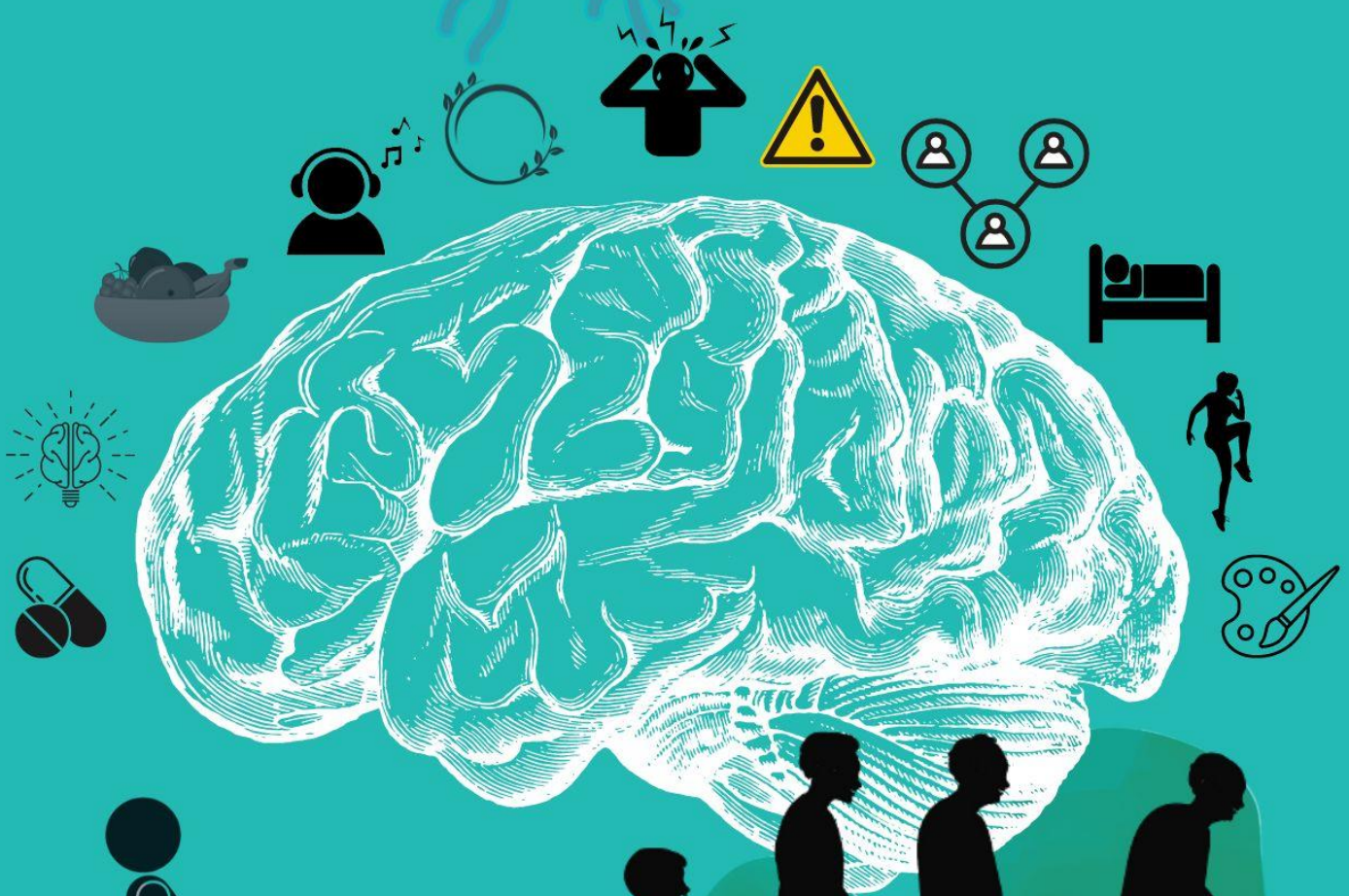
Short-term impact of anthropogenic environment on neuroplasticity

SHORT-TERM IMPACT OF ANTHROPOGENIC ENVIRONMENT ON NEUROPLASTICITY – A STUDY AMONG HUMANS AND ANIMALS

THE EXPOSOME ON NEUROPLASTICITY

BY KIRTHANA KUNIKULLAYA U



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Kirthana Kunikullaya U

Dedicated to my parents, in-laws, husband and daughter.

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List of Abbreviations

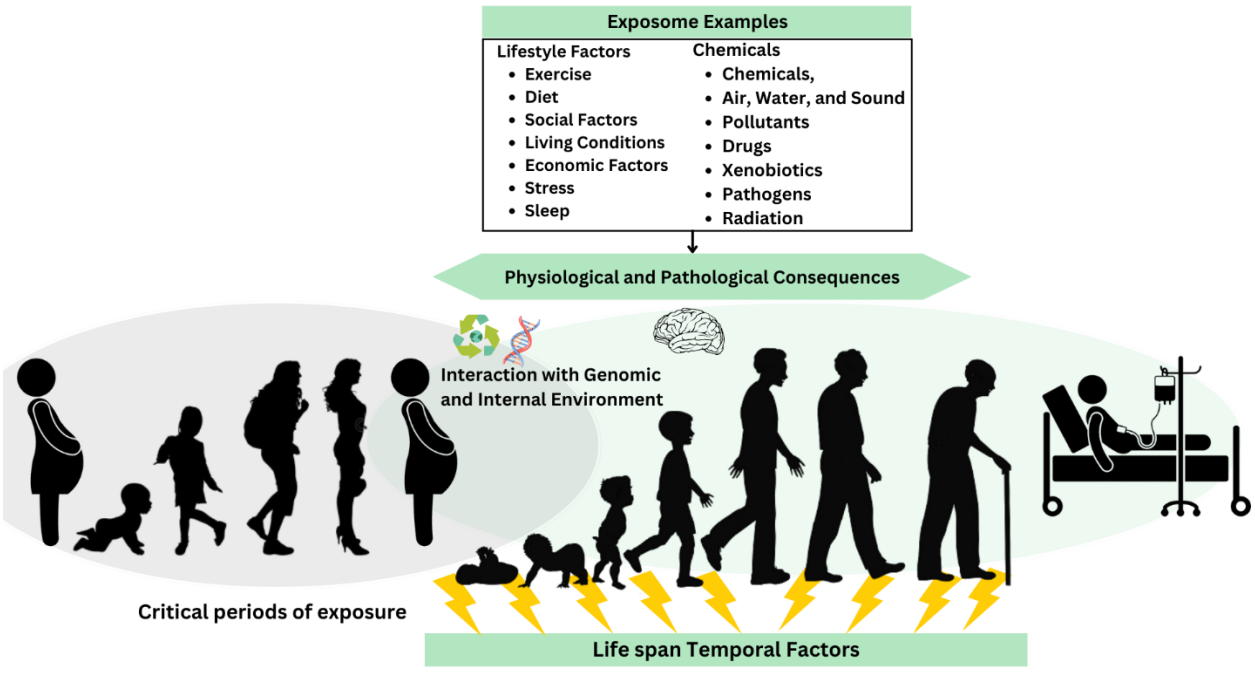
3β HSD	3β-Hydroxysteroid Dehydrogenase
5-HT	5-Hydroxytryptamine / Serotonin
AC	Adenylyl Cyclase
ACC	Anterior Cingulate Cortex
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic Hormone/Corticotropin
AD	Alzheimer's
ADI	Acceptable Daily Intake
AMP	Adenosine Monophosphate
AMPA	Amino-3-Hydroxy-5-Methyl-Isoxazole-4-Propionic Acid
AN	Arcuate Nucleus
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
aNSCs	Active Neural Stem Cells
AOEL	Acceptable Operator Exposure Level
ASR	Artifact Subspace Reconstruction
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BC	Basket Cell
BDNF	Brain-Derived Neurotrophic Factor
BLA	Basolateral Amygdala
BM	Basomedial Nucleus Of Amygdala
BMI	Body Mass Index
BNST	Bed Nucleus Of The Stria Terminalis
BP	Blood Pressure
CA	Cornu Ammonis
CE	Central Nucleus
CN	Cochlear Nuclei
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CO	Carbon Monoxide
CorrCA	Correlated Component Analysis
CP	Cortical Plate
CR	Cajal-Retzius
CREB	Cyclic Amp-Response Element-Binding Protein
CRH/CRF	Corticotropin-Releasing Hormone/Factor
DA	Dopamine
DAG	Diacylglycerol
DBP	Diastolic Blood Pressure
DCX	Doublecortin
DFA	Detrended Fluctuation Analysis

DG	Dentate Gyrus
DMH	Dorsomedial Hypothalamus
DMN	Default Mode Network
DMSO	Dimethyl Sulphoxide
ECG	Electrocardiogram
EDF	European Data Format
EE	Environmental Enrichment
EEG	Electroencephalogram
ELISA	Enzyme Linked Immunosorbent Assay
eNSCs	Embryonic Neural Stem Cells
Erα	Estrogen Receptor Alpha
Erβ	Estrogen Receptor Beta
ESC	Embryonic Stem Cells
EU	European Union
FFT	Fast Fourier Transformation
FSH	Follicle-Stimulating Hormone
GABA	Gamma Amino Butyric Acidergic
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GD	Gestation Day
GFAP	Glial Fibrillary Acid Protein
GlucRs	Glucocorticoid Receptors
GnRH	Gonadotropin-Releasing Hormone
GPER	G-Protein Coupled Estrogen Receptor
GR	Granule cells
GRH	Growth Hormone–Releasing Hormone
GW	Gestational Week
HF	High-Frequency
HPA	Hypothalamo–Pituitary-Adrenal
HR	Heart Rate
HRV	Heart Rate Variability
IC	Inferior Colliculus
ICMR	Indian Council For Medical Research
IGF-1	Insulin-Like Growth Factor
IN	Intercalated Neurons
INM	Interkinetic Nuclear Migration
IP3	Inositol 1,4,5-Triphosphate
IPCs	Intermediate Progenitor Cells
IQR	Interquartile Range
ISC	Intersubject Correlation
IZ	Intermediate Zone
LA	Lateral Nucleus
LD	Lactation Day
LF	Low-Frequency
LHo	Luteinizing Hormone
LH	Lateral Hypothalamus

LTD	Long-Term Depression
LTP	Long-Term Potentiation
M1	Primary Motor Cortex
mAChRs	Muscarinic Acetylcholine Receptors
MAP	Mitogen-Activated Protein Kinase
MCC	Middle Cingulate Cortex
MDD	Major Depression Disorder
ME	Medial Nucleus
MEG	Mangetoencephalography
MGB	Medial Geniculate Body
mGluR	Metabotropic Glutamate Receptor
MGN	Medial Geniculate Nuclei
MPTP	Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MR	Minerocorticoid Receptor
MRI	Magnetic Resonance Imaging
MS	Musical Stimuli
MZ	Marginal Zone
NAc	Nucleus Accumbens
nAChRs	Nicotinic Acetylcholine Receptors
NC	Deep Nuclei
NE	Norepinephrine
NECs	Neuroepithelial Cells
NF-kB	Nuclear Factor Kappa B Protein Complex
NMDA	N-Methyl-D-Aspartate Glutamate Receptor
NO2	Nitrogen Dioxide
NOAEL	No Observed Adverse Effect Level
nu	Normalized Units
O3	Ozone
OB	Olfactory Bulb
OFC	Orbitofrontal Cortex
PBPK	Physiologically Based Pharmacokinetic
PC	Purkinje Cell
PCNA	Proliferative Cell Nuclear Antigen
PD	Parkinson’s Disease
PDE	Phosphodiesterase
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
PI	Principal Investigator
PIH	Prolactin-Inhibiting Hormone
PH	Posterior Hypothalamus
PKA	Protein Kinase A
PKC	Protein Kinase C
PL	Paralaminar Nucleus
PM	Particulate Matter
PMC	Premotor Cortex

PND	Postnatal Day
PNS	Parasympathetic
POA	Preoptic Area
PPPs	Plant Protection Products
PRH	Prolactin-Releasing Hormone
PRL	Prolactin
PTSD	Post-Traumatic Stress Disorder
PVN	Paraventricular Nucleus
qNSCs	Quiescent Neural Stem Cells
qPCR	Quantitative Polymerase Chain Reaction
RCZ	Rostral Cingulate Zone
RG	Radial Glial
RGL	Radial Glia-Like
RGUHS	Rajiv Gandhi University Of Health Sciences
RM-ANOVA	Repeated Measures of Anova
RMS	Rostral Migratory Stream
RMSSD	Root Square of The Mean Squared Difference Of Successive NN intervals
Rsk	Ribosomal S6 Protein Kinase
sAA	Salivary Alpha-Amylase
SAM	Sympathetic–Adrenal–Medullary System
SBP	Systolic Blood Pressure
sCort	Salivary Cortisol
SDNN	Standard Deviation of NN Intervals
SEM	Standard Error of The Mean
SEZ	Subependymal Zone
SGZ	Subgranular Zone
SMA	Pre-Supplementary Motor Area
SNS	Sympathetic
SO2	Sulphur Dioxide
SON	Supraoptic Nuclei
SP	Subplate
STAI	State-Trait Anxiety Inventory
SVZ	Subventricular Zone
TP	Total Power
TRH	Thyrotropin-Releasing Hormone
TrkB	Tropomyosin Receptor Kinase B-Bdnf Receptor
TSH	Thyroid-Stimulating Hormone/Thyrotropin
UV	Ultraviolet Radiation
VAS	Visual Analog Scale
VGCC	Voltage-Gated Calcium Channel
VLF	Very Low-Frequency
VMH	Ventromedial Hypothalamus
VN	Vestibular Nuclei
VTA	Ventral Tegmental Area
VZ	Ventricular Zone

Chapter 1
Introduction



Chapter 1: Introduction

Human physiology and health are impacted by the environment. The field of environmental health has progressed over time, but evidence concerning the effects of the environment on health is based predominantly on epidemiological studies. The nervous system is highly sensitive to the environment and thus anthropogenic factors have a significant impact on brain plasticity. Although a large array of studies focus on the potential negative impact of environmental factors, it should be noted that some factors can have a positive impact.

In this chapter, the concepts of neuroplasticity and anthropogenic stimuli are introduced. This is followed by a brief overview of the latest literature on the factors that influence neuroplasticity in general. The exposure of humans and animals to different anthropogenic stimuli, and their effects on overall health, with specific emphasis on neuroplasticity, is then explained. Based on the focus of the current thesis, the effects of anthropogenic stimuli, one potentially positive (auditory) and one potentially negative stimulus (chemical) are detailed, with the need for this thesis.

1 Neuroplasticity

The concept of plasticity came from the term plastic, originally '*plasticus*' in Latin which comes from the Greek term '*plastikós*' or 'plastos' meaning 'molded, formed'. Applying this moldability capacity to the brain, the concept was introduced into neuroscience. Neuroplasticity (neural or brain plasticity) is a process where the brain can adapt itself **structurally and functionally** in response to intrinsic or extrinsic stimuli by reorganizing its morphology, functions, or connections at different levels. Cajal suggested that the brain could increase its capacity by augmenting the number of synapses (1). The stimuli can be physical, electrical, chemical, and psychological, including injuries, such as a stroke or trauma (2). A few classic examples of plasticity in humans are the observations: (a) sign language activating the auditory association areas in individuals who become deaf before language skills are fully developed, or, (b) better localization of sound in people who succumbed to early blindness (functional changes in the brain areas that are activated due to loss of function of a few areas); and (c) structurally, an increase in cortical (increase size of auditory areas for tones or altered somatosensory

representation of fingers) and cerebellar plasticity (increased size) in response to experience (training, learned precise finger movements) in musicians (violinists/ pianists) (3). This concept of 'activity-dependent learning' shows that neuroplasticity is influenced by **experience-based** stimuli that can be biological, and environmental. There also exists a **critical period** when the brain is developing and the time of stimulus exposure remains crucial. This is due to genetic influences as well as epigenetic influences one is exposed to in-utero during pregnancy or post-birth. These critical periods are observed in all animals, studied to date, including humans. Significant plastic abilities may be lost or limited if the stimulus does not occur during this critical period (4,5). This concept of critical periods was proposed by Charles Stockhard, who exposed fish embryos to different chemicals and observed the change in their development (6).

At a cellular level, short- and long-term plastic changes in the neuronal or synaptic function occur because of the history of repeated discharge at the synapse. A few examples of such plasticity are changes in dendritic morphology, habituation, sensitization, long-term potentiation, or depression. Neurogenesis is one of the postulated mechanisms involved in neuroplasticity (as seen in the hippocampus, and olfactory bulb). The neuroplastic processes also include dynamic reconfiguration of neural connections, cell shape, size, and myelination. Neuroendocrine plasticity, i.e., altering the hormonal levels, or proportion of secretory cells to meet the demands at different stages of life is an added example of plasticity (3).

In this chapter, the current knowledge of neurogenesis during development and adult neurogenesis, the chronological time points of their development in mice and humans, followed by a brief note on the mechanisms of neuroplasticity are introduced. Neuroplasticity in specific structures is introduced, followed by an overview of how these structures and their effects are integrated with the autonomic nervous system (ANS).

1.1. Development of the Brain and Neurogenesis

Neurogenesis involves the active production of new neurons, glia, and other cells, and neural lineages from undifferentiated neural progenitors or stem cells. It is most active in prenatal development and is responsible for the growth of the brain. It. After fertilization, the single-celled zygote undergoes multiple mitotic divisions from the morula and then transforms into the blastula (non-mammalian term) or blastocyst (human development).

During 3rd gestational week (GW) in humans, gastrulation occurs, transforming the blastula into a multilayered and multidimensional structure (7). Embryonic stem cells (ESC) can be isolated from the developing mouse blastocyst around embryonic day (E) 3.5. By E6.5, three germ layers can be observed, the endoderm, the mesoderm, and the ectoderm (8,9). The **ectoderm** is responsible for the formation of the neural tube, which gives rise to the brain, spinal cord, and posterior pituitary. The peripheral nervous system, on the other hand, originates from the neural crests (10). As the neural tube develops, it undergoes rostrocaudal and dorsoventral differentiation. The rostral part of the neural tube divides into three primary vesicles, namely the forebrain, midbrain, and the hindbrain. The inner cavities of these vesicles later fill with cerebrospinal fluid. Meanwhile, the caudal portion of the neural tube gives rise to the spinal cord, which is influenced by its immediate environment. Specifically, the dorsal side of the spinal cord receives sensory inputs, while the ventral side is responsible for motor signals (11).

Neurons and glial cells are formed from pseudostratified epithelial or **neuroepithelial cells (NECs)**, also known as embryonic neural stem cells (eNSCs), which line the cerebral ventricles. These NSCs, at various stages of development, begin to differentiate into distinct lineages that ultimately give rise to specialized neurons or glial cells (12). During early development, the NECs divide **symmetrically** to produce more NECs. Some of these NECs then develop into early neurons. As the brain epithelium thickens, the NECs become elongated and transform into **radial glial (RG) cells**. These cells remain in contact with both the pial and ventricular surfaces, and their cell bodies are situated within the **ventricular zone (VZ).** In mice, NECs become activated around E8 and develop into early RG cells around E14. The RG cells divide **asymmetrically** to produce **intermediate progenitor cells (IPCs).** These IPCs can give rise to neurons (nIPCs) or different types of glial cells, including oligodendrocytes (oIPCs) and astrocytes (aIPCs) (13). Around the same time, the tight junctional complexes that connect the neuroepithelial cells (NECs) change into adherens junctions (14). Additionally, the aIPCs form connections with the endothelial cells of the developing cerebral vasculature, thereby creating the blood-brain barrier (13). Similar to NECs, RG cells maintain apical-basal polarity, bordering the ventricles, and undergo **interkinetic nuclear migration**

(INM) - a complex mitotic behavior that helps maintain a pseudostratified epithelium within the VZ **(Fig 1)**.

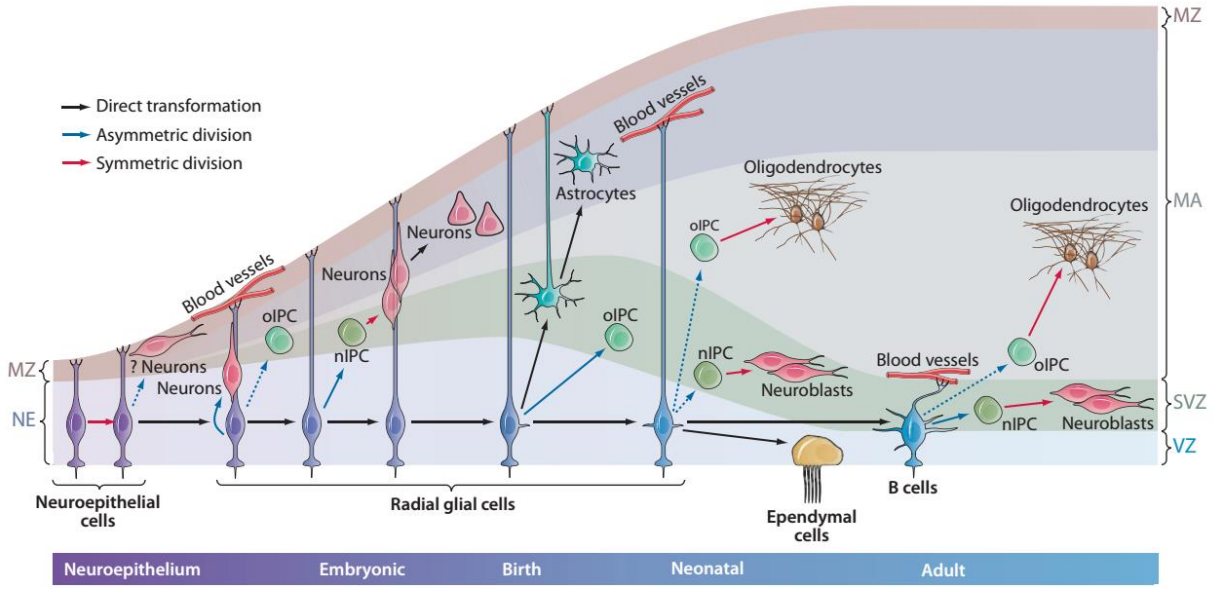


Fig 1: Modes of neurogenesis during cortical development. MZ, marginal zone; NE, neuroepithelium; nIPC, neurogenic intermediate progenitor cell; oligodendrocytes (oIPCs); VZ, ventricular zone; SVZ, subventricular zone (13).

Before describing neuroproliferation, the cell division phases are briefly introduced. Cell cycle/division is the process through which cells replicate and make two new cells. It has different stages called G1, S, G2, and M. **G1:** cell is preparing to divide. **S:** DNA replication occurs, making copies of the DNA for the daughter cells. **G2:** There is organization and condensation of the new genetic material, and preparation to divide. **M:** M stands for mitosis, where the cell divides producing two daughter cells each with its copy of the genetic material. After the M phase, based on the situation and factors, the cell cycle can begin again (15). During neurogenesis, cells undergo the S phase at the basal surface of the VZ and the M phase at the apical surface, while nuclei in G1 and G2 phases transition between the S and M phases in the mid-region. As the cortex develops, the length of the cell cycle increases mainly through the extension of the G1 phase. At the same time, cells start to undergo **asymmetric cell division**, and the fraction of cells that differentiate into **neurons increases**, while the percentage of cells remaining as **progenitors decreases**. Toward the end of cortical development, the majority of neural progenitors produce two daughter cells that differentiate into neurons, leading to a gradual

depletion of neural precursors (16). Therefore, cortical neurons are generated by RG cells through direct asymmetric division or indirectly via the generation of nIPCs and one round of amplification, or two rounds of division and further amplification. Most, if not all, neurogenic lineages in the nervous system are founded by RGs and a subpopulation of astrocytes. Once a cell has exited the cell cycle, it must **migrate out of the VZ** toward its final layers in the developing neocortex/**cortical plate (CP)** (the future layer 2 to 6 of the neocortex). Around E11, the initial set of neurons migrates to form the **preplate**, which is the first stage of corticogenesis before the development of the CP **(Fig 2)**. The CP comprises migrating and immature CP neurons that are densely packed with rudimentary cell processes and is separated from the germinal layer by an intermediate zone (IZ) of axons. The subsequent wave of neuronal migration (~E13) splits the preplate into two layers: the **more superficial marginal zone (MZ)**, which includes the **Cajal-Retzius (CR) cells**, and the **deeper subplate (SP)**, which comprises the rest of the primordial cells, with postmitotic SP neurons and well-developed cellular processes. The CR cells are a transient cell population of the central nervous system (CNS) that is critical for brain development. In the neocortex, they release **reelin** to guide the radial migration of projection neurons. Two types of migration have been identified during neurogenesis: **radial migration**, in which cells move from the progenitor zone towards the brain surface following the neural tube, and **tangential migration**, in which cells migrate perpendicular to the direction of radial migration (17). Later in neurogenesis (~E14.5-E16.5), many neurons are derived indirectly through divisions of IPCs in the SVZ. At the end of embryological development, RG cells reduce and disappear in most brain regions. In mammals, most RG cells transform into astrocytes.

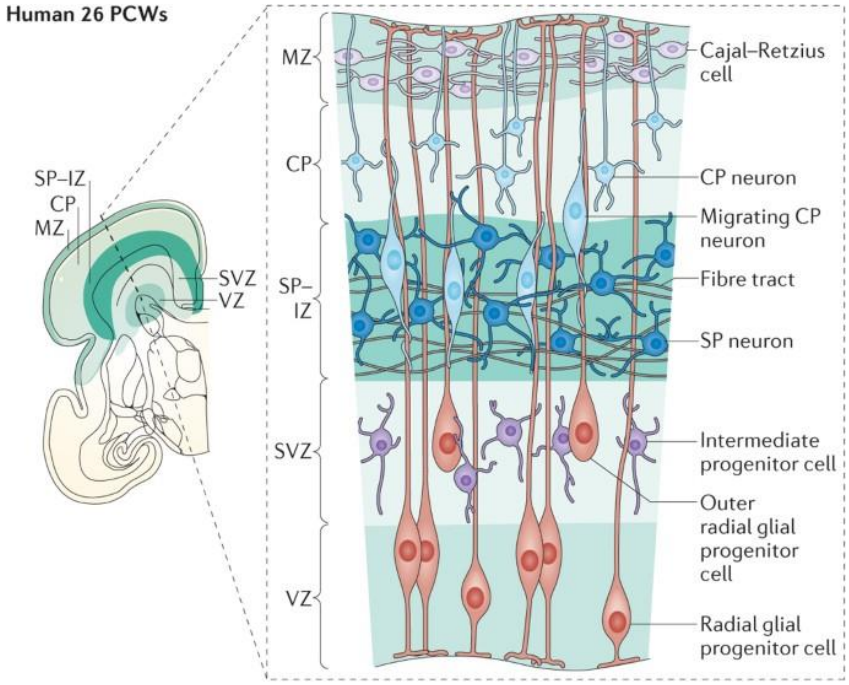


Fig 2: The developing human cerebral cortex schematic at 26th GW. The germinal zone is the VZ and SVZ in which cell divisions take place. The subplate (SP) and the intermediate zone (IZ) lie between the SVZ and the cortical plate (CP). The outermost layer is the marginal zone (MZ) (18).

Primates and humans provide extensive care to their young post-parturition and through early development, the critical periods **(Fig 3).** The **critical period** in brain development refers to a specific time during early childhood when the brain is particularly sensitive to environmental inputs, and is "critical" or "sensitive" experiences for the proper development of brain circuits. During this period, the brain is more malleable and capable of being shaped by experiences. The exact timing of the critical period varies depending on the specific brain circuit and the developmental process involved. For example, the critical period for language development, necessary for the development of language circuits, is thought to be between birth and approximately 5-7 years of age. If a child does not receive adequate exposure to language during this time, they may have difficulty acquiring language later in life. The critical period for visual development occurs between birth and approximately 2-3 years of age.

Synaptogenesis (the formation of new inter-neuronal connections) continues throughout the lifespan, with a certain degree of synaptic pruning based on the availability of trophic factors and experience. Early in brain development, synaptogenesis is at its peak, known as **exuberant synaptogenesis**. The brain undergoes significant growth and refinement during early childhood, with neural connections becoming stronger and more efficient. During this period, the brain increases in size (brains of infants is approximately 30% of the adult size), doubling within the first year of life (19), and continues to develop throughout early adolescence (20). The formation and **strengthening of synapses**, and **pruning** of unused synapses, contribute to changes in neural connections and overall neural architecture. Changes in **gene expression**, particularly through epigenetic mechanisms, can also contribute to neuroplasticity. **Experience-dependent plasticity**, such as through learning and sensory stimulation, can enhance the brain's adaptive capabilities. **Hormones** such as estrogens (17β-Estradiol) and androgens (testosterone) can impact brain development and plasticity during adolescence. Various **factors**, such as stress, exercise, and environmental enrichment, can modulate postnatal neuroplasticity in the human brain. Certain regions such as the prefrontal cortex (PFC), which is involved in higher-order thinking and decision-making, undergoes significant development and refinement during adolescence (21). Traumatic experiences during childhood and adolescence can have negative effects on brain development and plasticity, leading to long-term impacts on mental health and well-being. Thus, the whole process of neurogenesis, cellular migration early in development, critical periods, myelination, and synaptogenesis through adolescence, are significant vulnerable periods when the brain is susceptible to a variety of environmental stimuli, both positively and negatively impacting stimuli.

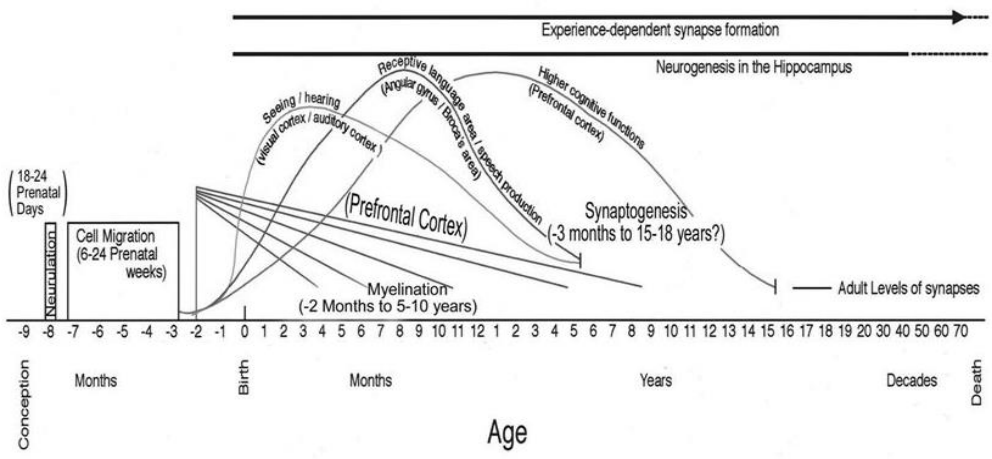


Fig 3: Brain development and neuroplasticity in humans (22).

1.2. Neuroplasticity in adults

The persistence of neurogenesis in adults is a remarkable example of brain plasticity. Although the idea of neurogenesis in adults was initially proposed by Joseph Altman in the 1960s, concrete evidence was not established until the 1980s, when Fernando Nottebohm researched the process in songbirds (23). It was difficult to accept the concept, as several scientists showed that there is a sharp drop in neurogenesis as the brain ages (24,25). However, neuroscientists reported that neurogenesis in the lateral SVZ and dentate gyrus (DG) of the hippocampus persists in aged human brains (26–29). After several years of research, the adult brain, although largely postmitotic, is now known to have dividing cells that can produce both glia and neurons. This process is also seen in rats, mice, songbirds, and nonhuman primates (30). To demonstrate the process, scientists treated cancer patients with bromodeoxyuridine (BrdU) and observed if new neurons were born in postpartum brain tissues, and found that the DG and SVZ had dividing progenitor cells. BrdU is a thymidine analog, that gets incorporated into the DNA of newly dividing cells (31).

It is worth noting that adult NSCs exist in two distinct stages in the adult SVZ: **quiescent (qNSCs) or active (aNSCs)**. The aNSCs are responsible for tissue replenishment, while the qNSCs serve as a backup to replace active stem cells. This strategy may be in place to avoid the depletion of the stem cell pool and prevent the accumulation of potentially tumorigenic mutations. The qNSCs can be activated by stochastic mechanisms, feedback signals resulting from the loss of active stem cells, or

extensive tissue damage. The presence of both of these different pools in the same region emphasizes the importance of the “neurogenic niche” that produces specific factors to maintain the cells' proliferative state (32). The regions that have been extensively studied for the process of adult neurogenesis are the ventricular-**subventricular or subependymal zone (SVZ/SEZ)** adjacent to the lateral ventricles and the **subgranular zone (SGZ)** of the DG of the hippocampus (33).

The qNSCs are also known as **radial glia-like (RGL)** cells due to their morphology and ontogeny. When activated, RGL cells can divide to self-renew and/or produce nIPCs, which undergo multiple rounds of proliferation before differentiating into **neuroblasts (type A cells)**. Unlike RGL cells, nIPCs usually have multipolar processes and do not remain in contact with the ventricle or pial surface, and they do not undergo INM (13). About 25% of these neuroblasts survive and mature to become DG granule neurons (34). Newborn cells derived from NSCs in the DG develop into excitatory, glutamatergic granule cells (GRs) that integrate into the pre-existing hippocampal circuits. It is worth noting that SGZ cells do not necessarily migrate, whereas SEZ cells differentiate and migrate toward the olfactory bulb (OB) through the rostral migratory stream (RMS). Direct neurogenesis is more common in the OB, while indirect neurogenesis is more common in the neocortex (see reviews (35,36). Neurotransmitters involved include dopamine, gamma amino butyric acid (GABA), and glutamate. Several genes are known to control the RG cells (see (37)).

Activated qNSCs/RGLs in the SGZ undergo (34):

1. Asymmetric division to self-renew and generate either an astrocyte or an IPC. This is the more common outcome in a healthy adult brain.
2. Symmetric self-renewal division to generate two RGLs (type B1 cells).
3. Symmetric division to produce two astrocytes or two IPCs without self-renewal. This is more frequent with age and leads to a reduction in NSCs in aged brains.
4. Differentiate directly into an astrocyte without undergoing cell division.

More recently, adult neurogenesis is shown to occur in other regions such as the **hypothalamus arcuate nucleus and the median eminence, striatum, substantia nigra, habenula, cerebellum, cortex, and amygdala** (38) **(Fig 4)**. While some studies have proposed the presence of endogenous stem cell pools within these regions to form

new neurons. Others have demonstrated that these are cells from the hippocampus, that have migrated to the PFC, striatum (39), substantia nigra (40), and amygdala (41), after deviating from the RMS. Adult neurogenesis has functional significance. For example, in the habenula, it is linked to the regulation of the circadian cycle and stress response, affecting the antidepressant effect of fluoxetine (42,43); in the cerebellum, new GRs and interneurons contribute to learning and adapting motor skills based on environmental cues (44). The process of neurogenesis can be modulated by various factors, including growth factors, cytokines, hormones, neurotransmitters, drugs, and environmental exposures. While stroke, seizures, and brain trauma have negative effects, exercise, and enrichment have positive impacts on the process of neurogenesis (23,38). Despite several lines of evidence, conflicting findings make it challenging to reach a definitive conclusion about the presence of adult neurogenesis in human brains (see reviews (45,46)).

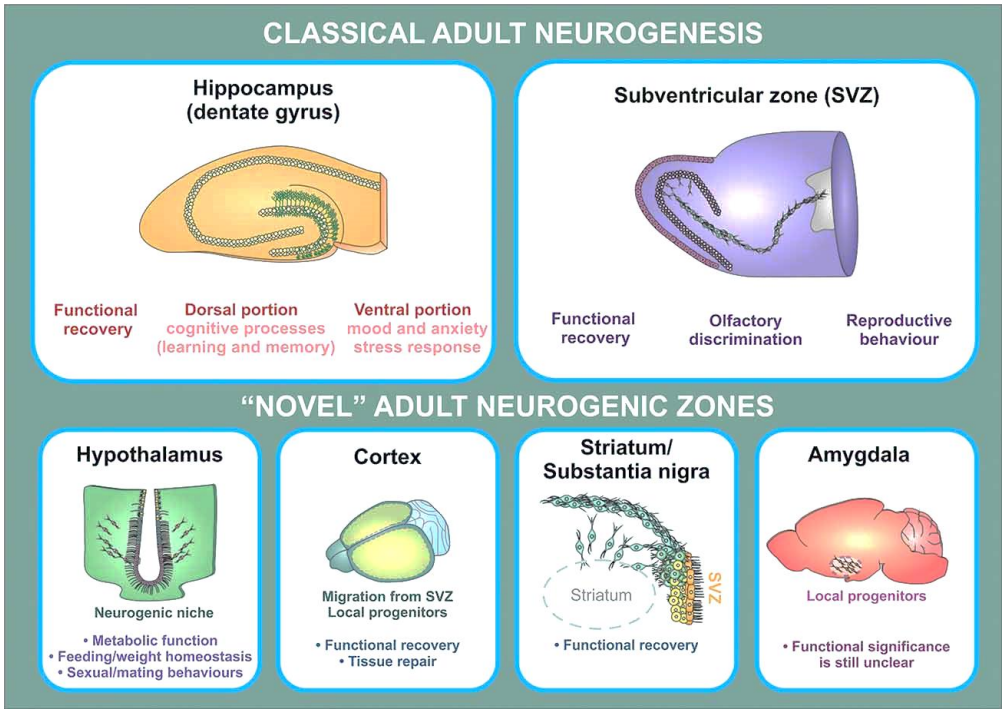


Fig 4: Postulated sites of adult neurogenesis and their functional significance - the hippocampal DG, SGV, SVZ, hypothalamus, PFC, striatum, substantia nigra, and amygdala (38)**.**

Adult neuroplastic processes reduce with ageing. The processes such as synaptic potentiation, synaptogenesis, and cortical map reorganization are reduced with ageing,

and associated with widespread neuronal and synaptic atrophy (47). There is thus cognitive decline that compensates for the age-related reduction in neuroplasticity.

1.3. Mechanisms of Plasticity

The mechanisms underlying neuroplasticity are diverse, encompassing neuronal sprouting, synaptogenesis, and neurogenesis, which may be inherent or acquired through experience. These mechanisms may contribute to both developmental and adaptive plasticity in response to injury. Beyond synaptogenesis and neurogenesis, molecular mechanisms such as angiogenesis (formation of new blood vessels) and gliogenesis also play a role in neuroplasticity (48). At a molecular level, neuroplastic changes occur via gene transcription, protein synthesis, and signaling pathways, with cascades of intracellular proteins transmitting signals from receptors to the DNA (49). The influx of calcium (Ca^{2+}) through depolarization or N-methyl-D-aspartate (NMDA) glutamate receptor and sodium (Na^{+}) through amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) glutamate receptor activates signaling pathways. These pathways include Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), extracellular regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase (MAPK), and the brain-derived neurotrophic factor/tropomyosin receptor kinase B receptor (BDNF/TrkB) pathway. Transient increases in calcium and cAMP levels trigger the necessary events for short-term synaptic plasticity. This then leads to the activation of cyclic AMP-response element-binding protein (CREB) or the nuclear factor kappa B protein complex (NF-kB) in the nucleus, which modulates gene transcription and protein synthesis, thereby initiating long-term plasticity processes (1,50). CREB is a major transcription factor implicated in both cellular and behavioral learning and memory models, affecting various gene targets (51), depending on the cell type, the length of stimulation, as well as the magnitude of stimulation. Neurotrophic factors are gene targets linked to learning, memory, and stress, and to study the effects of antidepressant treatment. Among these, BDNF is an abundant factor in the brain and is of particular interest. CaMKII, when autophosphorylated, remains active even when calcium levels drop, which is crucial for long-term plasticity. These and other mechanisms are discussed in detail in (52,53) **(Fig 5)**.

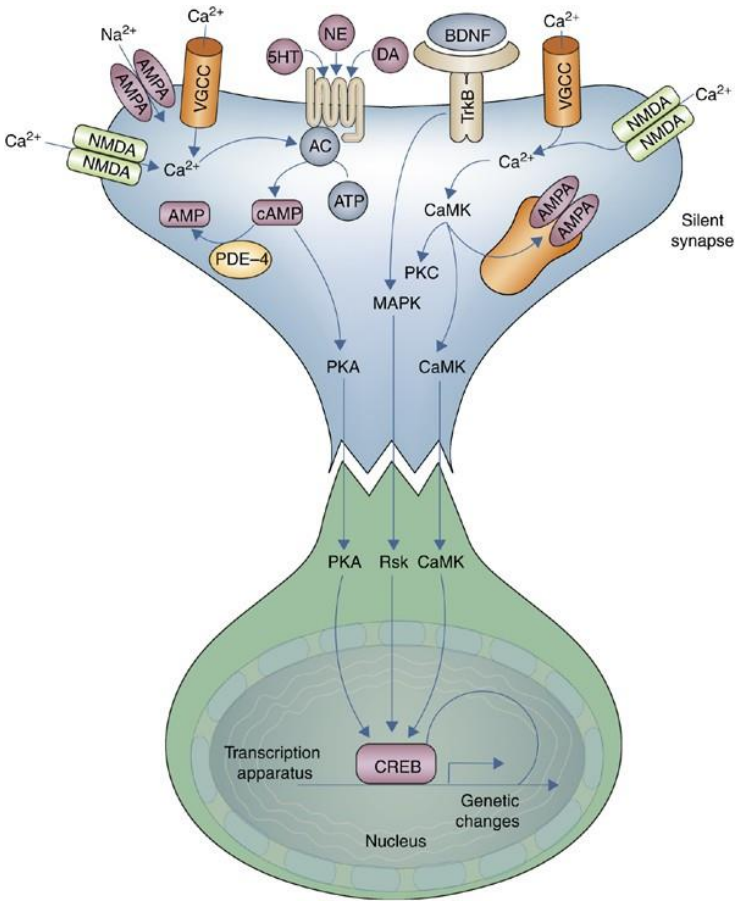


Fig 5: Molecular pathways involved in neuroplasticity (53); VGCC, voltage-gated calcium channel; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; DA, dopamine; AC, adenylyl cyclase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PDE, phosphodiesterase; PKC, protein kinase C; PKA, protein kinase A; Rsk, ribosomal S6 protein kinase.

At a cellular level, changes can be categorized as either **structural or functional**. Structural changes encompass neuronal plasticity, dendritic plasticity, and synaptic plasticity. Neurogenesis is an example of neuronal plasticity and occurs in distinct phases, as previously mentioned. Dendritic plasticity involves alterations in the number or complexity of dendritic spines, with a higher number of spines and more complex dendritic branches indicating greater synaptic strength (54,55). Dendritic spines mark the location of glutaminergic synapses and play a crucial role in synaptic plasticity (56). Rapid changes (acute neuroplasticity) in dendritic spine shape and number can occur within minutes to hours following Ca^{2+}/CaMKII-dependent glutamate release. Adaptations at the cellular level in response to experiences involve modifications in presynaptic and

postsynaptic elements. Consistent and repetitive usage of a peripheral organ can bring about neuroplastic changes in its corresponding brain region including the higher release of neurotransmitters and neurotrophins from the presynaptic neuron, reduced reuptake and breakdown of neurotransmitters in the synaptic cleft, and the addition of more receptors on the postsynaptic cell membrane (49). Neuroplasticity may also occur when neighboring healthy neurons take over the function of impaired neurons in the brain as a result of injury or disease (57).

Synaptic plasticity refers to the ability of synapses to change their strength through short- and long-term functional changes. **Habituation** occurs when a neutral stimulus is repeatedly presented, resulting in a decrease in the electrical response due to a decrease in intracellular Ca^{2+}. **Sensitization** is the prolonged augmented postsynaptic responses after a stimulus is paired with a noxious stimulus, resulting from presynaptic facilitation and a change in adenylyl cyclase. Scientists have long believed that neurons that activate in tandem are more likely to form a connection. This is the "Hebbian Principle," which provides a foundation for learning via synaptic connections (58). In contrast, the principle of "use it or lose it" also applies to neuroplasticity, with a decrease in the strength of the connection between neurons resulting from a lack of activity between them (59). Experience-dependent synaptic plasticity, including **long-term potentiation (LTP) and long-term depression (LTD)**, is key to learning and memory. LTP involves increased synaptic strength and requires activation of increased intracellular Ca^{2+}, glutamate receptors, and enhanced postsynaptic potential response last for days, while LTD results in a persistent decrease in synaptic strength produced by slower stimulation and is associated with a smaller rise in Ca^{2+} (60). Both involve protein synthesis and modulated growth of the presynaptic and postsynaptic neurons and their connections (3).

1.4. Specific brain regions, development, connections, functions, and neuroplasticity

The hippocampus and amygdala are among the most extensively researched brain regions in terms of their neuroplasticity. This thesis provides a brief overview of these regions, and their development, followed by a discussion of other plastic regions such as the cerebellum, cerebral cortex, and hypothalamus. Some of these regions are associated with sexual behavior since steroidal hormones exert their actions primarily in the brain. Additionally, these regions are integral parts of the limbic system, which

regulates various autonomic responses in the periphery. Thus, the integration of these neuroplastic regions with the endocrine, limbic, and ANS is briefly explored.

Hippocampus

The hippocampus, a curved brain structure connecting the septal nuclei of the forebrain and the temporal cortex, plays a crucial role in forming and storing episodic and semantic declarative memories. It has been one of the **most thoroughly investigated regions** since H.M.'s case, who lost the ability to form new declarative memories after hippocampal removal (61). The discovery of activity-dependent synaptic plasticity (62), and hippocampal place cells (63) further enhanced our understanding of its neurophysiology. The hippocampus can be divided into three main subdivisions, which are the DG, cornu ammonis (CA) CA1, and CA3, with an additional CA2. The entorhinal cortex sends both spatial and non-spatial information to the hippocampus, which then transmits the information through different pathways: from the EC to the DG, CA1, and CA3 via perforant path fibers, from the DG to CA3 pyramidal neurons via mossy fibers, from CA3 to CA1 pyramidal neurons via Schaffer collaterals, and from CA1 to the cortex in a unidirectional, feed-forward excitation manner. This creates the **tri-synaptic hippocampal circuit** (64) **(Fig 6)**. There exists a polysynaptic pathway that plays a role in semantic memory while the direct intra-hippocampal pathway has episodic and spatial memory functions (65). The CA1 region is the major output from the hippocampus. It sends projections to various regions of the brain, such as the anterior thalamus, hypothalamus, subiculum, and lateral septum through the fornix, ventral striatum, amygdala, PFC, and other areas of the limbic system. The hippocampus is important in emotional behavior through its connections with the amygdala and also helps to regulate hypothalamic functions (as explained later). Thus, the hippocampus serves as an additional pathway through which incoming sensory signals can trigger behavioral responses (66).

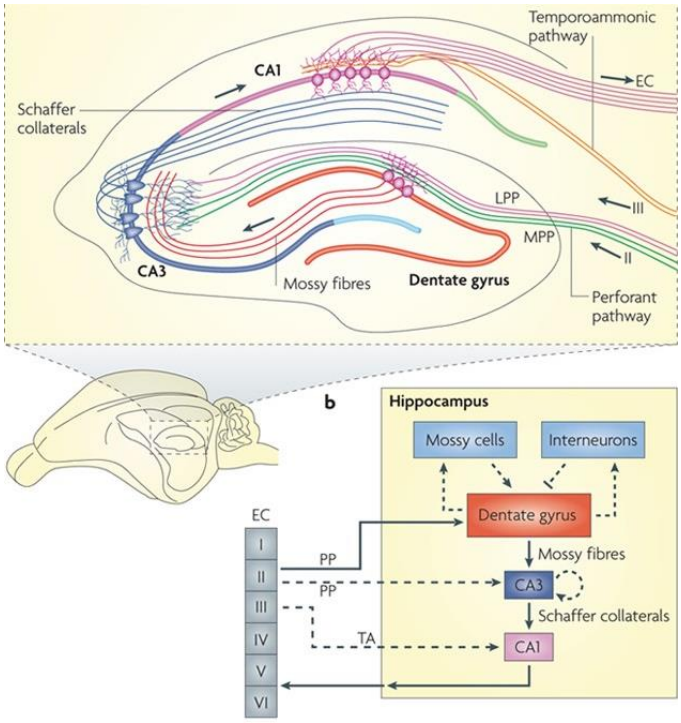


Fig 6: Schematic of the structure and circuits within the hippocampus (67).

The period of hippocampal neurogenesis in rodents is compressed within <10 embryonic days, from day 10 to 18. In humans, this occurs within 2 weeks, from 16 to 18th GW (68). Adult neurogenesis in the hippocampus, as explained before (also see (69)), can be influenced by various factors, such as exercise, enrichment, and growth factors, which promote neurogenesis, while neurotoxins and ageing-related neurodegeneration inhibit it (70). The hippocampus, through several genetic, molecular, and electrochemical mechanisms, plays a role in both long-term and short-term plasticity (see (71)).

Amygdala

The amygdala, situated in the medial temporal lobe is often described as the 'window' that allows the limbic system to perceive a person's position in the world (66). It plays a critical role in processing socially and emotionally relevant information and eliciting responses. The effects of medial temporal lobe lesions on monkey behavior were studied by Klüver and Bucy (72), which resulted in several behavioral changes (such as increased exploration, hypersexuality, and hyperorality) and psychic blindness, including the absence of fear and anger, and loss of social interactions. People with amygdala lesions exhibit emotional deficits, including impaired recognition of facial expressions and

abnormal social behavior and decision-making (73,74). In humans, the amygdala structure is discernible by the 8th GW and is mature by 8 months in utero. Structural connectivity across the cortex is seen by the 13th GW. The major amygdala nuclei are fully formed by the 15th GW. Postnatally, the amygdala undergoes rapid growth before 3 months of age (75–79). In mice, the cellular components of the amygdala are seen between E10-14 (80,81). Each nucleus of the amygdala receives reciprocal inputs from multiple brain regions, both cortical and subcortical **(Fig 7).** It is linked to emotional and social aspects of memory (82), attention (74), and perception (83) through its connections with the hippocampus, orbital and medial PFC, and sensory cortex.

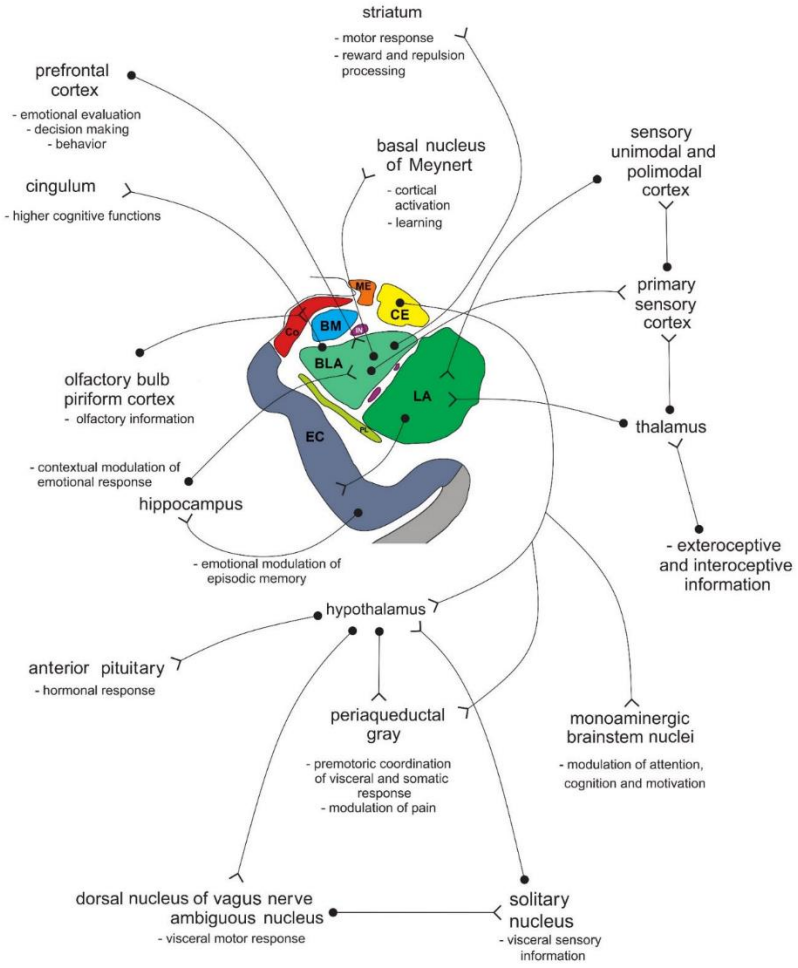


Fig 7: Connections of the Amygdala - BLA—basolateral (basal) nucleus; BM—basomedial (accessory basal) nucleus; CE—central nucleus; Co—cortical nucleus; IN—intercalated neurons; ME—medial nucleus; LA—lateral nucleus; PL—paralaminar nucleus (84)

The amygdala in primates consists of 13 nuclei and cortical fields. The **lateral nucleus** (LA) is the primary afferent structure that receives projections from the neocortex and transmits them to other amygdala nuclei and other parts of the LA. The basolateral amygdala (BLA) through its connections with the orbitofrontal cortex, the medial PFC, the ventral striatum, the nucleus accumbens (NAc), the bed nucleus of the stria terminalis (BNST), and the central nucleus (CE) forms sensory information flow loops between the amygdala and cerebral cortex (85). The CA1 neurons have bidirectional projections to and from the BLA (71,86). The basomedial (BM) amygdala projects into the CE, secreting corticotropin-releasing hormone/factor (CRH/CRF), enkephalins, and neurotensin, and expresses dopamine, estrogen and serotonin receptors (87), playing an important role in sex hormone-regulated motivational behavior. The paralaminar nucleus (PL) also has a high concentration of CRH receptors and is innervated by serotoninergic fibers (88,89). The medial nucleus (ME) mostly contains GABAergic neurons, and psychological stress activates it, which, in turn, leads to activation of the hypothalamo–pituitary-adrenal (HPA) axis and secretion of ACTH (90). The CE is the main source of efferent fibers of the amygdala and has a unique role in converting sensory information into a physiological response and behavior change (91), such as rage, aggression (66), social learning and memory, pheromonal processing, and reproductive-associated behaviors. The CE contains the second highest density of CRH/CRF in its GABAergic neurons, the first being the hypothalamus (92). The central part of the amygdala receives inhibitory projections from the PFC and orbitofrontal cortex, is linked to the hypothalamus and brain stem nuclei, and plays a role in regulating cardiac function and modulating emotional responses (see (93–95)). Stimulation of certain amygdaloid nuclei can also result in rage, escape, punishment, pain, and fear similar to the hypothalamus (96). The amygdala through its neuro-hormonal connections plays a chief role in emotional processing (97). It is also essential for memory association, and "records" the emotional aspect of memory to enable an individual to react effectively upon repeated exposure (98).

Adult neurogenesis was demonstrated in the amygdala of adult primates' rostral temporal lobe cells (41). Later several other species were also shown to elicit amygdala neurogenesis (for their effects on social behavior, hormonal release, and neurotransmission see review (96)) A volumetric growth of the human amygdala is seen

during adolescence, paralleled by an increase in neuronal cell number (99). A recent study using ^{14}C radiocarbon analysis demonstrated post-natal turnover of neurons in the human amygdala based on a quiescent and a cycling neuronal population with about a 35% fraction of renewing cells (100). Using stereological analysis of 52 human brains (24 neurotypical and 28 with autism spectrum disorder) of ages ranging from 2 to 48 years, it was found that the number of mature neurons increased in the basal and accessory basal nuclei of the amygdala from childhood to adulthood, while the immature neurons within the PL decreased. Additionally, individuals with autism had an initial surplus of amygdala neurons in childhood, which then reduced in adulthood across nuclei (99). Thus, the neurogenesis process is highly linked with the occurrence and development of neurological disorders.

Cerebellum

The cerebellum, situated beneath the temporal and occipital lobes of the cerebral cortex, is a part of the hindbrain composed of a thin, densely folded cerebellar cortex surrounding the deep cerebellar nuclei. In 1898, the cerebellum was named the 'head ganglion of the proprioceptive system' by Charles Sherrington due to its role in motor functions. The deep cerebellar nuclei consist of the dentate, globose, emboliform, and fastigial nuclei. The cerebellar cortex has 3 layers: an external molecular layer, a one-cell-thick Purkinje cell (PC) layer, and an internal granular layer. There are five types of neurons in the cerebellar cortex, which are the PC, granule (GR), basket (BC), stellate, and Golgi cells. GRs release glutamate, while the rest of the cells release GABA. The cerebellar cortex has two primary excitatory inputs: climbing fibers and mossy fibers. Climbing fibers originate from a single source, the inferior olivary nuclei, and project to the primary dendrites of the PC. The mossy fibers provide direct proprioceptive input from all parts of the body and input from the cerebral cortex via the pontine nuclei to the cerebellar cortex. They end on the dendrites of GRs in complex synaptic groupings called glomeruli (3) **(Fig 8).**

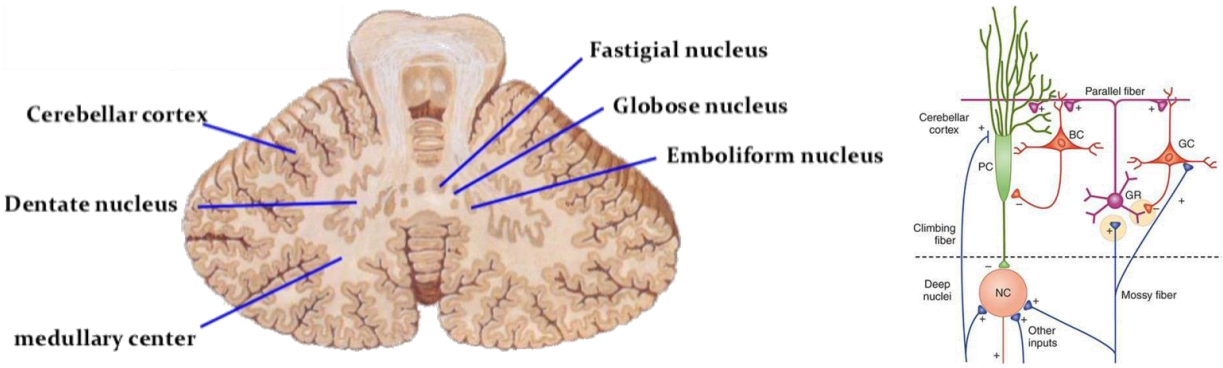


Fig 8: Diagram of nuclei and connections in the cerebellum. Plus (+) and minus (–) signs indicate whether endings are excitatory or inhibitory. In the fig - BC, basket cell; GC, Golgi cell; GR, granule cell; NC, Deep nuclei; PC, Purkinje cell (3).

Research conducted on mice can provide valuable insights into defining pathogenic mechanisms because the developmental mechanisms of the cerebellum are highly conserved between rodents and humans (101). In mice, PCs are born at E10.5–E13.5, GRs at middle to late stages (E13.5 onward), GABAergic interneurons at E10.5–E11.5, and Golgi cells at E13.5 (peak E14–E16) (101). Further, the cells migrate in different ways, radial, and tangential, through the embryonic period (see review (102)). In mice, the exponential proliferation of the GC progenitors occurs after birth, while, in humans, it starts at 35 to 42 embryonic days, achieving a peak at the 32nd GW and continues during postnatal age (103) until the second year (104). Interestingly, evidence of adult neurogenesis has been obtained in transgenic mice after cerebellar injury in the granular outer layer, rabbits during peripubertal ages in the subpial layer, and PC layer, though more research is needed to further confirm these findings (44,46,105).

The cerebellum plays a crucial role in motor control and learning. It has been proposed that the cerebellar cortex serves as a probable site for **motor memory storage**, and several molecular and cellular investigations support the hypothesis that the cerebellar cortex plays a crucial role in motor learning and could be a vital location for **learning-related plasticity** making the cerebellum a suitable model to study neuronal plasticity, learning, and repair (106,107). For instance, musicians have larger cerebellum than non-musicians due to the acquisition of precise finger movements. The plasticity of parallel fiber-PC has been extensively studied, indicating that general plasticity mechanisms may underlie cerebellar plasticity during learning (108). The mechanism of

learning may involve LTD of climbing fibers, leading to the decreased firing of parallel fibers. Metabotropic glutamate receptor (mGluR) activation can trigger an increase in intracellular inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) levels or a decrease in intracellular cAMP levels, resulting in cerebellar synaptic plasticity (3). The cerebellar vermis is involved in the retention of fear memory consolidation (109,110) (for a review see (111)). Evidence suggests that the cerebellum is also involved in non-motor functions such as language, cognition, and emotion. Integration with the hypothalamus (as discussed later) was revealed in the 1980s (112), and this integration, along with the cerebellum's many unknown functions, makes it a special organ.

Cerebral Cortex

Over the past two decades, there have been numerous reports of plasticity phenomena in the cerebral cortex following ischemic injury, both in animal models and in human stroke survivors. Experience is identified as one of the most powerful modulators of cortical plasticity, both structurally and functionally (113). The mechanisms underlying experience-dependent plasticity have been predominantly investigated in sensory cortices, with a focus on how sensory input regulates synaptic modifications, neural circuit reorganization, and cortical function (114). During critical periods, plasticity can be significantly modified by both environmental and genetic factors to optimize its function in that environment. Indeed, this plasticity plays a crucial role in shaping our perception of vision, hearing, touch, taste, and olfaction early in development. However, evidence of cortical remodeling is also observed in adults learning new skills, conditioning experiments, localized neural stimulation, and when there is a loss of peripheral input (as reviewed in (115)). Engaging in a sensory task that requires learning through repeated practice, attention, and engagement improves performance, indicating the involvement of higher-order frontal brain regions (116). The basal forebrain, which is rich in cholinergic neurons, is one of the main regions involved in attention-based plasticity. Stimulation of the nucleus basalis of Meynert paired with tones induces rapid plasticity in the primary auditory and visual areas (117). Another extensively studied form of plasticity is induced by sensory deafferentation. For instance, individuals who are deaf show enhanced visual and vibrotactile skills (118,119) **(Fig 9)**. Similarly, early visual deprivation results in cortical reorganization with stronger sound localization in the occipital cortex and reduced

activation of the medial temporal cortex of blind participants (120). Various molecular mechanisms have been proposed to underlie cortical plasticity, including dendritic, axonal, and synaptic plasticity (121). The development of the cerebral cortex occurs from E10-17 in mice and between 7-18 GW in humans (122).

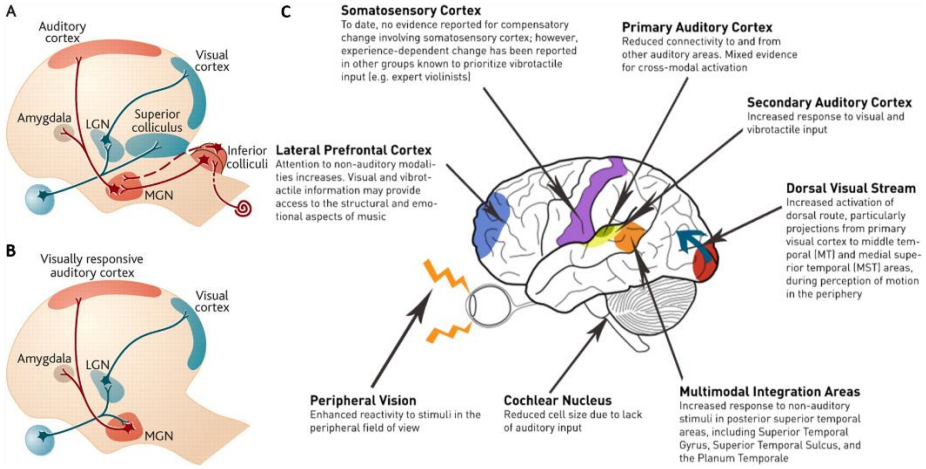


Fig 9: Representation of neuroplasticity in the cortex: A: Normal hearing sense - auditory inputs from the cochlea reach inferior colliculi, medial geniculate nuclei (MGN) in the thalamus, followed by relay to the auditory cortex. Visual stimuli normally relay onto the superior colliculus and lateral geniculate nuclei before transmitting signals to the visual cortices; B, C: Compensatory plasticity in a deaf individual, the auditory areas gradually respond to different other sensory stimuli (eg: visual stimuli) compensating for the auditory sense loss, and plasticity in different areas of the cerebral cortex in a deaf person listening to music (118,123).

Hypothalamus

The hypothalamus is a cluster of neuronal nuclei situated at the base of the third ventricle in the diencephalon, comprising just 1% of the brain mass. It is the "**master endocrine gland**," and "**head ganglion of the autonomic system**," as Charles Sherrington named it. Through its many nuclei **(Fig. 10)**, the hypothalamus plays a critical role in the regulation of arterial pressure, thirst and water retention, appetite and energy expenditure, temperature control, and endocrine regulation. The hypothalamus receives input from various sources, including sensory input from the external environment (visual, olfactory, auditory, and temperature), as well as visceral and pain receptors, and receptors in circumventricular organs that signal changes in blood levels of circulating

chemicals. The hypothalamic neurons (especially those in the paraventricular nucleus-PVN, dorsomedial hypothalamus-DMH, and perifornical area) then establish direct and indirect connections with autonomic preganglionic neurons (124). The dorsal forebrain bundle, medial longitudinal fasciculus, and mamillotegmental tracts are the primary pathways that facilitate communication between the hypothalamus and other regions of the brain to regulate the ANS. The **central autonomic network** comprises various regions in the cortex, amygdala, hypothalamus, midbrain, pons, and several nuclei in the medulla that receive inputs related to homeostasis, emotions, and sensations from other parts of the brain, the environment, and the body. All of these inputs are integrated to generate an appropriate response, which is rapidly transmitted to the heart through the ANS (125), as described later. The inputs from basal forebrain septal nuclei, and amygdala project into the hypothalamus via the medial forebrain bundle.

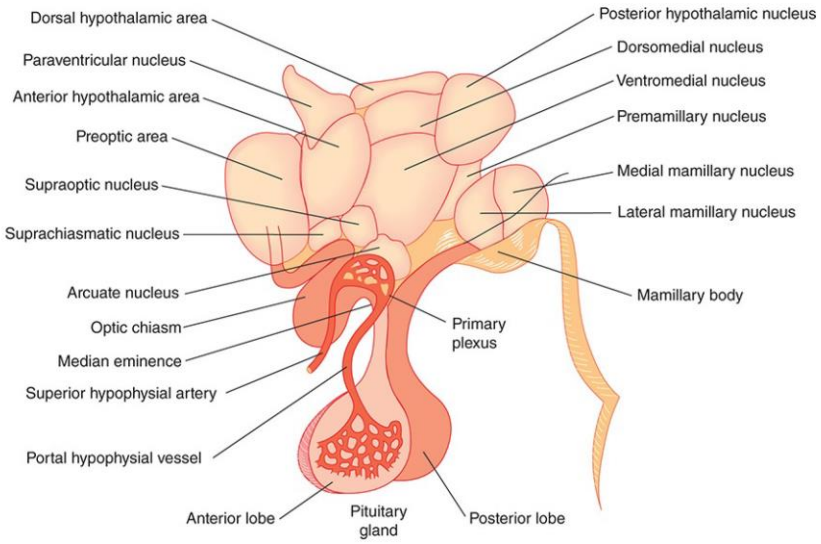


Fig 10: Group of nuclei in the Hypothalamus (3)

Hypothalamic neurogenesis occurs between E10-E16 in the mouse when one can identify genes responsible for neurogenesis to be enriched (126,127). However, gliogenesis and neuronal migration processes continue postnatally (Fig 12). In humans, hormone activity in the HPA axis can be detected between 8 to 12th GW (128). Different nuclear areas of the hypothalamus develop at different gestational periods. At 24 to 33rd GW, the fetal human hypothalamus takes an adult-like appearance (129). Novel neurogenic niches have been identified in the adult hypothalamic 3rd ventricular wall and

its vicinity, including PVN, ventromedial hypothalamus (VMH), arcuate nucleus (AN), and median eminence (130–134). The hypothalamus undergoes age-related changes, with some regions showing a decline in cell numbers while others remain active (supraoptic nuclei-SON and PVN) with evident sex-specificity (see review (135)). Tanycytes, the NSCs in the hypothalamus, can proliferate in certain conditions and divide symmetrically or asymmetrically to give rise to IPCs **(Fig 11)**. These IPCs differentiate into neurons, astrocytes, or oligodendrocytes and integrate into neural circuits to regulate physiological activities (see review (136)). The process is aided by factors like BDNF and ciliary neurotrophic factor (CNTF) (96,136). Studies have shown that neurogenesis and gliogenesis are linked with hypothalamic functions like control of metabolism (137), reproduction, thermoregulation, sleep, ageing, sensory information processing, and neuroendocrine regulation (136).

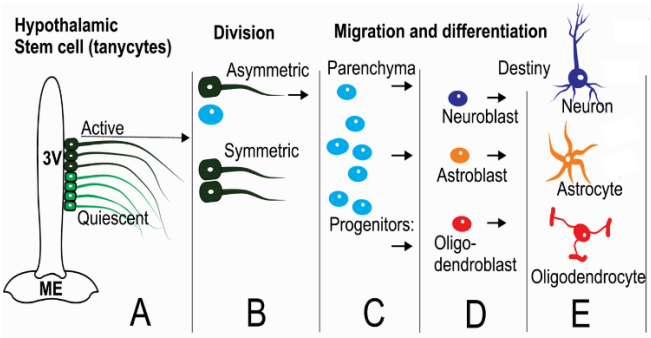


Fig 11: Schematic of hypothalamic neurogenesis and gliogenesis (136)

Sensory experience during the postnatal period, particularly mother-pup interaction, plays a crucial role in regulating neuroplasticity of the HPA axis throughout the animal's lifetime, leading to improved cognitive functions and reduced stress response (lower CRH expression, and increased hippocampal glucocorticoid receptors (GlucR) levels (138–142)). Hormones like estradiol can interact with energy balance disruptions and affect prenatal and adult neurogenesis (143,144).

The focus of this thesis is on the cardiovascular, endocrine, and limbic roles of the hypothalamus. In general, stimulation in the posterior hypothalamus (PH) and lateral hypothalamus (LH) increases the arterial pressure and heart rate (HR), whereas stimulation in the POA often has opposite effects (66). The PVN, the right anterior aspect of the LH/supraoptic nuclei (SON), and the AN have been proposed to have cardiovascular regulatory activity (145). There are six established **hypothalamic-**

releasing and inhibiting hormones. These are CRH, thyrotropin-releasing hormone (TRH), growth hormone releasing hormone (GRH), somatostatin, gonadotropin-releasing hormone (GnRH), prolactin-inhibiting hormone (PIH) and prolactin-releasing hormone (PRH). The **anterior pituitary,** under the influence of the hypothalamic hormones, secretes six hormones: the adrenocorticotropic hormone (corticotropin, ACTH) under the control of CRH, thyroid-stimulating hormone (thyrotropin, TSH) under the control of TRH, growth hormone under the control of GRH, follicle-stimulating hormone (FSH) and luteinizing hormone (LHo) both under control of GnRH, and prolactin (PRL) under the control of PRH/PIH. Thus, the hypothalamus through the network of the hypothalamo-hypophysial portal system of blood vessels (Fig 24), controls the release of anterior pituitary hormones. The hormones, oxytocin, and vasopressin, of the **posterior pituitary** gland, are synthesized in the cell bodies of the magnocellular neurons in the SON and PVN and are transported through the axons of the hypothalamohypophyseal tract system to their endings in the posterior lobe, where they are secreted in response to electrical activity in the endings (3). A few important functions of these hormones include metabolic functions, growth, development (GH, Insulin-like growth factor – IGF-1), gonadal functions and sexual behaviors (FSH, LHo, estrogens, progesterone), stress, inflammation, metabolic functions (ACTH, cortisol), metabolic functions, energy regulation (TSH, T3, T4), and water balance.

The HPA axis is involved in stress and hormone regulation. Its primary effectors are located in the PVN, anterior pituitary, and adrenal gland. When stressed, CRF triggers the release of ACTH into the bloodstream. ACTH's primary target is the adrenal cortex, where it stimulates glucocorticoid and mineralocorticoid secretion. Glucocorticoids interact with GlucRs that are widely distributed throughout the body, inducing physiological changes. The HPA axis is further modulated by glucocorticoids, acting via GlucRs in the hypophysiotropic neurons of the PVN, hippocampus, and PFC (layers II, III, and VI) through genomic, delayed feedback, and rapid nongenomic feedback systems. In contrast, the amygdala is thought to activate the HPA axis, promoting glucocorticoid synthesis and triggering stress responses. While glucocorticoids have adaptive effects, excessive HPA axis activation can contribute to the development of pathologies (146).

The regulation of emotions involves various nuclei in the hypothalamus, which has been established through experiments on mammals and observation of human diseases. Stimulation of the LH results in increased activity, aggression, and stimulation of the VMH induces satiety, decreased eating, and a sense of tranquility. The anterior and posterior hypothalamus regulates sexual behavior (66). In addition, the key brain regions responsible for rewarding sensations are situated along the path of the medial forebrain bundle in LH and VMH. Other brain regions, such as the septum, amygdala, specific thalamic areas, and basal ganglia, also participate in regulating rewards. Conversely, the regions of the hypothalamus's periventricular zones, hippocampus, and amygdala act as punishment centers.

Autonomic nervous system (ANS)

The ANS regulates a range of involuntary physiological processes, such as HR, blood pressure (BP), respiration, digestion, and sexual behaviors. It comprises three distinct anatomical divisions: the sympathetic (SNS), parasympathetic (PNS), and enteric nervous systems. The ANS involves a two-neuron chain that innervates the visceral target tissues, including cardiac muscle, smooth muscle, secretory glands, and immune system cells, through preganglionic neurons. The SNS is a thoracolumbar (T1-L2) system that originates from the intermediolateral cell column of the lateral horn of the spinal cord and acts through the chain of ganglia and collateral ganglia. The SNS is designed to elicit fight-or-flight reactions in emergencies. In contrast, the PNS, which is a craniosacral system, originates from brainstem nuclei associated with cranial nerves III, VII, IX, and X, and arises from the intermediate gray horn in the S2-S4 spinal cord. Connections from III, VII, and IX cranial nerves act through cranial nerve ganglia, while connections from the vagal system (X) and sacral system act through intramural ganglia in or near the target tissue. The PNS serves as a homeostatic reparative system (147) **(Fig 12)**. Preganglionic sympathetic and parasympathetic axons both use acetylcholine (ACh) as the neurotransmitter. Most sympathetic postganglionic axons release norepinephrine (NE) while the postganglionic parasympathetic axons release ACh. However, the postganglionic sympathetic nerve fibers to the sweat glands and very few blood vessels are cholinergic **(Fig 13)**.

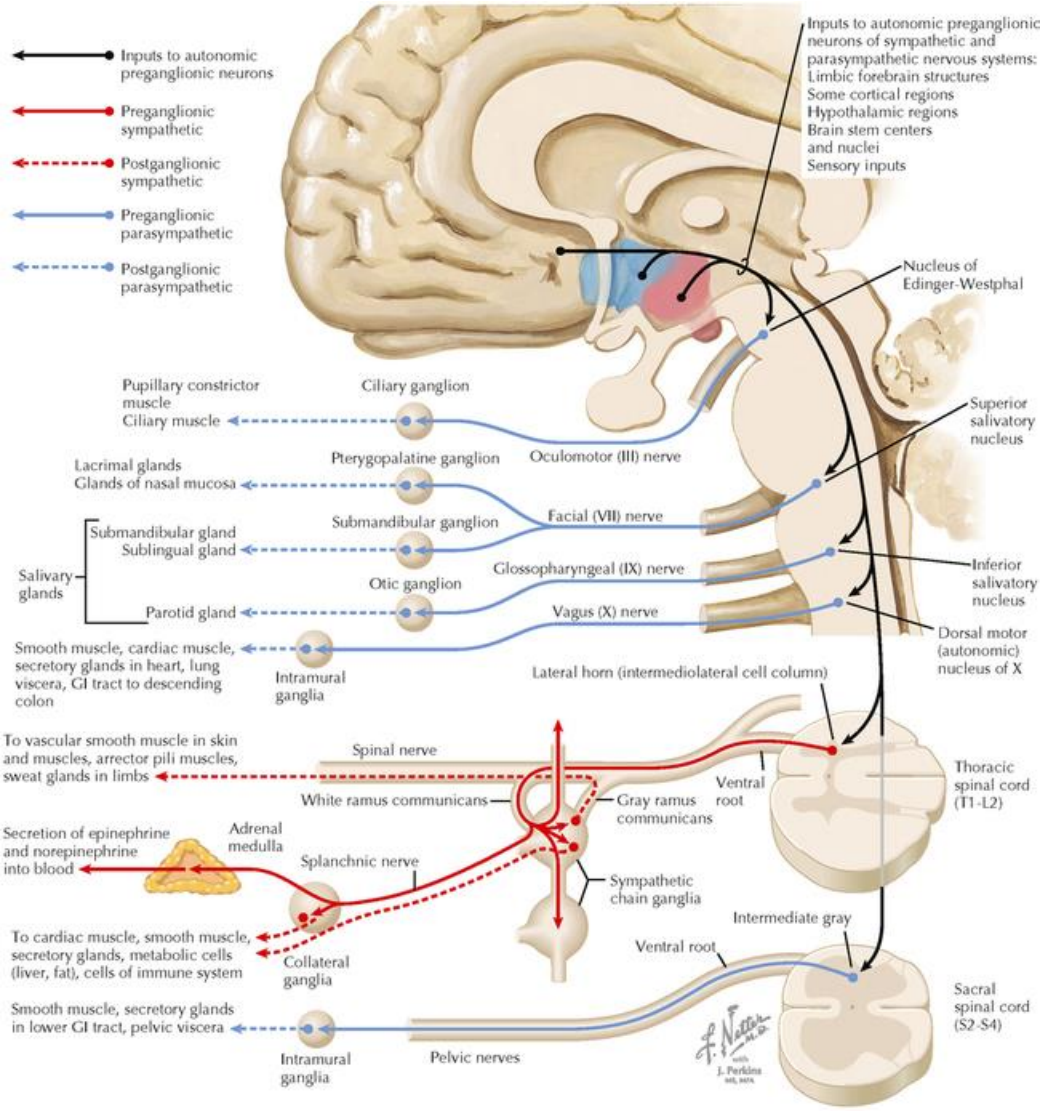


Fig 12: The general organization of the ANS. Note the autonomic nuclei in the brainstem, and intermediolateral horn of the spinal cord gives rise to preganglionic neurons. The sympathetic chain (red), parasympathetic outputs (blue), and the modified postganglionic sympathetic neurons - chromaffin cells in the adrenal medulla (147).

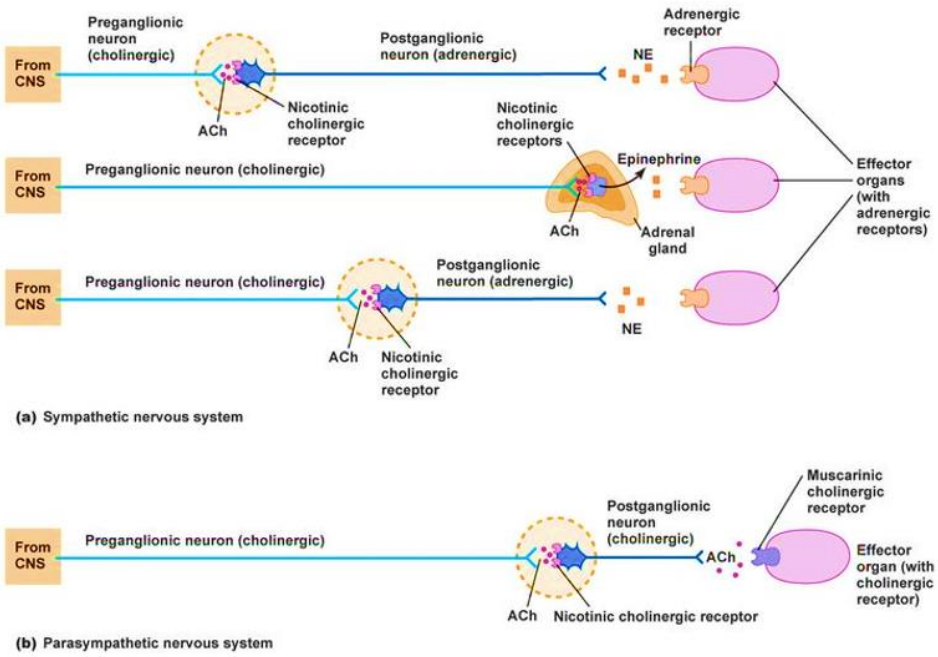


Fig 13: The autonomic nerve chains and neurotransmitters (66).

ACh acts on muscarinic (mAChRs) and the nicotinic acetylcholine receptors (nAChRs). ACh is then broken down by the extracellular Acetylcholinesterase (AChE). Each nAChR is made up of five subunits that form a central pore or channel. The five subunits designated as α, β, γ, δ, and ε are each coded by different genes. The brain nAChRs are composed of alpha and beta (α2-α7, α9–10, and β2–4) subunits that form homo- or hetero-pentameric ion channels with diverse pharmacological properties (148). The most common subunits in the brain are α7 and α4β2. The ACh binds to α subunits inducing a conformational change in the protein so that the channel opens, and permits the passage of Na^+ and other cations to produce a depolarizing potential. There are five types of muscarinic cholinergic receptors (M1–M5) (3). In this thesis, we used a chemical that binds to nAChRs and modifies its actions. These receptors are susceptible to neuroplastic effects, especially postnatally, which may be caused by early life stress and other environmental exposures (149,150).

The function of the ANS can be measured non-invasively from physiologic signals of HR, respiratory rate, and BP. Heart rate variability (HRV) **(Fig 14)** refers to the variation in the time interval between consecutive heartbeats (variability seen in each R-R interval on electrocardiogram recordings). It is a measure of the fluctuations in HR that occur due to changes in the ANS activity. After power spectral analysis of the waveforms, one gets

the high-frequency variability (0.15–0.4 Hz) that reflects parasympathetic function and is influenced by the respiratory rate, while low-frequency variability (0.04–0.15 Hz) is due to a combination of both divisions of ANS and baroreflex-induced changes in HR. Low HRV is associated with sympathetic dominance and high HRV is associated with parasympathetic dominance. HRV has been used as a tool for assessing various health conditions, such as cardiovascular disease, diabetes, and stress-related disorders. In addition, HRV biofeedback has been used as a therapeutic technique to improve ANS balance and overall health (see (151,152)).

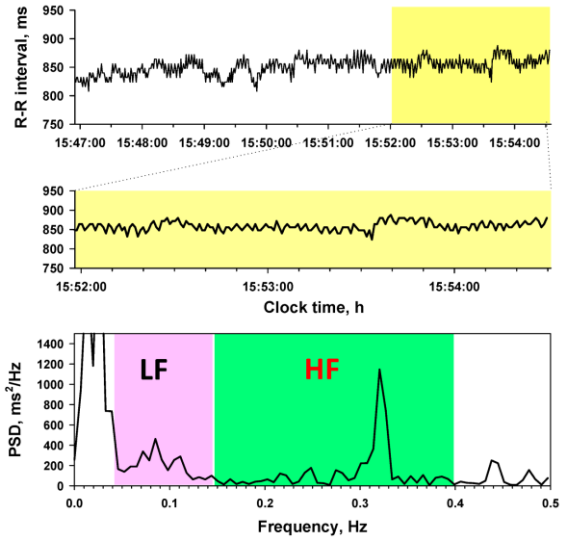


Fig 14: Analysis of HRV in sinus rhythm. HF = high-frequency, LF = low-frequency, PSD = power spectral density (153).

1.5. Integration of the regions

The HPA axis's activity is regulated by extrahypothalamic limbic structures, especially the hippocampus, and amygdala (154,155). The intricate interplay between the ANS and the limbic system provides a foundation for physical and emotional experiences that shape behavior, emotional, and neuropsychiatric health from prenatal development to adulthood. The limbic system is made up of several structures, such as the amygdala, thalamus, fornix, olfactory cortex, hippocampus, hypothalamus, and cingulate gyrus **(Fig 15)**. During early brain development, these structures develop multiple interconnections and connect to the brainstem ANS centers to regulate the ANS's outflow to the body and modulate visceral functions. This may be affected by the ‘intrauterine milieu’, including maternal preconception health and stress hormone levels during pregnancy (156). Over

a lifetime, these connections strengthen or weaken depending on the environment, stress, and other exposures (157).

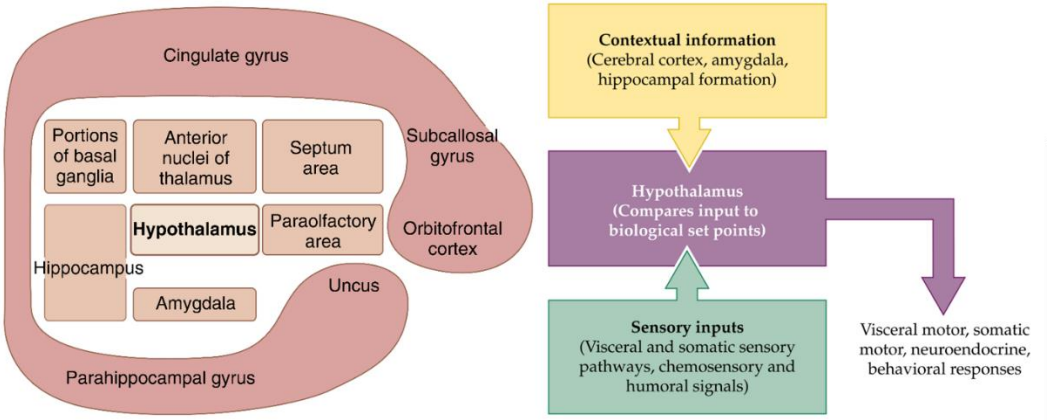


Fig 15: The limbic system, showing the key position of the hypothalamus. Note the inputs received by the hypothalamus from centers and periphery, which in turn manifests its actions through the ANS (66,158).

The activation of hippocampal neurons inhibits the HPA axis, while the amygdala exerts a significant facilitatory effect (154). The hippocampus expresses a high level of adrenal steroid receptors, both GlucR and mineralocorticoid receptors (MR), and is indirectly linked with the HPA axis through the amygdala e.g., hippocampus-modulated contextual fear memory-related autonomic responses (159). When stimulated, the hippocampus decreases neuronal activity in the PVN and inhibits glucocorticoid secretion, functioning as a negative feedback regulator of the stress response. But, prolonged and severe stress disrupts this control, resulting in stress-related damage (discussed later). On the other hand, the amygdala plays a critical role in fear, anxiety, and the activation of the HPA axis. The amygdala has a direct and indirect connection with the hypothalamus: a) BNST connects the amygdala with the preoptic area (POA) of the hypothalamus, b) The ventral amygdalofugal pathway situated in the medial forebrain bundle directly connects the CE and BLA with the hypothalamus, c) An indirect pathway consists of GABAergic projections from the amygdala CE to the BNST (disinhibition) (160) and the efferences which retro project to CRH cells in the hypothalamic PVN (161–163). A stressor or memory of a negative event activates the amygdala that sends stress response signals to the hypothalamus and brainstem sympathetic centers to elicit corticosterone secretion, increase arterial pressure and HR, decrease gastrointestinal

motility and secretion, defecation, micturition, increase pupillary dilation, piloerection, and secretion of anterior pituitary hormones, including gonadotropins (66) **(Fig 16)**. Other limbic structures and cortex, insular cortex, anterior cingulate cortex, and the infralimbic cortex have been implicated in descending control of the cardiopulmonary system (for more on cortical control over the ANS see (164)).

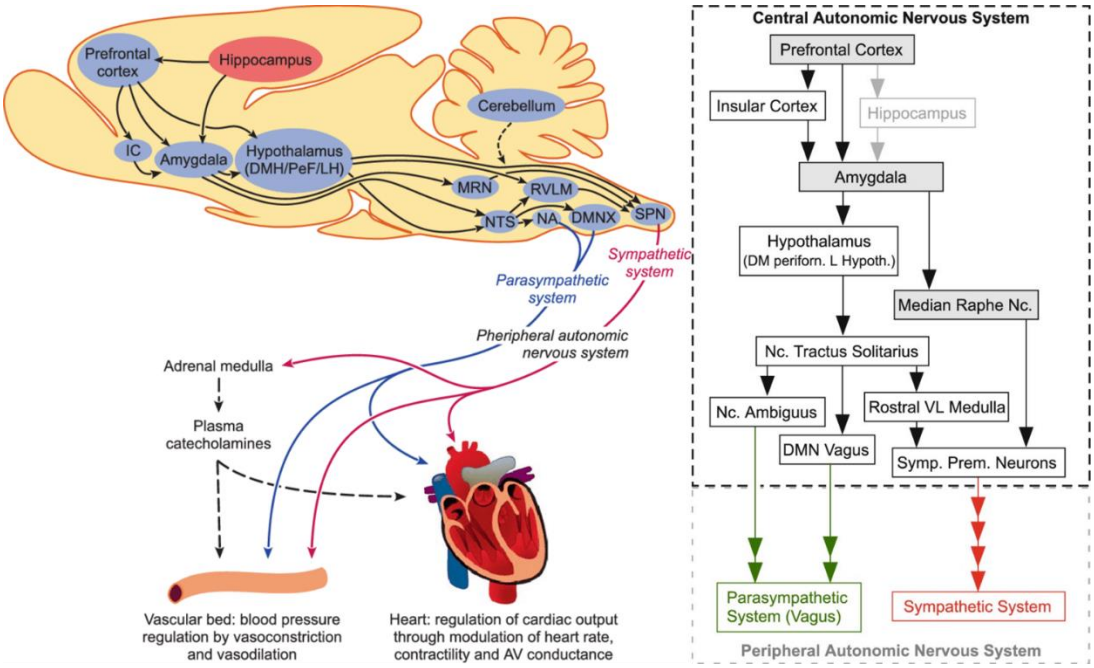


Fig 16: The integrated control of ANS a) Representation in a rodent brain b) Core areas involved in emotional modulation are depicted in gray. The hippocampus and cerebellum are only indirectly involved (165,166)). DMN Vagus – Dorsal Motor nucleus of the vagus; DM – Dorsomedial; Periforn – Perifornical; L.Hypoth – Lateral Hypothalamus; Nc – Nucleus; VL – Ventrolateral.

There is sizable evidence documenting that **integration** is one of the major functions of the **cerebellum**. For example, cerebellar influences on visceral functions have been described (see review (111)). The cerebellum, via its connection with the hypothalamus (167), is involved in controlling vasomotor reflexes, the somatic and autonomic manifestations of shame rage, pupil nictitating membrane, respiration, and gastrointestinal functions. The reciprocal cerebellar hypothalamic pathway has been suggested as the pathway that influences the ANS (111,112,168). The cerebellum is linked to emotional expression and behavior through its connections to the rostral basal forebrain (169). Molecular components of the circadian rhythm have been observed in

the cerebellar cortex (170). Interestingly, the hypothalamic histaminergic and orexinergic systems, which regulate the ANS, innervate the entire brain, including the cerebellum, and may modulate cerebellar neurons to participate in motor control and somatic-non somatic integration (171). The fastigial-hypothalamic glutamatergic transmission mediates the effect of cerebellar fastigial glutamatergic neurons on humoral immunity (172). Recent research has also identified non-motor functions of the cerebellum in fear responses, fear memory, social behavior, cognition, and spatial navigation, which occur indirectly through connections to the limbic areas mentioned above, in addition to medial PFC, thalamus, reticular formation, sensorimotor cortex, and medial septum (173).

2 Anthropogenic Factors

The term **“anthropogenic”** is used for referring to the environmental change caused by people, either directly or indirectly. It was Paul Crutzen who introduced the term "Anthropocene" in the mid-1970s and was first used by Russian geologist Alexey Pavlov and by British ecologist Arthur Tansley (174). Anthropogenic factors are those that occur due to human activities. It is more often used in the context of chemical or biological wastes that are produced and released into the **environment by human activities**. For example, carbon dioxide, methane, and ozone-depleting gas are one of the primary factors driving anthropogenic climate change (175). The rising levels of industrial chemicals in the soil, water, and air, depletion of essential minerals and gases from the soil, mining, deforestation, wastewater disposal of heavy metal contaminants, and drug waste from industries and medical settings have become a bottleneck to environmental sustainability (176).

How a **person's health is influenced by** the different types of exposure they are subjected to during their lifetime, introduces us to the term **“exposome”**, a concept presented in 2005 by Christopher Paul Wild, a cancer epidemiologist (177). The exposome is proposed to complement the genome, wherein an individual’s exposure begins before birth and includes insults from chemical, dietary, lifestyle, biological, and occupational sources. This exposome interacts with an individual’s unique characteristics such as genetics, epigenetics, and physiological outcome to impact health acutely but also in the long term **(Fig 17, Table 1).** Current epidemiological data suggest that only

10% of the occurrence of human diseases is linked to genetic anomalies, leaving the exposome a major factor influencing health (178,179).

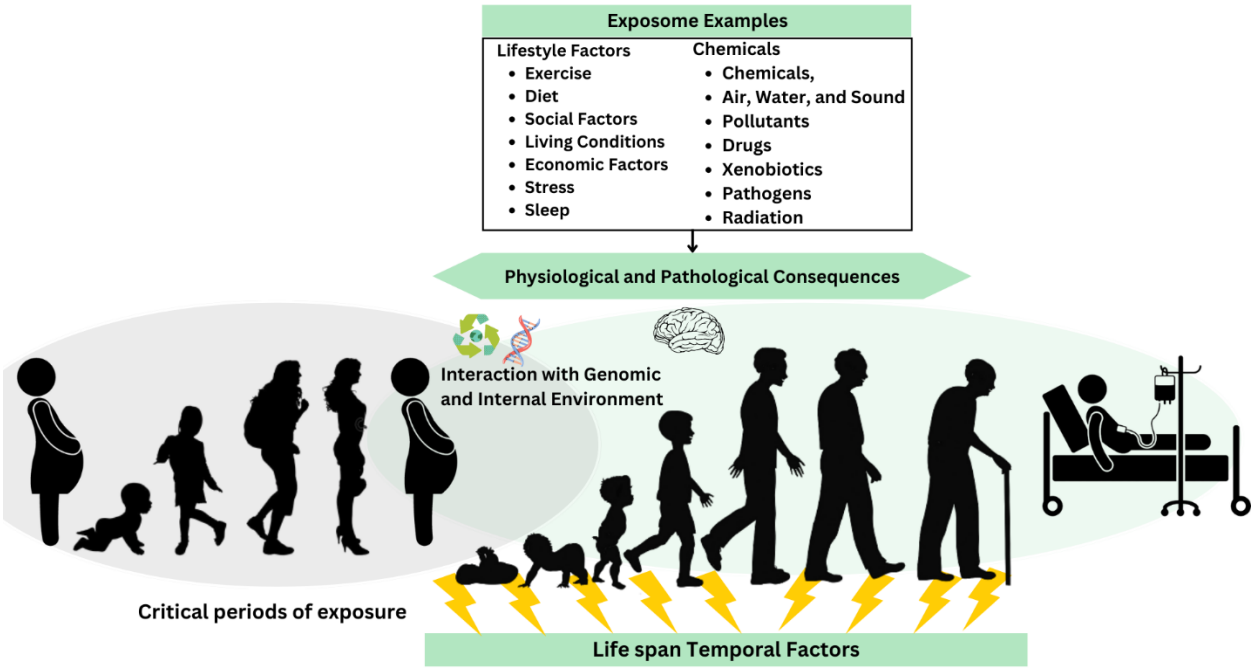


Fig 17: Exposome interact with one's unique genomic characteristics that in turn affect health and disease

Table 1: The exposome: Comprehensive list of environmental exposures potentially impacting human health

External	
- Meteorology	Climate change, temperature, humidity, wind, atmospheric pressure
- Outdoor exposures	Nitrogen dioxide (NO_2), Sulphur dioxide (SO_2), Carbon monoxide (CO), Ozone (O_3), Particulate matter (PM), radiation, Ultraviolet radiation (UV), traffic, pollen
- Built environment	Population density, building density, facilities, green space, walkability, neighborhood safety, accessibility to resources (e.g., hospitals, bus stations), **noise**
- Home environment	PM, NO_2, CO, aldehydes, metals, plasticizers, dust, pets, pests, allergen (e.g., house dust mites), mold, fungi, microbes, endotoxin
- Personal behavior	Diets, physical activity, tobacco smoke, alcohol, drugs, sleep, sex, cosmetics
- Social economic factors	Social factors, education, economy, psychological and mental stress
- Food and water contaminants	Fertilizers, metals, **pesticides**, plasticizers, flame retardants

- Medications	Medicines, surgeries
- Occupational exposures	Chemicals, dust, metals, virus, animal proteins, plants, heat/cold stress

Adapted from (180).

3 Exposome and Neuroplasticity

Brain plasticity is an adaptation to the environment with phylogenetic (eg: humans as a species) and ontogenetic (from birth to old age) evolutionary advantages. This adaptation allows an organism to change to survive in its environment by providing better tools for coping with the world. The complexity and the dynamic structural and functional changes of the nervous system linked notably to learning make the brain vulnerable to a variety of environmental insults. There is evidence that genetic and lifestyle factors can influence brain plasticity in humans and animals (181). The development of the brain is not solely determined by genetic inheritance from parents but is also influenced by a multitude of environmental, biochemical, and physical factors. These factors can include diet, stress, exposure to drugs, as well as sensory and motor experiences, which can shape the developing brain in various ways. Similarly, the adult brain is capable of plasticity and can be altered by a range of experiences including sensory and motor stimulation, task learning, prenatal experiences, exposure to neurotrophic factors, psychoactive drugs, diet, exercise, stress, and the process of ageing. Essentially, almost **every experience has the potential to induce changes in both brain structure and behavior** to some degree (182). Some factors can increase and decrease brain plasticity **(Fig 18)**. Below is a short review of each of these factors and how they influence neuroplasticity with a few examples.

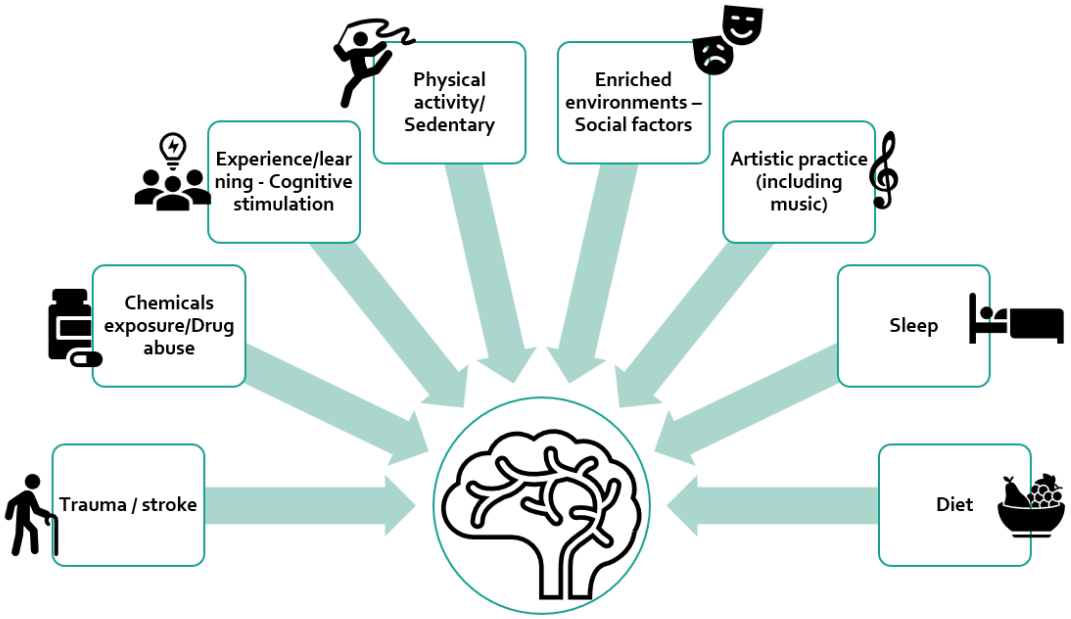


Fig 18: Factors that affect Neuroplasticity

Factors negatively impacting neuroplasticity

Environmental influences on neuroplasticity are not minor (183). For example, the synthesis of vitamin D is regulated by exposure to sunlight. The incidence of multiple sclerosis, a demyelinating disease of the spinal cord, is known to be associated with geography, with higher incidence rates observed farther from the equator. **Climate change** can also contribute to brain diseases by increasing exposure to environmental neurotoxins, infectious diseases, and diseases related to food and malnutrition (see review (184)). For instance, air pollution, particularly high levels of particulate matter, nitrogen dioxide, ozone, and carbon monoxide, has been linked to increased incidence of migraine and Parkinson's disease, with stronger effects seen on high-temperature days (185-187). Temperature changes can affect gene expression (188), neuronal structure, brain organization (189), and learning ability (190). Hyperthermia, for example, has been shown to increase the occurrence of epileptic seizures, induce neuronal injury in the amygdala and hippocampus (191), and interfere with GABA receptor signaling (192). Changing climates also favor the growth of weeds and pests, which in turn leads to more diseases in plants and a greater need for herbicides, pesticides, insecticides, and other chemicals that eventually enter the food chain and affect humans (193). Malnutrition

during early development is another example of how the environment can affect mental health by influencing neurodevelopment (194).

While **sedentary behavior** has been shown to have a negative impact, exercise has been consistently proven to have beneficial effects on neuroplasticity. Magnetic resonance imaging (MRI) images in sedentary middle-aged humans showed lower medial temporal lobe thickness (195), and in overweight or obese children lower gray matter volume in different brain regions was documented (196). **High-fat diet** results in insulin resistance, oxidative stress, neuro-inflammation, transcriptional dysregulation, impaired synaptic plasticity, loss of blood-brain barrier integrity, and reduced cerebral blood flow, overall resulting in the development of cognitive deficits, reviewed in (197). A multidisciplinary treatment program (diet restriction, cognitive behavioral therapy, and physical activity) in obese children showed an improvement in neuroplasticity (198). Several **social factors** influence neuroplasticity (see review (199)). Briefly, the review explains the factors that play a role from the perinatal period (early life stress, maternal care, or growing in nurturing environments) to the end of life. Moderate to severe **stress** affects the amygdala in ways opposite to that seen in the hippocampus and the PFC. Since this thesis dealt with early adversity through exposure to toxins and also alternative solutions to manage stress and anxiety, these two, i.e., stress and early life adversity and their effects on neuroplasticity will be briefly reviewed here.

To describe the strain on a particular structure or entity, **stress** is a broad term that is used in various fields. In life sciences, stress is defined as any perturbing life situations/events, including physiological and perceived psychological stressors that provoke adaptive bodily responses to maintain organisms' well-being or homeostasis (200). Stress is a natural response to certain situations or events that are perceived as challenging, threatening, or demanding. While stress is a regular part of daily life, excessive adverse experiences, and prolonged stress can lead to physical health problems such as cardiovascular, digestive, and metabolic diseases, as well as mental health issues such as anxiety, depression, post-traumatic stress disorder, schizophrenia, drug abuse and relapse in humans. Importantly, **stress pathways connect** the limbic network of areas, including the **hippocampus, amygdala, hypothalamus, the ANS, and the endocrine system** of the body. Stress significantly affects learning and memory in

terms of neuroplasticity, and the type, duration, and intensity of the stressor determine the effects. The best-known example of altered structural plasticity in response to stress is the atrophy of hippocampal neurons, which was first described by McEwen and colleagues (201,202).

Chronic stress can have deleterious effects on the brain, such as inducing hippocampal dendritic atrophy, exacerbating apoptosis, and suppressing neurogenesis, ultimately leading to lower hippocampal volumes, suppression of LTP, and memory impairments. In contrast, studies have shown that chronic stress can also cause hypertrophy of neurons in the amygdala, particularly the BLA (203). The emotional arousal produced by stress can enhance learning and memory via the synaptic plasticity of amygdala-dependent pathways, which may explain why traumatic events and post-traumatic stress disorder (PTSD) can lead to intense, long-term memories. The amygdala has connections with almost every cortical area, including the hippocampus (86). The HPA axis plays a crucial role in promoting resilience to stress. Stress and the release of CRH, ACTH, glucocorticoids (cortisol in humans and corticosterone in rodents), and their action on GlucR forming a closed-loop feedback system were explained before (204,205) **(Fig 19)**. Stress-induced changes resulting from GlucR activation can be long-lasting and alter future stress reactivity through epigenetic mechanisms, including loss of dendritic complexity in the hippocampus (206).

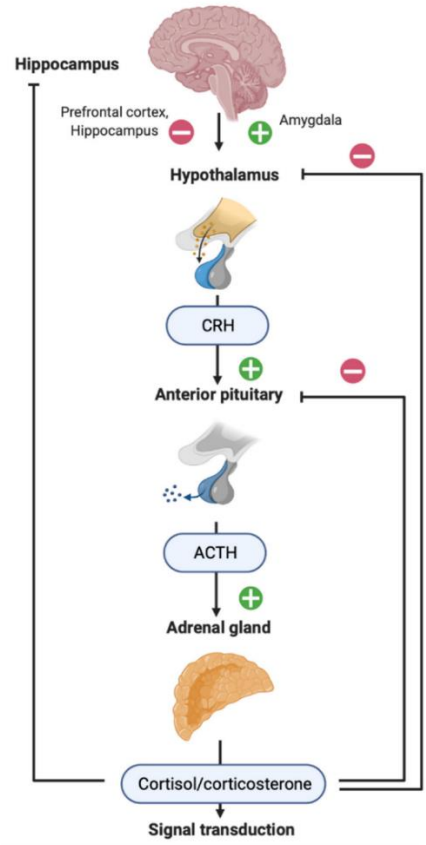


Fig 19: The hippocampus, PFC, and amygdala on the HPA axis. (207).

Studies have shown that in addition to the cortisol response, an increase in ACh release was also observed in the hippocampus after acute stress (208,209). Stress activates the septohippocampal cholinergic pathway within minutes, inducing gene expression changes and ACh-mediated neuroendocrine, emotional, and physiological responses by stimulating the HPA axis (210). ACh acts as a neuromodulator and alters the state of neurons in response to changing environmental stimuli, similar to glucocorticoids during stressful events (see review (211)). In the ventral tegmental area (VTA), activation of α4/α6β4-containing nAChRs modulates dopaminergic transmission, whereas α7 nAChRs modulate glutamate release and α4β2*nAChRs regulate the release of GABA (212) **(Fig 20)**. The mesolimbic dopaminergic pathway involves extra-hypothalamic structures such as the PFC, hippocampus, amygdala, NAc, and VTA, which modulate the stress-HPA axis and are innervated by basal forebrain cholinergic neurons (213). Addictive drugs such as nicotine and ethanol act via the central cholinergic system and the dopaminergic reward system of the brain. Nicotine can bind to the nAChRs, which

are expressed on neurons within the mesolimbic dopaminergic pathway, and also mediates the rewarding and reinforcing properties of ethanol. Ethanol does not directly modulate nAChRs but instead increases the release of ACh, which in turn modulates other neurotransmitters (214).

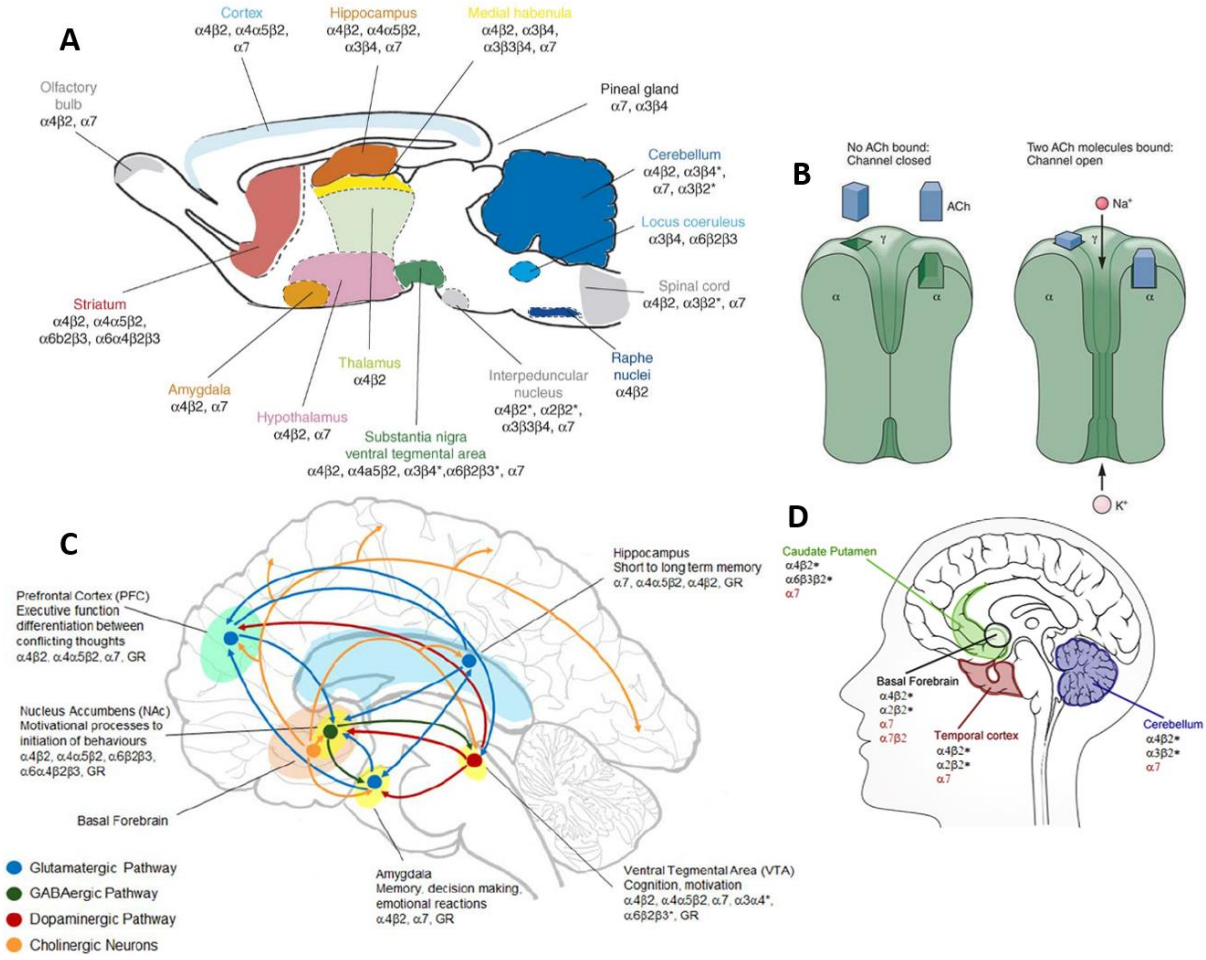


Fig 20: A, C: The expression and co-expression of nAChR subunits across different brain regions in rodents and humans. B: Model of the nicotinic acetylcholine-gated ion channel (3), D: nAChRs are also observed in the cerebellum and striatum (213,215).

The interaction of glucocorticoid with ACh in the brain is reviewed in (216). Optimal levels of ACh mediate sustained attention and facilitate learning and memory. Excess ACh increases the symptoms of anxiety, depression, and reactivity to stress. This was recently studied and reviewed in (217,218) **(Fig 21)**.

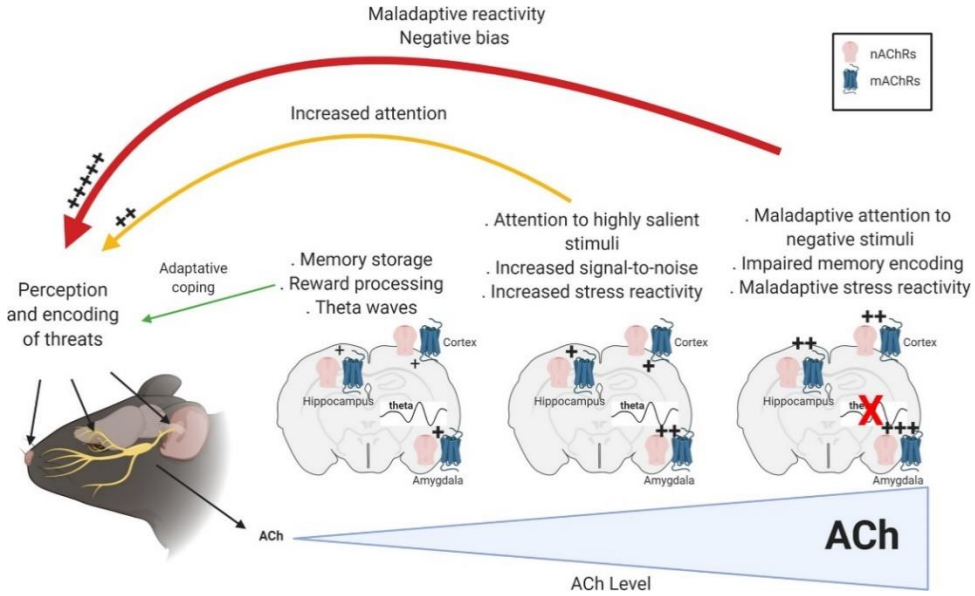


Fig 21: In passive coping, cholinergic afferents release little ACh. When there is moderate or brief exposure to stress, ACh and other neurotransmitters are transiently released, leading to increased activity of GABAergic interneurons and other neurons. This coordinated increase in activity enhances signal-to-noise in the network and promotes coping behaviors such as increased attention. However, if ACh release is prolonged or dysregulated, it can disrupt the balance between excitatory and inhibitory networks, causing asynchrony and maladaptive behavioral responses (217).

Early life adversity is another significant factor that increases the risk of developing mental illnesses like depression and PTSD and can also predict a poor prognosis. In rodents and higher primates, early adverse experiences such as prenatal maternal stress, maternal separation, variable foraging demand, or low maternal care can result in structural and functional changes in a network of brain regions that play a role in neuroendocrine control, autonomic regulation, and vigilance (219,220). Early life adversity thus causes lasting structural and regulatory adaptations in the neuroendocrine system predisposing to or sometimes protecting from stress-related diseases later in life (221–223). While studies have concentrated on psychological stress as a factor, **early exposure to neurotoxins (exposure to drugs/chemicals/drug abuse)** contributes equally to developmental neurological disorders and can have *a* devastating lifelong effect on the architecture of the brain. In a review published in 2006 (224), it was observed

that more than 200 chemicals were then known to be neurotoxic to adult human beings and there were many thousands of pesticides, solvents, and other industrial chemicals in widespread use that had never been tested for neurodevelopmental toxicity. Chemical exposure early in life is harmful due to the vulnerability of the fetus at this critical stage. The placenta fails to block the passage of all environmental toxicants, blood–brain barrier provides only partial protection, and postnatally, neurotoxins may be transferred through breastmilk (225,226). Neurotoxicants may lead to acute or chronic changes. Lead poisoning causes psychosis, myelin loss, and axonal degeneration. Long-term exposure to neurotoxicants often initiates neurodegenerative diseases, Alzheimer's (AD), and Parkinson's disease (PD). Exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes behavioral phenotypes similar to PD. Although genetic causes predominate among the etiological factors for AD, high aluminum and zinc exposure/consumption is proposed as a causative factor for AD. Other than MPTP, compounds including industrial gas, organophosphate insecticides, and certain pharmaceuticals, solvents such as trichloroethylene, methanol, ethanol, and industrial and home cleaners exposure to metals, and pesticides (paraquat and rotenone), metals such as lead, iron or manganese, have all been repeatedly implicated as risk factors for the development of neurological diseases are extensively presented and discussed in a large number of reviews (see for example (183,227)) **(Fig 22)**.

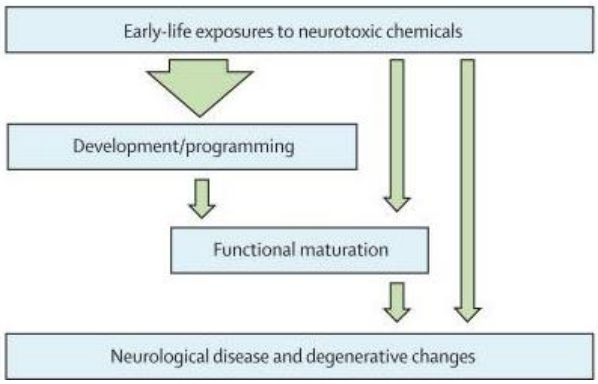


Fig 22: Early exposure to neurotoxins and their consequences (reviewed in (224)).

Factors positively impacting neuroplasticity

Exercise, diet, and sleep are three behaviors that represent essential pillars of mental health because of their impact on the structure and function of the brain. It is well

understood that good nutrition, regular exercise, and sufficient sleep are fundamental to maintaining a healthy lifestyle (228). A cross-sectional study revealed that sleep quality, adequate fruit and vegetable intake, and regular physical activity improve depressive symptoms and well-being in young adults (229). **Human studies** have shown that **exercise** can increase the levels of BDNF and other growth factors, stimulate neurogenesis, and improve mental performance while increasing resistance to brain insult (230). Epidemiological studies show that regular exercise reduces the risk of cognitive decline in ageing adults. Introducing exercise during mid-life has also been shown to reduce the risk of cognitive decline in late-life and dementia occurrence (231). Exercise attenuates neurodegeneration, including AD (232,233), improves mood in depressed individuals (234), and improves cognition and sensory-motor attention in children with attention deficit hyperactive disorders (235). **Animal studies** have also demonstrated the beneficial neuroplastic effects of exercise. Recently, enhancement of structural synaptic plasticity of the hippocampus and PFC was observed in AD models of mice after treadmill exercise for 12 weeks (236). After acrobatic exercise and acrobatic exercise along with a retention period of 8 weeks young male Wistar rats performed better on motor performance tests along with higher synaptic protein expressions in different limbic, striatal, and motor brain areas, compared to sedentary rats (237). The introduction of exercise intervention even after a brain insult often has been shown to improve the condition of the patient or animal models of disease. Intensive daily treadmill exercise in a mouse model of Parkinson's disease led to improved motor function and increased dopamine release and reduced clearance (238). Physical exercise as a therapeutic intervention in rehabilitation programs for stroke patients is routinely recommended, for its multiple benefits, including improvement in aphasia, balance, and cognition (239,240).

Many stimuli the human is exposed to are beyond the control of the individual, but nutrition is not. For positive effects of **diet** on neuroplasticity see reviews (241–243). Randomized clinical trials support the role of vegetarian or plant-based foods (citrus fruits, grapes, berries, cocoa, nuts, green tea, and coffee) in improving specific domains of cognition, in particular, the frontal executive function (244). Intermittent fasting, exercise, and followed recovery result in repeated metabolic switches and are shown to promote neuroplasticity (245). A recent meta-analysis revealed the overall positive effects of

whole-food diets on pain, with emphasis on more research required in this direction (246). **Sleep** rejuvenates the brain daily. Sleep aids in consolidating memory, the process of learning, and, mechanisms of neural plasticity, with a role in functional recovery from clinical conditions such as stroke, AD, and depression (247–249). In rat models, the sleep-wake cycle is shown to control the gene transcription in the cortex and hippocampus, promoting synaptogenesis (250). Sleep thus allows for homeostatic mechanisms to optimize the functioning of neural networks, which are important for memory, cognition, behavior, and information processing (251).

Cognitive therapy, and experience-dependent influences, such as language acquisition, mindfulness meditation, and learning music (discussed later), have shown significant positive effects on brain development and neuroplasticity in human and animal studies. One of the chief principles for neuroplasticity includes the "*Use it or lose it*" phenomenon, which explains how learning, repetition, time spent in a **cognitively stimulating** activity, and the intensity of engagement in such an activity can stimulate the process of neurogenesis, synaptic plasticity, dendritic growth and thus neuroplasticity (252). **Sensory experiences** (passive stimulation from the external environment) during critical periods rapidly organize our sensory-motor cortex. Plasticity during adulthood is tightly regulated by a variety of cellular and molecular processes, and is susceptible to brain insults, resulting in neurological disorders (dementia, depression, addiction) (253). As discussed before, the neural plasticity described in musicians versus non-musicians and the increase in the cortical representation of fingers among violinists is a classic example of learning-dependent neuroplasticity (254).

Environmental enrichment (EE) is defined as a combination of complex inanimate and social stimulation (255). For EE, lab animals are raised in spacious environments that are rich in stimuli, featuring a diverse array of frequently rotated objects with varying shapes. The objective is to enhance the well-being of animals by offering them various forms of sensory and cognitive stimulation, higher levels of physical activity, more opportunities for social interaction, encouraging exploratory behaviors. EE is found to have a significant impact on the CNS in terms of functionality, structure, and genetics, both during the critical period and in adulthood. Rats living in EE conditions exhibit higher levels of hippocampal LTP, which is related to synaptic plasticity and memory retention

(256). Accompanying the functional improvement observed, a significant increase in cortical thickness, enhanced dendritic arborization, a greater number of dendritic spines, higher synaptic density, and post-synaptic thickening was observed in several brain regions, including the hippocampus (257). Furthermore, exposure to EE results in increased hippocampal neurogenesis and integration of newly born cells into functional circuits (256). At the molecular level, EE leads to significant changes in the expression of numerous genes involved in neuronal structure, excitability, synaptic transmission, and plasticity (258). EE also modulates the synthesis and secretion of neurotrophic factors throughout the brain and affects the cholinergic, serotoninergic, and noradrenergic systems (259,260). Therefore, EE is currently being implemented as a rehabilitation treatment for stroke patients, and other clinical applications have been proposed (see reviews (261–263).

It is worth mentioning that the impact of anthropogenic parameters on brain neuroplasticity is mostly studied independently and very little information is available on the impact of the combination of multiple factors, i.e. exposome. This was further reinforced in a recent review (264), particularly when it involved toxicology. As introduced above, we find exposome acts as the common factor influencing neuroplasticity. It is therefore important to understand the link between exposure of human beings or animals to anthropogenic stimuli (positive or negative) and their effects on physiological parameters and neuroplasticity. In this thesis, the effect of **two anthropogenic stimuli: acoustic stimulus, and chemical exposure** among humans and animals was investigated. These two anthropogenic stimuli, with specific emphasis on their known neuroplastic effects will now be discussed.

4 Impact of acoustic stimuli on neuroplasticity in humans: Specific effects of Music

"Natural" refers to something existing or occurring in nature, without human intervention or manipulation. Natural experiences shape an individual mentally and physically, and there is evidence related to their beneficial effects. Natural sounds are considered the most complex sound types that provide a wide array of information such

as the species, season, and also temporal basis of the same (265). Forests, plain grasslands, and wetlands are composed of a diverse array of sounds produced by mammals, birds, amphibians, and insects (266). Added to this are the sounds of wind, rustling grass, rain, tree leaves, rivers, streams, or beach sounds. A detailed review of the beneficial effects of natural sounds can be found in (267). About 100 years ago, the German physician and Nobel Prize winner Robert Koch predicted that “one-day mankind will have to fight the burden of noise as fiercely as plague and cholera.” Among the auditory anthropogenic stimuli that humans and animals are exposed to, noise is deleterious to day-to-day functioning, delays maturation, and has side effects on the reproductive system, brain, and behavior (268–273). As the focus of the current thesis was on the positive impact of anthropogenic auditory input on humans, we chose music as the acoustic stimulus.

Music is a man-made entity. It is thus not wrong to call it anthropogenic. Music has a significant positive impact on humans and the ecosystem. Thus, music forms a meaningful intervention to be explored more in detail, from a neuroscientific perspective. In the current thesis, we focus on the effect of music on general anxiety, stress, ANS, and CNS. Music is an aesthetic stimulus that evokes a subjective experience in every individual involved with it, be it in the production of new music or a simple exercise such as listening to music. Music has been shown to reduce peri-operative and operative anxiety along with a reduction in BP, HR, and the respiratory rate in patients undergoing surgical procedures such as gastrointestinal endoscopy (274), colonoscopy (275), and cardiac patients (276). Music therapy is commonly used in health-related areas such as pain clinics, intensive care units, peri-operative set-ups, scan waiting rooms, and pediatric units (277). Newer studies emphasize the effect of different types of music in promoting relaxation and reducing anxiety and stress levels (278–280). Meditative classical music lowers the neuroendocrine markers of stress (281,282). A recent systematic review of eleven randomized trials consistently showed that music therapy (ranging from 15–60 minutes) reduced the anxiety and stress of critically ill patients (283).

Music may be a way to help young people reduce negative emotions (284). Young people report that they often have a collection of favourite ‘tunes’ that they listen to when they are feeling ‘stressed out’ (285). Many correlational research studies have been

conducted to determine the relationship between different genres of music and stress (286). Participants in bad moods often choose highly energetic, joyful music for longer periods and were more decisive in exercising their musical preferences. More than half of the time that was stipulated for the participants to listen, participants chose to listen to enjoyable music (285). In one study participants indicated that the melody was the most effective and instrumentation was the least effective among musical components in reducing State-Trait Anxiety Inventory (STAI) scores (287). A broad body of literature exists on the potential health benefits of Mozart's music and certain genres of music (288). Exploration of the effect of Indian music, melodic scales, scientifically on anxiety or stress is currently limited (289–292). Listening to classical Indian instrumental music reduced psychological distress (measured using questionnaires or galvanic skin response) in lab and clinical settings (289,293–295). Settling on the conclusions drawn from the outputs of available Indian scientific literature is difficult due to small sample sizes or inherent study design deficiencies (296,297). Thus, to address these lacunae, in this thesis, the physiological effects of different Indian melodic scales were tested on young healthy individuals.

Music and brain plasticity were further reviewed in (298) **(Fig 23)**. Multiple sensory-motor and cognitive capabilities of the brain are engaged when one is trained to play a musical instrument. **Playing music** induces feed-forward and feedback interactions between the multiple sensory inputs, motor output, as well as engages higher-order cognitive functions such as memory, attention, emotion, combining skills in auditory perception, kinaesthetic control, visual perception, and pattern recognition, and processing of the musical syntax (299–302). Learning highly rewarding or pleasurable musical stimuli drives neuroplasticity (303,304). It was shown that musicians had increased cortical excitability in the motor cortex and had larger and more stable auditory-evoked magnetic fields, compared to non-musicians (305–307). Significant neuroplastic changes occur with **musical training** as evident in neuroimaging studies (see reviews (298,308)).

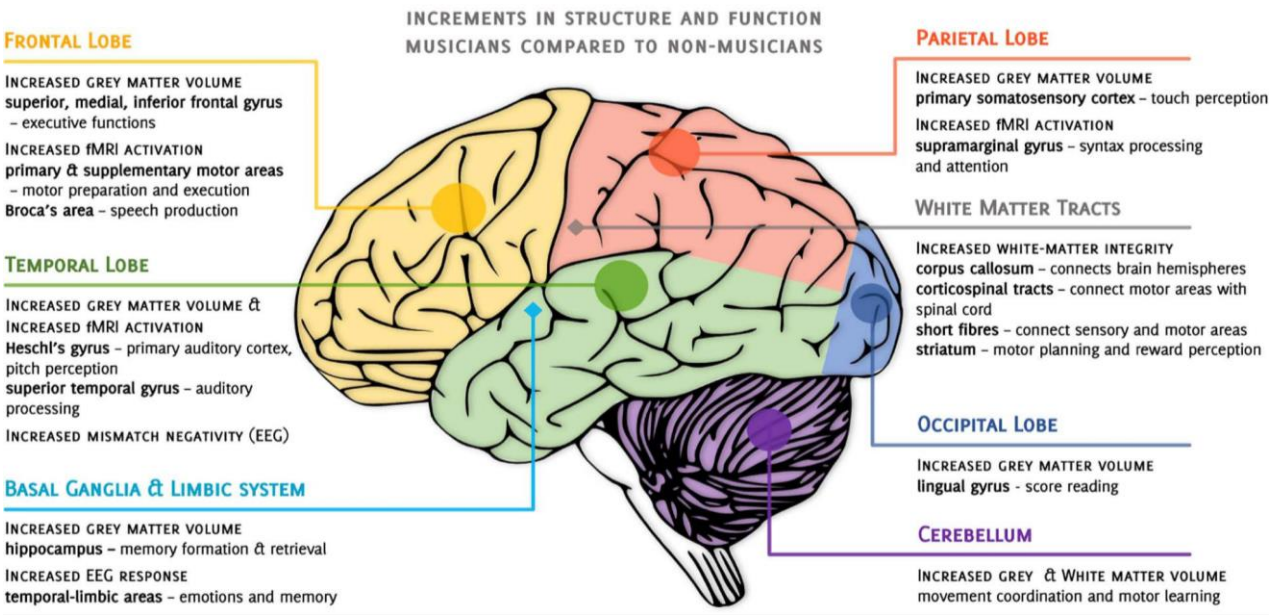


Fig 23: Musical training-induced neuroplastic changes (298).

Neural substrates that participate in music-evoked emotions are several of the limbic system structures. Previous works have shown that there is considerable overlap between the regions that give rise to everyday emotions and those that are responsible for music-induced emotions. The results of the meta-analysis of several studies showed involvement of the amygdala, the hippocampi, the ventral striatum (including the NAc) extending into the ventral pallidum, the head of the left caudate nucleus, the auditory cortex, the pre-supplementary motor area (SMA), the cingulate cortex and the orbitofrontal cortex (OFC) (309) **(Fig 24)**. Other than the emotional responses, music induces neuroendocrine plasticity, since many of the above-mentioned regions connect with the hypothalamus.

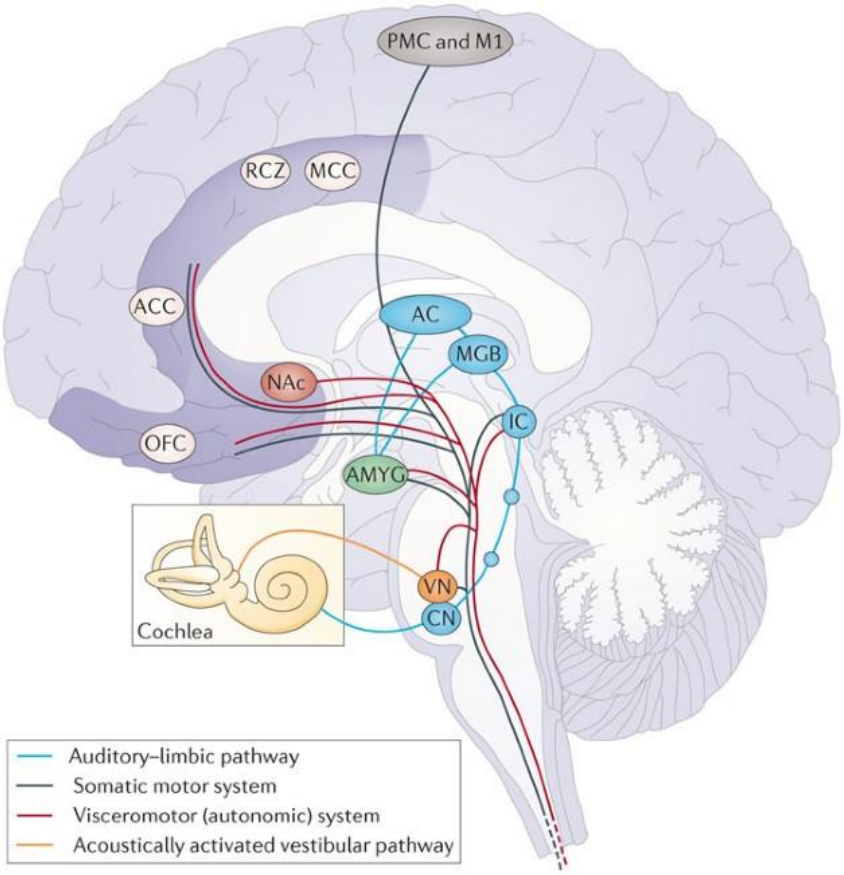


Fig 24: Brain correlates of music-evoked emotions (309). Note that the auditory cortex (AC) also projects to the orbitofrontal cortex (OFC) and the cingulate cortex. ACC, anterior cingulate cortex; CN, cochlear nuclei; IC, inferior colliculus; M1, primary motor cortex; MCC, middle cingulate cortex; MGB, medial geniculate body; NAc, nucleus accumbens; PMC, premotor cortex; RCZ, rostral cingulate zone; VN, vestibular nuclei.

Interestingly, not just playing music and active music training, **passive listening to music** (also called receptive music) can also induce neuroplastic changes. Fourteen participants with early-stage cognitive decline who underwent three weeks of daily music listening for an hour duration had significant cognitive improvement along with functional and structural brain changes. However, the change was more evident in musicians compared to non-musicians (310). A detailed review of the benefits of music listening may be seen in (311) and the positive effects of listening to music on fetal cognitive, social, and emotional development (312). Recent studies have provided evidence of how music-induced plasticity can be used to prevent and treat neurological impairments, including

psychiatric, neurodevelopmental disorders, and acquired brain injuries (stroke) (313,314) as well as in neurorehabilitation (315–317). For example, in a recent study, listening to vocal music in stroke patients, for 3 months, enhanced verbal memory, and selectively increased gray matter volume in left temporal areas and functional connectivity in the default mode network than listening to instrumental music or audiobooks (314). Listening to music is associated with neurochemical changes that are associated with reducing anxiety and stress. These neurochemicals include the increased release of dopamine, oxytocin, endogenous opioids, reduced cortisol, and beta-endorphin (318). Neural connectivity and functional neural plasticity also improve after listening to music as evidenced by Electroencephalogram (EEG) and fMRI studies. Evidence suggests preterm infants who listened to music from 33rd GW until term showed increased functional connectivity between the primary auditory cortex and the thalamus and the middle cingulate cortex and the striatum when listening again to the known music (319). A recent study observed that music not only induces relaxation and alertness but also improves functional connectivity across different brain regions (320,321). EEG power of most of the brain waves increased, indicating a 'mind-wandering effect', during passive listening to music (322). The power in the alpha-band in the parietal and occipital areas of both hemispheres was shown to rise during listening to music (popular classical symphonic pieces), with a decrease in the peak frequency of the alpha-band. However, on repeated listening, these changes were attenuated (323,324). Music is a complex combination of various features that include, pitch, tempo, dynamic contrasts, melodic scales, and so on, it is important to understand the effect of systematically combined musical features, to create the music that best suits one's needs, for therapeutic purposes. Importantly, how the brain responds to passive listening to different melodic scales, irrespective of training, remains to be elucidated in detail.

Other than the CNS, music affects the **ANS**. Since ANS is affected by several systemic chronic disorders (325) finding efficient strategies for the prevention of these disorders is important. The strategies for restoring the autonomic balance include behavioral interventions (e.g., meditation, yoga, physical activity, lifestyle modifications, smoking cessation, etc). Studies have demonstrated short-term improvement in autonomic tone during or after a single session of listening to music. Research suggests

the use of music for better long-term regulation of the autonomic tone (326,327). In our previous study on hypertensives, we showed that long-term passive listening to music can help in better regulation of BP and the ANS (328,329). Listening to music between the 32nd and 38th GW was shown to modulate the ANS function in the fetus in a positive direction (330).

Currently, the application of music in **experimental animals** is lagging. It is observed that different animals, including piglets, chimpanzees, and adult rodents have better cortical plasticity when grown in an enriched environment, including auditory enrichment (331–333). Perinatal exposure of mice to music was shown to have a positive influence on BDNF/TrkB signaling pathway targets, including PDK1, that improved learning and memory functions (maze learning task) (334). A recent review observed that music interventions among rodents led to positive structural and chemical neuroplastic changes along with improvement in animal behavior, and immune functions (335,336). There exists a hypothesis that music induces neurogenesis and is thus a promising therapy for patients with neurodegenerative diseases (337,338). After middle cerebral artery occlusion in rat models, the motor functions improved, the BDNF protein level of the ipsilateral hemisphere motor cortex was higher, and BDNF and Glial fibrillary acid protein (GFAP) accumulated at the damage boundary in a group that had 12 hours compared to 1-hour music sessions. Longer duration of therapy sessions was associated with longer cell synapses and better cell-to-cell connections, with mature activated astrocytes, indicating that music therapy was beneficial to improve poststroke motor function and promote neuronal repair in the long term (339). Previously, studies have shown that music exposure was associated with improved spatial memory performances and increased hippocampal and DG BDNF levels in adult rats (340,341). A recent study tested this hypothesis on ageing rats. They showed that after 4 months of music exposure spatial reference memory was not impaired in cognitively impaired middle-aged (14-month) rats and 8 months of music exposure improved both working memory and spatial reversal learning capacity. However, these changes were not associated with cell proliferation changes or levels of BDNF expression in the hippocampus and frontal cortex (342). Thus more studies are needed to confirm the positive neuroplastic effects of music, in animals.

Brain plasticity can be measured by neuroimaging and neurophysiological techniques. In this thesis, EEG was used to understand acute changes in the brain after the acoustic stimulus. EEG is a record of a brain's electric potential oscillation from 20 to 256 electrodes applied on the human scalp. The most commonly studied waveforms include alpha (8 to 12Hz), beta (13 to 30Hz), delta (0.5 to 4Hz), and theta (4 to 7Hz) (343) **(Fig 25)**. EEG is commonly used for diagnostic and prognostic purposes, in conjunction with other methods, and reflects cortical reorganization. EEG measurements indicate changes in power bands on exposure to environmental stimuli or a thought process that occurs within. EEG changes can also be used to investigate neural plasticity in neurological disorders, with a focus on cerebrovascular and neurodegenerative diseases (344).

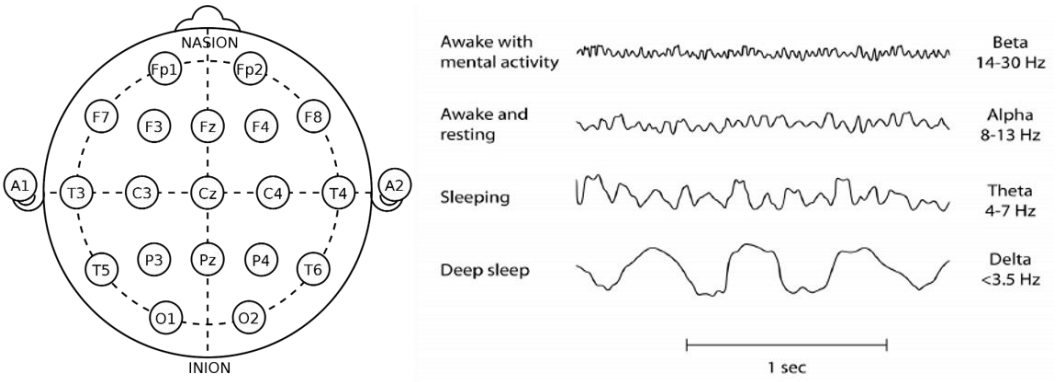


Fig 25: Electroencephalogram lead placement as per the international 10-20 system of electrode placement and the different waveforms (alpha, beta, theta, and delta) and their frequencies (343,345).

Music is a time-based stimulus and EEG is well suited to investigate the time-locked brain responses as the temporal resolution is sufficiently high. When a set of individuals are exposed to the same sensory stimulus, few individuals will have typical experiences and they are said to be engaged with the stimulus. This can be measured by **inter-subject correlation (ISC)**, which evaluates the similarity of an individual's brain over some time with that of another individual or a group, in a given region of the brain. ISC is often used with fMRI or EEG data from individuals visualizing a moving stimulus such as a movie clipping or listening to a speech. Essentially observation of synchronized responses, if any, in a group of participants listening to music indicates a socio-behavioral response to musical stimuli, having implications in the management of mental health

conditions. In the present thesis other than power spectral changes and correlated component analysis of EEG, the concept of ISC and engagement with Indian music modes was explored.

5 Impact of chemical stimuli on neuroplasticity in animals: Neonicotinoid Thiacloprid

Human-caused environmental changes are many and are driving global biodiversity loss. However, the effects of these anthropogenic changes on species composition (genetic alterations), physiology, and ecosystem functioning are poorly understood (346). **Pesticides** are individual substances or substance mixtures designed for preventing, destroying, or mitigating groups of harmful organisms (pests), including unwanted plants or animals. The term pesticides include insecticides, herbicides, rodenticides, fungicides, disinfectants, attractants, plant defoliants, swimming pool treatments, Plant Protection Products (PPPs), and plant growth regulators. Pesticides are used primarily in the agricultural sector to protect against crop deterioration and are essential for food production (347). It has been estimated that only about 0.1% of the pesticides reach the target organisms and the remaining bulk contaminates the surrounding components of the water, air, and soil ecosystem environment (348). The period of these anthropogenic stimuli ranges over millennia and not merely the last two centuries of industrialization (349). It is therefore important to study the influence of chemicals **persisting in the environment** despite a few being banned. **Neonicotinoids** are derivative insecticides of synthetic nicotinoids and are commonly used in agriculture, aquaculture (fish farming), pet treatment, and in urban pest control. Neonicotinoids are structurally related to nicotine and target nAChRs (350) **(Fig 26)**. These pesticides showed a strong affinity for insect receptors while exhibiting a very low affinity to vertebrate subunits (see (351,352)). Initial results revealed that neonicotinoids were less toxic to the handlers and non-target organisms in comparison to other insecticides such as organophosphate and carbamate (352).

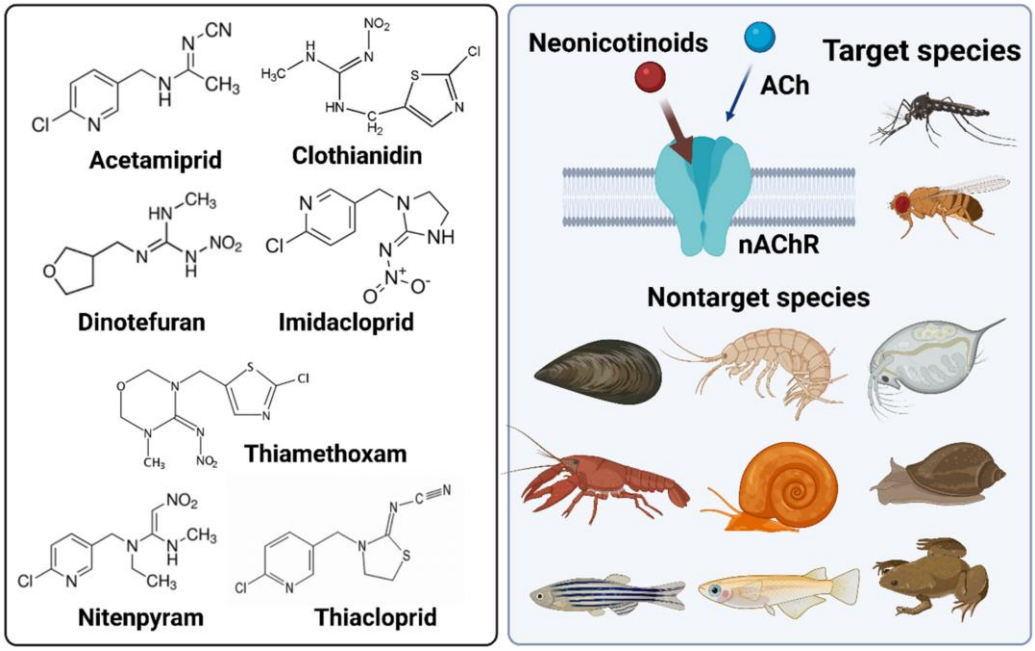


Fig 26: List of Neonicotinoids that mimic nicotine in activating nAChRs in target and non-target species (353)**.**

Pesticides in the soil are degraded either by physical, chemical, physical-chemical, or microbial degradation (354). They easily adhere to the surface of plants, animals, and human beings, and thus do not degrade as expected. Microbial degradation decides the fate of neonicotinoid insecticides in the soil (355,356). Further, studies suggested that several neonicotinoids, including imidacloprid, acetamiprid, and thiacloprid can readily cross the intestinal barrier (357,358) and the blood-brain barrier (358–362), and these pesticides and their metabolites were found in human biological samples, confirming human exposure (363–369). The European Union (EU) restricted the use of the neonicotinoids Clothianidin, Imidacloprid, and Thiamethoxam in plant protection and seed treatment products, due to their deleterious effects on pollinators, honeybees, and other non-target insects. Later **in 2020**, due to its impact on pollinating invertebrates and its endocrine disruptor effects in vertebrates, **thiacloprid was banned** (370). However, thiacloprid is still being used in other continents, and the intensive use of neonicotinoids and the persistence of the molecule in the plant and the environment contribute to the increased exposure of non-target invertebrates (honeybees and other pollinating insects) and vertebrates (371,372). Due to systemic distribution throughout the plant (350,372), the molecule is found in fruits and vegetables from around the world (373–378). This

chronic exposure to neonicotinoids and their potential bioavailability in the mammalian organism raises questions and concerns about potential adverse health effects in humans (379).

Among the different neonicotinoids, this thesis explored the **effect of thiacloprid** as most of the previous studies have concentrated on the impact of imidacloprid, acetamiprid, or clothianidin on the brain and the endocrine system, far less is known about the potential long-term effect of early exposure to thiacloprid (380), and the proportion of species affected, even at lower concentrations is much higher for thiacloprid than other neonicotinoids (381). Though several studies have looked into the deleterious effects of neonicotinoids on neuroplasticity (382), very little is known about the potential neuroplastic effects seen after **early exposure to thiacloprid, during the perinatal critical period of embryonic development**.

6 Need of this thesis

Due to the ease of study and predominant usage of vision for our experiences, visual stimuli, and their effects have been the focus of research. However, human beings being multisensory, the beneficial effects of other non-visual sensory experiences also become equally important. Based on a literature search, **acoustic stimulation** is one of the second most well-studied sensory stimuli. Only a complete understanding of our acoustic environment along with other sensory inputs will help us in deciding the enriching sensory inputs we wish to retain in comparison to the various negatively impacting anthropogenic stimuli that we may decide and take actions to reduce. It would be interesting to research more of such anthropogenic stimuli that are propounded to have a positive impact, to study potential physiological effects and their neuroplastic effects. **Music** is a promising intervention that is ubiquitous across human cultures. Music listening involves the sensory processing of acoustic stimuli (peripheral nervous system) followed by cognitive and emotional processing in a neural network (central system) producing pleasurable physical and emotional experiences. While there is a growing body of research on the effects of music on health, there is still much that is not fully understood. Furthermore, music is made up of many features. These include tempo, rhythm, lyrical component, percussion, timbre, harmony, tones, and modes (a combination of notes or tones called *ragas* in Indian music). It is not yet clear which

specific musical elements or characteristics are most beneficial for various health outcomes, and whether different types of music have different effects. Additionally, the mechanisms underlying the effects of music on the brain and body are not yet fully understood, although there is evidence that music can affect neurotransmitter systems, such as the dopaminergic and opioid systems. There is also ongoing research exploring the potential role of individual differences in musical preferences, as well as the influence of cultural and social factors on the effects of music on health. In this thesis, in chapters 2 and 3 (clinical studies), we chose to explore the short-term effect of listening to different modes on stress, anxiety, autonomic and central neuroplasticity.

Among the different anthropogenic stimulants that have a significant impact every time human or animal is exposed to them, are **chemicals**. Humans consume chemicals as drugs, for the treatment of any ailments, and this is usually of known concentration and composition for a given duration. Then there is 'addiction' in the form of drug abuse, where humans know the composition, and this usually lasts for a longer duration, with well-known side effects, and is very often treatable. Other than these, humans and animals are often exposed to chemicals such as **pesticides**, which are consumed unknowingly through water or food. These pesticides can affect the organ systems of humans and animals, depending on their composition, often having a synergistic effect with a combination of chemicals, and this consumption happens over a long time, with slow accumulation in the body. If we can identify the relevant exposures, we can potentially intervene to reduce the burden of disease occurrence. Future studies may combine both, i.e., one anthropogenic stimulus with proven positive impact and one with negative, to find if, the negative impact can be reversed in any potential way. We investigated the impact of chemical exposure on the CNS using animals. Most of the previous studies have been on imidacloprid, and not many have looked into the effect of **thiacloprid**, which is highly toxic as well. Some research has shown that thiacloprid can have developmental neurotoxic effects in animals. However, more research is needed to understand the precise mechanisms of thiacloprid's neurotoxicity and how exposure levels might impact human health, particularly in vulnerable populations such as developing fetuses and infants. Additionally, the long-term effects of thiacloprid exposure on neurological function and behavior have not been fully studied. In the current thesis,

the effects of perinatal chemical exposure to the neonicotinoid thiacloprid at different doses on neuroplasticity markers were investigated in zebrafish and mice using the quantitative Polymerase chain reaction (qPCR) approach, in chapter 4 (preclinical studies).

Given the 3Rs (i.e. reduction, refinement, and replacement) alternatives agenda, additional experimental model systems/technologies need to be further developed and incorporated into exposome studies. The final aim of the EU Directive 2010/63/EU was and is to replace all animal research with non-animal methods of research, such as organoids or computer simulations (383). There are several alternative models to animals for neurotoxin research, including a) **Cell cultures:** Cultured cells, such as neuronal cell lines, using mouse and rat primary neuronal cells (384,385) and neural precursor cells derived from human induced pluripotent stem cells (386). These include the two-dimensional (2D) functional, manipulatable networks that can incorporate many neuronal subtypes and other cell types (e.g., glia). However, cell lines and cultures may not fully represent the physiological characteristics of primary cells in the brain. They do not replicate the complex interactions and organization of cells in a living organism, limiting the ability to observe complex biological responses to neurotoxicants. Cell lines are often limited to a specific cell type, making it difficult to study interactions between different types of cells in the brain. Finally, cell lines are difficult to maintain in culture, and they can change over time, leading to inconsistencies in results between experiments. Overall, while cell lines offer a useful tool for neurotoxicity research, their limitations highlight the need for complementary approaches, such as organoids, to better recapitulate the complexity of the brain in vitro; b) **Organoids:** Organoids are structures that resemble mini-organs. The potential for the use of organoids to research human physiology and pathology range from human stem cell-derived organoids to study liver, gut, infectious diseases, and autoimmune diseases, to neurological diseases (387,388). These are three-dimensional (3D) networks that can be formed using scaffolds and microfluidic devices to include the features of 3D regionalization, while some organoids are scaffold-free and free-floating. Four-dimensional (4D) ‘assembloids’ expand 3D models by integrating vasculature, or combining other body regions, such as the gut to mimic the brain/gut axis (389). **Organoids** have emerged as a valuable tool in neurotoxin research

due to their ability to replicate the complexity of the brain. Advantages of using organoids include the ability to study human-specific effects, reproduce genetic variability, and investigate the impact of environmental factors such as toxins. One can test and screen for toxicity more cost-effectively and ethically than animal studies. Additionally, organoids can be genetically modified and studied at various stages of development, allowing researchers to identify key developmental windows during which exposure to toxins may be particularly harmful. Overall, the use of organoids in neurotoxin research holds great promise. Some limitations must be considered. First, organoids are not a perfect replica of the human brain, and there may be differences in the organization of brain regions. They are often too small to study complex brain functions and neural circuits and they lack the complexity of a whole organism. Finally, organoids require a complex and expensive experimental setup, including specialized equipment and personnel with advanced training in stem cell culture (390); c) **Computational models:** These simulate the effects of neurotoxins on the brain, allowing researchers to predict the potential outcomes of exposure. However, there are several limitations to using this; d) **Human studies:** Human studies can provide direct evidence of the effects of neurotoxins on the brain, but they are limited by ethical considerations and the difficulty of controlling exposure to toxins in a real-world setting.

While *in vitro* toxicity studies are cheap, quick, and easy, they poorly correlate with *in vivo* mechanisms and therefore the observations have limited translational value (see (391)). *In vitro* data are generally not decisive by themselves but, can enhance animal data but can be difficult to extrapolate to intact organisms because of out of context from their microenvironment and thus exhibition of different non-reliable responses (392). The long-term effects of developmental exposure to any chemical in a laboratory setting are difficult using in vitro models. This also means there is a lack of understanding of the effect on the structure of the brain, the normal physiology, including the impact of other organ systems such as the integration of hormones and microbiomes. Further, the detection of neurotoxicity is different from other toxicities because of the complexity of the brain (393). An *in vivo* model is useful in that way, as the early developmental exposure to a chemical will impact the physiological development of the brain, with the whole organism being intact, until the day of analysis. Zebrafish are promising candidates for

intermediate models. Mice are also another good model for developmental neurotoxicology research.

Zebrafish are often used for developmental neurotoxicity studies because they share many similarities with humans in terms of the development and organization of their nervous system (394). Zebrafish possess a high degree of genetic, morphological, and physiological homology with humans (395,396). Zebrafish are significantly more complex than cultured cells and other model systems, such as Drosophila melanogaster. Additionally, zebrafish embryos are transparent, which allows researchers to visualize and track the development of the nervous system in real-time. Zebrafish are also relatively easy to maintain and breed, and they have a short generation time. This allows for rapid screening of large numbers of compounds, by simple soaking of embryos (397) making zebrafish ideal for high-throughput screening (398). Another advantage of using zebrafish is that brain development occurs within 3 days post-fertilization (for more on zebrafish for studying neurotoxicity see (399,400)). According to the EU Directive, the 96 to 120 hpf zebrafish embryos and larvae, serve as an alternative to animal experimentation and thus do not under the regulatory frameworks dealing with animal experimentation. Mice are good models for several reasons: a) Mice nervous system is structurally and functionally similar to humans, including similarities in development, neurotransmitter systems, and neural circuitry (401); b) Mice have a relatively short gestation period and life span, making it possible to study the effects of neurotoxicants on multiple generations in a shorter period than in larger animals; c) Mice are relatively easy to breed and maintain in laboratory settings, making them a convenient and cost-effective model for developmental neurotoxicity studies; d) There are well-established protocols and assays for measuring neurodevelopmental outcomes in mice, allowing for standardized and reproducible studies across laboratories.

This thesis included mouse models in addition to zebrafish. The optic tectum is the predominant brain structure in zebrafish, while in mice and other mammals, it is the neocortex. Despite this structural difference, the two species share several similarities in terms of cellular and synaptic structure. Both the animals share the hypothalamo-pituitary-adrenal/intrarenal axis system in regulation of stress and hormones. However, when it comes to translating a specific chemical exposure protocol from one species to another,

pharmacokinetics must be taken into consideration. Mice are exposed to toxicants through their diet or by manual administration. The distribution in the fetal tissues is affected by the presence of the placenta during pregnancy. Zebrafish, on the other hand, absorb toxicants directly from their environment, including through a porous chorion that surrounds the embryo during early development. Additionally, both models exhibit neurotoxic effects after exposure to nicotine during development, which is due to the highly conserved cholinergic mechanisms present in both species (402). Since thiacloprid is a neonicotinoid that acts on the central cholinergic system, it was important to include both these species in this thesis.

7 Aims of this thesis

1. Explore the short-term effects of anthropogenic auditory stimuli on physiological parameters among healthy human beings.
2. Evaluate the neuroplastic effects of perinatal exposure to toxic anthropogenic stimulant neonicotinoid in animals.

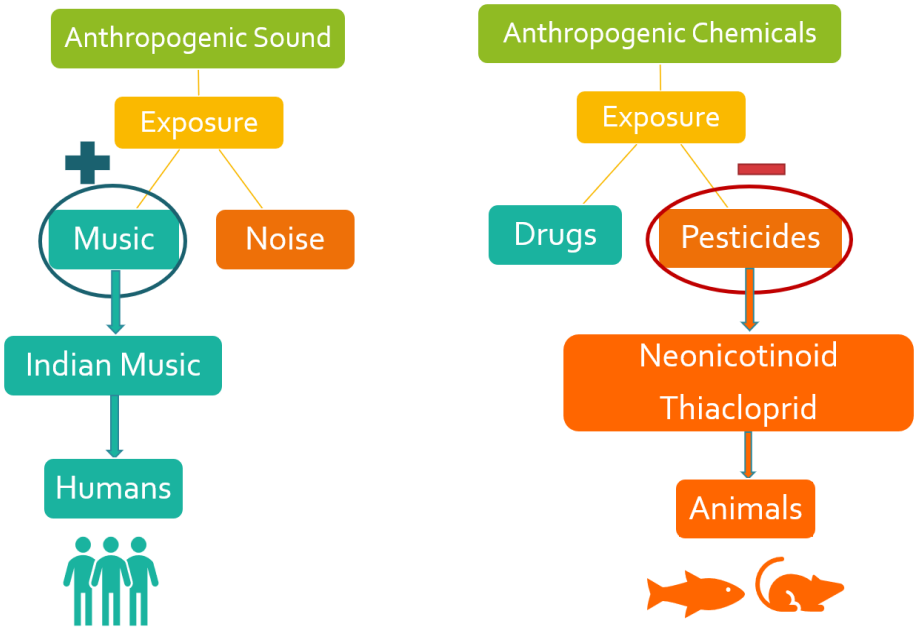


Fig 27: Theme of this thesis - Study of physiological effects of one positive stimulus (Music) in human beings and Study of Neuroplastic effects of one negative stimulant (Neonicotinoid) in animals (zebrafish, and mice)

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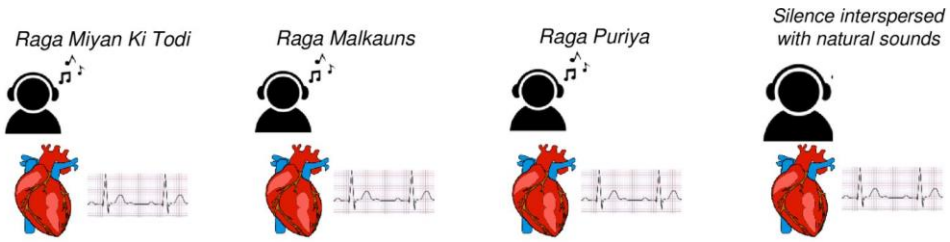
Chapter 2

Stress, cardiovascular and autonomic responses on exposure to environmental stimuli among humans



	Raga Miyan Ki Todi	Raga Malkauns	Raga Puriya	Control group
Anxiety	↓	↓↓	↓↓↓	No change
HRV				
During intervention	Arousal	Sustained ↑ Parasympathetic Parameters	Arousal	Sustained ↑ Parasympathetic Parameters
After intervention	Relaxation		Relaxation	

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Chapter 2: Stress, cardiovascular and autonomic responses on exposure to environmental stimuli among humans

Effect of Indian Music as an Auditory Stimulus on Physiological Measures of Stress, Anxiety, Cardiovascular and Autonomic Responses in Humans—A Randomized Controlled Trial

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Abstract

This study aims to explore the possible range of change after a single session of auditory stimulation with three different 'Modes' of musical stimuli (MS) on anxiety, biomarkers of stress, and cardiovascular parameters among healthy young individuals. In this randomized control trial, 140 healthy young adults, aged 18–30 years, were randomly assigned to three MS groups (Mode/Raga Miyan ki Todi, Malkauns, and Puriya) and one control group (natural sounds). The outcome measurements of the State-Trait Anxiety Inventory, salivary alpha-amylase (sAA), salivary cortisol (sCort), blood pressure, and heart rate variability (HRV) were collected at three time points: before (M1), during (M2), and after the intervention (M3). State anxiety was reduced significantly with raga Puriya ($p = 0.018$), followed by raga Malkauns and raga Miyan Ki Todi. All the groups showed a significant reduction in sAA. Raga Miyan ki Todi and Puriya caused an arousal effect (as evidenced by HRV) during the intervention and significant relaxation after the intervention (both $p < 0.005$). Raga Malkauns and the control group had a sustained rise in parasympathetic activity over 30 min. Future studies should try to use other modes and features to develop a better scientific foundation for the use of Indian music in medicine.

Keywords: Indian music; heart rate variability; stress; anxiety; STAI; state anxiety; trait anxiety; blood pressure; melodic modes

1 Introduction

The seven main functions of music are summarized to be background entertainment, recall of memories, diversion, emotion regulation, self-regulation, self-reflection, and social bonding. This umbrella review of the health effects of participation in performing arts, including music participation, reported positive effects in five domains (*auditory, cognitive, immune system, self-reported health/wellbeing, and social functioning)* (1). Though music is predominantly used as a form of entertainment the use of music for attaining health benefits dates back over centuries, probably since the Paleolithic period (2). Music Therapy is defined as the evidence-based use of music as an intervention as per the individual needs of the patient, be it physical, emotional, cognitive, or social, as per the American Music Therapy Association (3). It is seen that music, when used as an intervention, affects health (4,5) through different processes, which are yet to be well understood. Mechanisms put forth include the impact on the nervous system, the limbic system, (6) the autonomic nervous system (7), as well as synchronization of the body's natural rhythms (for example, heart rate or respiratory rate) with the rhythm of the music (1,2,8). Music is a safe, inexpensive, easily administered intervention that can be used for anxiety reduction and has proven to be beneficial in various diseases that include cardiovascular, neurological, and oncological diseases, as reviewed in (9,10).

The relaxation effects of music on stress, anxiety, and lowering of the neurohumoral markers have been evaluated in several research works (11–18). Active music intervention has proven to be beneficial in people afflicted with post-traumatic stress disorder (19,20). Young people report that music can help them relax and often have a collection of favorite 'tunes' that they listen to when they feel stressed out (15,21). In a cross-sectional study, we observed a significant drop in state anxiety after listening to Indian music (22), and in another follow-up study, we observed a significant reduction in state and trait anxiety after 3 months of Indian music intervention on 100 pre-hypertensives and hypertensives (23). Recent meta-analysis reports show that music is efficient in reducing anxiety levels (24,25), though some emphasized the need for additional research to endorse the same (26). Among the physiological measurements

that can be correlated with anxiety and stress are the cardiovascular parameters such as blood pressure (BP) and autonomic functions (that control the heart). Music-based interventions have largely been carried out on patients with hypertension (23,27–31) studying cardiovascular effects or perioperative conditions (25,32–35) studying anxiety. Listening to sedative music decreased heart rate (HR) and BP and was shown to work as effectively as benzodiazepines in reducing BP (36).

Heart rate variability (HRV) is a marker of cardiac autonomic functions (37) and has also been commonly investigated in music intervention studies (38). Several studies reported decreased HRV, indicating physiological relaxation (33,39–42), while some reported no change (43), and a few others showed an increase in HRV on listening to music (arousal effect) (40,44). The overall effect also depends on the mood (21), preferences (45), tempo, genre, and various other factors (40), for example, listening to preferred music caused an increase in sympathetic activity, regardless of the type of music (calming or stimulating) (46). It is important to understand the genre and features of the music used in a study before interpreting the HRV findings. It may also be observed that there is evidence of music's varied effects on stress, anxiety, the cardiovascular system (BP and HRV), and the mechanisms behind it. Although a subjective reduction in stress levels was recorded, objective measurements (47,48) or psychophysiological signals have not always shown the same (49,50). A systematic review and meta-analysis also failed to establish a cause-effect relationship between the intervention and BP reduction (51). It is thus important to have both subjective and objective measurements during music intervention to draw reasonable conclusions.

Among the music features, works on the effect of listening to different modes/melodic scales to elicit the difference in physiological effects, if any, are very few. Among the different genres, very little literature is available on Indian music as a scientific intervention, despite the rich repertoire of modes in this genre, for producing relaxation effects or health benefits (22,52–56). Indian music is broadly classified into Hindustani and Carnatic music, each having its system of modes (called *ragas*). Ancient literature on Indian music (*Gandharva Veda*, a part of the *Sama Veda* and '*Raga Chikitsa*' manuscript) mentions various modes that have health benefits (23,57,58). A '*raga*' (melodic mode) is

a set of musical notes presented in an orderly manner to generate a melody out of the same and has the “effect of coloring the hearts of men” (22,52,58,59). Each melodic mode is said to induce a specific emotion (called ‘*rasa*’) (58,60,61). Scientific studies that have analyzed emotions after listening to Indian classical music have observed that the tonality of the scale is an important factor that determines the emotions perceived (52,54,55). To the best of our knowledge, not many studies have included behavioral parameters with physiological measurements while listening to different modes of Indian music.

With this purview, this study tried to elucidate the effects of listening to Indian classical music on different behavioral and physiological parameters among young healthy individuals. Music is a complex stimulus that unfolds over time, it is important to understand the effect of systematically combined musical features during an average duration of listening. Our specific hypothesis was that distinct cardiovascular and behavioral responses would be associated with passive listening to each specific auditory stimulus, the response being specific to the melodic mode. For this, we chose three Indian modes (*ragasð Puriya, Malkauns*, and *Miyan ki Todi*). The primary outcome measure was to evaluate the state and trait anxiety levels, biomarkers of stress, blood pressure, and autonomic functions (HRV) after short-term listening to pre-recorded music in each of the three modes mentioned above.

2 Materials and Methods

2.1. Study Design

A prospective, parallel-group, triple-blinded, randomized controlled trial was conducted with an experimental study design, with a sample of 140, randomized into 4 groups, A to D, with a sample of 35 participants in each group. The four acoustic stimuli (stored as .mp3 files) were coded by a person uninvolved in the current study as A, B, C, and D, to be used as respective group interventions.

2.2. Ethical Approvals

The study protocol was approved by the institutional scientific committee on human research and ethical review board (Reference: MSRMC/EC/2017, dated: 25 July 2017). The study period ranged from 2019 to 2021 (June 2019—first recruitment and February 2021—last recruitment). The data presented here were taken from a larger experiment (full trial protocol: NCT03790462 on clinicaltrials.gov.in). The research was conducted following the Declaration of Helsinki guidelines (62).

2.3. The Basis for Sample Size

After music intervention, the State-Trait Anxiety Inventory-6 (STAI-6) anxiety scores changed from 33.3 (23.3–41.7) to 30 (20–40), respectively (Median (interquartile range—IQR)), in a previous study (63). Using these data, considering the minimum difference of 4 units in the STAI score before and after the intervention, with an effect size of 0.7, power of 85%, and an alpha error of 5%, the sample size was calculated to be 35 in each group.

2.4. Recruitment

The study participants were recruited from a group of educational institutions in the city of Bengaluru, Karnataka, India. Healthy Indian individuals aged 18–30 years were invited to participate in the study via an open call for participants for the study posted online (social media) and notice board advertisements across the institutions. Given the objectives of the study, to avoid cultural familiarity differences, only Indians were invited to participate in this study. Participants who responded to the call were sent an online questionnaire via Google forms.

2.5. Inclusion and Exclusion Criteria

Inclusion criteria were participants volunteering for the study, aged 18–30 years, of either gender and medically and surgically healthy individuals (initially self-reported—based on the online questionnaire and later confirmed on visiting the lab). The participants had to be non-smokers and non-alcoholics. Participants on any medication (based on drug intake history, drugs known to affect the BP or autonomic status of the individual)

were excluded from the study. Pregnancy and body mass index (BMI) >30 kg/m^2 were the other exclusion criteria.

2.6. Baseline Demographic Data Recording

A web-based questionnaire (Google forms) was designed and implemented for this study. This questionnaire contained details such as a unique identification number for each subject, subject's name, gender, socio-demographic details, education background, drug history, present report or history of non-communicable diseases if any, and family history of non-communicable disorders, and smoking and alcohol history. A questionnaire containing details inquiring about the participants' preference for any type of music and previous experience with music (instrumental or vocal training) was also included. After screening >300 individuals who responded to the call, 166 completed the Google form. Following the collection of data online, the participants were invited for further data collection in the lab. Out of 166 participants who answered the online questionnaire, 154 participants reported to the lab. The principal investigator (PI) and Co-PI enrolled participants in the study.

2.7. Randomization

The total sample size (n = 140) was randomized into 4 groups using a simple randomization technique where the random numbers were computer-generated using MS Excel (4 sets of 35 each). The numbers generated were kept in a sealed, opaque envelope which was opened by the research assistant after the baseline assessment of each participant who assigned them to each of the four groups (Consort diagram Figure 1).

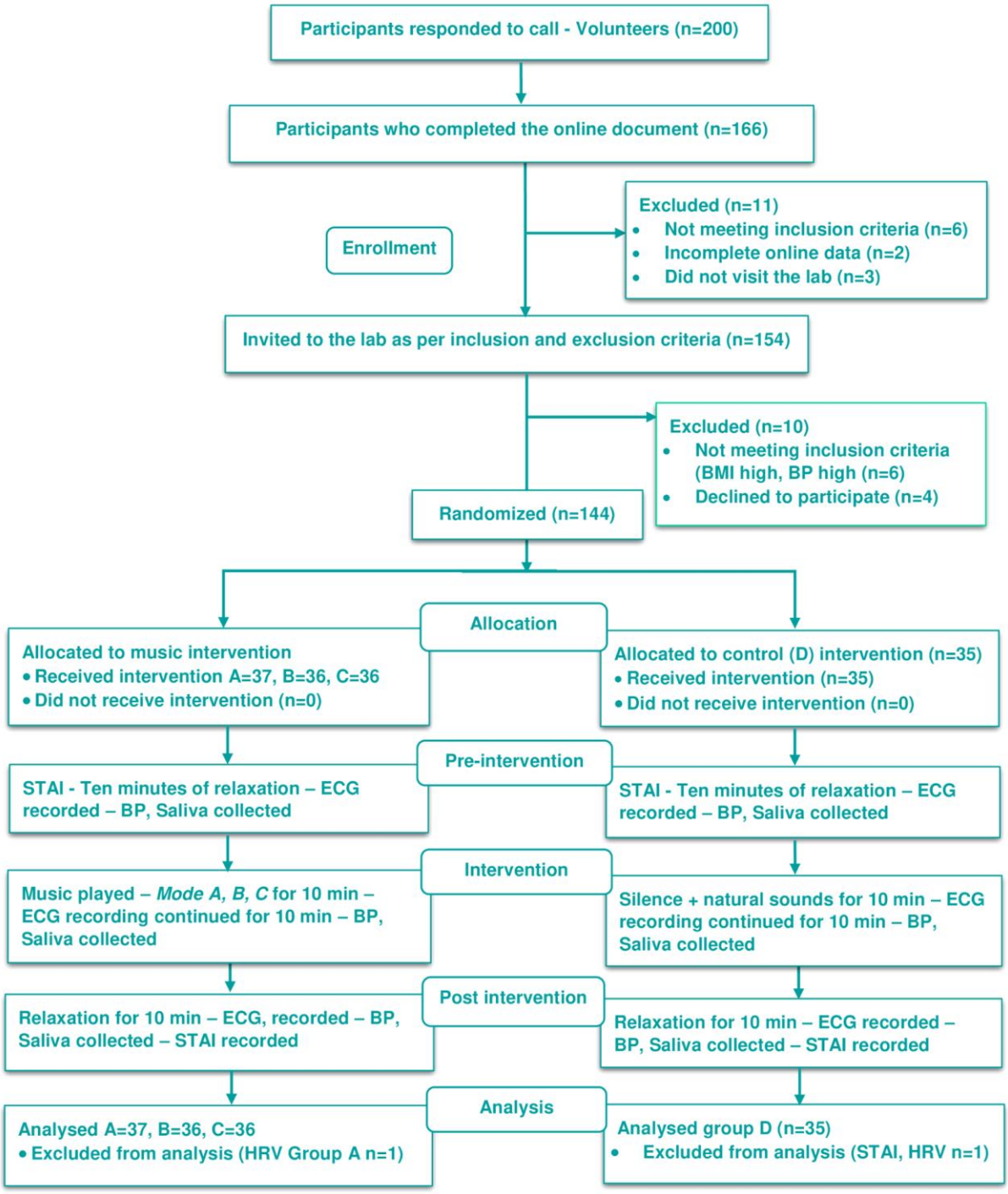

Figure 1. Consort diagram of participant recruitment, distribution, and follow-up.

2.8. Interventions

Three groups (A, B, C) received one of the *ragas*/modes as an acoustic intervention, while the fourth group (Group D/control arm) received natural sounds as an

acoustic stimulus (all audio clips were coded as A, B, C, and D by a person uninvolved in the study).

2.8.1. Music Intervention

Ten-minute, tailor-made instrumental renditions of 3 modes were digitally pre-recorded and played via headphones (64) connected to a laptop, at a uniform volume (50%). Group A received *raga Miyan ki Todi*, group B received *raga Malkauns*, and group C received *raga Puriya* (Table 1). These modes were chosen based on the criterium of having beneficial cardiovascular effects as per ancient music literature (23,57,61,65). The music was tuned to be at a frequency of 329.63 Hz (the tonic or '*Sa*' at Pitch E). Details about each mode used and the notes can be found in the supplementary file (S). The start and end time of the music was marked using an event marker in the software.

We instructed the participants to listen to this with eyes closed, and minds relaxed, for the duration it was played. The music was recorded by an eminent musician in India (exclusively for the present study) with the drone (*tanpura*) in tonic in the background and flute/*Bansuri* playing the respective *alaap* in the above-mentioned scales. A specific rhythmic structure or tempo was not there for this musical piece, and percussion instruments were avoided. The '*Bansuri*' is a flute in India made from a single hollow shaft of bamboo with six or seven finger holes, held horizontally while playing (66). As there was very little literature available on the most relaxing or soothing instrument, we chose *bansuri* for this study based on common instruments used commercially to produce relaxing music tapes. Instrumental music helped us avoid percussion (tempo) (67–74), lyrics, and the emotions or semantic processing due to them, and thus the music had minimal pitch dynamics, contrasts, and rhythm in it.

Table 1. The three chosen Indian melodic modes, the names of the notes in Hindustani music, and Western scale equivalents.

Svara/Note	Hindustani Name	Staff Note	Western Scale Interval Name
	Raga Miyan ki Todi (Scale A) (heptatonic, G appears in descent)		
S	*Shadja*	C	Perfect unison
r	*Komal Rishab*	D ♭	Minor second
g	*Komal Gandhar*	E ♭	Minor third
M	*Tivra Madhyam*	F#	Augmented fourth
P	*Pancham*	G	Perfect fifth
d	*Komal Dhaivat*	A ♭	Minor sixth
N	*Shuddha Nishad*	B	Major seventh
	Raga Malkauns (Scale B) Ascent and descent sameð pentatonic		
S	*Shadja*	C	Perfect unison
g	*Komal Gandhar*	E ♭	Minor third
m	*Shuddha Madhyam*	F	Perfect fourth
d	*Komal Dhaivat*	A ♭	Minor sixth
n	*Komal Nishad*	B ♭	Minor seventh
	Raga Puriya (Scale C) C, D ♯♭ E, G ♯♭ G, A/A ♯♭ B (hexatonic)		
S	*Shadja*	C	Perfect unison
r	*Komal Rishab*	D ♭	Minor second
G	*Shuddha Gandhar*	E	Major third
M	*Tivra Madhyam*	F#	Augmented fourth
D	*Shuddha Dhaivat*	A	Major sixth
N	*Shuddha Nishad*	B	Major seventh

2.8.2. Control Group Intervention

The control group (Group D) did not receive any music intervention, but since the complete recording lasted for 30 min duration, it was possible for the participants to feel sleepy (sleep is anxiolytic, which would alter the current objective). Thus, natural sounds (birds chirping and flowing river) were played for 10 s duration once every 2 min in the middle ten min (during intervention); a total of 50 s in the middle ten minutes. This also ensured uniformity of intervention between the groups.

2.9. First Visit to the Lab

Before visiting the lab, all participants were instructed to come after overnight fasting, with a light breakfast, to abstain from tea and coffee about 2 h before the recording, and abstain from exhaustive exercise, for the preceding 24 h. Female participants were asked to visit the lab during the follicular phase of their menstrual cycle. The study protocol and the rights to withdraw their participation from the study were explained to the participants, after which written informed consent to participate in the study was obtained. A general health check-up was done for all participants. The BMI was calculated, and BP was measured twice. The healthy cardiovascular system of the volunteers was defined by measuring BP, which confirmed their non-hypertensive state, and by measuring baseline HR, which confirmed their non-tachycardiac state. Normotensives were included as per inclusion criteria (excluded n = 10; Baseline systolic BP—SBP > 120 mm Hg). Healthy participants (n = 144) were recruited (Consort diagram Figure 1). Though 10.4% of participants were current alcoholics, their baseline BP was within the normal range and were included in the study after being instructed to abstain from smoking/alcohol 24 h before the recording.

2.10. Second Visit to the Lab

All the recordings were carried out between 09:00 and 11:00 a.m. in an isolated room at a stable temperature between 20 and 22 °C, in a noise-free atmosphere. After the participants responded to the STAI Form Y (between 0 to 5 min—T5, explained further), they were asked to relax in the supine position for 10 min before the tests, with their eyes closed. Participants were carefully monitored to ensure there were no significant respiratory or postural changes during the session. During this time (first 5 min), electrocardiogram (ECG) electrodes in lead II were applied, similar to previous studies (64), headphones were adjusted, and comfort with the pre-set volume (50% on laptop) was tested. The BP cuff was tied to the left arm of the participant and a reading was taken so that the participant understands the process of automatic cuff inflation and deflation. At the end of the first 10 min the baseline BP was recorded, and the protocol was begun. At the beginning of the protocol, one saliva oral swab was inserted into the

participants' mouths and kept sublingually. The first 10 min of baseline ECG recording commenced. At the end of 10 min (M1), digital measurement of BP (SBP, diastolic BP—DBP, and HR) was done (recorded as pre-intervention readings) and saliva samples were taken. This was repeated at 20 (M2) and 30 (M3) minutes later (see Figure 2 for the process of recording). After the 30 min protocol, the participants were asked to complete the STAI Form-Y (between 35–40 min—T35), recorded as post-intervention STAI scores, and rate the valence of intervention on a 10-point visual analog scale (VAS). All saliva swabs were stored at 4°C until centrifugation. The saliva samples were then centrifuged at 3000 rpm for 15 min and supernatant saliva was stored at −80°C until further analysis within 1 h of saliva collection. Pre, during, and post-intervention data analysis of BP, HRV, STAI, and salivary stress markers (salivary cortisol—sCort and salivary alpha-amylase—sAA (ELISA- Enzyme-linked immunosorbent assay)) was done.

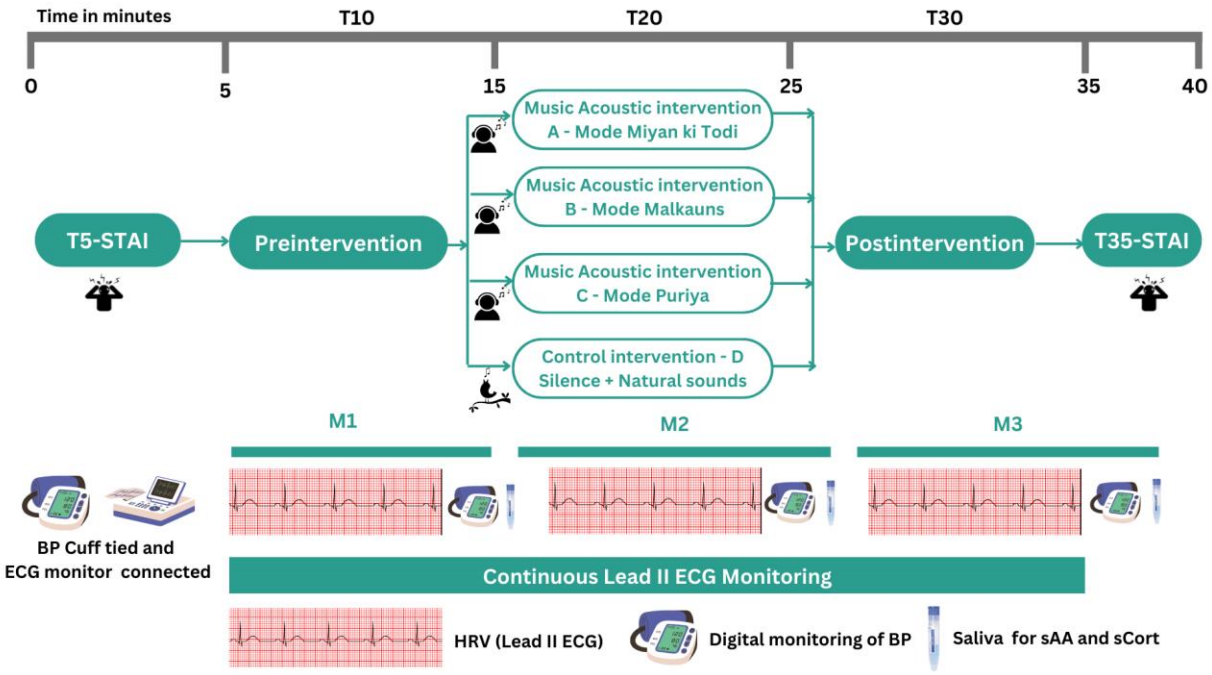

Figure 2. Study protocol; T5, T10, T20, T30, T35 is the time in minutes; STAI—State-Trait Anxiety Inventory; BP—Blood pressure; HRV—Heart rate variability; ECG—Electrocardiogram; sAA—Salivary Alpha-amylase; sCort—Salivary Cortisol.

2.11. Behavioral Measures

2.11.1 Measurement of Anxiety

The State-Trait Anxiety Inventory (STAI) (Form Y) for adults is a validated questionnaire (75) implemented in the current study, as explained in (22). Briefly, participants had to respond to 40 questions by rating themselves on a four-point Likert scale (1—Not at all, 2—Somewhat, 3—Moderately so, 4—Very much so), resulting in a range of possible scores between 20 to 80 on both the State and Trait subscales (75). It differentiates between the temporary condition of “State anxiety” (feeling at the moment, in Form Y 1) and the more general and long-standing quality of “Trait anxiety” (feeling in general, in Form Y 2) (75,76). The STAI has demonstrated good internal consistency (average as >0.89) and test–retest reliability (average r ¼ 0.88) at multiple time intervals. The reliability of the STAI in patients with an anxiety disorder is found to be between 0.87 and 0.93 (77,78). One of the STAI forms in group D had more than 3 missing values and was thus not included for further analysis.

2.12. Physiological Parameters

2.12.1 Saliva for Biomarkers of Stress

To measure free cortisol levels, which reflects hypothalamic–pituitary–adrenal system (HPA) activity, and salivary alpha-amylase (sAA), which indicates the activity of the sympathetic–adrenal–medullary system (SAM) (79–81), saliva was collected using the SalivaBio Oral Swab (Salimetrics LLC, State College, PA, USA) every 10 min (Figure 2). Salivary alpha-amylase was assessed using a Salimetrics Salivary Alpha-Amylase Assay Kit (Salimetrics LLC, State College, PA, USA), following the manufacturer’s guidelines. Results were expressed in U/mL. The intra-assay precision coefficient of variation (%) was 2.5–7.2%, and the inter-assay precision was 3.6–5.8%. Salivary levels of cortisol were assessed using the Expanded Range High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics LLC, State College, PA, USA), following the manufacturer’s guidelines. Results were expressed in µg/dL. The intra-assay coefficient of variation was 5.5–5.68%, and the inter-assay coefficient of variation was 6.3–6.7%.

2.13. Cardiovascular Parameters

2.13.1. Blood Pressure (BP) and Heart Rate (HR)

A standardized digital BP monitor was used (Omron HEM-7130-L, OMRON Healthcare Manufacturing Vietnam Co., Ltd., Sourced from Haryana, India) to measure BP (82). The measurements of SBP (in mm of Hg), DBP (mm Hg), and HR in beats per minute were noted once every 10 min (Figure 2).

2.13.2. Electrocardiogram Recording and Heart Rate Variability Analysis

The Electrocardiogram (ECG) was recorded in Lead II (sample rate of 1000 Hz) for ten minutes as this is twice the minimum window required for HRV analysis. The data were recorded using Power lab 15 T LabChart Pro 8 software (ADInstruments, Sydney, Australia) and analyzed as described in (37). Analysis of HRV was done by the same investigator to avoid sources of error. The HRV parameters analyzed using fast Fourier transformation (FFT size: 1024) were SDNN—the standard deviation of NN intervals, RMSSD—root square of the mean squared difference of successive NNs, NN50—number of pairs of successive NNs that differ by more than 50 ms, pNN50—the proportion of NN50 divided by the total number of NNs, spectral components such as Very Low-Frequency (VLF), Low-Frequency (LF), and High-Frequency (HF) components in absolute values of power (ms^2) and normalized units (nu), and LF/HF. Pre (M1), during (M2), and post-intervention (M2) parameters of HRV (as an average of a minimum of 5 min of recording, during each condition) were analyzed. During analysis, one of the HRV readings was not saved in group A and one in group D was too noisy, and they were thus not analyzed and were deleted from further processing.

2.14. Statistical Analysis

Analysis was conducted at three levels: (1) group-wise (within and between) behavioral analysis of anxiety scores and stress markers, (2) group-wise (within and between) analysis of cardiovascular responses, and (3) regression analysis to investigate the relationship between the acoustic stimulus used and the cardiovascular and behavioral responses. Data were analyzed using SPSS software version 18.0 software

(SPSS Inc. Released 2009. IBM SPSS Inc., Chicago, IL, USA). The continuous variables were analyzed using descriptive statistics such as mean and SD or median and interquartile range as per skewness of data. The qualitative/categorical variables were analyzed using frequency and percentage. The normality of the BP and HRV data was checked by applying the Kolmogorov–Smirnov Test. The categorical variables were tested for differences in proportion using the Chi-Square test of significance. Pre- and post-intervention data analysis of state and trait anxiety scores were compared using Wilcoxon's signed-rank test. The Independent t-test was used to compare the differences between the groups. Baseline comparisons were carried out using a one-way analysis of variance (ANOVA). BP, HRV, and salivary parameters were compared across different groups pre, during, and post-intervention using repeated measures of ANOVA (RM-ANOVA). The HRV parameter's absolute levels and log-transformed levels were compared using RM-ANOVA with sphericity assumption. Further, a two-way RM-ANOVA analysis was done to inspect the interaction between the intervention group and time. Analysis of covariance (ANCOVA) was used to assess the effect of various covariates, viz., age, age groups, gender, smoking, alcoholism, involvement in mind-body relaxation techniques, physical activity, and music training, on the change in STAI, BP, and HRV parameters over time. Apart from tabulation, data were also depicted graphically using box plots and line diagrams. A two-tailed *p*-value <0.05 was considered statistically significant at a 5% level of significance.

3 Results

3.1. Sociodemographic Data

The socio-demographic data showed that the groups were comparable (Table 2), except for their educational status. There were more graduate students in the music intervention groups compared to the control groups ($p < 0.001$). About 30 to 45% of participants were trained in music, but the distribution of participants across the groups was comparable. Participants were predominantly trained in Indian music, with more than 70% trained for more than a year. About 85% of participants considered themselves familiar with or experts in Indian classical music (supplementary file Table S1).

Table 2. Sociodemographic characteristics of participants.

Variables	Group A	Group B	Group C	Group D	*p*-Value
Sample	N = 37 (%)	N = 36 (%)	N = 36 (%)	N = 35 (%)	
Age (Years)					
<=18	9 (24.3)	5 (13.9)	6 (16.7)	4 (11.4)	0.171
19–21	18 (48.6)	18 (50.0)	15 (41.7)	19 (54.3)	
22–24	8 (21.6)	11 (30.6)	11 (30.6)	4 (11.4)	
>=25	2 (5.4)	2 (5.6)	4 (11.1)	8 (22.9)	
Age (years) Mean, SD	20.54, 2.5	20.75, 2.5	21.11, 2.6	21.26, 3.0	0.646
Gender					
Female	29 (78.4)	20 (55.6)	24 (66.7)	25 (71.4)	0.202
Male	8 (21.6)	16 (44.4)	12 (33.3)	10 (28.6)	
Education					
High school/ Intermediate	16 (43.2)	7 (19.4)	16 (44.4)	29 (82.9)	<0.001
Graduate/ Postgraduate	21 (56.8)	29 (80.6)	20 (55.6)	6 (17.1)	
Marital status					
Married	36 (97.3)	35 (97.2)	35 (97.2)	33 (94.3)	0.875
Single	1 (2.7)	1 (2.8)	1 (2.8)	2 (5.7)	
Diet					
Vegetarian	14 (37.8)	11 (30.6)	7 (19.4)	16 (45.7)	0.112
Non-vegetarian	23 (62.2)	25 (69.4)	29 (80.6)	19 (54.3)	
BMI (kg/m^2) Mean, SD	23.17, 3.96	22.96, 4.71	22.16, 3.47	22.47, 4.10	0.714
Music Training Yes/No (%)	17 (45.9)	14 (38.9)	11 (30.6)	12 (34.3)	0.562

[1] **Note:** N is the number of participants in each group; All the values of the two groups are in absolute values and parenthesis contain percentages; a *p*-Value of <0.05 is considered significant; P calculated using Chi-square test/Fisher exact test; Mean age and BMI comparison were done using ANOVA.

3.2. Behavioral Analysis

3.2.1 STAI

Pre-intervention levels of the state and trait score (T5), across the groups, were comparable (for the state, $F(3, 138) = 0.170$, $p = 0.917$; and for the trait, $F(3, 138) = 0.811$, $p = 0.490$). Comparison of state anxiety STAI scores between all three music intervention

groups showed statistical significance (reduction) within the group. The maximum reduction in state score was with *raga Puriya* (**mead difference—md** = 3.94, $p = 0.018$), the next being *raga Malkauns* (md = 3.83, $p = 0.057$), followed by *raga Miyan ki Todi* (md = 2.35, $p = 0.054$). The reduction in the control group was mild, with md being 0.32 (statistically not significant) (Table 3, Figure 3a,b). Between the groups, there was no significant difference in the T35 state score ($p = 0.696$). On comparison of the difference in means of pre–post values between the groups, there was no significant difference in state score ($p = 0.319$). A comparison of trait anxiety scores showed that group C (*raga Puriya*) had a statistically significant increase in trait score (increase by 2.33 mean level, $p = 0.011$) (Table 3, Figure 3c,d). Between the groups, the T35 trait score was not significant ($p = 0.660$). There were no significant differences in the pre–post values of trait scores between the groups ($p = 0.634$). On multivariate analysis, none of the confounding variables seemed to affect the change in STAI scores (both state and trait).

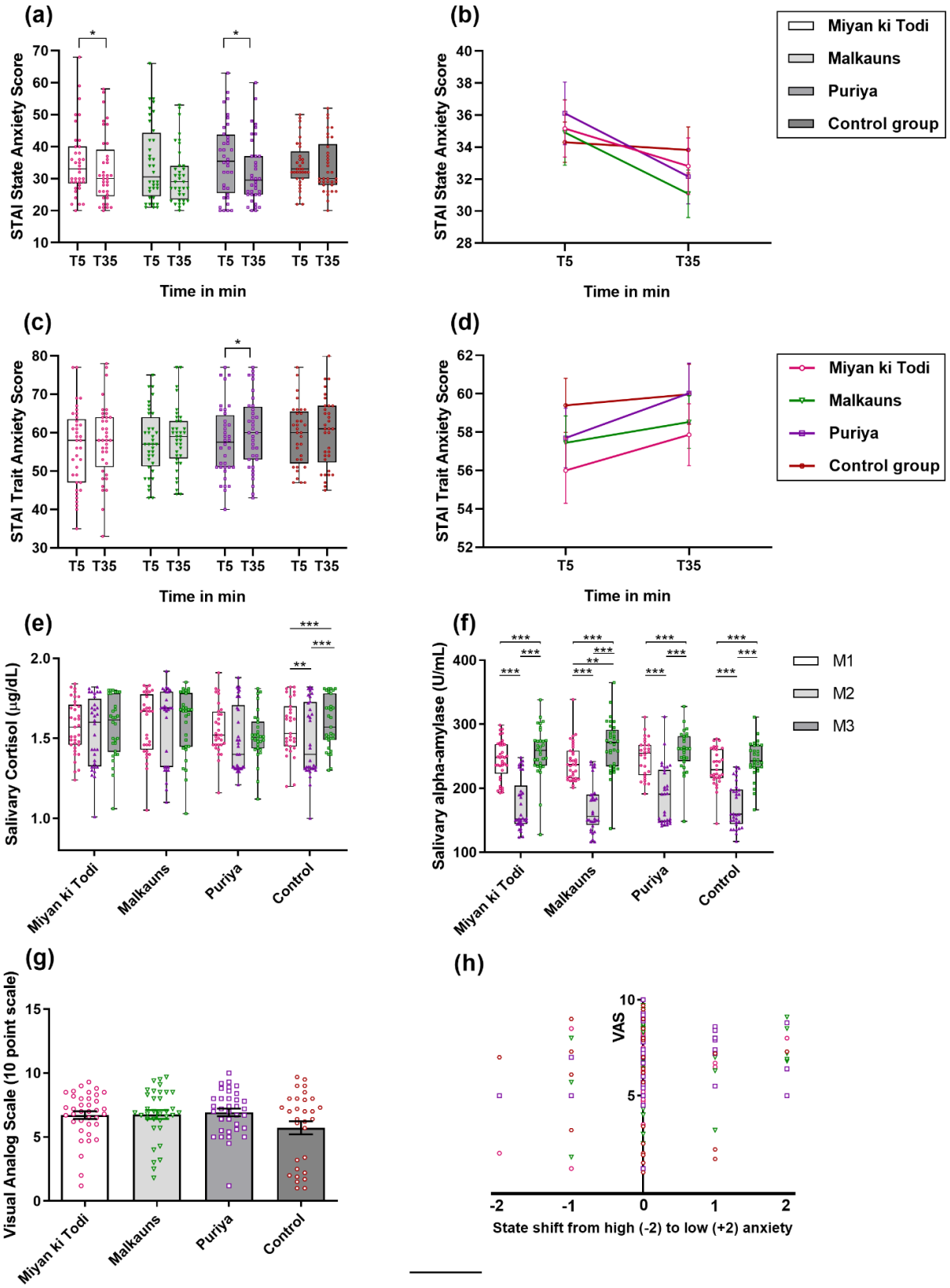
(a)
STAI State Anxiety Score
Time in min
T5 T35 T5 T35 T5 T35 T5 T35
(b)
STAI State Anxiety Score
Time in min
T5
T35
Miyan ki Todi
Malkauns
Puriya
Control group
(c)
STAI Trait Anxiety Score
Time in min
(d)
STAI Trait Anxiety Score
Time in min
(e)
Salivary Cortisol (µg/dL)
Miyan ki Todi
Malkauns
Puriya
Control
(f)
Salivary alpha-amylase (U/mL)
M1
M2
M3
(g)
Visual Analog Scale (10 point scale)
(h)
VAS
State shift from high (-2) to low (+2) anxiety

Figure 3. (a,b) Comparison of state score on STAI-Y1; (c,d) Trait score on STAI-Y1; (e) Salivary stress markers—Cortisol in µg/dL, (f) Alpha-amylase in U/mL, (g) VAS (valence rating), and (h) change in state anxiety score with VAS scoring—among the four groups at different time points (T5 is 5 min before the protocol began, and T35 is 35 min after the protocol or 5 min after the protocol was completed). Note: *: $p < 0.05$, **: $p < 0.01$, *** $p < 0.001$.

Table 3. Comparison of pre and post-intervention STAI scores between four groups.

Group		Mean	SD	md	Quartiles			*p*
					25	50	75	
STAI State Anxiety								
A (N = 37)	Pre	35.16	10.8	2.35	28.5	33	40	0.054
	Post	32.81	10.7		24.5	30	39	
B (N = 36)	Pre	34.92	12.3	3.83	24.5	30.5	44.3	0.057
	Post	31.08	8.9		23.5	29	34	
C (N = 36)	Pre	36.11	11.7	**3.94**	25.5	35.5	43.8	0.018
	Post	32.17	10.3		25	29.5	37	
D (N = 34)	Pre	34.21	7.2	0.32	30	32.5	37	0.781
	Post	33.74	8.3		28	30.5	40	
STAI Trait Anxiety								
A (N = 37)	Pre	56	10.4	−1.87	47	58	63.5	0.057
	Post	57.87	9.8		51	58	64	
B (N = 36)	Pre	57.44	8.4	−1.08	51.25	57	64	0.135
	Post	58.53	8.2		53.3	59	63	
C (N = 36)	Pre	57.69	9.3	−2.33	51	57.5	64.5	0.011
	Post	60.03	9.4		53	60	66.8	
D (N = 34)	Pre	59.32	8.0	−0.88	53	60	65	0.302
	Post	60.09	9.0		54	61	67	

Note: *p*-value < 0.05 was considered significant, calculated using paired *t*-test.

3.3. Physiological Parameters

3.3.1 Biomarkers of Stress

Pre-intervention levels of sAA and sCort (M1), across the groups, were comparable (for sAA, P, $F(3, 127) = 1.421$, $p = 0.240$; for sCort, $F(3, 126) = 0.197$, $p = 0.898$). Mean sCort levels reduced maximally in the control group ($F = 12.34$, $p < 0.0001$). Mean sAA levels reduced in all four groups significantly at M2, after which the levels increased slightly more than baseline levels. The drop in sAA was maximal at M2 with *raga Puriya* ($F = 67.01$, $p < 0.0001$), which increased at M3 to a level higher than within the group baseline and in comparison with other groups (Figure 3e,f; post hoc analysis in the supplementary file, Table S2). The visual analog score and corresponding state scores did not vary significantly across groups (Figure 3g,h).

3.4. Cardiovascular Parameters

3.4.1. Blood Pressure and Heart Rate

Pre-intervention levels of BP and HR (M1 levels), across the groups, were comparable (for SBP, $F(3, 139) = 0.463$, $p = 0.708$; for DBP, $F(3, 139) = 1.053$, $p = 0.371$; for HR, $F(3, 139) = 0.417$, $p = 0.741$). On RM-ANOVA analysis of the intervention effects, no significant differences were observed in SBP and DBP in any of the groups (Figure 4, explanation elaborated in Supplementary text S4). Heart rate increased with *raga Miyan ki Todi* intervention and reduced below baseline levels at M3 ($F = 3.645$, $p = 0.031$), with maximum difference seen between M2 and M3 (mean difference = 3.351 drops, $p = 0.073$) (detailed in the supplementary file, Table S3a,b). It may be observed that, in line with the STAI state anxiety, SBP and HR reduced maximally with *Raga Puriya*.

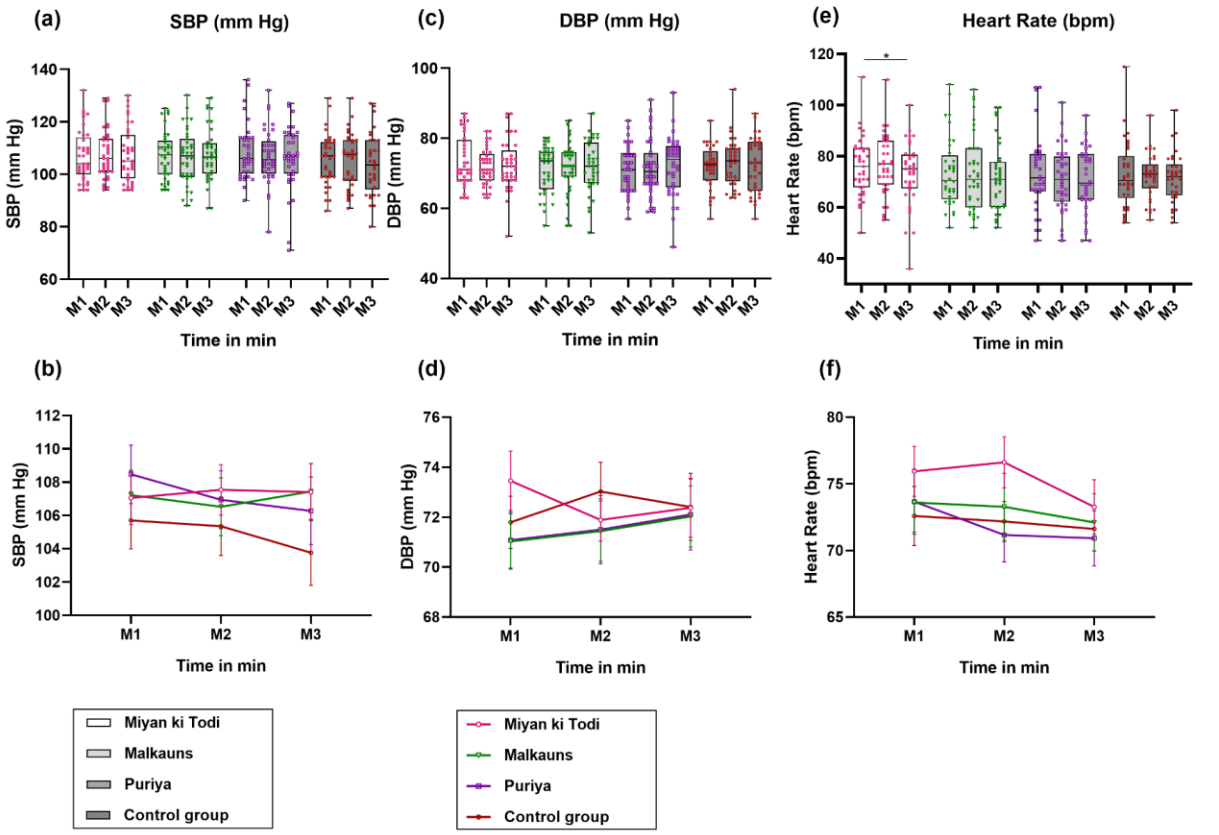

Figure 4. Comparison of (a,b) systolic BP (SBP), (c,d) Diastolic BP (DBP), and (e,f) Heart rate among the four groups at different time points (M1 is at the 10th minute, M2 is at the 20th minute, M3 is at the 30th minute). Note: *: $p < 0.05$.

On multivariate analysis, it was found that age and physical activity had a significant effect on change in the mean SBP. There was a statistically significant effect of age group on SBP, F (1, 132) = 5.572, p = 0.020, and involvement in physical activity on SBP, F (1, 132) = 4.664, p = 0.033. However, the percentage of variation in SBP that could be explained by the independent variables mentioned in the table was only 10% (R Squared = 0.107). On gender-wise and subgroup analysis based on involvement in physical activity, no significant differences were observed in BP or HR levels. The particular age group of 22–24 years old showed a significant effect (F = 3.308, p = 0.043), based on time (i.e., M1 vs. M2 vs. M3 SBP levels); however, group-wise means difference was statistically not significant. On comparison of DBP levels based on age groups, we observed that participants aged 18 years showed significant changes in DBP based on time (F = 7.337, p = 0.002) and interaction effect (time and group, F = 2.773, p = 0.024). A subgroup analysis based on training in music failed to show significant differences in BP or HR.

3.4.2. Heart Rate Variability

All pre-intervention HRV parameters across the groups were comparable (all HRV parameters had $p > 0.05$, data in Tables S4 and S5, supplementary file), except for VLF ms^2 (high in group C and low in group A, $F(3, 138) = 2.878$, p = 0.038). The comparison of intervention was done using RM-ANOVA (for actual values and statistics, see the supplementary file, Tables S4–S6).

3.4.2a Time-Domain Parameters of Heart Rate Variability

There was a continuous rise in mean NN among the music intervention groups through the 30-min protocol, but in the control group, the change was minimal at M3 (last 10 minutes, after intervention). Post hoc comparisons revealed a significant rise from M1 (first ten minutes before intervention) to M3 mean NN with *raga Miyan ki Todi* (difference of 22.67 ms; $p < 0.001$), *raga Malkauns* (difference of 33.15; $p < 0.001$), and *raga Puriya* (difference of 23.46; p = 0.01). Group listening to *Raga Malkauns* (difference of 24.78; $p < 0.001$) and the control group (difference was 18.74; $p < 0.001$) had a significant rise from M1 to M2 (min ten minutes, during intervention) (Figure 5a,b). The mean HR change was statistically significant in all the groups, with results inverse to that of mean NN. The

maximal significant change was with *raga Malkauns*, where HR reduced by a value of 2.05 bpm ($p < 0.001$) from M1 to M2 and 2.86 bpm ($p < 0.001$) from M1 to M3. The next maximal significant change was observed with *raga Puriya*, with a drop of 1.92 bpm ($p = 0.01$), and *raga Miyan ki Todi*, with a drop of 1.79 bpm ($p = 0.01$) from M1 to M3. The control group had a significant drop from M1 to M2 by about 1.7 bpm ($p < 0.001$) (Table S4, Figure 5c,d). The SDNN change was significant in all the groups. The maximal significant change in SDNN was with *Puriya*, where SDNN increased by 12.24 ms ($p < 0.001$), followed by *raga Miyan ki Todi*, where the level of the rise was 10.03 ms ($p < 0.001$) from M2 to M3. A significant M1 to M3 SDNN increase was seen with *raga Malkauns* (difference of 7.19; $p = 0.05$) and the control group (difference of 9.51; $p < 0.001$) (Figure 5e,f). The mean RMSSD, similar to SDNN, reduced during M2 with *Puriya* and *raga Miyan ki Todi*, but increased beyond baseline during M3; the change was statistically significant in both these groups. The maximal significant change in RMSSD was with *Puriya*, where the RMSSD increased by 9.70 ms ($p = 0.06$), and *Raga Miyan ki Todi*, where the level of the rise was 9.49 ms ($p = 0.04$) from M2 to M3. In the control group, the RMSSD increased significantly from M1 to M3 (7.97 units ($p = 0.03$)) and M2 to M3 (4.36 units ($p = 0.04$)). Group B did not show a significant change in RMSSD (Figure 5g,h). The pNN50 (%) between M1 and M3 was statistically significant only with *raga Puriya* (Figure 5i,j; Table S4, supplementary file).

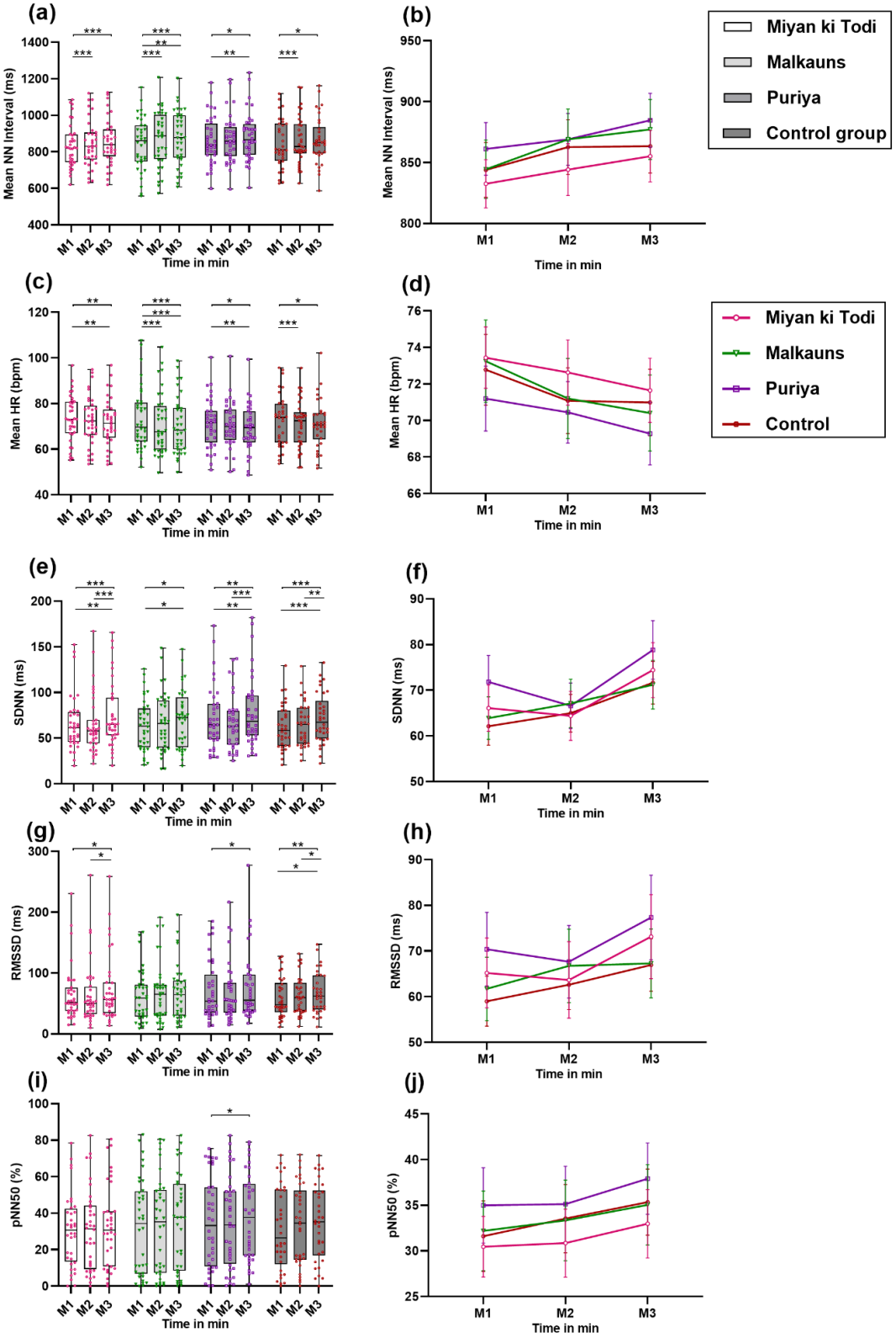
(a)
Mean NN Interval (ms)
Time in min
(b)
Mean NN Interval (ms)
Time in min
Miyan ki Todi
Malkauns
Puriya
Control group
(c)
Mean HR (bpm)
Time in min
(d)
Mean HR (bpm)
Time in min
Miyan ki Todi
Malkauns
Puriya
Control
(e)
SDNN (ms)
Time in min
(f)
SDNN (ms)
Time in min
(g)
RMSSD (ms)
Time in min
(h)
RMSSD (ms)
Time in min
(i)
pNN50 (%)
Time in min
(j)
pNN50 (%)
Time in min
M1
M2
M3

Figure 5. Comparison of Time domain parameters of HRV among the four groups at different time points ((M1, M2, M3 is the measurement of HRV pre-intervention (T10), during the intervention (T20), and post-intervention (T30)). (a,b) Mean NN interval in ms; (c,d) Mean HR (bpm); (e,f) SDNN in ms; (g,h) RMSSD; (i,j) Percentage of NN50 in %. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4.2b Frequency-Domain Parameters of Heart Rate Variability

In line with the findings of time-domain HRV parameters, TP reduced during M2 with *raga Miyan ki Todi* and *raga Puriya*, and increased beyond baseline levels at M3, while in groups B (*raga Malkauns*) and D (control), TP continuously increased. The maximal significant change in TP (ms^2) was with *Puriya*, where TP increased by 2211.1 units ($p = 0.04$), followed by *raga Miyan ki Todi*, where the level of the rise was 1597.1 units ($p = 0.01$) from M2 to M3. A significant TP increase was seen from M1 to M3 with *raga Miyan ki Todi* (difference of 1414.3; $p = 0.01$) and *raga Malkauns* (difference of 1379.9; $p = 0.03$) (Figure 6a,b). There was a significant difference in mean VLF power (ms^2) with *raga Malkauns* and *raga Puriya* ($p = 0.013$ and 0.007, respectively). The power spectrum of HRV in the VLF range reduced significantly at M2 (by 459.96 units; $p = 0.05$) and tended to increase at M3 (by 652.62 units; $p = 0.03$) with *raga Puriya*. VLF change was also significant, with *raga Malkauns* showing a continuous rise in VLF power, the M1 to M3 difference being statistically significant (630.87 units change; $p = 0.03$) (Figure 6c,d). Like SDNN, RMSSD, and TP, the LF in ms^2 also reduced during M2 with *raga Miyan ki Todi* and *raga Puriya* and increased beyond baseline levels at M3. In that, the change between M2 to M3 was statistically significant with *raga Miyan ki Todi* (rise by 551.22 units; $p = 0.04$), while the change between M1 and M3 was significant with *raga Puriya* (rise of 457.38 units; $p = 0.01$) (Figure 6e,f). The LF (nu) reduced significantly only with *raga Miyan ki Todi* ($p = 0.014$) and increased beyond baseline levels post-intervention (pairwise comparison of M2 to M3, 4.26 units; $p = 0.03$) (Figure 6g,h). A significant rise was seen in HF (ms^2) only in the control group ($p = 0.041$) (Figure 6i,j). There was a significant change in the LF/HF ratio observed only with *raga Miyan ki Todi* ($p = 0.028$), wherein the LF/HF ratio reduced slightly during the intervention and later increased beyond baseline levels post-intervention (Figure 6k,l; Table S5, supplementary file).

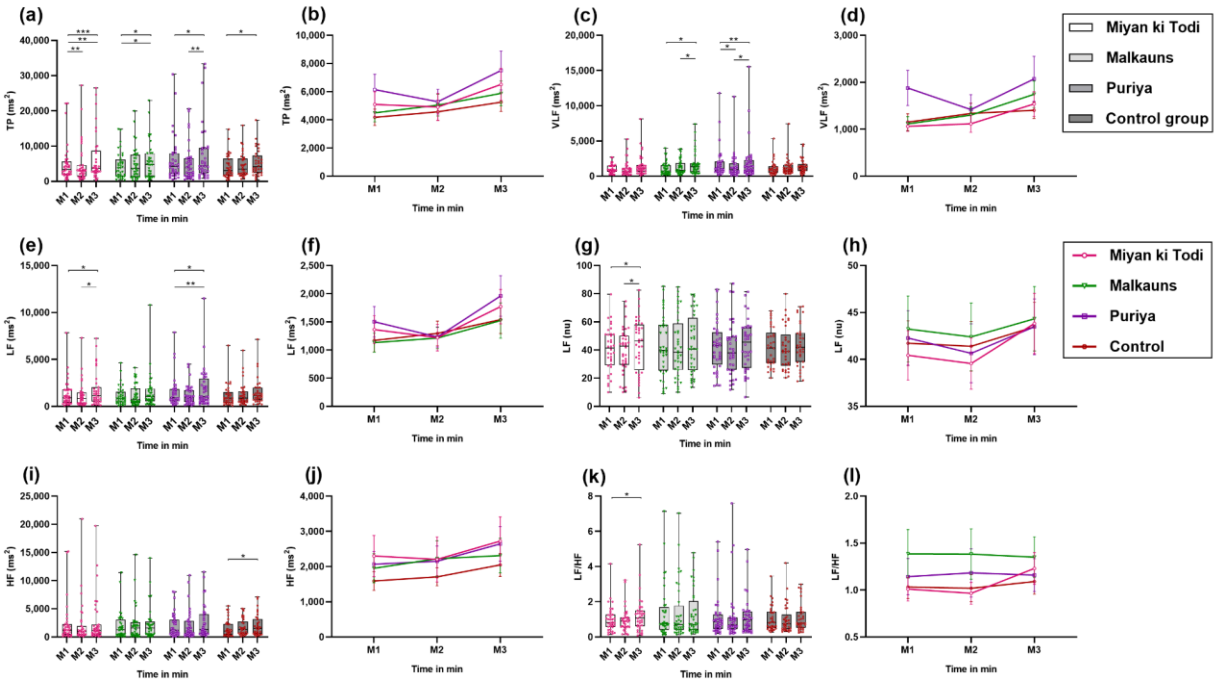

Figure 6. Comparison of frequency-domain parameters of HRV among the four groups at different time points ((M1, M2, M3 is the measurement of HRV pre-intervention (T10), during the intervention (T20), and post-intervention (T30)). (a,b) TP in ms²; (c,d) VLF in ms²; (e,f) LF in ms²; (g,h) LF in nu; (i,j) HF in ms²; (k,l) Ratio of LF/HF. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

On univariate analysis of HRV parameters, none of the confounding factors (based on questionnaire data) were found to be associated with the change in the HRV parameters, except VLF ms^2. Alcoholism history seemed to affect the VLF ms^2 difference: $F(1, 131) = 4.844$, $p = 0.029$ (log-converted VLF ms^2 difference $F(1, 131) = 7.880$, $p = 0.006$). It was observed that there was a significant effect of time (M1, M2, M3) on participants who were non-alcoholics ($F = 11.315$, $p < 0.0001$), compared to alcoholics. Group-wise, no difference was observed. However, the percentage of variation in VLF ms^2 that could be explained by the independent variables (Supplementary Table S6) was only 9–10%.

4 Discussion

In this study, we assessed the effect of passive listening to three different acoustic stimuli (Indian classical music modes) on the cardiovascular electrophysiological effects and subjective behavioral responses (anxiety and stress) among normal healthy individuals and compared them with a control group listening to natural sounds. Three different modes/ragas of Indian classical music used as interventions were *Miyan ki Todi* for group A, *Malkauns* for group B, and *Puriya* for group C. Those in the control arm (group D) relaxed for 30 min while listening to intermittent natural sounds for a very short duration. Sociodemographically, the groups matched, except for educational status, with more graduates or postgraduates in the three intervention groups compared to the control group. All groups matched based on their musical training as well.

4.1. Behavioral Analysis

4.1.1 Anxiety

As listening to music can initiate a multitude of cognitive processes in the brain (83), it might be assumed that music also influences stress-related cognitive processes and, as a consequence, physiological responses (12). Anxiety was measured in the current study using a standard validated State-Trait Anxiety Inventory (STAI) Form Y. The three intervention groups showed a significant drop in state anxiety, while the control group had an insignificant mild drop. The maximum reduction in the state score was with *raga Puriya*, followed by *raga Malkauns* and *raga Miyan Ki Todi*. This reduction in state anxiety indicated a relaxation response to listening to music. In contrast, trait anxiety increased in all four groups, which could be due to chance or the boredom that set in after answering multiple questions (trait anxiety formed the last 20 questions). A shorter version of the STAI might have been a better tool to assess the trait anxiety after 30–40 min of the protocol. Furthermore, a reduction in trait anxiety might occur after a few weeks or months of music intervention, as we observed previously among pre-hypertensives after listening to *raga Bhimpalas* for 15 min a day, for a minimum of 5 days a week, followed up after 3 months duration (23,38). In comparison, the three other modes (*raga Ahir Bhairav, Raga Kaunsi Kanada, and Raga Bhimpalas*) reduced the state anxiety

levels, with *raga Kaunsi Kanada* causing maximal reduction (22). The current study's findings are similar to this, wherein any music intervention reduced anxiety levels, but the level of reduction depended on the melodic mode and, probably, its features. A reduction in anxiety after listening to music is the most consistent finding reported in field studies with patients (84–86) and laboratory-based studies (15,84,85). Music may be a way to help young people reduce negative emotions (15). Another study suggested listening to relaxing music (based on the music rating scale); in particular, classical music led the listener to experience positive emotions (STAI-Y and Relaxation Rating Scale) and an increase in parasympathetic nervous system arousal (physiological assessment of HR, respiration, and skin conductance) (15).

4.2. Biomarkers of Stress

In the current study, we observed that the mean sCort levels reduced in all three music intervention groups during the intervention, but maximally in the control group, which was statistically significant. This is similar to previous research that found lower sCort in the music group when compared to the control groups, but the levels were lowest when participants listened to the sound of rippling water (used as an acoustic control condition) (12). In the current study, the control group received natural sounds for about 50 s in the mid ten min, indicating that natural sounds have a higher impact on cortisol levels. Several studies have documented either no change or a drop in sCort levels either after listening to music or passive listening to music during a stress task (87–90). A decrease in serum cortisol levels was found to be better among men compared to women upon listening to music by Mozart and Strauss (91). A recent study also observed that the listening environment mattered for this change in cortisol levels or emotions, in that, the cortisol levels were generally lower at home than in the laboratory, though it reduced both in home and lab settings after listening to music (92). In the current study, the mean sAA levels reduced in all four groups significantly during the intervention, after which the levels increased slightly more than baseline levels. Post hoc analysis showed a significant maximal drop in sAA with *raga Malkauns*, followed by *raga Miyan Ki Todi* and then *raga Puriya*. The rise post-intervention was maximal with *raga Malkauns*, followed by *raga Miyan Ki Todi* and *raga Puriya*. This observation is in line with the HRV changes during

and post-intervention, where *Malkauns* exhibited a parasympathetic response during the intervention, while *Miyan Ki Todi* and *Puriya* showed sympathetic responses. A study observed an association between sAA and music-induced arousal, with energizing music increasing and relaxing music decreasing sAA. They proposed that the best effect of music was recorded when participants listened to the music with the intent of 'relaxation', which might lead to reduced sAA and sCort levels (47). In addition to this, another study revealed that listening to Tibetan music before surgery reduced the state score and sAA levels, while in the control group (who wore headphones with no sounds), the state score remained unchanged and the sAA level increased (93).

4.3. Cardiovascular Parameters

4.3.1. Blood Pressure

The changes in SBP and DBP were insignificant in all the groups of the present study. A large change in BP was not expected, as all individuals were healthy, aged 18 to 30 years in the current study. Subtle differences in BP were observed in the music intervention groups compared to the control group and also between different modes. Most music-based research has used music as an intervention among hypertensives (23,31), or during or after stressful tasks (94), with very few among healthy individuals in the absence of a task. A meta-analysis showed that music therapy, which is a more intensive intervention than the present study, led to a significant reduction in SBP, DBP, and HR compared to those who did not receive music therapy (29). Thus, with a longer duration of listening, across multiple days or sessions, differences in SBP and DBP would be evident. Self-selected sedative music induced both aroused and sedative emotions and a slight but significant increase in HR (95). In the present study, participants were not given a choice to select their music, or their preference was not taken into account during the planning of the intervention. In addition, continuous monitoring of BP fluctuations might have aided us to obtain more conclusive results.

4.3.2. Heart Rate Variability

In the present study, we found that the mean NN interval increased and the mean HR reduced significantly in all four groups. The maximal significant change in HR was

with *raga Malkauns*. When looking at the time and frequency-domain measurements we observed that *raga Miyan ki Todi* (group A) and *Puriya* (group C) caused arousal effects in the form of a drop of SDNN, RMSSD, TP (ms^2), VLF (ms^2), LF (ms^2), and HF (ms^2) during the intervention, with significant relaxation after the intervention was stopped. SDNN and RMSSD are strong indicators of parasympathetic activity (37,96). This shows that *raga Miyan ki Todi* and *Puriya* caused sympathetic arousal during music while increasing the parasympathetic response after the music has stopped. This seems similar to previous studies showing increased sympathetic activity, regardless of the type of music (calming or stimulating) (46,97), and a classic paper by Bernardi et al., where a pause after playing music for 2 min exhibited the maximal relaxation response (40). In contrast, the *raga Malkauns* results went hand-in-hand with the control group, wherein a sustained increase in parasympathetic response was observed over 30 min. These findings are partly in sync with the BP results quoted above, suggesting that the changes observed between the cardiovascular and the autonomic system were in parallel. A recent review observed that, out of 29 randomized trials and pre to post-intervention studies, 26 studies suggested a significant positive impact of music on HRV (50). In a study where *raga Malkauns* were used as an intervention in different forms (vocal rendition, sitar recital, and *Rabindra sangeet*), *Rabindra sangeet* had the most relaxing effect. In addition, *alaap* delivered at a fast tempo increased excitement, while, like the current study, *alaap* at a slow tempo resulted in calming the mood (56). Unlike in the current study, meditative music has been shown to reduce state anxiety and HR, and increase the HF norm of HRV (98). These results also confirm the importance of analysis of temporal changes in physiological parameters when using music as an intervention, as the music unfolds over time (99). Other studies on HRV using music intervention have been detailed before in (38,50). Regarding the mechanism behind the effect of auditory stimulation and cardiac autonomic regulation, it was hypothesized that pleasurable songs induce dopamine release in the striatal system, which is involved in autonomic regulation, and this topic has been well-reviewed in (100).

4.4. Future Directions

The current study used a triple-blinded, randomized control trial design and showed that listening to three different Indian modes caused behavioral and cardiovascular modifications among healthy adults. This is the first study of its kind to focus on how Indian melodies may alter physiological measures related to stress, arousal, and anxiety. Clinically, this study promotes the idea of the use of music, and particular modes, to facilitate relaxation, prevent cardiovascular disease, and provide an alternative treatment strategy. Future studies may find it beneficial to expand the present findings to other melodies, provide longer periods of music listening, and more closely investigate in both males and females how reproductive steroid hormones may play a role in the physiological measures assessed. It would also be interesting to investigate factors related to perception and emotion, such as personality and music preferences, in future work. Further analysis of the musical features and the components (e.g., temporal analysis of note/tonal variations, pitch, tempo, dynamics, contrast) of the music used may enhance our understanding of the physiological effects.

5 Conclusions

Among the different relaxation therapies known to us, music is an important modality, as it is an easy-to-follow, easy-to-use, inexpensive mode of relaxation. This study provides evidence that listening to music for just 10 min can have an acute reduction in anxiety and improvement in cardiovascular parameters, depending on the mode. Future studies may try to elucidate the role of music after intervention over a longer duration or a few months of intervention, as done in (1,2). Though all three modes (*ragas*) reduced state anxiety scores, *raga Puriya* caused a maximal reduction in state anxiety scores, followed by *Malkauns* and *Miyan ki Todi.* Cardiovascular effects went in hand with the behavioral recordings, in that *raga Puriya* and *raga Miyan ki Todi* produced an arousal effect during music intervention but caused significant relaxation after the intervention was stopped. In contrast, *raga Malkauns* reduced state anxiety, significantly increased the mean NN interval, and reduced HR. This proves that listening to music, in general, cannot be said to produce a relaxation effect; rather, the timing of the effect, the notes/tones present in the music given, and the combination of notes (modes) as a whole

that produces a particular effect. Future studies need to emphasize the health benefits of various aspects of different acoustic stimuli, including other types and genres of music, and establish solid evidence for the usage of the same in different medical disorders.

6 Supplementary Information

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ejihpe12100108/s1 (101,102,103,104,105).

S1. Music intervention – Melodic scales in detail

***Miyan ki Todi*, the *raga* A of this study,** is a Hindustani classical raga that gave its name to the *Todi thaat*, one of the ten modes of Hindustani classical music, also known as *Darbari Todi*, and sometimes Shuddha Todi, is amongst the more popular morning ragas of Hindustani music. The scale of *Miyan ki Todi* is *Arohana:* S r g m d N S' or 'd 'N S r g m d N S' or S r g m d P, m d N S' or S r g m P, m d N S' and *Avarohana:* S' N d P m g r S or S' N d P m d m g r g r S. *Vadi* and *Samavadi* are *Komal Dha* and *Komal Ga*. *Re, ga,* and *dha* are intoned slightly low, and *tivra ma* is very sharp. Bhatkhande pronounces *Komal Dha* as *Vadi* (primarily dominant), but some musicians accord this status to *Komal Ga*. According to him, *Komal Ga and Komal Re*, are candidates for the status of *samvadi* (secondary dominant). *Todi* is a *Raga* of the late morning. The prescribed time for the raga is the first three-hour slot after sunrise. The equivalent *raga* in Carnatic music is *Shubhapantuvarali. Miyan ki Todi* predominantly is mostly pervaded by a pensive, mournful mood which is then relieved in the *drut* (faster tempo) part, by a festive piece, possibly to alleviate the heavy pathos in the earlier stages of rendering, though not always. The composition is such as to afford an artist of high caliber to mold it in either the inherent pensive mood or to entirely present a festive mood. Despite this, the raga has attained a decent presence in the classicist as well as romanticist genres of Hindustani music. The common phrases used in this scale are: N. N. S r g / r g r / r g M^ P or r g M^ g P/ g M^ d P / M^ g M^ d / N d P / d d N S’ [or] M^ d N S’/ N S’ r’ g’ r’/ d N S’ r’ g’ / r’ g’ r’ S’/ N r’ N d P / M^ P d M^ g [or] N d M^ g / r g r S [1]. Popular songs based on this *raga* are: *Bhini bhini bhor (Asha Bhosle's Album Dil Padosi Hai), aeri mai to prem diwani mera dard na jane koi (A meerabai bhajan from the movie ï Meera), Watan pe jo*

fida hoga (movie ï Phool bane angaare)[2], *oora serabahude neenu (title track of T N S e e t h a r a m K a n n a d a s e r i a l ó m a g a l u j a a n a k i ô) .*

Raga Malkauns belongs to *Kalyan thaat*, and is a majestic and somewhat introverted pentatonic raga. Ma is the pivotal tone of this *raga* and the tone in which the first string of the *tanpura* is usually tuned. *Ga, Dha,* and *Ni* may slightly oscillate. *Malkauns* should be performed in a slow and dignified manner, and to bring out its ethos the notes should be linked by glides, in particular N / D, D / M, and M / G [3]. Time: Late night, 12 - 3. *Aarohan* (ascending scale): S G M D N M D S*; *Avaraohan* (descending scale): S* N D M G M G S, D S; * indicates a higher (third) octave. The *Rishabh* and the *pancham* are skipped in the scale. It is an *audhav - audhav* (5 notes in ascent and descent of the scale) *vakra (nishad* is rarely employed in *avaroh)*. The *vaadi samavaadi swaras* for this raga are d and g. The *vishranti sthaan* for this scale are G; D; S'; - D; G;. Example of *sanchar* (move/ phrases / flow) through this *raga*, S; G M D G M G ; M G ; G S ; ,D ,D S ; ,N ,M ,D S ; S G M D ; G M G ; M D S' ; N M D ; G M M G ; S ; ,D ,D S;. It is this preponderance of the *tivra madhyam,* thus intense training is required to perform this raga. Time for best effects is between (12 night - 3 am): 3rd *prahar* of the night (*Ragas* are divided into *prahaars* whereby each *raga* has a specific period of the day when it is performed). The popular Hindi film songs based on *raga Malkauns* include *Aaye Sur Ke Panchhi Aaye (Movie - Sur Sangam), Adha Hai Chandrama Raat Adhi and Tu Chhupi Hai Kahan (Navrang), Man Tarapat Hari Darshan Ko (Baiju Bawra)* [3]. **Malkauns was the *Raga* B in this study.**

Raga Puriya is a major hexatonic raga (*Shadhav ï Shadhav*) of Hindustani classical music, belonging to the *marwa thaat*. Best performed just after sunset (2nd prahar of the night). What is common among all types of *Puriya raag* are *komal* (flat) *Re*, *shuddha* (natural) *Ga*, *tivra* (sharp) *Ma*, and *shuddha* (natural) *Ni.* Aarohan: N r G M D N r S and avarohan: S N D M G r S N or r N D M Gg, M G r S. *Pancham Varjya. Rishabh Komal, Madhyam Teevra*. Rest all *Shuddha Swaras*. Mandra Saptak Nishad is the Nyas swar in Puriya. Illustrative combinations are: N r G ; G r ,N ,D ,N; ,N ,M ,D S; G M D N; N M G; G M D G M G; r S; G M D N D S'; N r' N M G ; G M D G M G r S [4]. In this *raga*, N-M and D-G *sangati* is observed. Nishad is often skipped in Aaroh like G M D N D S'. *Raag*

Puriya is often referred to as king of night *ragas*. The rasa / emotions related to this raga are *Shanti* (equanimity/peace) and *Gambhir* (seriousness) [5]. ***Puriya* was the *Raga* C in this study.** Pure *Puriya* has not been very commonly used for film music.

S2. Music exposure and Training:

Of the participants who were trained, 41 of them had received training for more than a year. Concerning musical training, all groups were comparable, with statistically no significant differences between them.

Table S1: Music preference questionnaire descriptive statistics

Variables	Group A	Group B	Group C	Group D	P-Value
	N=37 (%)	N=36 (%)	N=36 (%)	N=35 (%)	
Training in Music					
Yes	17 (45.9)	14 (38.9)	11 (30.6)	12 (34.3)	0.562
No	20 (54.1)	22 (61.1)	25 (69.4)	23 (65.7)	
The trained genre of music					
Indian	14 (82.4)	11 (78.6)	9 (81.8)	10 (83.3)	0.898
Western	2 (11.8)	2 (14.3)	1 (9.1)	1 (8.3)	
Both	1 (5.9)	1 (7.1)	1 (9.1)	1 (8.3)	
Duration of training in music					
≤1 year	2 (11.8)	4 (28.6)	3 (27.3)	2 (16.7)	0.619
> 1 year	15 (88.2)	10 (71.4)	8 (72.7)	10 (83.3)	

Note:

a) N is the number of participants in each group.
b) All the values of the two groups are in absolute values and in parenthesis are in percentages.
c) P value < 0.05 was considered significant
d) P calculated using Chi-square / Fisher exact test.

S3. Salivary Stress markers

Table S2: Post-hoc analysis of sAA and sCort

	Group			Mean Difference (I-J)	Std. Error	P Value.[b]	95% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
Salivary Cortisol in U/mL	D	Pre	Dur	.079*	.024	**.007**	.019	.139
			Post	-.021	.019	.807	-.069	.027
		Dur	Post	-.100*	.021	**.000**	-.155	-.046
	A	Pre	Dur	66.154*	6.542	**.000**	49.456	82.852

Salivary Alpha Amylase in U/mL		Dur	Post	-80.228*	10.720	**.000**	-107.589	-52.866
	B	Pre	Dur	73.108*	7.078	**.000**	54.995	91.220
			Post	-26.663*	8.962	**.019**	-49.597	-3.729
		Dur	Post	-99.771*	10.417	**.000**	-126.427	-73.115
	C	Pre	Dur	63.538*	6.149	**.000**	47.541	79.534
		Dur	Post	-74.661*	7.942	**.000**	-95.322	-54.001
	D	Pre	Dur	60.913*	5.104	**.000**	47.851	73.974
		Dur	Post	-72.714*	7.504	**.000**	-91.917	-53.510
	Based on estimated marginal means							
	*. The mean difference is significant at the .05 level.							
	b. Adjustment for multiple comparisons: Bonferroni.							

S4. Blood pressure and Heart Rate

On comparison of pre-intervention levels of all SBP, DBP, and HR, across the groups, it was observed that they were comparable (SBP, P = 0.708, DBP, P = 0.371, and HR, P = 0.741). Between the groups, we did not find any statistically significant difference (SBP: group A, P=0.794, B, P=0.416, C, P=0.234, D, P=0.215; DBP: group A, P=0.208, B, P=0.484, C, P=0.622, D, P=0.429). No significant differences were observed in SBP in any of the groups (See figure in main text 4a,4b). However, there was a continuous trend of reduction in SBP in groups C and D. In group B, SBP was reduced only during the intervention, and returned to pre-intervention levels after the intervention. In group A, SBP increased slightly with music. Similar to SBP, DBP change was statistically not significant in any of the groups (See figure in main text 4c,4d). A trend of sustained rise was observed in groups B and C with and after the intervention. While in group A, DBP reduced with music and returned to baseline after the intervention. In the control group, DBP increased slightly with the intervention, followed by a mild reduction after the intervention. In groups, B, C, and D a sustained drop in HR was observed throughout the 30 minutes duration, with group C having a higher drop in HR during the intervention (See figure in main text 4e,4f).

Table S3: Pairwise comparison of HR in group A based on time (Pre=1, Dur=2, Post=3)

RANDOM GROUP	(I) TIME	(J) TIME	Mean Difference (I-J)	Std. Error	P value[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
A	1	2	-.676	1.084	1.000	-3.397	2.045
		3	2.676	1.403	.193	-.846	6.197
	2	1	.676	1.084	1.000	-2.045	3.397
		3	**3.351**	1.424	**.073***	-.225	6.928
	3	1	-2.676	1.403	.193	-6.197	.846
		2	-3.351	1.424	.073	-6.928	.225
Based on estimated marginal means							
a. Adjustment for multiple comparisons: Bonferroni.							

S5. Heart rate Variability

Table S4: Comparison of time-domain parameters of HRV between the four groups

			Mean	SD	Min	Max	Percentiles			$F(df_{time}, df_{error}) = F$ value	P
							25	50	75		
Mean NN (ms)	A	Pre	832.57	118.5	620.38	1086.49	743.07	821.55	895.36	(2,70)=8.854	**<0.0001**
		Dur	844.19	127.7	632.18	1121.28	758.83	830.08	905.14		
		Post	855.23	126.7	619.8	1125.15	776.01	839.84	922.31		
	B	Pre	844.49	143.8	557.96	1151.72	746.13	860.74	945.53	(1.585,53.874)=16.908	**<0.0001**
		Dur	869	149.3	573.24	1207.65	760.84	887.42	1002.58		
		Post	877.22	147.6	607.72	1202.98	768.23	877.55	1000.37		
	C	Pre	861.24	129.7	598.65	1179.46	782.3	837.65	953.48	(2,70)=3.642	**0.031**
		Dur	869.06	127.2	595.91	1196.59	776.75	859.66	935.82		
		Post	884.7	133.1	603.84	1233.71	783.54	865.35	951.85		
	D	Pre	843.94	131.3	626.76	1119.1	751.33	811.11	953.43	(1.247,41.143)=5.844	**0.015**
		Dur	862.68	131.2	628.03	1154.47	790.99	828.34	952.19		
		Post	863.42	128.4	587.25	1162.77	790.9	849.58	934.38		
Mean HR	A	Pre	73.44	10.1	55.22	96.71	67.01	73.03	80.75	(2,70)=5.256	**0.008**
		Dur	72.63	10.7	53.51	94.91	66.29	72.28	79.07		
		Post	71.65	10.5	53.33	96.81	65.06	71.45	77.33		
	B	Pre	73.24	13.5	52.1	107.54	63.46	69.72	80.42	(1.625,55.249)=14.987	**<0.0001**
		Dur	71.2	13.2	49.68	104.67	59.85	67.61	78.87		
		Post	70.41	12.5	49.88	98.73	59.98	68.37	78.1		
	C	Pre	71.2	10.7	50.87	100.23	62.94	71.63	76.7	(2,70)=3.907	**0.025**
		Dur	70.44	10.1	50.14	100.69	64.13	69.8	77.25		

		Post	69.28	10.2	48.63	99.36	63.05	69.34	76.58		
	D	Pre	72.78	11.3	53.61	95.73	63.21	74.16	79.97	(1.177,38.837)=5.109	**0.024**
		Dur	71.08	10.5	51.97	95.54	63.39	72.58	76.61		
		Post	70.99	10.6	51.6	102.17	65.4	70.68	75.88		
SDNN	A	Pre	66.07	30.5	19.76	152.49	45.92	61.3	78.39	(2,70)=11.208	**<0.001**
		Dur	64.38	32.1	21.94	167.05	44.35	57.71	69.56		
		Post	74.41	36.1	19.89	165.59	53.12	64.9	94.15		
	B	Pre	63.9	27.9	20.49	125.52	40.1	63.3	82.46	(2,68)=4.066	**0.022**
		Dur	67.11	31.9	16.26	148.53	39.36	66.13	91.04		
		Post	71.2	31.9	19.46	147.24	40.29	72.8	94.73		
	C	Pre	71.83	34.7	28.58	173.05	48.66	64.57	87.48	(2,70)=9.068	**<0.001**
		Dur	66.59	29.9	25.19	136.92	42.75	63.22	79.99		
		Post	78.82	38.5	30.6	182.07	53	68.44	96.62		
	D	Pre	62.12	24.4	20.56	129.43	41.66	58.32	80.07	(2,66)=8.580	**<0.001**
		Dur	64.97	24.1	25.33	128.67	43.92	65.06	83.2		
		Post	71.63	27.2	22.26	132.79	49.61	67.44	90.62		
RMSSD	A	Pre	65.2	45.8	14.76	230.66	37.74	51.57	75.6	(2,70)=4.619	**0.013**
		Dur	63.67	50.3	10	260.82	32.57	50.06	77.09		
		Post	73.15	55.2	13.58	258.84	34.08	56.69	84.53		
	B	Pre	61.71	41.7	8.91	167.28	28.73	58.43	81.11	(2,68)=3.207	0.05
		Dur	66.74	48.3	7.47	191.13	29.97	64.91	80.94		
		Post	67.27	45.3	10.57	195.64	29.56	64.39	87.41		
	C	Pre	70.39	48.3	13.14	185.39	34.76	53.4	97.38	(2,70)=3.34	**0.041**
		Dur	67.64	47.7	14.79	216.71	34.63	54.36	83.76		
		Post	77.34	55.7	17.36	277.13	38.03	55.32	97.57		
	D	Pre	58.99	32.0	11.4	127.27	35.37	48.15	83.45	(2,66)=6.31	**0.003**
		Dur	62.6	31.5	12.18	131.82	37.51	59.58	83.91		
		Post	66.96	33.7	11.4	147.01	40.22	62.03	95.94		
NN50	A	Pre	226.19	132.6	1	486	110.75	253.5	310.75	(2,70)=1.363	0.263
		Dur	213	140.1	0	460	71	213	302.5		
		Post	231.56	144.2	1	484	85.5	231.5	347		
	B	Pre	223.31	185.6	0	566	52	223.5	391.5	(1.435,48.794)=0.248	0.707
		Dur	215	162.9	0	528	68.5	213.5	330		
		Post	219.94	160.1	1	530	74	215	312		
	C	Pre	258.42	190.6	2	664	92.25	198.5	425.5	(1.66,58.243)=0.034	0.946
		Dur	257.28	171.7	3	536	94	270.5	436.25		
		Post	254.64	142.4	12	544	125.5	243	376.25		
	D	Pre	236.47	183.1	1	890	98	201.5	356.5	(1.178,58.352)=0.204	0.789
		Dur	246.94	162.0	2	746	118.5	255	341		
		Post	247.15	137.2	0	496	135.75	280	344		
pNN50	A	Pre	30.45	19.9	0.12	78.41	13.46	30.6	42.34	(2,70)=1.588	0.212
		Dur	30.86	22.4	0	82.64	9.31	31.47	44.11		
		Post	32.97	22.5	0.1	80.66	10.86	30.63	41.06		
	B	Pre	32.19	26.2	0	83.07	6.84	34.25	52.07	(1.393,47.375)=2.147	0.142
		Dur	33.34	26.5	0	80.4	7.33	35.1	52.76		

		Post	35.04	26.1	0.1	82.66	8.44	37.5	55.97		
	C	Pre	34.99	24.7	0.21	75.43	10.87	33.18	54.16	(1.756,61.469)=3.128	0.06
		Dur	35.13	24.9	0.31	82.59	12.33	33.62	52.07		
		Post	37.92	23.4	1.15	79.07	16.76	37.53	56.02		
	D	Pre	31.61	22.5	0.1	71.77	12.06	26.27	52.85	(1.235,40.765)=3.088	0.078
		Dur	33.54	21.9	0.19	72.01	14.58	34.54	52.39		
		Post	35.34	21.1	0	71.62	16.78	35.3	52.54		

Table S5: Comparison of frequency domain parameters of HRV between the four groups

			Mean	SD	Min	Max	Percentiles			$F(df_{time}, df_{error})$ = F value	P
							25	50	75		
TP (ms2)	A	Pre	5095.95	5419.2	420.16	22146.9	2011.06	3380.3	5599.52	(2,70)=7.291	**0.001**
		Dur	4913.12	5759.5	427.13	27246.6	1433.55	3048.35	4568.31		
		Post	6510.2	6838.6	335.16	26477.4	2637.89	3680.8	8644.46		
	B	Pre	4495.09	3770.9	338.84	14823.3	1449.76	3737.17	6117.14	(1.748,61.167)=4.421	**0.020**
		Dur	5048.44	4646.1	258.78	19947.9	1326.04	3668.72	7551.1		
		Post	5875	5286.2	303.78	22966.3	1488.67	4798.3	7988.89		
	C	Pre	6149.24	6509.2	539.63	30386.7	2190.71	4216.17	7983.97	(2,70)=4.702	**0.012**
		Dur	5291.66	5276.2	546.98	20559.6	1510.47	3755.9	6523.42		
		Post	7502.7	8286.2	815.09	33352.4	2270.37	4306.67	9433.66		
	D	Pre	4177.44	3342.3	395.44	14789.2	1641.55	3070.9	6440.53	(2,66)=3.8	**0.027**
		Dur	4567.27	3465.4	645.93	15867.7	1993.28	3449.98	6487.22		
		Post	5255.51	3774.2	432.59	17376	2409.59	4113.7	7291.77		
VLF(ms2)	A	Pre	1060.76	641.9	194.13	2695.84	644.46	902.33	1479.56	(1.595,55.824)=3.127	0.063
		Dur	1117.27	1101.3	157.76	5277.33	448.31	776.43	1164.49		
		Post	1537.73	1568.3	98.11	8131.27	599.09	1127.61	1575.58		
	B	Pre	1113.18	935.5	146.12	3957.64	402.58	746.13	1552.25	(2,70)=4.595	**0.013**
		Dur	1300.72	1047.0	119.82	3847.22	547.56	866.94	1876.43		
		Post	1744.05	1689.5	69.21	7374.79	574.94	1321.68	1798.24		
	C	Pre	1878.68	2249.1	196.83	11816.5	641.15	1129.85	2063.46	(2,70)=5.313	**0.007**

		Dur	1418.72	1908.4	93.97	11297.7	464.35	886.17	1785.35		
		Post	2071.34	2887.3	142.9	15538	625.55	1335.72	2295.61		
	D	Pre	1151.48	1038.4	77.69	5336.71	489.1	892.08	1402.54	(2,66)=0.806	0.451
		Dur	1336.48	1264.7	320.42	7449.73	633.41	1068.22	1632.61		
		Post	1404.73	1012.3	100.14	4537.77	736.91	1225.15	1683.94		
LF ms2	A	Pre	1364.42	1474.0	103.11	7839.03	388.75	924.06	1784.1	**(1.330,46.538)=4.71**	**0.026**
		Dur	1220.28	1409.4	142.11	7303.4	321.76	851.67	1464.24		
		Post	1771.5	1830.6	134.03	7245.26	524.82	1140.08	2012.94		
	B	Pre	1131.91	1010.8	61.49	4601.4	427.35	857.15	1556.1	(1.439,50.378)=2.502	0.108
		Dur	1214.31	1135.3	81.89	4121.94	381.04	709.15	1927.15		
		Post	1524.86	1883.8	167.12	10786.4	571.66	1047.09	1897.06		
	C	Pre	1503.04	1628.1	159.18	7876.05	585	907.87	1820.27	**(1.284,44.942)=4.559**	**0.029**
		Dur	1232.27	1063.5	111.19	4491.08	470.35	980.56	1726.92		
		Post	1960.42	2150.8	257.78	11504	642.69	1050	2973.81		
	D	Pre	1171.37	1188.6	93.36	6494.83	420.6	877.64	1518.69	(1.555,53.393)=1.87	0.172
		Dur	1295.99	1266.7	168.93	5964.73	486.54	881.23	1587.72		
		Post	1539.95	1426.8	217.33	7140.09	663.82	1156.17	2002.34		
LF nu	A	Pre	40.44	15.8	10.04	79.63	29.14	41.43	51.12	**(2,70)=4.535**	**0.014**
		Dur	39.56	16.3	10.1	74.73	29.34	42.78	50.25		
		Post	43.83	19.3	6.18	82.62	25.72	46.46	58.05		
	B	Pre	43.25	21.1	9.16	85.14	25.55	39.75	57.9	(2,70)=0.746	0.478
		Dur	42.41	21.7	9.94	84.62	25.86	38.38	58.96		
		Post	44.33	20.7	13.5	79.48	25.8	40.67	62.77		
	C	Pre	42.29	17.4	14.6	83	29.69	42.93	52.42	(2,70)=2.141	0.125
		Dur	40.66	18.8	11.87	87.23	25.97	37.84	49.43		
		Post	43.48	17.8	6.55	81.41	27.44	45.74	55.97		
	D	Pre	41.73	13.9	20.24	67.72	30.71	41.35	52.37	(2,66)=0.87	0.424
		Dur	41.4	15.4	20.31	79.86	28.86	39.13	51.02		
		Post	43.48	15.1	17.8	70.97	31.55	41.91	52.16		
HF ms2	A	Pre	2300.53	3508.0	102.7	15196.9	407.58	1223.75	2255.82	(2,70)=2.106	0.129

		Dur	2205.29	3845.6	98.3	20973.8	411.45	977.11	1922.42		
		Post	2724	4135.8	91.38	19746.4	492.49	1151.97	2155.6		
	B	Pre	1952.82	2494.1	33.24	11426.6	394.03	1232.09	3079.13	(2,70)=1.677	0.194
		Dur	2223.82	3020.5	22.34	14610.3	323.41	1621.43	2601.11		
		Post	2312.33	2959.0	47.96	13970	399.83	1592.69	2725.47		
	C	Pre	2069.48	2154.3	49.18	8027.73	502.94	1177.53	3043.25	(2,70)=2.227	0.115
		Dur	2150.6	2591.3	97.16	10952.5	476.94	1084.4	2833.33		
		Post	2645.22	2961.8	158.65	11548.2	534.86	1313.53	4035.13		
	D	Pre	1591.07	1545.0	75.73	5533.11	398.95	1067.53	2321.29	**(1.190,39.274)=4.203**	**0.041**
		Dur	1709.74	1479.9	87.44	5035.98	567.68	1302.84	2704.98		
		Post	2047.83	1897.6	97.09	7072.91	558.5	1513.38	3154.41		
HF nu	A	Pre	49.14	14.6	14.43	76.68	40.59	49.39	59.21	(2,70)=2.402	0.098
		Dur	49.43	15.4	22.45	78.69	40.78	49.36	59.43		
		Post	46.47	17.1	12.87	78.73	35.35	41.79	57.72		
	B	Pre	48.8	19.1	11.92	82.58	34.39	51.84	59.4	(2,70)=0.401	0.672
		Dur	50.02	19.1	12.05	78.64	34.13	54.58	63.74		
		Post	49.19	18.8	16.65	80.95	32.09	53.98	64.43		
	C	Pre	50.02	16.2	15.33	80.01	40.03	49.87	62.66	(2,70)=1.759	0.18
		Dur	52.01	18.0	11.5	81.19	43.68	53.86	65.6		
		Post	49.38	17.4	16.37	81.26	38.23	47.1	65.53		
	D	Pre	49.34	14.3	17.34	72.88	35.2	51.35	59.64	(1.716,56.627)=0.775	0.448
		Dur	51.37	14.8	19.02	71.32	38.57	52.97	64.92		
		Post	49.44	14.0	25.14	74.29	36.69	52.51	60.39		
LF/HF	A	Pre	1.01	0.8	0.15	4.16	0.57	0.83	1.26	(1.457,51.004)=4.385	**0.028**
		Dur	0.97	0.7	0.14	3.24	0.57	0.89	1.11		
		Post	1.23	1.0	0.08	5.25	0.61	1.09	1.5		
	B	Pre	1.39	1.6	0.12	7.14	0.41	0.76	1.71	(1.474,51.579)=0.039	0.922
		Dur	1.38	1.6	0.13	7.02	0.41	0.7	1.77		
		Post	1.35	1.3	0.17	4.77	0.4	0.73	2.05		
	C	Pre	1.14	1.2	0.19	5.42	0.47	0.86	1.27	(1.672,55.165)=0.043	0.936
		Dur	1.18	1.5	0.18	7.59	0.44	0.67	1.1		
		Post	1.16	1.1	0.22	4.97	0.42	0.95	1.44		
	D	Pre	1.03	0.7	0.28	3.45	0.5	0.82	1.43		0.746

		Dur	1.02	0.8	0.3	4.2	0.44	0.75	1.27	(1.676,58.663)=0.242	
		Post	1.09	0.8	0.24	2.99	0.52	0.79	1.42		

Table S6: ANCOVA analysis of VLF ms^2 – based on drinking history

	Alcoholism history	**F**	**P value time**	**F**	**P value Time*group**
VLF (ms^2)	Yes	0.833	0.445	**2.828**	**0.027**
	No	**11.121**	**<0.0001**	0.836	0.534
Log VLF (ms^2)	Yes	0.200	0.820	1.438	0.233
	No	**11.315**	**<0.0001**	1.893	0.083

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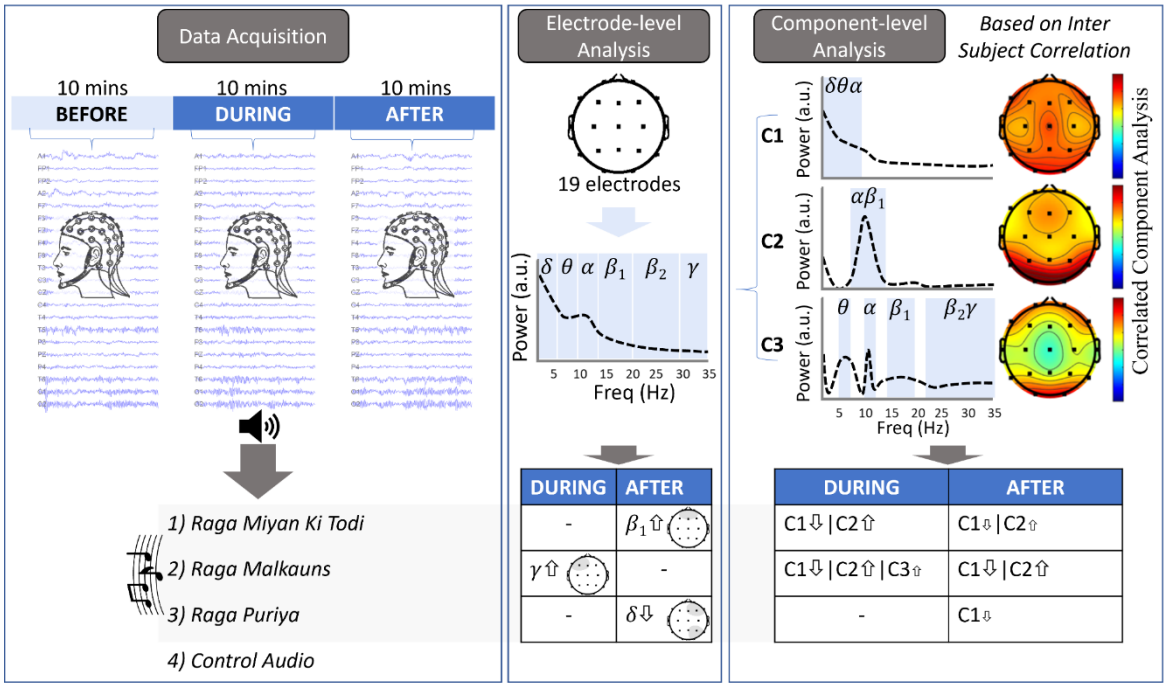
Chapter 3

Neural and cognitive responses to environmental stimuli among humans



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Chapter 3: Neural and cognitive responses to environmental stimuli among humans

Acoustic stimulation with different modes of Indian music on electroencephalographic correlated components and intersubject correlation – a randomized controlled trial

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1 Abstract

Electroencephalogram (EEG) studies evaluating the correlated brain changes and inter-subject correlation (ISC) have often used western music as the acoustic stimulus. The current study employed Indian music to understand inter-brain synchronization. This randomized controlled triple-blind study with four groups (three music interventions: mode or raga Miyan ki Todi, Malkauns, and Puriya and one control group) involved 35 participants in each group. EEG power spectrums before (BI), during (DI), and after acoustic interventions (AI), were measured and analyzed for correlated components (C1, C2, and C3), as well as scores of ISC. A hierarchical general linear model with cluster statistics to the electrode-level data and robust ANOVA with post hoc tests were applied. Results of the study showed that left frontal gamma power increased with raga Malkauns DI, while raga Miyan ki Todi showed a frontal increase in beta1 power, and raga Puriya showed a decrease in right frontoparietal delta power AI. The raga Malkauns and raga Miyan ki Todi groups showed a decrease in C1 (globally distributed low-frequency activity) and an increase in C2 (posteriorly dominant alpha-beta1 activity) power, while raga Puriya showed a weak decrease in C1. In ISC scores, the raga Puriya group showed a marginal drop in C3 (peripherally dominant broad-band activity) after the intervention. These findings demonstrate specific mode-dependent correlated EEG components that persist after the listening period. The short-term functional neuroplastic effects were postulated to be due to default-mode network activity and autobiographical memory. Overall, this study adds to our understanding of the effects of Indian music on brain activity and inter-brain synchronization.

Keywords: Indian music; melodic modes; electroencephalogram; inter-subject correlation; correlated component analysis.

2 Introduction

Studies have shown that exposure to natural environments can reduce stress and induce positive attitudes through stimulation of the sensory systems (1–3). One of the universal ways a human connects with the environment is through engagement, which is defined behaviorally as a commitment to attend to the stimulus. Similar to other sensory experiences, acoustic stimulus, especially music, engages a person and helps to induce and perceive different emotions (4,5). Music not just entertains an individual but affects cognition (6,7), modulates emotions (8–10), and reduces stress (11–13). These responses can be recorded using subjective measures such as emotion questionnaires, valence, and arousal (14) and objective measures such as electrocardiography (15), electroencephalograph (EEG), and functional magnetic resonance (fMRI) (16,17). Previously studies that have recorded EEG with music have predominantly concentrated on the groupwise or stimulus-specific power spectral changes (17–19). It was observed that listening to Indonesian Gamelan music increased EEG beta power, bilateral temporal region blood flow, and freshly recruited the posterior portions of precuneus bilaterally (cerebral blood flow positively correlated with the beta power in these regions) (20), indicating music-evoked memory recall or visual imagery (21). Listening to instrumental music (*raga Desi Todi*), for 20 days (30 min each day) reduced anxiety and increased alpha power on EEG (22). Using multifractal detrended fluctuation analysis (DFA) of EEG, it was shown that listening to a drone instrument (tanpura) increased the complexity of the frontal alpha and theta power (23). Music therapy for stress reduction increased posterior theta power and decreased mid-frontal beta and posterior alpha power, along with a reduction in the degree of anxiety (24). It is important to note that the duration of the music stimulus in several studies ranged from a few seconds to about 3 minutes. A Digital music study conducted in 2019 examined music consumption across people aged 16–64 years, in different geographical locations in India to conclude that Indians spend approximately 19.1 hours of music per week (higher than the 18 hours average in remaining parts of the world) (25). This would make up to about two hours a day. Thus, in a natural setting one listens to music for longer durations. Previously we have shown that there is an overall effect on the EEG power spectrum while listening to different modes of

Indian music, lasting for durations over ten minutes. The highlight of this study was that we noted significant temporal differences within short bins of time intervals (every 2 minutes) that were analyzed as the music was heard over this duration (17). Therefore, while studying engagement to a stimulus such as music, which in itself is a time-based stimulus that unfolds over time, it is important to give music that lasts for relatively natural durations.

Further, when a set of individuals are exposed to the same sensory stimulus, few individuals will have typical experiences and they are said to be engaged with the stimulus. However, the experience of few remains atypical, which is said to be due to the typicality of their stimulus-evoked brain activity (26). This can be measured by inter-subject correlation (ISC), which evaluates the similarity of an individual's brain over some time with that of another individual or a group, in a given region of the brain. Recently, this concept of brain-to-brain synchrony or ISC was reviewed (27), and for a tutorial see (28). ISC is often used with fMRI or EEG data from individuals visualizing a moving stimulus such as a movie clipping or listening to a speech (26,29–33). ISC has been explored using an fMRI approach with relatively natural listening conditions as well as emotion-inducing music stimuli (such as sad music) (34,35). While the advantage of music-related fMRI investigations is source localization, the constraint of fMRI, which may have resulted in an insufficient grasp of music-induced brain responses, is that the conventional experimental setups mandate repeated, and concise stimuli to simulate the anticipated hemodynamic response, thus making the music stimuli given, last for shorter durations. It will be interesting to understand music-based engagement and ISC through the recording of EEG for two reasons, one, Music is a time-based stimulus and EEG is well suited to investigate the time-locked brain responses as the temporal resolution is sufficiently high; second, the melodic modes or scales of music are made up of a set of organized tones at particular frequencies implicated to induce specific emotions, and thus varied brain responses. Essentially observation of synchronized responses, if any, in a group of participants for music indicates a socio-behavioral response to musical stimuli, having implications in the management of mental health conditions generating atypical socio-behavioral network responses (autism, schizophrenia). ISC of neural responses is said to be well-suited for measuring musical engagement (36). Music-induced EEG changes and ISC patterns are now more often studied to identify the unmodelled neural patterns in response to listening or playing music (37–41). A recent EEG-based music study showed that ISC can be modulated by musical training and familiarity with

the musical genre (39). Although previous studies have investigated the brain responses during continuous listening to relatively simple musical stimuli or controlled auditory paradigms (18,42,43), there is a dearth of parallel studies that examine how the human brain processes the multitude of musical features present in naturalistic musical stimuli.

To date, there has been little discussion on changes in EEG power and ISCs on receptive listening to Indian music. Similar to western music, the Indian musical system has several scales or modes (44), which are made of a given set of tones or frequencies, presented in an orderly manner, to generate an emotion of their own. Examining the short-term plastic changes in the brain on exposure to complete musical pieces, made up of specific modes, that may be inducing different emotions, can assist in comprehending the distinctive functions of specific neural regions spatially and temporally in portraying emotional encounters. It is still unknown if ISC varies with different musical/melodic modes within a particular genre. In the current study, we evaluated the engagement of participants while listening to different acoustic stimuli, using different modes of Indian music through recording of EEG changes. This study is part of a larger study where cardiovascular and other physiological parameters were reported after intervention with three modes of Indian music i.e., *raga Miyan ki Todi, raga Malkauns*, and *raga Puriya*. We observed that the state anxiety and salivary stress measures were reduced with all three modes of music. The autonomic changes as measured using heart rate variability, were specific to the mode heard and the time of intervention (45). In the current manuscript, we report the detailed EEG findings, ISC, and correlated component analysis. The main objective was to analyze the spectral variations of EEG power and the cortical response to music during listening to three different melodic modes, in addition to ISCs. We also explored if the effects lasted beyond the intervention period. We hypothesized that different modes of music would induce different electrophysiological changes and would produce specific correlated components of cortical frequency as per the mode heard and due to the repetition of the phrases during the elaboration of a given melodic mode (in the given duration) the overall ISC may reduce. Based on previous studies (30,38), we took an exploratory approach and calculated ISC from the maximally correlated components. We found that each mode induced specific power spectral changes in EEG. Correlated component analysis of the EEG, during and after the intervention, revealed that there was a decrease in low-frequency and an increase in high-frequency activity on listening to *raga Malkauns* and *raga Miyan ki Todi*, while *raga Puriya*

showed a weak decrease in low-frequency activity. We did not observe significant changes in ISC scores. This indicated mode-dependent short-term functional cortical effects that persisted after the listening period.

3 Materials and Methods

3.1 Study design & ethics

This was a prospective randomized controlled trial with a triple-blind design (part of a larger trial (45)) with 140 participants who were randomly divided into 4 groups, with 35 participants in each group. Though triple-blinded in design, once the participants were given the intervention they knew if they were in the music or the control group. Albeit the participants were familiar with Indian modes and tones due to their cultural background, none of them had been exposed to this musical piece before. Each group received one of the acoustic interventions, group A received *raga Miyan ki Todi*, group B received *raga Malkauns* and group C received *raga Puriya*, with group D as a control group. We recorded EEGs during three tasks: silence, music listening, and silence (each lasting 10 minutes) and compared them with the control (no music). The study period ranged from 2019 to 2021 (June 2019 - first recruitment and February 2021 - last recruitment). The data presented here were taken from a larger experiment (full trial protocol: NCT03790462 on clinicaltrials.gov.in). The research was conducted following the Declaration of Helsinki guidelines. The study protocol was approved by the institutional scientific committee on human research and the ethical review board (Reference: MSRMC/EC/2017, dated: 25/07/2017).

3.2 The basis for sample size

The sample size was calculated based on a previous study where the change in State-Trait Anxiety Inventory-6 (STAI-6) anxiety median and IQR scores was from 33.3 (23.3–41.7) before music intervention and to 30 (20–40) after music intervention. Considering the minimum difference of 4 units in the STAI score, with an effect size of 0.7, power of 85%, and an alpha error of 5%, the sample size was calculated to be 35 in each group (46).

3.3 Recruitment

The study participants were recruited from a group of educational institutions in the city of Bengaluru, Karnataka, India. Healthy right-handed aged 18–30 years were invited to participate in the study via an open call for participants for the study posted online (social media) and notice board advertisements across the institutions. Given the objectives of the study, to avoid cultural familiarity differences, only Indians were invited to participate in this study. Participants who responded to the call were sent an online questionnaire via Google forms, as explained in (17,45). They had to be fluent in English, with normal hearing, and without cognitive or decisional impairments, and those who were not smokers or alcoholics were invited to participate in the study. Participants with any past or current medical or surgical disorders, and self-reported BMI of over 30 kg/m2 were excluded from the study (Fig 1). The rest of the participants were invited to the lab for further recordings.

3.4 Randomization

A simple randomization technique was used to randomly select participants into four groups (Fig 1). The random numbers were generated using MS Excel (4 sets of 35 each) and sealed in an opaque envelope. The serial number of the participants was written on the top of the envelope. The envelope was opened by the research assistant after the baseline assessment of each participant and then they were assigned to their respective arms. The participants knew understood that they were in the music intervention group once the intervention began, though they did not know the mode. All the investigators who did the outcome assessments were blinded to the interventions.

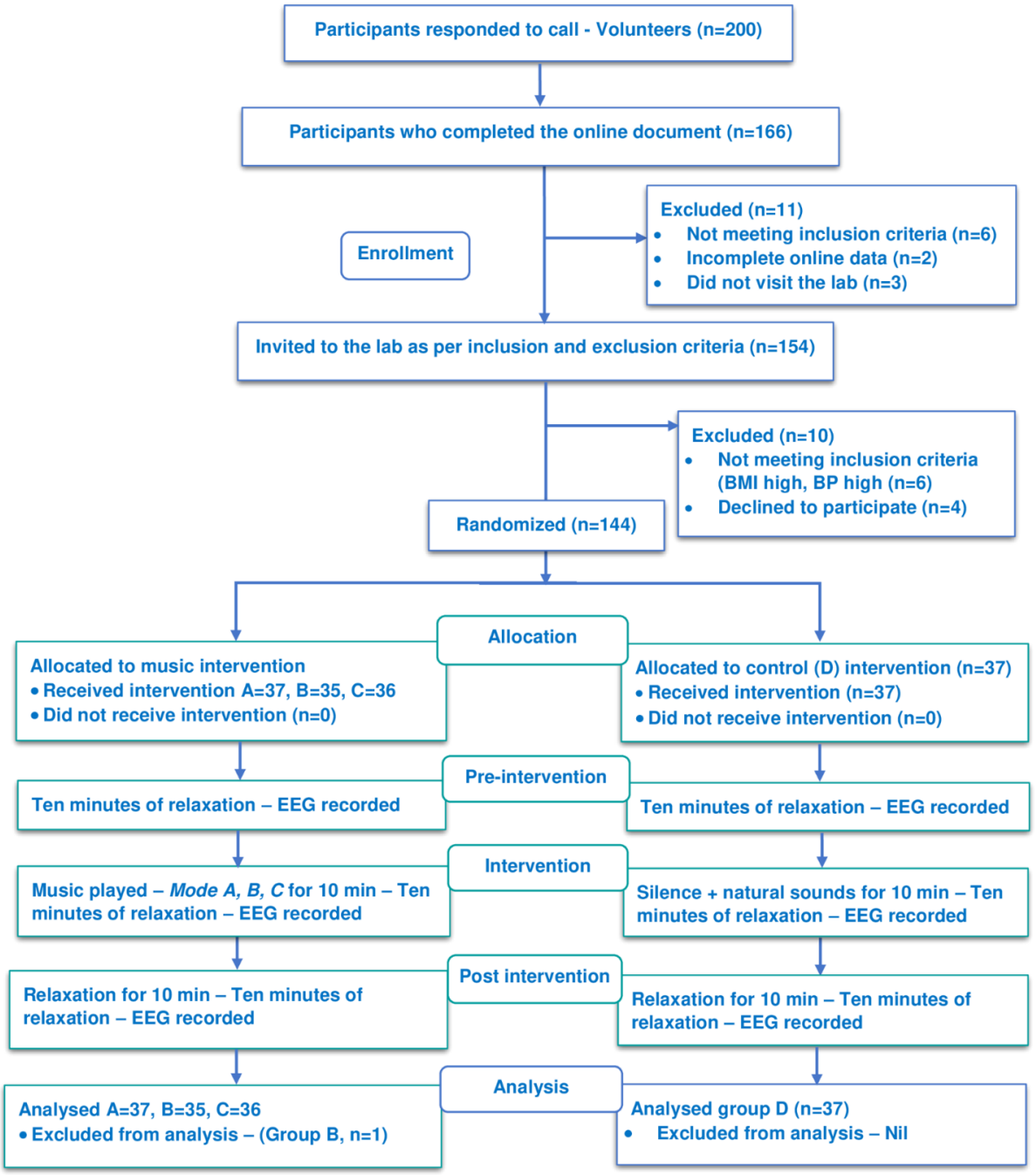

Figure 1: Consort diagram of participant recruitment, distribution, and follow-up

3.5 Intervention

All participants listened to the acoustic intervention through headphones [considered ideal according to review (47)], connected to a laptop, at a uniform volume (50%). The acoustic stimulus was coded as A, B, C & D by a person uninvolved in the

project. We instructed the participants to listen to this with their eyes closed, and minds relaxed, for the duration it was played (10 to 12 minutes). Three melodic scales (modes/ragas) were chosen and implemented as interventions. Indian classical melodic scales/modes (*ragas*) *Malkauns* (pentatonic: C, E ♭, F, A ♭, B ♭), *Puriya* (hexatonic: C, D ♭, E, F#, A, B) & *Miyan ki Todi* (heptatonic: C, D ♭, E ♭, F#, G, A ♭, B) were chosen based on our previous work [Table 1], where we standardized the music (11,12,45). In this study, solo instrumental Indian music modes were used, without percussion or lyrics, ensuring uniformity of the intervention. As repetition is said to reduce ISC values (30–32), the acoustic stimuli were not repeated within the same individual or between groups.

Table 1: The three chosen Indian melodic modes, the names of the notes in Hindustani music, and Western scale equivalents.

Svara/Note	Hindustani Name	Staff Note	Western Scale Interval Name
	Raga Miyan ki Todi (Scale A) (heptatonic, G appears in descent)		
S	*Shadja*	C	Perfect unison
r	*Komal Rishab*	D ♭	Minor second
g	*Komal Gandhar*	E ♭	Minor third
M	*Tivra Madhyam*	F#	Augmented fourth
P	*Pancham*	G	Perfect fifth
d	*Komal Dhaivat*	A ♭	Minor sixth
N	*Shuddha Nishad*	B	Major seventh
	Raga Malkauns (Scale B) Ascent and descent sameð pentatonic		
S	*Shadja*	C	Perfect unison
g	*Komal Gandhar*	E ♭	Minor third
m	*Shuddha Madhyam*	F	Perfect fourth
d	*Komal Dhaivat*	A ♭	Minor sixth
n	*Komal Nishad*	B ♭	Minor seventh
	Raga Puriya (Scale C) C, D ♭ E, G ♭ G, A/A ♭ B (hexatonic)		
S	*Shadja*	C	Perfect unison
r	*Komal Rishab*	D ♭	Minor second
G	*Shuddha Gandhar*	E	Major third
M	*Tivra Madhyam*	F#	Augmented fourth
D	*Shuddha Dhaivat*	A	Major sixth
N	*Shuddha Nishad*	B	Major seventh

The music was tuned to a frequency of 329.63 Hz (the tonic or '*Sa*' at pitch E). The music used for this study was 10 to 11-minute-long instrumental (Flute/*Bansuri*) music recorded by an eminent musician (exclusively for the present study), playing the improvisation in the respective scales [named *alaap* (12,45,48) in Indian music]. We chose the flute as the instrument to play the melodic scales, as only musical components such as pitch, intensity, rhythm, and timbre would be present, without the lyrical and percussion components. Participants in group D (the control group) were given headphones similar to the intervention group. The audio clip consisted of predominant silence for over 10 minutes. The participants in the control group had to lie down in a supine position, with the complete recording lasting for over 30 min duration, it was possible for the participants to feel sleepy (sleep is anxiolytic, which would alter the current objective, and would induce other sleep-related EEG changes). Thus, natural sounds (birds chirping and flowing river) were played for 10 s duration once every 2 min in the middle ten min (during intervention); a total of 50 s in the middle ten minutes. This also ensured uniformity of intervention between the groups, similar to our previous study (17,49–57).

3.6 Process of recordings

Recordings of EEG were done in a supine position with eyes closed, with the first five minutes utilized for attaching EEG electrodes. The participant was asked to relax with eyes closed for the next 30 – 35 minutes when the EEG was continuously recorded, with the event marking artifacts such as eye movement, jaw movement, and acoustic intervention played through headphones, in the mid 10 minutes (Fig 2). We concentrated on the effects that would naturally occur during acoustic stimulation, without the participation of the participants in any particular cognitive task (58). Subsequently, the participant's heads were cleaned and relieved. The recordings were made using a 19-channel EEG system (Galileo Mizar Lite, EB Neuro, Italy), with silver chloride electrodes (Ag-Cl) placed on the scalp after the 10-20 international system of electrode placement [active electrodes were placed in Fp1, Fp2, F7, F3, Fz, F4, F8, T3, C3, Cz, C4, T4, T5, P3, Pz, P4, T6, O1, O2 and the reference electrode in the ear lobes (A1 & A2)].

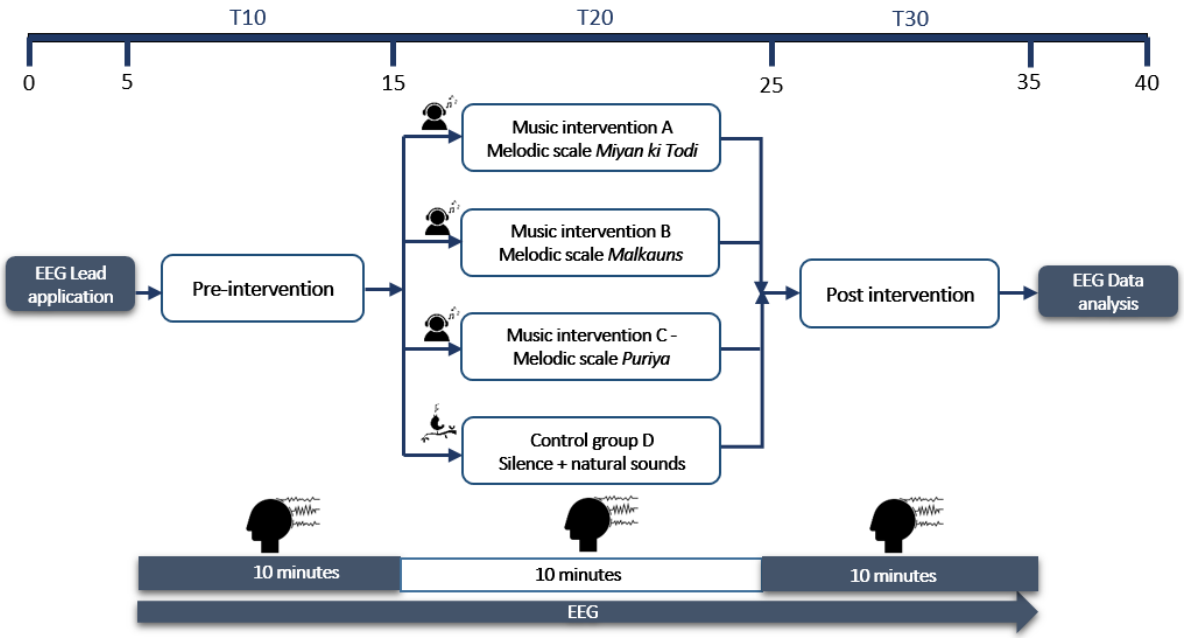

Figure 2: Flow chart describing the process of recording

3.7 Electroencephalographic Data Analysis

Raw recordings of 3 conditions were marked before, during, and after interventions and stored in GaINT software (EB Neuro, Italy). These were then converted to the standard European Data Format (EDF). As the EEG data exported from the device did not have markers, a researcher manually recorded the timings for each of the 3 study conditions (i.e., Before, during, and after the intervention) in the device software. These timings were carefully corrected for data clipped off during acquisition pauses (there was at least one pause per subject). Using custom code written in MATLAB software, event marker files (.evt files required for the next step) were generated for each EEG file, denoting the 7-minute continuous segments within each of the 3 study conditions (skipped the initial 1 minute of each condition to avoid artifacts during transitions) (17). These data were then subjected to pre-processing using EEGLAB (59) functions (version: 2021). This included: 0.5-40Hz bandpass filtering, automated bad channel and bad segment removal by artifact subspace reconstruction (ASR) approach (60), bad channel spline interpolation, and average re-referencing - all done with custom written codes in MATLAB software (version: 2021b). The cleaned EEG data would be discontinuous with multiple short bad segments removed (varied across participants) and ‘boundary markers' added

to handle boundary effects during further analysis (which do not require long continuous EEG segments). Therefore, these were further visually inspected and only data from participants who had a total of at least 3 min of good quality EEG for all three portions were chosen for further analysis.

Band power was evaluated using FFT-based power spectral analysis on each of the EEG segments (with 2 seconds nonoverlapping Hanning windowed subepochs giving 0.5Hz resolution) and grouped in standard frequency bands (Delta: 1-4Hz, Theta: 4-7Hz, Alpha: 8-13Hz, Beta: 13-30Hz, and Gamma: 30-45Hz), for all 19 EEG channels, across all files. The gamma power was limited to <45Hz) due to the low sampling rate (128Hz) and potential filter effects in this frequency band.

As multi-channel EEG captures spatially distributed activity of prominent brain networks, instead of analyzing individual electrodes, we decided to use a linear combination electrode-level spectral activity that captures EEG spectral activity most correlated between participants. Correlated component analysis (CorrCA) helped achieve this and is conceptually based on canonical correlation analysis. This approach has been used in prior studies on time-domain data to extract multi-electrode EEG components related to music listening and video viewing (30,39). We used frequency domain data, i.e., the average power spectral data within each 10 min condition, for CorrCA. CorrCA also gives the forward model topography and time series of the components which could be used for spectral and other analyses that are typically done on single electrode data. For CorrCA, we used the code available at http://www.parralab.org/isc/. For the current analysis, we performed CorrCA on compiled spectral data from each group separately to capture the components most prominent to each intervention and selected the first three components per group with the highest ISC values at the group level. The subject-level total power across the spectra of each component was examined for the intervention effect between groups. Furthermore, the ISC values at the subject level were compared as a measure of engagement within the conditions. For statistical analysis, the data during and after the intervention were subtracted from the data before the intervention and then used for comparisons between groups. The results were also compiled into a Master

Chart for further processing in a statistical tool using other physiological, psychological, and socio-demographic variables.

3.8 Statistical analysis

Data were analyzed using SPSS software version 18.0 (SPSS Inc. Released in 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.). The continuous variables were analyzed using descriptive statistics and the qualitative/categorical variables were analyzed using frequency and percentage. For statistical analysis of electrode-level EEG spectral data, we applied a hierarchical general linear model with cluster statistics to the electrode-level data using functions of the LIMO toolbox in MATLAB software. For statistical analysis of component-level spectral data and ISC scores, we applied robust one-way repeated measures ANOVA on trimmed mean followed by posthoc Yuen's trimmed mean test (20% trimming) (61) and p-values adjusted using Holm's correction (62), using Jamovi software written in R language. Two-tailed p value ≤0.05 was considered statistically significant at a 5% level of significance.

4 Results

All the sociodemographic characteristics were comparable between the groups as we reported in (45), except educational status, which was adjusted for during physiological parameters analysis. There were no differences in familiarity with music or training between the groups.

4.1 Electrode-Level Band Power Changes Relative to Baseline

We calculated the power changes across the scalp for each subject, relative to the baseline power before the intervention, for each frequency band. These power changes were obtained as first-level beta values of a hierarchical general linear model approach described in the Methods section. During the intervention, the most prominent changes were a global decrease in alpha power for all intervention groups and a frontocentral increase in beta/gamma for two of the music intervention groups (*raga Malkauns* and *raga Miyan ki Todi*) (Fig 3). After the intervention, the most prominent change was a frontal decrease in delta power and a frontal increase in beta1 power in most groups (Fig 4).

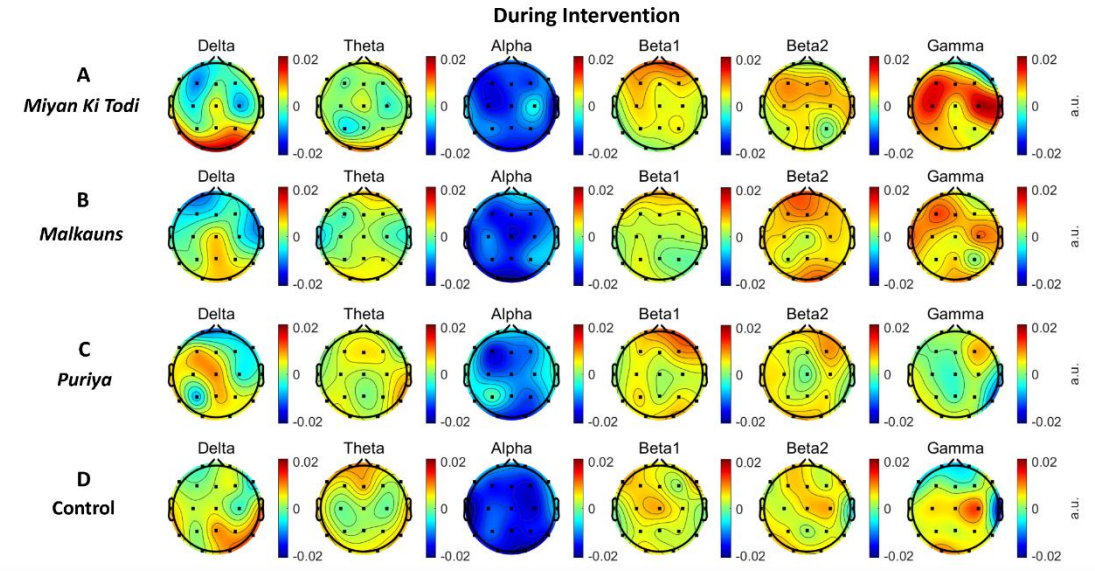

Figure 3: Scalp maps representing the average changes in electrode-level band power during the intervention

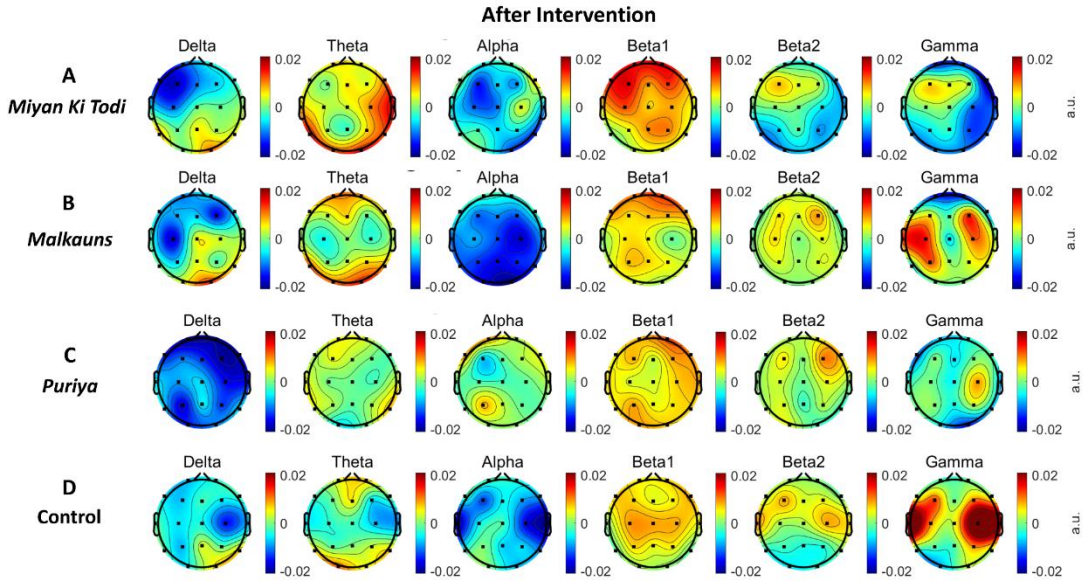

Figure 4: Scalp maps representing the average electrode-level band power changes after intervention

To evaluate the group differences, a second-level analysis was done where each music intervention group was statistically compared with the control group and cluster statistics (tfce) was employed to determine the significant changes. Based on this analysis, the group listening to *raga Malkauns* showed a significant increase in gamma power in the

left frontal regions during the intervention (Fig 5). While the group listening to *raga Puriya* showed a right frontoparietal decrease in delta power and those to *raga Miyan ki Todi* showed a frontal increase in beta1 power, after the intervention (Fig 6).

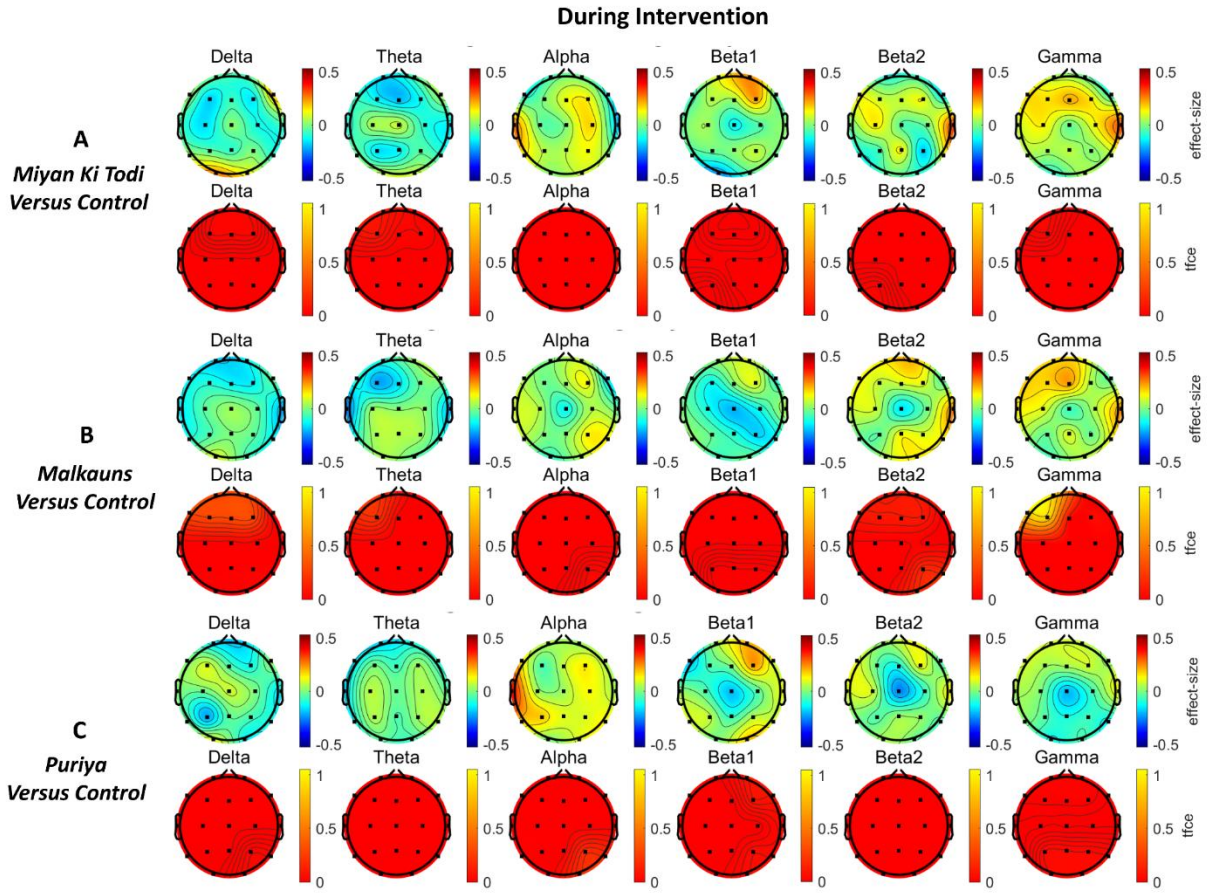

Figure 5: Scalp maps showing the differences between the groups in electrode-level band power between the intervention and control groups during the intervention.

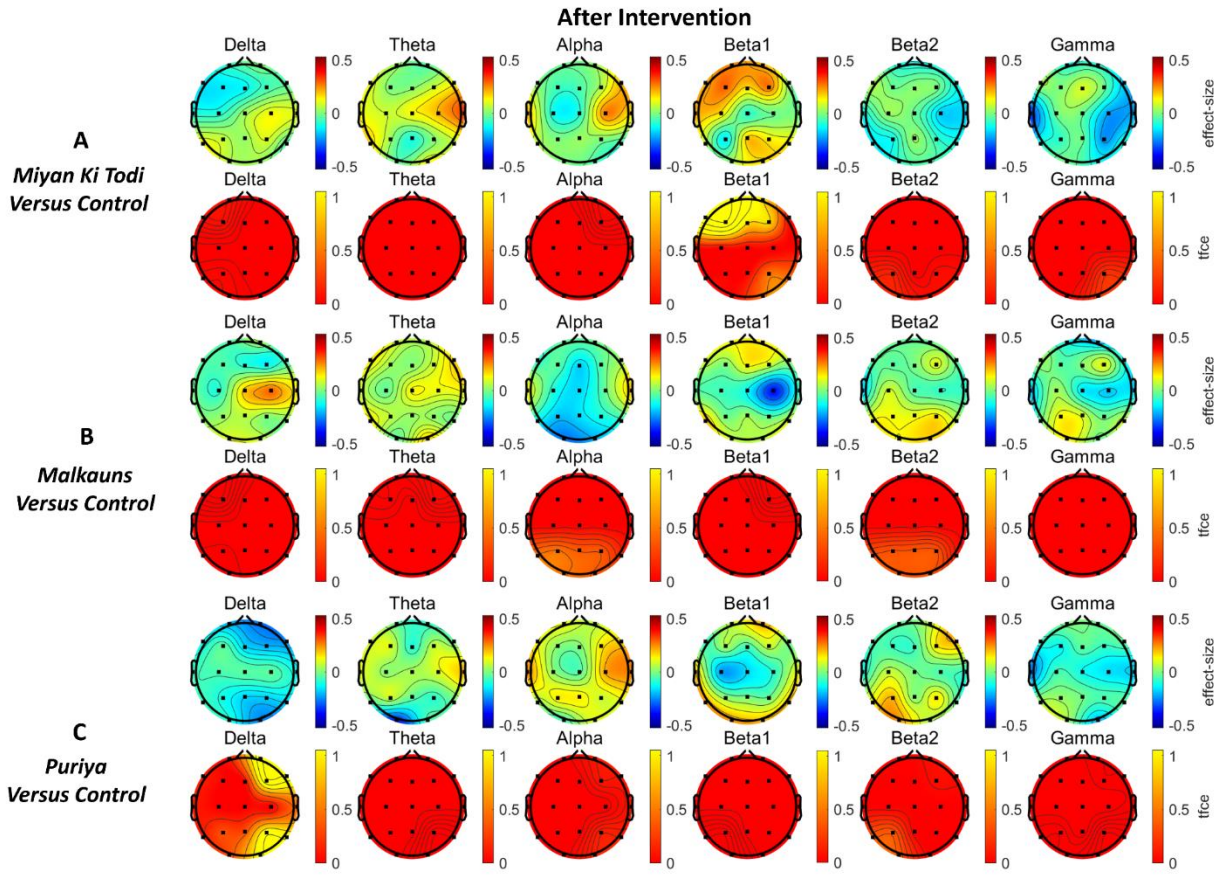

Figure 6: Scalp maps showing group differences in electrode-level band power between the intervention and control groups after intervention.

4.2 Correlated component analysis (CorrCA) based on Inter Subject Correlation (ISC)

To explore the most temporally consistent EEG pattern for the different conditions and groups, we performed a Correlated Component Analysis (CorrCA), which extracts the components correlated between subjects. As our EEG segments were not well timed for intervention stimuli, we used frequency domain data (average power spectral data within each 10 min condition) for CorrCA. Based on the spectral distribution of the first three most correlated components, the first component is globally distributed low-frequency activity (C1), the second component represents posterior dominant alpha-beta1 activity (C2), and the third component represent peripherally dominant broad-band activity (C3) (Fig 7).

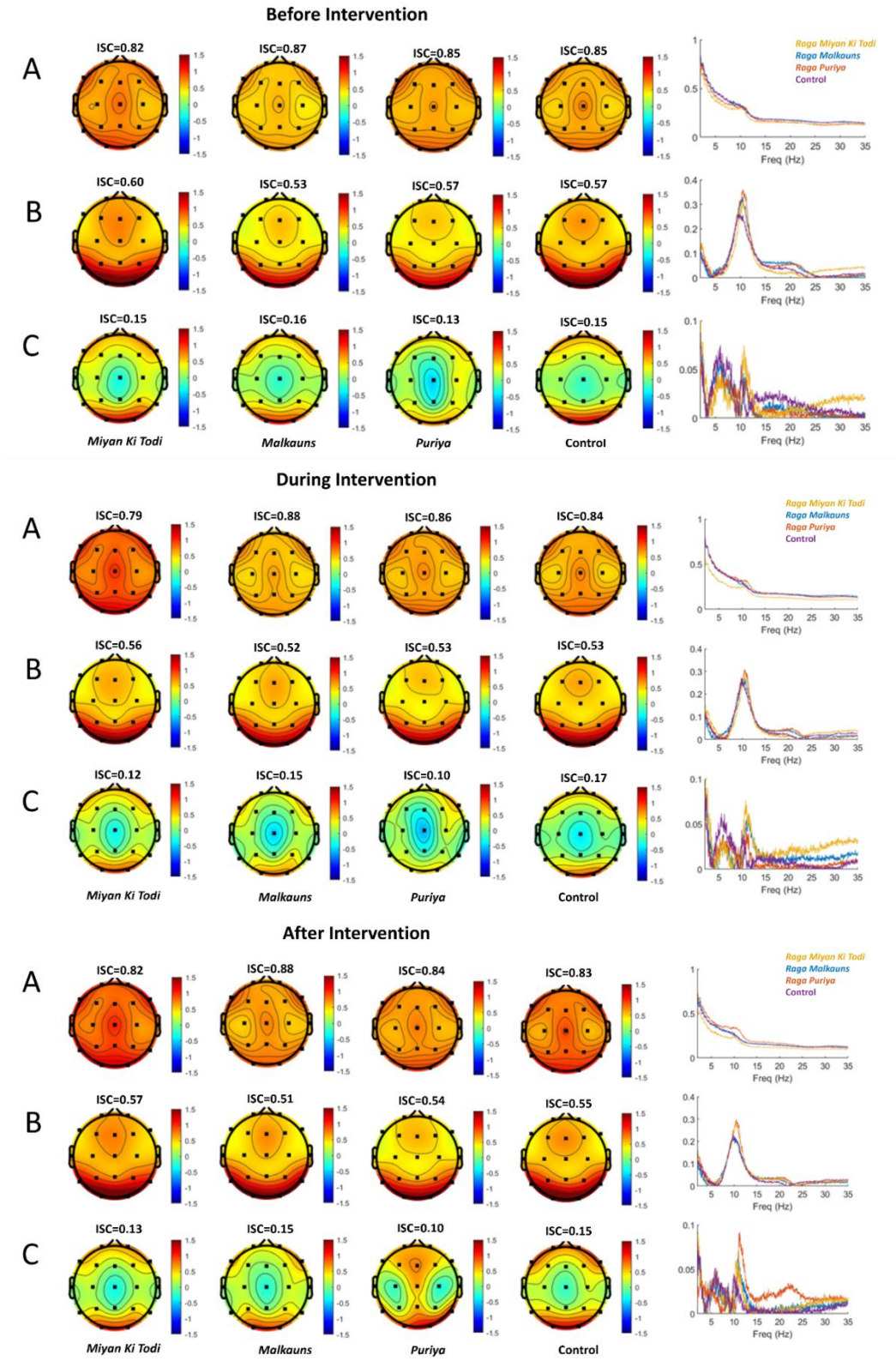

Figure 7: Scalp distribution and spectral pattern of the first three components (C1, C2 & C3) based on CorrCA before (7a), during (7b), and after (7c) intervention.

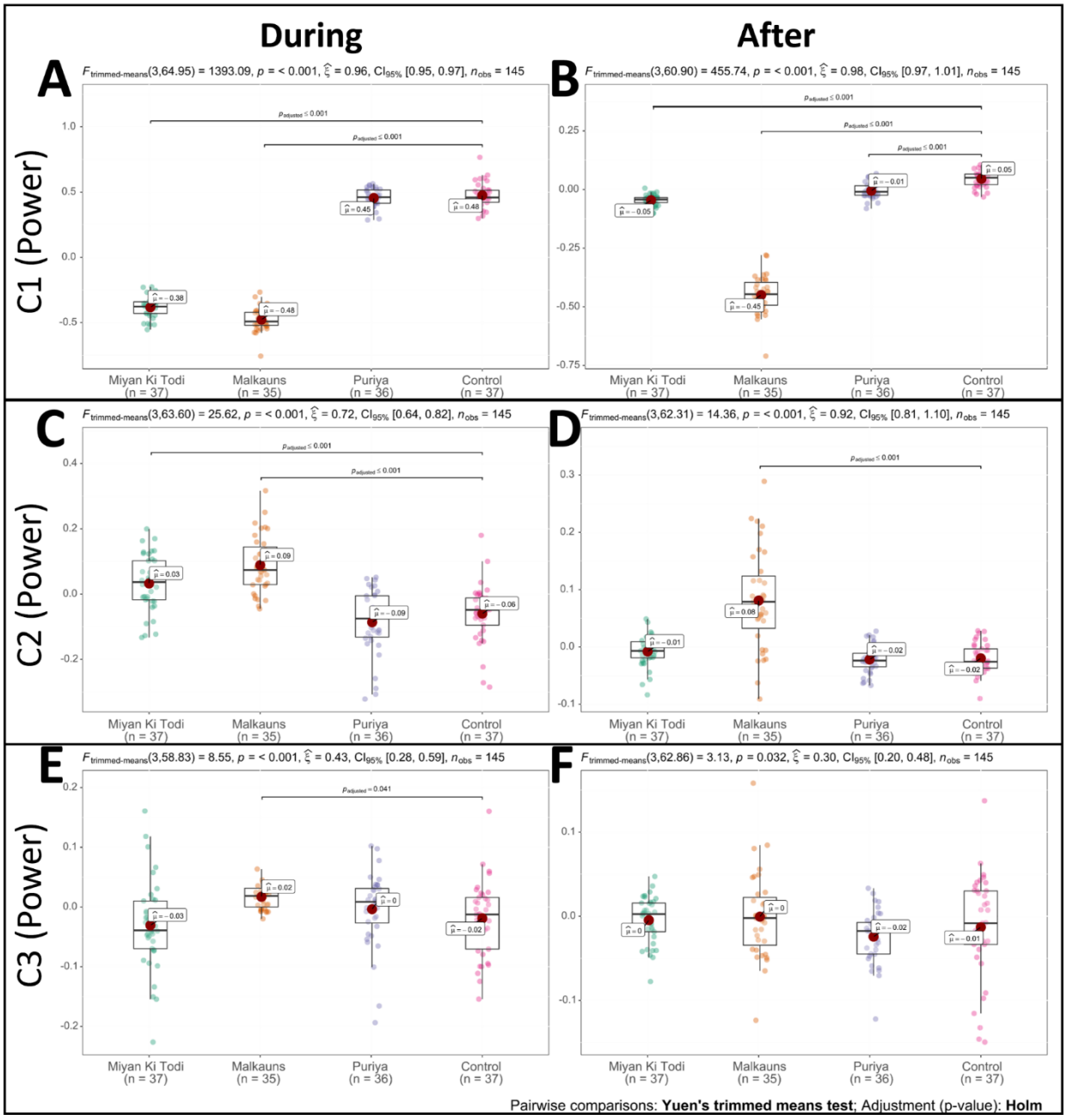

Figure 8: Change in the total spectral power of the components (C1, C2, & C3) between the four groups during and after the intervention, relative to before the intervention [C1 during (8A) C1 after (8B) C2 during (8C) C2 after (8D) C3 during (8E) C3 after (8F) intervention].

In terms of the spectral dynamics of the CorrCA components, both *raga Malkauns* and *raga Miyan ki Todi* groups showed a similar pattern of decrease in C1 power and increase in C2 power during intervention relative to baseline when compared to the control group

(Fig 8). Even after the intervention, this pattern was strong for *raga Malkauns* but weaker for *raga Miyan ki Todi*. Whereas *raga Puriya* showed only a weak decrease in C1 (after intervention), compared to the control group.

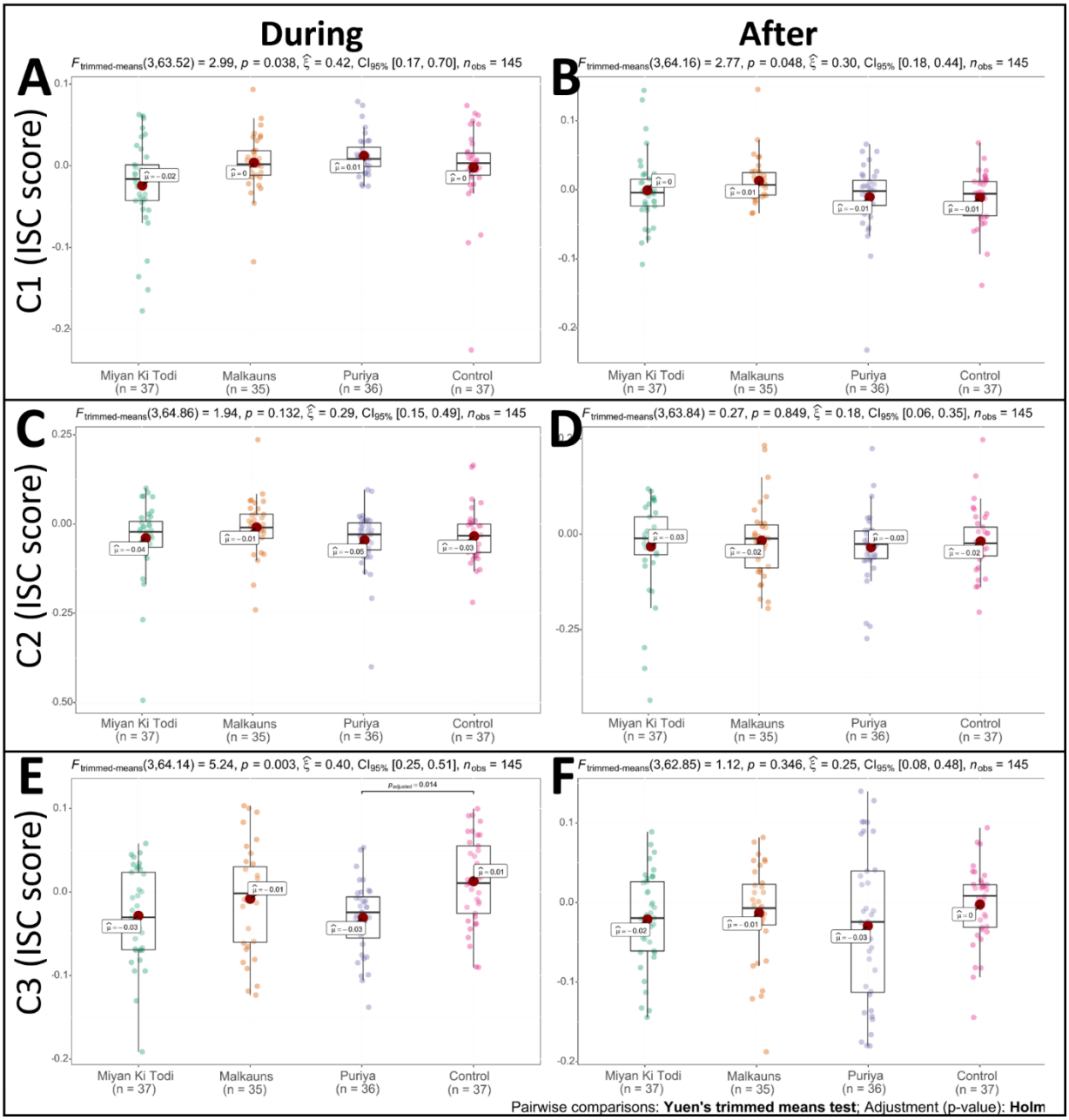

Figure 9: Change in the inter-subject correlation scores of the components (C1, C2, & C3) between the four groups during and after the intervention, relative to before the intervention [C1 during (8A) C1 after (8B) C2 during (8C) C2 after (8D) C3 during (8E) C3 after (8F) intervention].

ISC scores were comparable between groups, except for *raga Puriya*, which showed a marginal drop in C3 after intervention (Fig 9).

5 Discussion

In this study, we evaluated the spectral effect of EEG of acoustic stimuli (three Indian music and one control stimulus) in healthy young participants, the intervention lasting 10 minutes. The three musical stimuli consisted of instrumental music based on different ragas of Indian classical music (*raga Miyan ki Todi, raga Malkauns, and raga Puriya*) that were chosen based on Indian music ancient literature (44,63). The socio-demographic data was comparable across the groups (45). In the electrode-level analysis of EEG, during the intervention, the *raga Malkauns* group showed a significant increase in left frontal gamma power. After the intervention, the *Puriya raga* group showed a decrease in the right frontoparietal delta power, and the *Miyan ki Todi raga* group showed a frontal increase in beta1 power. Exploring this further, the component-level analysis showed a decrease in C1 power (globally distributed low-frequency activity) and an increase in C2 power (posterior dominant alpha-beta1 activity) with *raga Malkauns* (strong both during and after intervention) and *raga Miyan ki Todi* (strong during and weak after intervention), whereas *raga Puriya* showed only a weak decrease in C1 (after intervention), compared to the control group.

5.1 EEG power spectral patterns

On electrode-level power spectral analysis, we saw a decrease in delta power and an increase in beta1 and gamma power, and no significant change in alpha power, in music intervention groups relative to the control group.

Several EEG studies report conflicting evidence of electrode-level power spectral changes (decrease or increase, or null responses) when listening to music. For example, an EEG study that used Mozart's K.448 music (64), reported a significant drop in alpha power during listening globally (F3-C3, F4-C4, C3-T3, C4-T4, T3-O1, and T4-O2) which persisted in posterior sites (T3-O1, T4-O2, O1-C3, and O2-C4) post-music, compared to pre-music alpha power. This study also observed a significant decrease in theta (at T3-

O1 & O1-C3) and beta (at T3-O1, O1-C3 & O2-C4) power during listening, which partially persisted after music. Alpha power is often associated with active inhibition and therefore a drop in alpha power could indicate an overall increase in the level of brain activation (or disinhibition) that occurred when listening to the acoustic stimulus (65) or when actively participating in processing or anticipating a stimulus (66). But since alpha power drop co-occurred with drop-in closer spectral bands (theta and beta) while listening to music, the spectral specificity mentioned before may not be true. Another EEG study that involved listening to popular classical symphonic pieces has shown that the alpha power increased in the parietal and occipital areas of both hemispheres during listening and that the maximum frequency of the alpha band was significantly reduced (19). The authors concluded that the level of brain activation was reduced on listening to music.

A previous study observed a reduction in delta power after slow-wave sleep brain-wave music, and this the authors interpreted to be a positive effect on sleep quality (67). Alternatively, conflicting evidence exists that this reduced delta could be due to lesser sleep (68). Listening to natural music has been found to drive the beta, theta, and delta activity (69), especially when the rhythm of the music falls in these frequency ranges. A MEG study of responses to pain induced during listening to preferred music versus personalized entrainment music found that preferred music reduced delta power in the cingulate gyrus, while entrainment music led to changes in gamma power in the somatosensory regions (70). The drop in delta power after stopping *raga Puriya* in the current study could be due to alertness or divergent thinking after stopping the intervention (71). Other frequency bands found to change when listening to a Mozart musical piece, Sonata K.448, were reduced theta power in the left temporal area; increased beta in the left and right temporal, the left frontal, and increased alpha1 power in the left temporal region (72). It was found that the power of low-frequency brain waves increased in the auditory cortex with a gradual increase in theta and alpha power in the amygdala and orbitofrontal cortex (probable higher analysis of music) with time along with an increase in the power of alpha, theta and beta1 waves in the orbitofrontal cortex while listening to consonant sounds (18). Taken together, there could be a globally distributed alpha activity that decreases along with other frequency bands (like delta, theta, and beta) and a more posterior dominant alpha activity that increases while listening to engaging

music. This is possibly what our component-level analysis captured as C1 power decreases and C2 power increases during and after music listening. Similarly, studies have found a co-occurrence of power changes in higher frequency bands. In one study, enhanced spatial performance after exposure to music was associated with a lower alpha 2 (10.5-11.97 Hz) and a higher beta 1 (12.02-17.97 Hz) (72). In another study, there was a uniform reduction of alpha power and an increase in the gamma high power localized in the electrodes over or nearby the auditory-cortex brain regions (e.g. FT7, TP7, FC3, FT8, T4), during music listening (58). This may be captured by the slight increase in C3 power during listening in the *raga Malkauns* group.

Alpha activity in EEG often increases with an increase in task demands (for example, answering questions about stimuli) (73). The beta and alpha rhythms are seen in the awake state and in that, beta rhythm is usually associated with increased alertness, cortical integrity, stress, strong emotions, and cognitive processes (74,75). As reported before, a significant increase in EEG beta power during music listening, positively correlated with regional cerebral blood flow (20). This was proposed to be due to active cognitive sound processing, within the premotor-posterior parietal framework. Similar to beta, an increase in gamma activity is associated with selective attention, working memory, and conscious stimulus recognition (76,77). Beta rhythms are shown to predict listener-specific neural signatures in naturalistic music listening (78). Gamma activity has been implicated in the perceptual binding of musical features at the sensory level and the matching of external acoustic information to internal thought processes to form meaningful concepts (79,80). Gamma is also found to be higher in trained musicians, reflecting improved binding of musical features (81–83) and may be related to musical expectations (84). A previous study observed that dancers had strengthened theta and gamma synchrony during music relative to silence and silent dance. Musicians in contrast had decreased alpha and beta synchrony to music (85). A recent study recorded lower gamma event-related synchronization among dancers, during listening to preferred music, said to be due to selective attention to stimuli while probably planning/imagining dance movements. Alternatively, the rise in gamma and beta was postulated to be due to familiar music, which increases emotional provocation (86). The same study also showed a significant decrease in alpha activity for their expertise-related music compared to other

music, probably due to inhibition release (87) and cortical involvement in listening to their own music. Therefore, the decrease in globally distributed low-frequency activity (C1), the increase in posterior dominant alpha-beta1 activity (C2), and the weak increase in peripherally dominant broad-band activity that includes beta/gamma (C3), shown most prominently by the group *raga Malkauns*, (and insignificantly by *raga Miyan ki Todi*) could be due to attention modulation, increased alertness, binding of music features or may also serve as a reliable indicator of liking to music (88).

5.2 Significance of Component-Level Analysis Based on Intersubject Correlation

In the current study, ISC scores were comparable between the groups, except with raga Puriya which showed a marginal drop in C3 power (peripherally dominant broad-band activity) after the intervention. ISC examines shared brain responses between participants, the degree to which their responses match each other, and musical stimuli, it represents the degree to which the music is gripping their brains and is driving their experience (39). The ISC of EEG has been used as an index of engagement with naturalistic stimuli such as movies, stories, speeches, and music. The ISC has both lower-level processes, such as sensory processing, and higher-level processes, such as memory retention (29,31). However, ISC is shown to be affected by attention (30,89) and is capable of tracking musical engagement even though the behavior is not recorded. When attention is diverted away from sensory processes, the resulting ISC is shown to be smaller among participants (89,90). Thus, when attention is allocated to the stimulus, ISC is increased. Our results strengthen the notion that ISC is linked to engagement with the stimulus. A recent study found that ISC reduced after repeated listening to familiar music compared to unfamiliar classical music pieces (stimuli of 60 – 90 seconds) and that slower music is associated with higher rates of mind-wandering (39). Consequently, slower tempos might cause decreased focus on the music, resulting in greater differences in physiological responses among individuals and ultimately lower ISC as shown in previous studies (29,91,92). It is important to note that all the participants were Indians, and the music was exclusively recorded for the current study (not familiar with the clippings used). But, we cannot exclude the participants' prior exposure to these tones or

combinations of tones. Concerning familiarity, in a previous study, the ISC of the relative component (RC1) in EEG was highest for a foreign language narrative and lowest for an English narrative among fluent English speakers (32).

As our EEG segments were not very well time-locked to intervention stimuli, we used frequency domain data (average power spectral data within each 10 min condition) for CorrCA, based on the spectral distribution of the first three most correlated components. In the current study, we used the first three components with the highest ISC values in each group, and their ISC values were statistically comparable and showed similar patterns. This means that we captured spatiospectral components that showed comparable engagement during the session and their spectral pattern might better represent the intervention-related changes. Previous studies have observed that passive listening to natural music evoked significant intersubject and stimulus-response correlations, suggesting distinct neural correlates of musical engagement (37). Another recent study also showed significant ISC during periods of escalated tension with natural music (cello, strings) but at the high point, there was no significance (38). These authors have also recorded that EEG ISCs are highest for the remix stimulus that had more attention-catching musical events and lowest for our most repetitive manipulation, Tremolo (40). Our hypothesis that repetition of the phrases may reduce ISC was partially true only for the group that listened to *raga Puriya*. Further, the music used in the current study did not have peaks, tension, or emotionally charged variations as in previous studies (35,38). The music was created to produce an overall relaxing effect which might have resulted in more groupwise comparable results of ISC.

Our EEG spectral findings, especially those from component-level analysis, align with findings related to default mode network (DMN) activity and its relation to imagery or self-referential thoughts. To support this notion, a high-density EEG study that intermittently switched the attention of participants from internal (autobiographical remembering) to external processing (GO-NO-GO task) processing, found that autobiographical remembering was associated with an increase in spectral power in alpha and beta and a decreased in the delta band (93). At the source level, the alpha power increase was localized to regions of DMN. The decrease in delta power is more

pronounced when the autobiographical contents have positive emotions. Furthermore, the more posteriorly distributed alpha increase would suggest a relatively higher activity in the anterior DMN hub, which is involved in mostly conscious modeling, planning, and control functions, and relatively lower activity in the posterior hub, which is involved in mostly unconscious processes that include self-representation, emotion, and salience detection (94). However, there are some methodological limitations in this study. Due to the lower density of the EEG electrodes, we cannot verify the relation of our finding to the DMN activity through source localization analysis. The contribution of acoustic salience, which is the acoustic features in the stimulus itself that might have contributed to the increased perceptibility of the listener and also the top-down attention of based on previous knowledge, and current goals as one of the causes of the observed changes cannot be ruled out (95). A recent meta-analytic review on music perception and attention involvement observed that the perception and production of music relied on the auditory and sensorimotor cortices, while music imagery involved the parietal regions, indicating the recruitment of different brain structures in musical processing with the interaction between the environment (bottom-up) and internal thoughts (top-down) (96). Furthermore, we had not collected phenomenological reports after the intervention sessions that could be subjected to structured analysis and correlated with the EEG findings. These limitations should be addressed in subsequent studies.

To corroborate these findings with autonomic changes and anxiety levels, all three music interventions were found to reduce state anxiety levels, along with a reduction in salivary alpha-amylase (45). During the intervention, the *raga Miyan ki Todi* and *raga Puriya* groups had a significant drop in the parasympathetic parameters of heart rate variability (HRV), while after the intervention these two modes led to a significant increase in the parasympathetic response. In contrast, the *raga Malkauns* group showed a sustained rise in parasympathetic tone, similar to that seen in the control group. However, raga Malkauns caused a significant reduction in anxiety levels that was not observed in the control group. Though we did not find any difference in the visual analog scale for the liking of the music, it may be observed that functional neural plasticity could occur even when not subjectively perceived. In this study, the electrocardiogram data, from which HRV was derived was computed for a minimum of 5 minutes to a maximum of 10 minutes

for each condition, which meant that the participants had heard most of the phrases of the musical piece. It would be interesting to understand whether these autonomic changes also exhibit ISC as done in a previous study (92), and are modified after controlling for baseline data from the control group, as seen in the current EEG study.

6 Conclusion and Future Perspectives

To our knowledge, this is the first time that three acoustic stimuli in the form of Indian melodic modes have been studied systematically and scientifically as acoustic interventions for their short-term neuroplastic effect on the EEG power spectrum, with ISC-based component-level analysis, among a comparatively larger sample of healthy young individuals. Reduction in globally distributed low-frequency activity and increase in posterior dominant alpha-beta1 activity may be characteristic of passive listening to relaxing Indian modes, which may persist even after 10 minutes of the listening period. Among the modes, *raga Malkauns* showed this effect most prominently, followed by *raga Miyan ki Todi* and least by *raga Puriya*. As the increase in posterior alpha and low beta power is associated with DMN activity and a decrease in delta power with positive emotional memory, the spectral pattern we observed may indicate the observation of positive autobiographical memory while listening to musical modes and thus contribute to a relaxing experience. Further studies may include phenomenological reports to support these findings and build a stronger scientific foundation for the use of music in medicine. As ISC-based brain activity is modulated by training, studies may try to explore the effect of musical training, exposure to different genres, correlation with music features, and genre familiarity aspects. Different musical stimuli that are known to be emotionally stimulating can be studied, as ISC is said to vary with time-based emotional stimuli such as stories or movies. To exactly know the neural substrates activated within and between participants passively listening to the different scales, it is better to use higher-density EEG or fMRI data.

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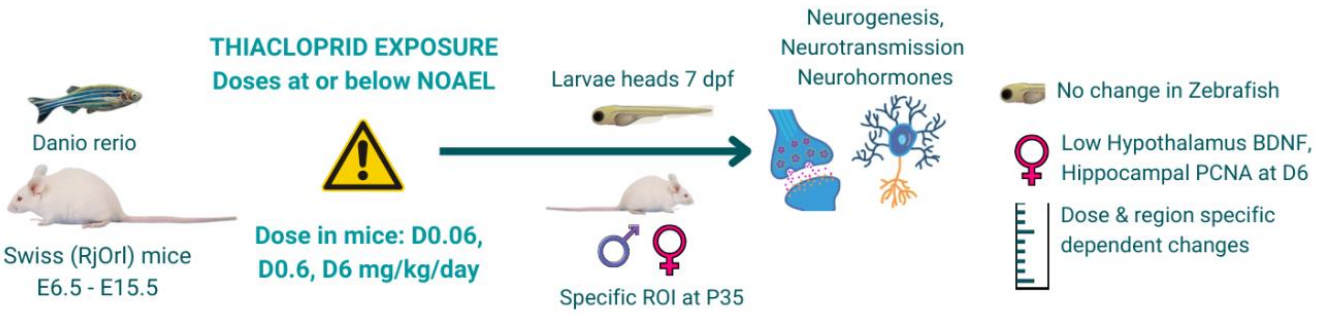
Chapter 4

Short-term impact of anthropogenic environment on neuroplasticity using animals



Manuscript to be submitted to Neurotoxicology.

Chapter 4: Short-term Impact of anthropogenic environment on neuroplasticity using animals

Perinatal exposure to the neonicotinoid thiacloprid impacts neuroplasticity and neuroendocrine system in vertebrates

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1 Abstract

Neonicotinoids are a set of insecticides until they were recently banned as they targeted nicotinic receptors of the cholinergic system of non-target vertebrates including fish. Chronic persistence of these insecticides in the soil, drinking water, and edible substances led to this study where we investigated the effects of a neonicotinoid thiacloprid on the nervous system in parallel in zebrafish and mouse models. Zebrafish embryos were exposed from 1 day post fertilization (dpf) to 6 dpf to 10^{-6} M to 10^{-8} M thiacloprid. Mice were treated during gestation, from embryonic day 6 to 15 (0, 0.06, 0.6 mg/kg/day or 6 mg/kg/day of thiacloprid). Whole heads of 6dpf zebrafish eleutheroembryos and 4 brain regions from thirty-five days old mouse offspring were investigated for neurogenesis [(doublecortin (DCX), proliferative cell nuclear antigen (PCNA), nestin)], neuronal [neurogenin, brain-derived neurotrophic factor (BDNF) synaptophysin, and synapsin Iia] and endocrine (estrogen receptors alpha, beta, and aromatase) markers by RT-qPCR. In the zebrafish, exposure to different doses of thiacloprid did not affect any of the markers. This could be due to the dose used or receptor specificity indicating the need for further investigations on the effects of neonicotinoids in the developing vertebrate brain. In mouse offspring, with 6 mg/kg/day of thiacloprid, a significant main effect of dose with an increase in DCX (amygdala), decrease in hypothalamic ERβ, nestin, synapsin IIA, hippocampal DCX, PCNA, neurogenin, aromatase, nestin, and synaptic markers was observed. Sex-specific reduction in BDNF in the hypothalamus and PCNA in the hippocampus was observed. Significant main effects of dose with an increase in DCX, PCNA (at D0.06 in the amygdala), PCNA (D0.06 and D0.6 in the cerebellum), synaptophysin (D0.06 in the

hypothalamus), ERα, ERβ, aromatase, nestin, neurogenin (D0.6 in the hippocampus) and reduction in aromatase (amygdala), hypothalamic neurogenin, aromatase, nestin, BDNF (at D0.06 and D0.6) was observed. Prenatal exposure to thiacloprid resulted in the dose and sex-dependent alteration in the neuronal and steroid markers in specific brain areas only in mice, not in zebrafish. This could be due to the dose used or receptor specificity indicating the need for further investigations on the effects of neonicotinoids in the developing vertebrate brain.

Key Words: thiacloprid, zebrafish, mouse, neurogenesis, neuroplasticity, Aromatase, hippocampus, hypothalamus, amygdala

2 Introduction

Neonicotinoids are insecticides commonly used in agriculture, aquaculture (fish farming), pet treatment, and in urban pest control. Neonicotinoids are structurally related to nicotine and target nicotinic cholinergic receptors (nAChRs), the membrane receptors sensitive to the neurotransmitter acetylcholine (1). Each neonicotinoid exhibits distinct binding to the nAChRs (2,3) and, likely, the specificity of these subunits is also species-dependent in vertebrates. These receptors are functionally present as homo- or hetero-pentameric receptors, a combination of alpha subunits α1 to α9 and non-alpha subunits (β1 to β4, δ, ε, or γ), and are largely distributed throughout the organism, being present at the neuromuscular junction and in the central and peripheral nervous system. The most commonly expressed nAchR subtypes on which neonicotinoids are shown to act are the heteromeric α4β2, α3β4, and homomeric α7 types of nAchRs (4–7). The structural differences between invertebrate and vertebrate nicotinic receptors led to the development of neonicotinoids and these pesticides showed a very strong affinity for insect receptors while exhibiting a much lower affinity to vertebrate subunits (for review:(2,8)). Initial results revealed that neonicotinoids were far less toxic to the handlers and non-target organisms in comparison to other insecticides such as organophosphate and carbamate (2).

However, the intensive use of neonicotinoids and the persistence of the molecule in the plant and the environment contribute to the increased exposure of non-target invertebrates (honeybees and other pollinating insects) and vertebrates (9,10). Indeed, due to systemic distribution throughout the plant, the molecule is found in fruits and

vegetables from around the world (11–16). A few studies suggest that several neonicotinoids, including imidacloprid, acetamiprid, and thiacloprid can readily cross the intestinal barrier (17,18) and the blood-brain barrier (18–22), and these pesticides and their metabolites are found in human biological samples, confirming human exposure (23–29). Due to chronic exposure to neonicotinoids and their potential bioavailability in the mammalian organism, questions and concerns were raised about potential adverse health effects in humans (30).

A large number of studies highlight the impact of several neonicotinoids such as acetamiprid, imidacloprid, and clothianidin on vertebrate locomotor activity and behavior via a direct impact on the nervous system. For example, imidacloprid or clothianidin was shown to significantly increase locomotor activity during various behavioral tests in mice and rats, independently of the dose and the exposure period (31–37). Interestingly, the opposite effect on locomotor activity was observed in aquatic vertebrate species, including amphibians (37,38) and zebrafish (38–40). In addition to locomotion, learning, and memory were also affected in rodents following exposure to some neonicotinoids (41–43). These behavioral alterations are linked to the impact on neurons and neurotransmission in the peripheral and/or central nervous system, as shown by in vitro and in vivo studies (39,42,44–49). These alterations of the nervous system could be linked to the misactivation of central nicotinic receptors as the cholinergic system stemming from the basal forebrain and brainstem innervates the entire central nervous system (50,51). Neonicotinoids are shown to reduce the expression of α7 receptors in the hippocampus (19). Treatment with acetamiprid was shown to significantly reduce the levels of glutamate and its N-methyl-d-aspartate (NMDA)-like receptor subunits, which could translate into significant memory deficits (43). The neurotransmission effects of neonicotinoids depend on the receptors that are activated as well. Clothianidin led to striatal dopamine release via an exocytotic-, vesicular-, and Ca+2-dependent mechanism that required the activation of α4 or α7 subunits of nAChRs and not the β2 subunit. The authors also noted the dependence on the activation of muscarinic acetylcholine receptors (mAChRs) (49,52). Imidacloprid facilitated tyrosine hydroxylase transcription by acting as a partial agonist at α3β4 and α7 receptors, causing long-term activation of second messenger systems (CREB-PKA-ERK and Rho cascade) (53). Previous studies

have shown that nAChR α7 and β2 subunits with clothianidin binding affinity were seen in the dentate gyrus neural progenitor cells (54) and that stimulation of α7 nAChR using nicotine-cultured hippocampal cells activated ERK 1/2, which promotes the proliferation of neural progenitor cells (55). It should also be noted that more recent studies report the potential endocrine-disrupting action of neonicotinoid as suggested by a decline in fertility rate (56–58), impact on steroidogenic enzymes such as aromatase (59–61), and changes in circulating sex hormones, including FSH, estrogens, and testosterone (62–64). The brain itself is a major steroidogenic site and neurosteroidogenesis is fundamental for brain development and physiology (for reviews: (65–69)). Any change in brain steroid synthesis and bioavailability during development, including endocrine disruptor exposure, leads to significant long-term defects in brain plasticity and behavior (see for example reviews (69–73)).

While the majority of studies on neonicotinoids focused on the impact of imidacloprid, acetamiprid, or clothianidin on the brain and the endocrine system, far less is known about the potential long-term effect of early exposure to thiacloprid [(Z)-thiacloprid (3-((6-Chloro-3-pyridinyl) methyl)-2-thiazolidinylidene) cyanamide (74), another widely used neonicotinoid (non-renewal of approval on 3 February 2020 in Europe, but repeated emergency authorizations for use in sugar beets and berries, see (75)). Thiaclopride shows the same mode of action as the other neonicotinoids although its lethal concentration (LC50) is slightly lower in various aquatic invertebrates (76). We, therefore, aimed at investigating the impact of early thiacloprid exposure on local steroid action in the brain, and link these effects to potential changes in neuroplasticity, including neurogenesis and synaptic changes. We also considered potential differences between vertebrates and studied the exposure to thiacloprid on zebrafish and mice. Our goal was to further highlight potential sex differences in mice, as the majority of studies investigating the long-term impact of neonicotinoids on the brain were performed on males only (see (77)) while brain neuroplasticity is sexually differentiated (78–80).

3 Material and Methods

3.1 Animals

Zebrafish (Experiment 1: AB Strain) and mice (Experiment 2a and 2b: Swiss Strain) were handled and euthanized in agreement with the guidelines for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare. The animal facilities used for the present study are licensed by the French Ministry of Agriculture (Zebrafish: Biosit ARCHE: agreement number B35-238-40 and Mice: IRSET agreement number D35– 238–19). All animal procedures were performed according to the Ethics Committee of the Ministry of the Research of France (agreement number: 17473-2018110914399411). All experimental procedures followed the ethical principles outlined in the Ministry of Research Guide for Care and Use of the Laboratory Animals and were approved by the local Animal Experimentation Ethics Committee (C2EA-07).

3.2 Experiment 1. Eleutheroembryo exposure to thiacloprid in Zebrafish

Adult Zebrafish (cyp19a1b: GFP (73)) were housed in our facility in a recirculation system (Zebtec, Tecniplast, Italy) under standard conditions of photoperiod (14 h light and 10 h dark) and temperature (28°C). Eggs obtained from zebrafish (reproduction ratio 2 males:1 female) were collected immediately after spawning and grown in E3 medium at 28°C in glass Petri dishes. Within 4 hours post-fertilization (hpf), developing embryos were randomly distributed into 4 groups of approximately 100 eggs: 3 groups were exposed to 10-6 M, 10-7 M, or 10-8 M thiacloprid dissolved in DMSO and the control group was exposed to DMSO only (4 µL in 40 mL E3). The exposure medium was changed every day for 6 days. On day 6, 50-60 eleutheroembryos per group were terminally anesthetized with MS222 (50 mg/L). Whole heads were collected, immediately frozen in liquid nitrogen, and stored at −80°C before RNA extraction and quantitative real-time PCR [Fig 2]. This protocol was repeated 7 times such that each experimental exposure represents one biological sample and the final number of biological samples is 7 (n=7). Each sample was sonicated for 15 sec in 250 µl of Nucleozol Reagent (Macherey-Nagel) and RNA extractions were performed using the NucleoSpin RNA Plus kit (Macherey-Nagel) (81) following the manufacturer's instructions.

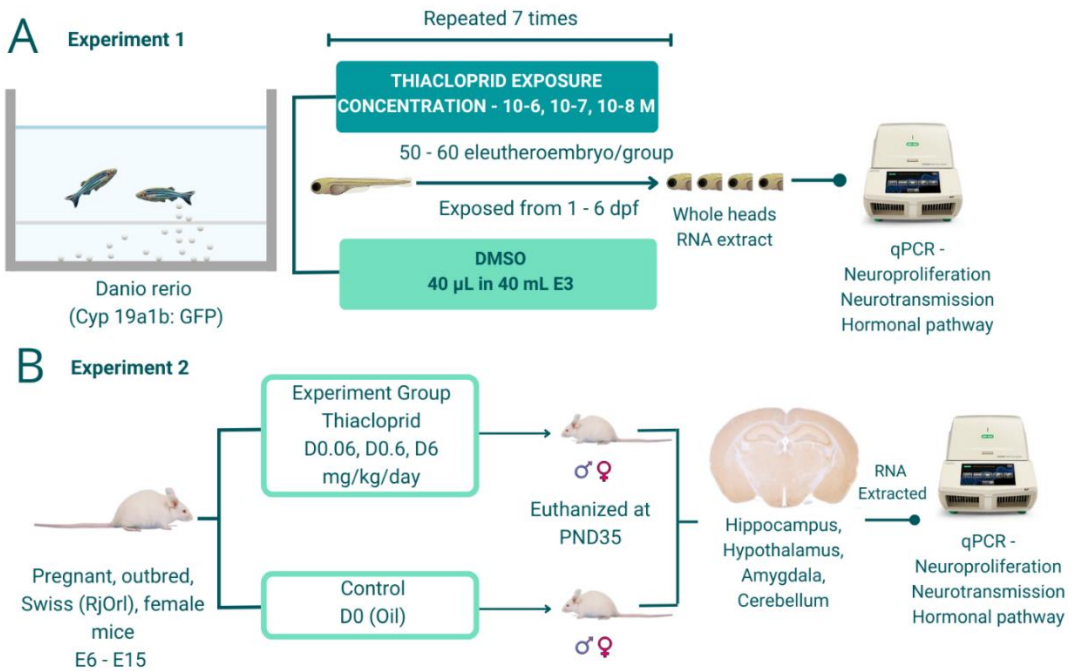

Figure 1: Protocol followed for (A) zebrafish (Experiment 1) and (B) mouse (Experiment 2a, 2b). In Experiment 2a D0 = Control group and D6 = 6 mg/kg/day thiacloprid, oral gavage. In Experiment 2b D0= Control, D0.06 = 0.06mg/kg/day thiacloprid, and D0.6 = 0.6 mg/kg/day thiacloprid.

3.3 Experiment 2. Exposure to thiacloprid in mice

3.3.1 Mice Treatment and Dissection

Outbred Swiss mice (RjOrl) were purchased from Janvier, France, and acclimatized in our facilities for one week before random assignment to the treatment group. Animals were kept under standard laboratory conditions in a 12:12-h light/dark schedule with access to standard mouse chow and tap water ad libitum. Females were then bred and the vaginal plug was checked in the morning. The day of the vaginal plug was considered embryonic day 0.5 (E0.5) and pregnant females were placed in individual cages. From embryonic day E6.5 until E15.5, female mice were treated with 6mg/kg/day (experiment 2a), 0.6 or 0.06mg/kg/day (experiment 2b) thiacloprid suspended in olive oil via oral gavage (150 microliters, see (58)). The 6mg/kg/day is a dose just around the NOAEL in mice and rats during developmental neurotoxicity and carcinogenicity studies (75). The control mice received only oil (D0). We treated 5 pregnant females per group except 4 for 0.6mg/kg/day. The male and female progeny were weaned on the 21st day

and 4 siblings of the same litter were housed per cage. F1 generation male and female mice (maximum 2 per litter) were euthanized at the age of 35 days (postnatal day PND35) after blood collection from the retro-orbital vein. The brain was dissected and placed immediately on dried ice and stored at -80 °C until use.

Brains were cut into 300 µm thick sections with a cryostat (Microm HM560), and bilateral punches were collected using the Stoelting brain punch set (diameter 1.25 mm) from 3 areas of interest: hypothalamus, hippocampus, and amygdala. The cerebellum was also collected and analyzed in Experiment 2b. Total RNA was extracted using the NucleoSpin kit for Nucleozol (Macherey-Nagel) and quantity and quality were determined on Thermo Scientific NanoDrop 8000.

3.4 RT-qPCR

RNA (1µg) from zebrafish and mice was reverse transcribed using M-MLV reverse transcriptase from Promega following the manufacturer's protocol and using random primers. Quantitative PCR was performed using Syber Green (iTaq SYBER, Biorad). We targeted cell proliferation (Proliferative cell nuclear antigen, PCNA), neuronal differentiation [Nestin, Neurogenin, doublecortin (DCX)] (51,56), neuronal markers [Brain derived neurotrophic factor (BDNF), Synaptophysin, Synapsin IIa] and neuroendocrine-linked proteins (estrogen receptors ERα and ERβ, and aromatase). Activated caspase 3 was also tested for zebrafish only [See supplementary table 1 for primer sequences]. Housekeeping genes used were the ef1 for zebrafish and GAPDH for the mouse. The threshold cycle (Ct) was determined for each gene and a melting curve was obtained for each sample to confirm specificity. Relative gene expressions were calculated using the $2^{-\Delta\Delta Ct}$ method for relative quantification (82). The fold induction/inhibition was determined and expressed as a fold change compared to the normalized control condition (the male control group in mice experiments).

3.5 Statistical analysis

Data are represented as the mean ± standard error of the mean (SEM). Outliers, defined from values outside the mean ± 2 standard deviations, were removed from the analysis (the number of animals remaining is plotted in the graphs). The treatment effect was analyzed with a one-way analysis of variance (ANOVA) for Experiment 1 (zebrafish) and 2-way ANOVA, for Experiment 2 (mice) with sex and dose as factors, for each brain

region (Statistica Version 13 (Dell Inc.)). Post hoc analysis was performed using the Tukey post hoc test where appropriate. The values were considered statistically significant if p was <0.05. The figures were generated using GraphPad Prism (Version 6).

4 Results

4.1 Experiment 1: Zebrafish exposure

In fish, developmental exposure to 3 concentrations of thiacloprid (10-8, 10-7, 10-6 M) for 6 days did not increase the developmental mortality of the embryos (data not shown). In addition, we did not observe an impact of any of the 3 different concentrations of thiacloprid on the transcription of any of the markers used in our experimental conditions when compared to control samples (p's >0.05; Figure 2).

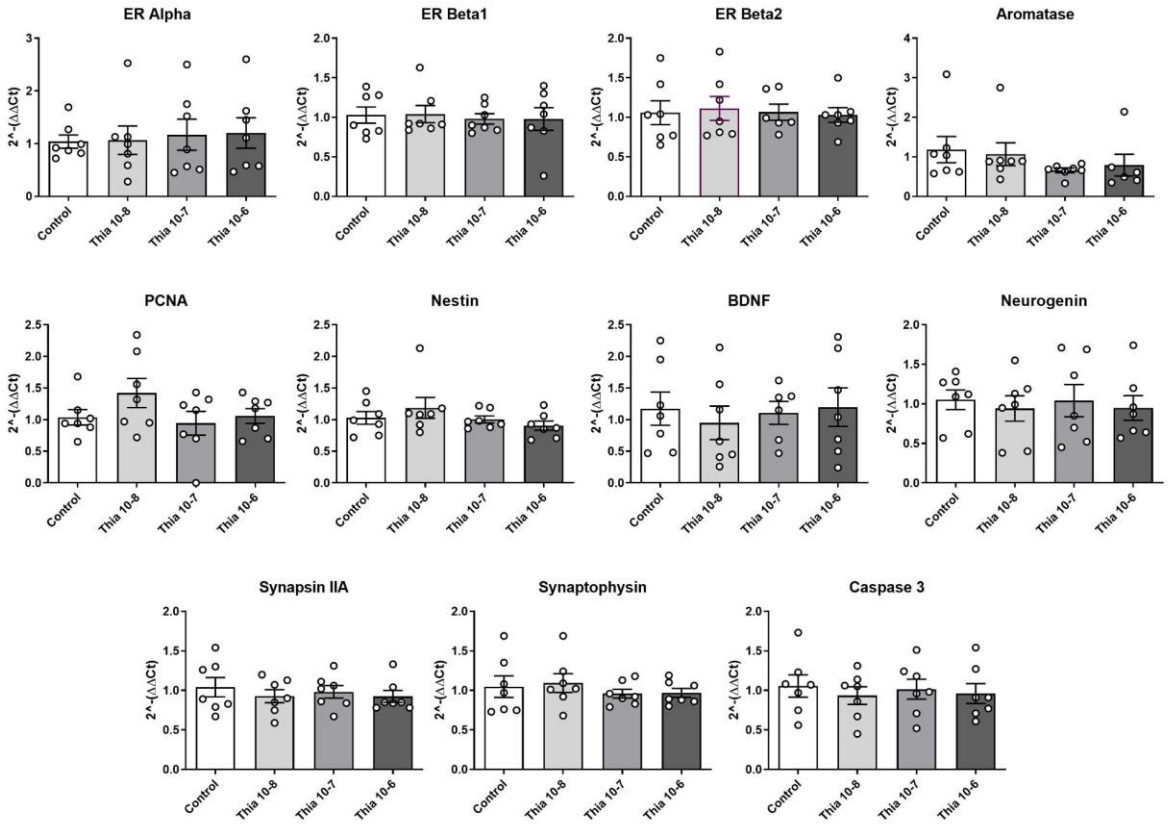

Figure 2: Mean (± SEM) fold change ($2^{-\Delta\Delta Ct}$ values) and individual transcription levels of 11 genes in zebrafish eleutheroembryos heads following 6 days of exposure to three different concentrations of thiacloprid (10^{-8}, 10^{-7}, 10^{-6} M). No statistically no significant difference was observed.

4.2 Experiment 2: Mouse exposure

4.2.1 Experiment 2a

The first experiment was performed to determine whether in utero exposure to 6 mg/kg/day thiacloprid, a dose just below the NOAEL (75), would affect neuroendocrine and neuroplasticity markers in adolescent male and female mice offspring (PND 35). Three regions of interest were investigated: the hypothalamus, the hippocampus, and the amygdala.

4.2.1a Amygdala

We found a significant main effect of treatment on DCX transcription ($F(1, 24)=35.806$, $p<0.0001$) with an increase after 6 mg/kg/day thiacloprid exposure **(Fig 3)**. There was no other significant main effect or interaction.

4.2.1b Hypothalamus

We found a statistically significant effect of thiacloprid exposure on ER beta ($F(1, 26) = 5.041$, $p=0.033$), nestin ($F(1, 25) = 11.339$, $p= 0.002$), and synapsin IIa ($F(1, 26) = 6.021$, $p= 0.021$) transcription in the hypothalamus **(Fig 3)**. It can be noted that there was a trend toward an interaction between sex and treatment on nestin transcription ($F(1,25) =3.047$, $p=0.09$), where the mean fold change was reduced in thiacloprid-exposed females compared to the other groups. We also found a significant effect of sex on BNDF transcription ($F(1, 25)=4.6810$, $p=0.040$) with females exhibiting a reduction compared to males and a tendency toward the main effect of treatment ($F(1, 25)=3.744$, $p=0.064$) but no interaction between treatment and sex, although females seemed to be most affected. No other difference was observed in the hypothalamus (see table 1).

4.2.1c Hippocampus

We found a significant main effect of thiacloprid exposure, with a reduction of DCX ($F(1, 23) = 4.988$, $p=0.036$), aromatase ($F(1, 27) = 68.360$, $p<0.0001$), neurogenin ($F(1,24) = 10.903$, $p=0.003$), nestin ($F(1, 24) = 23.649$, $p<0.0001$), synapsin IIa ($F(1, 27) = 106.908$, $p<0.0001$), synaptophysin ($F(1, 25) = 32.413$, $p<0.0001$) and PCNA ($F(1, 26) = 31.671$, $p<0.0001$) transcription. PCNA transcription was impacted by sex ($F(1, 26) = 5.643$, $p= 0.025$) with a reduction in females compared to males but no interaction between the 2 factors. There was a trend toward an interaction between sex and treatment on PCNA transcription ($F(1,26) =3.008$, $p=0.095$), where the mean fold change

was reduced in thiacloprid-exposed females compared to the other groups. No other difference was observed in the hippocampus **(see Fig 3 and Table 1)**.

Table 1: Factorial ANOVA of neuroendocrine markers tested in mice during Experiment 2a (D0 versus D6) in the amygdala, hippocampus, and hypothalamic regions

		Amygdala		Hypothalamus		Hippocampus	
		F	**p**	**F**	**p**	**F**	**p**
DCX	**Thiacloprid**	**35.806**	**.000**	.418	.523	**4.988**	**.036**
	Sex	.166	.687	.083	.775	.251	.621
	Sex * Thiacloprid	1.610	.217	.004	.949	.670	.422
Neurogenin	**Thiacloprid**	.665	.423	.491	.491	**10.903**	**.003**
	Sex	1.111	.302	4.063	.057	.058	.812
	Sex * Thiacloprid	.522	.477	.871	.361	.437	.515
PCNA	**Thiacloprid**	3.298	.081	1.814	.190	**31.671**	**.000**
	Sex	.871	.359	2.238	.147	**5.643**	**.025**
	Sex * Thiacloprid	.953	.338	1.182	.287	3.008	.095
ER Beta	**Thiacloprid**	.591	.449	**5.041**	**.033**	1.297	.265
	Sex	.273	.606	.310	.582	.410	.528
	Sex * Thiacloprid	.135	.716	.919	.347	.009	.926
Aromatase	**Thiacloprid**	1.646	.211	2.329	.140	**68.360**	**.000**
	Sex	.742	.397	3.919	.059	.682	.416
	Sex * Thiacloprid	.423	.521	1.136	.297	.234	.633
ER Alpha	**Thiacloprid**	.971	.333	.912	.349	2.207	.149
	Sex	.570	.457	.443	.512	.000	.994
	Sex * Thiacloprid	.507	.483	.338	.566	.130	.721
Nestin	**Thiacloprid**	.207	.653	**11.339**	**.002**	**23.648**	**.000**
	Sex	.158	.694	2.402	.134	.091	.765
	Sex * Thiacloprid	.204	.656	3.047	.093	.949	.340
BDNF	**Thiacloprid**	1.822	.189	3.744	.064	.087	.770
	Sex	1.921	.178	**4.681**	**.040**	.060	.809
	Sex * Thiacloprid	1.543	.226	1.424	.244	.124	.727
Synapsin IIA	**Thiacloprid**	1.533	.227	6.021	.021	**106.908**	**.000**
	Sex	.130	.722	.029	.866	.084	.774
	Sex * Thiacloprid	1.557	.223	.952	.338	.003	.959
Synaptophysin	**Thiacloprid**	.034	.856	.012	.913	**32.413**	**.000**
	Sex	.423	.521	2.990	.096	.007	.934
	Sex * Thiacloprid	.326	.573	.072	.791	.047	.830

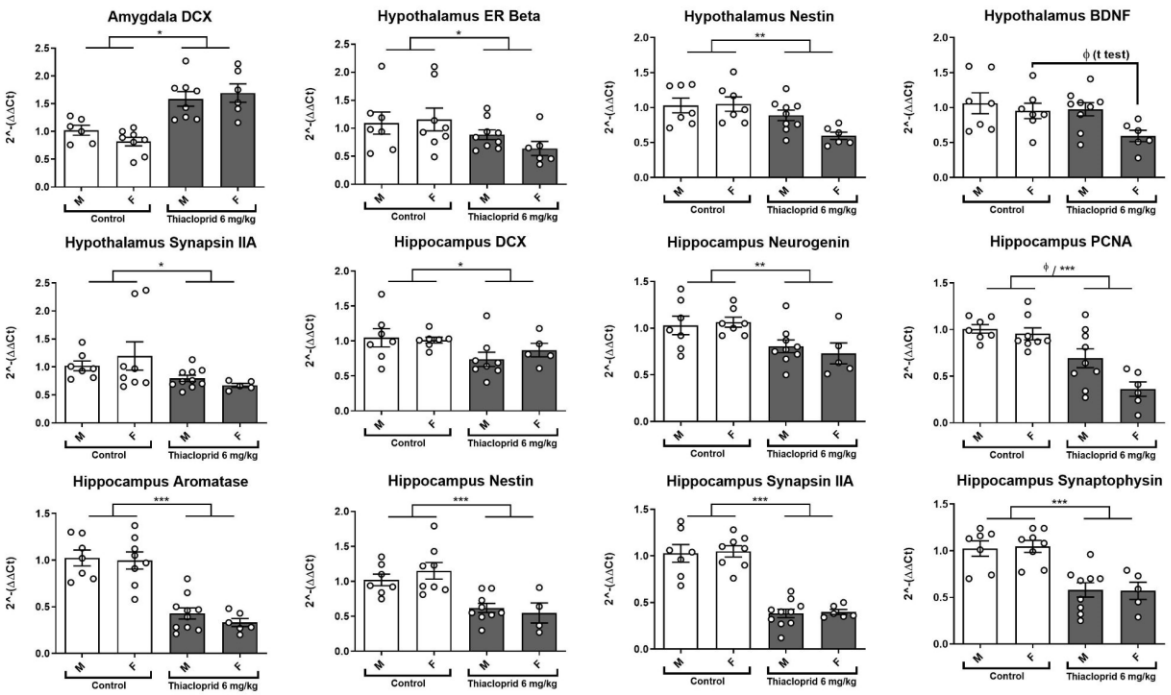

Fig 3: Mean (± SEM) fold change (2^- ΔΔCt values) and individual transcription levels in the amygdala, hippocampus, and hypothalamus in male (M) and female (F) mice offspring (PND35) following in utero exposure to thiacloprid (6mg/kg/day); ***p<0.001 **p<0.01 *p<0.05; ϕ Significant reduction in female mice (a posteriori analysis).

4.2.2 Experiment 2b

We next investigated the impact of lower doses of thiacloprid (0.6mg/kg/day and 0.06mg/kg/day) on neuroplasticity and neuroendocrine markers in the amygdala, hypothalamus, hippocampus as well as cerebellum.

4.2.2a Amygdala

We observed a statistically significant effect of thiacloprid on DCX ($F(2, 19) = 4.065$, $p= 0.034$), PCNA ($F(2, 19) = 4.441$, $p= 0.026$), and aromatase ($F(2, 19) = 4.116$, $p= 0.033$). Post Hoc analysis showed that the lowest dose of 0.06 mg/kg/day significantly increased DCX ($p=0.021$) and PCNA ($p= 0.016$) transcription compared to the control group while the dose of 0.6 mg/kg/day significantly reduced aromatase transcription in comparison to the control group ($p= 0.042$) **(Fig 4)**. There was no sex difference or interaction between treatment and sex. No other difference was observed for the other transcript in the amygdala.

4.2.2b Hypothalamus

We observed a statistically significant effect of the treatment with a reduction of nestin ($F(2,19) = 11.914$, $p= 0.018$), neurogenin ($F(2,19) = 8.349$, $p= 0.003$), BDNF ($F(2,19) = 6.771$, $p= 0.006$) and aromatase ($F(2, 19) = 6.135$, $p= 0.009$) transcription. Post-hoc analysis showed that both doses of thiacloprid (0.6 and 0.06 mg/kg/day) led to a significant reduction compared to the control group in the above-mentioned markers. There was a significant main effect of thiacloprid on hypothalamic synaptophysin ($F(2,19) = 5.773$, $p=0.011$), with posthoc showing a significant increase in transcription at the lower dose of 0.06 mg/kg/day ($p=0.006$) as compared to the control group **(Fig 4)**. There was no sex difference or interaction between treatment and sex. No other difference was observed for the other transcripts in the hypothalamus.

4.2.2c Hippocampus

We found a significant main effect of thiacloprid with an increase in transcription levels of the neural markers nestin ($F(2,18) = 10.308$, $p=0.001$) and neurogenin ($F(2,18) = 11.258$, $p=0.001$). Post-hoc analysis showed this impact of thiacloprid due to the higher expression level in the group exposed to 0.6 mg/kg/day compared to the control group (Nestin: $p=0.002$; Neurogenin: $p=0.002$) and to the group exposed to 0.06mg/kg/day (Nestin: $p=0.002$; Neurogenin: $p=0.001$; see figure 4). Similarly, there was a statistically significant increase in the transcription of ERalpha ($F(2,18) = 8.598$, $p=0.002$), ERbeta ($F(2,18) = 9.106$, $p=0.002$), and aromatase ($F(2,18) = 7.508$, $p=0.004$), again with the 0.6mg/kg/day group significantly higher than the control group (ER alpha: $p=0.011$; ER beta: $p=0.012$; Aromatase: $p=0.028$) and the 0.06 mg/kg/day (ER alpha: $p=0.003$; ER beta: $p=0.002$; Aromatase: $p=0.004$). There was no sex difference nor the interaction between treatment and sex. No other difference was observed for the other transcript in the hippocampus (see Table 2).

4.2.2d Cerebellum

We observed a statistically significant effect of thiacloprid exposure on PCNA transcription ($F(2, 19) = 9.025$, $P = 0.002$). There was a statistically significant increase at the low dose of 0.06 mg/kg/day ($p=0.002$) and with the higher dose of 0.6 mg/kg/day ($p=0.011$, Figure 4) compared to the control group. There was no sex difference or

interaction between treatment and sex. No other difference was observed for the other transcript in the cerebellum (see supplementary table 2).

Table 2: Factorial ANOVA of neural markers tested among mice during Experiment 2b (D0, D0.06, D0.6) in the amygdala, hippocampus, hypothalamus, and cerebellar regions

		Amygdala		Cerebellum		Hypothalamus		Hippocampus	
		F	p	F	p	F	p	F	p
DCX	**Thiacloprid**	**4.065**	**.034**	1.491	.250	1.843	.186	.589	.565
	Sex	.014	.908	1.419	.248	.952	.341	1.815	.195
	Sex * Thiacloprid	.624	.546	.896	.425	.402	.675	.363	.701
Neurogenin	**Thiacloprid**	1.021	.379	2.124	.147	**8.349**	**.003**	**11.258**	**.001**
	Sex	.435	.517	1.310	.267	.008	.928	.068	.798
	Sex * Thiacloprid	.318	.732	1.808	.191	.144	.867	.046	.955
PCNA	**Thiacloprid**	**4.441**	**.026**	**9.025**	**.002**	1.671	.215	.548	.588
	Sex	.813	.379	2.424	.136	.301	.590	1.188	.290
	Sex * Thiacloprid	1.035	.374	.948	.405	1.937	.171	.214	.809
ER Beta	**Thiacloprid**	1.661	.216	.673	.522	.833	.450	**9.106**	**.002**
	Sex	.011	.916	.157	.696	3.641	.072	.048	.829
	Sex * Thiacloprid	.123	.885	1.717	.206	.312	.736	.152	.860
Aromatase	**Thiacloprid**	**4.116**	**.033**	1.032	.375	**6.135**	**.009**	**7.508**	**.004**
	Sex	1.306	.267	.265	.613	.000	.990	.000	.990
	Sex * Thiacloprid	.356	.705	1.324	.289	.158	.855	.084	.920
ER Alpha	**Thiacloprid**	1.174	.331	.728	.496	.356	.705	**8.598**	**.002**
	Sex	.397	.536	.012	.916	1.962	.177	.029	.866
	Sex * Thiacloprid	.050	.951	1.227	.315	.059	.942	.036	.965
Nestin	**Thiacloprid**	3.164	.065	1.413	.268	**11.914**	**.000**	**10.308**	**.001**
	Sex	.064	.804	.509	.484	.418	.526	.104	.751
	Sex * Thiacloprid	.012	.988	1.865	.182	.163	.851	.072	.931
BDNF	**Thiacloprid**	1.109	.350	.961	.400	**6.771**	**.006**	1.072	.363
	Sex	.309	.585	.651	.430	.120	.733	.500	.489
	Sex * Thiacloprid	.252	.779	1.986	.165	.010	.990	.968	.399
Synapsin IIA	**Thiacloprid**	1.782	.195	2.722	.091	1.691	.211	.678	.520
	Sex	.935	.346	.196	.663	.181	.675	1.528	.232
	Sex * Thiacloprid	.574	.573	.466	.634	1.043	.372	.160	.854
Synaptophysin	**Thiacloprid**	1.942	.171	.137	.873	**5.773**	**.011**	.089	.916
	Sex	.437	.517	.397	.536	.025	.876	1.003	.330
	Sex * Thiacloprid	1.857	.183	2.058	.155	1.305	.294	.218	.806

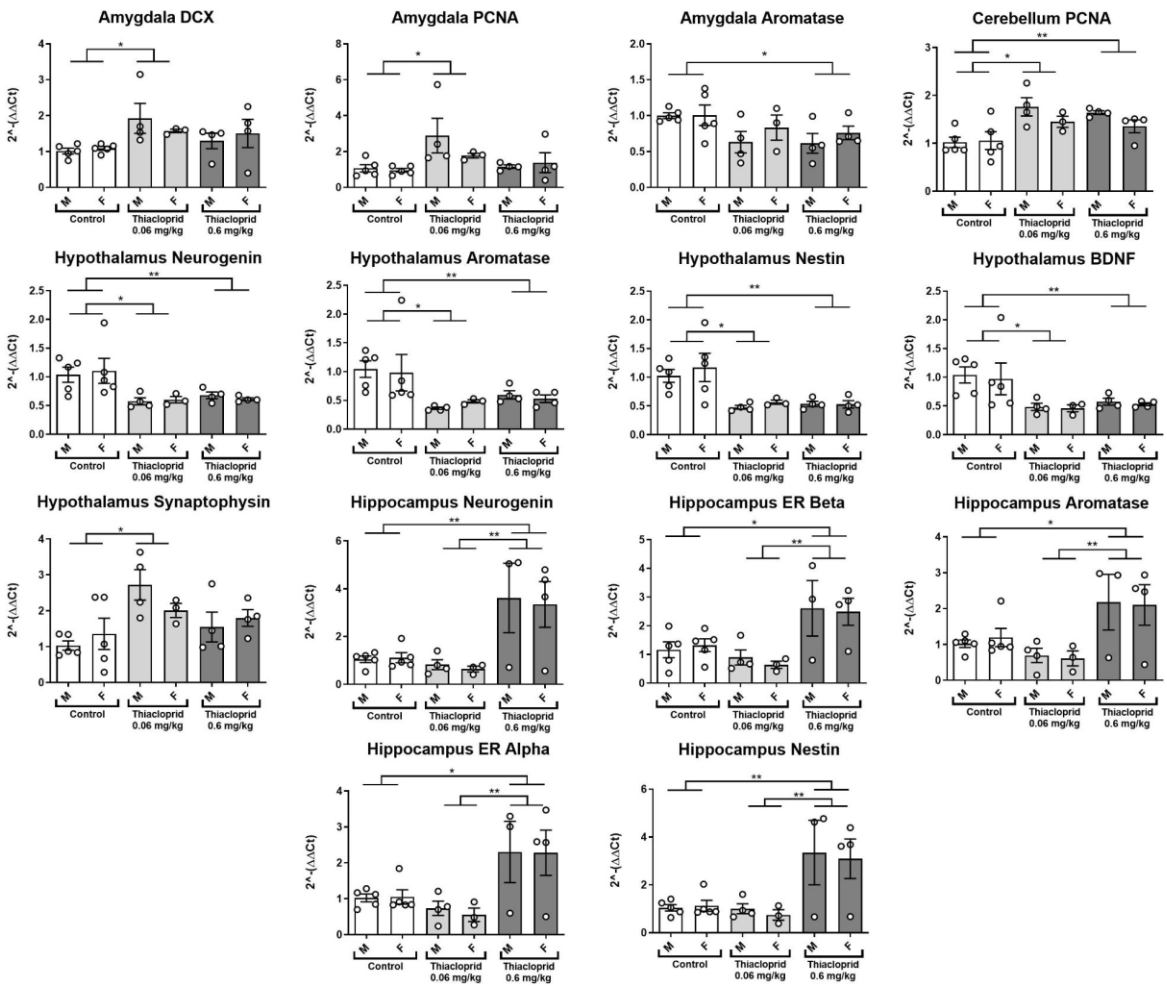

Fig 4: Mean (± SEM) fold change (2^-ΔΔCt values) and individual transcription levels of markers (shown as 2^-ΔΔCt values) in the amygdala, hippocampus, hypothalamus, and cerebellum in male (M) and female (F) mice offspring (PND35) following in utero exposure to thiacloprid (0.06 and 0.6 mg/kg/day) in Experiment 2b; ***p<0.001 **p<0.01 *p<0.05.

5 Discussion

Our objectives were to better define the potential impact of the neonicotinoid thiacloprid on neuroplasticity and the neuroendocrine markers in aquatic (zebrafish) and terrestrial (mouse) vertebrates. We did not see any impact of thiacloprid on zebrafish at the concentrations and developmental stages investigated, while specific brain regions were impacted in mice.

5.1 Zebrafish

Our results show that 120 hours-exposure of zebrafish at three different low concentrations of thiacloprid did not affect the mortality nor gene transcription in the whole head of eleutheroembryos. These results are to be added to the increasing literature investigating the potential impact of various neonicotinoids on the vertebrate central nervous system. Zebrafish brains express eight nicotinic AChRs subunits (α2, α3, α4, α6, α7, β2, β3, and β4) (83,84) but the potential direct interaction of these subunits with thiacloprid, or other neonicotinoids, has not been characterized to our knowledge. However, several in vivo studies suggest the direct impact of neonicotinoid exposure on the early developmental stage of zebrafish. Indeed, high concentrations of neonicotinoids, including thiacloprid, have significant deleterious effects on zebrafish, including teratogenic effects, heart rate modulation, increased DNA damage, oxidative stress (85–90), endocrine disrupting effects (91) and neurobehavioral consequences in zebrafish (92–98). For example, early exposure to 45 or 60 mM imidacloprid reduced swimming activity and increased startle response in juvenile and adult zebrafish (38). Similarly, lower concentrations (0.5mM) of imidacloprid as well as thiacloprid acutely reduced locomotor activity in eleutheroembryos (40) but these deleterious effects were reversible, independently of the window of exposure (99,100). Xie et al (95) found that concentrations above 100 microgram/L (about 0.4 10^{-6}M) affected locomotion and gene transcription linked to neurotransmitter systems (acetylcholine, but also GABA and 5-HT). It should be noted that the sensitivity to neonicotinoids is not only species-dependent (101–104) but also strain-dependent in zebrafish (38,105). In addition, it is likely that neonicotinoids, including thiacloprid, will impact specific targets in the zebrafish central nervous system and more global approaches should be integrated to study the potential impact of neonicotinoids on vertebrates, especially during development. Furthermore, the concentrations used in the majority of studies, including ours, are much above environmental concentrations (11.493 ± 5.095 ng L^{-1} (106,107)) albeit higher concentrations were observed locally during specific events (1.4microgram/l, or 0.5 10^{-8}M) and future work should investigate the impact of pesticides on fish at environmentally relevant concentrations.

5.2 Mice

In parallel to zebrafish, we investigated the impact of developmental exposure to 3 doses of thiacloprid on female and male mice offspring during the juvenile stage, focusing on several brain regions important for behavior, cognition, social interaction, and emotion. The cholinergic system, and more precisely the nicotinic receptors, are already functional as early as gestational day 10 in the mouse cortex and day 11 in the mesencephalon (108). To highlight their functional importance, a large number of studies investigating the impact of cholinergic alteration, including early exposure to nicotine or acetylcholinesterase inhibitors such as organophosphate or carbamate have shown a long-term impact on neurobehavioral outcomes (109). We show here, in support of other studies, that neonicotinoids impact the development of the central nervous system of vertebrates, and these alterations are not reversed in the juvenile stage. More importantly, we are the first to show that prenatal exposure to low doses of thiacloprid specifically impacts neurogenesis, neuroplasticity, and neuroendocrine functions in a dose-dependent and region-dependent manner.

5.2.1 Neurogenesis

Neurogenesis is predominant during development but is also observed during adolescence and even later in adulthood in the mammalian hippocampus as well as in the subventricular zone. More recent data suggest that other brain regions, including the amygdala and the hypothalamus, show a significant level of postnatal neurogenesis (110,111), including in humans (see for example (112,113)). We found here that in utero exposure to various doses of thiacloprid modulates biomarkers of neurogenesis, during adolescence, including PCNA (proliferation), Nestin (neural progenitor), neurogenin (neuronal specification), and DCX (immature neuron), in both male and female mice. The impact of nicotinic receptor agonists or antagonists on neurogenesis is in itself not surprising, as the cholinergic system is one of the many neurotransmitter systems regulating neurogenesis, both during development but also in adults (review in (114–116)). Previous studies have shown that neonicotinoids can impact neurogenesis in the neonatal cortex or the hippocampus (22,45,117–120). It should be mentioned that most of these studies were performed with relatively high doses/concentrations of

neonicotinoids However, there is little information on the impact of early cholinergic alteration by neonicotinoids on postnatal neurogenesis.

A few studies suggest that the impact of early exposure to molecules such as chlorpyrifos (121) or neonicotinoids including thiacloprid (40) on neurobehavioral parameters is transient while other reports suggest otherwise (for example (32,46)). Early exposure to thiacloprid may affect the neural progenitor pools and/or their local environment (stem cell niche). Further investigation at the cellular level should define how in-utero neonicotinoid exposure affects adolescent and adult neurogenesis, in males and females. Evidence using thiacloprid and other neonicotinoids in different non-target species does suggest impaired learning and memory (122–124).

5.2.2 Effect of Thiacloprid on neuroendocrine markers

Our results suggest thiacloprid may act through these estrogen receptors in a region and dose-specific manner to impair the above-mentioned functions in the hypothalamus while facilitating the functions of the hippocampus at the mid-dose range. At a high dose of thiacloprid, probable neuronal/glial cytotoxicity leads to a drop in most of the gene expressions.

Imidacloprid was shown to interrupt steroidogenesis by inhibiting 3β-HSD and 17β-HSD enzyme activities (57). Rabbits treated with thiacloprid had a significant decrease in serum levels of reproductive hormones and histopathological changes in reproductive organs (125). A few studies have shown the induction of aromatase expression in human H295R adrenocortical carcinoma cells by two neonicotinoids (thiacloprid and thiamethoxam) (61). A recent study showed that thiacloprid, thiamethoxam, and imidacloprid affect aromatase expression and activity and significantly increase estradiol and estrone production (by co-culture models of fetoplacental steroidogenesis of H295R and BeWo cells) at 0.1 and 0.3 μM (59). Another recent study evaluated the effect of thiacloprid, and imidacloprid (>63 μM), and observed that thiacloprid and imidacloprid induced estrogenic activity at the highest concentrations (126). This was similar to results with imidacloprid at concentrations above 10−5 M (127), while some studies have shown that thiacloprid and other neonics did not induce any estrogenic activity (128–130). The plausible explanation for these variations is the difference in cell models used. These

contrasting results strongly indicate the need for further work in this area to confirm the findings using in vitro and in vivo models.

5.2.3 Effect of thiacloprid on synapses

Exposure to high doses of 6 mg/kg/day of thiacloprid reduced the transcription of synapsin IIA and synaptophysin markers significantly. While only synapsin IIa was reduced in the hypothalamus, both Synaptophysin and Synapsin IIa were reduced in the hippocampus. However, in experiment 2b, at a dose of 0.06 mg/kg/day, synaptophysin in the hypothalamus increased.

It is important to keep in note that these synaptic markers are not specific to the cholinergic system and the observed effects might have taken place in other neurotransmitter systems. Indeed, the cholinergic system is interconnected with other neurotransmitter systems, such as the glutaminergic or dopaminergic system. Future studies should surely include immunostaining procedures during developmental and later stages in mice models to ascertain these structural changes, if any, seen at neuronal and synaptic levels after gestational exposure to different doses of neonicotinoids. Combining behavioral analysis will help in confirming the neurological phenotypic pathologies associated with developmental neurotoxicity caused by thiacloprid and other neonicotinoids.

5.2.4 Sex differences in thiacloprid exposure

We observed that the highest dose of thiacloprid in our experiment induced a stronger reduction of BDNF and nestin in the hypothalamus and PCNA in the hippocampus in females. We cannot define whether the lowest dose could have a sex-dependent impact as the number of animals per sex was not sufficient. The importance of biological sex on physiological responses to chemical exposure is relatively common but males are usually more sensitive to environmental stress (when males and females are investigated (131–133)).

Sex is an important variable to be included in the study of neonicotinoids as they act through nAchR which are known to be modulated by sex steroids, in particular estrogens. Sex differences in the impact of neonicotinoids were previously highlighted. For example, A recent study with non-gestational exposure to 5 or 50 mg/kg of clothianidin observed sex-specific neurotoxic effects. Males had a more apparent decrease in

locomotor activities, elevated anxiety-like behaviors, impairment of short- and long-term learning memory, increased c-fos positive cells in the paraventricular thalamic nucleus and the dentate gyrus of the hippocampus, and higher concentrations of clothianidin and most metabolites in blood and urine. Before this study authors also noted that there were no studies on the effect of clothianidin on female animals (134). A single intraperitoneal (i.p.) dose of 337 mg/kg of ~99.5% pure imidacloprid to pregnant Sprague–Dawley rats on gestation day (GD) 9 increased plasma cholinesterase activity in male offspring only, but no sex difference were observed in the brain (77). Clothianidin exposure at different doses during gestation did not result in any sex difference (34). A critical review of the developmental neurotoxicity of neonicotinoids has tried to cover published and unpublished EPA data as well. Acetamiprid (99% purity) given by gavage from GD 6 to Lactation day (LD) 21 noted that at a high dose of 45 mg/kg/day, acetamiprid decreased body weight gain in P-females and F1 animals, decreased the acoustic startle response in F1 males and was associated with a marginally significant increase in the number of errors in the Biel maze in F1 males just after weaning. Caudate-putamen width reduced modestly (but significantly) in F1 female rats at 750 ppm (highest dose) of imidacloprid on PND72, however, as per the authors, this minimal change could have been unrelated to treatment per se. Similarly increased thickness of the hippocampal gyrus (+9%) and cerebellum height (+10%) on PND 11 and decreased thickness of the hippocampal gyrus (5%) observed in F1 females after clothianidin exposure at 1750 ppm was proposed to be spurious and unrelated to treatment (22). Gestational exposure to acetamiprid showed that males in the low-dose group (1 mg/kg) had a significant increase in sexual and aggressive behaviors, while females remained unaffected (135). Gestational imidacloprid exposure led to sex-specific changes with reduced body weight and elevated motor activity in treated male mice (32). Similarly, dinotefuran (136) and clothianidin (34) exposure induced increased motor activity in adult male mice. Nicotine exposure was shown to decrease the expression of the steroidogenic acute regulatory protein (StAR), in CA1, CA3, and dentate gyrus regions of the hippocampus in female rats compared to the control group and male rats (137).

Human PET imaging revealed that the binding level of α4β2 nAChRs was higher in all brain regions in women than in men (138). Furthermore, among female rats and

mice, the basal expression of α4β2 nAChRs is shown to be higher in most brain regions, with repeated nicotine treatment reversing this expression, with higher upregulation in males (139,140). In fact, at the receptor subunit level, the presence of β2 and α7 nAChRs in the dentate gyrus was shown to play a crucial role in offering an advantage to the male sex in spatial learning, and memory tasks (141,142). In a recent epidemiological study in China, women had higher urinary and blood concentrations of most neonicotinoids (143), probably due to greater daily exposure to neonicotinoids.

5.2.5 Mice and physiological barriers

Though this study was carried out on a physiological scale, it is important to ask how thiacloprid may have had these effects on the transcription of different genes, and in particular, if it reached the nAChRs receptors present in the brain. In mice, the blood-brain barrier (BBB) begins to set up with the onset of angiogenesis, from the 10th embryonic development day (85). It was therefore not present at the beginning of exposure to thiacloprid and was not fully formed till the end of it. We can therefore comment that BBB is not a factor limiting the access of thiacloprid to the nAChRs receptors of the central nervous system. The placental barrier may also be involved. To the best of our knowledge, very few studies have demonstrated the possibility of thiacloprid passing the placental barrier. However, the observed effects imply the ability of thiacloprid to pass through some of these barriers but the observed effects may also be due to indirect effects of the treatment. For example, metabolites derived from thiacloprid that were not investigated in the current study could also have caused the observed effects. Some studies have already supported metabolites as a possible explanation for the toxicity of these molecules (14,20,21,59) and also for observed sex differences (134). Furthermore, neonicotinoids including thiacloprid, acetamiprid, nitenpyram, and imidacloprid could freely pass through the BBB and could be detectable in the brain of mice (19,144).

In the current study, four brain regions and three doses of thiacloprid were investigated in mice. The cingulate gyrus and other parts of the brain that express cholinergic receptors could bring other elements of understanding the effects of thiacloprid. The brain is also connected to other organs in the body, such as the liver and gonads, via a reciprocal feedback mechanism. The effect of exposure to thiacloprid may

also be linked to the inflammatory processes occurring in the other organs. Thiacloprid neurotoxicity was also suggested to be due to its functioning as both an antagonist and agonist at the nAChR (145,146), which needs to be confirmed by future studies. Knowing that the different generations of neonicotinoids do not have the same properties (absorption, metabolism, interaction with receptors, etc.), working with other molecules would provide complementary data. Likewise, the results differ according to the species and sex/gender studied, emphasizing thus the difficulty in defining a unique and appropriate model in toxicology studies. Furthermore, it remains unclear whether the observed changes in the expression of all the above-mentioned genes translate into protein in the brain regions of interest.

These results also indicate that thiacloprid may not be toxic to all cell types but affects each brain region differently and in a dose-dependent, sex-dependent manner. This may also be due to the specific subunit of nAchR present in these regions, their distribution, and the connection with other neurotransmitter systems.

5.2.6 Strengths and Limitations

The acceptable daily intake (ADI) of thiacloprid is 0.01 mg/kg body weight per day based on the NOAEL of 1.2 mg/kg body weight per day for liver histopathology and eye effects from the 2-year rat study and applying a standard uncertainty factor (UF) of 100. The acceptable operator exposure level (AOEL) is 0.02 mg/kg body weight per day based on the NOAEL of 2 mg/kg body weight per day for the decreased maternal and fetal weight from the developmental toxicity study in rabbits; this NOAEL was supported by the rat developmental toxicity study for the increased incidence of pelvic dilation and skeletal variations. No correction for oral absorption was needed and a UF of 100 was applied (75).

As per the review paper by Sheets et al (22), our study followed the different guidelines laid out to understand the toxic effects of chemicals, such as a minimum of six animals per treatment condition that is required for minimal confidence in the results (147). The developmental periods also modeled the ideal neural development time during gestational periods in human beings. Dose–response evaluations included more than two dose levels and statistical analysis was robust.

However, as mentioned before with every section of the discussion, there are several aspects that this study did not look into. One such example is long-term neurological changes, be it at the molecular level or behavioral analysis. Further neuronal changes may differ from glial responses to thiacloprid. Also, the dose for zebrafish needs to be titrated further to see the neurotoxic effects, if any. Treatment via the oral gavage of the pregnant dam and water solution for zebrafish embryos was used to model dietary exposure in humans during pregnancy and lactation. Although oral gavage is considered somewhat relevant to human exposure conditions, literature has not always supported this. For example, it was observed that the oral gavage method increased blood pressure, and corticosterone levels (148), and desired concentrations of the drug or chemical of interest were not reached in the blood or brain after oral gavage (149). It is important to further characterize the cholinergic system in the developing brain of rodents and zebrafish, in addition to detailed studies of receptor subunits, location, and distribution analysis. Future studies may try to elucidate other mechanisms of action of this chemical.

Nevertheless, this study is important as neonicotinoids are still the most commonly used insecticides that get incorporated into the physiology of the plants and thus cannot be cleaned off just by washing or cooking before consumption like surface insecticides. Insecticide safety should be determined and risks publicized before registration and introduction to the market. Testing for developmental neurotoxicity and general toxicity must be routine for all chemicals determined for human use. In general, the world must be encouraged to restrict or prohibit the use of synthetic products in the production of food (150). Alternatives to insecticide use must also be explored. For example, insecticides that do not affect the physiological parameters of non-target species, including neural activity are to be explored. Compounds that disrupt insects' respiratory energy production or alter growth and development (151) or genetic modification of crops to express endotoxins (biological insecticide) such as the Cry protein (δ-endotoxin) produced by the Bacillus thuringiensis are toxic to a wide variety of insects (152). Biosolarization is a fumigation alternative that employs soil amendments, solar heating, microbial activity, and anaerobiosis to create soil conditions that are lethal to pests, but safe for humans (153). Plant-derived substances such as corn gluten, black pepper, and garlic compounds can be used as biopesticides to control insects. Insect hormones may

act as biopesticides to repel bugs, disrupt mating and affect growth. Certain food-grade oils such as castor, cinnamon, clove, corn oil, etc are also eligible to be minimum-risk pesticide products. Organic pesticides include soaps, hydrogen peroxide, sodium hypochlorite, and diatomaceous earth (154).

6 Conclusions

Prenatal exposure to thiacloprid resulted in the dose and sex-dependent alteration in the neuronal and steroid markers in specific brain areas only in mice, not in zebrafish. This could be due to the dose used or receptor specificity indicating the need for further investigations on the effects of neonicotinoids in the developing vertebrate brain.

7 Supplementary Information

Supplementary Table 1: Oligonucleotide sequences used in real-time quantitative polymerase chain reaction experiments

Primer name	Forward	Reverse
Zebrafish		
ef1	5'-AGC AGC AGC TGA GGA GTG AT-3'	5'-CCG CAT TTG TAG ATC AGA TGG-3'
esr1 (ER Alpha)	5'-CTG GAG ATG CTG GAC GCT CA-3'	5'-GCT GCA GCT CCT CCT CCT GG-3'
esr2a (ER Beta2)	5'-GAT CCT CCT GAA CTC CAA CAT G-3'	5'-CCA GCA GAC ACA GCA GCT TGG A-3'
esr2b (ER Beta1)	5'-GAT CCT GCT CAA CTC TAA TAA C-3'	5'-CCA GCA GAT TCA GCA CCT TCC C-3'
cyp19a1b (Aromatase)	5'-TCG GCA CGG CGT GCA ACT AC-3'	5'-CAT ACC TAT GCA TTG CAG ACC-3'
Nestin	5'-ATG CTG GAG AAA CAT GCC ATG CAG-3'	5'-AGG GTG TTT ACT TGG GCC TGA AA-3'
Neurogenin 1	5′-TGC ACA ACC TTA ACG ACG CAT TGG-3′	5′-TGC CCA GAT GTA GTT GTG AGC GAA-3′
BDNF	5′-TTA CGA GAC CAA ATG CAA CC 3′	5′-CAC GTA AGA CTG GGT TGT CC-3′
PCNA	5'-CTCACAGACCAGCAACGTCG-3'	5'-GGACAGAGGAGTGGCTTTGG-3'
Synapsin IIA	5'-GTG ACC ATG CCA GCA TTT-3'	5'-TGG TTC TCC ACT TTC ACC TT-3'
Synaptophysin	5'- ATG CAA AGA GCT GCA CGA AC -3'	5'-CCC TGA GAG CTG GCA TAC TG -3'
Caspase 3	5'- CCG CTG CCC ATC ACT A -3'	5'- ATC CCT ACA CGA CCA TCT -3'
Mice		
GAPDH	5'- GCA TGG CCT TCC GTG TTC C-3'	5'- ACC ACC CTG TTG CTG TAG CC-3'
Erα	5'- AGG CAA AAG GGA TTC CAG GG-3'	5'- TTG CTG AGG CTT CCT CTT GG-3'
Erβ	5'- TTT AGC CAC CCA CTG CCA AT-3'	5'- CCT TCA CAG GAC CAG ACA CC-3'
Aromatase	5'- ATG AGG ACA GGC ACC TTG TG-3'	5'- GAG GTT CAC GCC ACC TAC TC-3'
PCNA	5'- GCC AGA CCT CGT TCC TCT TAG-3'	5'- CGT GAG ACG AGT CCA TGC TC-3'
DCX	5'- GAC CTG ACC CGA TCC TTG TC-3'	5'- ACG TTG ACA GAC CAG TTG GG-3'
Synaptophysin	5'- ATC AAC CCG ATT ACG GGC AG-3'	5'- TCT CTT GAG CTC TTG CCC AC-3'
BDNF	5'- TTG TTT TGT GCC GTT TAC CA-3'	5'- GGT AAG AGA GCC AGC CAC TG-3'
Nestin	5'- GTG ACC CTT GGG TTA GAG GC-3'	5'- AGA GCA CCT GCC TCT TTT GG-3'
Synapsin IIa	5'- GAG ACC ATC CGG AGC TTG AG-3'	5'- TCA AGT CAT GGG ACA TCG CC-3'
Neurogenin 1	5'- CGC TTC GCC TAC AAC TAC ATC-3'	5'- TAC TGG GGT CAG AGA GTG GGT-3'

Supplementary Table 2: Tukey's posthoc test Experiment 2a tested in mice during Experiment 2a (D0 versus D.6) in the amygdala, hippocampus, and hypothalamic regions

Tukey's			Equal variances	Levene's Test for Equality of Variances		t-test for Equality of Means						
	Markers	Sex				t	df	Sig. (2-tailed)	Mean Difference	S.E Difference	95% CI of Difference	
			Y/N	F	Sig.						Lower	Upper
Amygdala	DCX	M	Y	2.546	.137	-3.296	12	.006	-0.57	0.17	-0.94	-0.19
			N			-3.554	11.503	.004	-0.57	0.16	-0.92	-0.22
	DCX	F	Y	3.541	.084	-5.188	12	.000	-0.87	0.17	-1.24	-0.51
			N			-4.758	7.144	.002	-0.87	0.18	-1.30	-0.44
	Synapsin IIA	F	Y	.451	.516	-2.343	11	.039	-0.23	0.10	-0.45	-0.01
			N			-2.285	9.066	.048	-0.23	0.10	-0.46	0.00
Hypothalamus	Synapsin IIA	M	Y	.385	.544	2.298	15	.036	0.22	0.10	0.02	0.43
			N			2.173	10.430	.054	0.22	0.10	0.00	0.45
	Nestin	F	Y	3.539	.087	3.734	11	.003	0.45	0.12	0.19	0.72
			N			3.931	8.977	.003	0.45	0.12	0.19	0.71
	BDNF	F	Y	.206	.659	2.517	11	.029	0.36	0.14	0.04	0.67
			N			2.594	10.577	.026	0.36	0.14	0.05	0.66
Hippocampus	Neurogenin	F	Y	1.988	.189	2.923	10	.015	0.33	0.11	0.08	0.58
			N			2.658	5.843	.039	0.33	0.12	0.02	0.64
	PCNA	M	Y	6.886	.020	2.557	14	.023	0.31	0.12	0.05	0.58
			N			2.818	10.957	.017	0.31	0.11	0.07	0.56
		F	Y	.038	.848	5.958	12	.000	0.59	0.10	0.38	0.81
			N			5.916	10.624	.000	0.59	0.10	0.37	0.81
	Aromatase	M	Y	.552	.469	5.834	15	.000	0.59	0.10	0.37	0.81
			N			5.641	11.469	.000	0.59	0.10	0.36	0.82
		F	Y	3.446	.088	5.850	12	.000	0.66	0.11	0.42	0.91
			N			6.546	9.634	.000	0.66	0.10	0.44	0.89
	Nestine	M	Y	.250	.625	3.893	14	.002	0.40	0.10	0.18	0.62
			N			3.840	12.305	.002	0.40	0.10	0.17	0.63
		F	Y	.155	.702	3.051	10	.012	0.60	0.20	0.16	1.04
			N			3.229	7.086	.014	0.60	0.19	0.16	1.04
	Synapsin IIA	M	Y	2.222	.157	6.716	15	.000	0.64	0.10	0.44	0.85
			N			6.082	8.650	.000	0.64	0.11	0.40	0.89
		F	Y	4.926	.046	8.819	12	.000	0.65	0.07	0.49	0.81
			N			9.871	9.619	.000	0.65	0.07	0.50	0.80
	Synaptophysin	M	Y	.141	.713	3.875	14	.002	0.44	0.11	0.20	0.69
			N			3.907	13.419	.002	0.44	0.11	0.20	0.69
		F	Y	.053	.822	4.345	11	.001	0.48	0.11	0.24	0.72
			N			4.229	7.900	.003	0.48	0.11	0.22	0.74

Supplementary Table 3: Tukey's posthoc test Experiment 2b tested among mice during Experiment 2b (D0, D0.06, D0.6) in the amygdala, hippocampus, hypothalamus, and cerebellar regions

Tukey's Markers	Dosage		Mean Difference	S.E	p	95% Confidence Interval	
						Lower Bound	Upper Bound
Amygdala							
DCX	0	0.06	-0.72	0.24	.021	-1.35	-0.10
PCNA	0	0.06	-1.40	0.45	.016	-2.55	-0.25
Aromatase	0	0.6	0.32	0.12	.042	0.01	0.62
Cerebellum							
PCNA	0	0.06	-0.59	0.15	.002	-0.96	-0.22
		0.6	-0.46	0.14	.011	-0.82	-0.10
Hypothalamus							
Neurogenin	0	0.06	0.49	0.13	.005	0.14	0.82
		0.6	0.43	0.13	.010	0.10	0.75
Aromatase	0	0.06	0.60	0.18	.010	0.14	1.06
		0.6	0.45	0.17	.046	0.01	0.89
Nestin	0	0.06	0.58	0.14	.002	0.22	0.94
		0.6	0.57	0.14	.001	0.22	0.91
BDNF	0	0.06	0.53	0.16	.010	0.12	0.94
		0.6	0.45	0.16	.023	0.06	0.85
Synaptophysin	0	0.06	-1.22	0.34	.006	-2.10	-0.35
Hippocampus							
Neurogenin	0.6	0	2.38	0.59	.002	0.88	3.88
		0.06	2.71	0.64	.001	1.09	4.34
ER Beta	0.6	0	1.30	0.40	.012	0.28	2.31
		0.06	1.75	0.43	.002	0.65	2.86
Aromatase	0.6	0	1.03	0.36	.028	0.11	1.95
		0.06	1.48	0.39	.004	0.48	2.48
ER Alpha	0.6	0	1.25	0.38	.011	0.28	2.23
		0.06	1.64	0.42	.003	0.57	2.70
Nestin	0.6	0	2.12	0.54	.002	0.76	3.49
		0.06	2.31	0.58	.002	0.83	3.79

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Chapter 5
Discussion & Summary

Chapter 5: Discussion and Summary

About 10.7% of the global population suffers from a mental disorder. Concerning anxiety disorders, globally, 301.39 million prevalent cases were estimated in 2019 (1). Neuroscience research reveals that different mental health disorders such as stress, anxiety, depression, and even neurodegeneration and their forthcoming behaviors are associated with alterations in neuroplasticity. Thus, it is important to collect evidence regarding the etiological factors and if possible, design prevention and treatment strategies for this altered neuroplasticity. The plasticity of the human brain is adaptive to structural and functional changes based on experience and training leading to functional recovery (2).

Among the various factors that affect neuroplasticity, human physiology and health are strongly influenced by the environment. Several factors modulate neuroplasticity in a positive and negative direction. The field of environmental health has evolved significantly in the recent past but is still based on epidemiological studies. Most studies have focused on the potential negative impacts of environmental factors (mainly social stress and exposure to chemicals), but some factors have positive effects. Thus, it was planned to study one positive and one such negative stimuli on neuroplasticity in this thesis. In the first chapter **(Chapter 1)**, the concepts of neuroplasticity, anthropogenic factors, and exposome are introduced. The process of neurogenesis during development and adulthood is then discussed, indicating the vulnerability of the developing brain to exposome. Then the exposure of human beings and animals to different anthropogenic stimuli is introduced. The factors that influence neuroplasticity are detailed. Specific emphasis is laid on the impact of acoustic and chemical stimuli on neuroplasticity. Our objective was to gain more knowledge about the physiological impact of potentially positive (auditory stimulation: music) and negative (chemical exposure: thiacloprid) stimuli on the peripheral and central nervous systems.

Among the different stimuli that have shown beneficial effects, music is a promising intervention that is ubiquitous across human cultures. Music is a powerful sensory stimulus that produces physiological, psychological, and social effects. Both listening to music and music playing lead to structural and functional neuroplastic changes that are utilized in the management of different non-communicable disorders.

Music listening involves the sensory processing of acoustic stimuli (peripheral nervous system) followed by cognitive and emotional processing in a neural network (central system) producing pleasurable physical and emotional experiences. However, the association between acoustic intervention with music, its effects on overall health, and the mechanisms behind it remain unclear, and earlier studies reported contrasting findings. Thus, we asked the question: **What are the short-term effects of positive anthropogenic auditory stimuli (music) on physiological parameters among healthy human beings? In Chapters 2 and 3,** the effects of auditory stimuli (music) were studied on the central and autonomic nervous system, through the analysis of subjective measures of stress and anxiety, and of physiological parameters (electrocardiography, blood pressure, and electroencephalography recordings) in humans.

Chapter 2 details the study where we used a triple-blind, randomized control trial design and showed that listening to music led to behavioral and cardiovascular modifications among healthy young adults. This chapter demonstrated the effect of different musical acoustical stimuli (three modes of music stimuli, named *Miyan ki Todi, Malkauns, Puriya* in Indian music), compared to natural sounds in the environment (stimulus given to the control group), on measures of stress, anxiety, blood pressure control, and autonomic nervous system tone (measured employing heart rate variability) among healthy human beings. Modes are a combination of notes in a given scale of music woven together to produce a melody. The melody is the linear succession of musical tones that vary in pitch. We showed that all modes of music reduced the levels of anxiety and stress to different degrees. The control group did not show a change in anxiety levels. However, using heart rate variability measures, we observed that during the intervention with two modes (*Miyan ki Todi, Puriya)*, there was an arousal response, while after the intervention an improvement in parasympathetic tone (relaxation response) was observed. The third mode of music (*Malkauns)* caused a sustained increase in parasympathetic tone, like that observed in the control group. It indicated that although musical acoustic stimuli reduced anxiety and stress measures, the timing of autonomic changes varies with the mode used as the stimulus.

Dysautonomia refers to a group of medical conditions caused by problems with the autonomic nervous system (ANS). Worldwide, it affects more than 70 million people. Dysautonomia can affect ANS functions such as control of blood pressure, heart rate, respiration, gastrointestinal functions, all visceral organ functions, and temperature control. Secondary dysautonomia can result from different diseases such as Cancers, Diabetes, PD, Rheumatoid arthritis, Amyloidosis, and many such disorders. Currently, there is no cure for this condition except for symptomatic management of blood pressure (for example for low hypotension, more water and salt intake is prescribed) or other autonomic symptoms. The literature review suggests the use of supportive therapy modalities, including music as an intervention for the prevention or treatment of autonomic dysfunction (3). Interventions based on music therapy, traditional Chinese medicine-related treatments, exercise, relaxation, and myofascial release techniques are found to be beneficial. Many randomized controlled trials have reported that music possesses anxiolytic and analgesic properties, and is associated with decreased heart rate, respiration rate, and blood pressure in palliative care settings or perioperative periods. Regarding the mechanism behind the effect of auditory stimulation and cardiac autonomic regulation, it was hypothesized that pleasurable songs induce dopamine release in the striatal system, which is involved in autonomic regulation, and this topic has been well-reviewed in (4).

This is the first study to focus on how Indian modes can alter physiological measures related to stress, arousal, and anxiety. Clinically, this study promoted the idea of the use of music, and particular modes, to facilitate relaxation, and provide an alternative treatment strategy. Future studies may find it beneficial to expand the present findings to other melodies, investigate during live music concert sessions, analyze temporal variations in heart rate variability during the intervention, and more closely investigate gender differences to understand if reproductive steroid hormones can play a role in the physiological measures assessed. It would also be interesting to investigate factors related to perception and emotion, such as personality and music preferences, in future work. Further analysis of the musical features and the components (e.g., temporal analysis of note/tonal variations, pitch, tempo, dynamics, and contrast) of the music used may enhance our understanding of the physiological effects.

Musical stimuli can induce a variety of emotions in individuals. The mode is one of the most important structural features that constitute the expressional characteristics of music. The mode is a structural feature embodied in the structural relationship among the tones that constitute the basic series of music. Two of the modes are natural diatonic scales, the Ionian (Natural Major Scale) and the Aeolian (Natural Minor Scale). The remaining commonly used modes are Phrygian, Dorian, Mixolydian, and Lydian. In Indian music, modes are named *ragas*, and there are about 72 parent modes called the *Janaka ragas* (containing the 7 notes, *Sa Ri Ga Ma Pa Dha Ni*, similar to *Do Re Mi Fa So La Ti* of western music), derived from 22 music tones. From these modes are the thousands of other modes derived by either eliminating, adding, skipping a few notes, or by different permutations and combinations of the tones. The structural difference between the major and the minor mode produces distinct sound effects. It is important to understand these modes as the literature survey repeatedly suggests that the major mode tends to induce positive emotions, while the minor mode tends to induce negative emotions. For more on this see (5–7) which was also shown to be true using Indian music ragas (8,9). In the current study, though physiological effects varied with the modes heard, emotional ratings or valence recordings were not included to conclusively comment on the emotional experience the participants might have had. Thus, future studies may include studying modes of music and the emotional experience of the participant simultaneously.

In recent years, a lot of research has focused on the physiological effects of music. Electroencephalography (EEG) is often used to verify the influence of music on human brain activity. Music is considered a powerful brain stimulus, as listening to it can activate several brain networks. Music of different kinds and genres may have different effects on the human brain. Furthermore, the brain activity of multiple subjects has been shown to synchronize during salient moments of natural stimuli, suggesting that the correlation of neural responses indexes a brain state operationally termed 'engagement'. This is classically studied as Inter-subject correlation (ISC) using fMRI and has recently been used to analyze EEG signals on intervention with narrative stimuli, with a temporal structure such as a story, movie, or music.

In **Chapter 3** we analyzed the brain wave changes and frequency distribution spectral analysis (measured by EEG) by listening to the same set of acoustical stimuli used in chapter 2. On analysis of frequency components across the whole scalp during the intervention, a global drop in alpha power in all the groups and a frontocentral increase in beta (after intervention) and gamma power (during intervention) were noticed with modes *Miyan ki Todi* and *Malkauns* respectively. This change in beta was postulated to be due to attention modulation, and higher alertness. In EEG, the alpha rhythm is said to be associated with global cognitive engagement while beta and theta rhythms point towards specific functions, for example, working memory, and there is de-synchronization in alpha and beta bands during mental imagery (10). The rise in gamma was probably indicating the binding of music features or may also be due to liking the music based on previous literature. Group-based cluster statistics revealed a rise in left frontal gamma power during intervention with mode *Malkauns.* Group-based cluster statistics revealed a drop in the right frontoparietal delta (which could be due to alertness or divergent thinking following the stopping of the intervention) and a rise in frontal beta1 with mode *Miyan ki Todi*. With the mode *Puriya,* after the intervention, a widespread drop in delta power and a rise in frontal beta1 were observed.

It was further demonstrated that there existed three most correlated components, the first component representing delta or theta power (band1), the second component alpha or beta1 power (band2), and the third beta2 or gamma power (band3), with both modes *Malkauns* and *Miyan ki Todi* showing similar patterns of decrease in the lower frequency band (band1) and increase in mid-band (band2) during the intervention, compared to baseline and mode *Puriya* being similar to the control group, with an increase in the lower frequency band (band1) and decrease in mid-band (band2) during the intervention, compared to baseline. Shared brain responses among the participants as captured by Inter-subject correlations (ISC) were also studied in this chapter. Reduction in globally distributed low-frequency activity and increase in posterior dominant alpha-beta1 activity may be characteristic of passive listening to relaxing Indian modes, which may persist even after 10 minutes of the listening period. Among the modes, *raga Malkauns* showed this effect most prominently, followed by *raga Miyan ki Todi* and least by *raga Puriya*. As the increase in posterior alpha and low beta power

is associated with Default Mode Network (DMN) activity and a decrease in delta power with positive emotional memory, the spectral pattern we observed may indicate the observation of positive autobiographical memory while listening to musical modes, and thus contribute to a relaxing experience.

In a recent study, preferred music (called favorite music) and researcher-selected music (called relaxing music) were used as stimuli to study their effects on the brain. A better soothing effect was achieved by using relaxing music in that study, and it was also observed that longer periods of listening to relaxing music can cause a more significant change (11), which was further confirmed (12). When music pieces were played with two instruments, top-down modulations consistently enhanced or better reconstructed the relevant instruments than irrelevant ones during the segregation task. This wasn't the case with the integration task as listeners probably employed heterogeneous strategies. These findings are similar to those seen with speech and polyphonic music perception (13). Familiarity with music is another confounding variable in music studies. Human listeners exhibit marked sensitivity to familiar music. Pupil responses showed a greater and faster dilation rate to familiar music, consistent with a faster activation of the autonomic salience network. EEG showed a later differentiation of the tunes, from 350 ms after onset. Interestingly the cluster pattern identified in the EEG was very similar to that found in the classic memory retrieval paradigms, suggesting that the recognition of brief, familiar music snippets, drew on similar processes (14). Music listening has also been used to differentiate responses in EEG between major depression disorder (MDD) and healthy individuals. During music perception, MDD patients exhibited altered functional connectivity in delta and beta bands. MDD patients did not exhibit a lateralized effect while healthy people showed a left hemisphere-dominant phenomenon. These responses facilitate a new direction toward a diagnosis of connectivity disorders in depressed patients using music perception paradigms (15). Music of different valences has been shown to alter the EEG activity in emotion-specific regions (16). Music therapy and emotion-guided music decisions may be used for improving clinical depression and anxiety (17). Music has also been used to understand and elicit emotions in mental health conditions such as bipolar disorder, autism, and Alzheimer's disease (18–20). Recently EEG power with a higher beta band and gamma band at the O2 and P4

electrodes was used as evidence to conclude that sad music may alleviate pain (21), which also has potential value for clinical use. In PD patients EEG power differences are partially reduced by listening to music. Music slightly improved the connectivity differences, particularly the frontotemporal inter-hemispheric communication which might underlie music's beneficial effects on PD pathophysiology and should be further investigated (22). Brain development and morphological changes with age also influence the way sounds are processed, with studies showing an age-related increase in inter-subject variability (23). Neuroplastic effects of music-based interventions and their usage for neurorehabilitation were recently reviewed in (24).

The importance of studying changes in EEG on listening to music is thus not a new subject. In our current study, we showed that every mode of music can have a different physiological effect as observed on HRV (Chapter 2) or EEG recordings (Chapter 3). Further studies may include phenomenological reports to support these findings and build a stronger scientific foundation for the use of music in medicine. As ISC-based brain activity is modulated by training, studies may try to explore the effect of musical training and genre familiarity aspects. Different musical stimuli that are known to be emotionally stimulating can be studied, as ISC is said to vary with time-based emotional stimuli such as stories or movies. Studies may also include the emotion ratings for a better understanding of the exact emotions that might have caused these physiological changes. To exactly know the neural substrates activated within and between participants passively listening to the different scales, it is better to use higher-density EEG or fMRI data.

In the present thesis, we tried to understand the neuroplastic effects of one negative anthropogenic stimulus (insecticide) - **Chapter 4.** Industrialization of the agricultural sector has increased the chemical burden on natural ecosystems. Pesticides are agricultural chemicals used in agriculture, public health programs, and urban green spaces to protect plants and humans from various diseases. The intensive use of pesticides (eg: organophosphates, carbamates, pyrethroids, neonicotinoids) and the persistence of the molecule in the environment have contributed to the increased exposure of non-target invertebrates and vertebrates, including humans. For experimental studies, animals are commonly used to understand the negative impact of

anthropogenic stimuli on neuroplasticity. Among the various pesticides, though a handful of neonicotinoids were banned by the European Union, they continue to be used in some countries and they continue to persist in the environment. For the current thesis, we chose to study one of the most toxic, persistent insecticides, thiacloprid (**Chapter 4)**, where we highlight the developmental neurotoxic potential of this insecticide. Though studies have looked into the potential of neonicotinoids from a developmental neurotoxic potential point of view, not many have studied the same in the way that the results of those can be applied to human health as per European Protection Agency guidelines for the study of insecticides. The drawbacks of previous works were the absence of studying both sexes, at different doses and the absence of statistical analysis. Different areas of the brain, in particular, the cholinergic areas of the brain, that are the prime targets for neonicotinoids, for their gene transcription had not been explored before. Thus we asked the question, **what are the potential short-term neuroplastic effects (as evidenced by gene expression) after perinatal exposure to different doses of thiacloprid, a toxic anthropogenic stimulant neonicotinoid in animals, zebrafish, and mice** (Chapter 4)?

In Chapter 4, we evaluated the effect of perinatal exposure to different doses of thiacloprid, a neonicotinoid insecticide, on neuronal markers from whole heads of zebrafish larvae and specific brain regions (amygdala, hippocampus, cerebellum, hypothalamus) in mouse models. Perinatal exposure to thiacloprid resulted in the dose and sex-dependent alteration in the neuronal and steroid markers in specific brain areas only in mice, but not in zebrafish. In mouse offspring, a significant main effect of dose with an increase in DCX, PCNA (amygdala), PCNA (cerebellum), synaptophysin (Hypothalamus), and a decrease in hypothalamic ERβ, nestin, synapsin IIA, BDNF, Aromatase, hippocampal DCX, PCNA, neurogenin, aromatase, nestin, and synaptic markers was observed. The sex-specific difference in BDNF transcription in the hypothalamus and PCNA in the hippocampus was observed. Dose-dependent change (from 0.06 to 0.6 mg/kg/day) with an increase in synaptophysin (hypothalamus), ERα, ERβ, aromatase, nestin, neurogenin (hippocampus), and reduction in aromatase (amygdala), was observed. This work shows that alteration of the cholinergic system by neonicotinoid pesticide impacted the neuroendocrine system and the consequences of

this alteration should be further investigated in the central (limbic) and peripheral nervous system and on the pathophysiology in vertebrates, including humans.

Our objective was to define the potential impact of the neonicotinoid thiacloprid on neuroplasticity and the neuroendocrine system in two vertebrates: the zebrafish and the mouse. In our study, we did not see any impact of thiacloprid on zebrafish, independent of the dose while specific brain regions in mice were impacted by early exposure to this pesticide. Though several genes are expressed during the developmental stages of zebrafish, none of the gene expressions changed using three different concentrations of thiacloprid. The probable reasons this could be the dosage used (which was low compared to previous studies), the duration of exposure, the temperature during exposure, species sensitivity difference for the chemical, receptor affinity, dynamic regulation of the cholinergic system, or presence of chorion. Analysis of the effect of thiacloprid and other such neonicotinoids on cholinergic system transcripts and subunits of the receptors is proposed to be taken up in future studies. Oxidative stress was proposed to be one of the chief mechanisms for developmental neurotoxicity with neonicotinoids. Further studies in this regard will be valuable. Also, the dose for zebrafish needs to be titrated further to see the neurotoxic effects, if any. Long-term effects at the molecular level in the brain and behavior remain to be explored.

In mice, thiacloprid caused opposite effects on the hippocampus and the amygdala, regions chiefly involved in emotional behavior, memory, learning, fear, and stress responses. Further studies may try to elucidate the long-term behavioral modifications on developmental exposure to thiacloprid. Inflammatory response mounted by these regions may also be further elucidated in future studies. By thiacloprid effects on Estrogen receptors and aromatase, we postulated that thiacloprid may act through these ER receptors in a region and dose-specific manner to impair the functions in the hypothalamus while facilitating the functions of the hippocampus at the mid-dose range. At a high dose of thiacloprid, probable neuronal/glial cytotoxicity leads to a drop in most of the gene expressions. Recently, neonicotinoids, clothianidin, acetamiprid, and dinotefuran were shown to activate G-protein coupled Estrogen receptors (GPER) in a dose-dependent manner and thus promote breast cancer proliferation (25). It would be interesting to see if GPER in different brain regions is affected by neonicotinoids in future

studies. It is important to study the role of thiacloprid and other neonicotinoids on sex-dependent behaviors and their involvement in the epigenetic causation of neurological diseases, such as Alzheimer's disease in long term through their action as endocrine disruptors. In the current study, we found a rise in synaptophysin expression in the hypothalamus, indicating a probable rise in synaptic activity at that dose, but due to lack of further details, it is difficult to comment about a particular nucleus within the hypothalamus that might have been affected, or the neural activity change or the eventual protein expression. Despite high concentrations of these synaptic markers in different regions of the brain, we did not observe similar alterations in the expression in the various regions on exposure to thiacloprid. Future studies should include immunostaining procedures during developmental and later stages in mice models to ascertain these structural changes, if any, seen at neuronal and synaptic levels after gestational exposure to different doses of neonicotinoids. Combining behavioral analysis will help in confirming the neurological phenotypic pathologies associated with developmental neurotoxicity caused by thiacloprid and other neonicotinoids. Sex differences in exposure to neonicotinoids have often been ignored. Since several neurological diseases appear to occur in a sex-specific manner, studies on neonicotinoids must include sex as a biological variable. Furthermore, neonicotinoids could freely pass through the BBB and were detectable in the brain of mice (26,27). The results also indicate that thiacloprid may not be toxic to all cell types but affects each brain region differently and in a dose-dependent, sex-dependent manner. This may also be due to the specific subunit of nAchR present in these regions, their distribution, and the connection with other neurotransmitter systems. Differences between neuronal and glial responses to thiacloprid are yet to be elucidated.

This study is important as neonicotinoids are still the most commonly used insecticides that get incorporated into the physiology of the plants and thus cannot be cleaned off just by washing or cooking before consumption like surface insecticides. Furthermore, it should be noted that washing and peeling cannot completely remove residues. In the majority of cases, the concentrations do not exceed the legislatively determined safe levels. However, these 'safe limits' can underestimate the real health risk, as in the case of simultaneous exposure to two or more chemical substances, which

occurs in real-life conditions and can have synergistic effects. Pesticide residues have also been detected in human breast milk samples, and there are concerns about prenatal exposure and health effects in children. Insecticide safety should be determined and risks publicized before registration and introduction to the market. Testing for developmental neurotoxicity and general toxicity must be routine for all chemicals determined for human use. In general, the world must be encouraged to restrict or prohibit the use of synthetic products in the production of food (28). Alternatives to insecticide use must also be explored, such as the Cry protein (δ-endotoxin) produced by the *Bacillus thuringiensis*, Biosolarization, Plant-derived substances, Insect hormones, Certain food-grade oils, and other organic pesticides. We need to try to understand the impact of chemicals on non-target species and help to regulate the use of these molecules and find alternatives if (neuro)toxicity is present.

Future perspectives

Exposome research can facilitate the identification of particular environmental factors that contribute to the onset of neurological disorders. The impact of chemical exposures currently surpasses biological exposures, and until recently scientists mainly focused on the acute consequences of biological exposures. However, a crucial constraint of various exposome and health investigations so far is that they concentrate on the connections between individual components of the external exposome and unfavorable health outcomes. The mechanistic comprehension of the relationship between exposure and disease is disregarded in both epidemiological and toxicological studies. The recent exposome framework has propelled the domain of molecular toxicology by offering the necessary mechanistic examination of the exposome's effects on health (29). Understanding the mechanisms involved in developmental neurotoxicity should be used to develop focused therapeutic interventions. Furthermore, guidelines for testing developmental neurotoxicity may require re-evaluation. There is also a necessity to create and verify innovative sets of alternative models and tests for developmental neurotoxicity (see a recent review on risk assessment, alternate models, and recommendations (30)).

Neurotoxic effects are often observed as a result of exposure to toxic substances during pregnancy, nursing, early childhood, and adolescence. Although these effects

may manifest after only a brief period of exposure, research suggests that it may take months or even years for the detrimental effects of toxic substances to become clinically detectable. Therefore, similar to polio, tuberculosis, or any infectious disease detection or vaccination campaigns, it is crucial to conduct thorough epidemiological studies to establish risk assessments and possible causal associations between chemical exposures and developmental abnormalities in humans. To achieve this, it is necessary to quantitatively determine the relationship between internal exposure and exposome. Physiologically based pharmacokinetic (PBPK) and quantitative in vitro to in vivo extrapolation models can be used for this purpose (30). This will also aid in the early detection and treatment of neurological disorders, as well as the prevention of exposure risks. This involves analyzing how neurotoxicants interact with an individual's genetic susceptibility and exposure to other environmental factors. By understanding the interplay between various environmental factors and their impact on neurological health, exposome research can contribute to developing personalized prevention and precision medicine approaches that consider an individual's unique environmental exposures when treating neurological disorders. However, identifying silent neurotoxicity and subclinical changes remains a challenge, necessitating further research to develop tools for the early identification of exposure risks. It is essential to note that treating developmental neurotoxicity is often a multifaceted process that requires a team of healthcare professionals from various disciplines, including neurology, pediatrics, rehabilitation, and psychology. Exposome research can offer valuable insights to policymakers, enabling them to develop more effective public health policies that address environmental factors contributing to neurological disorders.

Although music therapy is effective in treating various neurological conditions such as stroke, traumatic brain injury, and Parkinson's disease, its efficacy in treating developmental neurotoxicity is uncertain. Developmental neurotoxicity can affect different aspects of neurological function, including cognitive, motor, and sensory processing abilities. Music intervention may help address certain areas such as improving motor coordination, communication, and socialization skills. However, the effectiveness of music therapy for developmental neurotoxicity would depend on the specific symptoms and underlying condition of the individual. Music can be used as an

adjunctive therapy alongside other treatments for the rehabilitation of individuals exposed to neurotoxins, providing a more comprehensive approach to treatment. Moreover, music has the potential to promote neurogenesis and neuroplasticity and thus may be beneficial for treating certain aspects of developmental neurotoxicity. Nevertheless, further research is necessary to establish the effectiveness and optimal use of music therapy in this context. Recent epigenetic studies have shown that music listening may upregulate microRNAs related to neuroplasticity, indicating a potential positive impact of music therapy as a way to counteract the negative impact of anthropogenic stimuli with positive ones (31). Future studies should look into the neuroplastic effects of music as an adjuvant in the management of people exposed to chemicals, including psychoactive substances.

Summary

- **In human studies,** we observed that musical acoustic stimuli have specific effects on the autonomic nervous system, stress, and anxiety levels. Specific modes *Miyan Ki Todi* and *Puriya* caused arousal during the intervention while improving the parasympathetic tone after the intervention, while mode *Malkauns* led to a sustained rise in parasympathetic tone, as observed in the control group receiving natural sounds as stimuli.
- Neurophysiological study of electroencephalogram during the different modes of musical acoustic stimuli showed a higher level of engagement and attention during modes *Miyan Ki Todi* and *Puriya,* while mode *Malkauns* led to divergent thinking after the intervention. These studies confirmed the acute neuroplastic effects of auditory stimuli in human beings.
- **In animal studies,** we have observed that chemical environments, such as exposure to pesticides, can be causally linked to the alteration of the central nervous system. As shown in the present work and adding to the current literature, the impact of pesticides will depend on the animal model, the brain regions, and the sex of the model, reflecting the complexity of studying the consequences of chemical exposure on the nervous system and behavior and its extrapolation to the human when required.

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Chapter 6
Societal Impact - Valorization

Chapter 6: Valorization

In universities, new methods are being used to assess the economic or social value of research. Establishing possible valorization routes in project planning brings great benefits to society and researchers. From a valorization point of view, this thesis targets different groups of scientific and non-scientific individuals in society, as briefly discussed below.

Neurological disorders caused by trauma or illness are a heavy burden not only for patients but also for their families, be they those impairing cognitive, sensory-motor functions, or autonomic nervous system (ANS) functions. New strategies to prevent and manage such neurological diseases must always be sought to reduce the risk and burden of disease. The empirical work presented in this thesis first and foremost aimed to better understand the short-term effect of **one positive anthropogenic auditory stimulus (music) on humans** and the mechanisms that underlie the observed changes. The focus was on neuroplastic changes, including the central and peripheral nervous systems (autonomic nervous systems). Therefore, the present findings are primarily of interest to the **auditory neuroscience community**, studying the neuroscience of music to appreciate the varied effects of using different modes of music (Indian classical genre) on subjective measures of stress, anxiety, cardiovascular, central nervous, and the ANS. At the time of writing this thesis, approximately 35,008 articles have been published using the MeSH word ‘Music’ and about 243 articles using ‘Indian Music’ on PubMed. The community of music neuroscience researchers has been growing in recent years for the exciting possibility of using music intervention to prevent and treat neurological problems, involving cognitive, motor, and sensory deficits. In addition to this, this thesis will capture the attention of **psychotherapists** who use cognitive behavioral therapy, mindfulness-based meditation, or different modes of relaxation therapy to reduce stress and improve the mood in emotionally or psychologically affected individuals. It is important for **music psychologists and therapists** who can decide on the number of music features and modes of music for patient-centric therapy, based on existing evidence, and that given in the current thesis. Research into emotions induced by music may also be further explored. Additionally, an improved understanding of the neuronal mechanisms that

support the auditory perception of music is of interest to other domains of cognitive neuroscience, such as **language and vision**. Language and music share common processing systems (1,2).

Although the research discussed here has been conducted in healthy volunteers, advances in its knowledge can be used to unravel more general **pathophysiological deficits** of stress, anxiety, ANS plasticity, functional and structural cortical neuroplasticity, and auditory cognition. For example, Alzheimer's disease (AD) is often associated with loss of memory and autonomic dysfunction (3). Studies using music as an intervention has shown that music often stimulates autobiographical memory centers (4), and restoration of autonomic balance is one of the main mechanisms of action through which music has an effect in Alzheimer's patients (5). Thus, the current thesis is important for **physicians, and neurologists** treating disorders with impaired neuroplasticity or ANS disorders and those involved in **neurorehabilitation and palliative care**. Importantly, the current hypothesis of neurological diseases is that stress activates the ANS, which in turn results in the activation of the neuroinflammation pathway, triggering a cascade of events that result in anxiety, depression, neurodegenerative, and neuroinflammatory disorders (6–8). Therefore, it is of utmost importance to **develop new strategies** for the prevention of ANS hyperstimulation and dysfunction. Given the significant side effects of several drugs and chemicals in the market (for example see(9)), it is high time, we analyze the non-pharmacological interventions available for the prevention and management of chronic diseases. **Non-pharmacological interventions** have been known since before modern pharmacology was developed. Recent systematic reviews declared that though non-pharmacological therapy showed promise high-quality evidence was lacking for the management of AD (10), and low to mixed-quality studies were observed for the management of pain in dementia patients (11) indicating the necessity of more rigorous design to validate the results. Our experiments in this thesis may benefit by adding to the current literature on the use of music (listening to music/playing instruments/singing) for any chronic systemic non-communicable diseases associated with autonomic dysfunction. Indeed, the inclusion of music into one's lifestyle is not a difficult task to achieve. **Artists** in collaboration with medical specialists can come up with music having

a combination of specific music features to target a particular physiological effect, and also explore long-term changes in neuroplasticity.

The second part of the thesis aimed to understand the short-term effect of **one negative anthropogenic chemical stimulus (insecticide-thiacloprid) on animals,** and the molecular mechanisms that underlie the observed changes. We observed significant changes in neuroplastic markers depending on the dose, sex, and species of animals. Pro-environmental behavior refers to acts that *benefit the natural environment, enhance environmental quality, or harm the environment as little as possible* (12). **Environmental social scientists** study human-environment interactions (also known as sustainability science and coupled human-natural systems research). This thesis contributes to understanding the relationships between humans and nature as affected not only by local and global factors but also by environmental policies. **Chemical usage and release policy** in the environment without prior analysis of its effects can harm society as a whole. The risks a chemical poses need to be identified, and publicized before registration and release into the environment. Although **agricultural workers** receive educational training through governmental and non-governmental institutions, the topics are usually around occupational acute poisoning prevention. It is necessary to educate them regarding the risks involved with long-term chronic exposure to pesticides and other chemicals, proper means of release and disposal of chemicals, and ways to reduce the exposure risk for the whole family. On the other hand, increased efforts aimed at reducing pesticides are mandatory. The findings of this study necessitate a need for regulatory action by the governmental and important international agencies to **promote alternatives for pest control** (or make them non-toxic/less toxic) eliminating the risk of pesticide exposure at source in humans and animals (particularly pregnant and children). Furthermore, **plant geneticists** can plan to investigate the probability of creating genetically modified plants as is already being tried. It is proposed through this thesis that like **clinical trials** in medicine, where every drug goes through different phases of testing for its safety and efficacy, anthropogenic chemicals that are currently present in the environment and those that are planned to be released into the environment should go through vigorous multiple steps of testing on not just target insects or pests, but also a set of non-target vertebrates, in land and aquatic environments (**preclinical trials**),

including trials in humans (at a milder dose). Only after these steps, should the **regulatory authorities** be allowed to utilize such chemicals for agricultural or home-based products. **Environmental quality standards** are required to be developed for all chemicals, and all this information should be made easily accessible to the common man. This thesis is also important for **policymakers** across the world who need to understand the impact of chemical exposures even at low concentrations on pregnant women and the developing fetus, survival, and biodiversity of animals in the land and water. This thesis highlights the neuroplastic changes caused by positive and negative stimuli, and long-term effects of developmental exposure to adverse stimuli. As a society, it is thus essential to cultivate and disseminate methods that enrich neuroplastic changes in the positive direction.

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Appendix

- About the Author
- List of Publications
- Acknowledgments

About the Author

Kirthana Kunikullaya U, MBBS, MD, DNB, was born in Mangalore, on 23rd January 1983. With an immense interest to reduce human illness, she pursued a Bachelor of Medicine, Bachelor of Surgery (MBBS) as a first-generation doctor (2000-2006). She then completed her MD in Physiology (2010) with a 1st rank for Rajiv Gandhi University of Health Sciences (RGUHS). Later she joined as an Assistant Professor in Physiology at a Medical School in India. Here she taught undergraduate and graduate-level courses in medical and paramedical streams. She was consistently best-rated >4.5 on 5 for her teaching. During this time she led research on - non-pharmacological interventions' influence on the central and autonomic nervous system in humans - as a Principal Investigator. This research was supported by the grants from the Indian Council of Medical Research (ICMR) and the RGUHS, Karnataka. She has published over 20 peer-reviewed articles in indexed, national/international journals. Her research was recognised and awarded by the Association of Physiologists and Pharmacologists of India (APPI) and the Indian Academy of Biomedical Sciences (IABMS). She was an invited speaker at several national and international workshops and conferences. She is also a reviewer of several scientific journals. She secured grants and organized the Neuroscience-2017, Science academies lecture workshop supported by the Indian Academy of Sciences, The National Academy of Sciences, and the Indian National Science Academy. Currently she is pursuing research in the area of molecular mechanisms of the brain, where is she is studying the interaction of neurotransmitter systems with neurosteroids in Thierry Charlier's lab at the University of Rennes 1, with the grants from Stratégie d'Attractivité Durable - Région Bretagne. In specific she is exploring the effect of estrogens on neuroplasticity, using transgenic and brain aromatase knock-out zebrafish models. She has been actively mentoring undergraduate, and graduate students on scientific research. Apart from this Kirthana is a trained Carnatic musician, singer and co-founder of Kalamshu Cultural Trust. More details can be found on https://kirthanaku.github.io/.

List of Publications

Publications:

1. **Kunikullaya Ubrangala K**, Kunnavil R, Sanjeeva Vernekar M, Goturu J, Vijayadas, Prakash VS, Murthy NS. Effect of Indian Music as an Auditory Stimulus on Physiological Measures of Stress, Anxiety, Cardiovascular and Autonomic Responses in Humans-A Randomized Controlled Trial. Eur J Investig Health Psychol Educ. 2022 Oct 19;12(10):1535-1558. doi: 10.3390/ejihpe12100108. PMID: 36286092; PMCID: PMC9601678.
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5. **Kunikullaya U K,** Kunnavil R, Vijayadas, Goturu J, Prakash VS, Murthy NS. Normative data and gender differences in heart rate variability in the healthy young individuals aged 18-30 years, a South Indian cross-sectional study. Indian Pacing Electrophysiol J. 2021 Mar-Apr;21(2):112-119. doi: 10.1016/j.ipej.2021.01.002. Epub 2021 Jan 19. PMID: 33482336.
6. **Kunikullaya KU**, Vijayadas, Kunnavil R, Goturu J, Prakash VS, Murthy NS. Gender-based comparison of salivary stress marker among healthy individuals after intervention with three melodic scales of Indian music – Exploration with a pretest-posttest design. Natl J Physiol Pharm Pharmacol.2021;11(2):160-168.doi:10.5455/njppp.2021.11.09245202008102020.

Manuscripts in Preparation:

1. Acoustic stimulation with different modes of Indian music on electroencephalographic correlated components and intersubject correlation – a randomized controlled trial.
2. Perinatal exposure to the neonicotinoid thiacloprid impacts neuroplasticity and neuroendocrine system in vertebrates.
3. Emotions induced by Indian Classical Music.

Conference Proceedings:

Oral Presentation

1. Invited talk - “Music and Physiology” - talk on 04 Sept 2021 - Meera Center for Music Therapy, Bengaluru, India (Virtual).
2. **Kirthana Kunikullaya Ubrangala**, Arun Sasidharan, Rakshith Srinivasa, Vijayadas, Radhika Kunnavil, Jaisri Goturu, Vadagenahalli S Prakash, Nandagudi Srinivasa Murthy. Influence of Indian melodic scales on EEG power spectrum among young healthy Indians – a randomized controlled trial. 16th International Conference on Music Perception and Cognition - 11th triennial conference of ESCOM July 2021 - https://sites.google.com/sheffield.ac.uk/escom2021/home and https://www.youtube.com/watch?v=oQ3sm7CTsG8&t=1s
3. **Kunikullaya U Kirthana**, Arun Sasidharan, Vijayadas, Anjani Bhushan Kumar, Mamta Sanjeeva Vernekar, Radhika Kunnavil, Jaisri Goturu, V S Prakash, N S Murthy. P-EG008. Electroencephalographic changes on passive listening to 3 Indian classical music scales. Clinical Neurophysiology, August 2021, 32(8):e79-e80 - Conference proceedings published (Virtual) - 7th Asian Oceanian Congress on Clinical Neurophysiology (AOCCN) organized by Malaysian Society of Neurosciences - Virtual Feb 2021.
4. **Kirthana Kunikullaya U**. Scientific study of subjective emotions on listening to melodic scales of Indian Music - An exploratory study. International Science Fiction Conference Nov 2020 (Virtual) - Organized by Bangalore University & Indian Association for Science Fiction Studies https://pimpon.in/international-science-fiction-conference/
5. Invited speaker - **Kirthana Kunikullaya U.** Specific group of emotions induced after passive listening to three melodic scales of Indian Classical Music - An exploratory study - Oct 2020 - Music & Psychology Research Conference: Virtually Together - Australian Music and Psychology Society (AMPS) - https://amps.org.au/2020/07/27/music-psychology-research-conference-virtually-together-9-october-2020/
6. Keynote speaker - Unique effects of melodic scales of Indian classical music on stress and electrophysiological parameters. International Conference cum Workshop on Rhythm in Speech and Music from Neuro-cognitive perspectives (ICRSM 2020) - Jan 2020.
7. Invited Speaker - National conference - Nadamanthana Conference - organized by Bharatiya Vidya Bhavan and Meera Centre for Music Therapy - Dec 2019.

Poster Presentation

1. **Kirthana Kunikullaya U**, Christine Kervarrec, Francois Brion, Elisabeth Pellegrini, Thierry D Charlier. Developmental exposure to 17α-Ethinyl Estradiol on

neuroplasticity in zebrafish. 27th annual meeting of the society for behavioral neuroendocrinology - June 26 to June 29, 2023 - https://sbn2023.colloque.inrae.fr/

2. **Kirthana Kunikullaya Ubrangala**, Zuzanna Baran, Valentine L'Estoile, Harry Steinbusch, Fatima Smagulova, Thierry Charlier. Effects of prenatal exposure to thiacloprid, a neonicotinoid on neuroplasticity in zebrafish and mouse. FENS 2022. https://forum.fens.org/abstract-e-book/
3. **Kirthana Kunikullaya U.** Scientific Analysis of Electrophysiological Effects And Anxiety Reduction With Three Different Indian Melodic Scales - A Randomized Control Trial. Future Physiology 2021, The Physiological Society UK. https://www.physoc.org/abstracts/scientific-analysis-of-electrophysiological-effects-and-anxiety-reduction-with-three-different-indian-melodic-scales-a-randomized-control-trial/
4. **Kirthana Kunikullaya U**, Arun Sasidharan, Radhika Kunnavil, Jaisri Goturu, Vadagenahalli S Prakash, Nandagudi Srinivasa Murthy. Temporal power spectral changes in electroencephalography during passive listening to improvisation of melodic scales. The Neurosciences and Music - VII - Connecting with music across the lifespan, Aarhus, Denmark - June 2021 https://www.fondazione-mariani.org/images/NMVII/ProgramNMVIIwithposters.pdf
5. **Kirthana Kunikullaya**, Arun Sasidharan. EEG Spectral changes with passive listening to Indian melodic scales - May 2020 - Brain. Cognition. Emotion. Music. (BCEM 2020) held at the University of Kent Canterbury - DOI 10.17605/OSF.IO/37F6B

Pre-prints:

1. Acute effects of passive listening to Indian musical scale on blood pressure and heart rate variability among healthy young individuals, a randomized controlled trial bioRxiv 2020.05.03.073916; doi: https://doi.org/10.1101/2020.05.03.073916 - Citation - 1
2. Effect of specific melodic scales of Indian music in reducing state and trait anxiety – a randomized clinical trial – Psyarxiv - 10.31234/osf.io/gujkd
3. Electroencephalographic power spectrum and intersubject correlation on acoustic stimulation with modes of Indian music: a randomized controlled trial. bioRxiv 2022.12.09.519709; doi: https://doi.org/10.1101/2022.12.09.519709

Books/Chapter:

1. Chapter - Indian music intervention as a tool in medicine – Publisher - Dr. Kalyan Gangarde; New Man Publication, Parbhani – 2019

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Kirthana Kunikullaya U