Noise trauma-induced auditory processing deficits and anxiety

by

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To those who grace life with their beauty and kindness…

ABSTRACT

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Hearing is integral to human life, playing crucial roles in safety, communication, social interaction, and overall well-being. Noise exposure significantly contributes to the prevalence of hearing loss, which impacts 19.5-21.2% of the global population. Current treatments for noise trauma-induced hearing loss and tinnitus lack FDA-approved medications, with existing therapies often being insufficient. Moreover, hearing deficits are associated with increased anxiety, yet the molecular mechanisms linking noise-induced auditory processing deficits and anxiety are poorly understood. The aim of this project is to examine the roles of parvalbumin-expressing (PV) interneurons and tumor necrosis factor alpha (TNF-α) receptors in noise-induced auditory processing deficits and anxiety. For this purpose; behavioral, molecular, and electrophysiological approaches were utilized using a transgenic mouse model (PV^{cre}/$TdTomato^{+}$). Noise-exposed mice showed reduced locomotor activity and exploration in anxiety-related behavioral tests and auditory processing deficits in acoustic startle response-based tests. Our findings reveal distinct roles of TNFR1 (Tumor Necrosis Factor Receptor 1) and TNFR2 (Tumor Necrosis Factor Receptor 2) signaling in the ventral hippocampus (vHPC) and primary auditory cortex (AI). TNFR1 signaling exacerbates, while TNFR2 signaling mitigates, noise trauma-induced auditory processing deficits and anxiety. Noise-induced deficits and their impact on the vHPC were dependent on TNF signaling, with TNFR1 knockdown ameliorating deficits by preventing PV neuron loss in the AI. Additionally, the knockdown of TNFR1 in PV neurons in the AI reduced noise trauma-induced auditory processing deficits, preserving auditory function. Conversely, the knockdown of TNFR2 disrupted neuroprotective signaling, leading to microglial

deramification. Furthermore, TNF-α signaling modulated synaptic transmission, with the knockdown of TNFR1 protecting against noise-induced synaptic changes, while the knockdown of TNFR2 exacerbated synaptic alterations. This thesis project elucidates the differential roles of TNFR1 and TNFR2 in noise-induced auditory and anxiety-related deficits, providing insights into potential therapeutic targets for mitigating the adverse effects of noise trauma.

ÖZETÇE

Gürültüye bağlı işitsel işlemleme eksiklikleri ve anksiyete

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İşitme, insan yaşamının ayrılmaz bir parçasıdır; güvenlik, iletişim, sosyal etkileşim ve yaşam kalitesinde önemli bir rol oynar. İşitme kaybı dünya nüfusunun %19,5-21,2'sini etkiler ve gürültü maruziyeti işitme kaybı ve tinnitusun başlıca sebeplerindendir. Gürültüye bağlı işitme kaybı ve tinnitus için FDA onaylı bir ilaç yoktur ve mevcut tedaviler genellikle yetersiz kalmaktadır. Dahası, işitme bozuklukları artan anksiyete ile ilişkilidir ancak gürültünün neden olduğu işitsel işlemleme bozuklukları ve tinnitus ile anksiyetenin birbiri ile ilişkisini açıklayan moleküler mekanizmalar tam olarak anlaşılamamıştır. Bu tez projesinin amacı, parvalbumin eksprese eden (PV) internöronların ve tümör nekroz faktör alfa (TNF-α) reseptörlerinin gürültüye bağlı işitsel işlemleme bozuklukları ve anksiyete patofizyolojisindeki rollerini araştırmaktır. Bu amaçla transgenik fare modeli (PV^{cre}/$TdTomato^{+}$) kullanılarak davranışsal, moleküler ve elektrofizyolojik yaklaşımlardan yararlanılmıştır. Gürültüye maruz kalan farelerde, anksiyete ile ilişkili davranış testlerinde lokomotor aktivitede ve keşif davranışlarında azalma; akustik irkilme tepkisine dayalı testlerde ise işitsel işlemleme eksiklikleri görülmüştür. Bulgularımız, ventral hipokampusta (vHPC) ve primer işitsel kortekste (AI) TNFR1 (Tümör Nekroz Faktörü Reseptörü 1) ve TNFR2 (Tümör Nekroz Faktörü Reseptörü 2) sinyal yolaklarının farklı rollerini ortaya koymaktadır. Gürültü maruziyetinden kaynaklanan bozukluklar ve bunların vHPC üzerindeki etkisinin, TNF sinyal yolaklarına bağlı olduğu gösterilmiştir; TNFR1'in susturulmasının, gürültü maruziyetinin neden olduğu PV nöron kaybını önlediği rapor edilmiştir. Ek olarak, AI ‘da yer alan PV nöronlarındaki TNFR1'in susturulması, gürültü

travmasının neden olduğu işitsel işleme eksikliklerini azaltarak işitsel işlevi korumuştur. Öte yandan, TNFR2'nin susturulması nöroprotektif sinyallemeyi bozarak mikroglial deramifikasyona yol açmıştır. Ayrıca, TNF-α sinyal yolağı sinaptik iletimi modüle etmiş; TNFR1'nin susturulması, gürültünün neden olduğu sinaptik değişikliklere karşı koruma sağlarken, TNFR2'nin susturulması sinaptik iletim bozuklukları şiddetlendirmiştir. Bu tez projesi, TNFR1 ve TNFR2'nin gürültüye bağlı işitsel işlemleme eksiklikleri ve anksiyete üzerindeki farklı rollerini açıklayarak, akustik travmanın olumsuz etkilerini hafifletmeye yönelik potansiyel terapötik hedeflere ilişkin kanıtlar sunmuştur.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAF	anterior auditory field
ABR	auditory brainstem response
ACh	Acetylcholine
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ACTH	adrenocorticotropin
AI	primary auditory cerebral cortex
AII	secondary auditory cortices
APA	American Psychological Association
ASHA	American Speech-Language-Hearing Association
AVCN	anteroventral cochlear nucleus
BLA	basolateral nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CN	cochlear nucleus
CNS	central nervous system
CRH	corticotropin-releasing hormone
dB	decibel
DC	dorsal cortex of the inferior colliculus
DCN	dorsal cochlear nucleus
DNLL	dorsal nucleus of the lateral lemniscus
dTT	3,6'-dithiothalidomide
FDA	US Food and Drug Administration
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GD	gap detection
GPIAS	gap-induced inhibition of the acoustic startle

HG	Heschl's gyrus
HPA	hypothalamic-pituitary-adrenal
i.p.	intraperitoneal administration
IBA1	ionized calcium-binding adaptor molecule 1
IC	inferior colliculi
ICC	central nucleus of the inferior colliculus
ICX	external inferior colliculus
IFN-γ	interferon-gamma
IHC	inner hair cell
IL-2	interleukin-2
IL-4	interleukin-4
ILD	interaural level difference
ITD	interaural time difference
kHz	kilohertz
LA	lateral nucleus of the amygdala
LDB	light/dark box test
LSO	lateral superior olivary nuclei
LTP	long-term potentiation
MCP-1	monocyte chemoattractant protein-1
mEPSCs	miniature excitatory postsynaptic currents
MGB	medial geniculate body
mIPSCs	miniature inhibitory postsynaptic currents
MLR	middle latency response
MMN	mismatch negativity
MSO	medial superior olivary nuclei
NFT	neurofibrillary tangle
NIHL	noise-induced hearing loss
NMDA	N-methyl-D-aspartate
NMDG	N-methyl-D-glucamine
OCT	optimal cutting temperature
OFT	open field test

OHC	outer hair cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPI	prepulse inhibition
PT	planum temporale
PV	parvalbumin
PVCN	posteroventral cochlear nucleus
PVN	paraventricular nucleus
RANCH	Road traffic and aircraft noise exposure and children's cognition and health
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
ROS	reactive oxygen species
ROS	reactive oxygen species
RT	room temperature
SGN	spiral ganglion neuron
SOC	superior olivary complex
SPL	sound pressure level
STG	superior temporal gyrus
STP	superior temporal plane
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TNFR2	tumor necrosis factor receptor 2
VCN	ventral cochlear nucleus
vHPC	ventral hippocampus
VNLL	ventral nucleus of the lateral lemniscus

CHAPTER 1

INTRODUCTION

In daily life, our sense of hearing alerts us to potential dangers, such as approaching animals or vehicles, alarms, and warning signals. Being able to hear and process the sounds allows us to respond quickly and appropriately, helping to keep us safe. Hearing makes it possible to participate in life, communicate with people, maintain relationships, listen, perceive speech and music, laugh, experience and enjoy many things that help shape our quality of life. In addition, we can perceive and interpret vocal cues that can profoundly influence our emotional responses in social interactions. The tone, pitch, and intonation of someone's voice can convey a wealth of emotional information. Hearing also helps us determine the direction and proximity of sounds, facilitating our navigation in the environment and enabling interaction with objects and people around us. For children, hearing is vital for speech production, language development, the maturation of cognitive abilities and academic achievement (Köse et al., 2022; Lieu et al., 2020). Protecting and preserving our hearing abilities is essential for maintaining a fulfilling and enriching existence.

Auditory processing in the peripheral and central auditory pathway involves a complex network of neuroanatomical connections that transform sound waves into meaningful auditory information. Auditory processing deficits generally indicate challenges in the processing of auditory inputs. These deficits can become more pronounced in challenging acoustic settings and may lead to adversities in listening, comprehending speech and acquiring language (Jerger & Musiek, 2000). Impairments in the central auditory pathway can occur alongside peripheral hearing loss (Ahn et al., 2020; Calcus & Rosen,

2024; Jerger & Musiek, 2000; Ji et al., 2023), moreover, coding deficits in signal processing can develop with noise-induced synaptopathy (Chen et al., 2019). Tinnitus, characterized by sound perception (e.g., ringing, whistling or hissing) without any stimulation, affects approximately 9 to 28% of the population in Europe, with 2% experiencing severe symptoms (Biswas et al., 2022; Hoare et al., 2011; Jarach et al., 2022). Typically, tinnitus is linked with hearing loss and cochlear damage. In addition to hearing loss substantially affects quality of life and impacts 19.5-21.2% of the global population, 16% of cases are due to occupational noise exposure (Haile et al., 2021). Since hearing loss can impair verbal communication, increase social isolation and loneliness, and may affect cognitive skills, individuals with hearing loss may experience an increased sense of threat if they are unable to comprehend their surroundings properly or effectively communicate about their needs (A. Shukla et al., 2020). Studies comparing hearing-impaired and normal-hearing individuals reported a higher incidence of anxiety disorders or symptoms in the hearing-impaired group (Shoham et al., 2019). APA characterizes anxiety as an emotion that involves feelings of tension, anxious thoughts, and physiological alterations such as elevated blood pressure. This feeling can occur without real danger, even with the perception that there is a danger, and it detrimentally impacts the quality of life. A link is reported to exist between tinnitus, which frequently accompanies hearing loss, and anxiety, depression, reduced sleep duration, and higher rates of missed workdays among adults (Bhatt et al., 2017). Underlying mechanisms by which hearing deficits lead to behavioral and emotional disorders are still unknown.

1.1. Auditory Processing

In accordance with the ASHA, central auditory processes involve functions and mechanisms within the auditory system that underlie various behavioral aspects, including:

- Temporal aspects of hearing, such as:
  - temporal resolution
  - temporal masking
  - temporal integration (ability to merge series of sounds into coherent and meaningful combinations)
  - temporal ordering (ability to sequence sounds)

- Identifying the location and direction of sounds
- Auditory discrimination
- Pattern recognition
- Handling challenges in perception when multiple sounds compete
- Performance with degraded acoustic signals

In addition to behavioral aspects, auditory processing deficits is characterized by deficiencies in the neurobiological activity that underpins this perceptual processing. These deficits involve difficulties in various facets of auditory perception, comprising spectral, temporal, and binaural hearing, along with the ability to sequence and group sounds (Miller, 2011; Moore, 2006).

1.1.1. Signal Processing in the Cochlea and Firing in the Auditory Nerve

Sound signals enter the outer ear, after being amplified and matched in impedance by the middle ear, are transformed into electrical signals in the inner ear. When cochlea is viewed in cross-section, it reveals three fluid-filled chambers: “the scala vestibuli, the scala media, and the scala tympani” (Figure 1.1.A).

The organ of Corti, situated within the scala media, comprises outer hair cells (OHCs) and inner hair cells (IHCs). The stereocilia located in the apical ends of the HCs project beyond the reticular lamina into the endolymphatic fluid. Tips of the OHCs terminate within the gel-like material of the tectorial membrane (Figure 1.1.B). Hair cells, acting as mechanoreceptors, respond to subtle shearing motion at sub-micrometer levels. When mechanical vibration of the low-frequency sounds enter the scala media of the inner ear through the fluid and membrane movement, the traveling wave moves alongside the basilar membrane and progresses toward the apex of the cochlea. Conversely, high-frequency sounds resonate at the base of the cochlea (Von Békésy, 1960).

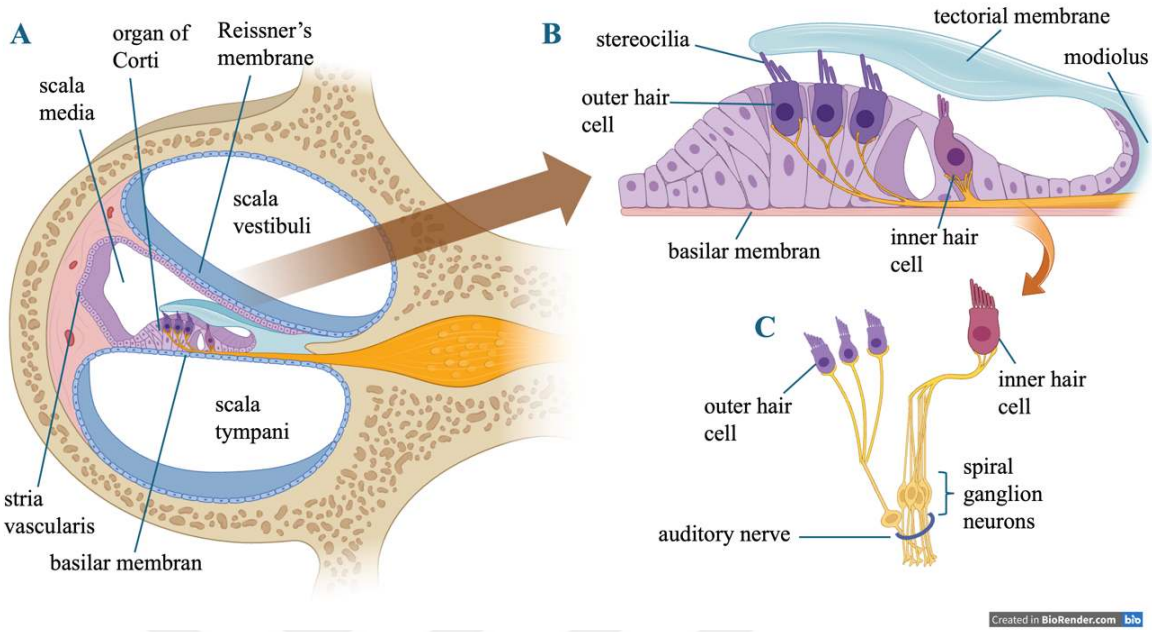


Figure 1.1: A. The three scalae of the cochlea, B. organ of Corti, and C. innervation of hair cells. The figure is adapted from (Bear et al., 2020) and created in BioRender.

The OHCs contribute to frequency tuning and sensitivity, acting as amplifiers linked to basilar membrane motion (Pickles, 1998). OHCs react to transduction currents triggered by the opening of channels in their stereocilia primarily in two ways. While they release neurotransmitter at their base, this function is considerably less significant than that of the IHCs. Instead, their primary response involves rapid changes in their stiffness and length, which is directly influenced by the transduction current (Raphael & Altschuler, 2003). This movement amplifies the vibrations within the organ of Corti, explicitly enhancing the transduction efficiency of IHCs at particular locations along the cochlear spiral, thus boosting both the sensitivity and specificity of hearing. The adjustments in OHC length result from structural changes in a motor protein situated in the cell's lateral wall (Frolenkov et al., 1998; Kakehata et al., 2000). This novel type of motor protein, named prestin, converts the electrical voltage into mechanical force by using cytoplasmic anions as voltage sensors and operates at microsecond speeds to alter OHC length in reaction to changes in membrane potential (Dallos & Fakler, 2002). While the IHC body itself likely remains stationary, the movement of its stereocilia in relation to each other activates mechanically gated ion channels. This process relies heavily on the movement of fluid and the tectorial membrane

above the IHC stereocilia. This movement is modulated, sharpened, and amplified by the vibrating region of the organ of Corti, particularly the region housing the OHCs (Raphael & Altschuler, 2003). Thanks to these mechanisms, the cochlea effectively conducts a Fourier analysis of the sound waveform, enabling the brain to interpret relative frequency amplitudes (Fettiplace, 2011). This process establishes the tonotopic organization in the early auditory system stages. Tonotopy is then transmitted from the cochlea through various intermediate stages to the AI.

The IHC establishes a crucial connection known as the "recepto-neural junction" along with the afferent auditory nerve. The afferent auditory nerve comprises spiral ganglion neurons whose dendrites connect with the IHCs of the cochlea (Figure 1.1.C), while their axons exit through the internal auditory meatus to reach the cochlear nuclei. Each spiral ganglion neuron collects input from a single IHC, with neurotransmitter glutamate likely involved (Wichmann & Moser, 2015). Auditory nerve cells function independently, inheriting properties from IHCs, resulting in a characteristic frequency sensitivity. Spike discharge patterns in auditory neurons are influenced by hair cell/afferent fiber synapse physiology, exhibiting phase-locking to basilar membrane motion for low frequencies. Auditory nerve cell activity reflects stimulus energy presence, amplitude, and timing within their narrow frequency passband (Musiek & Chermak, 2013; Rauschecker, 2021).

1.1.2. Tonotopic Maps on the Cochlear Nucleus (CN)

Central auditory pathways exhibit a tonotopic organization from the CN of the brainstem to the auditory cortex, aligning with the tonotopic organization observed in the cochlea and auditory nerve (Musiek & Baran, 2018). The auditory nerve fibers establish their initial synaptic connections within the CN located in the brainstem. The CN consists of three main nuclei: the ventral cochlear nucleus (VCN), which can be further divided into dorsal (DCN), posteroventral (PVCN) and anteroventral (AVCN) nuclei. When auditory nerve axons enter the CN, they split into two branches, with one branch going to the AVCN and the other to the PVCN before reaching the DCN. This early branching allows for parallel processing, enabling different target nuclei to simultaneously process the same input. Within these nuclei, each cochlear site is systematically innervated, resulting in comprehensive representations of

the cochlear partition. Various functional specializations exist within the CN, particularly in the AVCN, where spherical bushy cells respond with high temporal precision to low-frequency inputs via specialized synapses known as “endbulbs of Held” (Rhode, 1991). Conversely, “octopus cells” in the PVCN exhibit broader frequency tuning curves and precise responses to stimulus onsets (Rhode, 1991). The VCN contains neurons finely tuned to specific sound frequencies, processing auditory information in a linear fashion, while neurons in the DCN integrate a broader spectrum of frequencies, displaying more complex spatial and spectral tuning (Oertel & Young, 2004; Rauschecker, 2021; Yu & Young, 2000). Fusiform cells in the DCN, the output neurons, show sensitivity to specific frequency-intensity combinations and are influenced by local inhibitory circuitry. The inhibitory response areas surrounding the excitatory ones at the cell's characteristic frequency contribute to sensitivity to stimulus bandwidth and non-monotonic spike rate-intensity functions (Young & Brownell, 1976). Moreover, the predominance of responses to stimulus onsets in higher auditory processing areas prompts inquiries into the encoding of stimulus parameters and the roles of forebrain and hindbrain mechanisms in the perception of loudness (Phillips, 2000).

1.1.3. The Processing of Interaural Time (ITD) and Level Differences (ILD) in the Superior Olivary Complex (SOC)

The SOC comprises bilateral structures located in the caudal pons, housing distinct cell groups characterized by their cytoarchitecture, connections, and roles. Of particular significance are the medial (MSO) and lateral (LSO) nuclei, essential for encoding the binaural cues used in sound localization (Musiek & Baran, 2018). Both nuclei exhibit tonotopic organization and receive inputs from both ears, allowing for comparisons of stimuli from different directions based on their frequencies (Rauschecker, 2021). The ITD and ILD serve as primary cues for sound localization, influenced by factors like head size, position and the spectral composition of the sound (Phillips, 2000).

The medial superior olive (MSO) receives bilateral inputs directly from the AVCN. Notably, the cells in the AVCN contributing to this connection often receive direct input from the endbulbs of the auditory nerve, providing precise phase information about sounds (Oertel

et al., 1988). MSO cells detect temporal coincidences in the spike trains from both cochlear nuclei, allowing them to encode the relative phases of sounds heard by both ears. This neural sensitivity to interaural phase is reflected in a cyclical relationship between MSO cell spike count and interaural phase, with the cycle period matching the sound period perceived by both ears. The specific encoding of interaural disparities at each frequency enhances localization accuracy, especially for broad-spectrum sounds, providing detailed information regarding the direction of the sound source (Yin & Chan, 1990).

1.1.4. Inhibiting the Cochlear Gain and Selective Attention: The Role of Medial and Lateral Olivocochlear System

Olivocochlear efferent fibers arise from the MSO and LSO, travel to the cochlea along with the vestibulocochlear nerve, and reach the basal turn of the cochlea before terminating in the organ of Corti. The auditory efferent system serves multiple functions to modify the functioning of the cochlea throughout active and passive listening, including inhibiting the cochlear gain, enhancing selective attention, and improving the signal-to-noise ratio (Ciuman, 2010; Lopez-Poveda, 2018). Also, it promotes frequency selectivity by altering the micromechanical properties of OHCs.

Activated nicotinic-like ACh-receptors result in the hyperpolarization of the hair cell membrane and a decrease in afferent firing (Guth & Norris, 1996). At the synapse between efferent fibers and OHCs, activated acetylcholine alpha 9/alpha 10 receptors trigger calcium influx into the hair cell. This hyperpolarizes the cell membrane, altering the resting membrane potential and the gain of the OHCs. $GABA_A$ receptors linked to chloride channels in the postsynaptic OHC membrane mediate hyperpolarization. Hyperpolarization results in the expansion of prestin molecules, elongating the OHCs (Plinkert et al., 1993). Additionally, dopaminergic olivocochlear efferents facilitate gain control; disability of these efferents may indicate early signs of excitotoxicity (Ciuman, 2010).

The olivocochlear bundle also has an essential role in selective attention as an inhibitor of OHC activity, leading to reductions in auditory nerve response, basilar membrane motility, and the amplitude of otoacoustic emissions upon stimulation. Activation of the crossed olivocochlear bundle leads to evoked responses both ipsilaterally and contralaterally.

When attention is focused on a particular side, the amplitude of evoked otoacoustic emission increases in the corresponding ear. Individuals with an impaired efferent system experience diminished capacity to concentrate attention within specific frequency ranges (Ciuman, 2010).

1.1.5. The Major Axonal Fiber Tract, Lateral Lemniscus (LL)

The axons of the lateral lemniscal pathway, originating from the CN and SOC, convey finely tuned frequency data, with some fibers extending to the inferior colliculi (IC). The axonal outputs from the MSO primarily target the dorsal nucleus of the lateral lemniscus (DNLL) and the central nucleus of the inferior colliculus (ICC) on the same side. It's notable that many fibers originating from the CN and SOC bypass the nucleus of the LL. The nucleus of the lateral lemniscus (NLL) maintains sensitivity to ITD (Musiek et al., 2020). Tonotopic organization of the ventral nucleus of the lateral lemniscus (VNLL) is not as structured as in lower auditory nuclei or even as in the DNLL. There are emerging findings proposing a helicoid arrangement resembling a corkscrew in the VNLL, adding complexity to its tonotopic organization (Merchán & Berbel, 1996). In contrast, the DNLL represents high frequencies ventrally and low frequencies dorsally (Helfert & Aschoff, 1997).

One of its most significant functions is its central role in producing wave V of the auditory brainstem response (ABR), which is the most prominent and consistent wave in the ABR (Møller, 2012).

1.1.6. Temporal Processing and Intensity Coding in the Inferior Colliculus (IC)

The IC is the largest among the ascending brainstem auditory nuclei, located in the midbrain and consists of three divisions: the central, dorsal, and lateral nuclei. While the central nucleus is pivotal in auditory processing, the other divisions also contribute to auditory function. The IC receives major inputs from lower nuclei in the pons, both on the same side and across. Its central nucleus, characterized by stellate and disc-shaped cells, primarily projects ipsilaterally to the medial geniculate body (MGB) via the brachium, with some contralateral projections via the commissure of the IC (Musiek & Baran, 2018).

The ICC acts as a vital relay station for auditory information within the midbrain, organized based on frequency and surrounded by pericentral and external nuclei. The ICC receives significant input from various sources, including the lateral superior olive (LSO) on both sides, the MSO on the same side, and the DNLL on both sides (Oliver et al., 1997). Tonotopically, the central nucleus organizes from dorsolateral to ventromedial for low to high characteristic frequencies, with sharp tuning curves and some exhibiting double peaks, indicating complex frequency coding. Intensity functions in the IC vary, with some fibers showing a "roll over" at low sensation levels (Popelár & Syka, 1982). The IC is proficient in temporal processing, though its phase-locking ability is less precise than lower auditory nuclei. It contains a significant population of neurons responsive to tone modulations and ITD and ILD, contributing to binaural hearing and sound localization (Musiek et al., 2020).

1.1.7. Parallel Processing in the Medial Geniculate Nucleus of the Thalamus

The MGB includes three major subnuclei: the ventral division, organized tonotopically, sends outputs to core areas of the auditory cortex and serves as the primary nucleus for analyzing sound tones; the dorsal division projects to nonprimary areas of the auditory cortex engaged in spatial processing; and the medial subdivision, projects to multiple targets including the basal ganglia, amygdala, and limbic association areas, likely modulates emotional aspects of the sound signals (Keifer Jr et al., 2015; Winer & Schreiner, 2010).

Functionally, the ventral MGB is characterized by a tonotopic organization where high frequencies are represented medially and low frequencies laterally. Its tuning curves are varied—broad, narrow, multi-peaked, and occasionally atypical (Musiek & Baran, 2018). Unlike lower auditory nuclei, the intensity coding in the MGB predominantly involves nonmonotonic responses, with less activity at higher intensities. The MGB's phase-locking capabilities are decreased compared to other nuclei, mainly locking onto low-frequency stimuli. The ventral MGB is also responsive to ITD and ILD, enhancing sound localization. Both excitatory and inhibitory interactions within the MGB support this localization processing for contralateral and ipsilateral stimulations, respectively (Musiek & Baran, 2018). Moreover, the MGB participates in the middle latency response (MLR), though other thalamic and cortical areas may also play roles in this auditory response (McGee et al., 1992).

1.1.8. Classical and Non-classical Ascending Pathways to the Auditory Cortex

The ascending auditory pathways exhibit greater complexity compared to those of other sensory systems. They are divided into two main pathways: the classical and the non-classical. The classical pathways also termed the tonotopic system, involve relay nuclei like the CN, the contralateral ICC, and the lateral portion of the ventral division of the contralateral MGB. These nuclei process auditory information before transmitting it to the primary auditory cerebral cortex (AI) and other cortical areas (Figure 1.2.A). Abundant bifurcations of auditory nerve fibers and pathway fibers enable parallel processing, leading to an increased number of connections from the periphery to the cortex. Several fibers of the ascending pathways branch collaterals to other nuclei, such as those of the SOC and the NLL, contributing to intricate processing. Numerous fiber tracts link neurons in nuclei on both sides of the ascending auditory pathways, with major connections occurring between the nuclei of the SOC, the IC, and the auditory cerebral cortices.

The non-classical ascending auditory pathway diverge from the classical pathway, primarily at the ICC. These pathways connect, through the dorsal part of the MGB, to cortical areas other than AI, including the anterior auditory field (AAF) and the secondary auditory cortices (AII), bypassing AI processing. Neurons in the dorsal MGB also establish subcortical projections to the nuclei of the amygdala, following a "low route" that circumvents the lengthy cortical neuron chain required in classical pathways (Figure 1.3). Additionally, some neurons in non-classical pathway integrate signals from other sensory systems.

The primary and secondary areas of the auditory cortex's boundaries remain subjects of debate and evolution. Brodmann's area 41 has historically been deemed the primary auditory region, often overlapping with Heschl’s gyrus (HG). Although cytoarchitectonic and functional imaging studies suggest the AI extends further, covering the medial two-thirds of HG (Hackett, 2015; Hall et al., 2003), defining precise borders remains challenging. Additionally, areas traditionally labeled as secondary may exhibit more activity than the primary area in specific auditory processes. Despite this complexity, when necessary, alternative terms like "core" or "primary" are employed to differentiate specific cortical areas. Researchers have seen a growing interest in categorizing the primary zones of the auditory

cortex into "belt" or "core" regions, drawing insights from research on both primates and humans (Hackett et al., 2001; Kaas et al., 1999). This classification method holds appeal as it integrates findings from studies on both primate and human brains. These belt and core regions are primarily situated on the superior temporal plane (STP) and superior temporal gyrus (STG) and encompass significant anatomical features like the planum temporale (PT), HG, and the region immediately in front of HG. The core is essentially regarded as the "primary" auditory region, situated within the posterior two-thirds of the temporal plane and gyrus from an anterior–posterior perspective. These regions have been delineated based on investigations involving macaque monkeys and human subjects. The core–belt framework will be further explored later concerning its tonotopic organization.

A lateral view of the auditory cortex reveals structures integral to auditory functions. The Sylvian fissure, which segregates the frontal and temporal lobes, serves as a prominent landmark. It traverses anterior to posterior, sometimes changing course posterior to HG. Components such as the posterior two-thirds of the STG, supramarginal gyrus, and others contribute to auditory processing. The majority of the human auditory cortex resides on the STP, with HG and the PT being key features. However, anatomical variations, such as multiple Heschl's gyri and asymmetries in size and number, add complexity to identifying these structures. Recent MRI studies highlight left-sided dominance in gray matter volume within HG, especially pronounced in professional musicians (Guadalupe et al., 2021; Rus-Oswald et al., 2022). These findings underscore the intricacies of auditory cortical anatomy and its variability among individuals. The left HG is usually larger than the right (Musiek & Reeves, 1990). Positioned directly posterior to HG and anterior to the supramarginal gyrus is the PT, which also tends to be larger on the left side (Geschwind & Levitsky, 1968). Similarly, the insula, located medial to the mesial temporal lobe and characterized by a series of longer and shorter gyri and adjacent sulci, is typically larger on the left side (Mesulam & Mufson, 1985). The insula is also considered a crucial auditory structure (Bamiou et al., 2003). Another perspective on the auditory cortex's anatomy proposes a core-belt arrangement (Kaas et al., 1999), where the core is the primary auditory area encircled by the belt and parabelt regions. Connections from the core to other brain areas predominantly occur via the parabelt region (Musiek et al., 2020).

The traditional view of the tonotopic organization in HG posited low frequencies on the lateral aspect and high frequencies on the medial-posterior aspect. However, recent functional imaging studies suggest a more intricate tonotopic map, indicating high frequencies at both anterior and posterior ends with mid and low frequencies situated between (Figure 1.2.C) (Dick et al., 2012; Herdener et al., 2013; Humphries et al., 2010; Langers, 2014; Musiek et al., 2020; Saenz & Langers, 2014). As sound intensity increases, generally more fibers within the gyrus activate and fire at a higher rate, although some fibers exhibit a "roll over" effect where they decrease their firing rate. There are also interactions between inhibitory and excitatory fibers affecting how intensity changes are represented in the cortex.

Several evoked potentials are relevant to the functioning of both the auditory cortex and subcortex. The MLR arises from activity in the thalamocortical pathway and auditory cortex. Late potentials, including N1 and P2, also originate from the auditory cortex. While event-related potentials like P300 and mismatch negativity (MMN) are likely generated by various sources, the auditory cortex is involved in these responses as well (Musiek & Baran, 2018).

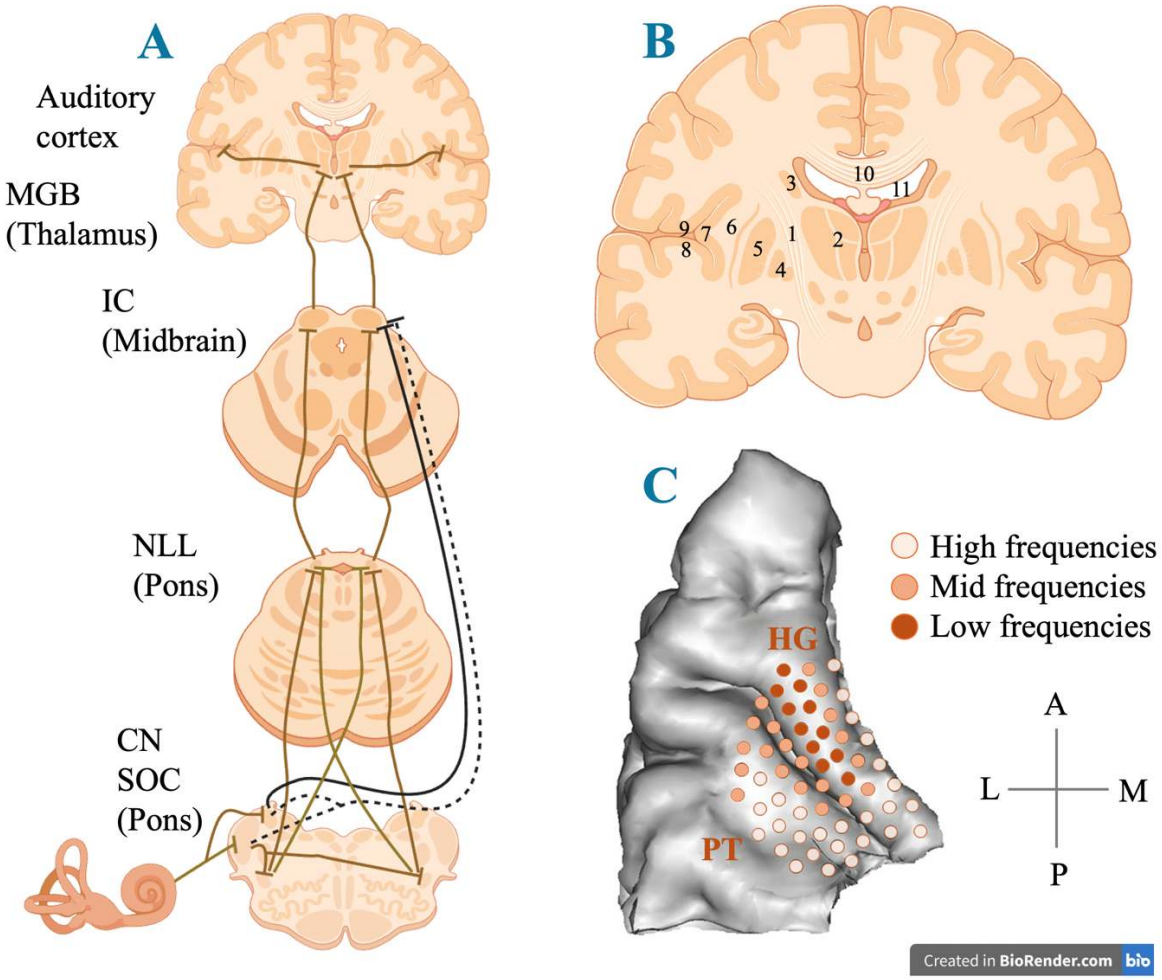


Figure 1.2: A. Schematic illustration of the main ascending auditory pathways from the left inner ear demonstrating the nuclei and their connections (CN: cochlear nucleus, SOC: superior olivary complex, NLL: nucleus of the lateral lemniscus, IC: inferior colliculus, MGB: medial geniculate body), B. Schematic illustration of the human cerebrum from the coronal view (1: internal capsule, 2: thalamus, 3: caudate nucleus, 4: globus pallidus, 5: putamen, 6: external capsule, 7: insula, 8: superior temporal gyrus, 9: Sylvian fissure, 10: corpus callosum, 11: lateral ventricle) C. The tonotopic arrangement of the auditory cortex (HG: Heschl's gyrus, PT: planum temporale) based on publications by (Dick et al., 2012; Herdener et al., 2013; Langers, 2014; Langers & van Dijk, 2012; Musiek & Chermak, 2013; Saenz & Langers, 2014). The figures are adapted from (Bear et al., 2020; Musiek & Baran, 2018; Musiek et al., 2020) and created in BioRender.

1.2. Neuronal Connectivity Between Auditory Regions and Limbic System

Auditory information has a broad impact on various brain regions. It interacts with the limbic system, the emotional center of the brain, through two distinct pathways. Auditory input to the amygdala can trigger fear responses, facilitated by both non-classical and classical pathways. The classical pathway takes a longer route through primary and secondary auditory cortices and association cortices, while the non-classical pathway offers a more direct, subcortical path to the amygdala (Moller, 2000; Musiek & Baran, 2018). Subcortical communications between auditory pathways and limbic structures play a significant role, mediating information not under conscious control (Figure 1.3). On the other hand, the non-classical pathways project extensively to the reticular formation, which regulates wakefulness (Moller, 2003).

The limbic system comprises a network of brain regions that take a central role in cognition, memory, goal-directed behavior, emotions, and affective responses (Catani et al., 2013; Kötter & Meyer, 1992). Despite numerous efforts, a definitive consensus on the specific structures constituting the limbic system remains elusive. However, commonly recognized regions of the limbic system include the cingulate gyrus, amygdala, hippocampus, mammillary bodies, parahippocampal gyrus, nucleus accumbens, and hypothalamus (Rajmohan & Mohandas, 2007). These regions are characterized by dense intrinsic connections, facilitating their coordinated functioning (Mark et al., 1995).

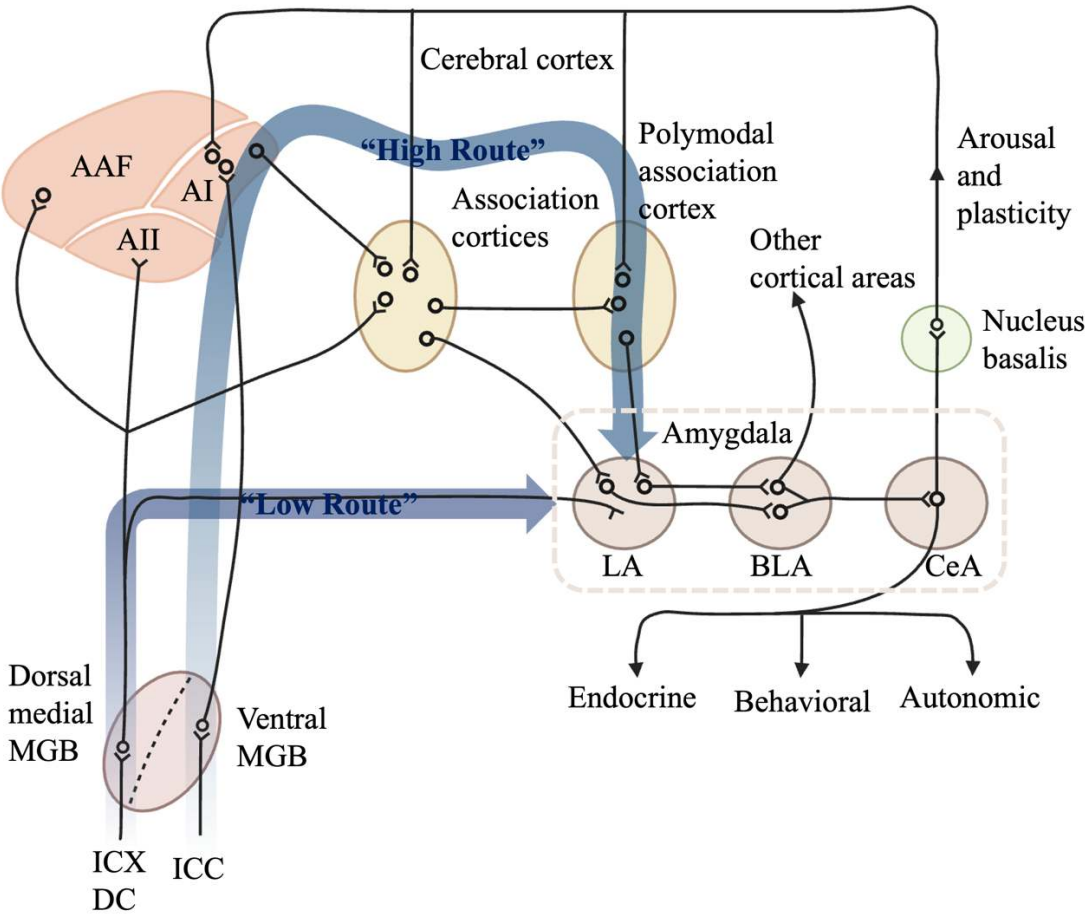


Figure 1.3: Schematic drawing of the pathways known as the "high route" and the "low route" from auditory regions to the lateral nucleus of the amygdala (LA), as well as the connections between the basolateral (BLA) and central nuclei (CeA) of the amygdala with other structures in the central nervous system (AAF: anterior auditory field, AI: primary auditory cerebral cortex, AII: secondary auditory cortex, MGB: medial geniculate body, ICX: external inferior colliculus, DC: dorsal cortex of the inferior colliculus, ICC: central nucleus of the inferior colliculus). The figure is adapted from (Møller, 2012) and created in BioRender.

Both MGB and the auditory cortex send neuronal outputs to the amygdala, which is highly sensitive to stimulation with emotional significance (Kraus & Canlon, 2012). Animal studies suggest the function of the amygdala in processing of life-threatening signals during unconscious states (Du et al., 2009). Observations in rats have shown that species-specific pain calls trigger neuronal responses in the LA even when the animal is anesthetized.

Functional magnetic resonance imaging (fMRI) studies have shown that both pictures and sounds can evoke changes in amygdalar activity regulated by their emotional theme (Anders et al., 2008). Furthermore, neuroimaging studies in humans reveal that music engages multiple brain structures involved in emotional processing, with the inclusion of the amygdala (Koelsch et al., 2006). Patients with Williams syndrome, characterized by increased emotional engagement in musical events, show heightened activation of the amygdala when listening to musical stimuli in comparison to age-matched control group (Levitin, 2005). Conversely, individuals with frontotemporal lobe degeneration, which affects regions implicated in encoding emotional meaning, containing the amygdala, exhibit weakened perception of emotional content in musical stimuli (Omar et al., 2011). The amygdala reacts to various aspects of music, including pleasant/unpleasant music, unexpected musical activities, and music combined with visual stimuli (Blood & Zatorre, 2001; Koelsch et al., 2008; Koelsch et al., 2006). These findings indicate that music can modulate emotional responses across different sensory modalities and enhance amygdalar activity, contributing to the overall emotional experience.

The hippocampus, buried in the medial temporal lobe, is a complex and critical structure that plays crucial roles in cognitive functioning, including emotion regulation, memory formation and spatial navigation (Knierim, 2015). It is also involved in processing of auditory sensory information, receiving inputs indirectly through the parahippocampal or perirhinal cortex, as well as from other forebrain regions like the medial prefrontal cortex, amygdala and insula or directly from auditory association cortices (Mohedano-Moriano et al., 2007; Munoz-Lopez et al., 2010). In return, the auditory association cortex gets indirect inputs from the hippocampus through the perirhinal or parahippocampal cortex (O'Mara, 2005), and a direct link exists from CA1 to both the AI and auditory association cortices, as shown in rats (Cenquizca & Swanson, 2007; Kraus & Canlon, 2012). This hippocampal-auditory pathway is crucial for creating long-term auditory memories. Hippocampal pyramidal neurons respond to noise, vowels, and pure tones, even during sleep (Billig et al., 2022; Vinnik et al., 2012). Bilateral severe hippocampal damage leads to impaired results in auditory recognition tests (Squire et al., 2001), and auditory cues have been reported to be critical for spatial memory in monkey studies (Tamura et al., 1990). fMRI analyses conducted in humans reveal that the hippocampus is active in processing both language and music. The

left hippocampus, for instance, is essential for the comprehension of spoken language (Davis & Johnsrude, 2003), activates in response to distorted sentences, and plays a role in adjusting to such distortions. It is also engaged in the learning of auditory-verbal materials, particularly in detecting novelty (Dolan & Fletcher, 1997). Furthermore, the left hemisphere regions, as well as the hippocampus, are active when recognizing familiar odors, visual objects, and acoustic elements like words and music (Plailly et al., 2007) while right hippocampus is specifically triggered by music memory retrieval (Watanabe et al., 2008), and processing the emotional aspects of music, showing increased fMRI activity in conjunction with the amygdala during sad music but not during happy or neutral tunes (Mitterschiffthaler et al., 2007).

1.3. Non-Auditory and Auditory Effects of Noise Exposure

As a result of the industrialization of contemporary society, noise pollution has emerged as a health hazard for humans. Environmental noise exists with highly variable intensities, temporal patterns, and exposure schedules in living conditions. Noise exposure can induce hearing deficit, causing cochlear damage (Pourbakht & Yamasoba, 2003), alter neural coding along the central auditory pathway (Pienkowski & Eggermont, 2010), and affect auditory perception and behavior (Eggermont, 2017). Noise-induced hearing loss (NIHL) can lead to hearing impairments such as tinnitus, sensory overload, and hyperacusis. However, the deleterious impacts of noise are not confined to the auditory system but also affect various other bodily systems (Figure 1.4). The most extensively studied non-auditory health outcomes associated with noise exposure include cognitive impairment (especially in children), sleep disturbances, perceived annoyance, and cardiovascular health issues (Basner et al., 2014).

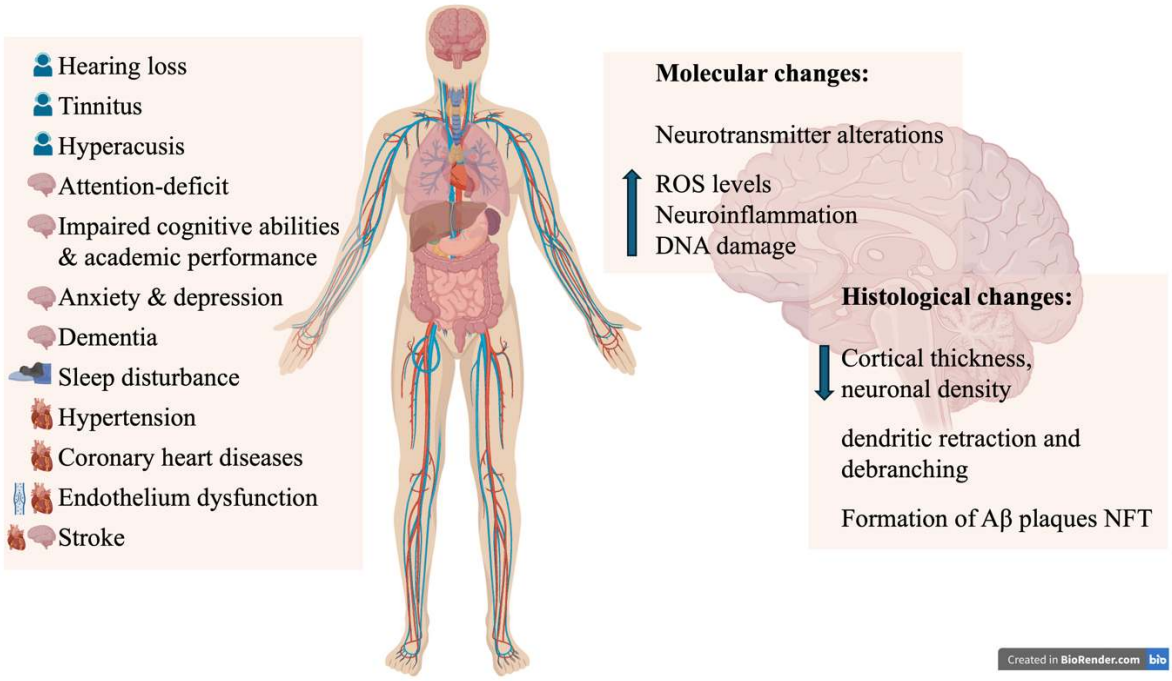


Figure 1.4: Auditory and non-auditory effects of noise exposure (NFT: neurofibrillary tangle; ROS: reactive oxygen species). The figure is adapted from (L. Yang et al., 2024) and created in BioRender.

Researchers have shown that exposure to environmental noise has a detrimental impact on children's cognitive abilities and academic performance (Evans & Hygge, 2007). Children subjected to constant aircraft, railway or road traffic noise at school tend to exhibit lower reading skills, memory, and standardized test scores compared to their peers in quieter environments (Bronzaft, 1981; Hygge et al., 2002; Lercher et al., 2003). The RANCH study, involving over 2800 children aged 9-10 from schools near airports in Amsterdam, London and Madrid, revealed a direct relationship between exposure to aircraft noise at school and children's reading perception and memory recognition, even after adjusting for socioeconomic factors. An increase of 5 dB in exposure to aircraft noise was linked to a delay of two months in reading age for UK children and one month for those in the Netherlands (Clark et al., 2006; Stansfeld et al., 2005). These results indicate that there is no threshold for noise-related effects, and reducing noise levels in the educational environment could potentially enhance the cognitive abilities of school-aged children.

Sleep disturbance, caused by environmental noise exposure, is considered the most harmful non-auditory effect since uninterrupted and sufficient sleep is crucial for health, quality of life, daytime alertness and performance (Muzet, 2007; Organization, 2011). Short-term consequences of noise-related sleep disturbance comprise impairments in mood, decline in cognitive performance and increased daytime sleepiness (Basner et al., 2014; Elmenhorst et al., 2010). Epidemiological studies suggest that nocturnal noise exposure may have a greater impact on long-term health outcomes like cardiovascular disease compared to daytime exposure (Jarup et al., 2008), likely due to less habituation of autonomic arousals to noise during nighttime (Basner et al., 2011).

Short-term exposure to noise triggers responses from the autonomic nervous and endocrine systems (Lusk et al., 2004). Researchers have consistently observed that noise exposure leads to elevated systolic and diastolic blood pressure, alterations in heart rate, and the release of stress hormones such as glucocorticoids and catecholamines (Jo, 2011). These reactions are explained by the general stress model, which posits emotional stress responses as a result of perceived discomfort (indirect path) and non-conscious physiological stress due to interaction within the central auditory system and other areas of the CNS (direct path). Long-term exposure can disrupt homeostasis (allostatic load), affecting metabolism and the cardiovascular system. This disruption leads to increases in well-established risk factors such as blood pressure, blood viscosity, blood lipid concentrations, and blood glucose concentrations for cardiovascular disease (Babisch, 2011). These changes heighten the risk of arteriosclerosis, hypertension, and are associated with serious events like myocardial infarction and stroke (Basner et al., 2014).

1.3.1. Noise-induced Hearing Loss, Auditory Processing Deficits and Tinnitus

NIHL is typically attributed to damage or loss of cochlear hair cells and cochlear spiral ganglion neural connections after acoustic over-exposure. Noise can cause structural degeneration in hair cells and spiral ganglion neurons (SGNs) and induce cellular loss (Wong et al., 2013). Because the cochlea has limited regenerative abilities in several mammalian species, any loss of cochlear cells is likely to result in permanent hearing impairment. Besides, synapses connecting hair cells and auditory nerve are especially susceptible to noise

(Liberman et al., 2016). When the IHCs undergo partial de-afferentation, also known as "hidden hearing loss," it impacts hearing capacity in challenging listening environments and objective measures of cochlear neural health without affecting the hearing thresholds in quiet.

Apart from direct damage to the cochlea, histological studies have shown that noise exposure is also associated with specific morphological changes and decreased neuronal density within the central auditory pathway (Moller, 2000; L. Yang et al., 2024). Studies have also demonstrated notable changes in neuronal structures, such as alterations in spine density and dendritic shape in the AI following exposure to noise (Bose et al., 2010). Moreover, researchers have indicated neuronal loss in various regions along the central auditory pathway, including the CN, IC, MGB, and AI, as a consequence of noise exposure (Basta et al., 2005; Sekiya et al., 2012). Besides, chronic noise stress can alter the GABA and glutamate neurotransmission and inhibitory/excitatory balance in the CNS (Cui et al., 2009; Cui et al., 2012; Kazi & Oommen, 2014). Various mechanisms contributing to tinnitus development within the DCN have been revealed. These include the downregulation of inhibitory neurotransmission involving glycine and GABA, reduced tonic potassium currents inhibiting neural activity, and heightened synaptic excitability due to postsynaptic long-term potentiation (LTP) (Li et al., 2015; Middleton et al., 2011; Tzounopoulos, 2008; Wang et al., 2009). Additionally, exposure to noise has been demonstrated to cause oxidative stress in the brain, which results in neuronal damage. After noise exposure, the accumulation of ROS) overwhelms antioxidant systems, causing widespread oxidation of lipids, proteins, and DNA (Kröller-Schön et al., 2018; M. Shukla et al., 2020). All these effects can contribute to noise-induced auditory processing deficits, tinnitus and hyperacusis.

Animal studies have also demonstrated that noise exposure leading to hearing loss may result in enhanced amplitudes in auditory-evoked cortical potentials (Sun et al., 2012; Syka & Rybalko, 2000). This enhancement may be associated with noise-induced tinnitus and hyperacusis. Moreover, it suggests that the processing of auditory information in the CNS is altered as a result of neural plasticity. Furthermore, exposure to environmental enrichment has been revealed to alter the frequency tuning of neurons in the AI, leading to increased frequency selectivity and sensitivity to quieter sounds (Engineer et al., 2004). These outcomes underline the complex impacts of auditory exposure on the nervous system, extending beyond the peripheral hearing structures to include significant CNS.

1.3.2. Involvement of the Limbic Regions

Exposure to noise may elicit feelings of annoyance and stress while also impacting cognitive functions, learning, working memory capacity, attention and mood. Although individual responses to noise can vary significantly and are influenced by the type of noise, there is general consensus on its negative impacts on the brain and health (Belojevic et al., 2003; Irwin et al., 2011).

Subcortical projections originating from the auditory system towards the amygdala may take a role in the generation of tinnitus (Moller, 2003). A neural pathway from the MGB to the amygdala could trigger an emotional response to noise-induced auditory processing deficits and tinnitus. The DCN typically exhibits heightened activity during tinnitus, potentially impacting emotion and attention through pathways involving the locus coeruleus, raphe nuclei and reticular formation (Kaltenbach & Godfrey, 2008; Langguth, 2011). A proposed common pathway for tinnitus includes brain regions activated across different cases of tinnitus, such as the amygdala, hippocampus, and insula (Lenhardt et al., 2008). Furthermore, noise exposure, especially at high levels or over prolonged periods, can lead to a series of neurobiological changes that impact the hippocampus, an essential element of the limbic system (Kraus & Canlon, 2012).

Noise exposure is known to activate stress responses leading to structural and molecular changes in the hippocampus, such as reduced neurogenesis and synaptic connectivity and altered cell morphology. Hippocampal dysfunction resulting from noise exposure can contribute to impaired LTP and plasticity, memory deficits and cognitive impairments. Also, the constant perception of tinnitus-related sounds may disrupt the hippocampus's normal function (Nadhimi & Llano, 2021).

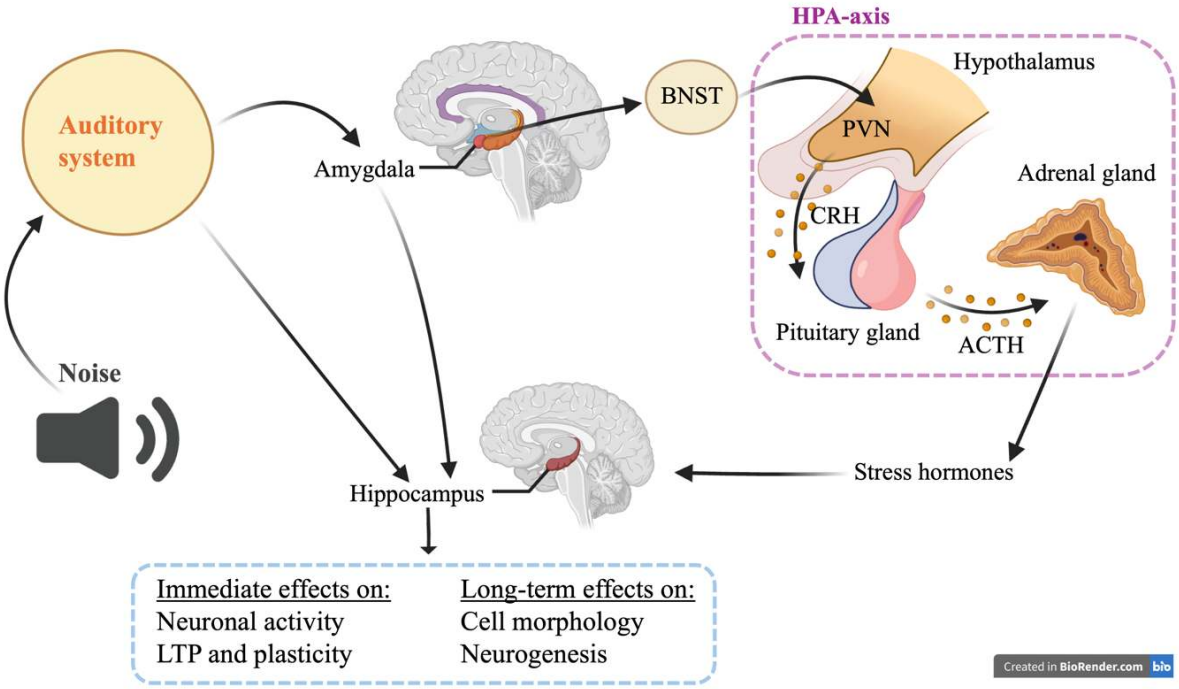


Figure 1.5: Effects of noise on limbic regions (BNST: bed nucleus of the stria terminalis; PVN: paraventricular nucleus; CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; HPA: Hypothalamic-pituitary-adrenal). The figure is adapted from (Kraus & Canlon, 2012) and created in BioRender.

Noise exposure can trigger the amygdala to stimulate the hypothalamic-pituitary-adrenal (HPA) axis, inducing glucocorticoid release (Figure 1.5). In animal studies, noise has been shown to activate cells in the hypothalamic paraventricular nucleus (PVN) that produce corticotropin-releasing hormone (CRH) (Helfferich & Palkovits, 2003). This activation prompts the pituitary gland to release adrenocorticotropin (ACTH), which in turn causes the adrenal gland to increase its production of corticosterone (the primary glucocorticoid in rodents) in response to the noise level (Burow et al., 2005; Tahera et al., 2007). Both ACTH and corticosterone levels remain elevated for as long as the noise continues, accompanying a behavioral stress response. These glucocorticoids, known as corticosterone in rodents and cortisol in humans, activate glucocorticoid receptors found in various tissues and organs, including the inner ear's hair cells and spiral ganglion neurons (Kraus & Canlon, 2012). Moreover, animal studies indicate that the connection from the auditory system to the HPA-axis passes through the MGB, amygdala, and the bed nucleus of the stria terminalis (BNST).

Studies have found that removing the auditory thalamus prevents the release of corticosterone (Campeau et al., 1997). Bilateral lesions in the central nucleus of the amygdala reduce the cardiovascular stress response to noise, which is connected to the secretion of stress hormones (Galeno et al., 1984; Ising & Kruppa, 2004). Additionally, lesions in the BNST reduce the activation of neuroendocrine cells in the PVN triggered by interleukin-1beta and the subsequent release of ACTH (Crane et al., 2003).

1.4. Hearing Loss, Tinnitus and the Risk of Anxiety

Psychiatric conditions, including cognitive decline, dementia, anxiety, and depression, have been linked to hearing impairments (Bhatt et al., 2017; Blazer & Tucci, 2019; Deal et al., 2017). A recently published population-based cohort study declared that the tinnitus prevalence was 28% and participants with tinnitus exhibited a significantly higher prevalence of anxiety (5.4%) compared to those without tinnitus (3.3%) (Hackenberg et al., 2023).

The mechanisms underlying the development of tinnitus remain unclear. It is suggested that tinnitus may stem from audiological dysfunctions, with cochlear damage from hearing loss potentially causing an imbalance (Czornik et al., 2022). The noise trauma-induced hearing loss might downregulate cortical inhibition and increase spontaneous firing rates, leading to hyperexcitability in the central auditory pathway, which is believed to contribute to the onset of tinnitus (Eggermont, 2016; Norena & Eggermont, 2003). The auditory cortex gets involved in the development of tinnitus, although it is not typically associated with anxiety disorders. Moreover, changes in activity within central auditory pathway are influenced by modifications in various neurotransmitters, including GABAergic, glutamatergic, and glycinergic transmission (Richardson et al., 2012; L. Yang et al., 2024). GABA, a primary inhibitory neurotransmitter, is also closely linked to anxiety networks, with reduced GABA neurotransmission leading to anxiety symptoms. Conversely, benzodiazepines, commonly used to treat anxiety, exert their effects through GABAergic pathways (Möhler, 2011).

Researchers offered the presence of three functional networks in tinnitus: memory network, distress network and salience network (Langguth et al., 2013; Pattyn et al., 2016). The memory network, primarily comprising the hippocampus and amygdala, is crucial for

learning and conditioning associated with threat behavior (Langguth et al., 2013). The distress network, activated by auditory stimuli, other sensations and pain involves limbic structures such as the amygdala, orbitofrontal cortex, insular cortex, cingulate cortex and hippocampus (Langguth et al., 2013). Meanwhile, the attention network, responsible for making individuals aware of the phantom auditory stimuli, includes regions like the cingulate cortex, insular cortex, hippocampus and amygdala (Møller, 2003). While these networks do not fully explain the initial perception of phantom auditory sensations, they are believed to play essential roles in the development and persistence of tinnitus, potentially leading to complications like anxiety disorders.

The ventral hippocampus (vHPC) is specifically implicated in emotional processing, including emotion regulation and anxiety (Fanselow & Dong, 2010). It is interconnected with other brain regions that are part of the limbic system, such as the amygdala and the prefrontal cortex (PFC). This network of interconnected brain areas is responsible for regulating emotions and responding to stress and threats. The projections between the vHPC and BLA are critical for the regulation of anxiety-related processes. Their communication pathways allow for the integration of contextual information, emotional processing, fear learning, and stress responses, all of which contribute to the complex experience of anxiety. Dysregulation in this circuitry can lead to heightened anxiety responses and contribute to the development of anxiety disorders (Hong & Kaang, 2022).

1.5. Animal Models of Hearing Loss and Tinnitus

Animal models emerged as valuable tools for objectively measuring hearing loss and tinnitus, offer several advantages over human clinical studies, including: (a) access to an extensive range of experimental methods spanning from behavioral to molecular approaches, (b) direct control over etiology and history, (c) the ability to use invasive methods if necessary, which are not feasible in humans, and (d) random assignment of subjects to control and experimental groups, facilitating the use of robust inferential statistics and causal attribution. The primary challenge encountered by animal models is ensuring their reliability and validity. However, inducing hearing loss and tinnitus in animal models is comparatively less

challenging because numerous causes identified in humans can be readily replicated in animals, making the induction process relatively straightforward (Brozoski & Bauer, 2016).

Table 1.1. Comparison of hearing range and sensitivity between human and animals (SPL: sound pressure level, [a] determined by the trough of the audiogram, [b] Data was extracted from the audiogram referenced, [c] Identified through behavioral assessment, [d] Identified through electrophysiological testing). The table is adapted from (Lue et al., 2023).

Species	Hearing range (at 60 dB SPL)	Greatest hearing sensitivity frequency range[a]	Reference
Human	31 Hz–8 kHz[c]	-10 dB SPL at 2–4 kHz[b,c]	(Jackson et al., 1999)
Chinchilla	50 Hz–33 kHz[c]	8 dB SPL at 500 Hz–4 kHz[b,c]	(Heffner & Heffner, 1991)
Monkey (Macaca fuscata)	28 Hz–37 kHz[c]	1 dB SPL at 4 kHz[c]	(Jackson et al., 1999)
Sheep	100 Hz–30 kHz[c]	-6 dB SPL at 10 kHz[b,c]	(Heffner & Heffner, 1990)
Pig	42 Hz–41 kHz[c]	9 dB SPL at 8 kHz[b,c]	(Heffner & Heffner, 1990)
Dog	67 Hz–45 kHz[c]	0 dB SPL at 2–8 kHz[b,d]	(Heffner, 1983)
Guinea pig	86 Hz–47 kHz[c]	12 dB SPL at 6–8 kHz[b,d]	(Heffner et al., 1971)
Rat	4–56 kHz[c]	35 dB SPL at 9 kHz[b,d]	(Syka, 2010)
Mouse	2–88 kHz[c]	7 dB SPL at 16 kHz[c]	(Heffner et al., 2006)
Cat	58 Hz – 75 kHz[b,c]	-3 dB SPL at 8–16 kHz[b, c]	(Heffner & Heffner, 1985)
Rabbit	360 Hz–42 kHz[c]	4 dB SPL at 2 kHz[b,c]	(Heffner & Masterton, 1980)

Successful outcomes observed in animal models are promising for translational research studies aimed at developing interventions to prevent NIHL and tinnitus. The most suitable animal model for investigating noise-induced hearing loss and tinnitus depends on the specific research question (Table 1.1). Commonly used animal models include mice, rats cats, guinea pigs, gerbils and chinchillas (Salvi & Boettcher, 2008). Chinchillas have a hearing range similar to humans but may be more susceptible to noise-induced damage (Miller, 1970). Cats are often preferred for long-term electrophysiological recordings due to their resilience (Liberman & Kiang, 1978). Guinea pigs and gerbils have slightly greater hearing ranges than humans, however, they are conducive to anatomical analysis and physiological recordings (Heffner et al., 1971; Mills et al., 1990). With advancements in molecular biology and genomics, rats and mice have become popular choices, particularly inbred strains that minimize genetic variability and genetically engineered mice that can be utilized to investigate underlying mechanisms (Davis et al., 2003; Ohlemiller, 2019). However, there are notable differences in susceptibility to noise-induced hearing loss and tinnitus among various strains and species. Mice, for instance, exhibit considerably larger threshold shifts in response to noise exposure compared to species like the guinea pig, which demonstrate greater resilience to auditory damage from loud sounds (Henton & Tzounopoulos, 2021).

Various techniques are employed to assess the effects of noise-induced auditory deficits in animal models. These techniques provide insights into questions such as the threshold for noise-induced damage, anatomical changes in the inner ear, physiological alterations associated with NIHL, and potential therapeutic interventions. Although there is no standardized animal model for tinnitus, they can be broadly categorized into operant and reflexive models (Galazyuk & Brozoski, 2020). Operant models involve probing animals about their auditory experiences, relying on voluntary behaviors influenced by the acoustic environment. These models mimic the evaluation process of human tinnitus patients and can tap into various brain functions beyond just auditory processing (Noreña & Farley, 2013). However, they require motivation management and training, which can be time-consuming and demanding. In contrast, reflexive models examine the acoustic startle reflex as indicative of a tinnitus state. These models don't require motivation management or training and are

rapid, making them suitable for tracking the progression of tinnitus (e.g., sound gap inhibition of acoustic startle- GPIAS). Despite their widespread use, reflexive models have limitations. They depend primarily on brainstem regions the neural generators involved may differ from those in operant models, which assess how animals evaluate their tinnitus consciously (Galazyuk & Brozoski, 2020; Galazyuk & Hébert, 2015).

Despite global efforts to comprehend underlying mechanisms of tinnitus and find effective treatments, it remains an incurable condition. Current approaches like sound masking, hearing aids, acupuncture, drug treatments and neuromodulation have not shown consistent benefits according to meta-analyses and systematic reviews (Hoare et al., 2011; Song et al., 2012). Cognitive behavioral therapy has shown some effectiveness in enhancing the quality of life for individuals with tinnitus, though it does not decrease the perceived loudness of the tinnitus (Hesser et al., 2011). This highlights the ongoing challenge posed by our incomplete understanding of tinnitus pathophysiology. Although the absence of any FDA-approved medications specifically for tinnitus, drugs still remain the preferred treatment option over devices or implants, according to patient surveys (Tyler, 2012; Zheng & Smith, 2024). Few drugs have undergone both animal studies and human clinical trials for tinnitus treatment. Since animal studies suggest a critical role of the imbalance between excitatory and inhibitory neurotransmission in tinnitus, it has captured primary attention in pharmacological treatment studies. Numerous studies have focused on NMDA (N-methyl-d-aspartate) receptor antagonists after finding these receptors upregulated in the cochlea and central auditory brain regions in animal models of tinnitus (Guitton et al., 2003; Hwang et al., 2013; Jang et al., 2019). Agents like MK-801, 7-chlorokynurenate, gacyclidine, and memantine have shown promise in animal studies for decreasing tinnitus-like behaviors, particularly at higher doses. However, memantine (an NMDA receptor antagonist), the only one of these tested in humans, did not improve tinnitus symptoms in a clinical trial (Figueiredo et al., 2008), likely due to much lower doses used in comparison to those effective in animals. Moreover, differences in drug administration methods between animal studies (injections) and human trials (oral intake) could account for lower effectiveness due to different bioavailability rates.

Additionally, the downregulation of GABAergic neurotransmission has also been implicated in tinnitus, as observed in the decreased expression of $GABA_A$ receptors and

glutamic acid decarboxylase-67 in auditory pathways after acoustic trauma (Browne et al., 2012; Zheng & Smith, 2024). This has led to exploring drugs with GABAergic-enhancing properties, like gabapentin and baclofen, which are already used for other neurological diseases. Gabapentin (acting as a $GABA_B$ receptor agonist) showed initial promise in animal studies and in a singular human case report (Zapp, 2001), but subsequent clinical trials yielded disappointing results, possibly due to heterogeneous patient groups and insufficient dosing protocols (Bauer & Brozoski, 2006). Overall, these findings highlight the complexities and challenges of translating successful animal model results into effective human treatments, pointing to the need for further research to refine drug types, dosages, and delivery methods to effectively treat tinnitus.

1.6. The Role of Parvalbumin-expressing (PV) Neurons

Parvalbumin is a protein categorized among calcium-binding proteins, also capable of interaction with magnesium ions, and is specific to vertebrates (Schwaller et al., 2002). PV neurons, which make up about 40% of inhibitory neurons in the cortex, are abundant in various brain regions, including the hippocampus (Hijazi et al., 2023). They are fast-spiking cells and they release the neurotransmitter GABA, which inhibits the firing of nearby neurons (Hu et al., 2014). This inhibition is crucial for preventing excessive neural activity and maintaining network stability. Also, PV neurons are known for their ability to synchronize the firing of pyramidal neurons in the hippocampus (Pelkey et al., 2017). They are crucial for maintaining the balance between excitation and inhibition in cortical circuits, thereby contributing to cognitive, social, and emotional regulation (Lim et al., 2018; Zaletel et al., 2016). Disruptions in the expression of PV neurons have been implicated in various neuropsychiatric disorders, including anxiety disorders (Pinna & Colasanti, 2021).

PV neurons serve various functions including enhancing temporal coding and improving signal-to-noise ratio and response timing, regulating neuronal gain, controlling developmental plasticity and critical period (Atallah et al., 2012; Hamilton et al., 2013; Nocon et al., 2023). In addition to the crucial role of PV neurons in maintaining the balance between inhibition and excitation in cortical circuits, researchers have revealed the functions of cortical PV neurons in the gap detection test (Keller et al., 2018; Weible et al., 2014).

Recent findings suggest that PV neurons are involved in encoding gaps within continuous background sounds, exhibiting more robust responses to gap onset compared to other neuron types (Keller et al., 2018). Neuronal activity of PV neurons is influenced by sensory input and sound deprivation causes a drop in PV interneuron-mediated inhibition within hours after the loss of auditory nerve fibers in adulthood (Resnik & Polley, 2017). Previous studies investigating PV neurons following noise exposure have reported conflicting effects on PV expression in the AI (Liu et al., 2018; Masri et al., 2021; Nguyen et al., 2017), possibly as a result of variations in noise exposure paradigms and experimental protocols, complicating the assessment of PV neuron involvement in noise-induced gap detection impairments. However, the specific contributions of PV neurons to behavioral deficits following noise-induced hearing loss remain unclear.

1.7. The Role of Neuroinflammation

Exposure to noise has been reported to stimulate immune cells and initiate inflammation within the brain regions (L. Yang et al., 2024). Microglia, the resident macrophages in the CNS, take an essential role in inflammatory responses, immune surveillance and maintaining tissue homeostasis. Diverse harmful stimuli can trigger microglial activation which leads to increased production of pro-inflammatory cytokines through a positive feedback loop. Noise exposure is among these insults known to cause microglial activation. Chronic exposure to noise has been demonstrated to elevate the expression of the microglial activation marker IBA1, in the hippocampus, with this elevation persisting for up to 14 days after noise exposure (Cui et al., 2015). This kind of prolonged microglial activation in the brain regions may promote the onset of neuroinflammation. Researchers have indicated that noise exposure induces neuroinflammation, evidenced by a considerable increase in levels of CD68 (abundantly expressed by macrophages) and MCP-1 (macrophage marker) in the serum of human subjects (Kröller-Schön et al., 2018). Additionally, subacute exposure to noise was found to increase pro-inflammatory cytokines like TNF-α, IFN-γ, and IL-2 while reducing levels of the anti-inflammatory cytokine IL-4 (Wankhar et al., 2016).

The principal origin of pro-inflammatory cytokine TNF-α is activated microglia, PV neurons in the AI also express both TNF-α receptors (Probert, 2015) which are involved in

neuroinflammatory processes and neurogenesis in the brain. There are two primary types of TNF-α receptors: TNFR1 and TNFR2. TNFR1 is the primary receptor responsible for mediating the inflammatory and cell-death- inducing effects of TNF-α (Holtmann & Neurath, 2004). When TNF-α binds to TNFR1, it can trigger apoptosis and proinflammatory signaling pathways. TNFR2 is generally associated with promoting cell survival, immune regulation, and tissue repair (Madsen et al., 2016). Besides, TNFR1 inhibits neurogenesis by inducing cell death and inflammatory responses in the neural progenitor cells, while TNFR2 supports neurogenesis by promoting proliferation, survival, and differentiation of neural progenitor cells (Iosif et al., 2006).

Noise-induced PV neuron loss occurs in the mouse AI and hippocampus and contributes to deficits in central auditory processing and possibly tinnitus (Deng et al., 2020; Masri et al., 2021; Zinsmaier et al., 2020). According to preliminary results, the surviving PV neurons had decreased TNFR1 expression and enhanced TNFR2 expression in the cortex of noise-exposed mice. This is consistent with the generally recognized roles of TNFR1 in neuroinflammation and necroptotic cell death, and TNFR2 in cell protection and survival (Probert, 2015; Vandenabeele et al., 2010). TNF-α signaling and its receptors may play a key role in noise-induced PV neuron loss because of the increased expression of TNF-α in the AI after noise trauma (Wang et al., 2019).

1.8. Aims and Hypothesis

Noise exposure can produce a range of molecular, cellular, synaptic and circuit alterations in the CNS, along with the central auditory pathway, including reduced inhibitory neuron function and neuroinflammation. These changes have also been reported as contributing factors to the development of tinnitus. However, the molecular pathways underpinning noise-induced parvalbumin-positive (PV+) neuron loss, specifically the roles of TNF-α receptors TNFR1 and TNFR2 and their relation to anxiety, remain poorly understood. In the present project, we utilized Cre-lox-based genetic tool to address these questions.

In this thesis, we focus on the roles of PV neurons and TNF-α receptors in noise-induced auditory processing deficits and anxiety by using behavioral, molecular and electrophysiological approaches in a transgenic mouse model (PV^{cre}/$TdTomato^{+}$). We used

tools such as the open field test, light/dark box test, prepulse inhibition test, and gap detection test for the behavioral assessment. We performed immunofluorescence stainings to evaluate parvalbumin-containing (PV+) neurons and microglial density and deramification. Also, we conducted whole-cell voltage clamp recordings to determine the electrophysiological properties of AI pyramidal neurons.

Our aims in this thesis project are:

- Examining the effects of noise and TNFR1/TNFR2 knockdown on behavioral assessment
- Investigating the roles of TNFR1 and TNFR2 signaling in noise-induced PV neuron loss and microglial deramification
- Determining the impact of TNFR1 or TNFR2 knockdown on noise-induced synaptic imbalance

We hypothesize that TNFR1 signaling impairs and TNFR2 signaling protects the surviving of PV neurons against noise trauma-induced synaptic deficits. We think differential activation of TNFR1 and TNFR2 in cortical and hippocampal PV neurons determines the fate of the PV neurons and microglial activation following noise trauma.

CHAPTER 2

MATERIALS AND METHODS

2.1. Ethics Statement

This project is funded by TUBITAK 2214-A (1059B142200098) grant and is carried out in collaboration with Prof. Shaowen Bao from University of Arizona. All procedures involving animal handling were sanctioned by the University of Arizona Institutional Animal Care and Use Committee (protocol number: 14-554) and Institutional Animal Care and Use Committees of Koç University (protocol number: 2022.HADYEK.021).

2.2. Animals

Animals weighing 26-30 g were housed under a 12 h/12 h light/dark cycle in the room with constant temperature (68–75°F) and humidity conditions and allowed access to water and food ad libitum. We utilized the Cre-lox-based genetic tool; male and female heterozygous PV-Cre-tdTomato mice were employed in the present thesis project. Genotyping experiments were conducted through PCR analysis of DNA extracted from the tail biopsies to confirm the existence of the Cre recombinase gene and the loxP-flanked sequences in animals. This confirmation ensures that the animals possess the necessary genetic components for Cre-lox-based recombination to occur.

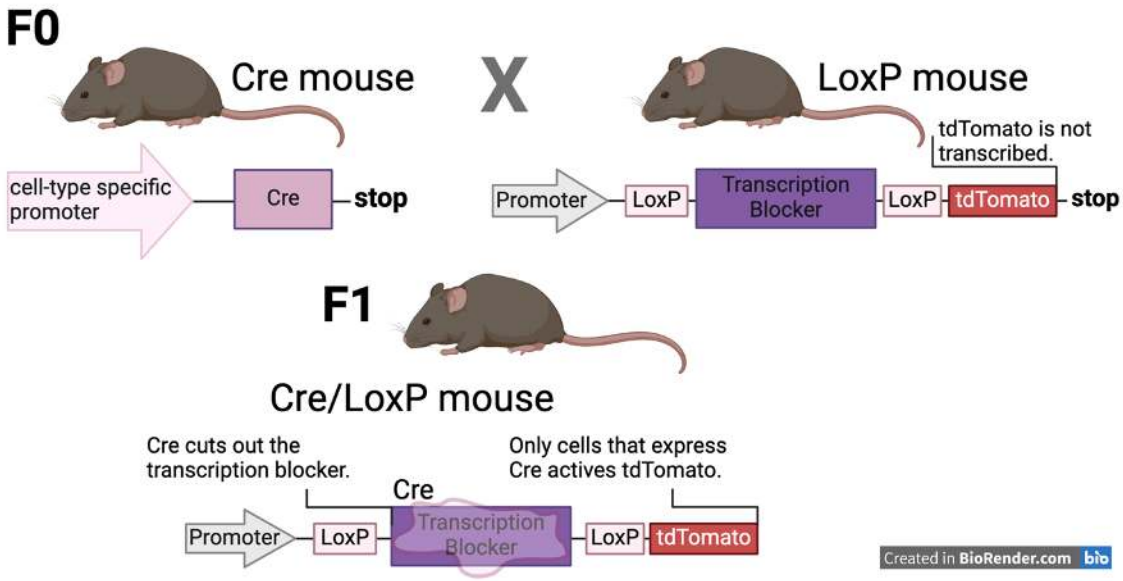


Figure 2.1: Schematic illustration of the Cre-LoxP recombination system. The figure is adapted from (Cazemier et al., 2016) and created in BioRender.

The Cre-Lox recombination system is a technology that allows genetic modifications in specific regions of the DNA. It allows DNA modification in a specific cell type. The system takes its name from the Cre enzyme that creates recombination and the LoxP, which is the binding site recognized by this enzyme. Cre recombinase is an enzyme derived from the P1 bacteriophage and it recognizes and recombines DNA at specific LoxP sites. LoxP sites are strategically inserted into the genome flanking the DNA segment to be manipulated. Allows precise spatial and temporal control over genetic modifications. The F0 generation consists of the original parent organisms that are bred together (Figure 2.1). Cre mouse expresses Cre through a cell-type specific promoter. In the LoxP mouse, tdTomato is not transcribed because of the transcription blocker. In the F1 generation, Cre recognizes the target gene between two LoxP, cuts out the transcription blocker, and marks target cells with a fluorescent protein (Cazemier et al., 2016). We marked PV neurons in red by using tdTomato in this thesis project. PV-Cre-tdTomato mice were generated in the breeding colony by crossing PV-Cre mice (B6;129P2-Pvalbtm1(cre)Arbr/J, Jackson Laboratory stock # 008069) with ROSA-tdTomato mice (B6.Cg-Gt(ROSA)26Sortm9(CAGtdTomato)Hze/J, Jackson Laboratory Stock # 007909).

2.3. Injections of the viral vectors

We injected custom-designed viral vectors (Figure 2.2) into the AI or vHPC for Cre-dependent expression of TNFR1 or TNFR2 shRNA in mice that express Cre recombinase in PV to knock down TNFR1 or TNFR2 in PV neurons. Animals were anesthetized using ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) with isoflurane (∼1.5% in a gas mixture) in the stereotaxic apparatus. Body temperature of the animals was maintained at 36.5°C by a homeothermic heating pad. The anesthetized mouse was placed into the stereotaxic frame (World Precision Instruments), which holds the head securely and allows for precise positioning. An incision was made on the scalp to expose the skull. The skin and connective tissue were carefully retracted to clear the area where the injection was made. The Paxino's and Franklin's Mouse Brain Atlas (Paxinos & Franklin, 2019) was used to determine the coordinates of the vHPC and AI. A small burr hole was created over the vHPC (Figure 2.3.A and 2.3.B; AP: -3.5 ± 0.2 mm, ML: 3.3 ± 0.2 mm, DV: 4.0 ± 0.2 mm) bilaterally or right AI (Figure 2.4.A and 2.4.B; AP: -2.91 ± 0.2 mm, ML: 4.1 ± 0.2 mm, DV: 0.8 ± 0.2 mm), and the virus was delivered through a small durotomy to knockdown of TNFR1 or TNFR2. Mice were received an injection in the bilateral ventral hippocampi or right AI with 0.5 µL of a designed viral vector through a glass micropipette (Drummond Wiretrol, 10 µL) connected with the stereotaxic injector. The volume and rate of injection were carefully controlled to minimize tissue damage and ensure accurate delivery. A non-gene-specific GFP virus was used as a control to account for possible damage from viral infection or microinjection into the brain tissue. After the surgery, the glass pipette was left in the same position for 10 minutes, subsequently, slowly withdrawn. The incision on the scalp was sealed using surgical adhesive (VetOne). The mouse was then allowed to recover from anesthesia under close monitoring. Analgesic (Ethiqa, Fidelis) was administered to manage pain, and the mouse was kept in a warm environment until fully recovered.

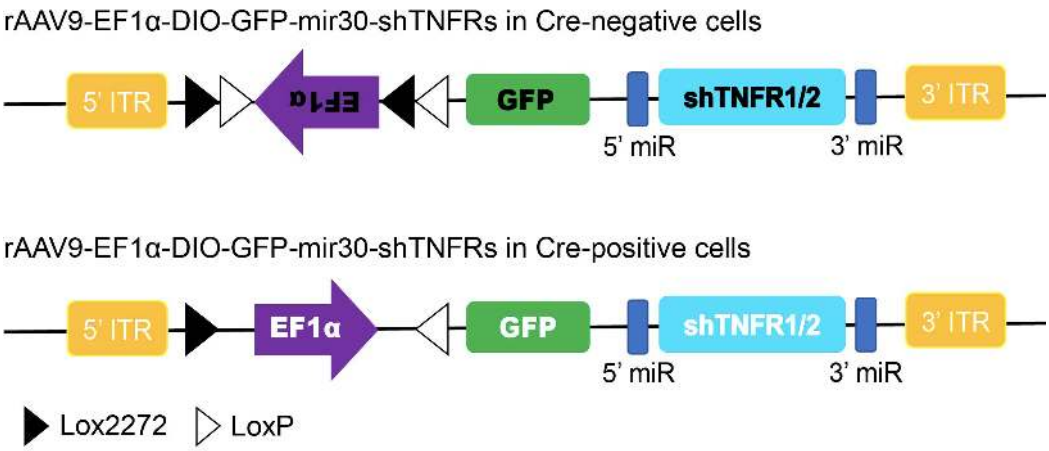


Figure 2.2: Schematic illustration of the viral vector design. The cre-dependent flip of EF1α promoter enables the expression of both green fluorescent protein (GFP) and shRNAs.

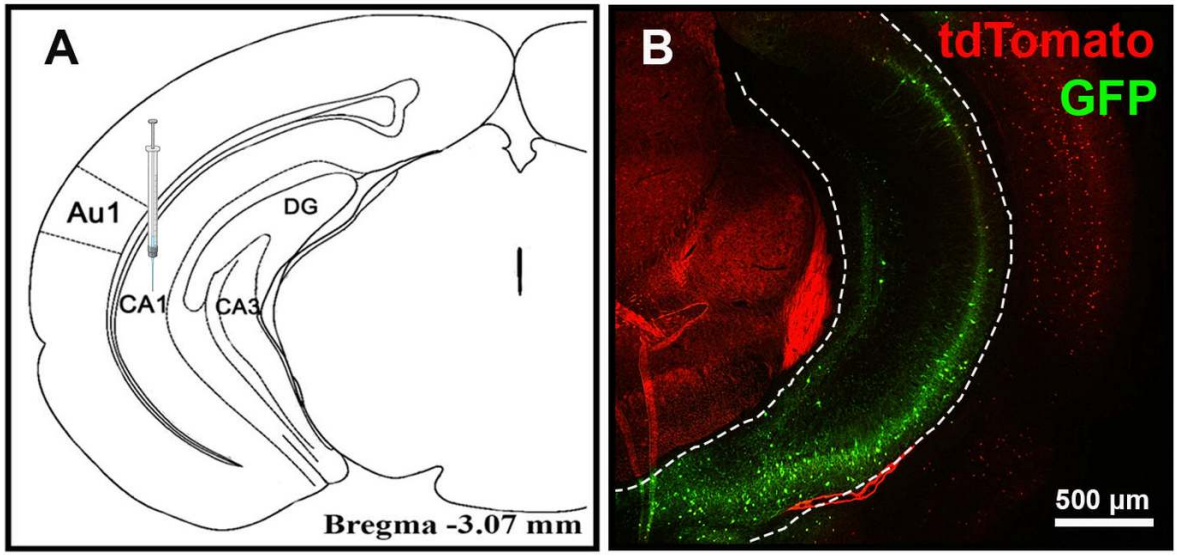


Figure 2.3: Schematic illustration for the coordinates of vHPC from the mouse brain atlas (A) and injection site (B). The green color represents viral injection and the red color represents cre reporter tdTomato.

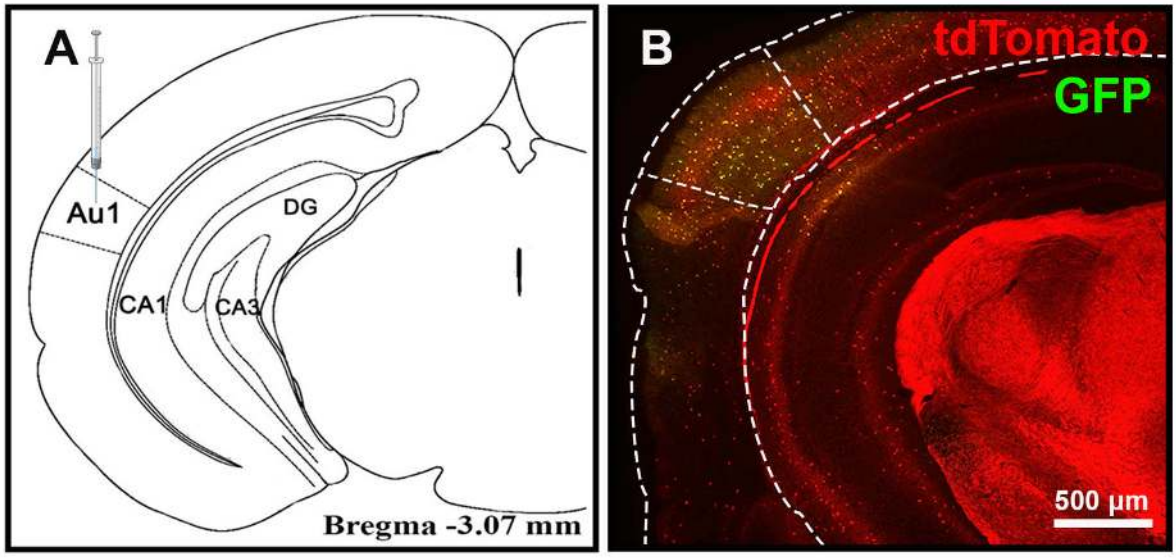


Figure 2.4: Schematic illustration for the coordinates of AI from the mouse brain atlas (A) and injection site (B). The green color represents viral injection and the red color represents cre reporter tdTomato.

2.4. Noise exposure

Animals were anesthetized using ketamine (100 mg/kg, i.p.) and isoflurane (~1.5% in a gas mixture) and temperature was kept at 36.5°C by the homeothermic heating pad. The level of sound was calibrated by way of a condenser microphone (Bruel and Kjaer, Denmark). NIHL and tinnitus were induced by exposing mice to continuous noise at 112-114 dB SPL with a center frequency of 8 kHz using piezoelectric earphone speaker for a duration of two hours. After two hours, the mouse was allowed to recover from anesthesia and kept in a warm and environment until fully recovered.

Animals that received injection into the ventral hippocampi were exposed to noise bilaterally. Animals that received injection into the right AI were exposed to noise unilaterally from the left ear, while the right ear was shielded using sound-attenuating clay. Prepulse inhibition test (PPI) and gap detection test were performed using animals injected with viral vectors into their right AI and exposed to noise unilaterally from the left ear. Behavioral assessment was performed before and after noise exposure in all experimental groups.

2.5. Auditory brainstem response (ABR) recordings

In order to record ABRs to tone burst, an animal was positioned on a homeothermic heating pad under anesthesia using a combination of air (1 liter/min) and isoflurane (~1.5% in a gas mixture). Three subdermal platinum electrodes were used, with the placed active electrode behind the ipsilateral ear, the reference electrode at the vertex of the skull and the ground electrode in the mouse's back (Figure 2.5). Tone burst stimuli at frequencies of 4, 8, 16, and 32 kHz (each with a duration of 10 ms) were produced using an RX5 multifunction processor and SigGenRP software. TDT BioSigRP software was used to record ABRs. The sound stimulus was carried through a tube placed in the external acoustic canal. The responses to the tone burst stimuli were averaged, and these average responses were recorded. The ABR threshold was determined as the minimum sound intensity at which a clear segment of the physiological waveform could be recognized. The latency of the first peak was measured at 70 dB SPL and 15 dB above the threshold using a 16 kHz tone, then switched to other frequencies. While testing, the contralateral ear was shielded using sound-attenuating clay. After completing the test, the electrodes were removed, the mouse was kept in a warm environment until fully recovered from anesthesia.

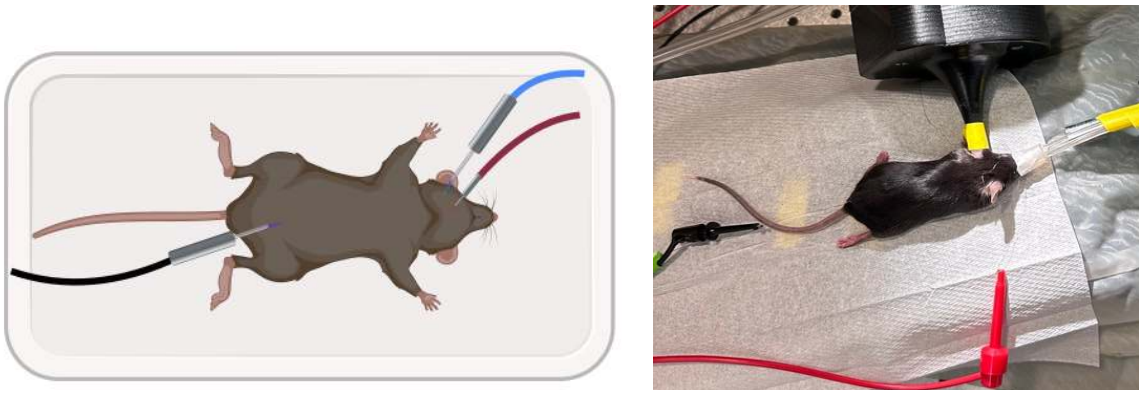


Figure 2.5: Schematic illustration (A) and the image (B) of the experimental setup for the auditory brainstem recordings. The illustration is created in BioRender.

2.6. Behavioral assessment

Behavioral assessment is employed to assess anxiety-like behaviors, exploratory activity, and responses to novel environments after injections of the viral vectors into the vHPC. The behavioral tests consisting of the OFT and the LDB were conducted using animals injected with viral vectors into their vHPC. Prepulse inhibition test (PPI) and gap detection test were performed to assess auditory processing deficits using animals injected with viral vectors into their AI. Behavioral assessment was performed before and after noise exposure in all experimental groups.

2.6.1. Open field test (OFT)

The OFT is a widely used behavioral assay designed to assess general locomotor activity, exploration, and anxiety-related behaviors in rodents (Kraeuter et al., 2019). Anxious mice tend to spend more time near the outer areas and avoid the more exposed center area. Their overall movement and exploration are often reduced. Additionally, anxious mice may exhibit increased freezing behavior, characterized by periods of immobility. Signs of stress, such as higher levels of defecation and urination, are also common (Kraeuter et al., 2019). We used OFT to assess the impact of knockdown of TNF-α receptors and noise exposure on their anxiety-related behaviors.

The OFT consisted of 40cm x 40cm x 40cm enclosed white plastic box. At the beginning of the test period, each mouse was positioned in the center of the area and allowed to explore the box for 10 min. Each trial was video recorded and the open field area was cleaned with 70% ethanol between a mouse and the next one. The distance traveled, time spent in the center or outer areas, distance ratio traveled in the center and the number of defecations were measured, analyzed and compared between groups using MATLAB and SPSS.

2.6.2. Light/dark box test (LDB)

In the LDB test, anxious mice typically exhibit a strong preference for the dark area over the light area (Bourin & Hascoët, 2003). The frequency of transitions between the light and dark compartments is markedly reduced, reflecting a reluctance to explore the aversive lighted environment. Additionally, the latency to first enter the light compartment is extended, indicating hesitation. While in the light compartment, the duration of exploration is minimized, and the mouse often exhibits a rapid retreat to the dark compartment (Bourin & Hascoët, 2003). We used LDB to assess the impact of knockdown of TNF-α receptors and noise exposure on their anxiety-related behaviors.

The LDB consisted of a black closed top compartment (27 × 15cm) that was not illuminated and a white illuminated compartment (27 × 29cm). The compartments were connected through an opening (10 × 7.5cm). At the beginning of the test, each mouse was positioned in the center of the light compartment and allowed 10 min to explore the apparatus. Each trial was video recorded and the test area was cleaned with 70% ethanol between a mouse and the next one. Time spent in the light and dark compartments, number of crossings between compartments, latency to first entry into the dark compartment, latency to reenter the light compartment, and number of defecations were measured and analyzed using MATLAB and SPSS.

2.6.3. Prepulse inhibition test

The PPI is a behavioral paradigm based on the acoustic startle reflex. The acoustic startle response is a reflexive, whole-body reaction to a sudden, intense sensory stimulus. In mice, this typically involves a jump in response to a sudden and loud sound stimuli/noise. It is an innate and primitive defensive mechanism that helps animals react quickly to potential threats in their environment. Although the fact that the influence of non-auditory inputs on the acoustic startle circuit should be taken into consideration, examining the magnitude of the acoustic startle reflex offers an efficient automated method for behavioral screening of auditory function in mouse (Lauer et al., 2017; Longenecker et al., 2016).

The testing apparatus for the PPI and the gap detection task was identical. We placed the mouse in a plastic container under a mesh lid and put the plastic container on top of a piezoelectric sensor positioned in the sound-attenuated test chamber.

For the PPI test, the animal hears fifty miliseconds pure tone pulse at seventy-five dB SPL after silence and we aim to test the animal's capacity to detect this pure tone. PPI performance of the animals was stabilized across two daily sessions prior to the commencement of data collection.

2.6.4. Gap detection (GD) test

We measured the animal's capacity to detect a silent gap in a constant pure tone in the GD test, which is a commonly used model for temporal acuity. This system provides information about auditory processing and it is also recognized as gap-induced inhibition of the acoustic startle (GPIAS).

Every trial commenced by a carrier pure tone, with the frequency pseudorandomly chosen from 5, 7, 10, 14, 20, 28, and 45 kHz, all presented at 75 dB SPL. During cued trials, a 50-millisecond silent gap in the background sound preceded the commencement of the loud noise burst by 100 milliseconds. The carrier tone was followed by startle stimulus—a duration of 50-ms white noise burst at 102 dB SPL in uncued trials. For the calculation of the startle response ratio in gap detection, the mean startle amplitude in the silent gap-cued trials was divided by the mean amplitude in the uncued trials. A lower ratio signifies more effective silent gap detection, while a ratio of 1 suggests failure to detect the gap (Figure 2.6). The results of the gap detection tests were stabilized across two daily sessions before data collection commenced.

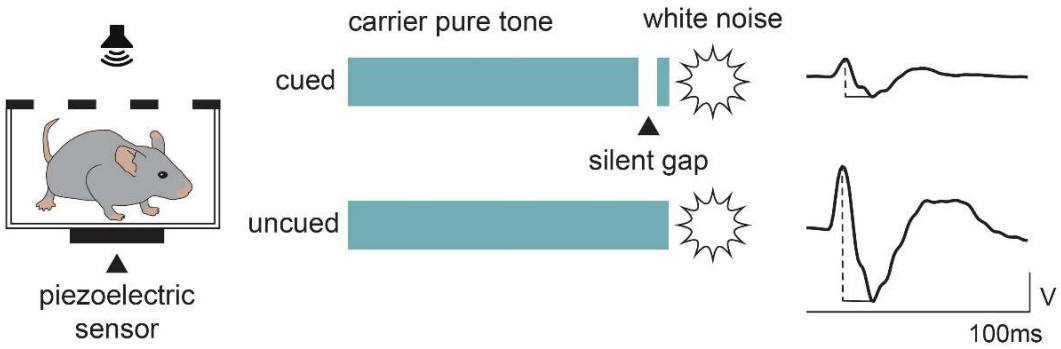


Figure 2.6: Schematic illustration of the gap detection test.

2.7. Immunofluorescence Staining, Imaging and Image Analysis

Following behavioral assessment, animals were deeply anesthetized using ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and fixed with open-chest cardiovascular perfusion of 50–75 ml of PBS solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain tissue was collected and subsequently fixed in the paraformaldehyde solution for 16 h at 4°C. Afterward, the tissue was immersed in 30% sucrose and dehydrated at 4°C until it settled at the bottom. It was then embedded in OCT compound and frozen at -80°C. The frozen tissue block was sectioned into 16 µm thick slices using cryostat microtome. Every third section was selected for further processing. The selected sections were blocked using 1x casein blocking buffer for 1 hour at RT and then incubated in the primary antibodies (anti-IBA1 polyclonal antibody; 09-19741, Wako Chemicals or anti-parvalbumin polyclonal antibody; PA1-933, Thermo Fisher Scientific) in the same buffer at 4°C. This was followed by incubation with secondary antibodies for 1 h at RT. All images for the same experimental condition were captured on the same day using identical parameters under the fluorescence microscope at 10× and 40× magnification (Olympus) with a digital microscope camera (Hamamatsu). The threshold levels and filter parameters were consistently maintained across groups for each experimental condition. The Paxino's and Franklin's Mouse Brain Atlas (Paxinos & Franklin, 2019) was used to determine the regions of interest. Analysis of the stainings and statistics were performed with MATLAB, ImageJ and GraphPad Prism. The

loss of PV and PV+ neurons were assessed by way of the Cre reporter tdTomato and stainings with anti-parvalbumin polyclonal antibody.

We refer to all neurons that are marked with tdTomato as PV neurons, and those that actively express PV as PV+ neurons. So, we could distinguish these two. According to the literature, variations in PV expression can occur within PV+ populations, and a decrease in PV+ neuron density might indicate either the death of PV+ neurons or a decline in PV expression (Filice et al., 2016; Nichols et al., 2018; Wang, 2022).

To investigate microglial deramification, we used images at 40× magnification from the AI and vHPC as previously described (Deng et al., 2020; Wang et al., 2019). Summarily, the entire cell body of the microglia and its soma were individually defined in ImageJ. Following this, the images were thresholded, and the suprathreshold pixels were quantified. This data was then used to calculate the soma-to-whole body ratio, which served as the indicator of microglial activation.

2.8. Slice preparation and whole-cell voltage clamp recordings

Tenth day following noise exposure, the mice were anesthetized utilizing isoflurane, and transcardial perfusion procedure was conducted using NMDG-HEPES (pH 7.3, 93 mM NMDG, 25 mM D-glucose, 3 mM Na-pyruvate, 2.5 mM KCl, 30 mM NaHCO3, 20 mM HEPES, 2 mM thiourea, 5 mM Na-ascorbate, 1.25 mM NaH2PO4, 10 mM MgSO4, and 0.5 mM CaCl2, 300 mOsm) . The brain tissue was isolated and placed in oxygenated (5% CO2 and 95% O2) ice-cold NMDG-HEPES solution for ~1 min. 300-μm thick sections were cut along the transverse plane and transferred into a custom-made tissue recovery chamber containing pre-warmed (32.5°C) oxygenated NMDG-HEPES solution for 30 min. A total of 4 mL Na+ spike-in solution was added gradually to the recovery chamber according to the protocol of (Ting et al., 2018) before transferring slices to the long-term holding chamber containing oxygenated HEPES-aCSF solution (125 mM NaCl, 20 mM D-glucose, 2.5 mM KCl, 26 mM NaHCO3, 1 mM Na-ascorbate, 1.2 mM MgSO4, and 2.4 mM CaCl2, 1.25 mM NaH2PO4). A fixed-stage upright microscope (BX51WI, Olympus) featuring differential interference contrast optics, 10× and 40× water-immersion objective, a Multiclamp 700B amplifier (Molecular Devices), micromanipulator, solution heating and perfusion system

were used to recordings of the pyramidal neurons in the AI. The sample chamber was constantly perfused with oxygenated recording aCSF solution (pH 7.3, 125mM NaCl, 2.5mM KCl, 1mM MgCl2, 25mM D-glucose, 25mM NaHCO3, 1.3mM NaH2PO4, and 2mM CaCl2) at a frequency of 2 mL/min.

Whole-cell recordings were carried out using 1.5-mm-diameter borosilicate glass micropipettes that had tip resistance of 3–5 MΩ when filled with an internal solution (pH 7.3, 135 mM CsMs, 10 mM HEPES, 10 mM CsCl, 4 mM ATP-Mg, 0.4 mM GTP-Na, 0.2 mM EGTA, 290mOsm). The resistance remained stable throughout the recording session and was constantly controlled by way of a brief voltage pulse. The data was discarded if the cell did not have a resting membrane potential of at least -68mV and a series resistance of 15–25 MΩ. Miniature inhibitory postsynaptic currents (mIPSCs) and miniature excitatory postsynaptic currents (mEPSCs) were recorded from pyramidal cells in oxygenated aCSF solution including 1 μM tetrodotoxin (TTX, the voltage-gated sodium channel blocker). Cells were held at -80 mV during the experiment for mEPSCs and at 0 mV for mIPSCs. Data collection and analysis were carried out using pCLAMP software (Molecular Devices) and MiniAnalysis program (Synaptosoft).

2.9. Experimental Plan

In order to systematically analyze the differential impacts and mechanisms of noise exposure and viral vector-mediated gene modulation in two key but functionally distinct brain regions, the project was divided into two distinct parts: ventral hippocampus and primary auditory cortex parts.

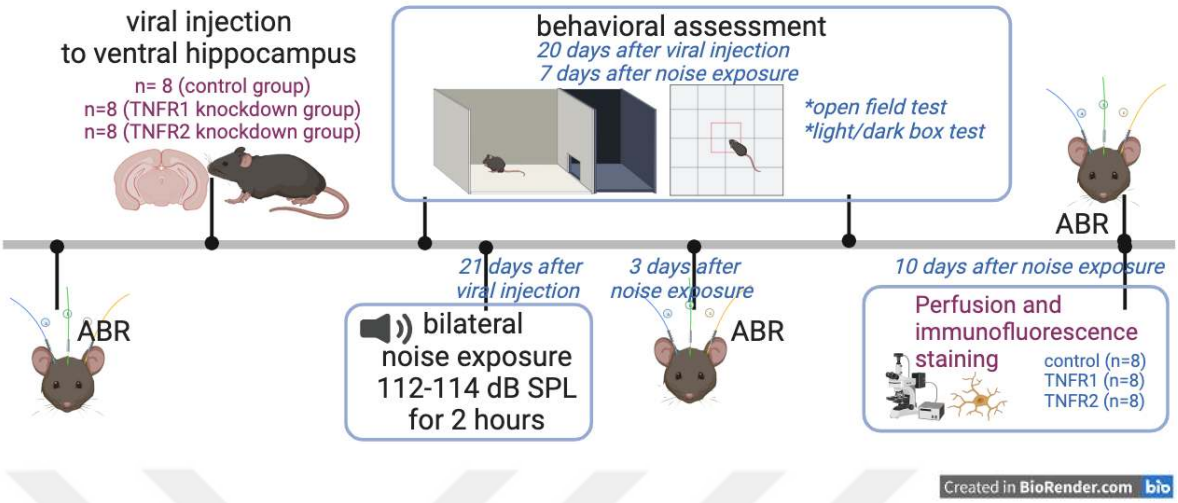


Figure 2.7: Experimental timeline of the vHPC part of the project. The figure is created in BioRender.

We used a total of 24 PV-Cre-tdTomato mice for the vHPC part of the project (Figure 2.7). After measuring auditory brainstem responses to indicate intact hearing, PV-Cre-tdTomato mice were injected with one of three viral vectors (with TNFR1 shRNA, TNFR2 shRNA or control GFP virus) in the bilateral ventral hippocampi. After a twenty days of incubation period, we assessed behavioral parameters indicating anxiety in mice. Then, we exposed mice to continuous 112-114 dB SPL white noise with a center frequency of 8 kHz to both ears for two hours. Three days after noise exposure, we tested hearing thresholds by measuring ABRs to make sure we induce hearing loss. After re-assessing behavioral parameters with OFT and LDB, ABR thresholds were recorded and mice were sacrificed for the immunohistochemistry.

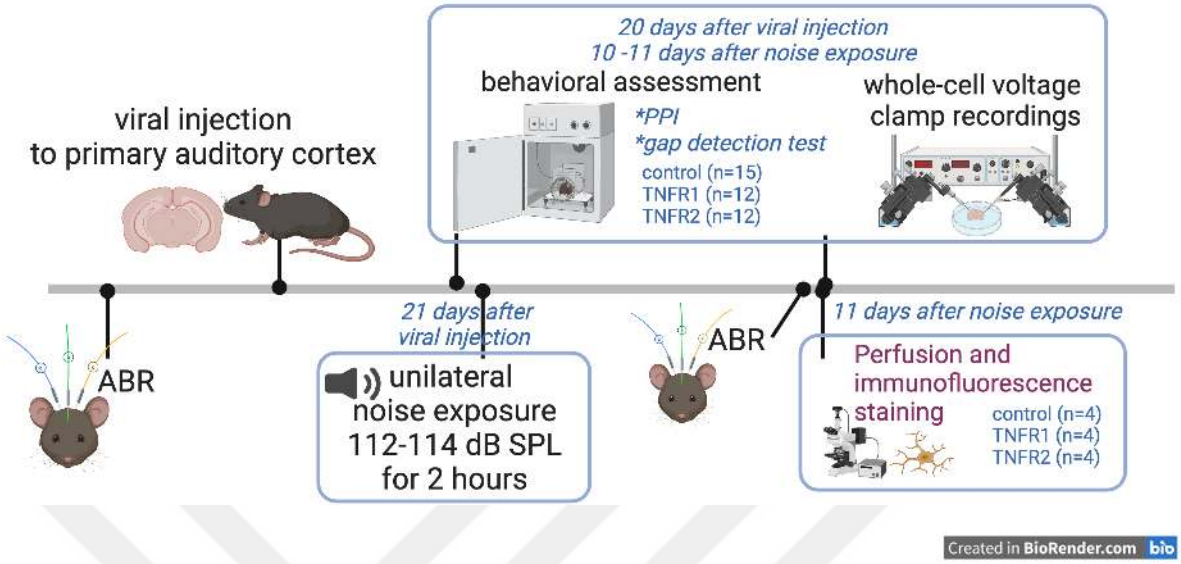


Figure 2.8: Experimental timeline of the AI part of the project. The figure is created in BioRender.

For the AI of the project (Figure 2.8), we used a total of 39 PV-Cre-tdTomato mice. We examined auditory brainstem responses to assess whether the animals had normal hearing. After viral injections into the AI, animals were housed in the facility for a twenty days incubation period to ensure that viral vectors were expressed. Then, we performed PPI and gap detection tests. Afterwards, we exposed the animals to noise unilaterally. Ten days after noise exposure, we repeated the behavioral experiments, re-evaluated auditory brainstem responses, perfused the animals, and collected brain tissue. Experiments for the whole-cell voltage clamp recordings were carried out before and after noise exposure.

Table 2.1. List of laboratory equipment used in experiments

Name	Company	Catalog number/model
Stereotaxic frame	World Precision Instruments	502650
Quintessential stereotaxic injector	Stoelting	53311
Micromotor high speed drill	Foredom	HP4-917
Drill bits	Hager & Meisinger GmbH	310-104001-001-005
Cold light source	Harvard Apparatus	W3 72-0215
Homeothermic heating pad and monitoring system	Harvard Apparatus	50-7220-F
Glass micropipette	Wiretrol	5-000-2010
Sterile latex surgical gloves	AmerisourceBergen	10119
Disposable filter unit	Thermo Scientific	564-0020
Vibratome	Leica	VT1000 S
Cryostat	Epredia	NX50
Glass bead sterilizer	CellPoint Scientific	101326-488
Micropipette puller	Sutter Instrument	P-1000
Water bath	Thermo Fisher Scientific	51221046
Lens paper	Fisher Scientific	11-996
Sterile syringe filters	LifeScience Products	CT-229746
Glass pipette for patch clamp	World Precision Instruments	PG10150-4
Camera controller	Hamamatsu	C2741-62
Multi-Manipulator Controller	Sutter Instrument	MPC-200
Manipulator	Sutter Instrument	ROE-200
Halogen Power Supply	Olympus	TH4-100
Automatic temperature controller	Warner Instruments	TC-324C
Mercury light source system	Olympus	U-HGLGPS
Peristaltic pump	World Precision Instruments	PERIPRO-4L
Fixed stage microscope	Olympus	BX51WI
Microelectrode Amplifier	Axon Instruments	MultiClamp 700

Low-Noise Data Acquisition System	Axon Instruments	Digidata 1550
Magnetic stirrer	Corning	PC-353
Ph meter	Mettler Toledo	01-912-345
Scale	Sartorius	BCE64-1S
Sound amplifier	Samson	SASA300
Anesthesia system	Kent Scientific	VetFlo
ABR recording rig	Tucker-Davis Technologies	RX5 Sys3
Open field speaker	Fostex	FT17
Surgical microscope	Zeiss	Opmi1
Incubator	VWR	1400E
Microscope	Olympus	BX40
Digital microscope camera	Hamamatsu	C11440
Cotton tipped applicators	VetOne	601020

Table 2.2. List of chemicals and consumables used in experiments

Name	Company	Catalog number/model
Povidone iodine solution	Priority Care	58829-120-32
Lactated Ringer's	VetOne	13985-803-60
Surgical adhesive	VetOne	510099
Artificial tears	Apexa	AP 704001
Absolute ethanol	Fisher Scientific	BP2818-500
Ethiqa	Fidelis	86084-100-30
Ketamine	VetOne	13985-584-10
Xylazine	VetOne	13985-704-10
Isoflurane	VetOne	13985-528-60
Sterile saline	VetOne	13985-807-60
20x phosphate buffered saline	Growcells	MRGF-6395
10x casein solution	VectorLabs	SP-5020
IBA1 polyclonal antibody	FUJIFILM Wako Chemicals	019-19741
Parvalbumin polyclonal antibody	Thermo Fisher Scientific	PA1-933
Alexa Fluor 647 secondary antibody	Thermo Fisher Scientific	A32733
Antifade mounting medium with DAPI	VectorLabs	H-200
Paraformaldehyde	Sigma	P6148
N-methyl D-Glucamine	TCI Chemicals	M0227
Potasium chloride	Sigma	P9541
Sodium phosphate monobasic	Sigma	S5011
Sodium phosphate dibasic	Sigma	S7907
Sodium bicarbonate	Sigma	S5761
HEPES	Sigma	H3375
Glucose	Sigma	G7528

Sodium l-ascorbate	Sigma	A4034
Thiourea	Merck	1.07979
Sodium pyruvate	Merck	P5280
Sodium chloride	Sigma	S3014
Calcium chloride dihydrate	Sigma	C3306
Magnesium sulfate	Fisher Scientific	M63-500
Tetrodotoxin citrate	Tocris Bioscience	1069

CHAPTER 3

RESULTS

3.1. Auditory Brainstem Response (ABR) Recordings

In this thesis project, we evaluated hearing thresholds with ABR recordings before and on the 1st, 3rd and 10th days following noise exposure. ABR recordings suggested that hearing thresholds of normal-hearing animals increased to 60-70 dB SPL in noise-exposed ear and thresholds increased temporarily in the protected ear (Figure 3.1).

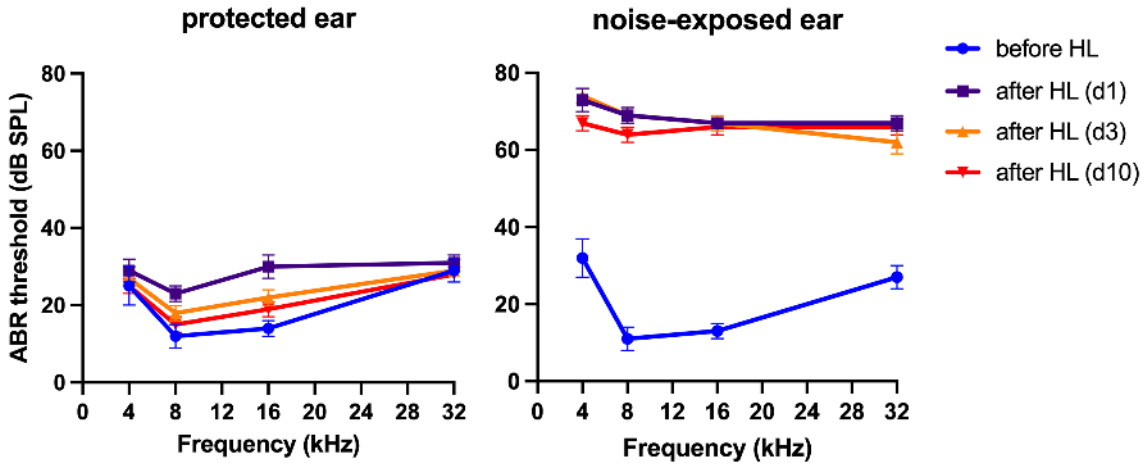


Figure 3.1: ABR thresholds of the animals before and after noise hearing lesion.

3.2. Results after Injection of the Viral Vectors into the Hippocampus and Noise Exposure

3.2.1. Open Field Test (OFT) Results

In the OFT, we compared the distance traveled by the animals, the time spent in the center area and in the areas outside of the center, the distance ratio traveled in the center by way of the animal tracks. Additionally, the number of fecal boli is counted (Figure 3.2).

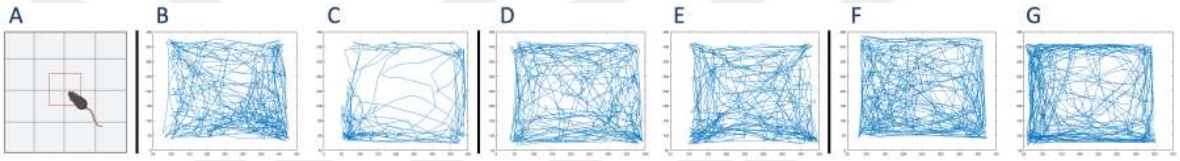
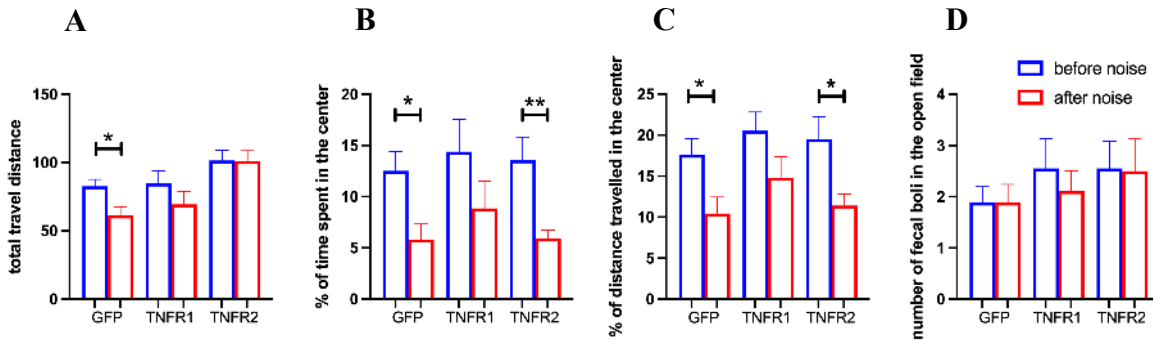


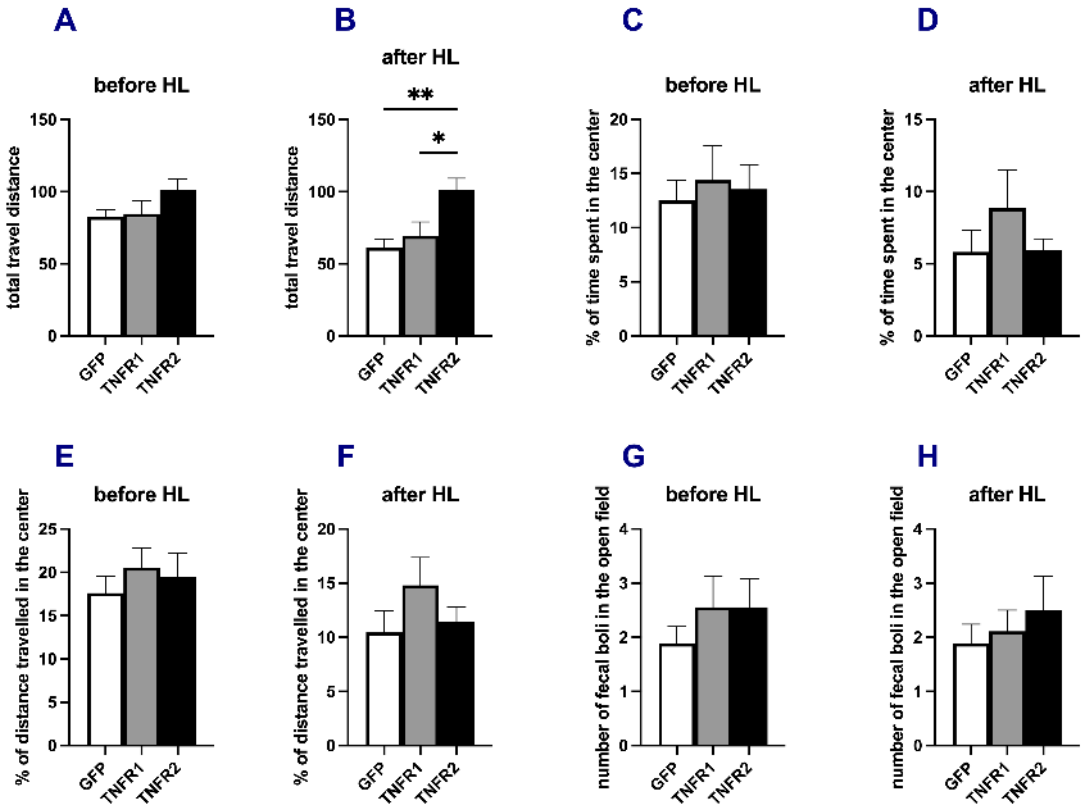
Figure 3.2: A) Schematic illustration of the OFT, B-C) Representative animal track for the control animal before and after the noise exposure, respectively. D-E) Representative animal track for the animal injected with TNFR1 knockdown virus before and after the noise exposure, respectively. F-G) Representative animal track for the animal injected with TNFR2 knockdown virus before and after the noise exposure, respectively.



The data were presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

Figure 3.3: Graphs for the total distance traveled (A), time ratio of center/outer area (B), distance ratio traveled in the center (C) and number of fecal boli in the open field arena (D), respectively.

We observed a statistically significant difference after NE in the control group compared to before NE in terms of the distance traveled ($p < 0.05$), while there was no change in the TNFR1 knockdown and TNFR2 knockdown groups (Figure 3.3.A). We calculated the time spent in the center before and after the noise; we showed a significant difference in the control group ($p < 0.05$) and TNFR2 knockdown group ($p < 0.01$), while we did not see any difference in the TNFR1 knockdown group (Figure 3.3.B). Further, we analyzed the distance ratio traveled in the center and demonstrated a significant difference in terms of the distance ratio traveled in the center before and after the noise in the control group ($p < 0.05$) and TNFR2 knockdown group ($p < 0.05$), while we did not see any difference in the TNFR1 knockdown group (Figure 3.3.C). A higher ratio is considered to mean that the animal is less anxious. A high number of fecal boli is also a sign of anxiety, but we did not observe any significant difference in any group in this comparison (Figure 3.3.D).



The data were presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

Figure 3.4: Graphs for the total distance traveled (A, B), time ratio of center/outer area (C, D), distance ratio traveled in the center (E, F) and number of fecal boli in the open field arena (G, H), before and after noise exposure, respectively.

When we analyzed the data to see differences between experimental groups before and after hearing lesion, the TNFR2 knockdown group showed a significant increase in total travel distance compared to the control group ($p < 0.01$) and TNFR1 knockdown group ($p < 0.05$) after noise exposure (Figure 3.4.B).

3.2.2. Light/Dark Box Test (LDB) Results

In the LDB, we compared the number of entry into the bright area, the time spent in the bright area, the distance from the door and the number of fecal boli. We examined the animal tracks in the light/dark box (Figure 3.5).

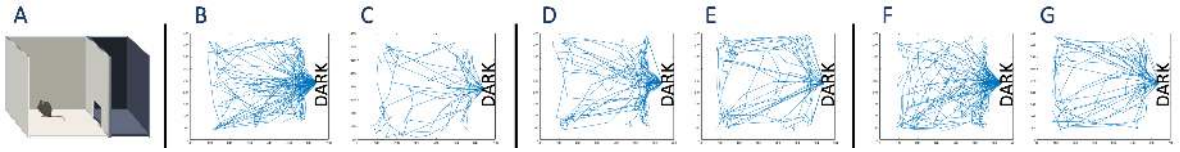
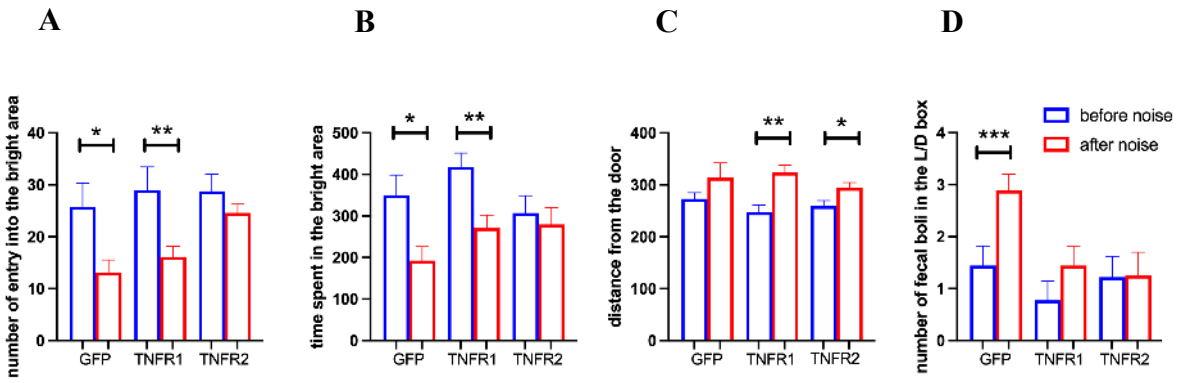


Figure 3.5: A) Schematic illustration of the LDB, B-C) Representative animal track in the light compartment for the control animal before and after the noise exposure, respectively. D-E) Representative animal track in the light compartment for the animal injected with TNFR1 knockdown virus before and after the noise exposure, respectively. F-G) Representative animal track in the light compartment for the animal injected with TNFR2 knockdown virus before and after the noise exposure, respectively.

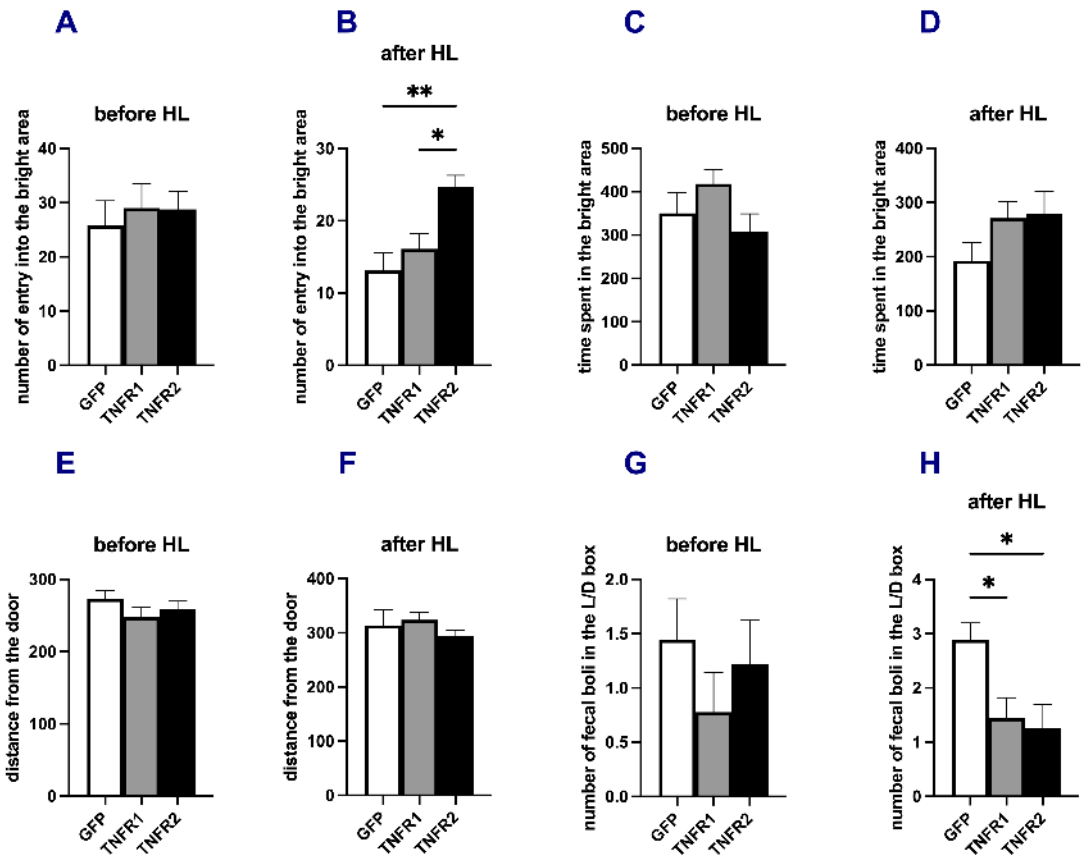


The data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.6: Graphs for the number of entry into the bright area (A), the time spent in the bright area (B), distance from the door (C) and the number of fecal boli (D) in the light/dark box.

We showed a statistically significant difference after noise exposure in the control ($p < 0.05$) and TNFR1 knockdown groups ($p < 0.01$) while there was no change in the TNFR2 knockdown group in terms of both the number of entry into the bright area (Figure 3.6.A) and the time spent in the bright area (Figure 3.6.B). In terms of the distance from the door, we demonstrated a significant difference after noise exposure in the TNFR1 knockdown group ($p < 0.01$) and TNFR2 knockdown group ($p < 0.05$), while we did not see any difference in the control group (Figure 3.6.C). Also, we observed a significant difference in the control group in terms of number of fecal boli ($p < 0.001$; Figure 3.6.D).

When we analyzed the data to see differences between experimental groups before and after hearing lesion, we observed that the TNFR2 knockdown group exhibited a significant increase in number of entry into the bright area compared to the control group ($p < 0.01$) and TNFR1 knockdown group ($p < 0.05$) after noise exposure (Figure 3.7.B). Also, we observed a significant difference in the control group compared to other experimental groups in terms of number of fecal boli following noise exposure ($p < 0.05$; Figure 3.7.H).

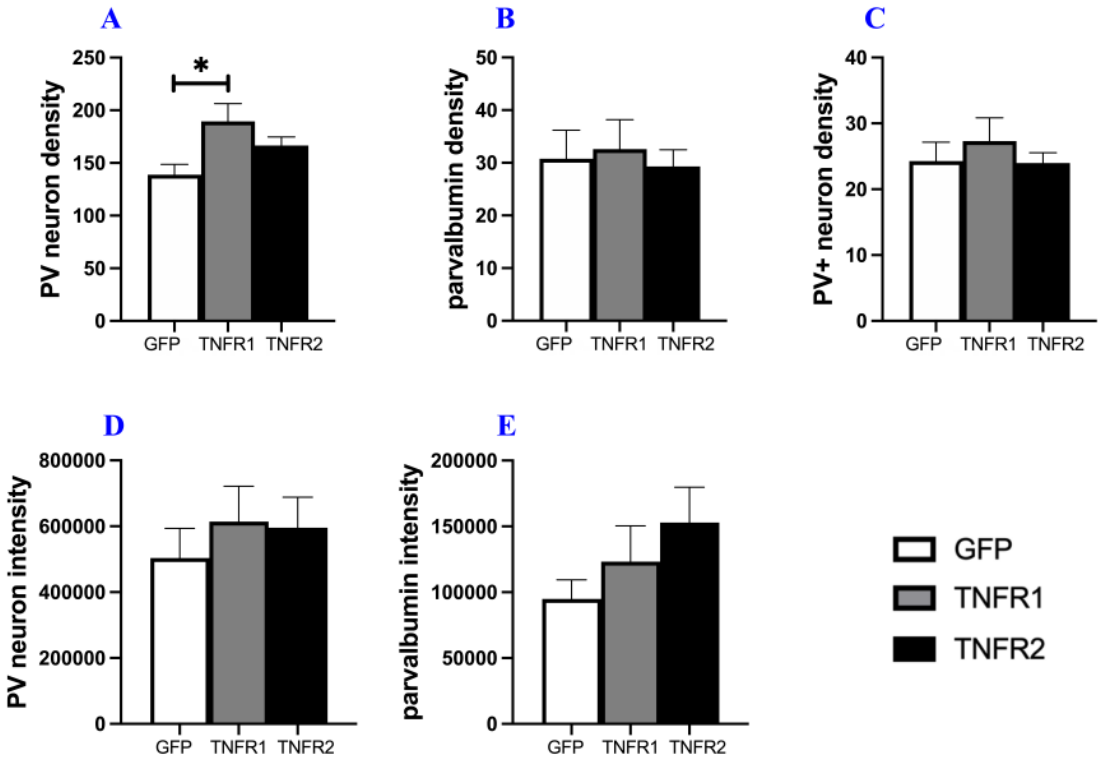


The data were presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

Figure 3.7: Graphs for the number of entry into the bright area (A, B), the time spent in the bright area (C, D), distance from the door (E, F) and the number of fecal boli (G, H) in the light/dark box, before and after noise exposure, respectively.

3.2.3. PV neurons in the AI

Images at 10× magnification from the AI were analyzed to investigate PV neurons. In the representative immunofluorescence images (Figure 3.9), red color marks PV neurons which are labeled in PV-Cre-tdTomato mice, the blue color marks cells stained with anti-parvalbumin antibody and the purple color marks colocalization. We refer to all neurons that are marked with tdTomato as PV neurons and those that actively express PV as PV+ neurons. According to the literature, variations in PV expression can occur within PV+ populations, and a decrease in PV+ neuron density might indicate either the death of PV+ neurons or a decline in PV expression.



The data were presented as mean ± SEM. * $p < 0.05$.

Figure 3.8: Graphs for the quantification of PV neuron density (A), parvalbumin density (B), PV+ neuron density (C), PV neuron intensity (D) and parvalbumin immunofluorescence intensity (E) in the AI after the viral injections to the vHPC and noise exposure.

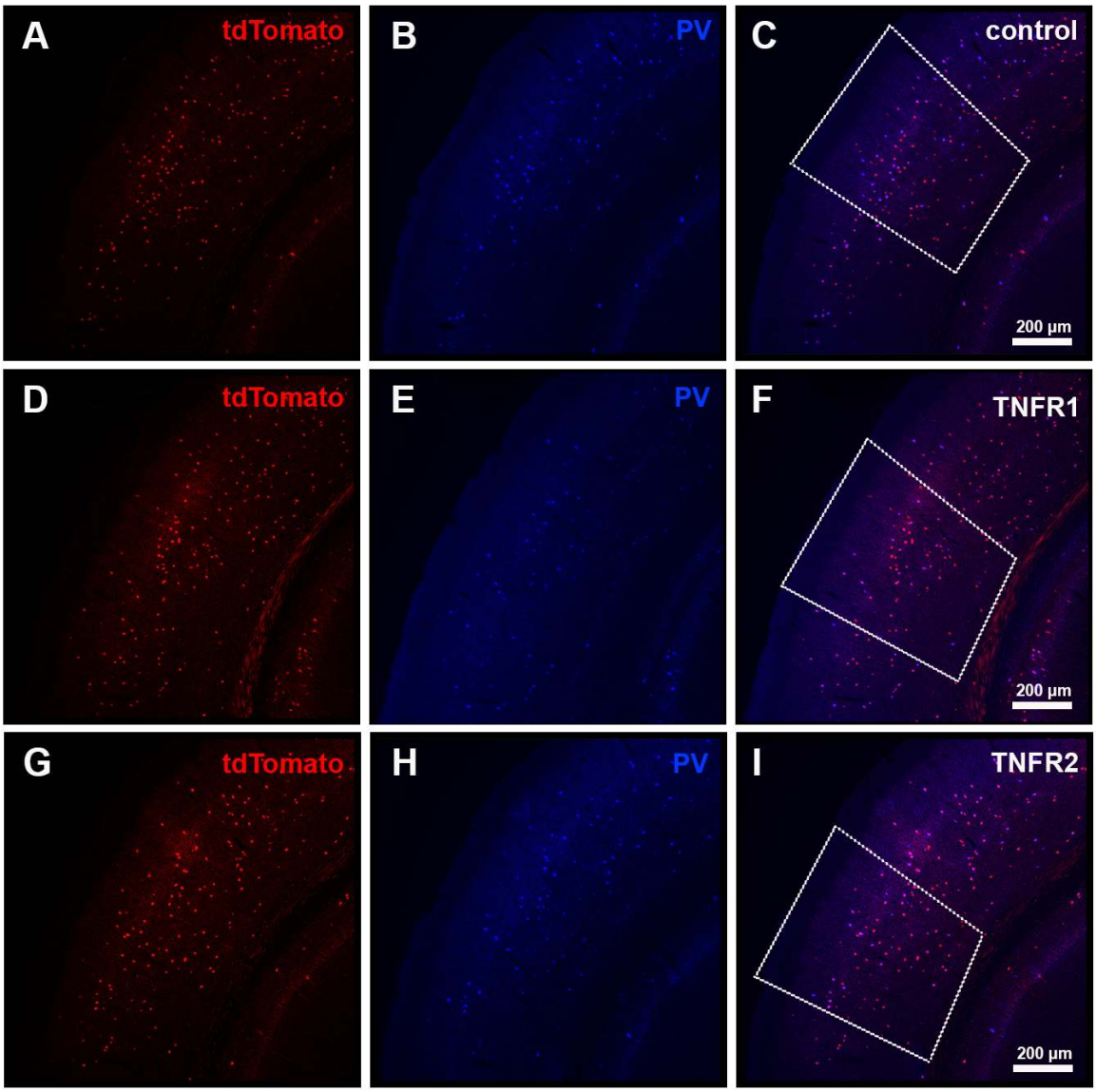


Figure 3.9: Representative immunofluorescence images from PV neurons in the AI of the control (A, B, C), TNFR1 knockdown (D, E, F) and TNFR2 knockdown (G, H, I) groups after viral vector injection into the bilateral vHPC. The red color represents cre reporter tdTomato (PV neurons), the blue color represents neurons stained with anti-parvalbumin polyclonal antibody and the purple color represents PV neurons those that actively express parvalbumin (PV+ neurons).

We showed that PV neuron density of TNFR1 knockdown mice was significantly higher than control group after noise exposure ($p < 0.05$; Figure 3.8.A). We did not see any other significant difference in terms of parvalbumin density, PV+ neuron density, PV neuron

intensity and parvalbumin intensity. We revealed that knockdown of TNFR1 hippocampal PV neurons prevented noise-induced decrease of PV neuron density in the AI but did not have any impact on parvalbumin expression.

3.2.4. Microglia Density and Intensity in the AI

Images at 10× magnification from the AI in the right hemisphere (Figure 3.10) were analyzed to investigate microglia density and intensity.

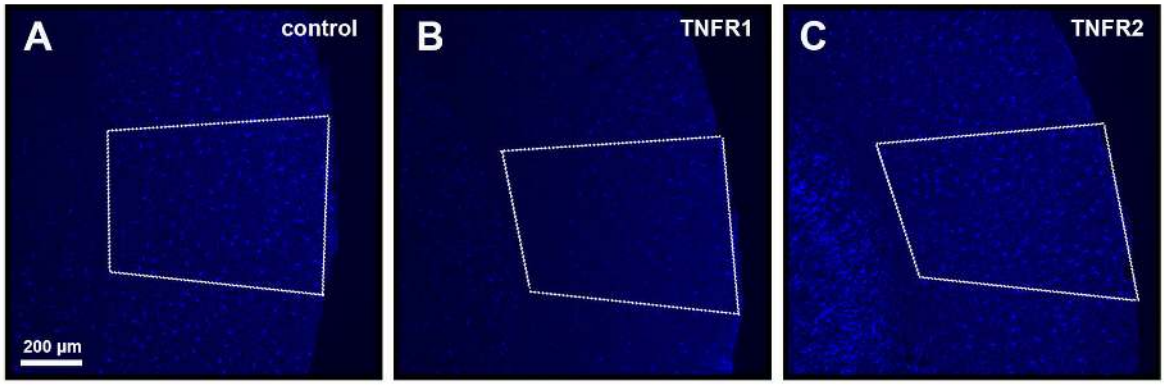
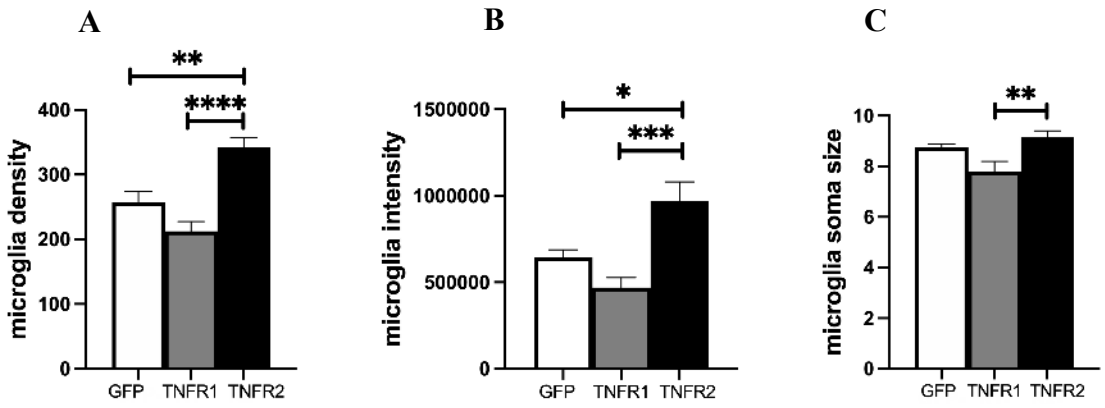


Figure 3.10: Representative immunofluorescence images from microglia in the AI of the control (A), TNFR1 knockdown (B) and TNFR2 knockdown (C) groups after viral vector injection into the bilateral vHPC and noise exposure. In the images, blue color represents microglia which are labeled with anti-Iba1 antibody.



The data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

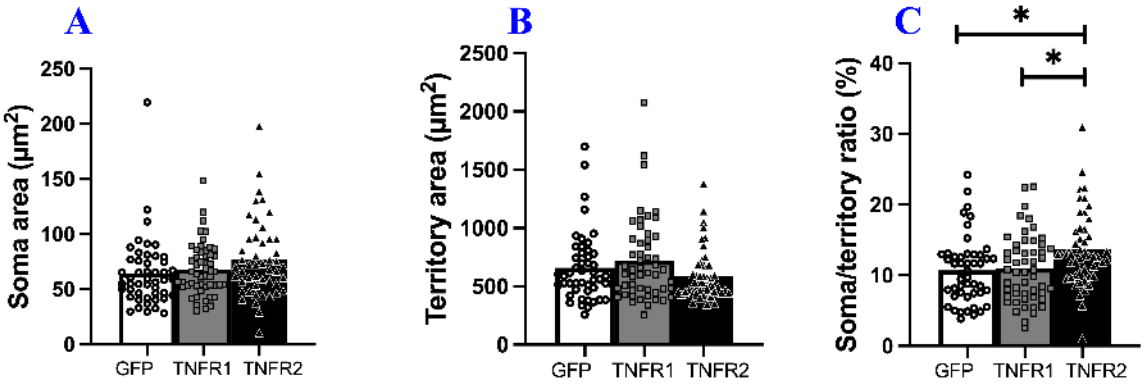
Figure 3.11: Graphs for the quantification of microglia density (A), microglia intensity (B) and microglia soma size (C) in the AI after the viral injections to the vHPC and noise exposure.

Microglia density of TNFR2 knockdown mice is higher than control ($p < 0.01$; Figure 3.11.A) and TNFR1 knockdown groups ($p < 0.0001$; Figure 3.11.A) after the viral injections to the vHPC and noise exposure. Also, microglia immunofluorescence intensity of TNFR2 knockdown mice is higher than control ($p < 0.05$; Figure 3.11.B) and TNFR1 knockdown groups ($p < 0.001$; Figure 3.11.B). In addition, soma size of the microglia increased in the TNFR2 knockdown group compared to TNFR1 knockdown ($p < 0.01$; Figure 3.11.C). We showed that knockdown of TNFR2 in hippocampal PV neurons exacerbates the increase of microglial density and intensity in the AI.

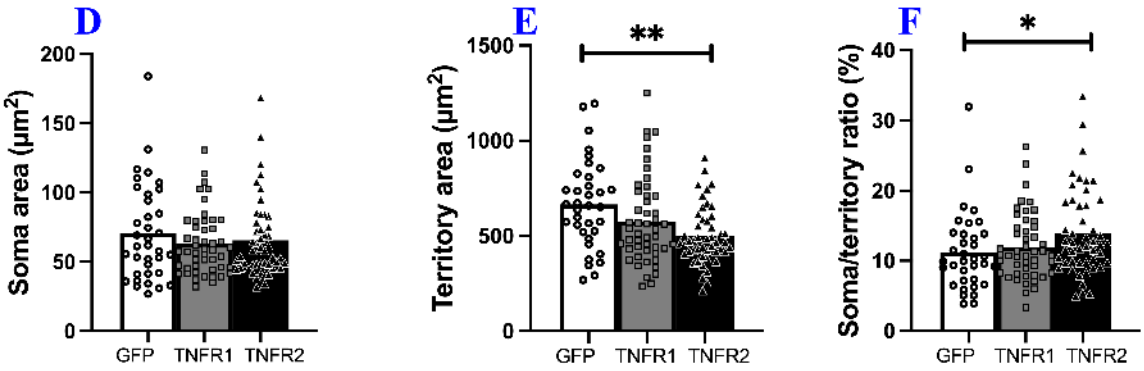
3.2.4. Microglial Deramification in the vHPC

Images at 40× magnification were analyzed to investigate microglial deramification in the vHPC. Soma/territory ratio in TNFR2 knockdown mice is higher than control ($p < 0.05$; Figure 3.12.C) and TNFR1 knockdown groups ($p < 0.05$; Figure 3.12.C) in the CA1 region after the viral injections to the vHPC and noise exposure. In the CA3 region or the vHPC, soma/territory ratio of TNFR2 knockdown mice is higher than control animals ($p < 0.05$; Figure 3.12.F). In addition, territory area of the microglia is decreased in the TNFR2 knockdown group compared to control mice ($p < 0.01$; Figure 3.12.E). We did not see any statistically significant difference in the DG region in terms of the soma area, territory area and soma/territory ratio. We showed that knockdown of TNFR2 in hippocampal PV neurons exacerbates the microglia deramification in the CA1 and CA3 regions of the vHPC following noise exposure.

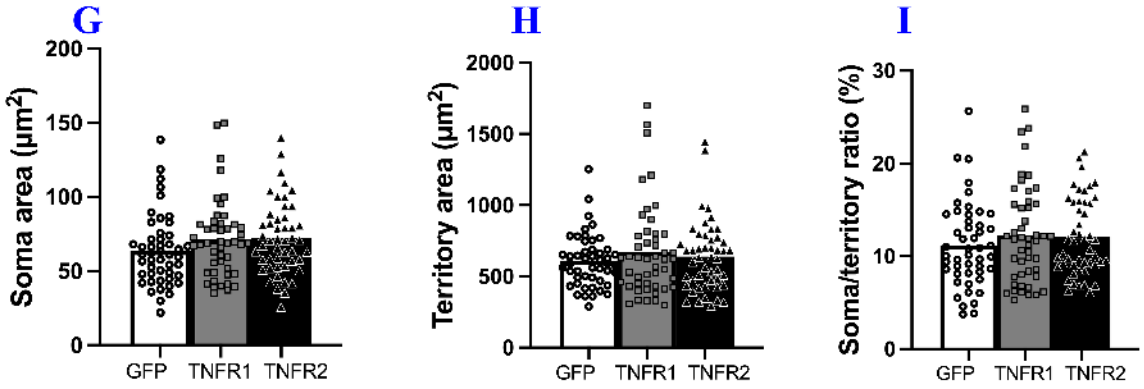
CA1



CA3



DG



The data were presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

Figure 3.12: Graphs for the quantitative measurements of the soma area, territory area, and soma/territory ratio for control, TNFR1 knockdown, and TNFR2 knockdown groups across CA1 region (A, B, C), CA3 region (D, E, F) and DG region (G, H, I) of the vHPC after the viral injections to the vHPC and noise exposure.

3.3. Results after Injection of the Viral Vectors into the AI and Noise Exposure

3.3.1. PPI Performance

In the PPI test, we quantified the acoustic startle response and compared the ratio before and after noise exposure between groups. We showed that PPI performance is not significantly altered by noise exposure in the PPI test (Figure 3.13). In addition, we compared the startle response ratio on frequency basis of all experimental groups before and after hearing loss. We demonstrated that PPI performance is not differ at all frequencies (5 kHz, 10 kHz, 14 kHz and 20 kHz) between control, TNFR1 knockdown and TNFR2 knockdown groups (Figure 3.14).

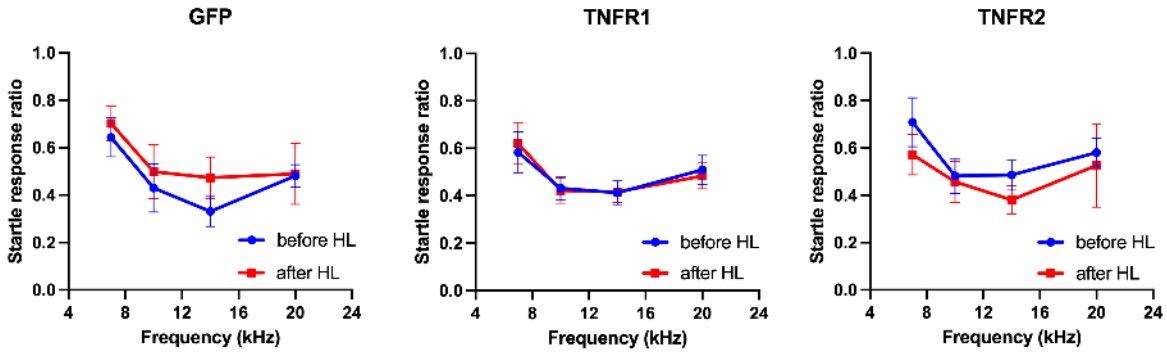


Figure 3.13: Startle response ratio of the control animals, TNFR1 knockdown and TNFR2 knockdown groups in PPI test before (blue line) and after (red line) hearing lesion.

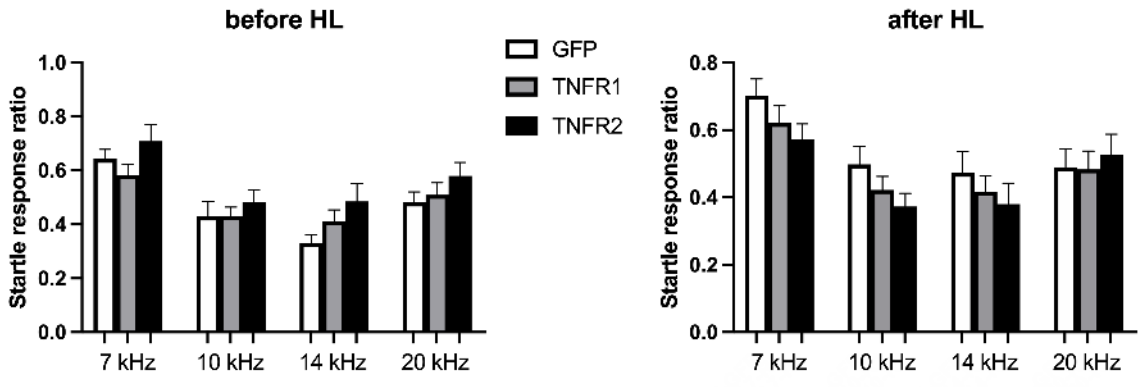
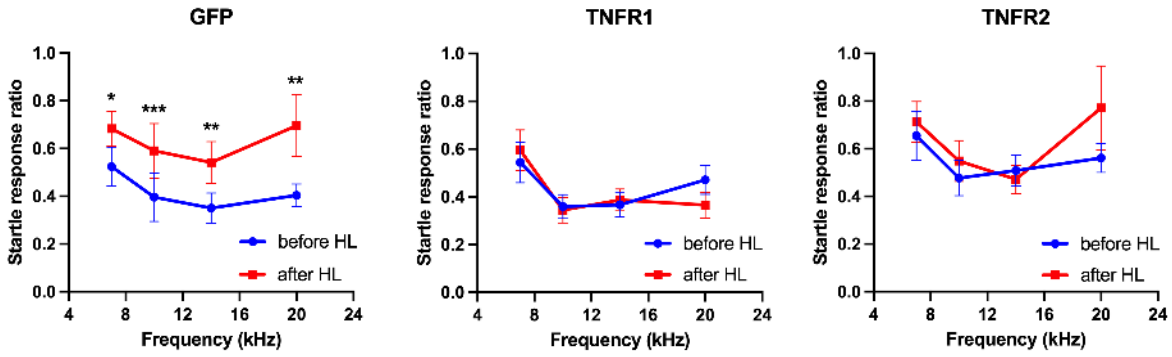


Figure 3.14: Startle response ratio on frequency basis of all experimental groups in PPI test before (left) and after (right) hearing lesion.

3.3.2. Gap Detection (GD) Results

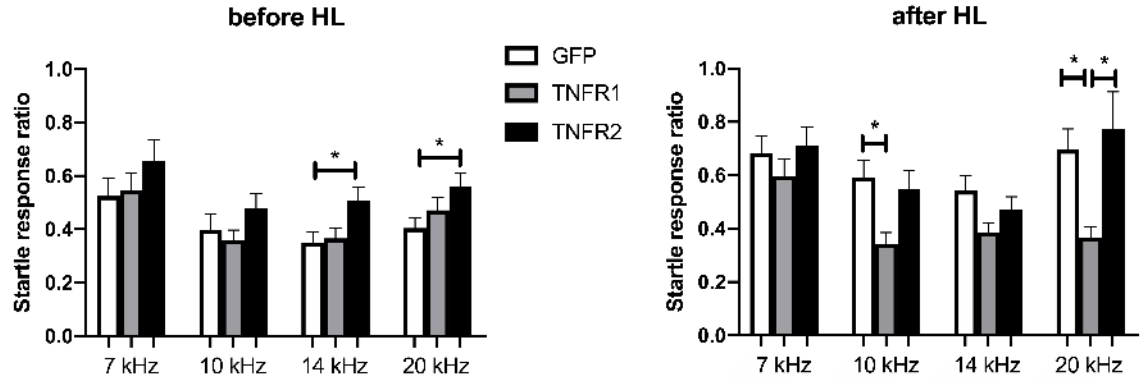
In the GD test, we quantified the acoustic startle response and compared the ratio before and after noise exposure between groups. We showed that startle response ratio of the control group is increased after hearing lesion at all frequencies (5 kHz, 10 kHz, 14 kHz and 20 kHz; Figure 3.15). There is no statistically significant difference in TNFR1 and TNFR2 knockdown groups after hearing lesion (Figure 3.15).

In addition, we compared the startle response ratio on frequency basis of all experimental groups in the GD test. Mice in the TNFR2 knockdown group have elevated startle response ratio compared to control group before hearing lesion at all frequencies; this difference is statistically significant at 14 kHz and 20 kHz (Figure 3.16, left side). When we looked at the GD results after hearing lesion, we saw that control group have increased startle response ratio compared to TNFR1 knockdown group at all frequencies and this difference is statistically significant at 10 kHz and 20 kHz (Figure 3.16, right side). Mice in the TNFR2 knockdown group have also increased startle response ratio compared to TNFR1 knockdown group at all frequencies but we showed statistically significant difference only at 20 kHz (Figure 3.16, right side).



The data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Figure 3.15: Startle response ratio of the control animals, TNFR1 knockdown and TNFR2 knockdown groups in GD test before (blue line) and after (red line) hearing lesion.



The data were presented as mean ± SEM. * $p < 0.05$.

Figure 3.16: Startle response ratio on frequency basis of all experimental groups in GD test before (left) and after (right) hearing lesion.

3.3.3. PV neurons in the AI

When we quantified PV neurons and the parvalbumin stainings and compared the results between all experimental groups (Figure 3.17), we saw that PV neuron density of TNFR1 knockdown mice was significantly higher than TNFR2 knockdown group after viral vector injection into the right AI and noise exposure ($p < 0.05$; and Figure 3.18.A). In addition, parvalbumin density ($p < 0.05$; Figure 3.18.B), PV+ neuron density ($p < 0.001$; Figure

3.18.C) and PV neuron intensity ($p < 0.05$; Figure 3.18.D) of TNFR1 knockdown mice was significantly higher than the control group. We did not see significant difference in terms of parvalbumin intensity. We revealed that knockdown of TNFR1 prevented noise trauma-induced PV+ neuron loss and reduction of PV expression in the AI.

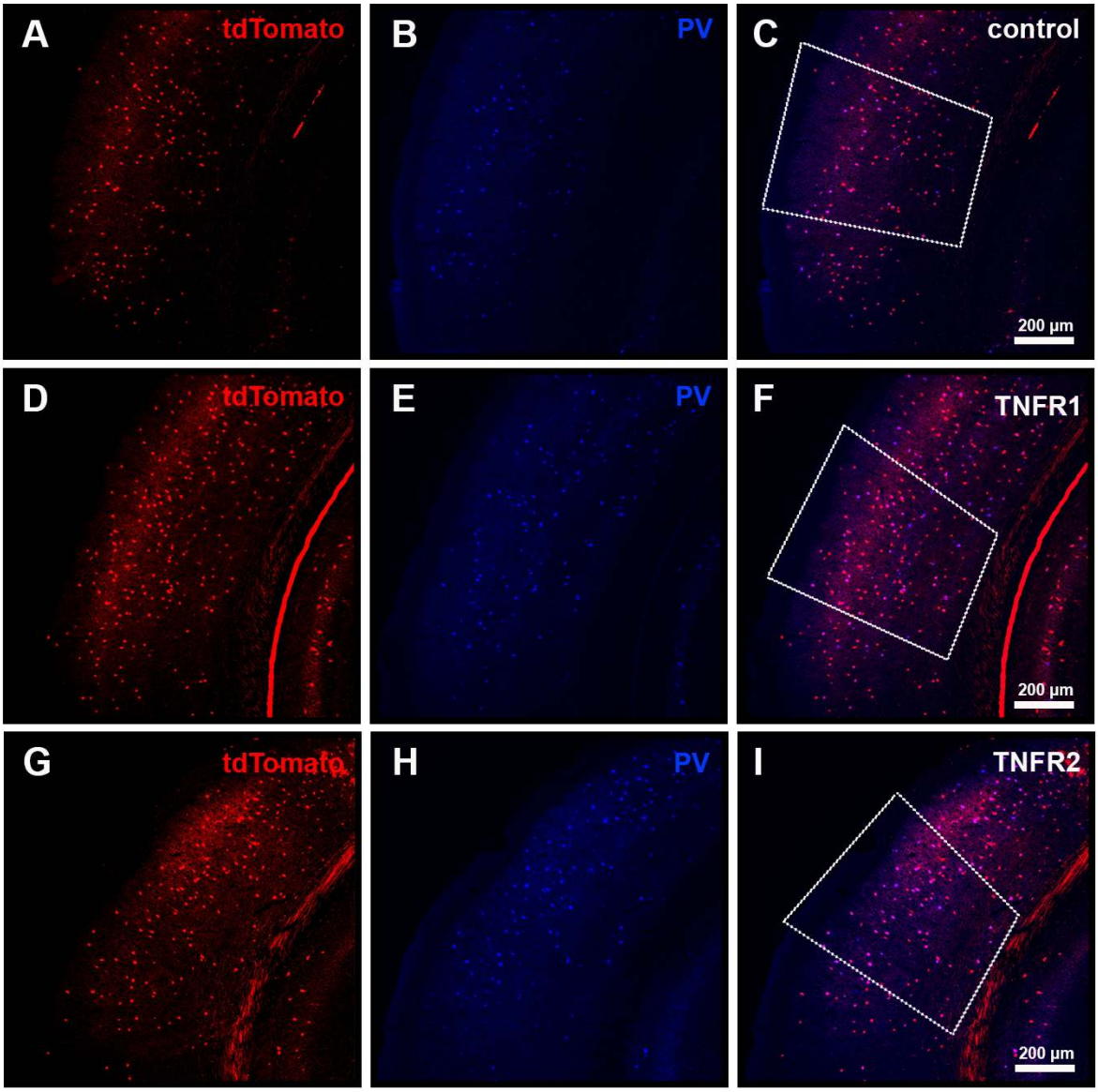
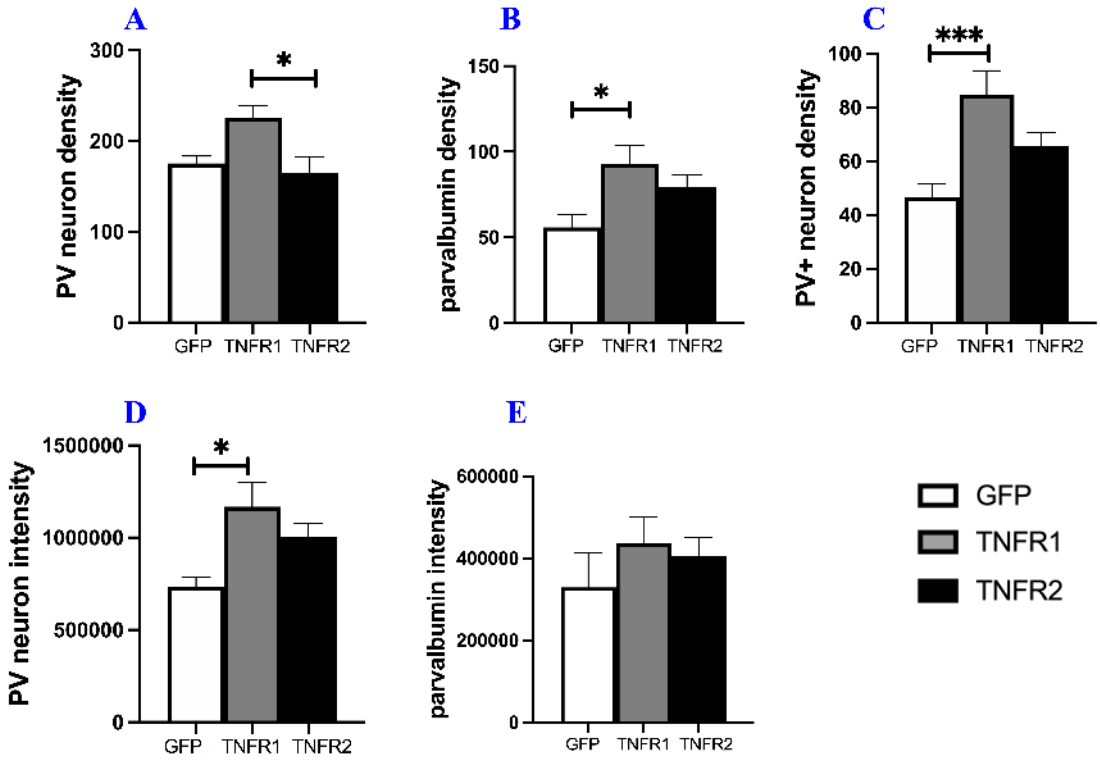


Figure 3.17: Representative immunofluorescence images from PV neurons in the right AI of the control (A, B, C), TNFR1 knockdown (D, E, F) and TNFR2 knockdown (G, H, I) groups after viral vector injection into the right AI and noise exposure. The red color represents cre reporter tdTomato (PV neurons), the blue color represents neurons stained with anti-parvalbumin polyclonal antibody and the purple color represents PV neurons those that actively express parvalbumin (PV+ neurons).



The data were presented as mean ± SEM. * $p < 0.05$ and *** $p < 0.001$.

Figure 3.18: Graphs for the quantification of PV neuron density (A), parvalbumin density (B), PV+ neuron density (C), PV neuron intensity (D) and parvalbumin immunofluorescence intensity (E) in the AI of the right hemisphere after the viral injections to the AI and noise exposure.

3.3.4. Microglia Density and Intensity in the AI

Microglia density in the AI of TNFR2 knockdown mice is higher than TNFR1 knockdown group after the viral injections to the AI and noise exposure (Figure 3.19 and Figure 3.20, left

side). However, we could not show statistically significant difference between TNFR2 knockdown and TNFR1 knockdown groups in terms of microglia intensity in the AI (Figure 3.19 and Figure 3.20, right side).

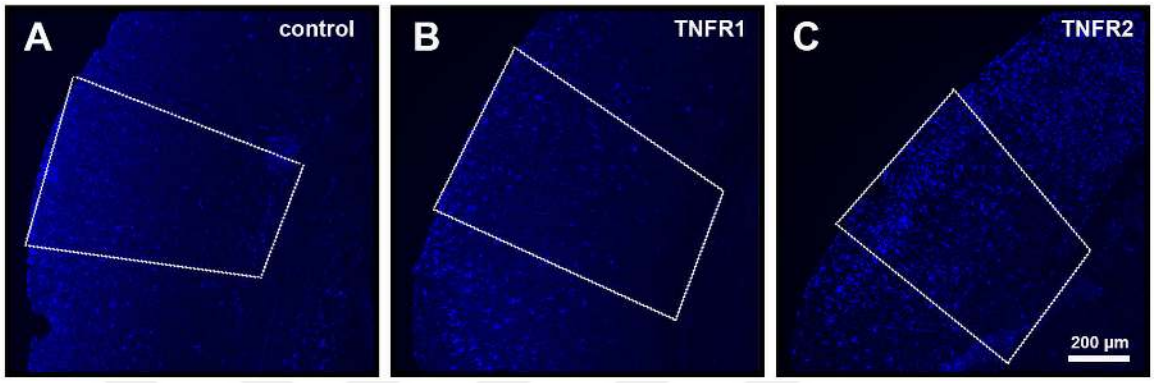
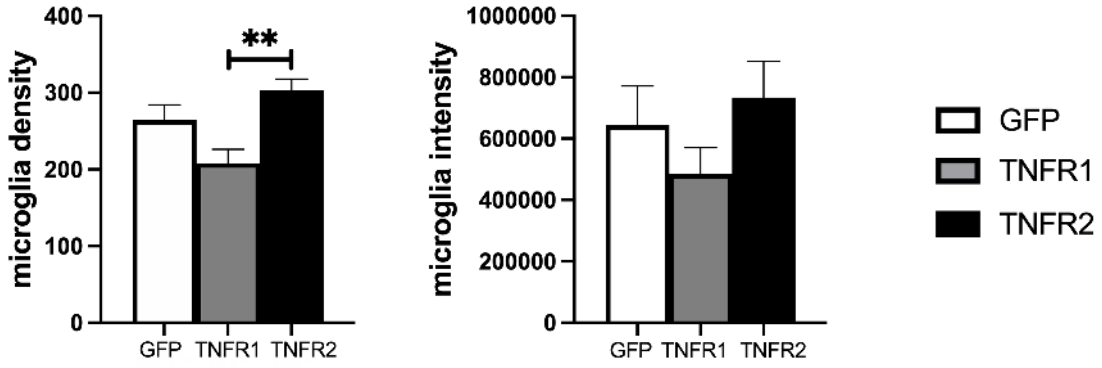


Figure 3.19: Representative immunofluorescence images from microglia in the right AI of the control (A), TNFR1 knockdown (B) and TNFR2 knockdown (C) groups after viral vector injection into the right AI and noise exposure. In the images, blue color represents microglia which are labeled with anti-Iba1 antibody.



The data were presented as mean ± SEM. ** $p < 0.01$.

Figure 3.20: Graphs for the quantitative measurements of the microglia density (left side) and intensity (right side).

3.3.4. Microglial Deramification in the AI

In addition to microglia and intensity, we measured microglial deramification in the right AI using higher magnification images from the control, TNFR1 knockdown and TNFR2 knockdown groups (Figure 3.21). Results indicated that soma area ($p < 0.05$; Figure 3.22.A), soma to territory ratio ($p < 0.01$; Figure 3.22.C), primary process number ($p < 0.01$; Figure 3.22.D) and diameter ($p < 0.05$; Figure 3.22.F) of TNFR2 knockdown mice is higher than control group. Also, there was a statistically significant difference between TNFR1 and TNFR2 knockdown groups in terms of soma area ($p < 0.01$; Figure 3.22.A), soma to territory ratio ($p < 0.01$; Figure 3.22.C) and primary process diameter ($p < 0.01$; Figure 3.22.F). Besides, primary process number of the control group is lower than TNFR1 knockdown group ($p < 0.05$; Figure 3.22.D) and primary process length of the control group is higher than TNFR2 knockdown group ($p < 0.05$; Figure 3.22.E). We revealed that knockdown of TNFR2 exacerbates the increase of microglial density and noise-induced microglial deramification in the AI.

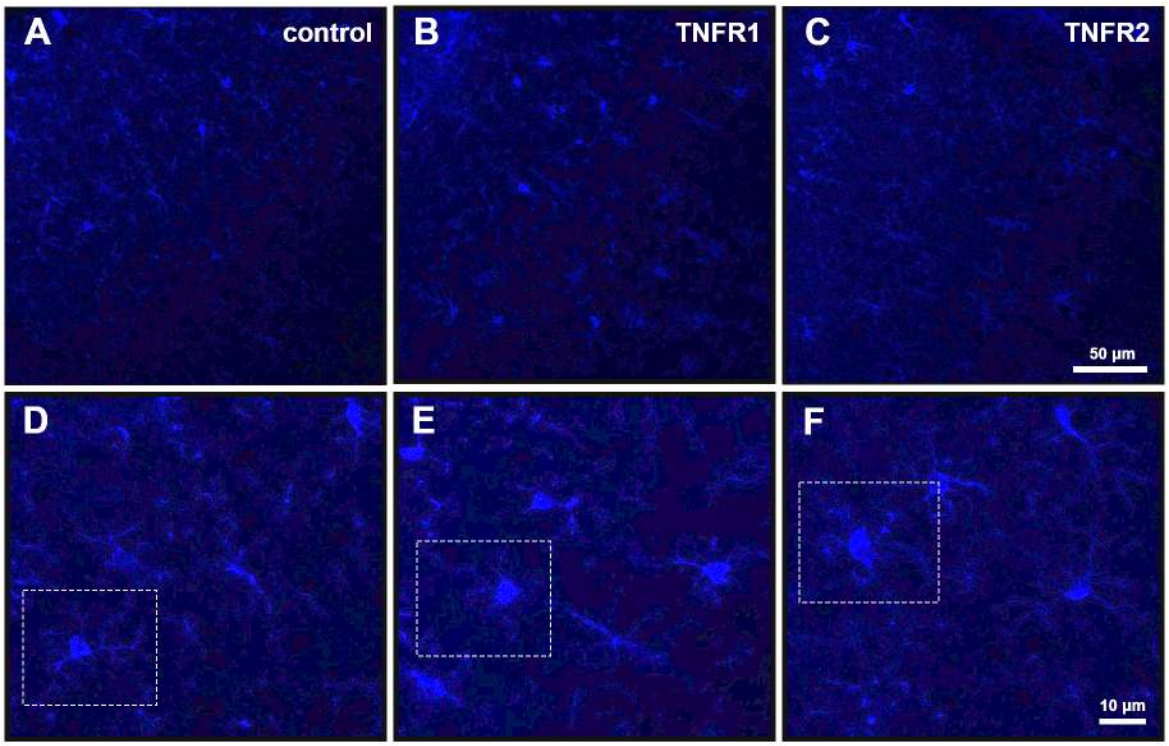
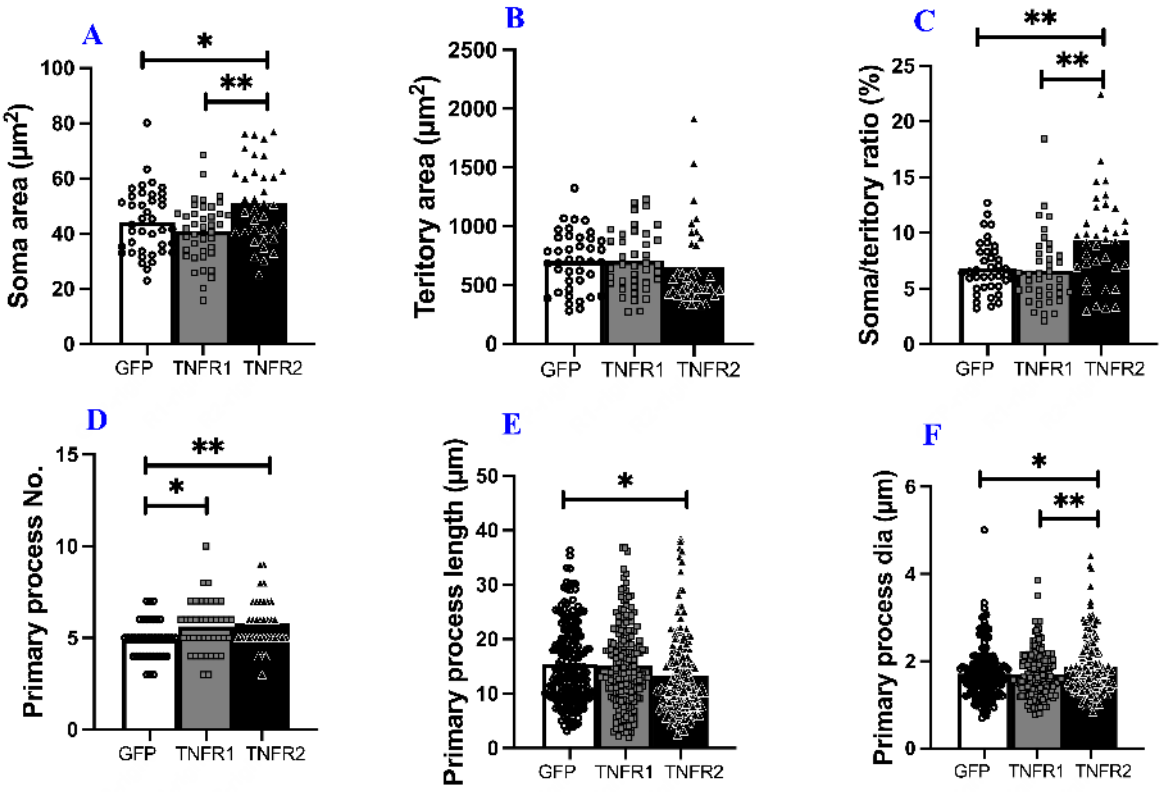


Figure 3.21: Representative higher magnification images from microglia of the AI in the control animals (A, D), TNFR1 knockdown (B, E) and TNFR2 knockdown (C, F) groups after the viral injections to the AI and noise exposure.



The data were presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

Figure 3.22: Graphs for the quantitative measurements of the soma area (A), territory area (B), and soma/territory ratio (C), number of primary processes (D), primary process length (E) and primary process diameter (F) for GFP, TNFR1 knockdown, and TNFR2 knockdown groups in the right AI after the viral injections to the AI and noise exposure.

3.3.5. Results of the Whole-cell Voltage Clamp Recordings

NIHL resulted in reduced inhibitory and increased excitatory synaptic transmission which was prevented by TNFR1 knockdown mice. We revealed that noise caused decreased mIPSC frequency in the control and TNFR2 knockdown groups, however, there was no statistically significant difference between before and after hearing lesion in TNFR1 knockdown mice (p

< 0.05; Figure 3.25.A). mIPSC frequency of the TNFR1 knockdown mice was significantly higher than control and TNFR2 knockdown mice after hearing lesion ($p < 0.01$; Figure 3.25.A). Moreover, the noise exposure produced an increase in mIPSC amplitude only in the TNFR2 knockdown mice ($p < 0.05$; Figure 3.25.B).

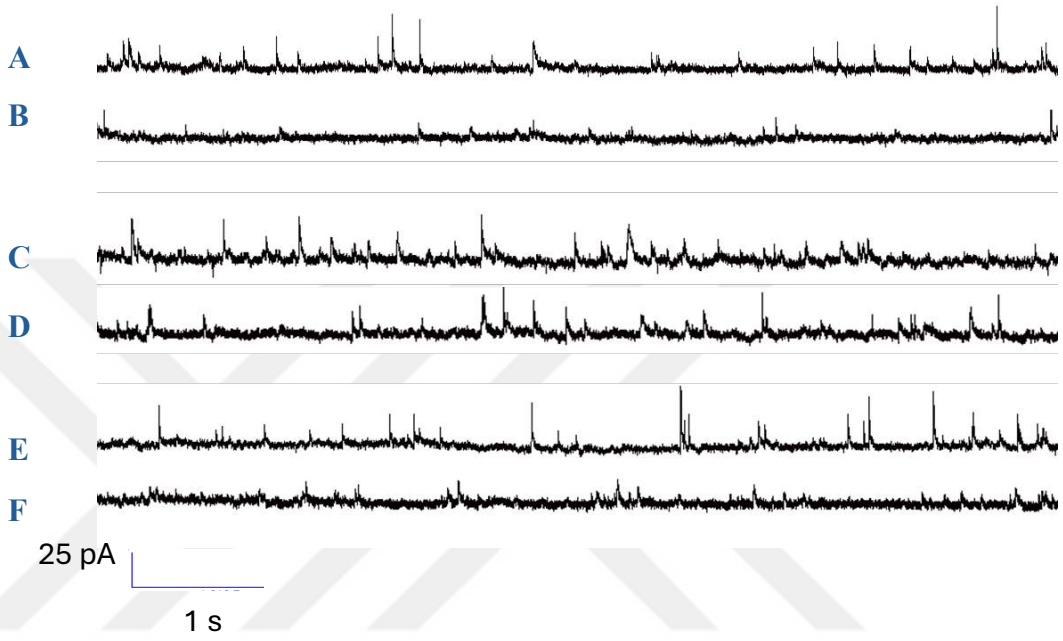


Figure 3.23: Representative traces from mIPSC recordings in AI of the control animals (A, B), TNFR1 knockdown (C, D) and TNFR2 knockdown (E, F) groups after the viral injections to the AI and before/after noise exposure, respectively.

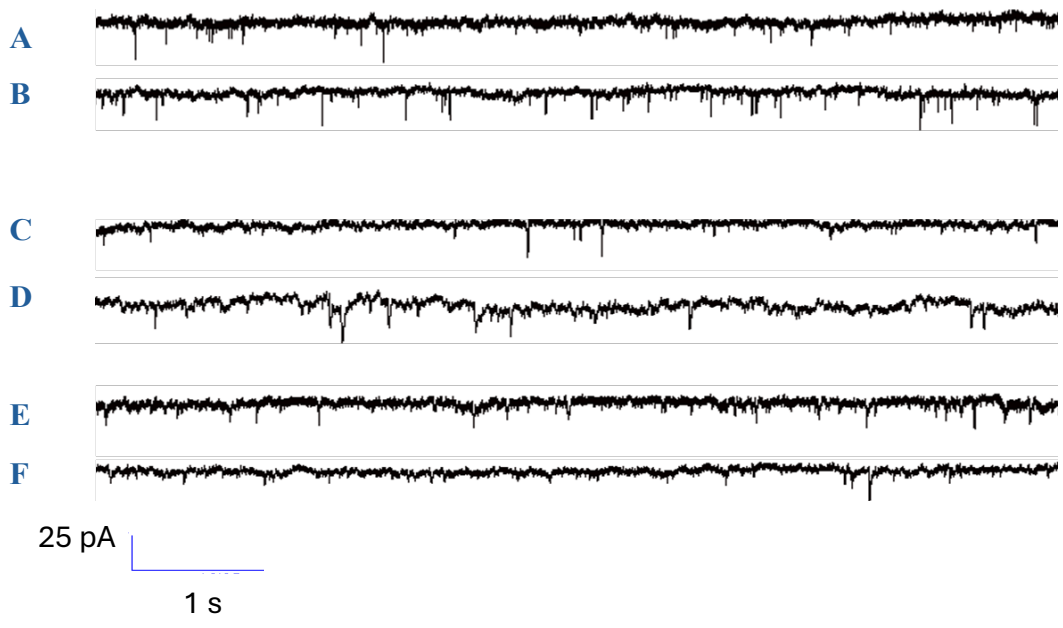
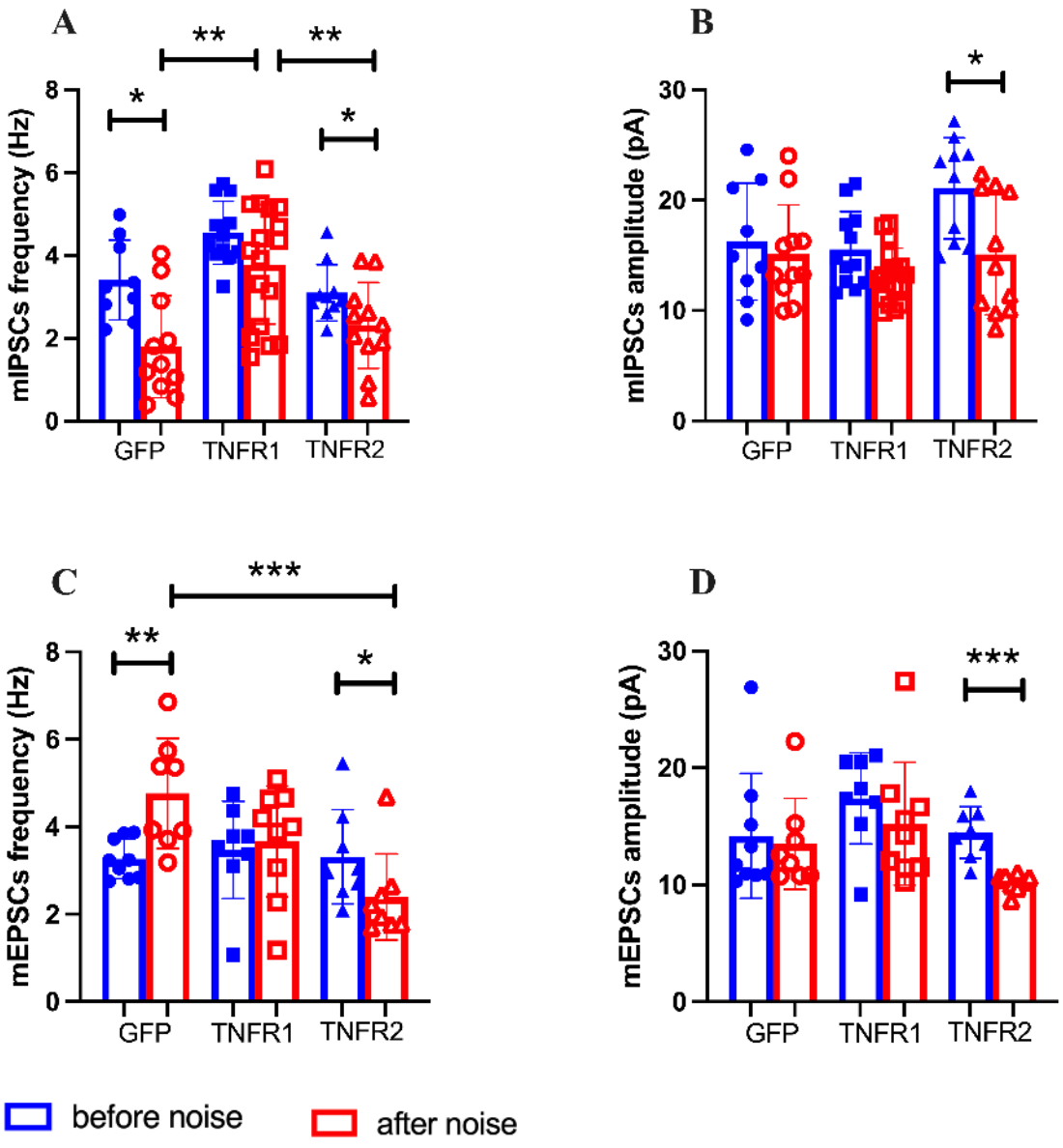


Figure 3.24: Representative traces from mEPSC recordings in AI of the control animals (A, B), TNFR1 knockdown (C, D) and TNFR2 knockdown (E, F) groups after the viral injections to the AI and before/after noise exposure, respectively.



The data were presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.25: Graphs for the mIPSC frequency (A), mIPSC amplitude (B), mEPSC frequency (C) and mEPSC amplitude (D) in the right AI of the control, TNFR1 knockdown, and TNFR2 knockdown groups after the viral injections to the AI and before/after noise exposure.

On the flip side, noise caused increased mEPSC frequency in the control group ($p < 0.01$; Figure 3.20.C) and decreased mEPSC frequency in the TNFR2 knockdown group ($p < 0.05$; Figure 3.20.C). , however, there was no statistically significant difference between before and after hearing lesion in TNFR1 knockdown mice ($p < 0.05$; Figure 3.20.C). mIPSC frequency of the control animals was significantly higher than TNFR2 knockdown mice after hearing lesion ($p < 0.0001$; Figure 3.20.C). Moreover, noise produced a decrease in mEPSC amplitude only in the TNFR2 knockdown mice ($p < 0.0001$; Figure 3.20.D).

CHAPTER 4

DISCUSSION

Hearing is essential in human life, serving multiple fundamental roles that impact safety, communication, social interaction, and overall well-being. Hearing enables the detection of sounds in the environment, which is vital for safety, such as alarms, approaching vehicles, or other warning signals that help prevent accidents. It is the primary means through which we engage in verbal communication, allowing us to understand and respond to spoken language. This capability is crucial for personal relationships, educational pursuits, and professional activities, as it enables clear and effective exchanges of information and ideas. In addition to hearing loss substantially affects quality of life and impacts 19.5-21.2% of the global population, 16% of cases are due to occupational noise exposure (Haile et al., 2021; Nelson et al., 2005). Even though hearing loss-related tinnitus is common and drugs still remain the preferred treatment option over devices or implants, according to patient surveys, there is no FDA-approved medication specifically for tinnitus (Tyler, 2012; Zheng & Smith, 2024). Moreover, anxiety disorder has been linked to hearing deficits (Bhatt et al., 2017; D. Yang et al., 2024) and the molecular pathways underpinning noise-induced auditory processing deficits and their relation to anxiety remain poorly understood. In this thesis project, we focus on the roles of PV neurons and TNF-α receptors in noise-induced auditory processing deficits and anxiety by using behavioral, molecular and electrophysiological approaches in a

transgenic mouse model (PV^{cre}/$TdTomato^{+}$). The results of this project revealed differential roles of TNFR1 and TNFR2 signaling in the vHPC and AI. Data from the current experimental study revealed for the first time that TNFR1 signaling impairs and TNFR2 signaling protects the surviving PV neurons against noise trauma-induced anxiety and synaptic deficits which take an essential role in auditory processing.

On mechanisms of noise trauma-induced anxiety

In this thesis project, we induced hearing loss and tinnitus by exposing mice to continuous noise at 112-114 dB SPL with a center frequency of 8 kHz for a duration of two hours. Parallel with the earlier experimental studies; we observed that exposure to loud noise caused long-lasting elevation of ABR thresholds in the exposed ear and temporary threshold shifts in the protected ear (Hirose & Liberman, 2003; Seist et al., 2021; Wang et al., 2019). Mice subjected to noise levels of 112-116 dB SPL for duration of two hours exhibit permanent elevation of ABR thresholds even two weeks post-exposure. The 112 dB SPL noise exposure lead to an average peak permanent threshold shift of approximately 60 dB especially for frequencies above 11.3 kHz, while mice exposed to 116 dB SPL noise for two hours are largely unresponsive across all test frequencies at ABR test (Hirose & Liberman, 2003). Additionally, experimental mice are more vulnerable to noise compared to humans due to several biological and anatomical differences between the species: Mice have specific genetic predispositions that make them more susceptible to NIHL. For instance, certain strains of mice show higher vulnerability due to genetic factors that affect their inner ear structures and functions. These genetic susceptibilities include variations in genes related to potassium ion recycling and heat shock proteins, which are crucial for noise resistance (Le et al., 2017). Also, the anatomy of the mouse ear is different from that of humans, particularly in terms of the size and structure of the cochlea. Mice have a smaller cochlea with a higher sensitivity to high-frequency sounds, which makes them more prone to damage from high-frequency noise exposure (Ohlemiller, 2019).

Blocking TNF-α expression using anti-TNFα drugs or via systemic injections has been shown to result in serious side effects including abnormal behaviors (Scheinfeld, 2004). However, preclinical studies indicate that modulating the TNF-α signaling through

manipulation of its receptors as a therapeutic target could affect anxiety-/ depression-like behaviors (Morgan et al., 2018; Patel et al., 2010). Target depletion of TNFR1 and TNFR2 receptor, such as using TNFR1−/− and TNFR2−/− mice demonstrated that selective inhibition could be efficient to address anxiety-/ depression-like behaviors (Simen et al., 2006). To be more specific, we injected custom-designed viral vectors into the vHPC and AI to knock down TNFR1 or TNFR2 only in PV neurons. Knockdown of TNFR1 and TNFR2 in hippocampal PV neurons did not lead to any significant difference in OFT and LDB before hearing lesion.

The association between noise-induced hearing impairments, such as hearing loss and tinnitus, and anxiety can be complex and bidirectional. Hearing loss can result in social withdrawal and isolation, cognitive load, changes in brain structure and functions that are significant risk factors for developing cognitive/emotional disorders (Lin & Albert, 2014; Pattyn et al., 2016). The persistent nature of tinnitus, coupled with its often unpredictable onset and intensity, can cause stress, leading to heightened anxiety levels (Hackenberg et al., 2023). Conversely, anxiety itself can exacerbate the perception of tinnitus and the influence of hearing loss (Moon et al., 2018; Yakunina & Nam, 2021). Even though anxiety disorder may not be a precondition for the onset and progress of tinnitus, its negative impact and the consequences of stress can influence the brain networks engaged (Pattyn et al., 2016). The findings of the current project demonstrated that noise-exposed mice in the control group showed reduced locomotor activity and exploratory behavior in the OFT and LDB tests. We know that anxious animals freeze/become more immobile and avoid spending time in center of the OFT area and tend to spend time in the dark and avoid entering bright areas in LDB (Bourin & Hascoët, 2003; Kraeuter et al., 2019). These results are interpreted as noise-exposed hearing loss is associated with reduced exploratory and locomotor activities, which are anxiety-/ depression-like behaviors, consistent with the previous studies (Lauer et al., 2018; Peng et al., 2023; Xu et al., 2022). Further, knockdown of TNFR1 prevented noise-induced anxiety in the OFT. In addition to the generally recognized role of TNFR1 in promoting inflammatory responses, the potential mechanism underlying inhibited anxiety-like behavior might be related to the role as T cell costimulatory molecule (Evangelidou et al., 2010). Interestingly, TNFR2 knockdown mice traveled more in OFT after noise exposure compared to the control/TNFR1 knockdown group, entered into the bright area frequently

and spent similar time in the bright area of LDB compared to after hearing lesion. These responses of TNFR2 knockdown mice in OFT and LDB tests might be interpreted in the context of fear and memory instead of anxiety since TNFR2 deficiency has been found to be associated with reduced freezing response in fear conditioning and impairments in cognition (Kawamoto et al., 2013; Naude et al., 2014). Taken together, these results confirm that the noise-induced deficits and their impacts on the ventral hippocampus is dependent on TNF signaling.

Noise exposure is known to trigger molecular changes in the hippocampus, such as impaired hippocampal LTP (De Deus et al., 2017) and neurogenesis (Tao et al., 2015), disrupted place cell responses (Goble et al., 2009) and phosphorylation of tau protein (Cheng et al., 2016). Besides, unpublished data from Shaowen Bao's Lab reported noise-induced PV neuron loss in the hippocampus thereby contributing to deficits in anxiety. PV neurons are a specific type of inhibitory interneuron found in various regions of the brain, including the hippocampus. They release the neurotransmitter GABA, which inhibits the firing of nearby neurons. This inhibition is crucial for preventing excessive neural activity, maintaining a balance between excitation and inhibition in cortical circuits and network stability (Hu et al., 2014). Parvalbumin neurons are known for their ability to synchronize the firing of pyramidal neurons in the hippocampus. This synchronization is essential for various cognitive, social, and emotional regulation (Hijazi et al., 2023; Zaletel et al., 2016). Recent studies also reported that optogenetically inhibition of PV neurons in the vHPC can lead to elevated activity of pyramidal neurons and inhibitory micro-circuits play an essential role in persistent anxiety (Forro et al., 2022; Volitaki et al., 2024). In our study, knockdown of TNFR1 in the vHPC ameliorated noise trauma-induced deficits by preventing noise-induced reduction of cortical PV neurons. This result is consistent with the generally recognized roles of TNFR1 in neuroinflammation (Probert, 2015; Vandenabeele et al., 2010) thereby leading to the release of additional pro-inflammatory cytokines and chemokines, creating a detrimental neuroinflammatory environment (Dostert et al., 2019). By knocking down TNFR1, this pathway may be interrupted, reducing microglial activation and subsequent inflammatory mediator release, thereby protecting PV neurons from inflammation-induced damage. Furthermore, TNFR1 is involved in necroptosis, described as programmed cell death characterized by cell membrane breakdown and the release of inflammatory content (Probert,

2015; Vandenabeele et al., 2010). The knockdown of TNFR1 in PV neurons may inhibit the necroptotic pathway by preventing the activation of RIPK1 and RIPK3 (Vandenabeele et al., 2010). While TNFR1 knockdown in PV neurons primarily affects neural processes, these changes may indirectly influence T cell function and behavior through a variety of neuroimmune mechanisms such as reductions in neuroinflammation, alterations in blood-brain barrier integrity, changes in neurotransmitter release (Evangelidou et al., 2010; Gimsa et al., 2012). Thus, knockdown TNFR1 in hippocampal PV neurons not only mitigates noise exposure-induced inflammation and cell death but also may support the functional integrity of brain regions involved in emotional responses, potentially preventing noise trauma-induced anxiety.

In addition to the roles of TNFR1 in neuroinflammation and necroptotic cell death, neuronal TNFR2 is known to be involved in neuroprotection and the regulation of neuroinflammation. Activation of TNFR2 has been shown to promote neuronal survival and protect against apoptosis and glutamate excitotoxicity through the induction of anti-apoptotic and anti-inflammatory pathways (Marchetti et al., 2004; Papazian et al., 2021). Additionally, TNFR2 contributes to neuronal regeneration and repair processes following injury, supporting the growth and maintenance of neuronal networks (Marchetti et al., 2004). By balancing pro-inflammatory and anti-inflammatory signals in the CNS, TNFR2 helps maintain neuronal health and prevents chronic inflammation (Papazian et al., 2021). A previous experimental study reported that blockade of TNF-α with intraperitoneal injections of dTT (3,6'-dithiothalidomide) prevented noise trauma-induced abnormalities in microglial morphology of the AI (Wang et al., 2019). However, the current project showed that the knockdown of TNFR1 in hippocampal PV did not cause a significant difference in terms of neuroinflammation; also, knockdown of TNFR2 disrupted neuroprotective and anti-inflammatory signaling, leading to an exaggerated microglial response to noise exposure. This includes increased microglial density and intensity in the AI and microglial deramification in the vHPC of animals in the TNFR2 knockdown group compared to both control and TNFR1 knockdown groups. Microglial deramification refers to the process where microglia change from a ramified (resting) state to a less branched, more amoeboid (activated) state. In their ramified form, microglia have long, branching processes. Upon activation by factors such as stress, these processes retract, and the microglia become more

amoeboid. Activated microglia release pro-inflammatory cytokines, which can disrupt neural processes, contributing to neuroinflammatory conditions and heightened anxiety (Ramirez et al., 2017). These effects might be driven by a combination of increased pro-inflammatory signaling, loss of regulatory control over microglia, and disturbances in neuronal network activity. The knockdown of TNFR2 can lead to an increase in the release of pro-inflammatory cytokines, which can induce microglial activation and morphological changes. Besides, PV neuron loss in TNFR2 knockdown group after noise exposure can disrupt excitatory/inhibitory balance, resulting in increased excitatory signaling and network hyperactivity. This imbalance promotes a pro-inflammatory environment, exacerbating microglial activation and structural changes.

In this thesis project, we investigated microglial deramification in subregions of the vHPC since the vHPC plays a crucial role in the regulation of anxiety-related behaviors, with its subregions contributing in distinct ways. Studies indicate that the ventral CA1 (vCA1) and ventral CA3 (vCA3) subregions are particularly involved in processing anxiety (Hong & Kaang, 2022; Hunsaker & Kesner, 2008). The vCA1 is implicated in the encoding and retrieval of contextual information that can elicit anxiety responses, likely through its projections to the mPFC (Adhikari et al., 2010), BLA (Felix-Ortiz et al., 2013) and lateral septum (LS) (Parfitt et al., 2017) which are key regions in the anxiety circuitry. The vCA3, on the other hand, is believed to contribute to the modulation of anxiety through its influence on memory formation and retrieval of contextual fear conditioning (Besnard et al., 2020). Additionally, lesion studies showed that DG of the vHPC plays a role related to exploratory behavior in the OFT and the elevated plus maze test, thereby influencing anxiety levels (Weeden et al., 2015). Collectively, these subregions interact with broader neural networks to mediate the balance between anxiety and appropriate behavioral responses to stressors. In the present study, we showed that knockdown of TNFR2 in hippocampal PV neurons exacerbates the microglial deramification in the CA1 and CA3 regions but not the DG of the vHPC after noise exposure. Microglial deramification, which involves the loss of their branched morphology, is typically a response to a heightened inflammatory state. Neuroinflammation in the vHPC has been increasingly recognized as one of the key elements in the development and regulation of anxiety (Won & Kim, 2020). Animals in the TNFR2 knockdown group exhibited inconsistent behaviors in anxiety-related tests after hearing

lesion. Compatible with the reduced freezing response in fear conditioning reported in the previous study (Kawamoto et al., 2013; Naude et al., 2014), they traveled more in OFT after noise exposure compared to the control/TNFR1 knockdown group, entered into the bright area frequently and spent similar time in the bright area of LDB compared to after hearing lesion. In contrast, they did not spend time in the center of OFT area compared to before noise condition. There is an inconsistency in fear responses of TNFR2 knockdown mice, possibly because of microglial activation. Noise exposure-related stress and other anxiety-inducing factors can activate microglia, leading to the release of pro-inflammatory cytokines that can disrupt synaptic plasticity, reduce neurogenesis, and alter neurotransmitter systems, contributing to heightened anxiety (Li et al., 2021). Further, activated microglia in the vHPC release inflammatory mediators that can impair neuronal function and plasticity. This neuroinflammatory response can disrupt the balance of excitatory and inhibitory signaling which promotes the pro-inflammatory environment. This positive feedback loop of neuroinflammation contributes to exacerbating anxiety symptoms.

On mechanisms of noise trauma-induced auditory processing deficits

PPI is based on acoustic startle reflex and serves as a quantitative measure of central auditory processing (Gómez-Nieto et al., 2020). The lack of significant change in PPI performance after noise exposure suggests that the unilateral noise exposure used in this study did not impair the sensory-motor gating mechanism in any of the groups which is consistent with previous studies (Deng et al., 2020; Wang et al., 2019). Further, the control group shows an increased startle response ratio at all frequencies after hearing loss, indicating a heightened startle reflex. A higher startle reflex ratio indicates that tinnitus fills the gap in the background noise and suggests an auditory processing deficit influencing the unconscious neural processing of GD (Wilson et al., 2019). This is expected because hearing loss can enhance the sensitivity of the auditory system to sudden sounds, a phenomenon often related to reduced auditory input leading to an increased central gain of the remaining auditory pathways. Moreover, noise trauma-induced PV neuron loss and impairments in their synaptic efficacy might cause a decrease in temporal acuity and dynamic gain control, which results in auditory processing deficits. We did not see any statistically significant difference in

TNFR2 knockdown group with hearing loss because animals in the TNFR2 knockdown group showed elevated startle response ratio compared to the control group before hearing loss, particularly at 14 kHz and 20 kHz. This suggests that TNFR2 may play a role in modulating auditory sensitivity or central auditory processing without the effect of noise exposure. Its knockdown may lead to heightened startle reflex, potentially due to PV neuron loss or dysregulated neuroprotective processes in the central auditory system. On the other hand, TNFR1 knockdown group has a decreased startle response ratio compared to the control group after noise exposure. This could mean that TNFR1 knockdown mitigates some of the effects of hearing loss, possibly by preventing PV neuron loss and reducing neuroinflammation in the auditory pathways.

PV neurons are crucial fast-spiking, GABAergic interneurons in the central auditory system, known for their function in ensuring precise timing and synchronization of neural activities. In the AI, PV neurons contribute to cortical gain control and enhance spatial tuning of auditory signals, which is essential for processing complex auditory inputs and temporal acuity (Keller et al., 2018). Dysfunctions in PV neurons have been linked to auditory disorders like tinnitus and hyperacusis, highlighting their role in maintaining the balance between excitatory and inhibitory signals in auditory circuits (Ferguson & Cardin, 2020). Thus, the proper functioning of PV neurons is essential for accurate auditory perception and for preventing auditory system-related pathologies. Even though there is an inconsistency in the literature in terms of alterations in PV neuron density after noise exposure (Liu et al., 2018; Nguyen et al., 2017), noise-induced PV neuron loss reported in the mouse AI (Deng et al., 2020; Masri et al., 2021; Zinsmaier et al., 2020) thereby contributing to deficits in central auditory processing and possibly tinnitus and hyperacusis. Besides, it is thought that TNF-α signaling and its receptors may play a key role in noise-induced PV neuron loss because of the increased expression of TNF-α in the AI after noise trauma (Wang et al., 2019). In the present study, knockdown of TNFR1 in the PV neurons of AI ameliorated noise trauma-induced auditory processing deficits evaluated with the GD test, possibly by preventing noise-induced reduction of cortical PV neurons. The intervention that targets TNFR1 in PV neurons likely reduced the inflammatory response that contributes to PV neuron loss, thereby preserving auditory function and highlighting the potential therapeutic value of modulating TNF-α signaling pathways in treating noise-induced auditory deficits. On the other hand, the

knockdown of TNFR2 in cortical PV neurons exacerbated microglial deramification in the AI after noise exposure. Microglial deramification is a sign of microglial activation, typically associated with neuroinflammation and neuronal damage (Kettenmann et al., 2013). Knockdown of TNFR2 likely disrupted these protective mechanisms related to cell survival and anti-inflammatory responses, leading to increased microglial activation and exacerbated inflammatory responses in the AI. This heightened state of microglial activation can contribute to further neuronal damage and exacerbate auditory processing deficits, indicating the critical role of TNFR2 in maintaining neuronal and microglial homeostasis in the face of acoustic trauma (Kettenmann et al., 2013; McCoy & Tansey, 2008).

TNF-α, through its receptors TNFR1 and TNFR2, can modulate both GABA and glutamate receptors, leading to significant changes in synaptic transmission (Stellwagen et al., 2005). TNFR1-mediated signaling can enhance the excitatory drive by increasing AMPA and NMDA receptor trafficking and decreasing inhibitory $GABA_A$ receptor expression, contributing to an imbalance in synaptic transmission (Olmos & Lladó, 2014; Stellwagen et al., 2005). The excessive influx of Ca^{2+} into neurons leads to neuronal death and the production of excessive reactive oxygen species. Experiments using an antagonist of TNFR1 indicated prevented excitotoxicity and cell death mediated by NMDA receptors and cognitive impairments (Ortí-Casañ et al., 2023). Although TNFR2 does not directly influence receptor trafficking, its activation helps to balance this process by promoting cellular mechanisms that protect against excitotoxicity and excessive synaptic excitation. This includes reducing the overactivation of NMDA receptors and subsequent Ca^{2+} influx that leads to neuronal death (Papazian et al., 2021). In addition to these roles of TNF receptors reported in the literature, in the current project, knocking down the TNF-α receptors only in PV neurons did not cause any statistically significant difference before noise exposure. NIHL significantly impacts synaptic transmission in the AI by disrupting the balance between excitatory and inhibitory inputs (Browne et al., 2012; Yang et al., 2011), leading to auditory processing deficits, tinnitus and hyperacusis. NIHL led to decreased inhibitory synaptic transmission, as evidenced by the reduced frequency of mIPSCs, consistent with the previous studies (Wang et al., 2019; Yang et al., 2011), primarily due to impaired function of GABAergic interneurons, such as PV neurons. Conversely, NIHL affected excitatory synaptic transmission by altering the frequency of mEPSCs in parallel with previous studies (Yang et

al., 2012; Zhao et al., 2016). Neuroinflammatory responses, particularly the activation of microglia and the release of pro-inflammatory cytokines like TNF-α and its receptors, play a crucial role in these synaptic changes. In the present study, the knockdown of TNFR1 appears to confer a protective effect against NIHL-induced synaptic changes. In TNFR1 knockdown mice, the inhibitory synaptic transmission remains relatively stable, with no significant decrease in mIPSC frequency after noise exposure. This suggests that blocking the pro-inflammatory and apoptotic pathways mediated by TNFR1 helps preserve synaptic function. However, TNFR2 knockdown mice show increased susceptibility to synaptic alterations due to the lack of neuroprotective signaling. These mice exhibit a decrease in mIPSC amplitude and frequency and alterations in mEPSC characteristics, highlighting the essential role of TNFR2 in maintaining synaptic integrity and function after noise-induced damage.

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVES

The present thesis project provides significant insights into the complex mechanisms underlying noise-induced auditory processing deficits and anxiety, through a particular focus on the roles of PV neurons and TNF-α receptors. Our research demonstrates that TNFR1 signaling impairs, while TNFR2 signaling protects, the survival of PV neurons against noise trauma-induced anxiety and synaptic deficits. These findings highlight the differential impacts of TNF-α receptors on neural and behavioral outcomes following noise exposure.

The study confirmed that noise exposure leads to permanent auditory threshold shifts and increased vulnerability to anxiety-related behaviors. Through the selective knockdown of TNFR1 and TNFR2 in PV neurons, we elucidated that TNFR1 knockdown ameliorates noise-induced anxiety by reducing neuroinflammation and protecting PV neurons from necroptotic cell death. Conversely, TNFR2 knockdown exacerbates microglial activation and neuroinflammation, leading to increased anxiety-like behaviors. These results emphasize the critical role of TNF-α signaling in the regulation of anxiety and highlight the potential of targeting TNF-α receptors for therapeutic interventions in noise-induced auditory and anxiety disorders. The differential roles of TNFR1 and TNFR2 in neuroinflammation and neuronal survival present promising avenues for developing targeted therapies to mitigate the detrimental effects of noise.

Future Perspectives

Future research should aim to elucidate further the molecular pathways involved in TNF-α receptor signaling and their specific contributions to auditory processing and anxiety. Exploring the potential of pharmacological agents that selectively modulate TNF-α receptor activity could lead to the development of novel therapeutic strategies. Given the differential impacts of TNFR1 and TNFR2 signaling, targeted therapies that inhibit TNFR1 while promoting TNFR2 activity may offer a balanced approach to protecting against neuroinflammation and neuronal damage.

The complex interplay between neuroinflammation, oxidative stress, and excitotoxicity and their collective impact on PV neuron loss after noise exposure remains poorly understood. Future studies should aim to dissect these interactions comprehensively. Also, it is essential to include necroptosis markers in future for understanding of cell death mechanisms in noise-induced PV neuron loss.

The connection between noise-induced hearing impairments and anxiety is complex and multifaceted. Future studies should investigate the long-term effects of noise exposure on cognitive and emotional health, considering factors such as social isolation, cognitive load, and changes in brain structure and function. Future studies should also aim to unravel the mechanisms within the auditory system and their connections to the vHPC and amygdala circuits. These regions play significant roles in processing auditory information, emotional responses and mechanisms of anxiety-related behavior. Integrating behavioral studies with neuroimaging and electrophysiological techniques can provide a comprehensive understanding of the neural correlates of anxiety in the context of auditory processing deficits. Furthermore, expanding research to include human studies will be crucial for translating these findings into clinical applications.

In summary, this thesis lays the groundwork for future investigations into the intricate interplay between auditory processing, neuroinflammation, and anxiety. By advancing our understanding of these mechanisms, we can move closer to developing effective treatments that enhance the quality of life for individuals affected by noise-induced auditory and anxiety disorders.

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