aural pressure. So, there are overlapping symptoms between these two diseases, and because of that, it can be challenging to make a diagnosis. There is not a single diagnostic test that can help to differantiate. MD's pathophysiology is still unclear, and the only pathological marker is ELH. ELH could also be seen with different etiologies, including VM . There is not much data on migraine's effect on the cochleovestibular system, and since MD's pathology is still unknown, MD could be thought of as a variant of VM. Clinical reports revealed that anti-migraine medications given to MD patients showed beneficial effects. There has recently been huge progress in the advancement of anti-migraine medications.

Aim: In this thesis project, we first aimed to investigate the possibility of ELH in migraine. Furthermore, we included a CGRP receptor blocker as the treatment group to study its effects specifically on the cochleovestibular system.

Methods: Our study included 30 male Wistar rats aged 2-5 months. Animals were divided randomly into three groups, 10 rats in each. Group 1 was the sham with saline injection. Group 2 was the migraine with diluted NTG injection at a dose of 10 mg/kg IP every other day for 9 days. Group 3 was the treatment group with Olcegepant in a volume of 1 mg/kg IP one hour and 15 minutes after the NTG injection every other day for 9 days. The hearing thresholds were determined by click and tone-burst ABR at 6, 12, 16, 20, and 32 kHz. DPOAE experiments were performed at 6, 8, 10, and 12 kHz. ECOG was performed, and SP/AP ratios were calculated. All the audiological evaluations were also done on day nine for the comparisons. The orofacial formalin test, OFT, tail hang reflex, and head circling tests were performed for behavioral analyses. Tail blood samples for measuring the levels of TNF-alpha, IL-6, and intracardiac blood samples for CGRP and CSF for the levels of HGMB1 were gathered on day nine. TG for the central, and organ of corti, saccule, stria vascularis for the peripheral immunohistochemical analyses were dissected after the perfusion. Half of the animal's cochleas (30 cochleas) were paraffin

embedded for H&E staining. And the other half of the animals' cochleas were immunostained after the whole mount dissection. Image J program was used for the image analysis.

Results: The immobility time that animals spent in OFT was measured and analyzed. A significant difference was detected between the migraine and the control group (p=0.001). The migraine group was more immobile, as expected. There was not a significant difference in the orofacial formalin test, and none of the groups had abnormal tail hang reflex or head circling.

DPOAE results were extracted, and db SPL calculations at 6, 8, 10, and 12 kHz were higher on day nine compared to day one in the migraine group with a statistically significant difference (p=0.0006, p=0.002, p=0.009, p=0.014). On the other hand, there was a significant decrease in all the above frequencies in the treatment group (p=0.002, p=0.028, p=0.018, p=0.006). There was also a significant difference at 6 kHz between day nine and day one of tone-burst ABR thresholds between the control and the migraine and the migraine and the treatment groups (respectively p=0.038, p=0.018). In addition, at 12 and 20 kHz, there was a significant difference between the control and the migraine groups (p=0.030, p=0.018). Tone burst ABR thresholds were lower on day nine than on day one in the migraine group. There was no difference in click ABR thresholds. SP/AP ratios were compared within the groups on day one and day nine; the treatment group had a statistically significant lower ratio on day nine than day one (p=0.049).

TNF-Alpha was significantly high in the migraine group (p=0.048). There was not any significant difference in IL-6 and CGRP results.

The PSM was calculated through the H&E staining of the cochlea sections, and we did not see a significant difference between the groups. The only statistically significant difference was in the CGRP count, and the Glutamate R2 count at the whole mount dissected and immunostained IHCs. The CGRP count was higher in the treatment group and significantly different from that of the migraine group (p=0.02) and the control (p=0.006). Also, the glutamate R2 was significantly higher in the treatment group compared to the migraine group (p=0.012).

Conclusion and Comment: NO has a beneficial effect on the central and peripheral auditory systems. The inhibition of NO lowers the SP/AP ratio. There is a signaling pathway of a reciprocal relationship of Glutamate with CGRP in the efferent system of LOC.

ÖZETÇE

Hayvanda Nitrogliserin İle Oluşturulmuş Migren Modelinde Endolenfatik Hidrops Olasılığı Ve Migren Tedavisinin Koklear Ve Vestibüler Sistem Üzerindeki Etkileri

Pelin KOÇDOR

Sinirbilim, Doktora

24 Eylül 2024

Giriş: VM, vestibüler semptomlar, vertigo, postural dengesizlik ve ayrıca kulak çınlaması, kulak basıncı, boğuk duyma gibi işitsel semptomlarla birlikte görülen farklı bir migren türüdür. MD, vertigo, dalgalanan işitme kaybı, kulak çınlaması ve kulak basıncı ile kendini gösterir. Bu nedenle, bu iki hastalık arasında örtüşen semptomlar vardır ve bu nedenle tanı koymak zor olabilir. Ayrımı yapmaya yardımcı olabilecek tek bir tanı testi yoktur. MD'nin patofizyolojisi hala net değildir ve tek patolojik belirteç ELH'dir. ELH, VM dahil olmak üzere farklı etiyolojilerle de görülebilir. Migrenin kokleovestibüler sistem üzerindeki etkisine dair çok fazla veri yoktur ve MD'nin patolojisi hala bilinmediğinden, MD, VM'nin bir varyantı olarak hipotezi öne sürülebilir. MD hastalarına verilen anti-migren ilaçlarının yararlı etkiler gösterdiğine dair klinik raporlar ortaya konmuştur. Son zamanlarda anti-migren ilaçlarının ilerlemesinde büyük bir ilerleme kaydedilmiştir.

Amaç: Bu tez projesinde, ilk olarak migrende ELH olasılığını araştırmayı amaçladık. Ayrıca, özellikle kokleovestibüler sistem üzerindeki etkilerini incelemek için tedavi grubu olarak bir CGRP reseptör blokeri ekledik.

Yöntem:. Çalışmamıza 2-5 aylık 30 erkek Wistar sıçanı dahil edildi. Hayvanlar rastgele üç gruba ayrıldı, her grupta 10 sıçan vardı. 1. Grup, serum fizyolojik enjeksiyonlu sham'dı. 2. Grup, 9 gün boyunca her iki günde bir 10 mg/kg IP dozunda seyreltilmiş NTG enjeksiyonlu migrendi. 3. Grup, 9 gün boyunca her iki günde bir NTG enjeksiyonundan bir saat 15 dakika sonra 1 mg/kg IP hacminde Olcegepant ile tedavi grubuydu. İşitme eşikleri 6, 12, 16, 20 ve 32 kHz'de klik ve tone-burts ABR ile tespit edilerek belirlendi. DPOAE deneyleri 6, 8, 10, 12 kHz'de gerçekleştirildi. ECOG yapıldı ve SP/AP oranları hesaplandı. Tüm odyolojik değerlendirmelerin enjeksiyonlar sonrası karşılaştırmaları için dokuzuncu günde de yapıldı. Davranışsal analizler için orafasiyal formalin testi, OFT, kuyruk sarkıtma refleksi ve baş dairesi testleri yapıldı. TNF-alfa, IL-6 seviyelerini ölçmek için kuyruk kan örnekleri ve HGMB1 seviyeleri için CSF, CGRP için intrakardiyak kan örnekleri dokuzuncu günde toplandı. Periferik immünohistokimyasal analizler için korti organı, sakkül, stria vaskülaris ve santral analizler için TG perfüzyondan sonra diseke

edildi ve immünoboyandı. Hayvanların koklealarının yarısı , 30 adet, H&E boyama için parafine gömüldü. Diğer yarısı whole-mount diseksiyon tekniği ile diseke edildi ve immün boyandı. Görüntü analizi için Image J programı kullanıldı.

Bulgular: Hayvanların OFT'de geçirdikleri hareketsizlik süresi ölçüldü ve analiz edildi ve migren ile kontrol grubu arasında anlamlı fark tespit edildi (p=0,001), migren grubu beklendiği gibi daha hareketsizdi. Orafacial formalin testinde anlamlı bir fark yoktu ve grupların hiçbirinde anormal kuyruk sarkıtma refleksi veya baş dairesi yoktu.

DPOAE sonuçları çıkarıldı ve 6, 8, 10 ve 12 kHz'deki db SPL hesaplamaları migren grubunda dokuzuncu günde birinci güne kıyasla istatistiksel olarak anlamlı bir farkla daha yüksekti (p=0.0006, p=0.002, p=0.009, p=0.014). Öte yandan, yukarıdaki tüm frekanslarda tedavi grubunda anlamlı bir azalma vardı (p=0.002, p=0.028, p=0.018, p=0.006). Ayrıca, kontrol ve migren ile migren ve tedavi grupları arasında tone-burst ABR eşiklerinin dokuzuncu ve birinci günleri arasında 6 kHz de değişimde istatistiksel olarak anlamlı bir fark vardı (sırasıyla p=0.038, p=0.018). Ek olarak, 12 ve 20 kHz'de kontrol ve migren grupları arasında anlamlı fark vardı (p=0,030, p=0,018). Tone- Burst ABR eşikleri migren grubunda dokuzuncu günde birinci günden daha düşüktü. Klik ABR eşiklerinde fark yoktu. SP/AP oranları gruplar arasında birinci ve dokuzuncu günde karşılaştırıldı ve tedavi grubu dokuzuncu günde birinci günden istatistiksel olarak anlamlı derecede daha düşük bir orana sahipti (p=0,049).

TNF-Alfa migren grubunda anlamlı derecede yüksekti (p=0,048). IL-6 ve CGRP sonuçlarında anlamlı bir fark yoktu.

PSM, koklea kesitlerinin H&E boyama yoluyla hesaplandı ve gruplar arasında anlamlı bir fark görmedik. İstatistiksel olarak anlamlı tek fark, whole-mount diseke edilmiş ve immüno boyanmış IHC'lerde CGRP sayısı ve Glutamat R2 sayısındaydı. CGRP sayısı tedavi grubunda daha yüksekti ve migren grubundan (p=0,02) ve kontrol grubundan (p=0,006) önemli ölçüde farklıydı. Ayrıca glutamatR2, migren grubuna kıyasla tedavi grubunda önemli ölçüde daha yüksekti (p= 0,012).

Sonuç ve Yorum: NO'nun sadece merkezi değil aynı zamanda periferik işitme sisteminde de yararlı bir etkisi vardır. NO'nun inhibisyonu SP/AP oranını düşürür. LOC'nin eferent sisteminde Glutamatın CGRP ile karşılıklı ilişkisinin olduğu bir sinyal yolu vardır.

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ABBREVIATIONS

ABR	Auditory Brainstem Response
AP	Action Potential
CGRP	Calcitonin-gene-related peptide
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	6-Diamidino-2-phenylindole
DB	Decibel
DPOAE	Distortion Product Otoacoustic emissions
ECOG	Electrocochleography
ELH	Endolymphatic hydrops
H&E	Hematoxylin and Eosin
HMGB1	High-mobility group box 1
IHC	Inner Hair Cell
IP	Intraperitoneal
IL-6	Interleukin-6
LOC	Lateral Olivocochlear
MOC	Medial Olivocohlear
MRI	Magnetic resonance Imaging
MD	Meniere's disease
NTG	Nitroglycerin
NO	Nitric Oxide
OFT	Open-field test
PFA	Paraformaldehyde
PBS	Phosphate-buffered Saline

PSM	Proportion of Scala Media
SHL	Sensorineural hearing loss
SM	Scala Media
SV	Scala Vestibule
Stria V	Stria Vascularis
SPL	Sound Pressure Level
SP	Summation Potential
TNF-α	Tumour Necrosis Factor-Alpha
VM	Vestibular migraine

Chapter 1

INTRODUCTION

Vestibular migraine (VM) is a different type of migraine that causes vestibular symptoms with or without headache. International Headache Society (IHS) and the Barany Society published the first well-established diagnostic criteria for VM in 2012 (Lempert et al., 2012), which is given below:

Vestibular migraine:

1) At least five episodes with vestibular symptoms of moderate[a] or severe[b] intensity, lasting 5 min to 72 h

2) Current or previous history of migraine with or without aura according to the International Classification of Headache Disorders (ICHD)

3) One or more migraine features with at least 50% of the vestibular episodes: a) headache with at least two of the following characteristics: one-sided location, pulsating quality, moderate[a] or severe[b] pain intensity, aggravation of routine physical activity; b) photophobia and phonophobia; c) visual aura

4) Not better accounted for by another vestibular or ICHD diagnosis

Probable vestibular migraine:

1) At least five episodes with vestibular symptoms of moderate or severe intensity, lasting 5 min to 72 h

2) Only one of the criteria B and C for vestibular migraine is fulfilled (migraine history or migraine features during the episode)

3) Not better accounted for by another vestibular or ICHD diagnosis

a- Usually interferes with daily activities. b- Usually prohibit daily activities.

1.1 Epidemiology

The prevalence of VM is 1 to 2.7%, and it is probably the most common neurological cause of vertigo in adults (Formeister et al., 2018). According to a study, two-thirds of patients previously diagnosed with VM were evaluated by a physician, and only 20% of them were diagnosed with VM (Neuhauser et al., 2006). Women are more affected than men (Benjamin et al., 2022). Frequently, patients report a family history of migraine, and the migraine vertigo combination in families is 4-10 times higher than the prevalence in the general population (Paz-Tamayo et al., 2020).

1.2 Clinical Features

VM presents with various vestibular symptoms, including vertigo, postural imbalance, head-motion dizziness, positional vertigo, and visually-induced vertigo. Many patients may not have headaches during an acute attack of VM. So, headache and vertigo may not occur together at all. That is why VM is often a challenge for physicians to diagnose. Frequently, other migraine features like photophobia, photophobia, and visual or other auras may accompany vertigo. Auditory symptoms like tinnitus (most common), aural pressure, muffled hearing, and ear pain may be seen in over two-thirds of patients with VM. The duration of the episodes is highly variable, but the main episode usually does not last more than 72 hours. Information about the migraine triggers, for example, menstruation, sleep deprivation, and specific foods, may help physicians with the diagnosis (Lempert & von Brevern, 2019), (Beh, 2022).

There is an overlapping relationship between VM and Meniere's Disease (MD). One-third of MD patients who suffer from vertigo, tinnitus, aural fullness, and hearing loss also have a history of migraine (Shepard, 2006). The clinical features of these two diseases overlap, and no specific diagnostic test can reliably distinguish them. Half of the MD patients present with migrainous features, such as a headache with photophobia or a positive family history of migraine (Radtke et al., 2002). Moreover, about a quarter of VM and MD patients meet both diagnostic criteria (Neff et al., 2012).

1.3 Clinical Findings

In general, the physical exam (neurologic and otologic) is normal in these patients. If a patient has a long history of VM, positional nystagmus and saccadic pursuit may be seen during neuro-ophthalmologic evaluation (Neugebauer et al., 2013). During acute attacks, imbalance is a regular finding, and patients may have spontaneous nystagmus, central positional nystagmus, or a combination of these (von Brevern et al., 2005).

VM is a clinical diagnosis, and neither in the acute nor in the interval is there a specific test finding that will point out the VM. However, a full neurotological exam and specific tests like an audiogram, videonystagmography (VNG), and brain magnetic resonance imaging (MRI) may help to exclude other conditions. For example, unilateral, low-frequency, progressive, or fluctuating sensorineural hearing loss (SNHL) is a typical audiometric finding of MD. In contrast, up to 20%of patients with VM may have mild SNHL and it is usually symmetric and likely represents presbyacusis (Beh, 2019). Bilateral caloric test weakness and unilateral canal paresis have been reported in patients with VM (Lempert & von Brevern, 2019).

1.4 Pathogenesis

The pathogenesis of VM is still unknown, but genetic, inflammatory, and chemical mechanisms related to sensory hypersensitivity, altered multisensory processing, and trigeminovascular and calcitonin-gene-related peptide (CGRP) effects have been suggested.

Migraine is probably caused by neuroinflammation of the trigeminovascular system, which is formed by trigeminal nerve endings near the dura and pia mater and cranial blood vessels. The trigeminal nerve mediates pain signaling by releasing neuropeptides like substance P, nitrous oxide, and CGRP (Pietrobon & Moskowitz, 2013). The release of these peptides causes vasodilation and inflammation. A theory has been proposed that unilateral release of these neuropeptides causes static vestibular imbalance, leading to vertigo, and bilateral release causes altered vestibular excitability, leading to motion sickness (Balaban, 2011). In another study, CGRP deficient mice showed impaired otolith activity and imbalance (Jones et al., 2018).

Another proposed mechanism for VM is the dysfunction of the ion channels in the inner ear and central vestibular structures (von Brevern et al., 2006). Even the

specific genetic alterations are not known at VM, an autosomal dominant inheritance pattern has been described (Neuhauser et al., 2006).

Depending on the clinical symptoms and findings, migraine may affect both peripheral and central vestibular systems together. Cortical spreading depression, the suggested mechanism for migraine aura, may produce vestibular symptoms when the temporoparietal junctions are involved. However, cortical spreading depression can not explain the complex nystagmus pattern of VM (Lempert & von Brevern, 2019).

Interestingly, a recent article showed endolymphatic hydrops of the cochlea by gadolinium-enhanced MRI in 3 out of 30 patients with VM (Sun et al., 2017). It is not clear if those patients were VM or had a variant of MD.

1.5 Differential Diagnosis

Vestibular and some non-vestibular disorders may mimic VM and should be differentiated. Table 1.1 lists these possible disorders and their key features (Lempert & von Brevern, 2019). Since MD is the most challenging one of all those disorders, it is analyzed in detail with upcoming pages.

Table 1.1 Differential Diagnosis of Vestibular Migraine

Disorder	**Key Features**
Benign Positional Paroxysmal Vertigo (BPPV)	Vertigo lasting up to 1 minute provoked by head movement. Positive positional torsional nystagmus
MD	Vertigo lasting 20 minutes to 12 hours with concurrent hearing loss, tinnitus, and aural fullness
Vertebrobasilar transient Ischemic Attack (TIA)	Vertigo, ataxia, dysarthria, diplopia, or visual field defects often associated with craniocervical pain

Schwannoma of the Eight Nerve	Rarely vertigo, the key symptom is progressive unilateral hearing loss and tinnitus
Anxiety Disorder	Vertigo exacerbation in specific situations (leaving the house, supermarket, etc.)

1.6 Meniere's Disease

MD is the most challenging differential diagnosis of VM. It is diagnosed by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) criteria, as shown in Table 1.2.

Table 1.2 The 1995 American Academy of Otolaryngology-Head and Neck Surgery Diagnostic Criteria for Meniere's Disease

Certain MD	Definite Meniere's disease plus histopathologic confirmation
Definite MD	Two or more definitive spontaneous episodes of vertigo 20 minutes or longer Audiometrically documented hearing loss on at least one occasion Tinnitus or aural fullness in the treated ear Other causes excluded
Probable MD	One definitive episode of vertigo Audiometrically documented hearing loss on at least one occasion

	Tinnitus or aural fullness in the treated ear Other causes excluded
Possible MD	Episodic vertigo of the Meniere's type without documented hearing loss or sensorineural hearing loss, fluctuating or fixed, with disequilibrium but without definitive episodes Other causes excluded

Nearly half of the VM patients may present with tinnitus, aural fullness and hearing loss (Chu et al., 2013). On the other hand, half of the MD patients may present with migranous features like headache, photophobia (Rassekh & Harker, 1992). As a consequence, establishing the correct diagnosis is challenging.

MD's pathophysiology is still unknown. The one of the typical histopathological hallmark, depending on the post-mortem temporal bone studies, for MD is endolymphatic hydrops (ELH). ELH is an increase of the inner ear fluid, endolymph, within the membranous labyrinth of the inner ear. It is believed that, hydrops results from altered endolymph homeostasis, or malabsorption, or both. ELH could be seen with different etiologies, such as trauma, viral infection, and autoimmune processes (Greco et al., 2012). In addition to this, there are definitions in the literature that patients may be with ELH without MD symptoms (Merchant et al., 2005).

Other than ELH, ischemia has been suggested as a possible reason for MD symptoms, acute attacks (Foster & Breeze, 2013). The terminal type of vascular supply of the inner ear is a potential target for hemodynamic instability which will end up with labyrinth ischemia and cause vertigo attacks (Pirodda et al., 2010).

There is an other theory for MD pathology related with inflammatory events. It has been proposed that, an inflammatory event may cause changes of the blood supply to the inner ear and that may affect the hair cells of the cochlea and the vestibule (Mohseni-Dargah et al., 2023).

There is a strong correlation of MD and VM triggers (Yeo et al., 2018). Stres, dietary intake and weather changes are among those triggers. Low atmospheric pressure has been linked to MD attacks and also can increase migraine frequency (Yilmaz et al., 2015) (Andersson et al., 1997). Fluctuations in estrogen level may contribute to both migraine and MD. Caffeine promotes vasoconstriction and decreased blood flow which provokes both migraine and MD (Sarna et al., 2020).

1.7 Animal Models of Migraine

Animal models of migraine have been leading to significant advances in understanding the mechanism of the disorder and antimigraine treatments. Unfortunately, none of the experimental animal models can reflect all the features of migraine (Chou & Chen, 2018).

A valid animal model for investigating human disorders should have five features, and these features are similarity of symptoms, observability and measurability of behavioral phenotypes, inter-observer reliability of assessment results, the similarity of response to treatment, and reproducibility of the system (Belzung & Lemoine, 2011). Migraine is a complex disorder, and since there are differences between animals and humans, completion of all the criteria is difficult. Besides, whether animals can experience migraine is another concern. Currently, available animal models of chronic migraine are given in Table 1.3. (Chou & Chen, 2018).

Table 1.3 Animal models of chronic migraine

Models	**Animals**	**Route and duration of administration**
Repeated dural stimulation with inflammatory soup	Rat	Epidural cannulation 4,7,>21,30 days

Nitroglycerin (NTG)	Rat, Mouse	Intraperitoneal; 11, 14 days
MOH(medication overuse headache) sumatriptan, and paracetamol	Rat	Intraperitoneal 7-30 days
Spontaneous trigeminal allodynia	Rat	-
Monogenic migraine mutation	FHM type 1 R192Q transgenic mice	-

1.7.1 Nitroglycerin as the experimental model of migraine

Nitroglycerin (NTG) is a highly lipophilic organic nitrate, a well-known vasodilator. NTG directly affects the endothelial wall and especially intracellularly forms nitric oxide (NO), which is responsible for vasodilation (Chung & Fung, 1993).

This drug has positive features that make it useful as a translational model for human migraine (Table 1.4) (Demartini et al., 2019).

Table 1.4 Features of the animal model of migraine induced by nitroglycerin

Positive Features	**Negative Features**
The majority of migraineurs develop an attack that fulfills the International Headache Society criteria for migraine	Not suitable for the study of aura phenomenon

Clear dichotomized response in patients and healthy controls	Not suitable for rare forms of migraine (e.g. Familial hemiplegic migraine)
Attacks prevented or aborted by common anti-migraine drugs	Variability between studies due to the different dosage/route of administration
Occurrence of typical migraine-associated symptoms	
Changes in the trigeminal and spinal nociceptive transmission and evoked cortical responses	
Activities of central areas comparable to those obtained during a spontaneous migraine attack	

NTG increases CGRP levels in migraineurs (Juhasz et al., 2003). NTG is a NO donor, and NO is an important signaling molecule involved in the synthesis and release of CGRP from trigeminal ganglion neurons (Bowen et al., 2006). To create a chronic migraine model, NTG injection every other day for 9 days was previously used, and progressive and sustained hyperalgesia was induced (Pradhan et al., 2014).

1.8 Treatment of Vestibular Migraine

Life style modifications such as; regular exercise, proper sleep, trigger avoidance are very important for VM patients' treatment. In addition to that, magnesium, and coenzyme-Q10 are effective supplements for VM (Beh, 2019). There are a few randomized clinical trials in VM prophylactic treatment. An unblinded clinical trial using flunarizine showed improved vertigo frequency and severity (Lepcha et al., 2014). Another study with venlafaxine and propranolol also reduced vertigo attacks (Salviz et al., 2016). Both VM and MD patients were treated with Verapamil for 3 months and had fewer vertigo attacks in a prospective study (Kaya et al., 2019). In a prospective study, topiramate was

successful in reducing the frequency and severity of vertigo and auditory symptoms (Carmona & Settecase, 2005).

Some well-known prophylactic migraine medications such as topiramate, propranolol, verapamil, amitriptyline, venlafaxine, acetazolamide, and benzodiazepines reduced the duration, frequency and intensity of vertigo attacks on different retrospective studies (Beh, 2019) (Power et al., 2018). There is no proven evidence that a prophylactic migraine medication is better than the other. Besides, there is no agreement on the duration of the drug treatment. In general, it is tapered slowly when VM is controlled for 6 months (Lempert & von Brevern, 2019).

In the last decade, Calcitonin Gene-Related Peptide (CGRP), an important neurotransmitter in migraine pathogenesis, has become the focus of interest for treatment (Digre, 2019). CGRP released from the meningeal vessels causes vasodilation and mast cell degranulation. This leads to the release of inflammatory agents that make trigeminal nociceptors sensitive and cause pain in migraine (Durham & Vause, 2010).

CGRP receptors are expressed by different cell types, such as neurons and glial cells in the peripheral and central nervous systems. These receptors were also found in cerebral blood vessels, meningeal smooth muscles, and mast cells in the dura mater (Petersen et al., 2005). The first potent and selective non-peptide antagonist of the human CGRP receptor was originally referred to as BIBN4096BS, and then renamed as olcegepant (Doods et al., 2000). Olcegepant works as a CGRP receptor antagonist by competing to bind the site of endogenous ligand CGRP and thus blocks the physiological and cellular effects of CGRP. There is another compound that was found and is a selective CGRP antagonist, MK-0974 has also been renamed as telcagepant. It is a highly selective, potent oral antagonist of the human CGRP receptor (Moore et al., 2009). Other than these receptor antagonists, there are three monoclonal antibodies directed toward the CGRP ligand or its receptor: erenumab, fremanezumab, and galcanezumab (Carmine Belin et al., 2020).

1.9 Rationale and Aims

In the literature, many studies showed an overlap of symptoms of MD and VM (Tabet & Saliba, 2017). Both diseases have proposed pathophysiological explanations. ELH is

known as a potential marker of MD, but it is also seen after trauma, viral infections, and autoimmune processes. There are small series and case reports that suggest ELH in VM (Redon et al., 2021). MRI results of 7 patients with VM and 7 patients with MD were compared and it was shown that 2 patients with VM had ELH unilateral or bilateral (Nakada et al., 2014). Other than ELH, there are common vascular mechanisms and inflammatory processes in VM and MD (Frejo & Lopez-Escamez, 2022). Current migraine treatments like anti-hypertensive drugs (calcium channel blockers, beta-blockers e.t.c.) have shown success in treating MD. Furthermore, diet modifications can promote not only VM but also MD patients. Steroids can manage acute attacks of MD and VM. Even a surgical treatment for MD, such as a tympanostomy tube, can alleviate VM inner ear symptoms (Sarna et al., 2020). Both disease VM and MD pathophysiology are multifactorial and still remain unknown.

CGRP is accepted as an initiator in migraine attacks and induces aura symptoms when it is released from the trigeminal nerve (Ramon et al., 2017). The inner ear, including stria vascularis, is innervated by the trigeminal nerve, and stria vascularis consists of a capillary network and is critical for the production of endolymph (Hegemann, 2021). Noise sensitivity, which is explained by disturbed cochlear efferent stimulation, is a symptom of migraineurs and could be caused by CGRP overproduction. Of course, this is not a very well-established fact, but it is a possibility.

In summary, we aim to enlight and uncover

1- The possibility of ELH in migraine

2- The auditory symptoms like hearing loss, noise sensitivity, and vestibular symptoms in migraine

3- CGRP role not only in the central neural system but also in the peripheral system especially in the inner ear.

We hypothesized that ELH could be seen in migraine, CGRP could answer unresolved questions of overlaps between VM and MD, and CGRP receptor antagonists may be an effective treatment and management for ELH.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

In this study, 30 male Wistar rats aged 2-5 months and weighing 240 ± 50 grams were included in the study. The rats were housed at 25 ºC with a 12-hour light-dark cycle, a background noise level of <50 dB, where they had ad libitum access to food and water. A total of three groups were formed according to the drugs and their doses administered. The rats included in the study were randomly allocated to groups with ten rats in each group. All experiments and procedures were applied according to the protocols and criteria approved by the Institutional Animal Care and Use Committee (IACUC) of Koç University in 2019.HADYEK.35/2021.HADYEK.14 approval numbers, according to Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes. All experiments were reported in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines.

2.2 Auditory Tests

Hearing thresholds were determined by obtaining electrophysiological auditory brainstem response (ABR) in all rats under general anesthesia. The ABR measurements were conducted in an environment where the background noise level was <50 dB and in the same room with the animals' cages, using a Tucker Davis Technologies RZ6 workstation and needle electrodes. Before all measurements, the patency of the external auditory canal was checked with an otoscopic examination. For general anesthesia, 50 mg/kg IP ketamine hydrochloride (Ketalar®, Eczacibasi Parke-Davis, Istanbul, Turkey) and 10 mg/kg IP Xylazine HCl (Alfazyn®, Alfasan International BV, Woerden, The Netherlands) were used. Distortion product otoacoustic emissions (DPOAE), ABR, and Electrocochleography (ECOG) recordings were carried out on each ear separately using

a BioSig program (Tucker Davis Technology). DPOAE examinations were performed at 6, 8, 10, and 12 kHz. To obtain the distortion product (2f1- f2), two pure tones were used at a ratio of f2/f1=1.22, presented as the mean intensity of 65 decibel sound pressure level (SPL) for f1 and 55 decibel SPL for f2. The study was carried out with the rats with emissions, and those (six rats in total) whose emissions were not obtained were excluded from the study.

Subdermal needle electrodes were inserted as follows: the recording electrode was inserted percutaneously along the contralateral mastoid bone. The reference electrode was placed intratympanic, and the ground electrode was placed on the vertex. Electrocochleography recordings were obtained through an insulated, stainless steel needle electrode, passed through the tympanic membrane with the guidance of an otoscope under general anesthesia. Stimuli (click) were created, and the electrophysiological response was recorded. The auditory stimulus was delivered from a speaker (free field), placed and positioned close to the test ear. The distance between the speaker and ear varied between 2 and 5 cm. The click stimulus was elicited by a 100 ms electrical square wave at 90 dB intensity and at a rate of 10/s. The resultant click waveform was analyzed by measuring both the deflection of the summation potential (SP) and the action potential (AP) from the baseline (Figure 2.1).

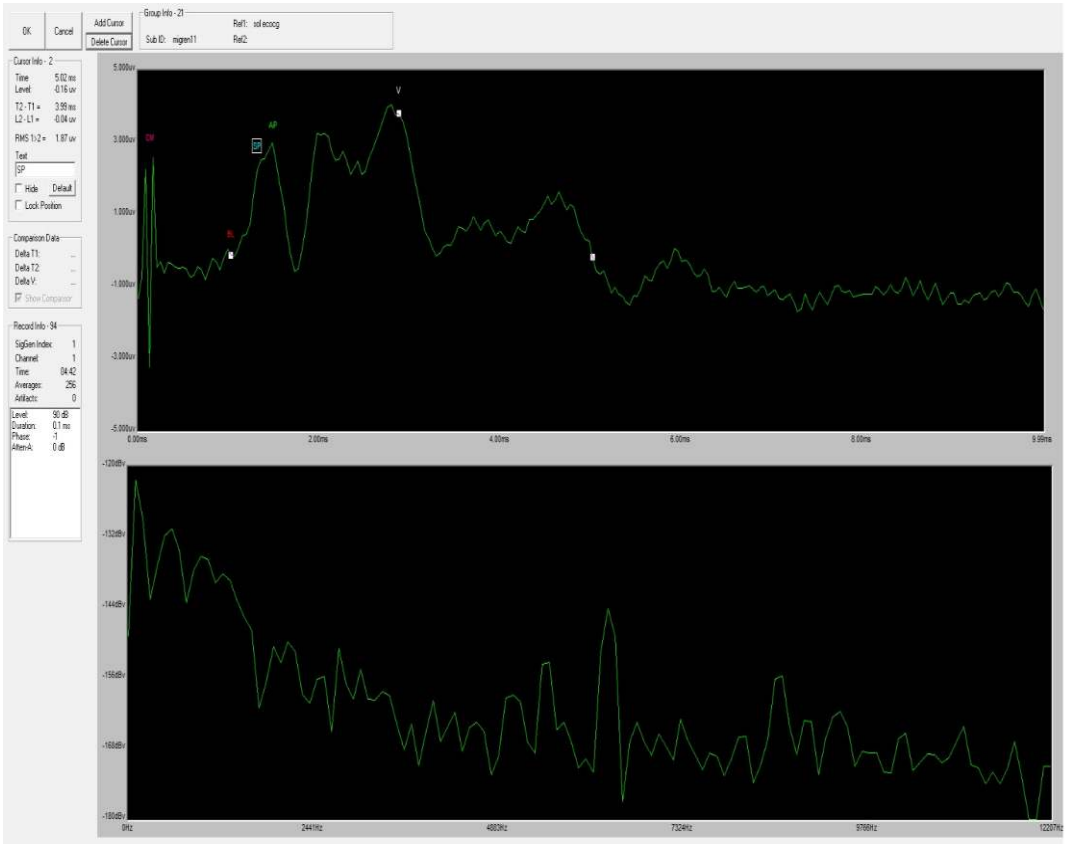

Figure 2.1 Representative click waveform with both the deflection of the summation potential (SP) and the action potential (AP) from the baseline

The electrodes for the ABR were inserted subcutaneously: one on the mastoid of the tested ear (reference), one on the vertex (active), and one on the contralateral mastoid (ground). The sound stimuli were presented directly to the ear canal. ABRs were determined using tone-burst stimuli at frequencies of 6, 12, 16, 20, and 32 kHz (10 ms duration, r/f time: 0.5 ms) and a click stimulus (0.5 ms duration, r/f time: 0.05 ms.). ABR-based hearing thresholds were detected by visual inspection at the lowest intensity at which a pronounced response was present (Figure 2.2). Seven or eight rats' auditory tests were done per day.

All rats' DPOAE, ABR, and ECOG measurements were repeated on day 10 of the study.

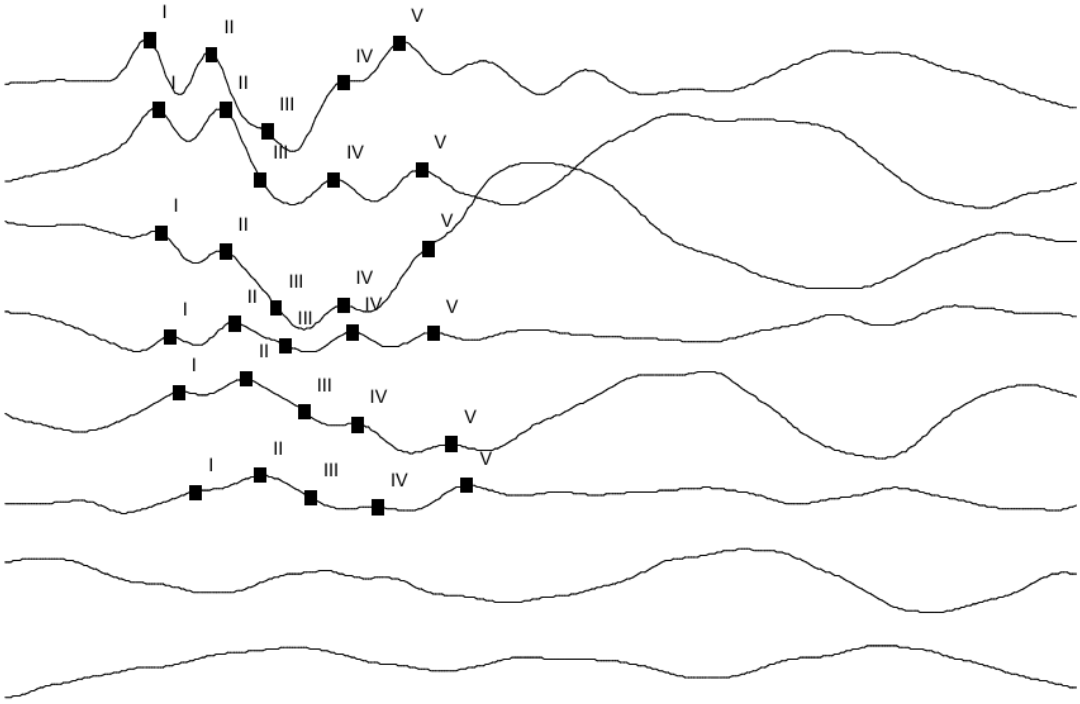

Figure 2.2 The representative image of an ABR waveform of one animal.

2.3 Study groups

Group I was the control with 0.25 ml saline injection. Group II was the chronic migraine model, and animals were injected with diluted nitroglycerin (NTG) (Sigma, 1466506) intraperitoneally (IP) at a dose of 10 mg/kg every other day for nine days (Pradhan et al., 2014). NTG was prepared from a stock solution of 5 mg/ml dissolved in water, 30% propylene glycol, and 30% alcohol. NTG was diluted to a dose of 1 mg/ml with saline, containing 6% propylene glycol and 6% alcohol. Group III, the treatment group, was injected with NTG in the same way as Group II. Olcegepant (Ab269696) was dissolved in DMSO (5%) and corn oil (95%) to a final concentration of 1.5 mg/ml and was administered IP in a volume of 1 mg/kg, one hour and 15 minutes later after the administration of NTG every other day for nine days (Greco et al., 2022).

2.4 Behavioral Tests

2.4.1 Orafacial Formalin Test

The subcutaneous injection of formalin (1.5%, 50 µl), an aqueous solution of 37% formaldehyde, was performed into the right upper lip with minimal animal restraints on day nine, two hours after the NTG injections. Immediately after the injection, each animal was placed into the observation box (30x30x30-cm glass chamber with mirrored sides), and rubbing behavior was recorded with a camera located 50 cm from the box for the off-line analysis (Greco et al., 2022). The recording was in 4 blocks of 3 minutes each, between 6th to 9th, 18th to 21st, 30th to 33rd, and 42nd to 45th minutes after the formalin injection. Pain-related behavior linked to the trigeminal activation was quantified by measuring the seconds the animal spent grooming the injected area (face rubbing) with the ipsilateral fore- or hind paw and how many times it groomed. The test consisted of 2 phases spaced by a latency period of 6–12 min: Phase I (0–6 min) refers to the acute pain, while Phase II (12–45 min) reflects the combined effects of afferent input and central sensitization. An investigator who is blinded to the animal's group assignment analyzed the rubbing behavior.

2.4.2 Open Field Test

The open-field test (OFT) is widely used to demonstrate the differences in locomotor activity. Several authors have commented on the influence of pain, particularly inflammation, on an animal's motor behavior, both in terms of general activity and its effect on the performance of a particular locomotor behavior. Animal activity measurements could then provide an objective measure for categorizing pain ((Whittaker & Howarth, 2014). Locomotor activity was assessed through measurements of the immobility time using the AnyMaze video-tracking software.

For OFT, The animals were placed in to a plexiglass box with 40x40x40cm dimensions (Seibenhener & Wooten, 2015). The activity of each animal in the arena was meticulously recorded for five minutes on day 5. This video recording was analyzed using the highly precise AnyMaze software (Stoelting Europe, Dublin, United Kingdom). The software first analyzed the total distance traveled by each animal in meters and then automatically detected and reported the duration of the time that the animal was not moving. The freezing time was reported as the percentage of the total test duration, providing a comprehensive view of the animal's activity. Other than animals' motor

activity, the animals were observed for specific activities that helped to evaluate the rats' vestibular function, like dyskinetic head movements and circling, and tail hang reflex (Zhang et al., 2020).

2.4.3 Tail-Hang Reflex and Head Circling

The tail-lifted rats' responses were rated as follows: 0 = straight body posture towards the ground with the extension of forelimbs, 1 = sporadic bending of the body ventrally, and 2 =persistently bending the body towards its tail (Zhang et al., 2020).

Rats were observed for head weaving or circling. Their response was rated as follows: 0 = neither dyskinetic head movements nor circling, 1 = sporadic dyskinetic head movements or circling, and 2 = frequent to persistent dyskinetic head movements or circling (Zhang et al., 2020).

2.5 Tail Blood Samples

Great care was taken during the blood sampling process. Animals were placed in a restrainer to ensure their comfort and safety while their tails were held. Blood was then sampled from the tail vein of each rat within an hour after the NTG infusion on day 9. The samples were put into a 5 ml lavender tube (BD vacutainer TM, Becton Dickenson, Plymouth, UK), and plasma samples were prepared by centrifugation (2000 rpm for 15 min) and then stored at − 80° C.

2.6 Cerebrospinal Fluid Samples

Under the influence of general anesthesia after the hearing evaluation, animals were placed through the external auditory canal to the rat brain stereotaxic instrument on day 9. The occipitocervical region of the rat was shaved, and a vertical incision was made by a #15 blade; muscles were dissected laterally, and an atlantooccipital membrane was seen. A tuberculin syringe was used to reach the cisterna magna through the atlanto-occipital membrane. Approximately 0.2-0.3 cc cerebrospinal fluid (CSF)was collected from the animals and was put into labeled Eppendorf tubes, centrifugated at 2000 rpm for 15 minutes, and then stored at − 80° C.

2.7 Perfusion

After the CSF collection, the animals were perfused with 50ml ice-cold PBS and then 50 ml of 4% paraformaldehyde (PFA). An intracardiac puncture was performed to collect the blood. A tuberculin syringe was used to do this, and 0.2-0.3 cc intracardiac blood was collected. Then, the blood was put into the tubes containing EDTA as an anticoagulant. Plasma samples were prepared by centrifugation (2000 rpm for 15 min) and then stored at − 80° C.

Afterward, the animals were decapitated; the skin was removed from the skull by pulling the skin toward the nose. The skull was split along the midsagittal plane by using sharp scissors. The brains with brainstems and trigeminal ganglions were removed. The brains with brainstems and trigeminal ganglions were incubated in 4% PFA for the following 24 hours at +4 °C separately. Then, they were consequently kept in 10%, 20%, and 30% sucrose solutions until they lost buoyancy. Finally, the samples were dried from excess water with tissue embedded in the cytomatrix and kept at -80 °C.

Once the brain and brainstem were removed, the inner ears (left and right sides) within the temporal bone were identified. The inner ears were dissected from the surrounding bone by using forceps. Both inner ears from the same animal were transferred to an Eppendorf tube containing 4% PFA for the following 24 hours at +4 °C. Then, they were washed with 1X PBS (phosphate-buffered saline), transferred to a different Eppendorf tube containing 1X PBS, and then stored at +4 °C.

2.8 Cochlear Decalcification and Organ of Corti and Vestibular Whole Mount Dissection

Six right-side cochleas and Seven left-side cochleas from each group were transferred to a 5 ml tube containing 5% EDTA (Sigma-Aldrich, catalog number: E7889) and incubated at 4 °C under gentle agitation. For good decalcification, EDTA was refreshed every other day for a month. A good decalcification was when the cochleae became soft at the touch with forceps and when the cochlea bone became transparent. The decalcified cochleas were washed with 1XPBS when the decalcification was complete. Then, they were transferred into an Eppendorf tube containing PBS and stored at +4 °C.

Six right-side cochleas from each group were gone into whole-mount dissection. The Leica Microscope (M125 C) was used for this purpose. First, the saccule was dissected with fine surgical tools #5 and #55 forceps under the microscope. Then, the otic capsule, the lateral wall, Reissner's membrane, and the tectorial membrane were removed using tools #5 and #55 forceps and Phaco blades. The organ of Corti was further dissected into a surface preparation and microdissected into individual turns. Five individual turns from each cochlea, and saccule were put into six separate PCR tubes containing cold 1xPBS and stored at 4 °C.

Note: The video of Liberman's laboratory shows a method of surface preparation dissection of the organ of Corti. http://www.masseyeandear.org/research/ent/eaton-peabody/epl-histology resources/video tutorial-for-cochlear-dissection/

2.9 Stria Vascularis Dissection

Four right-side cochleas from each group were separated for stria vascularis (Stria V) dissection; thus, they were not decalfied. #5 Forceps were used to break the cochlear bone from the apex under the microscope (Leica M125 C). Then, by using #55 forceps, the apical turn was scraped away, and gentle force was applied to break away small pieces of the outer bone layer from the apical, middle, and basal turn. The lateral wall and spiral ganglions were detached, and Stria V was dissected in smaller pieces. The Stria V pieces were put into PCR tubes containing cold 1XPBS and stored at 4 °C.

2.10 Tissue preparation for hematoxylin and eosin (H&E) staining of the cochleas

After fixation and decalcification processes, ten left-side cochleas from each group were blocked after paraffin infiltration. The tissue was sectioned with a microtome (Microm HM 360, Walldorf, Germany) in slices of 5-μm thickness along the mid-modiolar plane, and every fifth slide was mounted and stained with H&E. The volume of scala media (SM), and the scala vestibule (SV) is measured to quantify possible hydrops. Sections were scanned by the ultra-fast scanner (Philips, Netherlands), and images were transferred to a digital pathology software program (Philips, Netherlands) and exported. The Image J software program was used to analyze all images. A modified version of a technique described by Klis et al. was used to determine possible hydrops (Klis et al.,

1990). Every slide prepared from the rats' left ears was evaluated. The ones from which it was possible to measure the area of both SM and SV on both sides of the modiolus were studied. The proportion of the combined SM and SV area made up by SM was calculated. In formula: proportion of SM (PSM)=SM Area/ SM Area + SV Area. This is used to compensate for any slight deviations in the plane of the section between animals. PSMs were summed from each turn taken for each cochlea (Hott et al., 2003).

2.11 ELISA

Plasma samples and CSF were used for ELISA studies. The frozen samples were thawed at room temperature.

For determining the TNF-alpha and IL-6 levels, plasma samples from the tail blood, CGRP levels intracardiac blood samples, and HMGB1 levels CSF samples were used. The ELISA was conducted according to the manufacturer's instructions. The list of ELISA kits is listed below: Rat CGRP1(Calcitonin Gene Related Peptide 1) ELISA Kit (Elabscience, E-EL-R0135), Rat TNF-α(Tumor Necrosis Factor Alpha) ELISA Kit (Elabscience, E-EL-R2856), Rat IL-6(Interleukin 6) ELISA Kit (Elabscience, E-EL-R0015), Rat HMGB-1(High Mobility Group Protein B1) ELISA Kit (Elabscience, E-EL-R0505).

For the analysis, the link below was used.

GainData ELISA data analysis | free ELISA data calculator -arigobio - arigo Biolaboratories

2.12 Immune Fluorescence Staining

2.12.1 Trigeminal Ganglion and The Nucleus

The frozen brainstem and trigeminal ganglion samples embedded in resin (OCT medium) were sectioned 10µm with a cryostat on poly-l-lysine-covered glass slides. The staining protocol started with dipping in distilled water to eliminate resin (OCT) residues on tissues. Then, the slides were incubated for 10 minutes in 0.5% Triton X-100 in PBS for permeabilization and washed with PBS. After washing, the sections were incubated in superblock solution (Thermo Scientific™, PI37535) for 60 minutes at room temperature.

The primary and secondary antibodies were also diluted in the superblock. After blocking the nonspecific bondings, the slides were incubated with primary antibodies overnight at 4 °C. The primary antibody used in this study was rabbit anti-CGRP (Ab189786). After incubation with the primary antibody, the sections were washed with 0.025% Triton-X in PBS and incubated for 90 minutes at 37 °C as the appropriate secondary antibody. Finally, the secondary antibody was removed, and the slides were rewashed and mounted with 4 ‘, 6-diamidino-2-phenylindole (DAPI, Abcam). The fluorescent images were taken with the Leica DMI8 SP8 DLS/CS microscope.

2.12.2 Organ of Corti, Saccule, and Stria Vascularis

Tissue was placed in 0.5% Triton X-100/1XPBS for 10 min and then placed in a blocking buffer (Super Block, ScyTek Biotech life sciences, USA) for 1hr at RT, with gentle rotation. Tissue was incubated with primary antibody diluted in blocking buffer overnight with gentle rotation at 4 °C and then washed four times 5 minutes with 0.025% Triton X-100/1X PBS. All samples were then incubated with secondary antibody for 90 minutes at 37 °C then washed with 0.025% TritonX-100/1X PBS 4 times 5 minutes. Next, tissue was transferred to microscopic glass slides and mounted with Dapi, and closed by a coverslip. Later, the slides were imaged with the Leica DMI8 SP8 DLS/CS or STED (for imaging Anti-Myosin and Anti-CTBP2) microscopes (2 μm stacks) at Koc University Confocal Microscopy Core. Confocal image Z stacks were compiled, and images were created using LasX Software.

The primary Antibodies for the organ of Corti were;

1- Anti-Myosin VIIa/MYO7A antibody [EPR7497] ab150386

2- Anti-Ionotropic Glutamate receptor 2 antibody [EPR18115] ab206293

3- Alexa Fluor® 488 Anti-CTBP2 antibody [EPR7611(B)]

The primary Antibody for the organ of corti and saccule was Anti-CGRP (Ab189786).

The primary Antibodies for Stria vascularis were;

1- Isolectin GS-IB_4 From *Griffonia simplicifolia*, Alexa Fluor™ 488 Conjugate (I21411).

2- Anti-CGRP (Ab189786).

The secondary antibody for organ of corti, saccule, and Stria Vascularis was CY3 goat anti rabbit.

2.13 Image Analysis

The fluorescent images were processed using the LASX program (Leica, Wetzlar, Germany) and exported as TIF files. The number of inner hair cells (IHC), synaptic ribbons (glutamate for post-synaptic, CTBP2 for presynaptic), and CGRP dots were calculated from the images of the immunolabeled proteins using the Image J software (NIH, USA) program.

2.14 Statistical Analysis

All statistical analyses were done with GraphPad Prism 8 software (San Diego, USA) or IBM's SPSS statistical software (IBM, Armonk, NY, USA). Data were presented as Mean±SD if not mentioned otherwise. Normality assumptions were made using the Shapiro-Wilk normality test. The groups' comparisons were made using the One-way ANOVA or Kruskal-Wallis test. The Mann-Whitney U test was used to compare the two groups. Among the behavioral data, statistical analysis was performed for Time Immobile measurements. Normality and homogeneity of variance assumptions were tested with Kolmogorov-Smirnov and Levene's tests. Whenever the assumptions were met, multifactorial analysis of variance (ANOVA) test with factors of treatment having three levels (control/migraine/olcegepant), and time having two levels (Day1/Day5) followed by Bonferroni posthoc tests to adjust for multiple comparisons. A non-parametric Kruskal Wallis test followed by Mann Whitney U tests for pairwise comparisons with Bonferroni corrected p-values were used for the cases where the assumptions of normality or homogeneity of variance were violated. $p < 0.05$ was considered significant for all tests.

Chapter 3

RESULTS

3.1 Animals

Forty male Wistar rats aged 2-5 months weighing 240 ± 50 grams were recruited for the experiments. Ten out of 40 animals (25%) were excluded from the study because distortion product otoacoustic emissions (DPOAE) could not be obtained from those ten animals. When we started the experiments, there were thirty rats, and ten were divided randomly into each group.

One animal died before the last ABR in group three, but its tissues were still collected.

3.2 Evaluation of Auditory Tests

3.2.1 DPOAE

DPOAE thresholds were extracted from the data saved at the RZ6 auditory processor using tone stimuli at an 80-decibel sound pressure level (SPL) from 6 to 12 kHz. A statistically significant increase of db SPL at 6, 8, 10, and 12kHz in migraine group between day one and day nine was found (respectively, p=0. 0006, p=0.002, p=0.009, p=0.014). On the other hand, there was no difference in the control group. In addition, there was a significant decrease of dB SPL at the above frequencies in the treatment group between day one and day nine (respectively, p=0.002, p=0.028, p=0.018, p=0.006) (Figure 3.1, 3.2, 3.3).

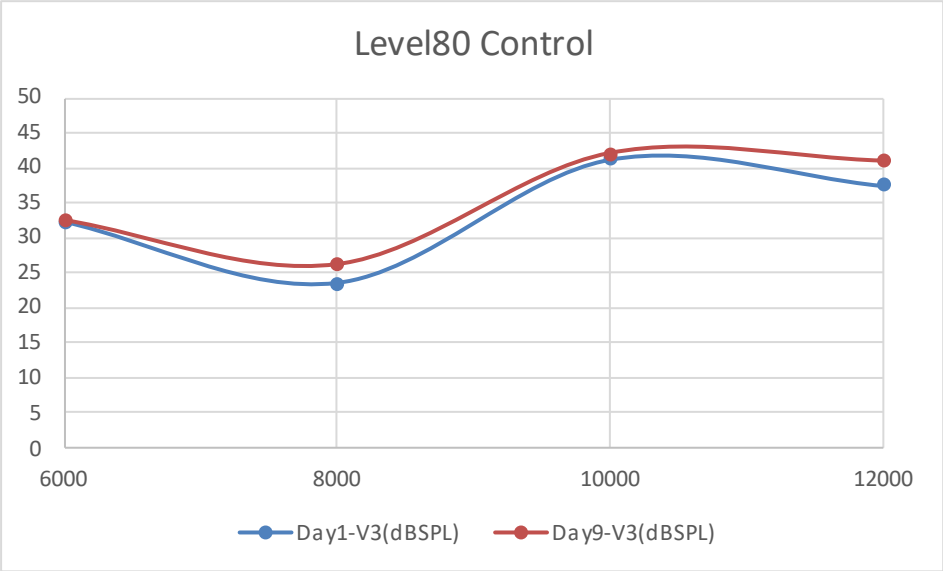

Figure 3.1 SPL thresholds of the control group according to frequencies on day one and day nine

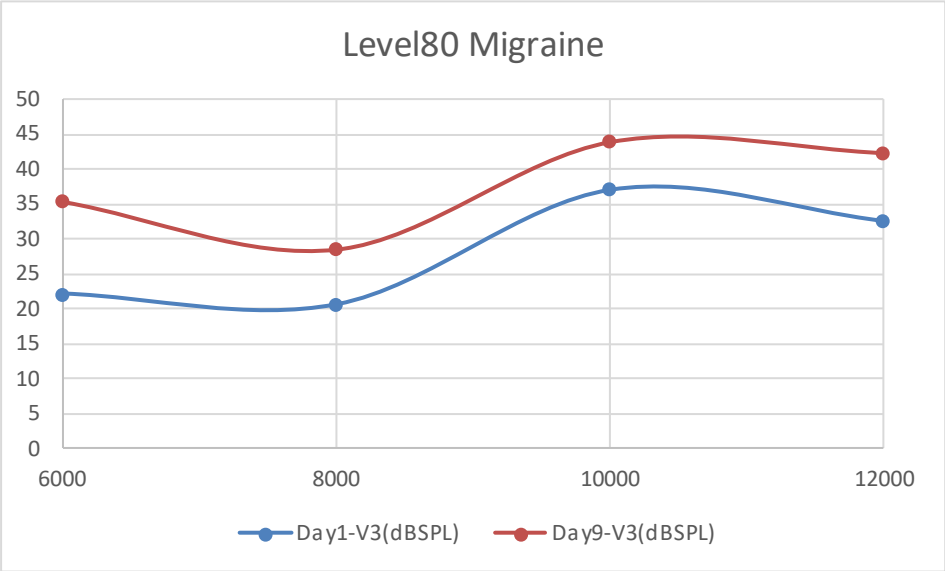

Figure 3.2 SPL thresholds of the migraine group according to frequencies on day one and day nine

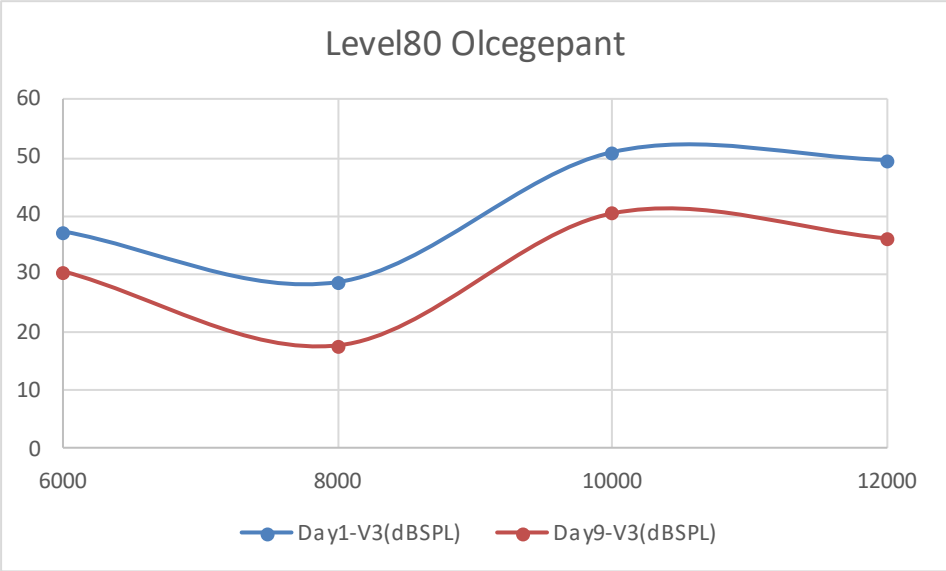

Figure 3.3 SPL thresholds of the olcegepant group according to frequencies on day one and day nine

3.2.2 ABR

3.2.2.1 Click ABR

The animals' click ABR mean thresholds were compared within the groups before and after the injections; in other words, the thresholds on day one and day nine were compared. There were no statistically significant differences within the groups, as shown in Figure 3.4.

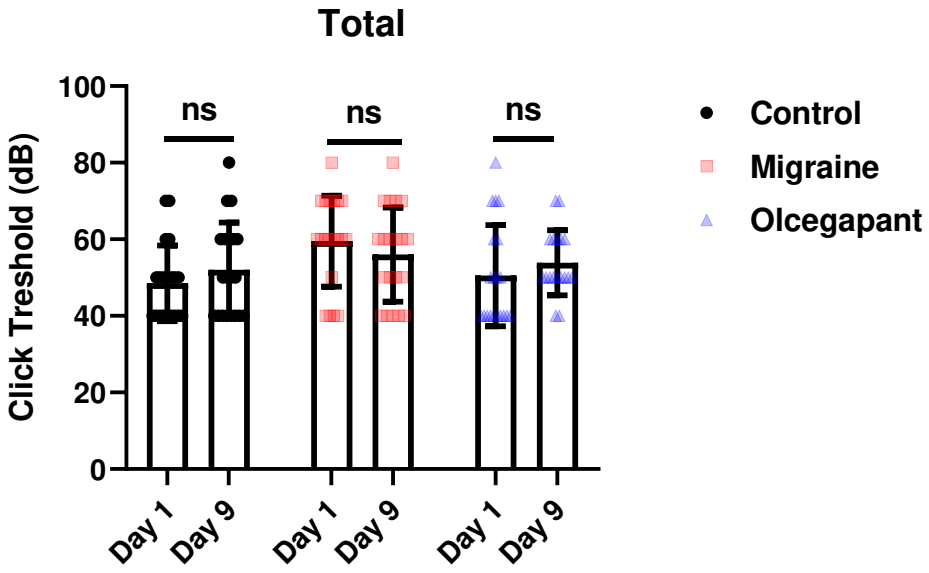

Figure 3.4 The comparison of the mean click ABR thresholds (right and left ears in total) within the groups before and after the injections

Table 3.1 The Mean Click ABR (decibel) Thresholds of the Groups

Group	**Click ABR (dB) Mean Threshold Day 1**	**Click ABR (dB) Mean Threshold Day 9**
Control	48.5	52
Migraine	59.5	56
Olcegepant	50.5	53.8

There was no statistically significant difference in the mean Click ABR threshold comparison between the groups between day one and day nine; however, the mean threshold was lower on day nine only in the migraine group compared to day one (Table 3.1).

3.2.2.2 Tone Burst ABR

The animals' tone burst mean ABR threshold changes between day one and day nine were compared in 6000, 12000, 16000, 20000, and 32000 Hertz separately between the groups. There was a statistically significant difference at 6000 Hz between the control and the migraine groups, the migraine and the treatment groups at multiple comparisons (respectively p=0.038 and p=0.003). In addition, at 12000 Hz, there was a significant difference between the control and the migraine group, p= 0.030, and also at 20000 Hz, between the same groups with a p-value of 0.018 (Figure 3.5).

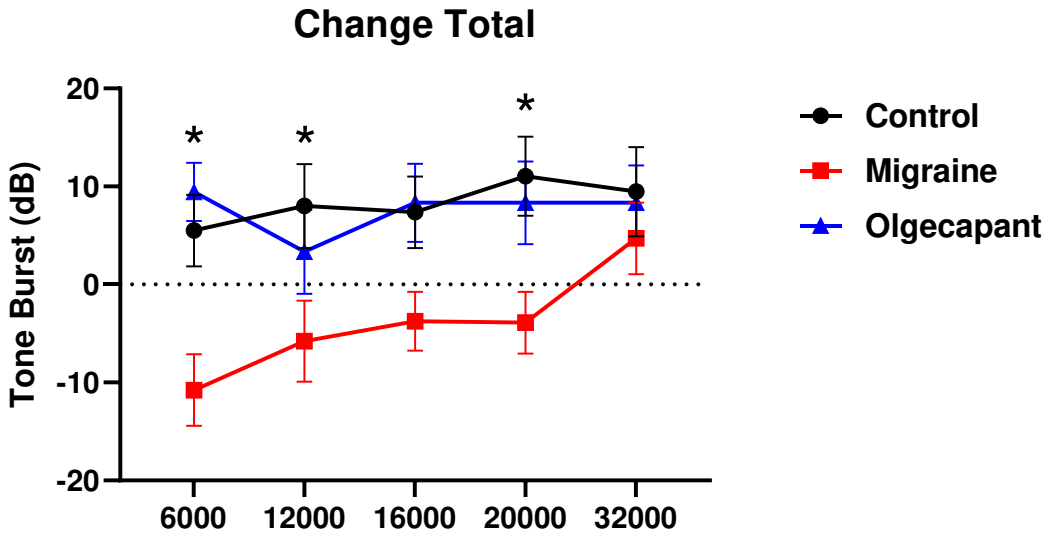

Figure 3.5. Tone Burst Changes between the groups

Table 3.2 gives the mean tone-burst ABR thresholds of the groups' at different frequencies.

Frequency	**6000 Hz**	**12000 Hz**	**16000 Hz**	**20000 Hz**	**32000 Hz**
Group 1 (Control)					
Day one	66 dB	52,5 dB	58,5 dB	58 dB	55,5 dB
Day nine	71,5 dB	60,5 dB	65,2 dB	68,9 dB	64,7 dB

Group 2 (Migraine)					
Day one	78,8 dB	67,5 dB	68,3 dB	71,5 dB	64,7 dB
Day nine	70,6 dB	62,1 dB	65,5 dB	68,4 dB	70 dB
Group 3 (Treatment)					
Day one	65,5 dB	58,5 dB	62,5 dB	60 dB	60 dB
Day nine	73,3 dB	61,6 dB	70 dB	68,3 dB	67,2 dB

3.2.3 ECOG

When SP/AP ratios were compared within the groups before and after the injections, on day one and day nine, the treatment group had a significantly lower ratio on day nine than on day one, p=0.049 (Figure 3.6).

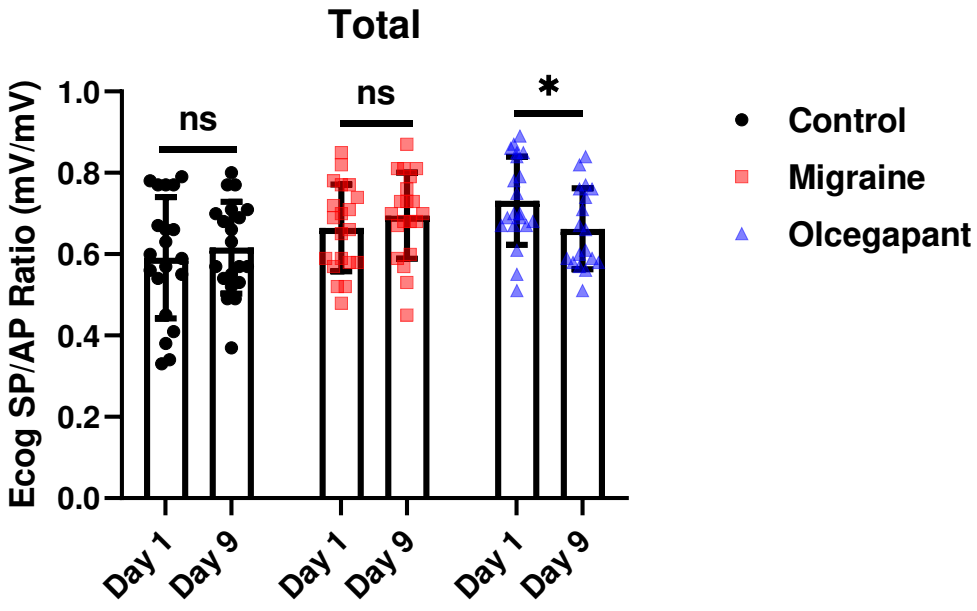

Figure 3.6 SP/AP ratio comparisons within the groups

The mean SP/AP ratios of the groups on day one and day nine are shown in Table 3.3 and Figure 3.7.

Table 3.3 The mean SP/AP ratios of the groups

SP/AP		
	Day One	**Day Nine**
Group 1	0,59	0,61
Group 2	0,66	0,69
Group 3	**0,73**	**0,66**

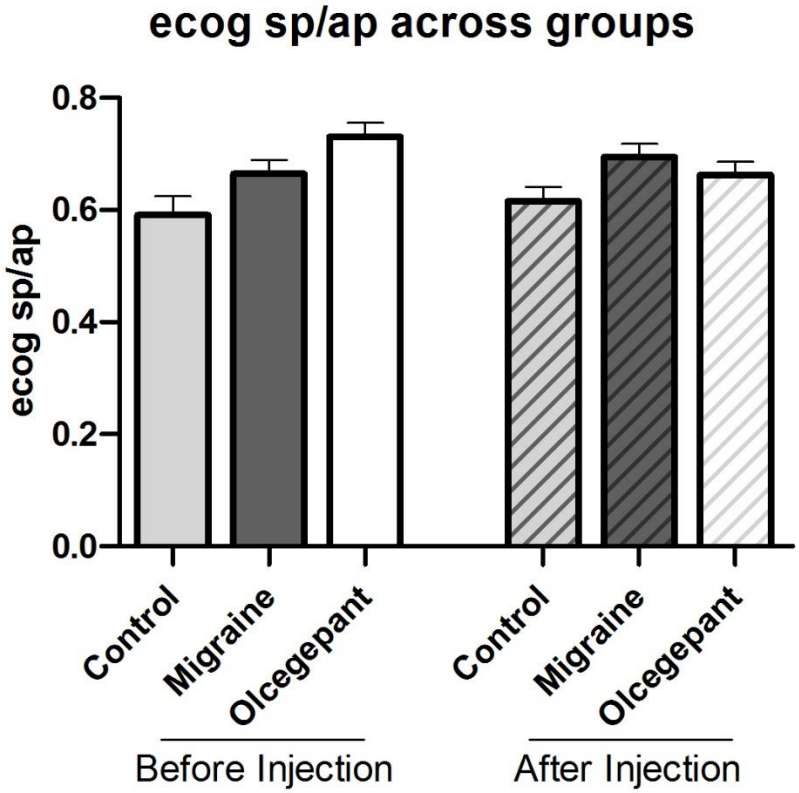

Figure 3.7 SP/AP Ratios across the groups.

There was a statistically significant difference between the migraine and the control group with a p-value of 0.054 and the control and the treatment group with a p-value of 0.018.

3.3 ELISA Results

To understand the mechanisms of inflammation in migraine, the tail blood plasma TNF-Alpha and IL-6 levels, CGRP levels in intracardiac blood plasma, and HMGB1 levels of the CSF were measured with the ELISA method.

When we compared the levels of TNF-Alpha between the groups, there was a statistically significant difference between the migraine and the control group (p=0.048). IL-6 was not statistically different between the groups (Figure 3.8)

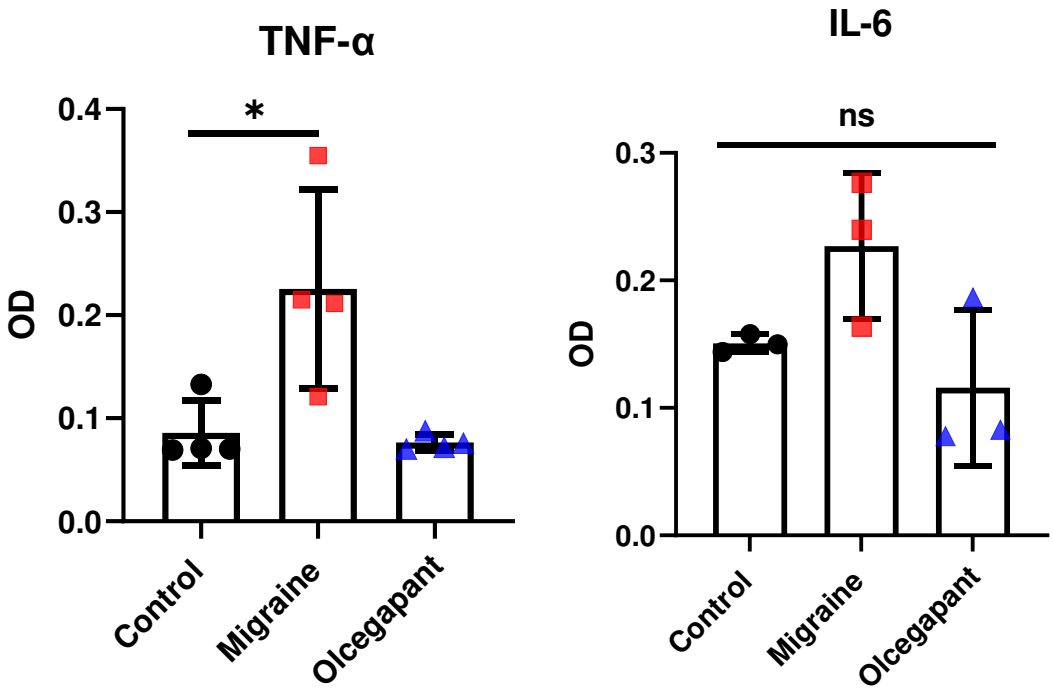

Figure 3.8 Tail blood TNFmeasurements. Data is presented as optical density volume

When we compared the levels of CGRP, no difference was found between the groups (Figure 3.9).

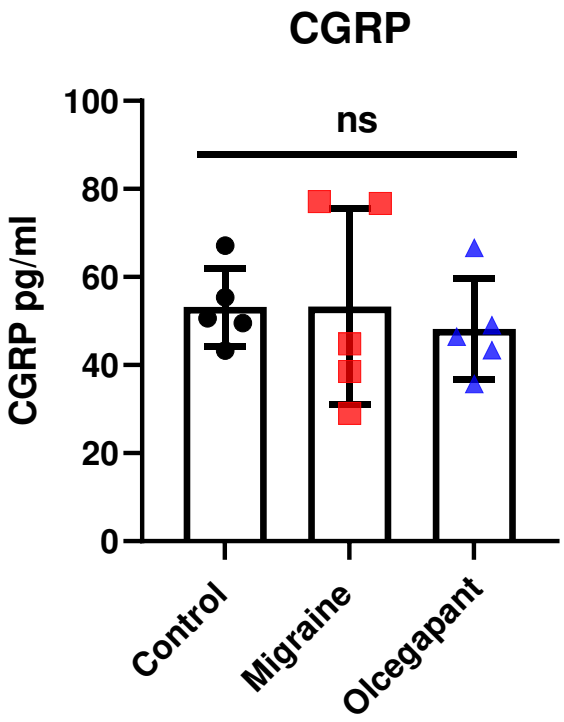

Figure 3.9 The comparison of CGRP levels in intracardiac blood samples

Since we could not obtain enough CSF samples for the ELISA analyses, we put the CSF of 5 animals from each group into 3 wells without dilution. This did not allow us to do a statistical comparison, but the concentration of HMGB1 levels differed between the groups, as shown in Table 3.4.

Table 3.4 CSF HMGB1 Concentrations

	n	CSF HMGB1 Concentration (pg/ml)
Control	*5*	1.76
Migraine	*5*	3.57
Olcegapant	*5*	2.15

3.4 Behavioral Evaluation

3.4.1 Open Field Test

The total locomotor activity of the animals was assessed with the open field test on day 1 and day 5. We preferred to look at the animals' activities on day 1 and day 5 instead of day 1 and day 9. The reason was that the last day of the experiments (day 9) was planned to be very busy with the hearing evaluations, the blood sampling, and the perfusions; thus, we chose day 5 as the last day of the behavioral tests.

A repeated measure two-way ANOVA test was applied. Statistical differences were observed for both the main effects of treatment ($F = 23.53$, $p < .001$) and between Day 1 and Day 5 measurements ($F=6.68$, $p=0.015$). Overall, immobility time was increased for all groups on Day 5 compared to Day 1. Further, Bonferroni post hoc tests for treatment comparisons showed that immobile time spent was significantly higher in the migraine group compared to the control group ($p=.001$) (Figure 3.10).

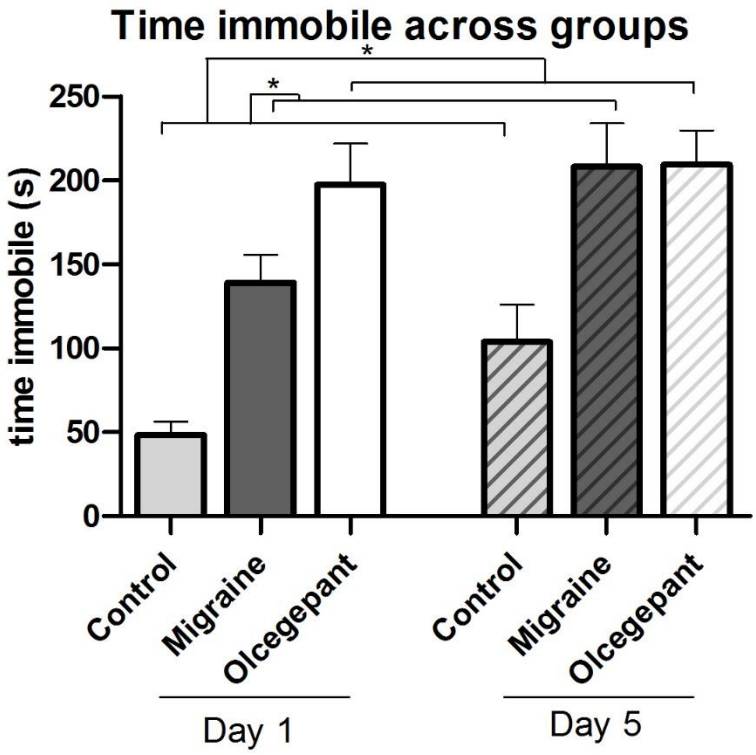

Figure 3.10 Time spent immobile across the groups on day one and day five.

Next, we calculated the immobility time differences between Day 1 and Day 5 (as Day 1 minus Day 5), converted the numbers to absolute, and compared the activity differences between Day 1 and Day 5 among the groups. Although the immobility was numerically increased in the migraine group compared to the control and olcegepant groups, no significant difference was found between groups ($F=1.85$, $p=0.18$) (Figure 3.11).

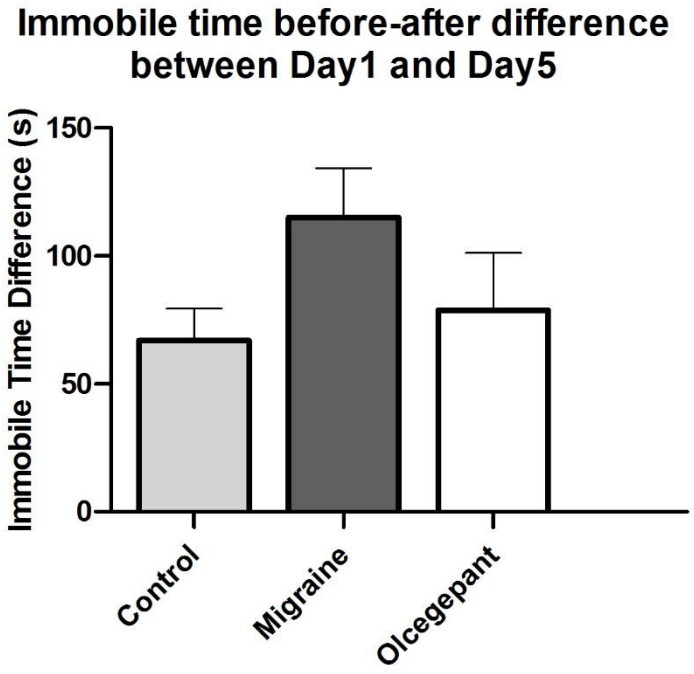

Figure 3.11 Immobility time difference between day one and day five within the groups.

3.4.2 Tail-Hang Reflex and Head Circling

All the rats on day one and day five showed straight body posture towards the ground with an extension of forelimbs, rated as 0, normal. No head circling was seen.

3.4.3 Orafacial Formalin Test

We calculated the total grooming time in seconds per number of grooming within the groups. Even though the time was longer in the migraine group than in the control group, there was no significant difference between them. On the other hand, there was a substantial difference between the control and olcegepant groups with a p-value of 0.001 and between the migraine and olcegepant groups with a p-value of 0.004 (Figure 3.12).

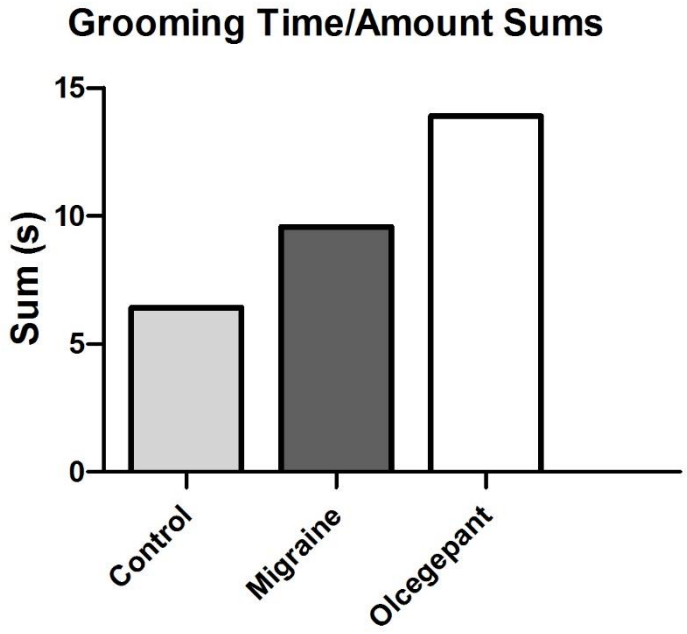

Figure 3.12 Grooming time per number across the groups

3.5 Hematoxylin-Eosin and Immunofluorescence Stainings

3.5.1 Hematoxylin-Eosin Staining of the Cochlea

PSM was measured and compared for all the groups. There was no statistically significant difference between the groups (p=0.47), but as seen in the figure below, the migraine group had the largest PSM (Figure 3.13).

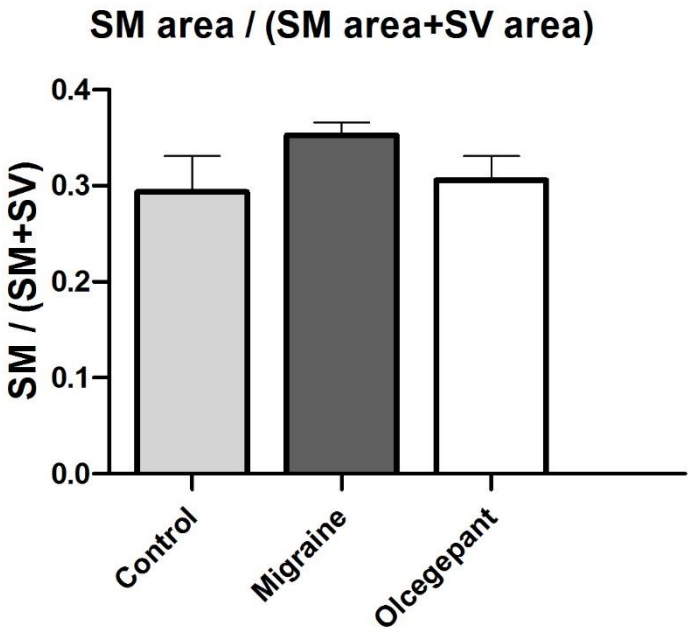

Figure 3.13 PSM across the groups

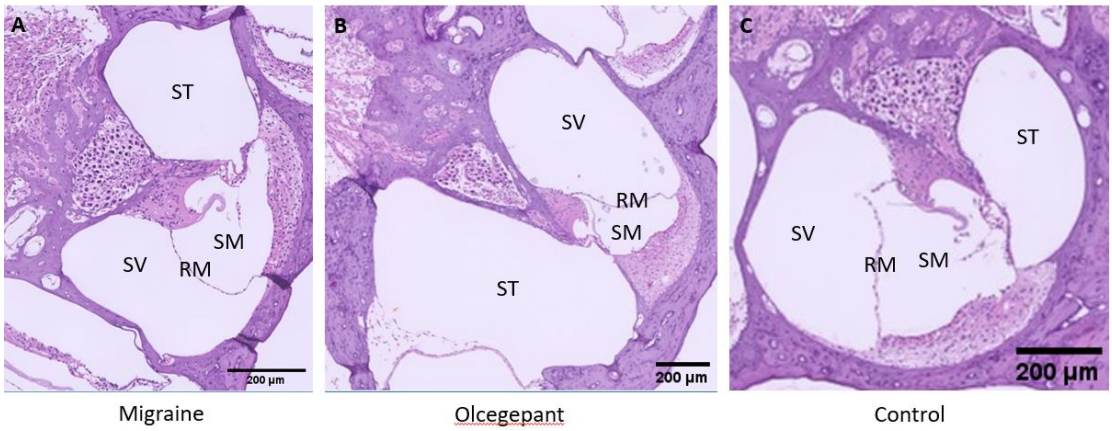

Figure 3.14 Representative images of the mid-modiolar plane stained with H&E. SV: Scala vestibuli area, SM: Scala Media area, ST:Scala tympani area, RM: Reissner Membrane

3.6 Immunofluorescence Stainings

3.6.1 Trigeminal Ganglion Staining

Trigeminal ganglions were stained with DAPI and anti-CGRP (Figure 3.15), and the mean ratio of CGRP dots to the number of nuclei was determined across the groups and compared. There was no significant difference between the groups, but the highest ratio was in the migraine group, as shown in the figure below (Figure 3.16)

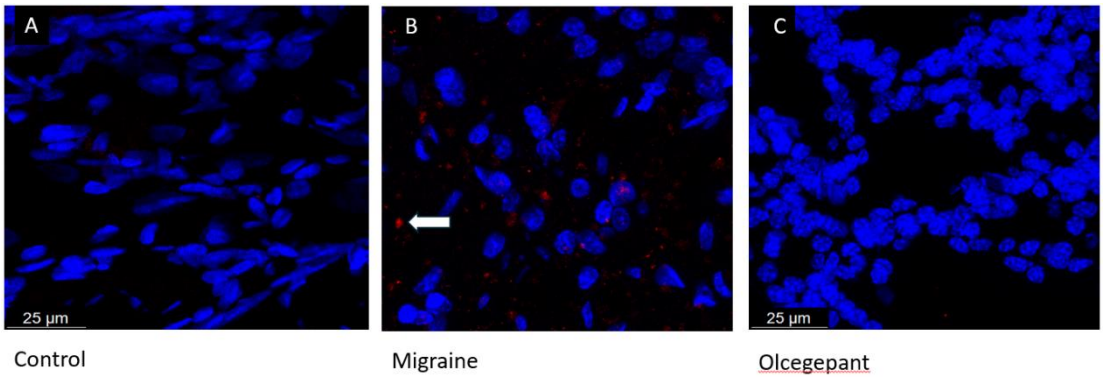

Figure 3.15 Representative images of immunostained TG of the groups. The white arrow shows CGRP dots (in red).

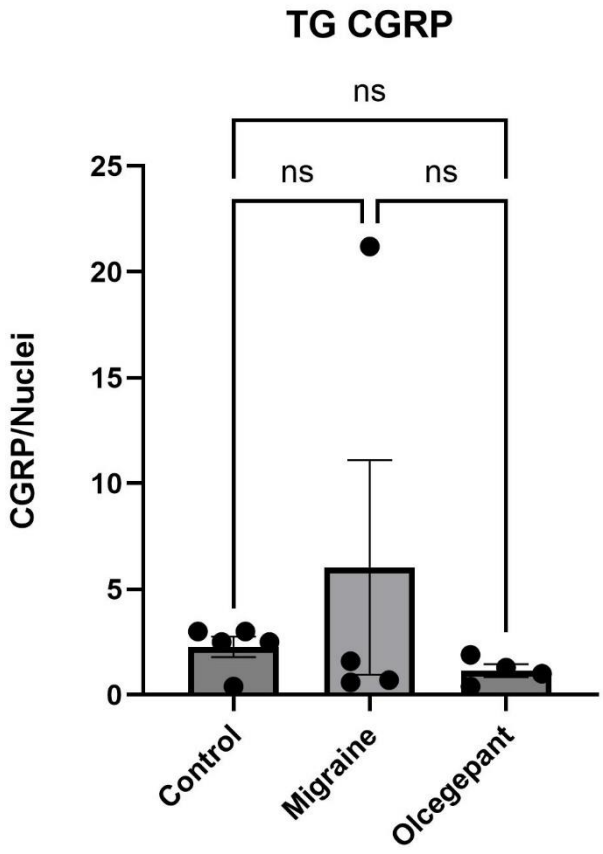

Figure 3.16 The mean ratio of CGRP per nuclei count at TG across groups (ns: not significant)

3.6.2 Stria Vascularis Staining

The distribution of CGRP around the vascular structure at stria vascularis was analysed with the Anti-CGRP and GS-IB_4 staining (Figure 3.17). The mean ratio of CGRP to vasculature length across the groups was measured, and even though there was not any significant difference between the groups, the highest ratio was in the migraine group (Figure 3.18)

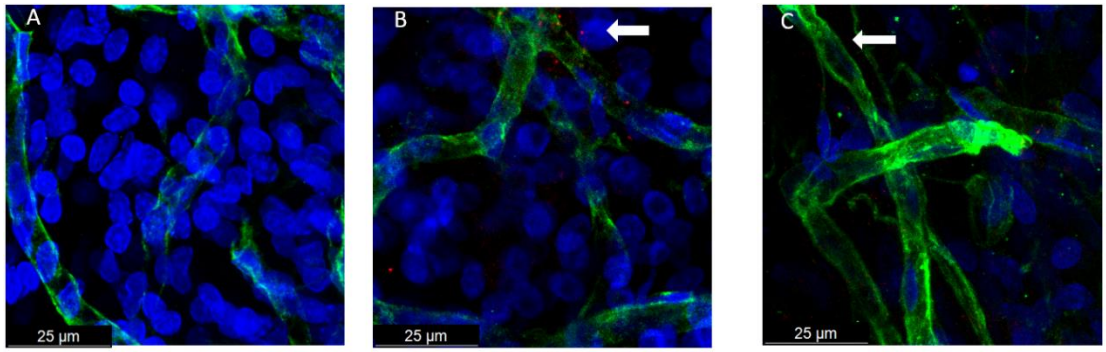

Figure 3.17 Representative images of stained stria vascularis of the groups. The white arrow in image B shows a CGRP dot (in red), and in image C shows the vascular structure (in green).

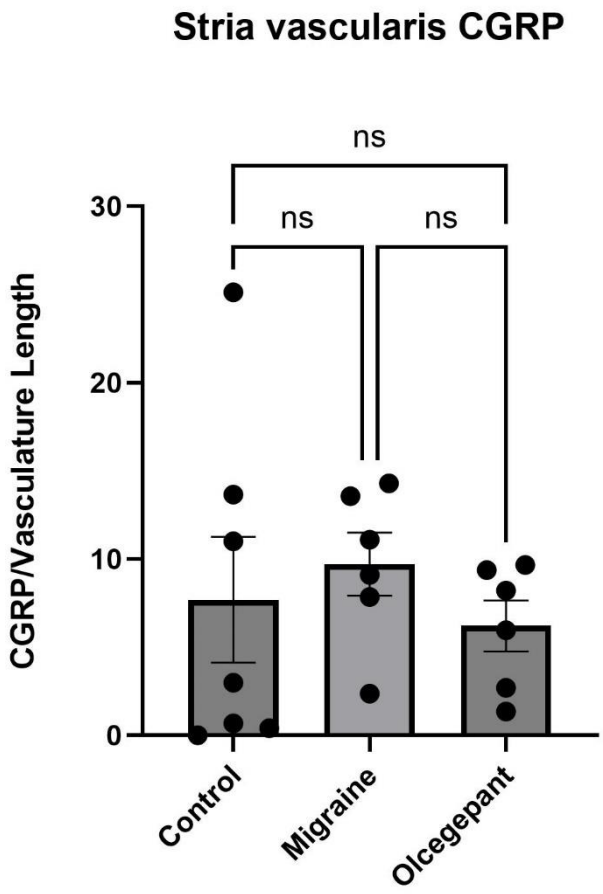

Figure 3.18 The mean ratio of CGRP to vasculature length at Stria V. across the groups

3.6.3 Saccule Staining

The saccule was stained with anti-CGRP (Figure 3.19), and the mean number of CGRP dots was higher in the migraine group. Still, there was no statistically significant difference between the groups (Figure 3.20).

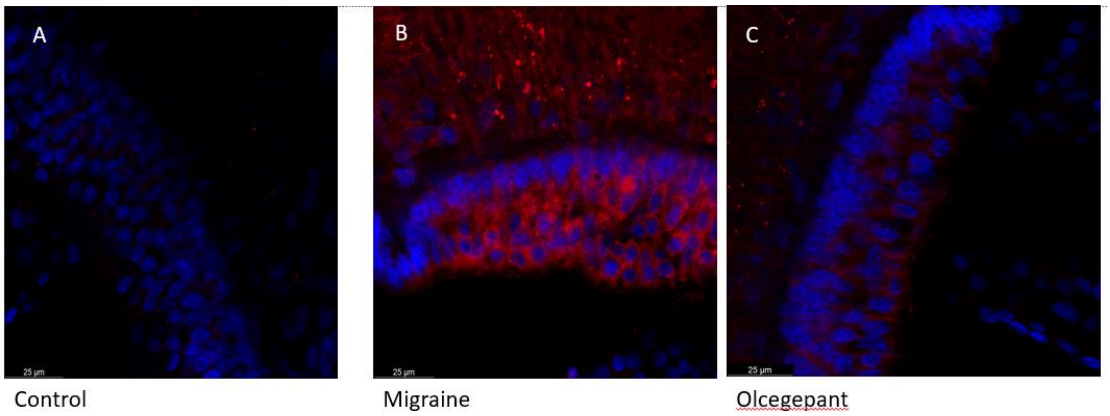

Figure 3.19 Representative images of the saccule stained with anti-CGRP (in red).

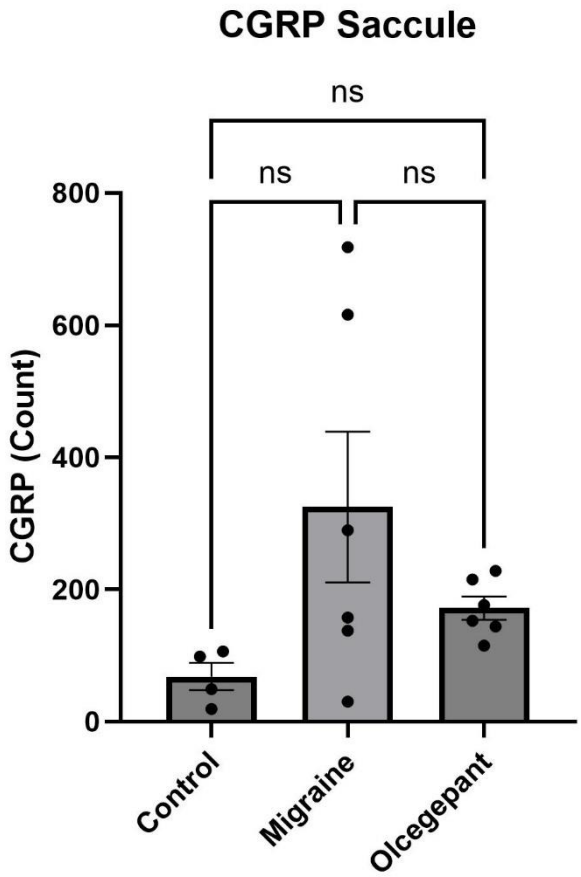

Figure 3.20 The mean CGRP count at the saccules in the groups.

3.6.4 IHC Staining with Anti-CGRP

The IHC was stained with anti-CGRP (Figure 3.21), and CGRP count per nuclei was calculated. Interestingly, the olcegepant group had a higher mean CGRP count compared to migraine and the control (respectively p=0.02, p=0.006), (Figure 3.22).

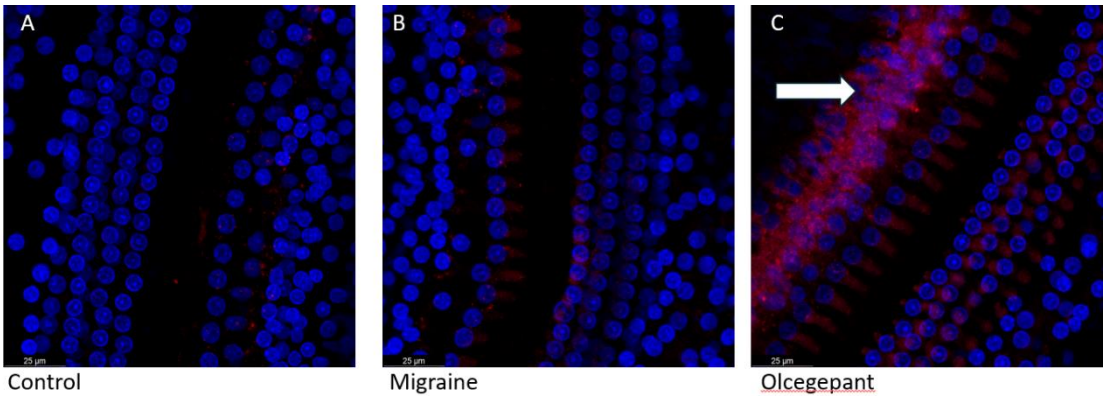

Figure 3.21 Representative images of the organ of Corti (at 6Khz) stained with anti-CGRP. The white arrow in image C shows CGRP dots around inner hair cells in red.

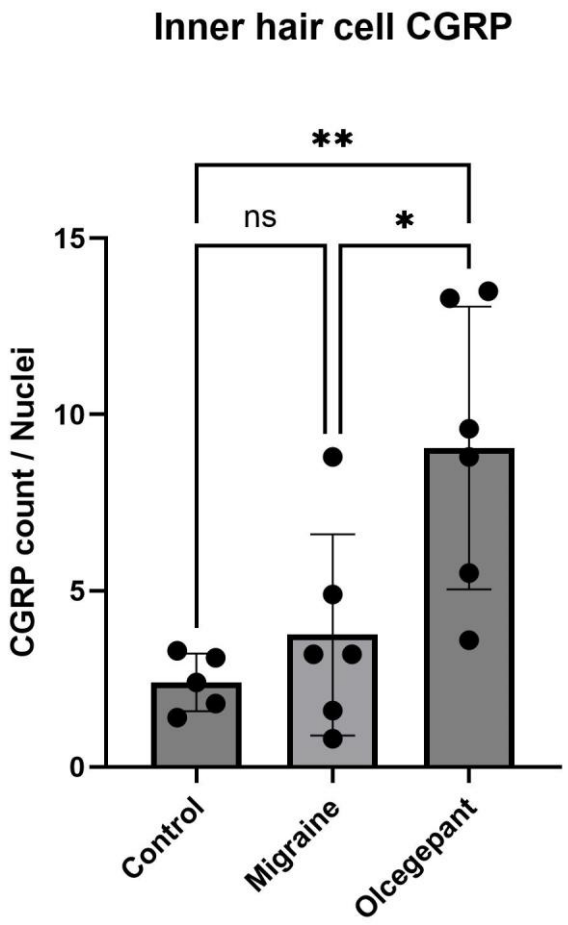

Figure 3.22 The mean CGRP count at the IHCs in the groups (* statistically significant)

3.6.5 IHC Staining with Myosin 7A

We stained the inner hair cells with the Anti-Myosin 7A antibody in the cochlea (Figure 3.23) and calculated the ratio of the hair cells to the animals' nuclei. The mean ratios of the group comparisons are shown below (Figure 3.24). There was no significant difference, but the lowest ratio was in the migraine group.

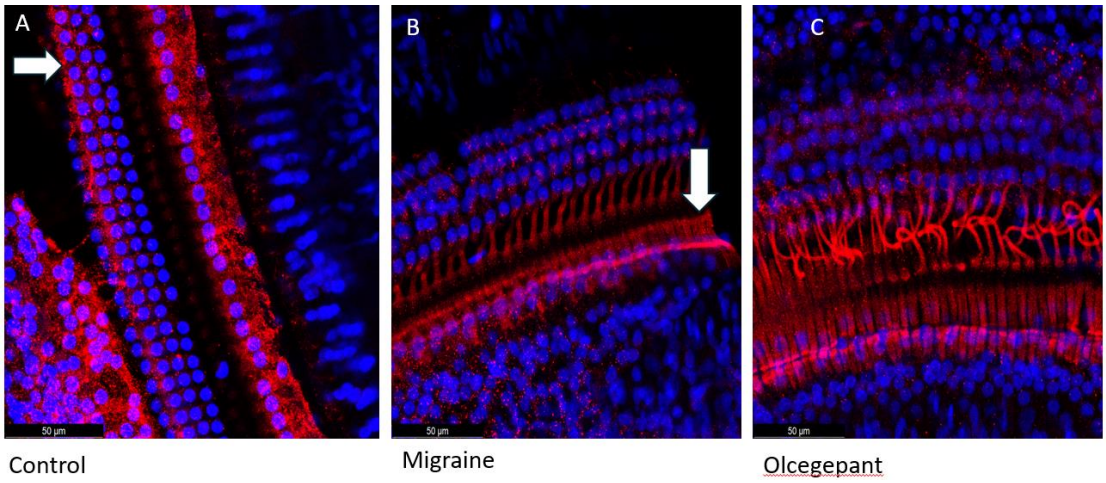

Figure 3.23 Representative images of the organ of Corti (12kHz) stained with Anti-myosin 7A antibody (red in color). The white arrow in image A shows Outer hair cells, and the arrow in image B shows the IHCs.

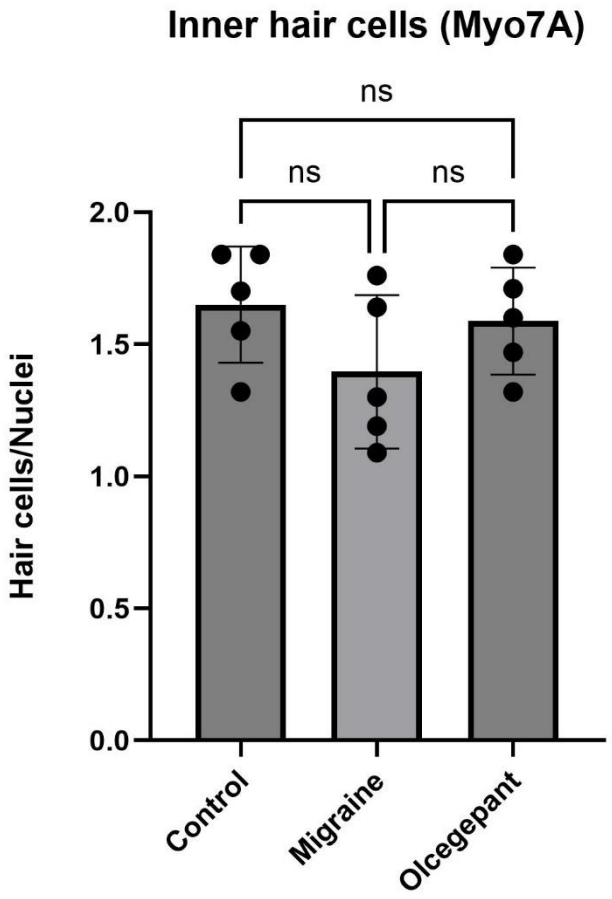

Figure 3.24 The mean IHC cell count ratios to the nuclei of the groups

3.6.6 IHC Presynaptic Ribbon Staining with Anti-CTBP2 Antibody

We then evaluated the number of afferent synapses in IHCs to label the presynaptic ribbon protein (CtBP2) (Figure 3.25). We found that the mean ratio of CtBP2 number to the nuclei was not different between the groups, but still, the lowest ratio was in the migraine group (Figure 3.26).

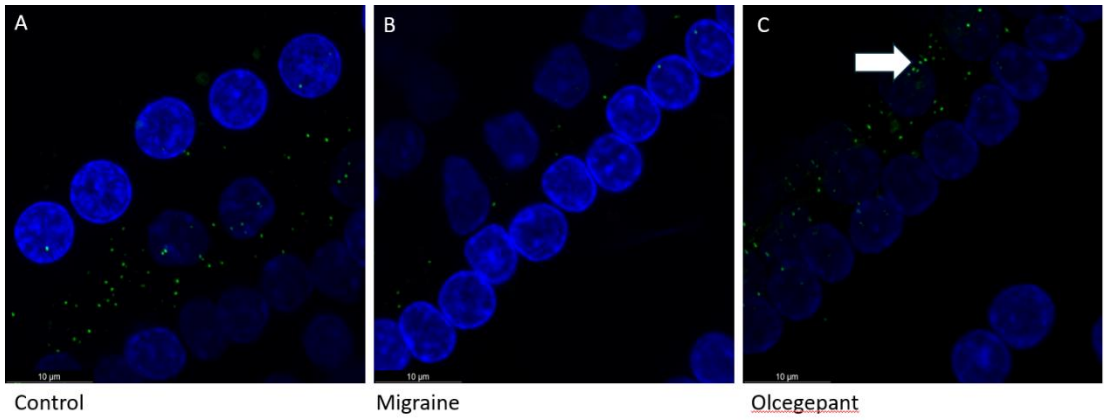

Figure 3.25 Representative images of the presynaptic ribbons in IHCs (20 kHz). The arrow in image C shows the presynaptic ribbons (in green).

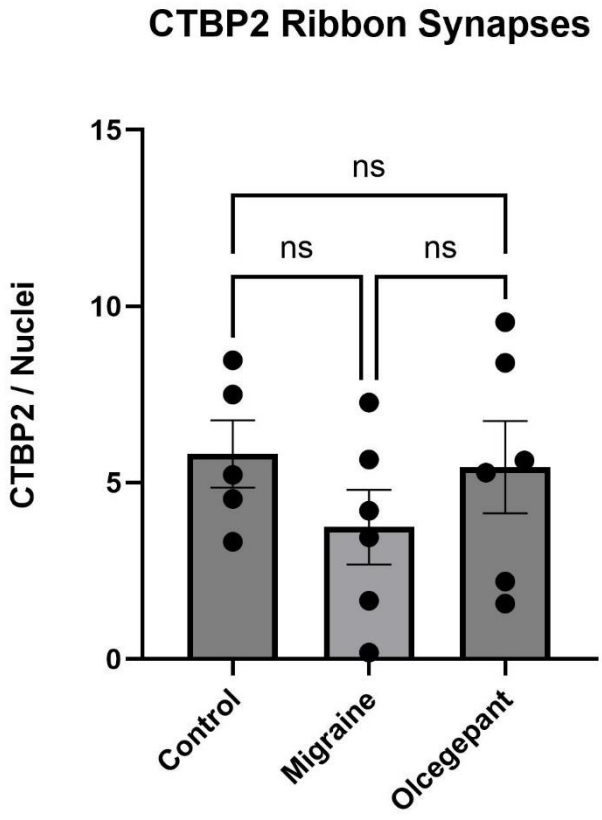

Figure 3.26 The mean ratio of CtBP2 number to the nuclei at the IHCs of the groups

3.6.7 IHC Postsynaptic Ribbon Staining with Anti-Ionotropic Glutamate Receptor 2 Antibody

We also evaluated the number of afferent synapses in IHCs using antibodies to label postsynaptic AMPA-type glutamate receptor GluR2 (Figure 3.27). The mean ratio of the number of Glutamate to nuclei per group was calculated, and there was a significant difference between the migraine and the olcegepant groups (p=0.012) (Figure 3.28). Like in CtBP2, the lowest number of glutamate was in the migraine group.

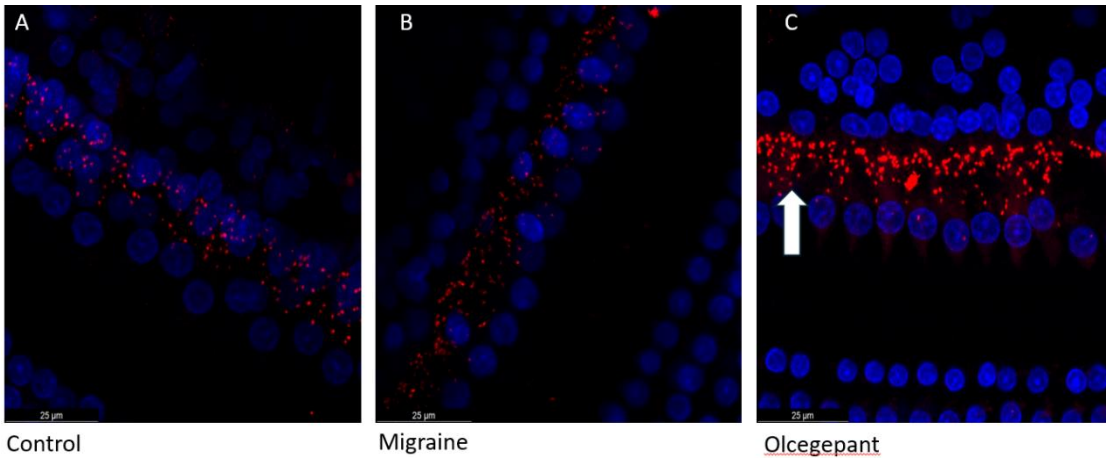

Figure 3.27 Representative images of the postsynaptic ribbons in IHCs (16 kHz). The arrow in image C shows the postsynaptic ribbons (in red).

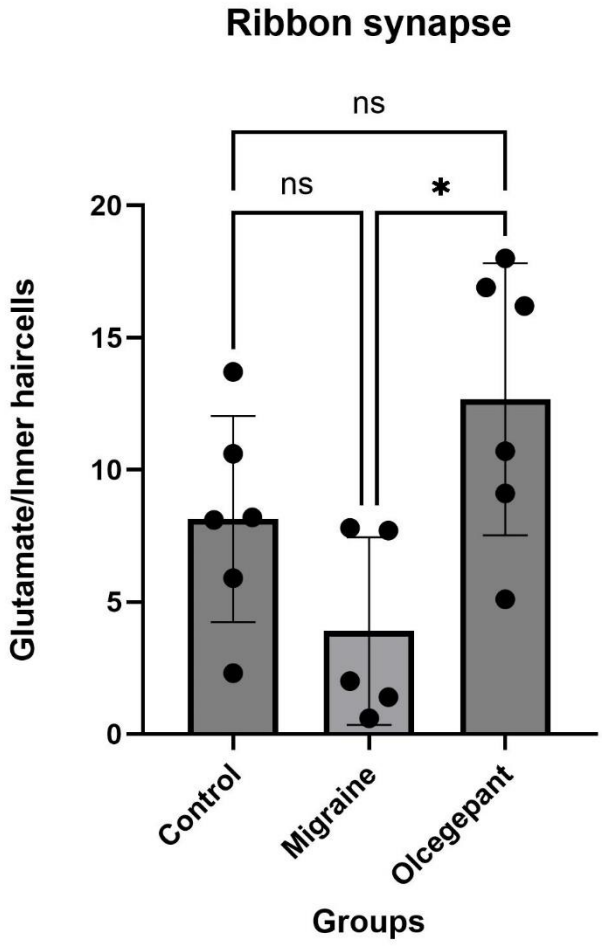

Figure 3.28 The mean ratio of the number of Glutamate to nuclei at IHCs in the groups.

Chapter 4

DISCUSSION

The mechanism that underlies migraine and MD remains unclear. VM patients and MD patients may present with the same symptoms. Since there is no diagnostic method that distinguishes both diseases, anti-migraine treatment has been tried to treat MD patients. The potential marker of MD is ELH, but there are also reported cases of migraine-associated vascular changes that cause ELH (Lee et al., 2000)Although the pathophysiology of these two diseases is unclear, we hypothesized that they could represent one syndrome rather than separate conditions. Therefore, to better understand the pathophysiology, our initial attempt was to establish an animal model of migraine, look for a well-known potential marker of MD, and uncover the effects of migraine and its treatment in the inner ear.

In our experimental model, NTG administration was chosen to create migraine model. Migraine is a multifactorial disease, and there is not a perfect animal model that reflects all the features of migraine (Chou & Chen, 2018). NTG acts through the formation of NO (Chung & Fung, 1993). Previously shown a close correlation between NO and CGRP in migraine pain (Messlinger et al., 2012), in addition, recent discoveries of drugs targeting CRRP and its receptors led us to choose this model. In the literature, it was previously shown that pain influences the general activity of animals, and activity measurements of the animals help to provide an objective evaluation (Whittaker & Howarth, 2014). Thus, In our study, OFT was used to evaluate the animal's behavior to measure their general activity and specific movements that show possible vestibular dysfunction, like circling of the head. Several studies evaluated the rats' vestibular function using balance beam walk, dyskinetic head movements and circling, retro-pulsion, and tail hang reflex. In these studies, usually, one group of the animals underwent a peripheral vestibular dysfunction by surgery or vestibulotoxic inner ear injections (Zhang et al., 2020) (Pericat et al., 2017). Our migraine model did not affect the vestibular

system as much as the surgically created vestibular dysfunction models, and that is probably why we did not see any circling or retropulsion in any of the groups. We also looked at their tail hang reflex, in which the tail lifted rats, and the response was rated. All the rats on day one and day rated as 0, normal. The locomotor activity is typically reduced in animal models of migraine (Chou & Chen, 2018), therefore we measured the immobility time on day one and on day five. We chose day five instead of the last day of the experiment because of the crowded workload on day nine with hearing assessments, orofacial formalin tests, and perfusions. Our findings showed that NTG was able to induce a migraine model. Even though all the animals were less mobile on day five compared to day one, migraine group was significantly less mobile compared to the control. The other interesting finding was that the treatment group, namely the olcegepant group, was the most immobile group on day one and day five. Although there was no significant difference in our orofacial formalin test results, face rubbing was longer in the migraine group than in the control group. Interestingly, the animals' face rubbing time in the olcegepant group was significantly longer than the migraine and the control group, which was unexpected. Greco R. et al. also used NTG for migraine and also olcegepant as the treatment in their study, and they found longer grooming in the NTG group compared to the control; in addition to that, they did not find a statistically significant difference in their treatment group compared to NTG, but they still found less grooming in the treatment group. Their olcegepant 2mg/kg applied group had less grooming time than the 1mg/kg group (Greco et al., 2022). We thought that since our treatment group is less mobile than the others, this could be a reason for their longer grooming time because less mobile animals may spend more time grooming.

Neurogenic inflammation is an important mechanism in the migraine pathophysiology. Anti-inflammatory and pro-inflammatory cytokines are involved in the trigeminal sensitization pathways. Two of the most studied pro-inflammatory cytokines are TNF-α and IL-6, and most studies reported increased levels of these two cytokines in migraine (Thuraiaiyah et al., 2022)The immune system also plays a role in MD. There is evidence of inflammation in inner ear diseases, including MD. ELH, the potential marker for MD, can be induced experimentally by injection of antigens. Clinical studies showed increased pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β in some MD patients (Frejo & Lopez-Escamez, 2022). Since there is common pro-inflammatory cytokines in the pathophysiology of both diseases, TNF-α and IL-6 were evaluated from the blood

samples taken from the tail blood in our study. Our results were compatible with the literature; both cytokines were high in the migraine group. CGRP is a potent vasoactive peptide distributed in the central and peripheral system and has a well-known role in the pathophysiology of migraine. On the other hand, CGRP is not a previously studied peptide in MD. Because of the close union between MD and migraine, it was thought that CGRP could enlighten the dark paths. A few studies in the literature measured CGRP levels in the blood of patients with migraine. In a study, CGRP was found high in the plasma taken from the external jugular vein, but in the same study, there was no change in the samples taken from the peripheral blood. On the other hand, another study showed an increase of CGRP in the plasma of peripheral blood of patients during an episodic attack (Ramon et al., 2017). On the contrary, there is another study that did not see any increase in CGRP levels from the samples taken from the external jugular vein or the peripheral blood (Tvedskov et al., 2005) When we look at the animal studies, trigeminal stimulation caused an increase of CGRP in the external jugular vein in cats in two studies. In one rat study, NTG failed to change CGRP levels in the jugular vein (Tfelt-Hansen & Le, 2009). In our study, tail blood samples were initially used to measure CGRP levels, and we did not detect CGRP in any of the groups. Afterward, we decided to look at CGRP levels in the plasma taken from the intracardiac blood samples. CGRP was detected this time, but there was no difference between the groups. This can not be taken as CGRP has no role in migraines, but the blood-brain barrier may prevent intracerebral CGRP from reaching the peripheral blood.

The effect of migraines on the inner ear remains unclear. A recent database analysis revealed that migraine was independently associated with an increased likelihood of subjective tinnitus and hearing loss (Benjamin et al., 2022). Sensorineural hearing loss has been described in up to 20% of patients with VM, which is generally mild and symmetric (Beh, 2022). A recent study of VM patients revealed that the hearing impairment was manifested as low-frequency hearing loss (Shi et al., 2022). The overlapping relationship between VM and MD is also seen in hearing evaluation because one of the distinguishing features of MD is low-frequency SNHL. Previous basic research studies on animal models of migraine have found the latency of ABR without a change in the threshold (Arakaki et al., 2014). Another study in which the animal model was created by postauricular NTG injection revealed an increase of click and 4 and 8 kHz tone burst ABR threshold with a prolonged latency (Qi et al., 2023). When we focus on the

studies looking for the DPOAE in migraine, there are a couple of clinical studies in the literature. In a case-control study, DPOAE scores were reported to be lower in migraineurs than in the control group. In contrast, another study showed no difference in DPOAE between patients with migraine and the control (Albanese et al., 2021). We performed a comprehensive hearing assessment using our model. Our DPOAE results presented a statistically significant increase of dB SPL at 6, 8, 10, and 12kHz in migraine group between day one and day nine. On the other hand, there was no difference in the control group. In addition, there was a significant decrease of dB SPL at the above frequencies in the treatment group between day one and day nine. DPOAE depends on OHC motility, and it asses functional integrity of the cochlea (Salvi et al., 2016). A robust DPOAE response (Db SPL) indicates good cochlear health; a lower response indicates the reduced function of the OHCs (Abdala & Visser-Dumont, 2001). The interpretation of our DPOAE result is that the migraine group responses got better on day nine compared to day one; on the other hand, the olcegepant group responses got worse on day nine compared to day one. In addition to DPOAE results, even though we did not see a significant threshold shift in click ABR, only in the migraine group did the hearing threshold decrease. Moreover, when we look at the change in the tone burst hearing thresholds at all the frequencies except 32 kHz between day one and day nine, only the migraine group's mean hearing threshold was reduced. The possible underlying mechanism of these findings could be the beneficial activity of NTG as a vasodilator. NTG acts through the formation of NO (Demartini et al., 2019). NO is involved in different pathological and physiological processes and is produced by nitric oxide synthases (NOS), which have three isoforms. Endothelial nitric oxide synthase (eNOS) is one of them and has been known for its protective effect via anti-inflammation, antioxidative, and anti-apoptotic features. It has been demonstrated by animal studies that eNOS is widely expressed within the cochlea, including the organ of Corti (Yang et al., 2022). NO has a broad impact on the cochlea and the other parts of the auditory pathway, like the cochlear nucleus. It increases firing rates, and high rates of auditory signaling arise from all neurons along the auditory pathway (Kopp-Scheinpflug & Forsythe, 2021). In a recent study, increased NO production by L-arginine supplementation could reduce noise-induced oxidative stress and preserve sensory hair cells (Yang et al., 2022). Some other studies in the literature implicate NO as a neurodestructive in the cochlea, but in these studies, for NO donor, sodium nitroprusside

was administered into the cochlea, and it released not only NO but also cyanide and Fe+, which was probably responsible for the destructive effect (Ruan et al., 2001).

ELH can be detected using ECOG. The literature generally uses the ratio of SP and AP to measure ELH. The sensitivity of this ratio is 62%, which is not high enough to be used regularly in the diagnostic algorithm of VM or MD. ELH is a marker for MD, but MRI studies also showed that patients with VM might also have ELH, and there are still higher rates of ELH in MD (Tabet et al., 2022). In a large study consisting of 523 migraineurs who were divided into four groups depending on their vestibular complaints, there was no difference in ECOG between the groups (Vitkovic et al., 2008). On the other hand, SP/AP ratios were reported as significantly high in VM compared to migraine patients in another study (Yollu et al., 2017). Some studies detected no difference between VM and healthy subjects (Martines et al., 2020). In the present study, the SP/AP ratio average of the control and migraine groups was higher on day nine compared to day one without a significant difference. Still, in the Olcegepant group, the ratio was significantly lower on day nine compared to day one. In addition to electrophysiological results, we also calculated the PSM through H&E sections, and there were no statistically significant differences between the groups. Still, PSM was the highest in the migraine group, and the control was the lowest. Combining the ECOG and the structural results we may propose that NO formed some hydropic changes and olcegepant might have reversed it. In an animal study in which ELH was created surgically, a type ii nitric oxide synthase inhibitor was applied, and they found a reduction in electrophysiological thresholds without a change in DPOAE and SP/AP ratios. They suggested that NO catalyzed by this enzyme plays a role in the pathogenesis of endolymphatic hydrops (Ikino et al., 2006).

CGRP plays a key role in migraine pathophysiology, and its' receptors are found throughout the trigeminovascular system on neurons and glia. The trigeminovascular system is associated with migraine. The trigeminal ganglion contains neurons that innervate the dura mater and intracranial vasculature peripherally. The activation of TG results in the release of CGRP (Eftekhari et al., 2015). NO is an important molecule that triggers the release of CGRP from TG neurons, and that is why NTG-induced models are generally used to explore the CGRP blocking agents to treat migraine (Bowen et al., 2006). CGRP has been shown in the autonomic pathways innervating the cochlear vasculature and the lateral and medial olivocochlear efferent systems. The lateral

olivocochlear efferent system targets the IHC, and the medial targets the OHC. CGRP immunoreactivity has also been shown in the efferent system of the vestibular end organs (Hara et al., 2005). The coexistence of Acetylcholine and CGRP in the efferent system of cochlea and vestibule was presented previously (Kong et al., 2002). Thus, it has been suggested that CGRP has a functional role in modulating ascending auditory signals (Le Prell et al., 2021). When we look at the immunohistochemical analyses in our study, even though there was not a statistically significant difference, the highest mean ratio of CGRP was in the migraine group, and the lowest ratio was in the olcegepant group at the TG, which is consistent with the literature. To the best of our knowledge, even though CGRP has been shown in different cell types within the nervous, cardiovascular, and immune systems, it has not been investigated around the stria vascularis, which makes our study unique from this perspective. As expected, the mean ratio of CGRP per vascular length was highest in the migraine group and the lowest in the olcegapant group, the same as the TG results. Unfortunately, there was also no significant difference in Stria V. measurements.

When we turned our attention to the vestibular organ saccule, the CGRP count did not statistically differ between the groups' Stria V. and TG, but again, the highest count was in the migraine group. Our study demonstrated a very interesting result in the measurements of CGRP and glutamate of the IHC immunostainings. CGRP count was significantly higher in the treatment group than in the migraine and the control group (respectively $p=0.02$, $p=0.006$). In addition, glutamate was significantly higher in the olcegepant group than the migraine group ($p=0.012$) but did not differ from the control group. Altogether, these observations suggest the possibility of a molecular pathway reactivity mainly working through the lateral olivocochlear efferent system. Olcegepant, a CGRP receptor blocker, reduces the CGRP release as expected, then this reduction reduces the effect of LOC inhibitory (modulating) effect on synapse and glutamate level increase. This rise in glutamate may stimulate the LOC reflex loop, and CGRP increases. We might not have seen this CGRP rise if only the dosage of olcegepant had been higher than our present dose. There is limited data regarding the intercellular signaling of glutamate receptors at ribbon synapses in the IHC of the cochlea (Klotz-Weigand & Enz, 2022). These synapses are interesting machines formed by sensory cells, and still less is known about group 2 and (mGluR2 and mGluR3) and group 3 (mGluR4, mGluR7, and mGluR8) types in the cochlea. A study proposed that the reason for the inhibition of

efferent dopaminergic signaling onto IHCs was the presence of group 2 mGluRs in the efferent LOC GABAergic fibers (Klotz et al., 2019). The gepant class of CGRP receptor antagonists has a high affinity for primate CGRP receptors but a negligible affinity for AM1 AM2 and calcitonin receptors. In rodent models, they need higher concentrations to obtain the antagonism effect (Russo & Hay, 2023). Altogether, our observations suggest a new possible intercellular signaling (Figure 4.1) induced by anti-migraine medication in the ribbon synapse, and it also shows the importance of the dose.

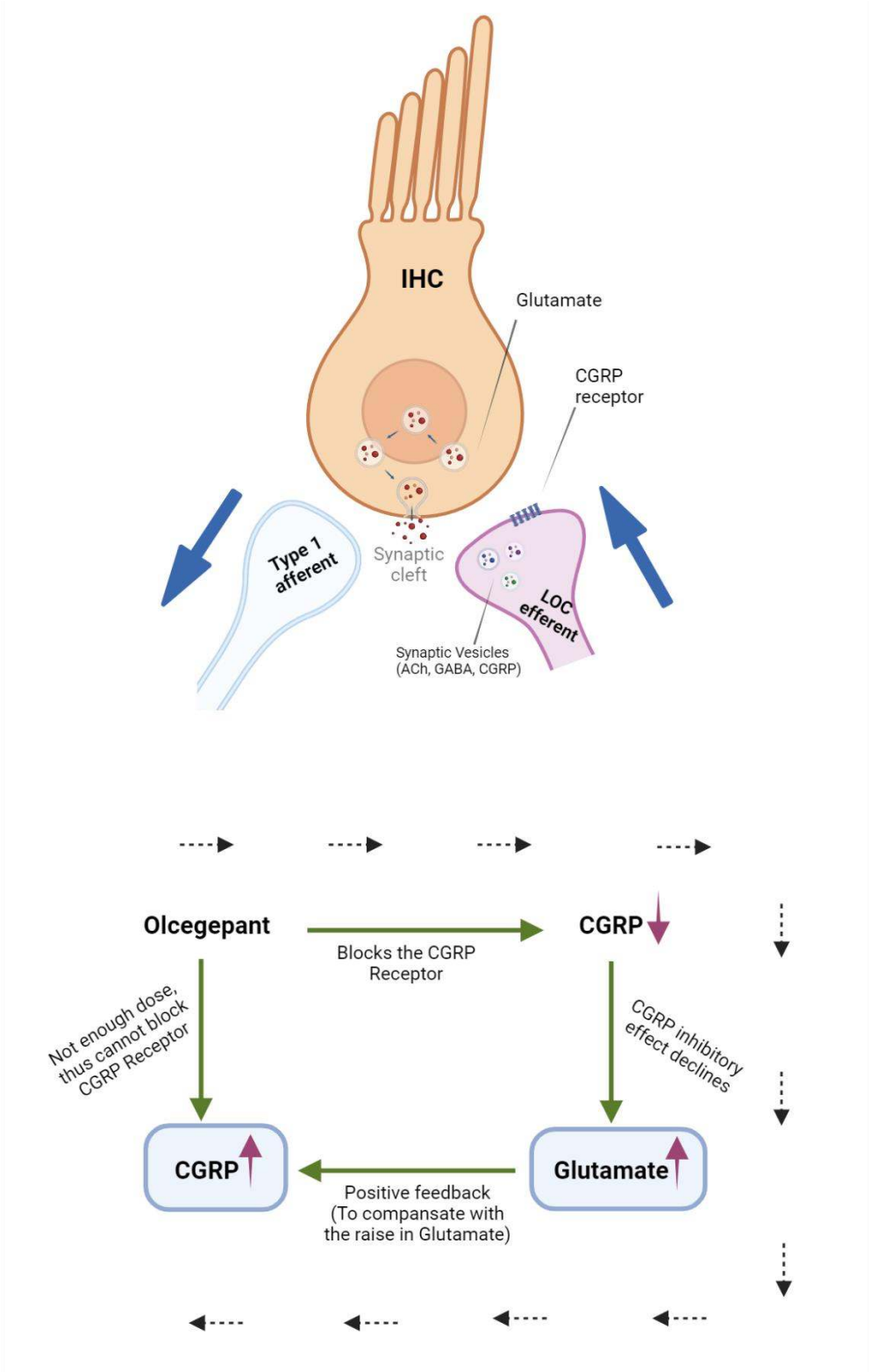

Figure 4.1. Our proposed intercellular signaling at the synaptic cleft between IHC and LOC efferent. Image created using BioRender.

Chapter 5

CONCLUSION

In this thesis project, we aimed to investigate the possibility of ELH in migraine and its auditory and vestibular symptoms. The key role of CGRP in migraine in the central nervous system is a well-known concept, but since there is not much data on the cochleovestibular system, we wanted to enlighten the pathophysiological process of migraine and the treatment with CGRP receptor blockers.

We hypothesized that ELH could be seen in migraine, CGRP could answer unresolved questions of overlaps between VM and MD, and CGRP receptor antagonists may be an effective treatment and management for ELH.

According to our aim, we used NTG for the animal model of migraine and olcegepant as the CGRP receptor blocker. We did not see any vestibular symptoms in the migraine group at all. Depending on our auditory data, there was a clear improvement in hearing in the migraine group, especially at lower frequencies on tone-burst ABR; also, OHC function in the migraine group was better in all frequencies, and on the contrary, the OHC functions were worse in the treatment group. This finding was not parallel with our expectations, but it could be clearly explained by the protective effect of eNOS. Moreover, our ECOG results presented that NO inhibition significantly lowers the SP/AP ratio. Our light microscopic results did not show distinct hydrops in the migraine group, but we also can not say that there were no hydrops and that shallow hydrops do not affect hearing. In parallel with our hypothesis, CGRP was detected in the saccule and was more detected in the migraine group. On the other hand, even though it was more seen in the cochlea of migraine group than in the control, it was significantly higher in the treatment group. Moreover, glutamate was found more in the treatment group than in the migraine group. In light of these findings, we proposed a signaling pathway in the efferent system of LOC, which puts forward the importance of the dosage of the olcegepant in rodents.

To the best of our knowledge, this is the first study that showed the systemic effect of NTG on the cochlea electrophysiologically and structurally, besides the CGRP receptor blocker effect on the cochlea. Our results open the door to working with CGRP knockout mice and with a higher dosage of olcegepant. So, according to our study, further studies are needed to clarify our cellular results and their value in clinical translation.

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