REPUBLIC OF TÜRKİYE
ANKARA UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

BEHAVIORAL AND GENETIC EVALUATION OF THE EFFECT OF PRENATAL STRESS ON FUNCTIONAL CEREBRAL ASYMMETRIES IN RATS

Sevim ISPARTA

DEPARTMENT OF GENETICS

DOCTORAL DISSERTATION

SUPERVISOR

Assoc. Prof. Bengi ÇINAR KUL

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Prof. Dr. Dr. h.c. Onur GÜNTÜRKÜN

ANKARA

2023

ETHICAL STATEMENT

To Ankara University

Directorate of the Graduate School of Sciences,

The doctoral dissertation entitled "Behavioural and Genetic Evaluation of the Effect of Prenatal Stress on Functional Cerebral Asymmetries in Rats" was written by me in accordance with scientific ethics and values. The idea/hypothesis of the thesis belongs entirely to my thesis supervisors and me. The experimental study/research in the thesis was conducted by myself, and all sentences and comments belong to me.

I declare that the above-mentioned statements are true.

Student's Name and Surname: Sevim ISPARTA

Date: 20.06.2023

Signature:

ACCEPTANCE AND APPROVAL

The thesis titled "Behavioral and Genetic Evaluation of the Effect of Prenatal Stress on Functional Cerebral Asymmetries in Rats" written by Sevim ISPARTA from the Department of Veterinary Genetics at Graduate School of Health Science, Ankara University has been accepted UNANIMOUSLY as a DOCTORAL THESIS by the PhD Dissertation Jury, whose members are listed below.

Defense Date: 07.07.2023

Prof. Dr. Mehmet Ali Ergün
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Ankara University
Reporter

The decision of the jury regarding the thesis has been approved by the Board of Directors of the Graduate School of Health Science, Ankara University.

Prof. Dr. Fügen AKTAN
Graduate School Director

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PREFACE

For many years, it was assumed that hemispheric asymmetries were characteristic of human beings. With the scientific advancements, it is very well now known that functional cerebral asymmetries are a common vertebrate feature. The most obvious representation of behavioural laterality is handedness in humans, and pawedness/paw preferences in animals. These asymmetries are driven by genetic and environmental factors and can be altered during the life span. The main aim of this study is to investigate the effect of prenatal stress on functional cerebral asymmetries in rats by using different behavioural and genetic analyses.

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SYMBOLS AND ABBREVIATIONS

%	Per cent
˚	Degree
˚C	Degree Celsius
~	Approximately
♀	Female
♂	Male
α	Alpha
µg	Microgram
µl	Microliter
ABS-LI	Absolute values of individual laterality index
AcBc	Nucleus accumbens
ACTB	Actin beta
AFE	Agilent Feature Extraction Image Analysis Software
ANOVA	Analysis of Variance
APOE	Apolipoprotein E gene
AR	Androgen receptor gene
BC	Background correction
BORIS	Behavioural Observation Research Interactive Software
CC	Corpus callosum
CCKAR	Cholecystokinin A receptor
cDNA	Complementary DNA
CNS	Central nervous system
CG	Control group
Cg1	Cingulate cortex, area1
Cq	Quantitative cycle
cRNA	Complementary RNA
CT	Cycle threshold
Cy3	Cyanine 3-CTP
ddCq	Delta-delta quantification cycle
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EPM	Elevated plus maze
ES	Enrichment score
F	Female
FCA	Functional cerebral asymmetry
FDR	False detection rate
FOXE1	Forkhead Box E1
FOXP2	Forkhead box protein P2

FWER	Familywise-error rate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GD	Gestational days
gDNA	Genomic DNA
GM	Geometric mean
GRINB2	Glutamate ionotropic receptor NMDA type subunit 2B
h	Hour
H_20	Dihydrogen monoxide
HSD	Honestly significant difference
Hz	Hertz
ICC	Intra-class correlation coefficients
IL	Infralimbic cortex
IRC	Inter-run calibrator
ITPR1	Inositol 1,4,5-Trisphosphate Receptor Type 1
i.e	id est
kg	Kilogram
L	Left
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz
LH	Left hemisphere
LI	Laterality index
LMO4	LIM Domain Only 4
LRRTM1	Leucine-rich repeat transmembrane neuronal 1
M	Molar
M	Male
M1	Primary motor cortex
M2	Secondary motor cortex
mg	Milligram
min	Minute
miRNA	Micro-ribonucleic acid
ml	Milliliter
Mm	Millimeter
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
MS	Maternal separation
N	No turning behaviour
NES	Normalized enrichment
ng	Nanogram
NOM p-val	Nominal p-value
NTC	No template-control reactions
P	Significance value
PCSK6	Proprotein convertase subtilisin/kexin type 6
PCR	Polymerase chain reaction
PD	Postnatal day
POLM	DNA Polymerase Mu

PrL	Prelimbic cortex
PSP	Prenatal stress protocol
Px	Pixel
qRT-PCR	Quantitative reverse transcription polymerase chain
R	Right
RH	Right hemisphere
r	Pearson's correlation coefficient value
RIN	Integrity of RNA
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	Real-time reverse transcription polymerase chain reaction
s	Second
SD	Standard deviation
SEM	Standard error of the mean
SETDB2	SET domain bifurcated 2
SG	Stress group
SV	Stability value
TGS	Total gene signal
TMT	Tympanic membrane temperature
UNG	Uracil DNA Glycosylase
USVs	Ultrasonic vocalization emissions
V	Volt
v/v	Volume/volume
X^2	chi-square
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
ZKF	Zentrum für Klinische Forschung
ΔCq	Relative quantity
ΔΔCq	Normalized expression

FIGURES

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1. INTRODUCTION

1.1. On Terms and Concepts

The symmetrical structure of the cerebral hemispheres provides left-right symmetry for the sensory and motor functions of the body. Nerve bundles connecting the two cerebral hemispheres exist in the brain. Sensory and motor centers are located symmetrically in the hemispheres and make cross-connections with the two halves of the body (Springer and Deutsch, 1998). The functional and structural specialization of the left and right hemispheres of the brain is referred to as cerebral asymmetry. Another term for this phenomenon is cerebral lateralisation, while functional cerebral asymmetry (FCA) is described as hemispheric specialization to control certain functions (Ocklenburg et al., 2014). Although it is well-known that brain hemispheres are anatomically and functionally asymmetrical, the molecular and genetic basis of hemispheric asymmetry has not yet been fully understood. While hand preference is the most obvious functional expression of brain lateralisation, various neuropsychiatric and developmental disorders such as depression (Denny, 2011; Logue et al., 2015), autism spectrum disorders (Rysstad and Pedersen, 2016), anxiety disorders (Logue et al., 2015; Lyle et al., 2013) or schizophrenia (Dragovic and Hammond, 2005; Hirnstein et al., 2010) in humans have been shown to be related with dominant left- hand use and ambilateral hand use. Thus, understanding the ontogeny of hemispheric asymmetries and hand preferences is crucial for obtaining vital information regarding the pathogenesis of these disorders.

Hemispheric asymmetries have long been believed to be a distinguishing feature of the human species. Contrary to this viewpoint, left-right asymmetries of brain structure and behaviour have been documented in all groups of vertebrates, including mammals (Corballis, 2009), reptiles (Bisazza et al., 1998), birds

(Güntürkün et al., 2000) and Osteichthyes (Vallortigara and Rogers, 2005). Moreover, it is well known that not only vertebrates but also various invertebrate species such as honeybees *(Apis mellifera)* (Frasnelli et al., 2010), Octopus vulgaris (Byrne et al., 2002; Frasnelli et al., 2019), cockroach *(Nauphoeta cinerea)* (Sreng, 2003) have left and right asymmetries in their brain and their behaviour. This ubiquity of brain and behavioural lateralisation likely confers evolutionary advantages and enhances survival ability. For instance, chicks exhibited better recognition of familiar birds with their left eye as compared to their right eye (Vallortigara, 1992), and responded more promptly to a predator approaching from the left as opposed to the right side (Vallortigara, 2006). This may signify decreased cognitive response selection time by reducing hemispheric neural conflict (Hirnstein et al., 2010). Rogers (2000) has declared that lateralised brains are capable of processing numerous forms of information from various sources simultaneously in both hemispheres. Consequently, it is reasonable to assume that lateralised individuals would possess greater perceptual, cognitive, and motor skills. This argument has been confirmed several times with the increasing of lateralisation studies conducted on different species, including marmosets (Piddington and Rogers, 2013), pigeons (Güntürkün et al., 2000), cats (Isparta et al., 2020) and songbirds (Yin et al., 2022).

Although it was assumed that functional cerebral asymmetries are often directed by genetic factors (Annett, 1996; Corballis, 1997) and are constant over time, it has been shown that FCAs have some degree of plasticity and may change over the life span of the individual as well as in short periods of time (Schlaug et al., 1995). There is steadily accumulating evidence that supports this conclusion that environmental factors such as stress are known to be linked to functional and structural alterations in cerebral asymmetries (Ocklenburg et al., 2016; Salgirli Demirbas et al., 2023). There are numerous studies investigating the relationship between FCA and stress in living organisms. These studies have shown that acute and/or chronic stress has an impact on FCA, and moreover, cerebral asymmetry plays a protective role in stress response (Backström et al., 2015; Culum et al., 2007; Ströckens et al., 2013). However, the effect of prenatal stress on brain organization

and the underlying molecular mechanisms have been poorly understood. Human and experimental animal studies have demonstrated that chronic prenatal stress may have a long-term effect on a neuronal source, which may cause different behavioural pathologies that can be detected in infancy and may continue into adulthood (Weinstock, 2001). Various psychiatric disorders, such as anxiety disorders and depression, have been linked to prenatal stress (Huizink et al., 2004). Weinstock (2001) has shown that the noise and light stress applied randomly in the prenatal period causes permanent changes in behavioural and neurochemical asymmetries in rat pups and these changes continue until adulthood. Studies have reported that prenatal stress may reduce cerebral lateralisation by altering the cortical and limbic system morphology of the developing embryo (Rogers, 1990). Moreover, it has also been argued that epigenetic changes caused by adverse prenatal exposure are associated with the common chronic diseases such as obesity, cardiovascular diseases, and endocrine disorders later in life (Gluckman et al., 2008; Waterland and Michels, 2007).

It has been suggested that the right hemisphere specializes in controlling emergency responses such as flight/avoidance and aggression with strong negative emotions and that the dominant hemisphere in processing information during stress is the right hemisphere (Lippolis et al., 2005; Vallortigara et al., 1999). Additionally, it has also been indicated that physiological responses such as pituitary-adrenal axis activation (Wittling and Pflüger, 1990) and escalated heart rate (Wittling et al., 1998) are also regulated by the right hemisphere of the brain. According to Bianki (1983), the left hemisphere of the brain is in charge of processing positive emotions and "sequential processing" which refers to the process that enables an individual to concentrate on and appraise a situation. Altered cerebral asymmetries resulting from heightened activity in the right hemisphere have emerged in humans under stress (Berretz et al., 2022; Cerqueira et al., 2008). Not only human studies but also animal studies revealed that stress is associated with the right hemisphere of the brain. Some evidence confirms this hypothesis: Stomp et al. (2021) have revealed that chronic stress in horses is positively correlated with gamma production in the right hemisphere. Another research investigation on marmosets found a link between

capture stress and the tympanic membrane temperature (TMT) of the right ear, an indicator of blood flow in the right hemisphere (Tomaz et al., 2003). In another study examining the acute stress responses of cats exposed to stressors such as transportation, restraint, and housing in an unfamiliar environment, a positive correlation was found between the TMT in the right tympanums with cortisolaemia levels (Mazzotti and Boere, 2009). Additionally, atypical leftward behavioural asymmetry was observed in rats because of stronger right hemispheric activity after chronic stress exposure (Mundorf et al., 2020). Furthermore, Perez-Cruz et al. (2009) revealed that under conditions of chronic stress induced by movement restriction, pyramidal neurons in the prelimbic cortex of rats underwent structural changes predominantly in the right hemisphere of the brain, while no alteration was observed in the corresponding cortical area of the left cortex. Furthermore, measuring FCAs in animals is of another great importance in veterinary medicine: animal welfare. A growing body of evidence has demonstrated that measuring FCA can serve as an alternative and non-invasive method to evaluate the welfare of the animals (Goursot, 2020; Marr, 2020; Leliveld et al., 2013; Rogers, 2023). Individuals exhibiting weaker or diminished lateralisation may display heightened reactivity to stress (Rogers, 2010). A recent study of dogs living in different environmental conditions revealed that increased ambilaterality (decreased FCA) may result from poor welfare. Considering the phenotypic plasticity feature of the brain organization, it has been suggested that diminished FCA may not be the cause but rather the consequence of emotional reactivity in dogs (Salgirli Demirbas et al., 2019). Hence, a better understanding of how FCAs interact with stress conditions may provide a great opportunity to enhance animal welfare.

Mice (*Mus musculus*) and rats (*Rattus norvegicus*) are the most common vertebrate species used in animal research around the world (Baumans, 2005). Through long ages, rodents' reaching skill has been used as a behavioural paradigm in a broad range of scientific areas, such as neural control of the forepaw (Kawai et al., 2015; Whishaw et al., 2008) assessment of brain damage (Alaverdashvili et al., 2008; Klein et al., 2012); functional recovery of forepaw function after neural injury (Ramanathan et al., 2006; Whishaw et al., 2008), assessment of motor function in

neurological dysfunction such as Parkinson's disease (Klein and Dunnett, 2012) and laterality (Collins, 1968; Whishaw, 1992). In addition, Whishaw (1992) claimed that rats and humans had analogous movements and the sequencing of actions while reaching for food. This finding has been corroborated by a more recent study revealed that rodents' reach and grasp movements are substantially similar to those of humans (Klein et al., 2012). Moreover, paw preference is regarded as the most prominent functional manifestation of cerebral asymmetries in rodents, as it has been found to be linked with hemispheric asymmetry at the functional (Hamani et al., 2010), molecular (Zimmerberg et al., 1974), and behavioural level (Soyman et al., 2015), akin to handedness in humans. In contrast to humans, rats do not exhibit population-level asymmetry for one direction but instead display individual paw preferences using either their right or left paws more predominantly (Manns et al., 2021).

The investigation of behavioural lateralisation in rodents dates back to the 1930s, once Tsai and Maurer (1930) conducted the first study. Since then, the predominant approach has been to use the food-reaching test methodology, such as the Collins' (Collins, 1968; Collins, 1969; Collins, 1975) and the Lateral Paw Preference Tests (Waters and Denenberg, 1991), which require animals to use their paws to reach food rewards. In later years, different test apparatus, such as the Pasta Matrix Reaching Test, was designed to investigate the reaching ability of animals more comprehensively, including the extent of the forelimb movement and ability to reach in a variety of directions and distances, and it was suggested that they could be used in laterality research in rodents (Ballermann et al., 2001). Following the initial investigations into the heritability of paw preference in mice conducted by Collins in 1968, the employment of this assay has gained wide popularity owing to its broad utility and resemblance to human manual lateralisation. A recent meta-analysis on the paw preference of mice and rats revealed that, unlike humans, neither species exhibits asymmetry at the population level. However, 81% of mice and 84% of rats exhibited individual-level asymmetry either to the left or the right side (Manns et al., 2021). In rodents, not only paw preference but also turning (Mundorf et al., 2020; Schwarting and Wöhr, 2012) and head-turning (Soyman et al., 2018) behaviours

have been used as behavioural markers of laterality. Soyman et al. (2018) reported that head-turning asymmetries might be an indicator of mood disorders such as anxiety or depressive-like behaviour in rats. Moreover, a recent study investigating the turning behaviour of rats using the elevated plus maze test has proposed that early life stress modulates left-turning rather than right-turning behaviour (Mundorf et al., 2020). In order to examine atypical asymmetries in psychiatric models, it has been proposed that turning behaviour analysis following a stress exposure could be a relevant model (Mundorf et al., 2020; Mundorf et al., 2021). Investigating behavioural laterality in rodents can provide important insights into biomedical studies (Manns et al., 2021), mental health and consequences of stress exposure (Mundorf et al., 2020), neurodevelopmental alterations (Alonso et al., 1991, Mundorf et al., 2021), and psychiatric disorders (Ecevitoglu et al., 2020; Soyman et al., 2018). In addition, studying rodent models of psychiatric and neurodegenerative disorders has substantially advanced the neurobiological understanding of cerebral asymmetries (Ocklenburg et al., 2022).

In early genetic models, it was assumed that a single gene with two alleles was responsible for hand preference and language lateralisation in humans. Thus, they have been referred to collectively as "single gene models" (Annett, 1964; McManus, 1985). Through advances in genetics and the successful culmination of the human genome project, it has been revealed that the molecular mechanisms underlying functional cerebral asymmetry are much more complex than previously thought (Schmitz, 2018). For instance, a study examining a genome-wide single nucleotide polymorphism (SNP) dataset from 3940 twins has refuted the simple genetic models of handedness since no locus was detected to be associated with hand preference in humans (Armour et al., 2014). Many studies have revealed that manual dexterity is not a monogenic trait determined by a single gene with two alleles but rather a polygenic model controlled by multiple genes, each with minor effects (Clyde Francks et al., 2002; Mcmanus et al., 2013). Very recently, the results of the most extensive GWAS study to date with 1,766,671 samples also indicated that hand preference is a highly natural polygenic (Cuellar-Partida et al., 2021). This study achieved this enormous sample size by conducting an analysis of participants from

the UK Biobank, 23andMe (accessed on 20 June 2023), and the International Handedness Consortium. The research findings revealed a total of 48 statistically significant associations, 41 of them were linked to left-handedness, while the remaining 7 were associated with ambilaterality. Other pivotal arguments of the study are that genetic variants cause a predisposition to leftward hand preference, which may partially explain the association between left-handedness and some psychiatric disorders, and microtubule genes may have a crucial role in lateralisation.

Sun et al. (2005) have found that cortical asymmetries have a molecular basis by investigating the gene expression levels in the human fetus's perisylvian cortex of both hemispheres. In this study, the human fetal brain was examined at 12 and 14 weeks of gestation, characterized by neuronal proliferation and migration, and at 19 weeks, when all these processes are completed. They identified 27 genes that were consistently asymmetrically expressed at 12 weeks of gestation, including LIM Domain Only 4 (*LMO4*), which was more expressed in the right hemisphere than in the left between weeks 12 and 14. In the following weeks 16 and 17, the expression level of the *LMO4* gene was found to be decreased between the hemispheres and, by the 19th week, symmetrically expressed in both hemispheres. In the same investigation, additional analyses were performed on the expression of *LMO4* in mouse brains to acquire a more comprehensive understanding of the dynamic alterations in *LMO4* expression throughout cortical development. It has been revealed that *LMO4* is asymmetrically expressed in the brain of each animal. In contrast to humans, mice exhibited an equivalent occurrence of asymmetric expression between the right and left hemispheres. Interestingly, it was stated that this finding may be in line with the previous findings from the anatomical and behavioural studies in mice, like paw preferences that were not biased on a population level but existed at the individual level (Collins, 1991; Signore et al., 1991; Riddle and Purves, 1995). As a follow-up study, Li et al. (2013) investigated the effects of unilateral *in-utero* electroporation (knockdown) of *LMO4* expression in the right anterior cortex, including the motor cortex. At the neuronal level, this unilateral modulation of *LMO4* expression in embryonic mice inhibits neurogenesis. This inhibition leads to asymmetric functional area formation, neuronal production,

and axonal projection. Moreover, the causative role of modified *LMO4* expression in functional laterality was also confirmed in this study by measuring the paw preferences of the animals. The gene expression analysis in the mature brains of human generated ambiguous results from two distinct investigations that discovered no differences in gene expression among corresponding brain regions throughout two hemispheres (Hawrylycz et al., 2012; Pletikos et al., 2014).

Candidate gene studies discovered several genes that are associated with handedness in humans. These genes contain the Leucine-rich repeat transmembrane neuronal 1 (*LRRTM1)* (Francks et al., 2007), Catechol-O-methyltransferase gene (*COMT)* (Savitz et al., 2007), Apolipoprotein E gene (*APOE)* (Bloss et al., 2010; Hubacek et al., 2013), Androgen receptor gene (*AR)* (Arning et al., 2015; Hampson and Sankar, 2012), Proprotein convertase subtilisin/kexin type 6 (*PCSK6*) (Arning et al., 2013; Scerri et al., 2011), and SET domain bifurcated 2 (*SETDB2)* (Ocklenburg et al., 2016). Moreover, Forkhead-Box-Protein P2 (*FOXP2)* (Ocklenburg et al., 2013a; Pinel et al., 2012), Cholecystokinin A receptor (*CCKAR)* (Ocklenburg et al., 2013b), Glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B)* (Ocklenburg et al., 2011) genes have been found to be associated with language lateralisation which is another hot topic of laterality research in humans.

Although most of the human population consistently prefers to utilise one hand over the other one for fine motor activities such as writing and drawing (Somers et al., 2015), there are no outwardly discernible differences in muscular, osseous, or peripheral nervous systems between two hands of individuals regardless of being left-handed or right-handed (Ocklenburg et al., 2015). Consequently, it has been implicated that hand preference originates from the central nervous system (Ocklenburg et al., 2013c; Ocklenburg et al., 2015). Nevertheless, the precise location of the handedness origin is a challenging inquiry to answer. Several authors have proposed the notion that structural asymmetries in grey matter regions may be responsible for handedness. However, empirical findings in this area have been notably inconsistent. While some studies have reported associations between

handedness and structural asymmetries in some areas, such as the left precentral sulcus (Amunts et al., 1996), planum temporale (Steinmetz et al., 1991), left central sulcus (Amunts et al., 2000) and Broca's area (Powell et al., 2012), other studies have yielded contradictory results regarding the presence of any correlations between structural asymmetries in these specific regions and handedness (Good et al., 2001). A functional magnetic resonance imaging (fMRI) study has documented that unilateral finger movements often resulted in ipsilateral motor cortex activation that was primarily observed in the left motor cortex in right-handers (Kim et al., 1993). Volkmann et al. (1998) have also suggested a hemispheric asymmetry in the functional activation of the motor cortex related to handedness in humans.

In the year 1870, the researchers Gustav Fritsch and Edvard Hitzig made a significant discovery related to the effects of electrical stimulation of the regions of the cerebral cortex generates and suppresses contralateral muscle movements in dogs (cited by (Gross, 2007; Taylor and Gross, 2003). The rodent motor cortex consists the Primary Motor Cortex (M1) and Secondary Motor Cortex (M2) involved in the execution of volitional motor control (Paxinos and Watson, 2006; Saiki et al., 2014). M1 is a dynamic hub responsible for overall motion control and decision making, which can be undergone consequential alterations following a neural stimulation (Bhattacharjee et al., 2021; Lee et al., 2022). The preservation of motor functions has been proposed to be associated with the functional connectivity between M1 and multiple brain regions. The M1 region of the brain is responsible for the integration of inputs from various regions of the brain. There are two main classifications of these inputs, specifically thalamocortical projections and corticocortical projections (Lee et al., 2022). Despite receiving inputs from various subcortical regions such as the globus pallidus, central amygdala, and dorsal raphe nucleus to M1, it was reported that the investigation of these inputs remained limited in the literature (Muñoz-Castañeda et al., 2021). The cortical regions that have been identified include posterior cortical areas that are presumed to code somatosensory signals, as well as frontal cortical areas, which are presumed to code motor signals (Hooks et al., 2013; Mao et al., 2011). In addition, it has also been reported that the frontal cortical regions that are in direct connection with the deep layers of M1 are

responsible for the regulation of general motion control (Hooks et al., 2013), as well as cognitive abilities (Siniscalchi et al., 2016). The M2, the other part of the frontal cortical areas, was suggested to be homologous to the primate premotor cortex. However, there has been a debate about whether they exhibit similarities in their functions (Papale and Hooks, 2018). Major advances have been achieved in comprehending the role and purpose of M2 with the developments in the fields of optogenetics and optical imaging, along with the emergence of comprehensive decision-making tests for rodents (Barthas and Kwan, 2017). According to a review by Barthas and Kwan (2017), comparing the various approaches, the primary function of M2 is to enable adaptive choice behaviour through flexible mapping of antecedent signals, such as sensory cues to motor movements. Furthermore, Saiki et al. (2014) proposed that the M1 and M2 regions form a two distinct system within the motor cortices, working together to effectively regulate voluntary movements by integrating crucial motor information from both peripheral and central information. Considering all these, in this study, M1 and M2 brain tissues of rats were chosen to be investigated to understand the molecular mechanism underlying functional cerebral asymmetry.

Early mammalian brain development is characterized by histogenesis and subsequent migration (Anderson et al., 2003). After conception and the formation of epiblast and hypoblast, the embryo will undergo gastrulation, during which time epiblast cells will differentiate into three primary stem cell lines, including neural progenitor cells, which will give rise to all cells that will make up the central nervous system (CNS) (Stiles and Jernigan, 2010). While cells migrate along the rostral-caudal midline of the embryo, signaling molecules released by the node induce differentiation into neural progenitor cells as well as a basic spatial organization of the nervous system. With the ensuing formation of the neural tube and the development of the first three primary, then five, brain pouches, the embryo has developed the primary organization of the CNS (Stiles, 2008). Subsequent neural patterning is strongly dependent on the concentration gradients of signaling molecules. For example, the signaling molecule Pax6, which has a high concentration in anterior lateral regions, induces progenitor cells to differentiate so

that they will form the motor cortex (Bishop et al., 2002). Experimentally induced increases in Pax6 concentrations lead to increased somatosensory and motor areas, while blocking Pax6 signaling leads to enlarged visual areas and smaller motor areas (Bishop et al., 2000).

During the process of neuron maturation, neurons branch out, start connecting, and their axons get myelinated (Sidman and Rakic, 1973). Following the initial construction of connections, immense morphological changes occur. A large proportion of neurons are naturally eliminated through apoptosis, and connections are reduced via synaptic pruning. Developmental processes during this period are highly susceptible to environmental influences, such as stress to the dam. While levels of the rats' stress hormone corticosterone are naturally elevated during pregnancy and play an important role in fetal development, abnormally high levels can potentially alter fetal development (Seckl and Holmes, 2007). In particular, stress in the late gestational period has been well-studied in rodents. Restriction stress during gestational days 15-21 decreases neurogenesis in the gyrus dentatus (Lemaire et al., 2000; Lemaire et al., 2006) and decreases dendritic branching (Barros et al., 2006).

During the postnatal period, connectivity maturation continues to develop (Bender and Baram, 2007), and overall brain growth peaks (Bandeira et al., 2009). During their first days, pups rely solely on the dam for nearly all vital functions, such as food intake and temperature control (Orso et al., 2019). Twenty-one days after birth, some developmental goals are reached. Synaptic density in the hippocampus has reached adult levels (Ribak et al., 1985) cerebellar neurogenesis is mainly complete (Marzban et al., 2015), and myelination has reached its maximum (Wiggins, 1986). This is also the time pups usually get weaned from their mothers (Zeiss, 2021).

Adolescence is described as a transitional period from childhood to adulthood, including puberty (Schneider, 2013). The peri-adolescent period is characterized by a second occurrence of neuronal activity, during which there is an excessive generation of axons and synapses followed by pruning (Teicher et al., 1995). According to (Gelbard et al., 1989), the striatal dopamine receptors in rats reach their peak levels at approximately 40 days of age. Adolescence is characterized by notable changes in brain development, specifically the proliferation of axons and synapses, which are subsequently pruned, resulting in the maturation of various brain regions. Defining the precise timing of adolescence is a considerable challenge in humans; the precise timing of this phase in laboratory animals is much more difficult, and divergent opinions exist regarding this specific time frame which needs to be described (Spear, 2000). It has also been suggested that the time period between postnatal day (PD) 28-42 is a prototypic adolescent stage, during which individuals from various breeding lines often exhibit adolescent characteristics. (Spear and Brake, 1983). It should be noted the identification of animals as adolescents is not limited to a certain age, as Spear (2000) pointed out. However, Schneider (2013) has suggested that adolescence covers the entirety of the developmental stage that occurs between childhood, specifically in the period shortly before puberty, and adulthood, which also includes the pubertal phase. During puberty, various neurodevelopmental changes take place, including the maturational processes in the cortical and limbic regions (e.g., amygdala). On the other hand, adolescence and puberty comprise the most critical periods of the postnatal process of brain maturation. During adolescence, there are notable alterations in the structure and function of neurons, leading to observable behavioural changes. Predictably, many neuropsychiatric disorders originate during exactly this time window. Taking into account all of these, adolescence and puberty play a pivotal role in comprehending the etiology and mechanisms underlying mental disorders in adulthood (Schneider, 2013).

In humans, adult brain weight is reached shortly before puberty, whereas this landmark is achieved well after puberty on PD60 in rats. The prefrontal cortex reaches maturity with complete synaptogenesis, and myelination occurs at

approximately 17 to 25 years of age in humans, compared to PD90 in rats (Zeiss, 2021).

In humans, the weight of adult braing is getting to level shortly before puberty. The prefrontal cortex achieves maturity upon the completion of synaptogenesis, while myelination typically takes place between the ages of 17 and 25 in humans. In contrast, myelination in rats occurs around postnatal day 90 (Zeiss, 2021).

1.2. Research Questions and Aims

The current study mainly aimed to address the following questions. The hypothesis and the aim of the study were designed within this scope.

- Does prenatal stress have any impact on maternal care behaviours of the dams?

- Does prenatal stress have an influence on the emergence of anxiety-related behaviours in rats in the postnatal period?

- Is there any effect of prenatal stress on motor laterality in rats?

- Is there any left-right asymmetry at the gene expression level in the stress response of the motor cortices of the brain?

The main hypothesis of the study was that prenatal stress alters functional cerebral asymmetries in rats. The main aim of this study was to investigate whether there are behavioural and molecular alterations induced by prenatal stress during different postnatal developmental periods in offspring. Microarray-based gene expression analysis aimed to elucidate the genetic basis of cerebral lateralisation and investigate how prenatal stress affects brain organization at the gene level, examining

the primary and secondary motor cortices RNAs from the left and right hemispheres of the brains in rats. Subsequently, Gene Set Enrichment Analysis (GSEA) was aimed to perform with microarray data in order to determine whether a specified gene set or biological pathway exhibits statistically significant differences between two experimental conditions. Another aim of the study was to conduct qRT-PCR to validate any significant differences between the stress and control groups observed at the hemisphere level for specific genes and/or gene families from the microarray analysis. The other aim of the study was to investigate sex-dependent differences in behavioural asymmetry and genetic regulation in offspring exposed to prenatal stress. It was also aimed to examine how prenatal stress affects the physiological conditions of the offspring and how it affects the mother-offspring interaction. In addition to paw preference determination tests, it was also aimed to investigate the effect of prenatal stress on different behavioural phenotypes utilising different behavioural laterality tests, such as turning behaviour frequency in the elevated plus maze test. It was also aimed to investigate whether prenatal stress plays a role in the emergence of anxiety-related behaviours in offspring during the postnatal developmental period.

2. MATERIAL AND METHODS

2.1. Animals and Husbandry

The subjects of the study consisted of 16 timed-pregnant Sprague Dawley rats and their offspring (N= 131). Timed pregnant Sprague-Dawley Rats that constitute the sample groups of the study were obtained either from Charles River Laboratories (Sulzfeld, Germany) or by breeding animals obtained there at Experimental and Molecular Psychiatry Laboratory of the Ruhr University/LWL University Hospital [located in Zentrum für Klinische Forschung (ZKF)]. Pregnant dams were individually housed in Euronorm Type IV plastic cages with *ad libitum* access to food and water under constant environmental conditions of temperature and humidity (22 ± 2°C and 55 ± 25%, respectively), which were in accordance with the regulations of the Federation of European Laboratory Animal Science Associations on the dark period of the 12-h light/dark cycle (between 11:00 and 23:00). Ethical approval for the present study was obtained from the animal ethics committee of the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of the state of Northrhine-Westfalia (81-02.04.2019.A361) prior to commencement, all of the experiments were carried out in compliance with the guidelines outlined of Germany's Animal Welfare Act.

2.2. Experimental Design

Eight pregnant rats underwent the restriction stress protocol described later, generating the stress group (SG), whereas the other 8 pregnant rats served as the control group (CG).

Four timed-pregnant rats from Charles River Laboratories aged between 10-11 weeks arrived at gestational days (GD) between 8 and 10 to ZKF and were randomly assigned to SG and CG of the study. To obtain the other 12 timed-pregnant rats, 2 female and 1 male rat were left to mate overnight; the next day was considered the GD0. A vaginal plug, a sign of occurrence of copulation, was checked at 9 o'clock at GD0. In the following days, the pregnancy status of the dams was confirmed by monitoring the weight gain. It was ensured that in-house breeding and ordered dams were evenly distributed between the groups.

Pups were sexed at PD2, and if there were more than pups, the number of offspring was reduced to 10 (if possible, the number of pups was reduced to 5 females and 5 males) on the same day. While determining the pups to be reduced, priority was given to those with the lowest weight. After the reduction, the brain exenteration of the excluded pups was performed and the brains were shock frozen with liquid nitrogen and stored at 80°C for further molecular analysis. Both dams and pups were included in the experiments. The pups were weaned on PD21. One female and one male offspring from each dam from each experimental group (stress and control) constituting Group PD21 were pair housed (2 animals per cage) in same-sex cages for the behavioural tests. The remaining PD40, PD60, and PD90 offspring were group-housed in same-sex caging up to two days before the behavioural tests. Afterward, these groups were also pair-housed in same-sex caging for two days before the behavioural tests to habituate with each other. In rare cases when the offspring of the moms did not show an equal distribution, the offspring housed as a maximum of three individuals in same-sex caging. Each developmental age group included one female and one male offspring from each mother. In cases where mothers did not have equal numbers of females and male offspring, more than one offspring from a mother, but not exceeding 2, was included in a group. For instance, 2 male pups from Mom11 were included in the P60 male group. The distribution of the number of offspring subjected to testing, categorized by age, group, and sex, is presented in Table 2.1.

Table 2.1. The distribution of the number of offspring tested per age, group and sex.
SG: Stress group; CG: Control group; F: Female; M: Male.

Age Groups	SG			CG			SG + CG
	F ♀	M ♂	Total (N)	F ♀	M ♂	Total (N)	Total (N)
PD21	8	6	14	7	7	14	28
PD40	8	7	15	10	8	18	33
PD60	7	9	16	8	10	18	34
PD90	9	9	18	9	9	18	36

2.3. Prenatal Applications

2.3.1. Prenatal Stress Protocol

The prenatal stress protocol (PSP) developed by Alonso et al. (1991) has been applied to 8 pregnant dams in this study. Within the scope of this procedure, pregnant dams were placed in a plexiglass apparatus for a total of 7 days, covering GD 15-21, and exposed to movement restriction stress for 2 hours. Although the animals were restricted, the apparatus was not narrowed to any physically invasive size, allowing the animals to turn freely from one direction to another. Prenatal stress protocol was conducted during the dark portion of a 12/12-h light/ dark cycle, which is their most active time. A 30-minute habituation period was avoided during prenatal stress application, except for the first session of the protocol.

2.4. Postnatal Applications

2.4.1. Measurement of Offspring Weight

Right before the maternal care assessment sessions performed on PD2, 6, 14, and 20, pups were sexed, and each pup was individually weighed. Three-way mixed ANOVA was performed on two conditions (stress or control), four-time points (PD2, PD6, PD14, and PD20), and sex status.

2.4.2. Maternal Care Assessment

The effect of prenatal stress on maternal care was assessed during the dark phase following the protocol developed by Bölükbas et al. (2020). Before each test, the animals were given at least 30 minutes to habituate to the experimental room. In the scope of this evaluation, the pups remained separated from their moms for a short term (only 10-15 minutes). During this short time frame, the weight of the pups was individually determined, and their health status was examined by the experimenter. Mother-offspring interactions of moms were recorded for 15 minutes after reunion with pups on 4 separate sessions before weaning on PD 2, 6, 14, and 20. Two GoPro Hero 7 action cameras were strategically positioned to capture footage from both sides of the maternal (home) cage. One of these cameras was placed on the back side of the cage while the other was placed on the side approximately 20 cm in distance. In addition to these cameras, another high-resolution camera (Sony, Handycam DCR-SR57) was placed in front of the cage in order not to miss any movement of the animals. The following behavioural parameters were scored: (1) licking and grooming pups (2) nursing, (3) retrieval of pups, (4) nest rebuilding (5) time spent on the nest, (6) self-grooming, (7) rearing and (8) searching for pups. The retrieval of the pups' state was initialized once the first pup was grabbed by the dam and only ended when all pups were gathered in one place. This location in the cage was defined as the nest for the time spent on nest time. On the PD20 assessment day, the

offspring is considerably larger than the infant size and highly mobile. Therefore, retrieval, nest rebuilding, and time spent on nest parameters were omitted from this evaluation day. A detailed description of all behaviours and their relevant categories were provided in the ethogram (Table 2.2). Additionally, ultrasonic vocalization emissions (USVs), the main means of communication used by rats to transmit their emotional state to their counterparts, were also recorded using an Ultramic 250k device (Dodotronic, Castel Gandolfo, Italy), which was placed approximately 10 cm above the cage. Ultrasonic vocalization emissions were recorded simultaneously with the video recordings.

Table 2.2. Ethogram for maternal behavioural parameters and related explanations
The asterisk (*) indicates that these parameters were also evaluated in addition to the behavioural parameters of Bölükbas et al. (2020) protocol.

Behavioural parameters	**Description**	**Behavioural Category**	**Type**
Licking/Grooming	Dam is licking/grooming pups (either head or anogenital region of pups)	Offspring-directed	Duration
Nursing	Arched-back nursing or passive nursing	Offspring-directed	Duration
Retrieval of pups	Retrieving the first pup	Offspring-directed	Time Point/Duration
Nest rebuilding*	Relocating objects or pups in/into the nest, digging in the nest	Offspring-directed	Duration
Time Spent on the nest*	Dam is sitting on the nest, regardless of what else she is doing simultaneously.	Offspring-directed	Duration
Self-grooming	Dam is grooming herself	Self-directed	Duration
Rearing	Dam is rearing with at least one of its paws off the ground	Self-directed and Explorative	Frequency
Searching for pups*	Sniffing and moving around outside the nest.	Explorative	Duration

Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016) was used to analyse videos obtained from maternal behaviour assessment by the experimenter blinded to the condition. BORIS allows to synchronize the starting points by adjusting the offset of each video and watching three recorded videos simultaneously (Figure 2.1). When the video coding was

completed, a behavioural time budget could be examined by selecting the required observation (Figure 2.2).

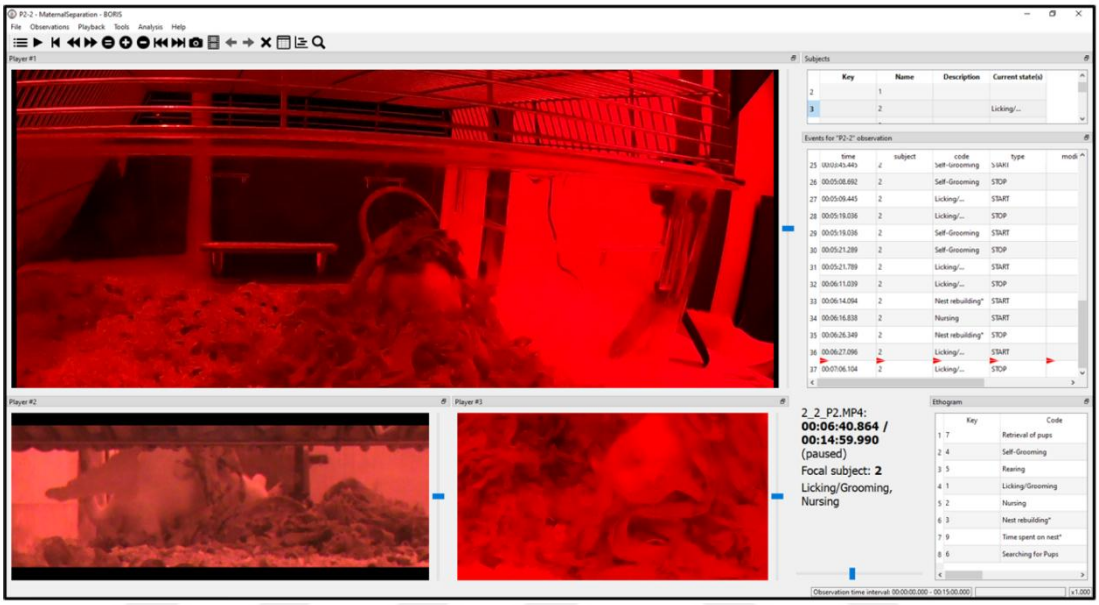

Figure 2.1. Example of BORIS while coding an observation.

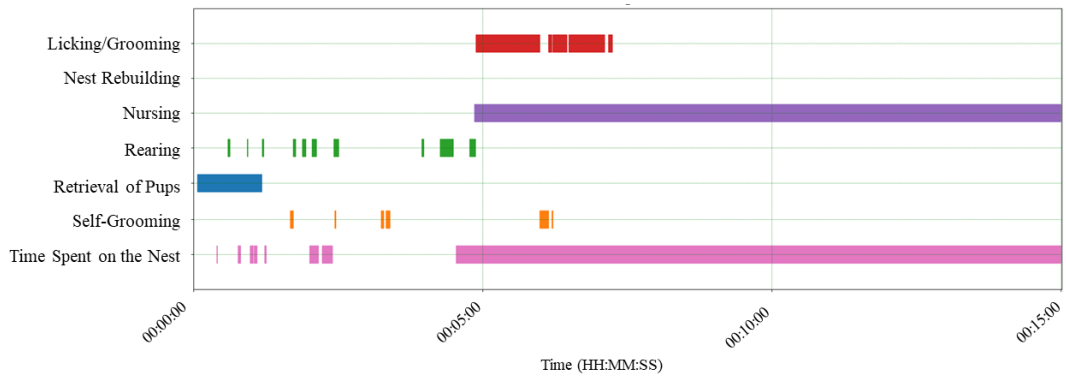

Figure 2.2. Example of time budget shown after coding an observation
The x-axis represents the time scale while the y-axis represents different behaviours.

The video footage of the rats obtained from the testing sessions assessing maternal care was analysed under blinded conditions. A second independent rater re-evaluated the video material, and the percentage of interobserver reliability has consistently exceeded 90%. Two-way mixed Analysis of Variance (ANOVA) was performed on two conditions (stress or control) and four-time points (PD2, PD6, PD14, and P20) for all behavioural parameters. Bonferroni correction for multiple comparisons was performed, and the limit of significance was determined as $p<0.05$.

2.5. Behavioural Tests

2.5.1. Experimental Setup

2.5.1.1. Elevated Plus Maze

The Elevated Plus Maze (EPM) test is widely used to assess rodent anxiety-related behaviour (Kraeuter et al., 2019). The apparatus/maze is 50 cm in height and comprises two open and two closed arms (50 cm x 10 cm) intersecting vertically at the center. The entire test apparatus is made of a cleanable material. A red LED light and an HD Webcam (C920 Pro, Logitech) plugged into a notebook were placed on top of the test apparatus, approximately 1 meter from the arms. Placing the red light precisely in the center point of the testing apparatus precluded any shadow formation which might adversely influence the animals during the test session.

2.5.1.2. Motor Laterality Tests

Considering that rats are neophobic creatures (Würbel et al., 2017) and the time required for habituation to each test setup, a novel testing apparatus that consists of motor laterality tests including Collins Paw Preference (Collins, 1968; Collins 1969; Collins, 1975) The Pasta Matrix Reaching (Ballermann et al., 2001), and Head-Turning Asymmetry (Soyman et al., 2018) tests were developed for this study

(Figure 2.3). The most remarkable feature of this novel test setup is that it is an adjustable apparatus that allows rats to be tested at different postnatal developmental stages regardless of their body size differences.

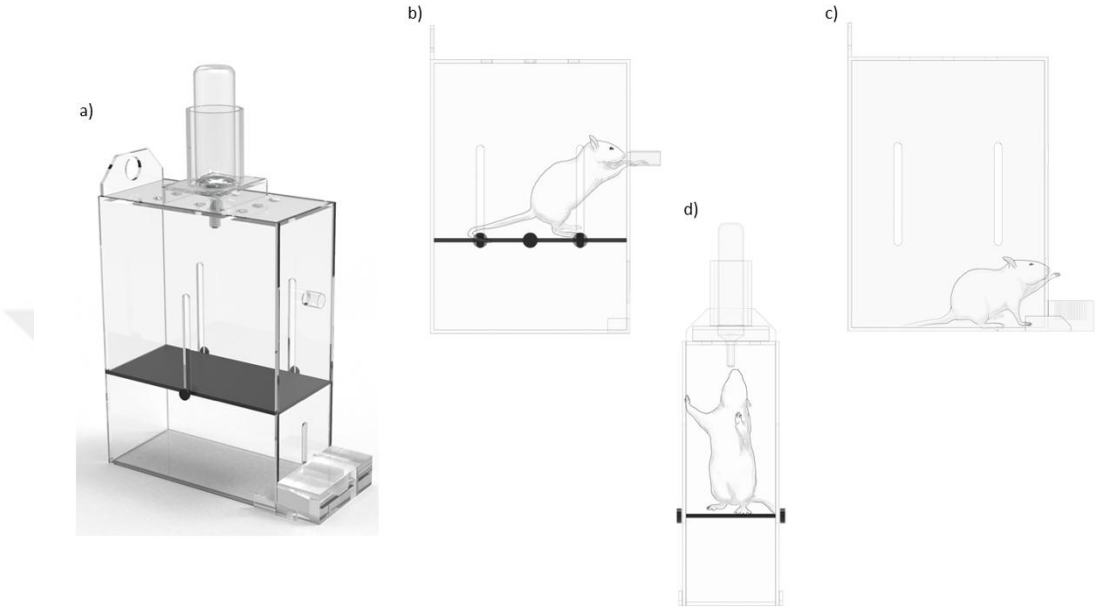

Figure 2.3. A novel testing apparatus for motor laterality tests in rats
Motor laterality testing apparatus by combining Pasta Matrix Reaching Test (Ballermann et al., 2001), Collins Paw Preference Test (Collins, 1968; 1969; 1975), and Head-Turning Asymmetry Test (Soyman et al. 2018) (a). A rat performing Collins Paw Preference Test (b); a rat performing Pasta Matrix Reaching Test (c); a rat performing Head-Turning Asymmetry Test (d).

The novel testing apparatus consists of the following sections:

i) Collins Test: A narrow, removable glass feeding tube was affixed to the frontal wall of the chamber, precisely equidistant to the left and the right wall of the chamber. Animals needed to insert their paw into the feeding tube to reach the food reward (Collins, 1968; Collins, 1969; Collins, 1975). Considering that the size of the animals in different developmental stages can differ, the height of this part of the test apparatus was designed to be lowered or raised as an option.

ii) Pasta Matrix Reaching Test: The test apparatus features a section on its front specifically designed for the pasta matrix reaching task, which includes a hole/slot measuring 1 cm in width.

There is a 1 cm wide hole/slot on the front of the section designed for the pasta matrix reaching task of the test apparatus. The rack (5.2 cm by 8.0 cm) contained an array of holes; 13 rows deep by 11 rows wide was taken placed next to this hole. The rows of holes were divided by 4 mm. Uncooked pieces of pasta (spaghettini, approximately 1 mm thick & 2.1 cm long) dipped in hazelnut cream were placed vertically to fill all the holes in the rack.

iii) Head-Turning Asymmetry Test: A window wide enough to place a water dispenser has been opened on the ceiling of the apparatus. The dispenser was placed exactly equidistant to the chamber's left and right walls. To drink hazelnut syrup water from this dispenser, animals need to stand up on their hind legs and extend their heads to reach it. Similar to the Collins test, the height of the apparatus is also adjustable in this test section.

2.5.2. Experimental Procedure

All behavioural assessments were conducted during the dark phase of a 12/12-h light/dark cycle under red light conditions since rats are nocturnal animals whose vision systems are more adapted to low-light conditions than brighter light conditions (Balcombe, 2010). Before each behavioural test, 30 minutes were given to the animals as a habituation period. All behavioural tests were conducted in the experimental room, free of disturbing elements such as noise and odors for the animal during the test sessions. Each animal was tested individually in the test apparatus in the experimental room. The height of the test apparatus was adjusted according to the age groups of the animals. All behavioural apparatuses were cleaned after each testing session with 2% antifect (Schülke, X89907.3), a disinfectant to

which the animals were accustomed. The application times of behavioural tests are given in Table 2.3.

Table 2.3. Application times of behavioural tests
The start and end days of behavioural tests are shown in bold. The asterisk (*) indicates the day of decapitation at which biological sampling was performed.

TEST SESSIONS	ANIMALS				BEHAVIOURAL TESTS
Habituation Day 1	PD19 + Dams	PD38	PD58	PD88	Demo Apparatus Introduction
Habituation Day 2	PD20 + Dams	PD39	PD59	PD89	Demo Apparatus Introduction
Day 1	**PD21 + Dams**	**PD40**	**PD60**	**PD90**	EPM + Collins Test
Day 2	PD22 + Dams	PD41	PD61	PD91	Collins Test / Pasta Test
Day 3	PD23 + Dams	PD42	PD62	PD92	Collins Test / Pasta Test
Day 4	PD24 + Dams	PD43	PD63	PD93	Collins Test / Pasta Test
Day 5	PD25 + Dams	PD44	PD64	PD94	Collins Test / Pasta Test
Day 6	PD26 + Dams	PD45	PD65	PD95	Collins Test / Pasta Test
Day 7	PD27 + Dams	PD46	PD66	PD96	Collins Test / Pasta Test
Day 8	PD28 + Dams	PD47	PD67	PD97	Collins Test / Pasta Test
Day 9	PD29 + Dams	PD48	PD68	PD98	Collins Test / Pasta Test
Day 10	PD30 + Dams	PD49	PD69	PD99	Head Turning Asymmetry Test
Day 11	PD31 + Dams	PD50	PD70	PD100	Head Turning Asymmetry Test
Day 12	PD32 + Dams	PD51	PD71	PD101	Head Turning Asymmetry Test
Day 13*	**PD33 + Dams**	**PD52**	**PD72**	**PD102**	Head Turning Asymmetry Test

2.5.2.1. Elevated Plus Maze

EPM test was administered to each pup belonging to 4 different developmental age groups only once on the first day of their test sessions. Dams were also tested in EPM on the weaning day (PD21). Animals were placed in the central area of the maze, facing a closed arm, and allowed to freely explore the maze for 5 min while being recorded using an HD Webcam camera from above in the test setup.

The videos obtained from EPM tests were analysed using DeepLabCut, an open-source Python toolbox using deep neural networks (Mathis et al., 2018). The raw video material was edited to be resized before analyzing via DeepLabCut, using FFmpeg, which is a free and open-source utility consisting of libraries and programs

that are utilized for editing, reformatting, and conversion of audio, video, and multimedia files (Tomar, 2006). In this manner, a total of 149 videos were prepared for DeepLabCut analysis, including videos of 16 dams and 131 offspring from the control and stress groups. The tracking of body positions on a frame-by-frame basis was performed using DeepLabCut, as presented in Figure 2.4.

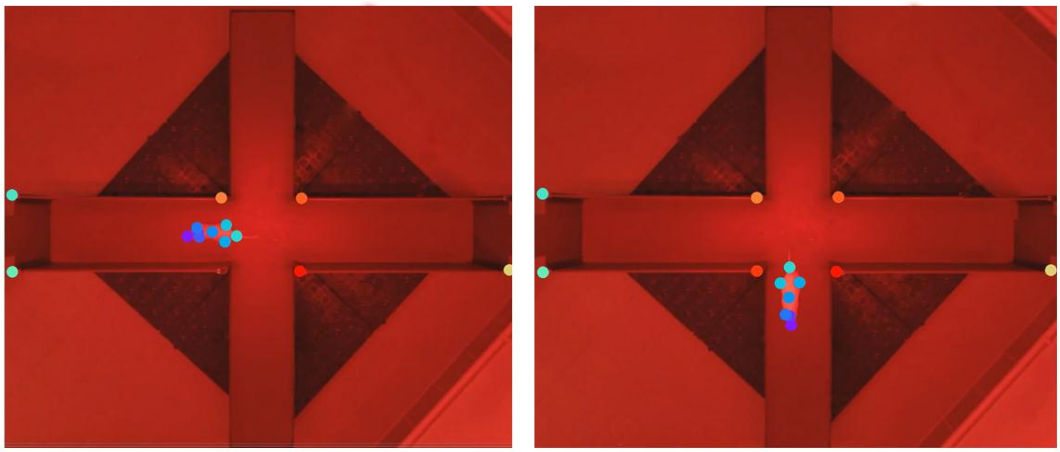

Figure 2.4. Frame-by-frame body positions of the rats were tracked with DeepLabCut
A rat entering the closed arm on the left side, while a rat entering the open arm on the right side.

To assess anxiety-related behaviours in rats, the time spent in the open and closed arms was calculated. In addition, the frequency of transitions to open or closed spaces was also analysed. Arm entry is counted when all four paws of the rodent are on the arm (Figure 2.4). Moreover, locomotive activities were quantified by calculating the overall average of moment-to-moment walking speed. The calculation of speed was as stated in Salgirli Demirbas et al. (2023) and Wittek et al. (2021) where it is defined by the subtraction of Euclidean distance obtained from two consecutive x-y frames.

To assess the general turning behaviour of the rats in EPM, angular movements were described by the rotation of body–head orientations using the same definition as Mundorf et al. (2021). To detect turning behaviour, angular speed was first calculated by calculating body-head orientation and subtraction between two

consecutive frames. The cumulative angular movements were then measured, and the occurrence of turning behaviour was counted when the cumulative angular movements were greater than 45˚. The left and right turning behaviours were separately counted.

2.5.2.2. Motor Laterality Tests

The real testing apparatus was not presented to animals prior the testing sessions to avoid any side bias learning effect on paw preference and head-turning. However, as a part of the habituation process, a demo and smaller version (11 x 12.5 x 21.5 cm) of the apparatus made of plexiglass material together with the food reward used in the Collins test, was presented to the animals in their home cages for a minimum of 15 minutes during the 2-3 days before the initial test session. In this way, the animals were allowed to roam freely in and around the apparatus and had the opportunity to explore the plexiglass material. At the same time, it was aimed to increase their motivation for the test setup by establishing a positive association between food reward and plexiglass material. Each rat was tested individually in the test apparatus in the experimental room. The behaviour of the rats was video recorded continuously during all motor laterality test sessions using a GoPro Hero 7 camera (1920 × 1440-pixel resolution). An example of motor laterality tests administration is presented in Figure 2.5.

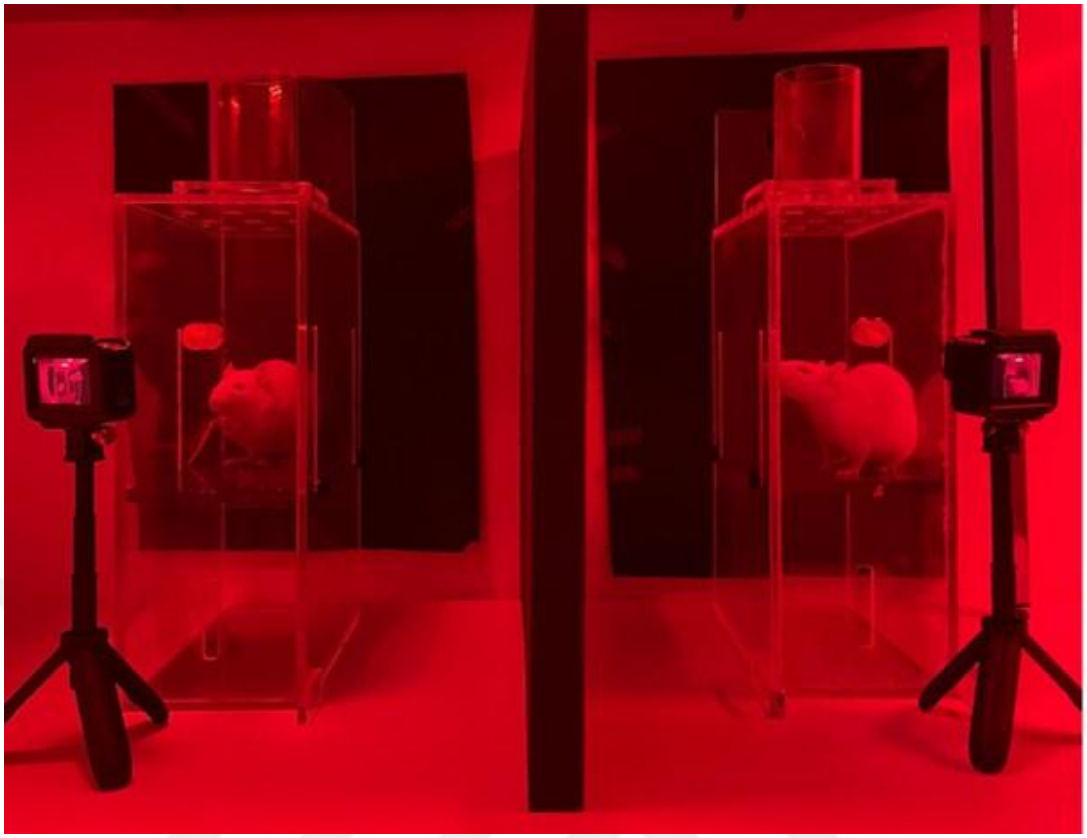

Figure 2.5. Simultaneous Collins Paw Preference Test application in the experimental room

2.5.2.2.1. Collins Paw Preference Test

The modified version of the Collins' paw preference test was used to determine the paw preferences of the animals. After the EPM application on the first day of the testing sessions, animals were given at least 1-hour break time before starting the Collins test to avoid any stress-related bias. A commercially available peanut snack (Erdnuss Locken) was used as a food reward. The animal could see and smell the food reward but could reach it only by using its paws. At the beginning of each testing session, a bite-sized piece of food was provided as a treat to introduce the food reward of the test and encourage the animals. The test sessions lasted 30 minutes, and animals were tested only once a day. During the first two days of the test sessions, the test session was terminated within 10 minutes if the animal showed no interest in the reward food and became inactive in the test apparatus. Collins test was terminated once the rats reached the total number of 50 paw responses that

counted as real-time by the experimenter. In the case of the rat did not reach 50 paw responses in a single session, the animal continued to be tested on subsequent days until it reached the required number.

Video analysis was carried out by the experimenter blinded to experimental conditions. To determine inter-rater reliability, randomly selected videos were further evaluated by a second independent and condition-blind observer. The percentage of agreement between the independent observer and the experimenter was consistently greater than 90 percent. The number of paws uses exhibited by rodents in their pursuit of the food reward was evaluated. The experimenter also documented the rat's initial attempt to retrieve the food reward from the feeding tube.

The present study employed binomial Z-scores for each rat to assess the presence of any directional bias for the paw preference at the individual level ([$z = (R - 0.5\ N)/\sqrt{(0.25\ N)}$]. As per the equation, the overall count of paw entries to the feeding tube for reaching the food reward was denoted by “N”. On the other hand, the variable “R” represents the count of right paw entries. Rats exhibiting a Z-score value above or equal to 1.96 were identified as R-pawed, while those with a Z-score value less than or equal to -1.96 were categorized as L-pawed. Rats that displayed Z-scores within the interval of +1.96 to -1.96 were classified as ambilateral (A). Laterality Index (LI) was computed utilizing the following classic formula, $LI = (R - L)/(R + L)$, to quantify individual paw preferences. The variable "R" represents the count of right paw entries to the feeding tube for reaching the food reward, whereas "L" denoted the count of reaching using the left paw. The LI values vary from -1.0 (left-pawed use only) to +1.0 (right-pawed use only). Furthermore, the absolute values of individual LI (ABS-LI) were also generated to assess the degree of lateralisation irrespective of animals' paw preferences towards a direction.

Statistical analysis was performed using SPSS Statistics 26 (Field, 2013) and $p<0.05$ was considered to be statistically significant. Initially, an evaluation was

conducted on the asymmetries of the subjects at both the population- and individual-levels. One-sample t-tests were applied to examine paw preferences at the population level for both groups, using the average LI as the dependent variable. To investigate the existence of paw preference at the individual level, the ABS-LI of each animal was computed. To this end, the average ABS-LI was tested to examine whether it was distinct from 0 using a one-sample t-test. Later, Chi-square analyses were conducted to determine whether there was an equal distribution of the number of left-preferred, right-preferred, and ambilateral rats. The effects of prenatal stress exposure, age, and sex as well as their interactions on LI and ABS-LI were analysed using Three-way ANOVA. Subsequently, a pairwise comparison test with Bonferroni correction for the factors which were found significant was conducted. Additionally, the effects of prenatal stress exposure, age, and their interactions on LI, were examined separately in both sex groups using Two-way ANOVA. Subsequently, a pairwise comparison test with Bonferroni correction was performed for the factors which were found significant. Thereafter, the effects of prenatal stress exposure on LI were analysed separately in age groups using an independent t-test. To evaluate the predictive potency of the first paw attempt on the overall paw preference direction of the animals, a between-subjects t-test was performed, with the direction of the first paw response serving as the group factor.

2.5.2.2.2. Pasta Matrix Reaching Test

The modified version of the Pasta Matrix Reaching Test was used to determine the paw preferences of the animals as an alternative limb preference test in the present study. The Pasta test was either applied when the animal completed the 50 paw responses in the Collins test or did not pay any interest to the food reward used in the Collins test after 4-5 testing sessions (Table 2.3). Each test session was 15 minutes. Since the Collins test is the primary and standard paw preference determination method in this study, the Collins test continued to be applied to the animals that continued to pay attention to the food reward and the feeding tube of the

apparatus until the last day of the testing period. However, no more than 2 test applications were conducted in a single testing day.

According to the experiences gained from the optimization trials performed before the experiments, pasta sticks (spaghetti) were dipped in hazelnut cream and presented to the animals to make the food reward more desirable for the animals in the current study. Before each session, as an introductory step, a small piece of hazelnut cream on the pasta stick was offered to the animal by the experimenter. Similar to the Collins Test, the animal could visually perceive and olfactorily detect the food reward; however, the only way to reach the food for the animal was using its paws. Except for the 15-minute duration of the Pasta test, the testing procedure, video analysis, and statistical analyses were identical to those of the Collins test.

2.5.2.2.3. Head and Body Turning Asymmetry Test

The simple drinking behaviour of the rats, which does not require motor learning or skill, was recorded to determine head- and body-turning asymmetries. Head and body turning asymmetry tests were applied in 4 consecutive sessions of 30 minutes over 4 days after the Collins and Pasta Tests had been completed. 5% hazelnut syrup water was used as a reward during the experiments. All test sessions were recorded via the GoPro Hero 7 placed in front of the apparatus.

To analyse the videos, around 1200 raw videos obtained from Head and Body Turning Asymmetry tests were edited and merged using FFmpeg before analyzing via DeepLabCut. Then, a DeepLabCut project was created according to DeepLabCut workflow (Nath et al., 2019), 2 frames were extracted from each video to increase the frame-to-frame variability between sessions and subjects instead of overtraining the model on single individuals. 26 different body parts of the rats (n=132) were manually labeled in a total of 810 frames by two researchers following the same

labeling procedure (Figure 2.6). Afterward, a ResNet-50 network was trained for 1000000 iterations on an NVIDIA RTX 3090 GPU with a 95% training/test split.

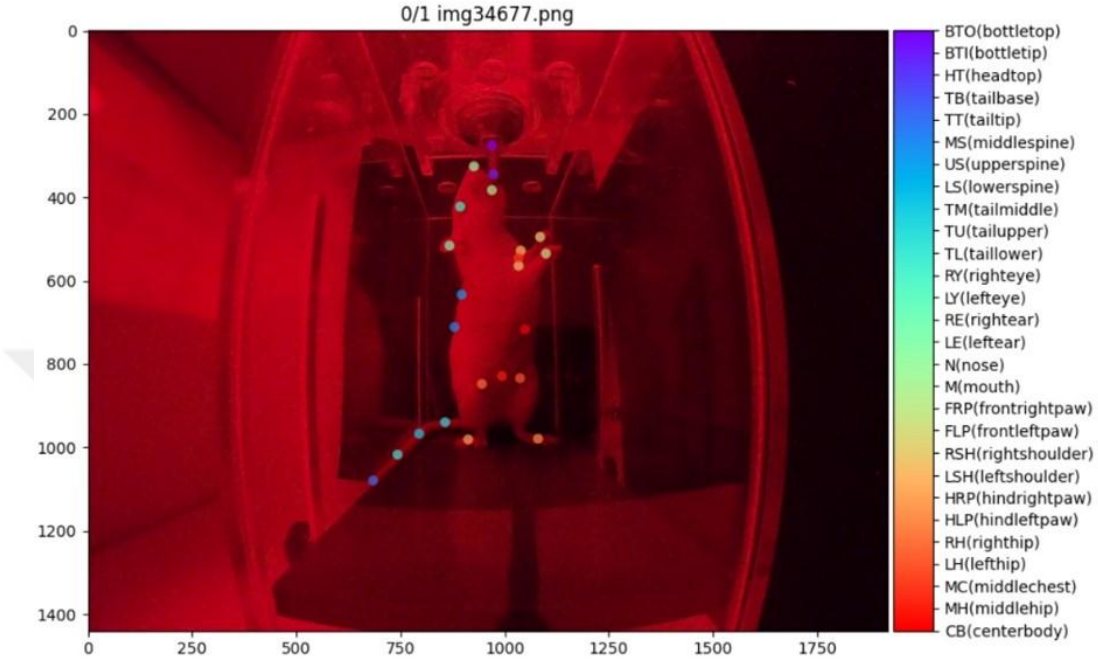

Figure 2.6. The labeling procedure of the extracted frames from the videos to train the network.

As explained in detail in the result section, after the video analysis approach failed in DeepLabCut (explained in the Result section), manual analysis became imperative. The present study, in line with the previous studies conducted in different animal species by Isparta et al. (2020) and Salgirli Demirbas et al., 2023), has demonstrated that the initial paw preference in animals can serve as a reliable indicator of their overall paw preference for the direction. To this respect, the first explorative approach to the bottle dispenser and the drinking behaviour of the animals were manually analysed to get an overall impression of whether there is a change in the turning direction due to prenatal stress exposure. The first explorative approach was described as the animal standing up and positioning their head towards the dispenser of the bottle, while the first drinking was defined as the first time the animal drinks from the dispenser of the bottle. The timestamps for each behaviour, the body position of the animal (front, back, cross-front left and right, cross-back left

and right, left side, right side), the turning direction of the body and the turning direction of the head were determined. The body position was determined based on the position of the hindlimbs and tail of the rats. The turning direction of the body was determined relative to the position of the body so that if the animal stands in a cross position, the body could still be neutral if the hindlimbs and upper body form one line. Head turning was determined in an equal manner. All video materials were evaluated by two independent raters. Intra-class correlation coefficients [(ICC): two-way random effects model) method was applied to compare the inter-rater reliability of video material in subjective body position and turning behaviours (Koo and Li, 2016). Further analysis was conducted solely on parameters that demonstrated a good inter-rater agreement with r=>0.70.

2.6. Molecular Analysis

2.6.1. Biological Sampling

After all behavioural test days were completed, biological materials of the animals, such as blood and brain, were collected and stored for genetic analyses. To that end, the animals were subjected to deep anaesthesia through an intraperitoneal injection of a ketamine and xylazine mixture (100 mg/kg and 10 mg/kg, respectively). Following this, the animals were sacrificed by decapitation for brain exenteration. Right after decapitation, intact brains were swiftly extracted from the skull, immediately treated with liquid nitrogen, and then placed into the freezer (−80 °C). To obtain whole blood samples for further studies, immediately after the decapitation, the trunk blood of the animal was collected into 50 mL Falcon tubes with the final concentration of EDTA adjusted to 4.1 mmol/L (1.8 mg/mL) and transferred into regular 1.5-ml Eppendorf tubes and subsequently stored at − 80 °C.

2.6.2. Tissue Preparation and Homogenization

Brain tissue, stored at -80°C, was placed on the filter paper-lined back surface of a Petri dish filled with a mixture of ice and dry-ice for dissection. The freezing mix provided better quality dissection, keeping the brain frozen by preventing melting and any rapid thawing that causes RNA degradation. The brains were cut into thin slices in the coronal plane using an 0.3 mm razor blade (Apollo-Herkenrath, Solingen, Germany) which was quickly pre-cooled on dry ice. The dissection protocol was performed under RNase-free conditions to avoid any RNA degradation in the brain tissues. To this end, all surfaces were cleaned with RNase AWAY (Carl Roth, Germany) prior to working. Moreover, to prevent cross-contamination, all surgical equipment was cleaned with 70% ethanol and RNase-free water and subsequently autoclaved at 121°C for 20 minutes under 2.1 bar to be sterilized.

In accordance with Paxinos and Watson's The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 2006), the motor cortex [Primary Motor Cortex (M1) + Secondary Motor Cortex (M2)] was dissected from Bregma 5.16 mm until Bregma 0.48 mm. As a result of dissection, approximately 18-30 mg of the motor cortex was obtained from each hemisphere.

Although only the motor cortex tissues were used for analysis in the study, different target areas detailed below were also dissected in addition to the motor cortex. The medial prefrontal cortex (mPFC) [Cg1 (cingulate cortex, area1) + Prelimbic cortex (PrL) + Infralimbic cortex (IL)] was taken from Bregma 3.00 to Bregma 2.52 mm. Both hemispheres were extracted together for the nucleus accumbens (AcBc) from Bregma 2.28 mm to Bregma 0.84 mm and corpus callosum (CC) from Bregma 1.44 mm until Bregma 0.36 mm. For AcBc extraction, 1 mm Biopsy Punch (Kai Medical) was used. The hippocampus was dissected from both hemispheres from Bregma -1.92 mm until Bregma -6.72 mm separately, while the entire cerebellum was taken from the slices. The tissues were placed in 2 mL tubes,

which were pre-cooled and kept on dry ice during dissection. All tissues were subsequently stored at -80°C.

Tissue homogenization was executed utilising Tissue Lyser LT (Qiagen, Hilden, Germany) for the purpose of RNA isolation. First, 5 mm stainless steel beads, pre-cooled at -80°C, were added to the 2 mL reaction tubes containing the dissected motor cortex tissue. The reaction tube was then kept at -80°C for further 15 minutes. Afterward, the sample was treated with 350 µl of Lysis Buffer RP1 from Macherey-Nagel's NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany) and 7 µl of 1 M dithiothreitol (DTT). Reagent DX (Qiagen, Hilden, Germany) was used for foam reduction during sample disruption and homogenization at a rate of 0.5% (v/v). The samples were then inserted in the Tissue Lyser LT and homogenized for 2 minutes at 30 Hz.

2.6.3. RNA Isolation and Quality Control

RNA isolation from the motor cortex was performed according to the manufacturer's protocol using the NucleoSpin® RNA Kit (Macherey-Nagel, Düren, Germany). Before proceeding to the washing steps, 95 µL of DNase reaction mixture was added to the column and incubated for 15 min at room temperature to digest the DNA. After incubation, the membrane was washed thrice. The spin column was placed in a new collection tube, and 40 µL RNase-free H_2O was added and subsequently centrifuged for 1 min to elute RNA. As a last step, the eluate was again applied onto the column for re-elution. Eluted RNA was immediately put and always kept on ice for optimal stability and placed in the -80°C freezer for long-term storage as soon as possible.

After the isolation process, the quantity and quality of RNA were evaluated using the NanoPhotometer® NP80 (Implen, Munich, Germany). The concentration was directly measured via the absorbance of the samples at 260 nm. The ratios of

absorbance at 260 and 280 nm (the A260/280 ratio) and 260 and 230 nm (the A260/230 ratio) for pure RNA are considered ~2.0 and in the range of 2.0-2.2, respectively (Wilfinger et al., 1997). In addition to spectrophotometric quality control, the integrity and purity of RNA were verified on denaturing gel containing 1.5% agarose, 5% formaldehyde, electrophoresis in 1X MOPS buffer at 120 V for ~20 min and stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany) to visualize 18S and 28S ribosomal RNA bands and detect the presence of genomic DNA (gDNA). To this end, the RNA integrity of the female samples in the P60 group used in the microarray analysis and randomly chosen samples from male individuals in both SG and CG were also representatively run in the gel for RT-PCR reactions. Each RNA sample per lane was diluted with nuclease-free water to 1µg RNA in a total volume of 8 µl, then mixed with 2µl 5x RNA Loading Buffer. Subsequently, the mix was incubated at 65°C for 5 minutes before loading to the gel. Then gel imaging was performed using Syngene G: BOX Chemie XT 4 Chemiluminescence Fluorescence Gel Imaging System (Syngene, UK).

RNA integrity is an essential precondition for obtaining accurate gene expression data. Utilizing intact RNA is essential for the success of microarray and RT-PCR analyses. Hence, randomly chosen RNA samples from female and male subjects from stress and control groups were also representatively tested using the 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany) based on the capillary electrophoresis technique. The integrity of RNA (RIN value) was evaluated using RNA 6000 Pico-Kit (Agilent Technologies, Waldbronn, Germany). Testing was performed according to the manufacturer's instructions. According to the numbering system of Agilent Expert Software, RIN values range from 1 (completely degraded) to 10 (most intact), high-quality RNAs reflecting with RIN values greater than 8, while partially degraded RNAs reveal a RIN within the range of 5 to 7. Strongly fragmented RNAs reflect a RIN value of around 3 (Mueller et al., 2004). Therefore, RIN values above a cut-off of 8 were considered evidence of non-degraded RNA extraction techniques (Schroeder et al., 2006) and the RNA Integrity Number of the RNA utilized in the microarray study was >8. In addition, the ratio of

28S:18S was checked by using the gel images, which were within the range of 1.8-2.0 as an indicator of intact RNA.

2.6.4. Microarray-based Gene Expression Analysis

Considering the results of the Collins Paw Preference test, female individuals of the P60 group (stress n=6, control n=6), in which the effect of prenatal stress was more prominent, were used in microarray gene expression analysis to investigate neurobiological alterations induced by prenatal stress. Within this scope, RNA gene pools were created from the RNAs obtained from the motor cortex of the right and left hemispheres of female offspring in CG and SG to be used in gene expression analysis. Thus, four different pooled RNA samples were created from the females in P60: (i) Stress-right, (ii) Stress-left, (iii) Control-right, and (iv) Control-left. Each pool contained equivalent concentrations of the RNA extracted from the motor cortex of the associated six individual samples (Figure 2.7).

100 ng of total RNA was used to generate Cyanine 3-CTP (Cy3) labeled cRNA (complementary RNA) using the Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies) along with Agilent's One-Color RNA Spike-in Kit, according to the manufacturer's instructions. The labeled/amplified cRNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). Subsequently, cRNA concentration (ng/µL) and Cyanine 3 dye concentration (pmol/µL) was measured using NanoPhotometer® NP80 (Implen, Munich, Germany). The yield of cRNA and the specific activity of the Cy3, respectively, were calculated based on the measuring results using the following equations.

$$\frac{(Concentration\ of\ cRNA)\ x\ 30\ \mu L\ (elution\ volume)}{1000} = \mu g\ of\ cRNA$$

$$\frac{Concentration\ of\ Cyanine\ 3}{Concentration\ of\ cRNA} \text{ x } 1000 = \text{ pmol Cy3 per } \mu g\ cRNA$$

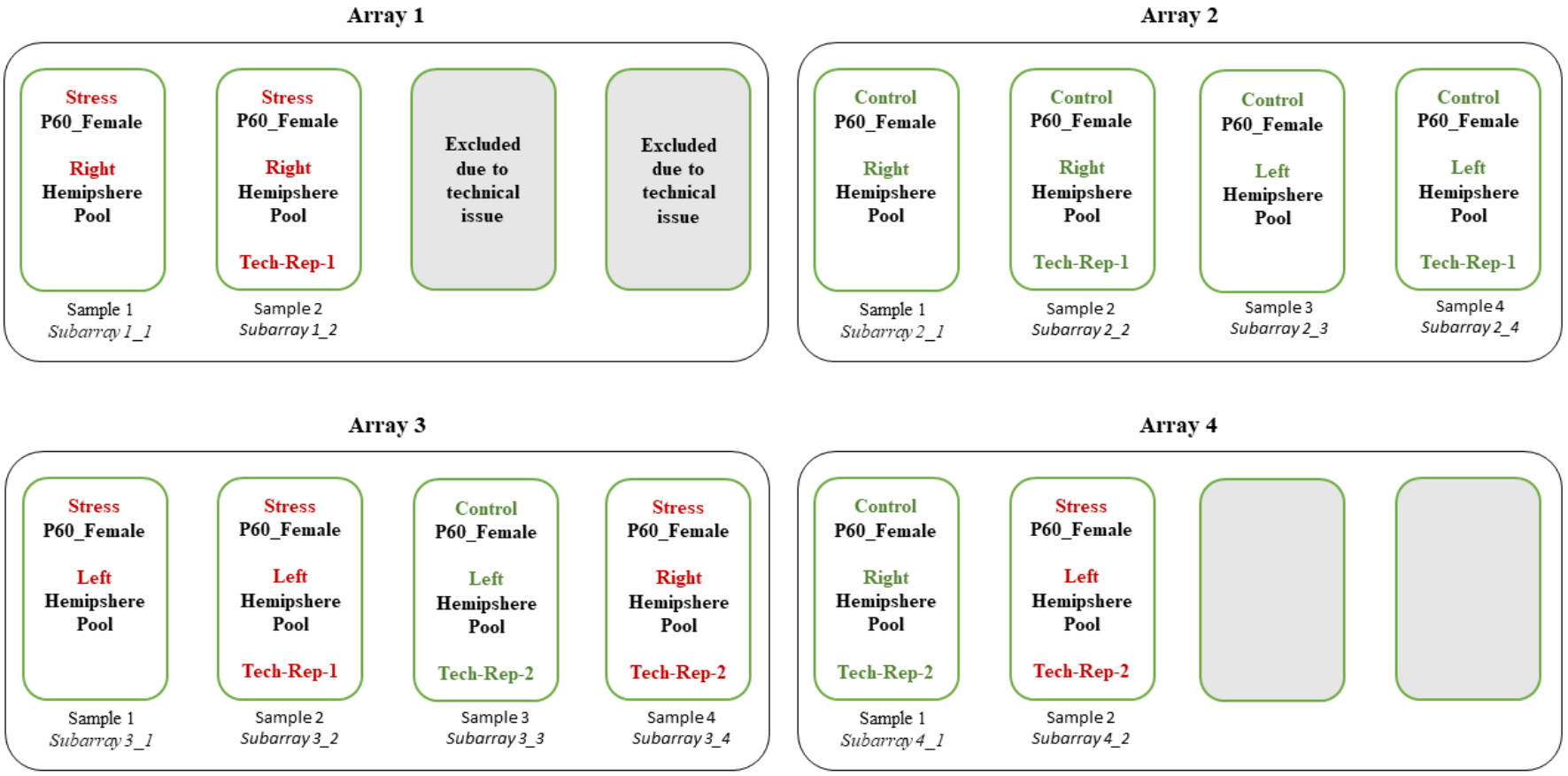

Figure 2.7. Experimental design of the Microarray gene expression analysis.

In line with the guidelines provided by the manufacturer, the recommended cRNA yield and specific activity for the four-pack microarray format used in the present study are 1.65 µg and ≥6 pmol Cy3 per µg cRNA, respectively. Following quantification, 100 ng of labeled cRNA was fragmented and hybridized on a Rat Gene Expression 4 × 44K (V3) Microarray (Agilent Technologies, G2519F-028282) using the Gene Expression Hybridization Kit (Agilent Technologies), following the guidelines. The hybridization step was carried out at 65°C at full rotation speed in a hybridization oven for 17 hours. The arrays were washed using a Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned using a DNA Microarray Scanner with SureScan High-Resolution Technology (Agilent G2505B). An experimental workflow illustrating the microarray analysis procedure is depicted in (Figure 2.8).

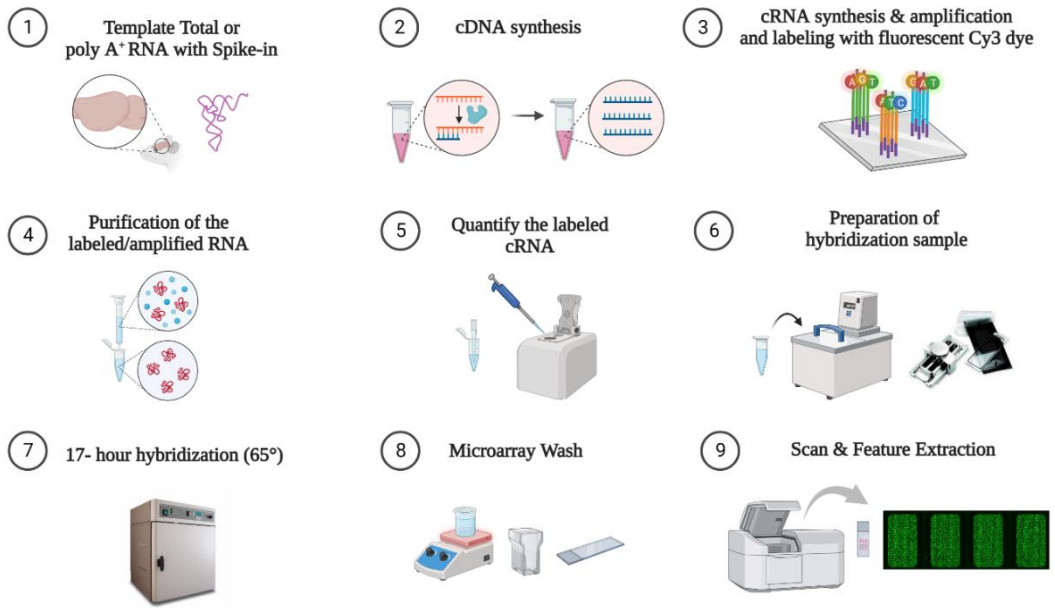

Figure 2.8. Visualization of microarray procedures
The procedure from sample preparation to array scanning, according to the Agilent One-Color Microarray-based Gene Expression Analysis. Numbers indicates the steps of the microarray procedure. Figure created using BioRender.com.

Agilent Feature Extraction software (AFE, version A.10.7.3.1) was used for image analysis and data extraction with default settings and protocols. The total gene signal (TGS) was obtained by the AFE algorithm generating a single intensity

measure for each feature and was used for further analyses. The normalization of the data was performed by the quantile method that is implemented in the R package Limma version 3.50 (Smyth, 2004). Bioconductor's RnAgilentDesign028282.db_3.17 annotation package (Carlson, 2016) was used to annotate the chip probes. The data was filtered regarding the normalized expression values, and the moderated t-test was used to identify differentially expressed genes. According to Benjamini and Hochberg [false detection rate - FDR], the p-values were corrected for multiple testing, and the limit of significance was determined as $p<0.05$. Afterward, the mRNAs with a fold change of more than 1.7 in the microarray analysis were considered. Later, GSEA software v4.0.1 (Subramanian et al., 2015) provided by the Broad Institute of the Massachusetts Institute of Technology and Harvard University (http://www.broad.mit.edu/gsea/ accessed on 14 June 2023) was used for gene set enrichment analysis with default parameters. This study utilized the hallmark gene sets (h.all.v2022.1). Additionally, gene set permutation was performed and FDR q-value ≤ 0.05 were considered statistically significant.

2.6.5. Quantitative Real-Time Polymerase Chain Reaction

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) is widely used and the most reliable tool to validate or confute microarray-generated data (Provenzano and Mocellin, 2007). Not all samples collected at selected time points for each age group were examined by the qRT-PCR analysis. In light of the data obtained from the microarray gene expression analysis, only RNAs of the females (n=15) and males (n=19) at PD60 in CG and SG were used for qRT-PCR analysis. A total of 2000 ng RNA from the motor cortices of each hemisphere was first reversed into cDNA using the High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scientific, Darmstadt, Germany) to quantify mRNA levels by an Eppendorf 6333 Nexus MasterCycler Thermal Cycler (Eppendorf AG, Hamburg, Germany) using the following PCR conditions: incubation at 37°C for 60 minutes, reverse transcriptase deactivation at 95°C for 4 minutes, and holding at 4°C,

according to the manufacturer's instructions. Based on the data obtained from microarray-based gene expression analysis, a novel panel of 11 candidate reference genes, including some traditional ones such as Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH)* and Actin Beta (*ACTB*), was generated based on their low variability and high stability in expression from microarray data and current literature to select the best reference genes for the present study. According to RefFinder web-based tool that integrates four main computational programs containing geNorm, Normfinder, BestKeeper, and the comparative ΔCt method to extensively rank the tested candidate genes (Xie et al., 2023), two genes were chosen as reference genes for the qRT-PCR: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ)* and Inositol 1,4,5-Trisphosphate Receptor Type 1 (*ITPR1)*. Forkhead Box E1 (*FOXE1)* and DNA Polymerase Mu (*POLM)* genes were selected as target genes. qRT-PCR was performed using the TaqMan system (Applied Biosystems, Thermo Fisher Scientific, USA) using probesets for *FOXE1* (Rn00594363_s1), *POLM* (Rn01443281_m1), *YWHAZ* (Rn00755072_m1) and *ITPR1* (Rn01425738_m1). The Bio-Rad CFX Connect Real-Time PCR System was utilized according to the manufacturer's instructions. The total reaction volume (20 µL) consisted of the following: 2 µL cDNA (100 ng), 10 µL TaqMan Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA) using, 1 µL of each TaqMan Gene Expression Assay, and 7 µL Nuclease-free water. The cycle parameters were as follows: Initial step of Uracil DNA Glycosylase (UNG) incubation at 50°C for 2 minutes, DNA polymerase activation at 95°C for 20 minutes, and PCR with 40 cycles of denaturation at 95°C for 3 seconds followed by annealing and elongation at 60°C for 10 seconds. The cDNA concentration was calculated based on a 1:1 reverse transcriptase efficiency assumption relative to the initial RNA amount, and a total cDNA of 100 ng was obtained in a 20 µL reaction.

Throughout the optimization steps no template-control reactions (only nuclease-free water) (NTC) and negative control (without the reverse transcriptase enzyme) were carried out to assess the efficiency and robustness of the reverse transcription and PCR reactions. All samples and gene assays were run in duplicate. Moreover, to preclude between-plate variation, a single sample, with its technical

duplicates on each plate, was utilized as a specific calibrator (Ruiz-Villalba et al., 2021). This inter-run calibrator (IRC) was loaded to each plate in duplicate. Plate design is depicted in Figure 2.9.

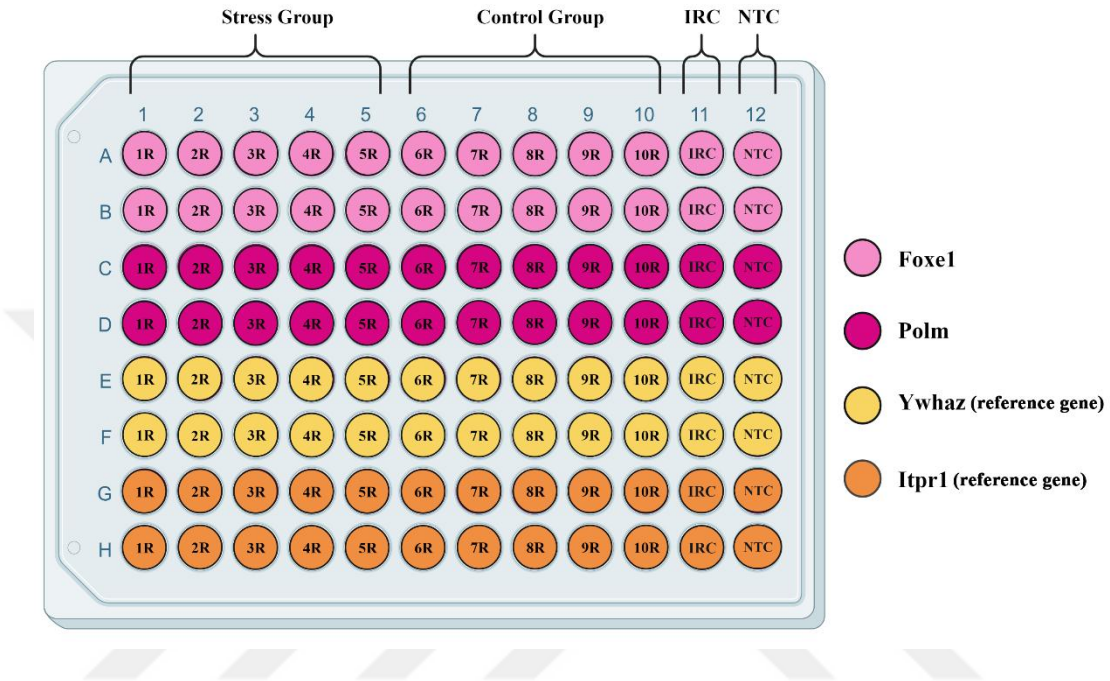

Figure 2.9. Scheme of the qPCR 96-well plate for 12 samples including inter-run calibrator (IRC) and no template-control (NTC).

Shapiro-Wilk normality test was performed to determine whether the normalized gene expression data for each experimental group were normally distributed. A p-value higher than 0.05 value indicates that the data is normally distributed.

Gene expression analyses of the samples for each target genes, inter-run calibration of plates to correlate data from multiple plates and reference gene stability analysis were performed using Bio-Rad CFX Maestro Software Version 2.3 (Bio-Rad, Hercules, CA, USA). Threshold determination, baseline correction, and normalization based on the reference genes (*YWHAZ* and *ITPR1*) were performed using automated settings. The quantitative cycle (Cq) was used to quantify the real-time PCR reaction (Bustin et al., 2009). Pfaffl method (Pfaffl, 2001) was used to

calculate relative expression values, in which PCR primer efficiencies of the genes were calculated by using LinRegPCR software, based on the kinetics of the fluorescene obtained from the amplification curve of each individual sample (Untergasser et al., 2021). Within this scope, normalized expression (ΔΔCq) was performed, and an unpaired t-test was applied for statistical analysis of gene expression data. The GeNorm algorithm-based Reference Gene Selector tool, available in Biorad CFX Maestro™ Software, was utilized to calculate the mean stability (M value) of individual reference genes across all samples in multiple plates (Vandesompele et al., 2002; Zhou et al., 2017). M value and gene expression stability are inversely proportional, and stably expressed genes usually have an average M value that is less than 0.5 in homogenous sample set (Vandesompele et al., 2002; Zhang et al., 2005). $P<0.05$ was considered to be statistically significant for the gene expression differences.

The formula for relative quantity (ΔCq) for any sample (GOI) is as follows:

$$\textbf{Relative Quantity}_{\textbf{sample (GOI)}} = \boldsymbol{E}_{\textbf{GOI}}{}^{(\boldsymbol{C_q}\,\textbf{(min)} - \boldsymbol{C_q}\,(\boldsymbol{sample}))}$$

$$\mathrm{E} = Efficiencies\ for\ the\ primer\ and\ probe\ set$$

$$GOI = Gene\ of\ interest\ for\ per\ target$$

$$Cq\ (sample) = AverageCq\ for\ the\ sample$$

$$Cq(min) = Average\ Cq\ for\ the\ sample\ with\ the\ lowest\ average\ Cq\ \ for\ GOI$$

The formula of the normalized expression (ΔΔCq) uses the calculated Relative Quantity (RQ):

$$\textbf{Normalized Expression}_{\textbf{sample (GOI)}} = \frac{\textbf{RQ}_{\textbf{sample (GOI)}}}{(\textbf{RQ}_{\textbf{sample (Ref 1)}}\ \textbf{x}\ \textbf{RQ}_{\textbf{sample (Ref 2)}}\textbf{x...x}\textbf{RQ}_{\textbf{sample (Ref n)}})^{\frac{\mathbf{1}}{\mathbf{n}}}}$$

$$RQ = Relative\ quantity\ of\ a\ sample$$

$$Ref = Reference\ targets\ for\ each\ sample$$

The formula for expression and relative quantity for biological groups is as follows:

$$\textbf{Expression biological group} = \sqrt[n]{\textbf{Exp}_1 \bullet \textbf{Exp}_2 \bullet \ldots \bullet \textbf{Exp}_n}$$

$$Expn = Relative\ quantity\ or\ normalized\ expression\ of\ the\ samples$$

$$n = Number\ of\ samples\ in\ the\ biological\ group$$

Fold change is a measure of the increase or decrease in the expression of a target for an experimental versus a control sample or biological group and is determined as follows:

If Expression (experimental) > Expression (control) :

$$\textbf{Fold Change} = \frac{\text{Expression (experimental)}}{\text{Expression (control)}}$$

If Expression (experimental) < Expression (control) :

$$\textbf{Fold Change} = -1 \,/\, \left(\frac{\text{Expression (experimental)}}{\text{Expression (control)}}\right)$$

In addition, pooled RNA samples obtained from the motor cortices of the right and left hemispheres of female offspring in CG and SG were also analysed by qRT-PCR for comparing microarray data.

To investigate the correlation between laterality index (LI) and target gene expressions in the left and right hemispheres, ΔCq values were utilized. The relationship between the strength, and direction of lateralisation and the expression status of target genes (ΔCq) in both hemispheres was analysed by the Spearmen correlation coefficient analysis using SPSS Statistics 26.

3. RESULTS

3.1. Behavioural Analyses

3.1.1. Measurement of Offspring Weight

The results of the ANOVA revealed main effects of time assessment and group condition on the weight of the offspring ($F(3,470) = 4785.279$; $p<0,001$; $F(1,470) = 28.008$; $p<0,001$, respectively). As predicted, the offspring increased in weight over time in both groups (all p's <0.001). Similarly, there was a main effect of sex status on the weight of the offspring and with the weight of the male offspring being statistically higher than their female counterparts ($F(1,470) = 5.297$; $p=0.022$). Moreover, the day × experimental condition interaction was also found to be statistically significant ($F(3,470) = 14.628$; $p<0.001$), while the day × sex interaction was not statistically significant ($F(1,470) = 0.699$; $p=0.553$). In both conditions, the days were different from each other (all p's <0.001). However, a statistically significant difference was found between the control and stress groups only at PD20 ($p<0.001$). Lastly, the day × condition × sex interaction was not statistically significant ($F(1,470) = 0.105$; $p=0.957$) as well.

3.1.2. Maternal Care Assessment

3.1.2.1. Licking and Grooming

Day Effect: The main effect of days on licking and grooming was statistically significant ($F(3,55) = 3.189$; $p=0.031$). Dams at PD20 exhibited significantly less licking behaviour than dams at PD14 ($p=0.05$).

Stress Effect: There was no significant main effect of prenatal stress on licking and grooming behaviour ($F(1,55) = 0.271$; $p=0.605$).

The prenatal stress × day interaction on licking and grooming behaviour was also not statistically significant ($F(3,55) = 1.549$; $p=0.212$). The results of the licking and grooming behaviour of the dams with their mean and standard error of the mean (SEM) values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.1.

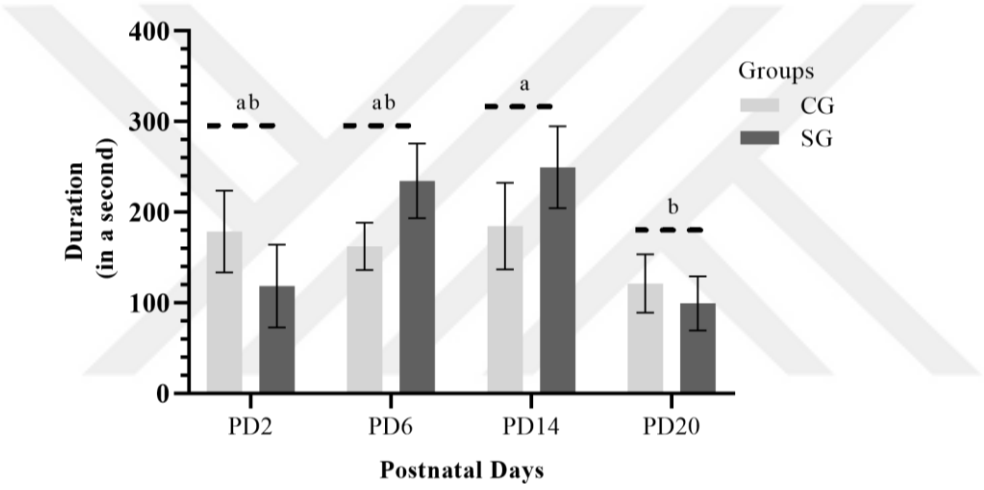

Figure 3.1. Licking and grooming behaviour of the dams
The mean ± SEM for the licking and grooming behaviour of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Lowercase letters (a,b) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. Dams at PD20 displayed substantially less licking behaviour compared to the dams at PD14. Figure created using GraphPad Prism Software.

3.1.2.2. Nursing

Day Effect: A significant main effect of the days was obvious with $F(3,55) = 3.582$, $p=0.019$, as the nursing behaviour of the dams differed significantly at PD20 compared to PD14 ($p<0.05$).

Stress Effect: There was no significant main effect of prenatal stress on nursing behaviour ($F(1,55) = 0.669$; $p=0.417$).

The prenatal stress x day interaction on nursing behaviour was not statistically significant ($F(3,55) = 0.736$; $p=0.535$. The results of the nursing behaviour of the dams with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.2.

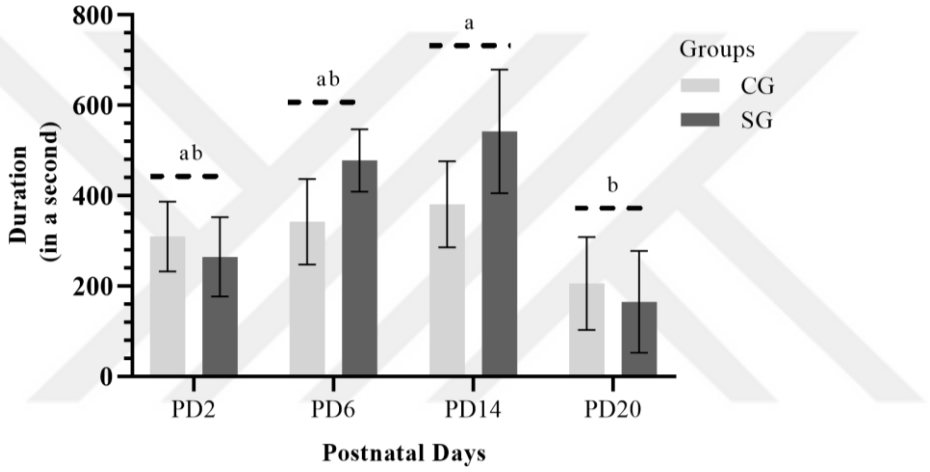

Figure 3.2. Nursing behaviour of the dams
The mean ± SEM for the nursing behaviour of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Lowercase letters (a,b,ab) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. The nursing behaviour of the dams was significantly less at PD20 compared to PD14. Figure created using GraphPad Prism Software.

3.1.2.3. Retrieval of Pups

3.1.2.3.1. The Duration of Retrieval of Pups

Firstly, the duration elapsed between the retrieval time of the first pup to the time to retrieve the complete litter was analysed.

Day Effect: The main effect of day on retrieval behaviour was statistically significant ($F(2,42) = 3.638$; $p=0.035$). Dams at PD14 exhibited significantly less retrieval behaviour than dams at PD6 ($p=0.031$).

Stress Effect: The main effect of prenatal stress on retrieval behaviour was not statistically significant ($F(1,42) = 0.271$; $p=0.605$). There was no significant difference between stress and control groups.

The prenatal stress x day interaction on retrieval behaviour was not statistically significant ($F(2,42) = 0.043$; $p=0.958$). The mean ± SEM for duration for retrieval of pups at PD2, PD6, and PD14 in control and stress groups are depicted in Figure 3.3.

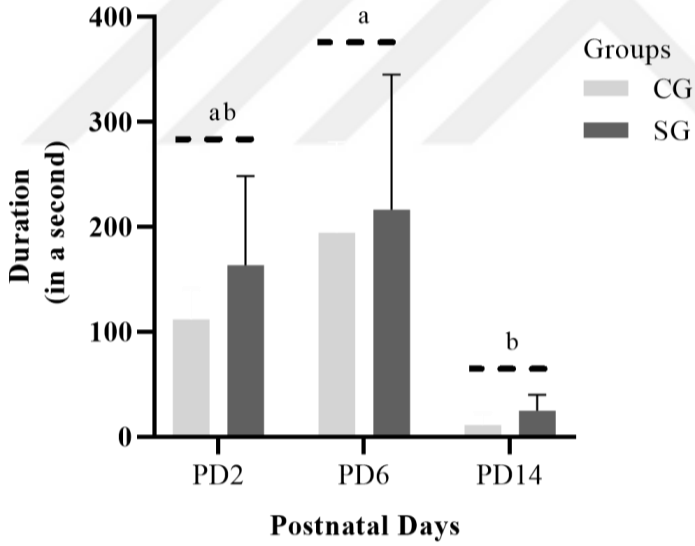

Figure 3.3. The duration of retrieval of pups
The mean ± SEM for the duration of retrieval of pups at PD2, PD6, and PD14 in control and stress groups were shown. Lowercase letters (a,b) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. Figure created using GraphPad Prism Software.

3.1.2.3.2. Time to Retrieve the First Pup

Day Effect: The main effect of the day in time until the first pup was retrieved was not statistically significant (F(1,27) = 2.930; p=0.098).

Stress Effect: The main effect of the prenatal stress in time until the first pup was retrieved was not statistically significant (F(1,27) = 2.051; p=0.164).

The prenatal stress x day interaction in time until the first pup was retrieved was not statistically significant (F(1,27) = 0.882; p=0.356). The time to retrieve the first pup at PD2 and PD6 in control and stress groups are depicted in Figure 3.4.

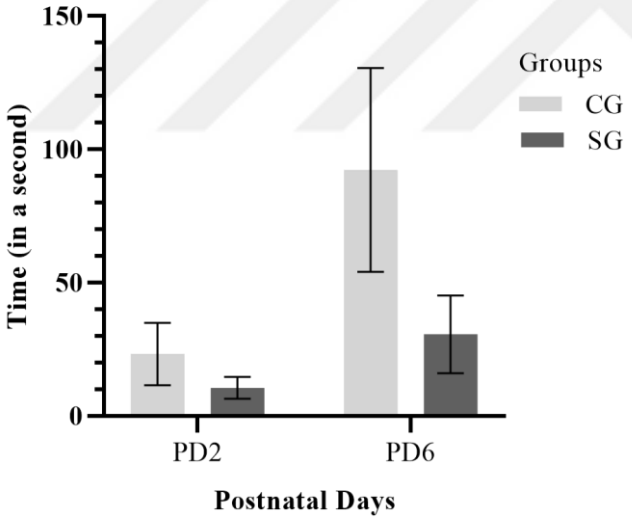

Figure 3.4. Time to retrieve the first pup
The mean ± SEM for the time for the first pup retrieval at PD2, and PD6 control and stress groups were shown. Figure created using GraphPad Prism Software.

3.1.2.3.3. Time to Retrieve the Complete Litter

Day Effect: The main effect of the day in time to retrieve the complete litter was not statistically significant (F(1,27) = 0.836; p=0.369).

Stress Effect: The main effect of the day in time to retrieve the complete litter was not statistically significant ($F(1,27) = 0.000$; $p=1.000$).

The prenatal stress x day interaction in time to retrieve the complete litter was not statistically significant ($F(1,27) = 0.206$; $p=0.654$). The results of the retrieval time of the complete litter at PD2 and PD6 in control and stress groups are depicted in Figure 3.5.

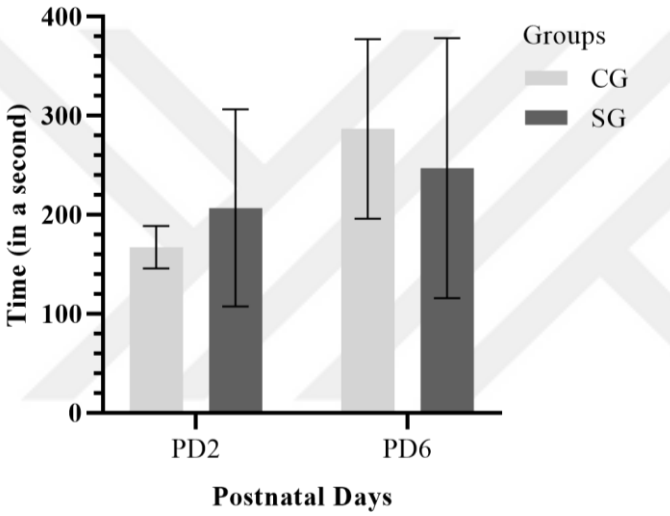

Figure 3.5. Time to retrieve the complete litter
The mean ± SEM for the time to retrieve the complete litter at PD2, and PD6 control and stress groups were shown. Figure created using GraphPad Prism Software.

3.1.2.4. Nest Rebuilding

Day Effect: Statistical analysis revealed a main effect of the day on nest rebuilding behaviour ($F(2,42) = 15.995$; $p<0,001$), indicating lower nest rebuilding behaviour at PD14 than at PD2 and PD6 (respectively, $p<0.01$ and $p<0.05$)

Stress Effect: The main effect of the prenatal stress on nest rebuilding failed to reach significance ($F(1,42) = 3.240$; $p=0.079$).

The prenatal stress x day interaction on nest rebuilding behaviour failed to reach significance ($F(2,42) = 0.944$; $p=0.397$). The results of the nest rebuilding behaviour of the dams with their mean ± SEM values at PD2, and PD6 in control and stress groups are depicted in Figure 3.6.

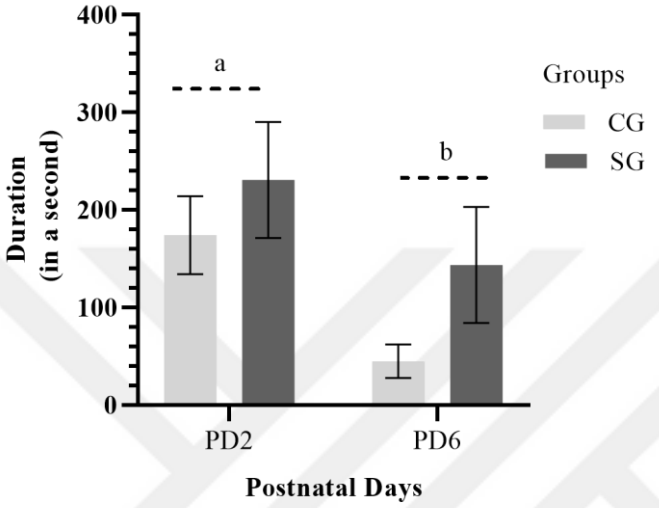

Figure 3.6. The nest rebuilding behaviour of the dams
The mean ± SEM for nest rebuilding behaviour of the dams at PD2 and PD6 in control and stress groups were shown. Lowercase letters (a,b) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. Figure created using GraphPad Prism Software.

3.1.2.5. Time Spent on the Nest

Day Effect: The main effect of day on time spent on the nest was statistically significant ($F(2,42) = 23.634$; $p<0,001$), indicating dams spent less time on the nest at PD20 than at PD6 and PD14.

Stress Effect: The main effect of prenatal stress on time spent on the nest was not statistically significant ($F(1,42) = 0.509$; $p=0.479$).

The prenatal stress x day interaction on time spent on the nest failed to reach significance (F(2,42) = 1.333; p=0.0275). The results of the time spent on the nest with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.7.

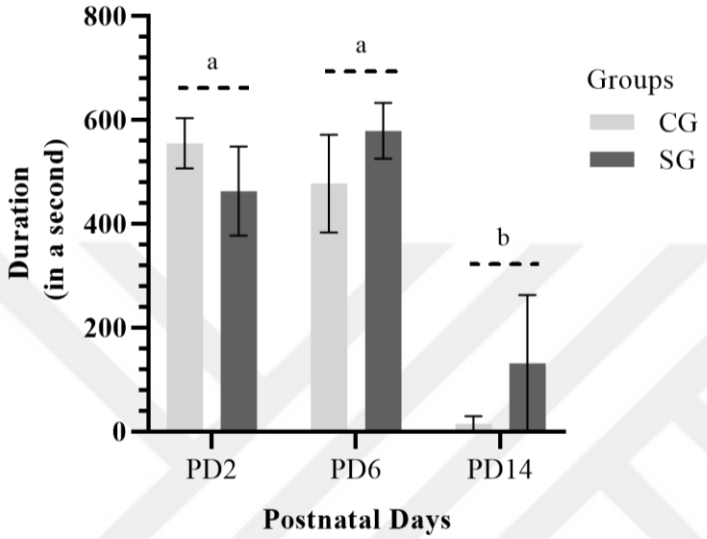

Figure 3.7. The time spent on the nest for dams
The mean ± SEM for the time spent on the nest for dams at PD2, PD6, and PD14 in control and stress groups were shown. Lowercase letters (a,b) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. Figure created using GraphPad Prism Software.

3.1.2.6. Self-grooming

Day Effect: The main effect of day on self-grooming was not statistically significant (F(3,55) = 1.682; p=0.181).

Stress Effect: The main effect of prenatal stress on self-grooming was not statistically significant (F(1,55) = 3.347; p=0.073).

The prenatal stress x day interaction self-grooming behaviour was also not statistically significant (F(3,55) = 1.669; p=0.184). The results of the self-grooming

behaviour of the dams with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.8.

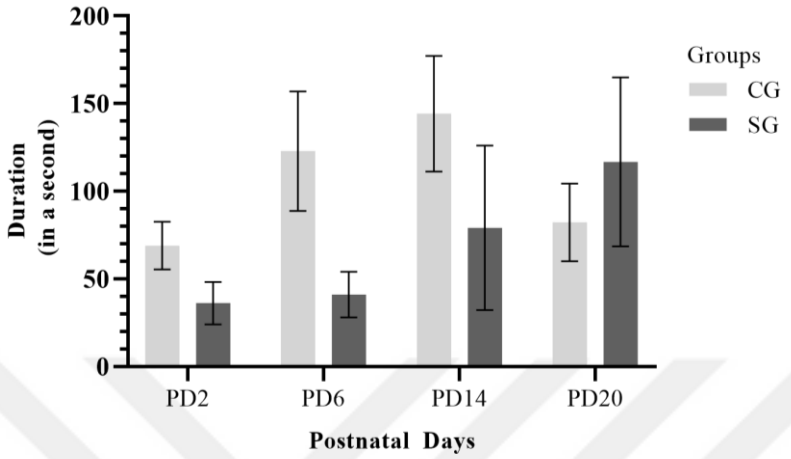

Figure 3.8. Self-grooming behaviour of the dams
The mean ± SEM for self-grooming behaviour of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Figure created using GraphPad Prism Software.

3.1.2.7. Rearing

Day Effect: The results indicated that the main effect of day on rearing behaviour was not statistically significant ($F(3,55) = 1.692$; $p=0.179$).

Stress Effect: The main effect of prenatal stress on rearing behaviour failed to reach significance ($F(1,55) = 0.308$; $p=0.581$).

The prenatal stress x day interaction on rearing behaviour also failed to reach significance ($F(3,55) = 0.460$; $p=0.711$). The results of the rearing behaviour of the dams with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.9.

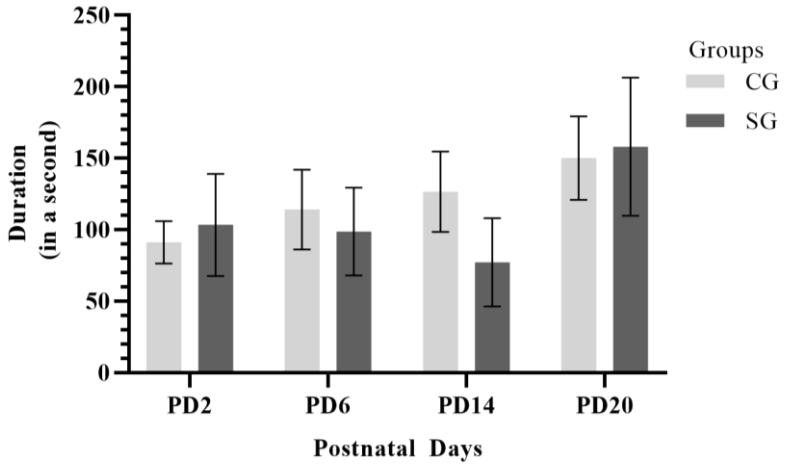

Figure 3.9. Rearing behaviour of the dams
The mean ± SEM for rearing behaviour of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Figure created using GraphPad Prism Software.

3.1.2.8. Offspring-directed Behaviours

Offspring-directed behaviours category included the sum of the duration dam exhibits licking and grooming, nursing, retrieval of pups, nest rebuilding behaviours, and time spent on the nest.

Day Effect: The results of the statistical analysis revealed a main effect of the day ($F(3,56) = 21.003$; $p<0,001$), indicating dams spent less time on offspring-directed behaviours at PD14 and PD20 than at PD2 and PD6 ($p<0.001$).

Stress Effect: The main effect of prenatal stress on self-directed behaviours failed to reach significance ($F(3,56) = 1.071$; $p=0.305$).

The prenatal stress x day interaction on offspring-directed behaviours also failed to reach significance ($F(3,56) = 1.159$; $p=0.333$). The results of the offspring-directed behaviours of the dams with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.10.

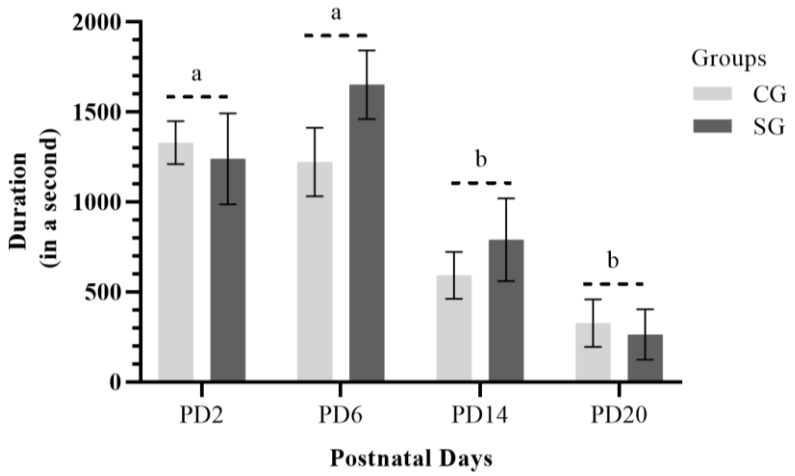

Figure 3.10. Offspring-related behaviours of the dams
The mean ± SEM for offspring-directed behaviours of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Lowercase letters (a,b) indicate whether there is a difference between age groups regardless of stress and control conditions. The alpha value for significance was assigned to 0.05. Dams at PD14 and PD20 spent significantly less time on offspring-directed behaviours compared to PD2 and PD6. Figure created using GraphPad Prism Software.

3.1.2.9. Self-directed Behaviours

The self-directed behaviours category included the sum of the duration dam exhibits self-grooming and rearing behaviours.

Day Effect: The main effect of day on self-directed behaviours was not statistically significant ($F(3,56) = 2.042$; $p=0.118$).

Stress Effect: The main effect of prenatal stress on self-directed behaviours was not statistically significant ($F(1,56) = 3.260$; $p=0.076$).

The prenatal stress × day interaction on self-directed behaviours also failed to reach significance ($F(3,56) = 1.778$; $p=0.162$). The results of the self-directed behaviours of the dams with their mean ± SEM values at PD2, PD6, PD14, and PD20 in control and stress groups are depicted in Figure 3.11.

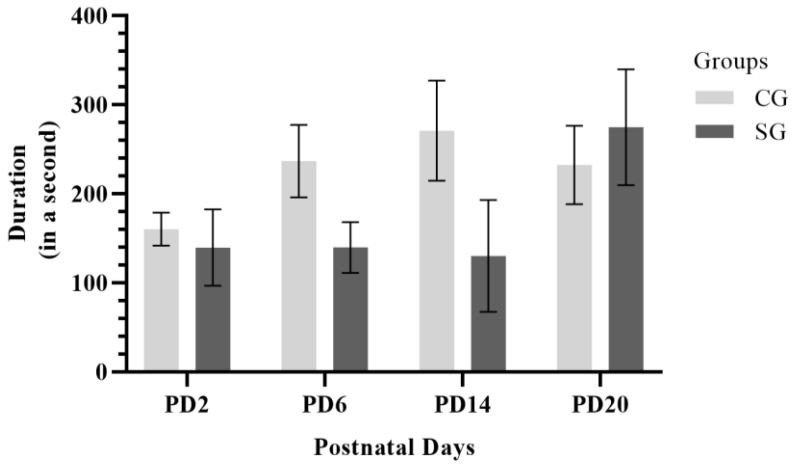

Figure 3.11. Self-directed behaviours of the dams
The mean ± SEM for self-directed behaviours of the dams at PD2, PD6, PD14, and PD20 in control and stress groups were shown. Figure created using GraphPad Prism Software.

3.1.3. Elevated Plus Maze

The data of dams and offspring were analysed separately. To determine the effects of experimental manipulation (i.e. restriction stress) for dams, the time spent by the dams in the control and stress groups in the open and arms of the EPM was compared using an independent t-test. Dams in the stress group spent statistically more time in the closed arms than the control group ($t_{(13.56)}$ = -0.83; p=0.41). The results obtained from the open arms were also in line with these results, and the control group spent more time in the open arms than the stress group ($t_{(13.73)}$ = 1.27; p=0.224) (Figure 3.12). These results showed that the time spent in both arms was influenced by restriction stress exposure.

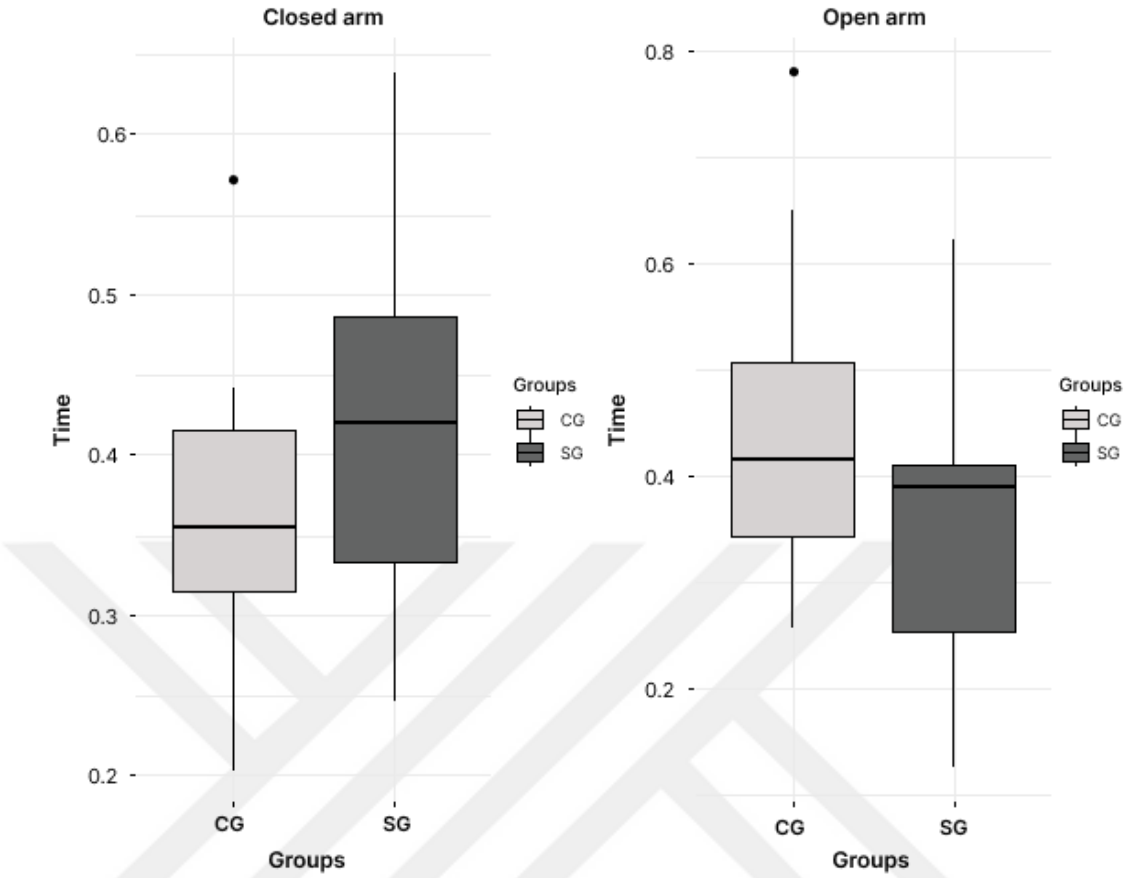

Figure 3.12. Comparison of time spent in open and closed arms between stress and control dams
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. Control group (CG); Stress group (SG).

To investigate the effect of prenatal stress on offspring, the time spent in the open arm and closed arm was separately analysed using 2 × 4 univariate ANOVA with the between-subjects factors of the experimental group (SG and CG) and age group (PD21, PD40, PD60, PD90). The main effect of the experimental group in the open arm failed to reach significance ($F(1,108) = 0.241$; $p=0.624$), while the main effect of the age group was statically significant ($F(3,108) = 2.857$; $p=0.040$). Subsequently, age groups were compared using the Bonferroni corrected pairwise t-test, and no significant difference was found among the groups (all p's >0.05). However, the experimental group × age group interaction reached a significance ($F(3,108) = 3.503$; $p=0.018$), indicating that control group spent more time in open arm compared to the stress group at PD60 ($p=0.04$) (Figure 3.13).

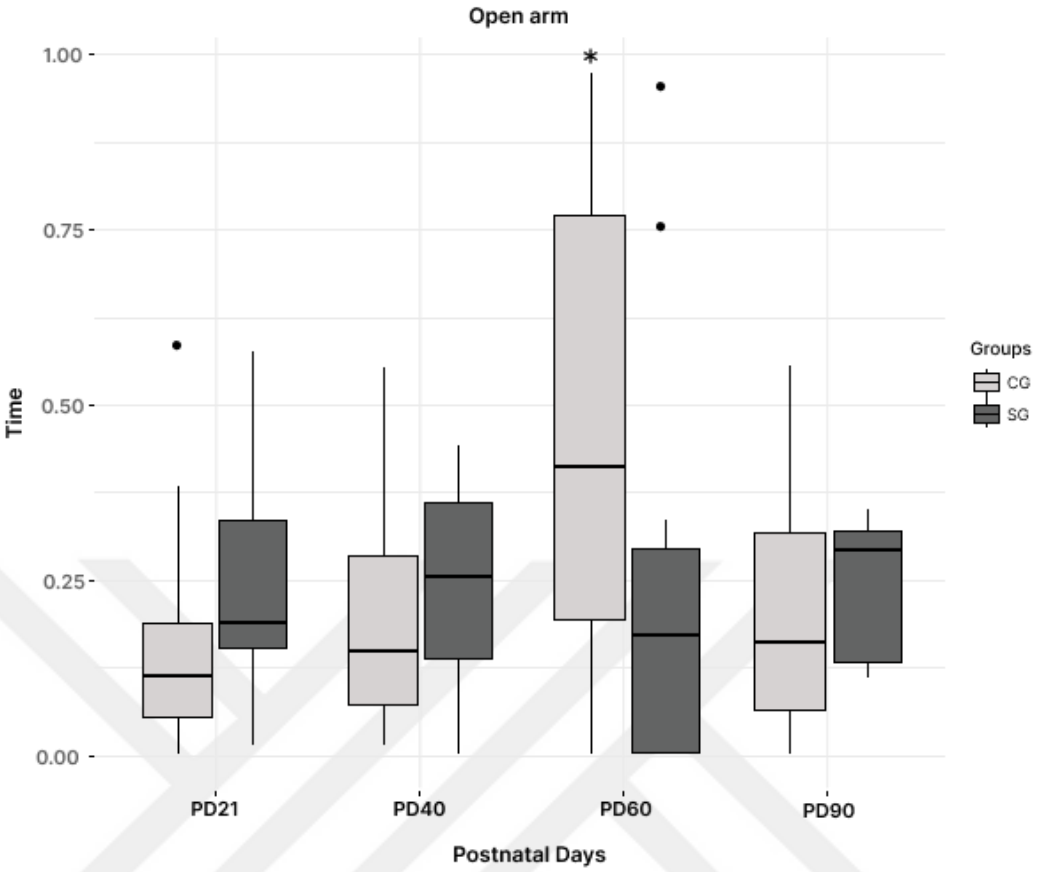

Figure 3.13. Time spent in the open arms of the offspring in CG and SG
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. Asterisk (*) represents the significant difference ($p<0.05$). CG: Control group; SG: Stress group.

The main effects of the experimental and age groups on the closed arm did not reach significance [respectively, $F(1,108) = 0.699$; $p=0.405$ and $F(3,108)= 0.879$; $p=0.455$)]. The experimental group × age group interaction was statically significant ($F(3,108) = 3.885$; $p=0.011$). According to this finding, experimental groups in each age group were compared using the t-test, and statistically significant differences were found between the stress and control groups in the PD60 and PD90 age groups [($t_{(28)} = -2.092$; $p=0.04$); ($t_{(17)} = 2.669$; $p=0.01$), respectively] (Figure 3.14).

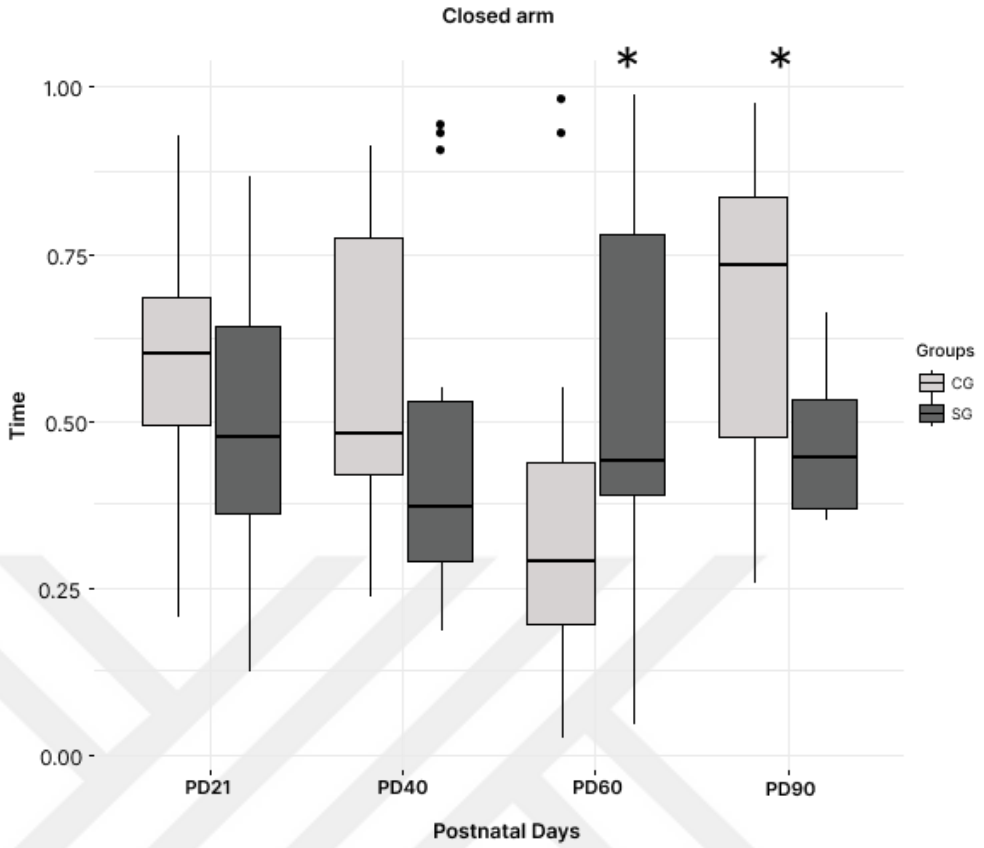

Figure 3.14. Time spent in the closed arms of the offspring in CG and SG
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. Asterisk (*) represents the significant difference ($p<0.05$). CG: Control group; SG: Stress group.

The number of entries (transitions) of the dams in stress and control groups to each region was analysed using an independent sample t-test. There was no significant difference between the groups in the transition from the closed arm to the open arm and from the open arm into the closed arm for the dams (both $p >0.05$). The number of entries of offspring into each zone (crossings) was analysed using ANOVA (type III) between-subject factor group (Condition: SG/CG; Sex: F/M) and the within-subjects factor condition (Age: PD21, PD40, PD60, PD90). The main effect of the experimental group and sex on the number of entries into the open arms from the closed arms failed to reach significance [($F(1,102)=0.010$; $p=0.921$); ($F(1,102)=0.160$; $p=0.690$), respectively], while the main effect of the age was statistically significant ($F(3,102)=6=3.839$; $p=0.012$). Accordingly, multiple pairwise comparisons were performed using a t-test among age groups, indicating the number

of transitions from the closed arm to the open arm for the offspring in the PD21 group was significantly lower than the offspring in PD40 and PD90 groups (p=0.02 and p=0.003) (Figure 3.15). Three-way interaction for the transition from closed arms to open arms was also found to be significant (F(3,102)=2.735; p=0.047). Therefore, the simple main effect tests were applied to each pair grouped by sex and age. However, no pair showed a significant effect of the stress condition (all p's>0.05).

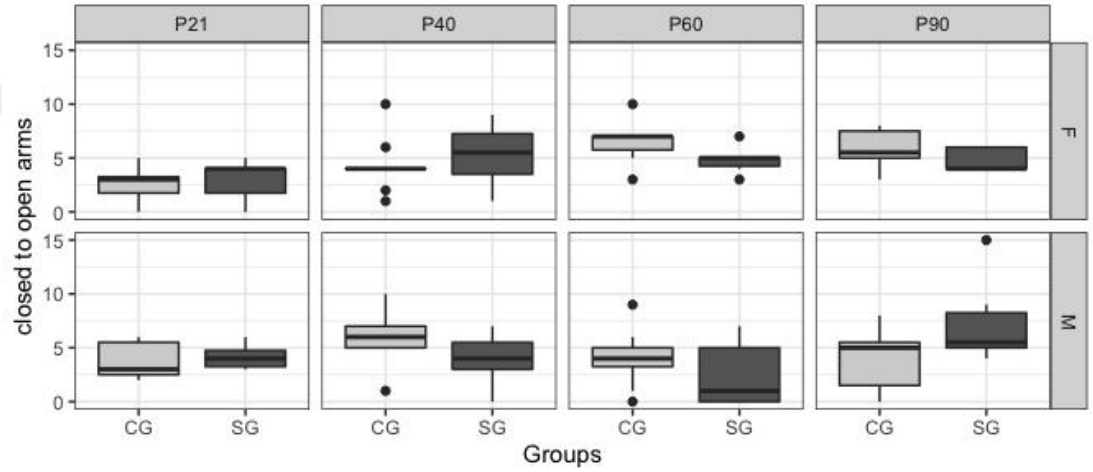

Figure 3.15. The frequency of transitions from closed arms into open arms
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. CG: Control group; SG: Stress group; F: Female; M: Male.

In the analyses of the frequency of the transition from the open arm to the closed arm, the main effect of the experimental groups and sex failed to reach the significance [(F(1,102)=0.119; p=0.731); (F(1,102)=0.216; p=0.643)]. However, the main effect of the age group was found to be statistically significant (F(3,86)=6.803; p<0.001). Accordingly, multiple pairwise comparisons were performed using a t-test among ages groups, indicating the number of transitions from the open arm to the closed arm for the offspring in the PD21 group was significantly lower than the offspring in PD40 and PD90 groups (p=0.02 and p=0.002) (Figure 3.16). Three-way interaction for the transition from open arms to closed arms was also found significant (F(3,102)=2.750; p=0.047). Therefore, the simple main effect tests were

applied to each pair grouped by sex and age. However, no pair showed a significant effect of the stress condition (all p's>0.05).

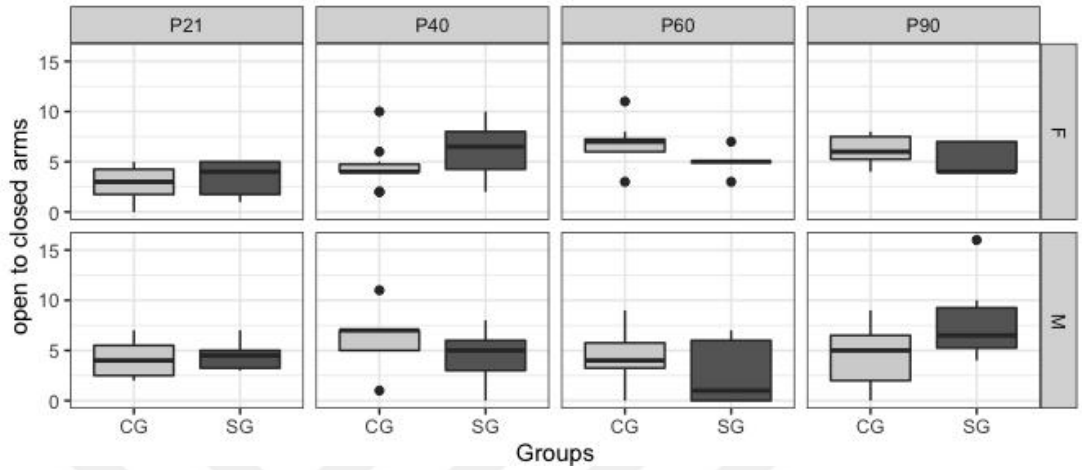

Figure 3.16. The frequency of transitions from open arms into closed arms
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. CG: Control group; SG: Stress group; F: Female; M: Male.

In order to assess the locomotive activity of the animals, average of moment-to-moment walking speed was analysed using ANOVA (type III) with the same factors and conditions. The main effects of the experimental group and sex failed to reach significance [$F(1,102)= 0.753$; $p=0.388$; $F(1,102)=1.833$; $p=0.179$; respectively]. The main effect of the age was found to be statistically significant $F(3,102)= 10.785$; $p=0.03$) , indicating that the moment-to-moment walking speed in PD21 age group was significantly lower than the PD40, PD60, and PD90 age groups (all p's<0.001) according to multiple pairwise comparisons. Moreover, the moment-to-moment walking speed in PD40 and PD60 did not differ from each other ($p=0.74$), while both were significantly lower than in the PD90 age group ($p=0.03$ and $p=0.01$, respectively) (Figure 3.17).

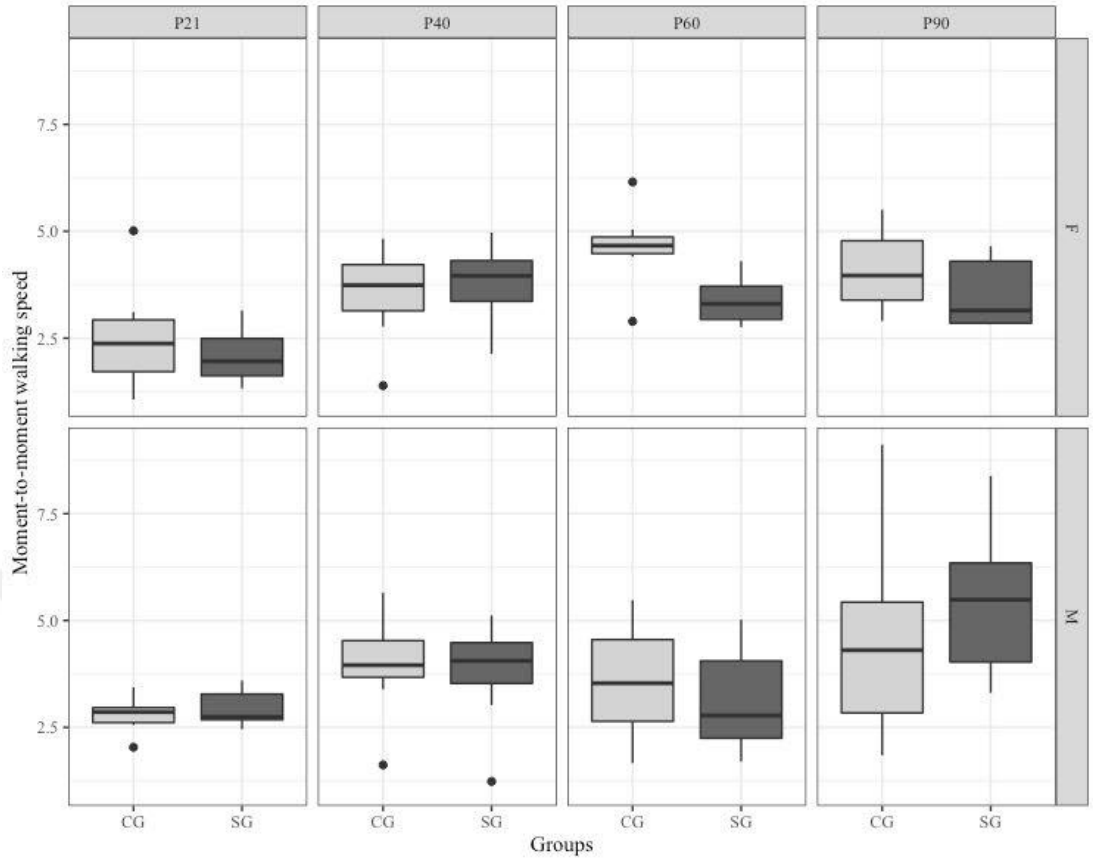

Figure 3.17. The movement-to-movement walking speed of the offspring
The first quartile (25th percentile) and the third quartile (75th percentile) are represented by the bottom and top boxes, respectively. The lower and upper bounds for data that exclude outliers are represented by whiskers. CG: Control group; SG: Stress group; F: Female; M: Male.

In order to determine whether there were any general variations in turning behaviour frequency, it was investigated whether there was a difference in the total number of turns between age and experimental groups. To fulfil this objective, a 2 x 4 univariate ANOVA with the between-subjects variables of the experimental group (SG and CG) and age group (PD21, PD40, PD60, PD90) was used to examine the overall number of turns for each animal. The main effects of the experimental group, age group, and sex failed to reach significance [respectively, $F(1,101)= 0.008$; $p=0.928$; $F(3,101)= 0.619$; $p=0.604$; $F(1,101)=0.243$; $p=0.623$, as did the experimental group × age group interaction ($F(3,101= 0.437$; $p=0.727$). Lastly, the experimental group × age group × sex interaction in turning frequency (both of the sides) was also not statistically significant ($F(3,101) = 1.089$; $p=0.357$). Therefore, no significant differences existed in the overall number of turns in any conditions.

3.1.4. Collins Paw Preference Test

3.1.4.1. Population-level and Individual-level Asymmetries

The Collins test was applied to 131 animals in total and of which 84 [PD21 (n=24); PD40 (n=22); PD60 (n=20); PD90 (n=16)], used their paws to reach the food reward through the feeding tube. According to the results of this test, 35.7% of the rats were right-pawed, 23.8% were left-pawed, and 40.4% were ambilateral in the control group. In the stress group, 21% of the rats were right-pawed, 47.3% were left-pawed, and 31.5% were ambilateral. The distribution of paw preferences in the Collins test based on prenatal stress condition, age groups, and sex is presented in Table 3.1.

Table 3.1. Individual paw preferences in rats in Collins Paw Preference Test according to Binomial Z-score.
Groups: (SG: Stress group; CG: Control group); Sex: (F: female; M: male); Paw preference (R: right paw; L: left paw; A: ambilateral).

PD21 (n=24)											
F ♀ (n=11)						M ♂ (n=13)					
SG (n=5)			CG (n=6)			SG (n=6)			CG (n=7)		
L	R	A	L	R	A	L	R	A	L	R	A
1	2	2	1	0	5	2	1	3	0	2	5
PD40 (n=22)											
F ♀ (n=12)						M ♂ (n=10)					
SG (n=6)			CG (n=6)			SG (n=5)			CG (n=5)		
L	R	A	L	R	A	L	R	A	L	R	A
2	2	2	2	2	2	3	1	1	3	1	1
PD60 (n=20)											
F ♀ (n=12)						M ♂ (n=8)					
SG (n=6)			CG (n=6)			SG (n=4)			CG (n=4)		
L	R	A	L	R	A	L	R	A	L	R	A
3	1	2	0	5	1	3	1	0	1	1	2
PD90 (n=16)											
F ♀ (n=10)						M ♂ (n=4)					
SG (n=4)			CG (n=6)			SG (n=2)			CG (n=2)		
L	R	A	L	R	A	L	R	A	L	R	A
2	0	2	2	4	0	2	0	0	1	0	1

The mean LI in the control group was 0.084 ± 0.09. The results of a one-sample t-test showed that the LI was not significantly different from zero ($t_{(41)}$ = 0.901; p=0.373), indicating that the control group did not show population-level asymmetry towards one side in Collins Test. This was also supported by Chi-Square analysis of the proportions of ambilateral, right-preferent, and left-preferent animals, since there was no deviation from equal distribution of them ($\chi 2$ = 1.85; p=0.395). Meanwhile, the mean LI in the stress group was 0.084 ± 0.09. In the stress group, an one-sample t-test revealed that LI was significantly different from zero ($t_{(37)}$ = -2.166; p=0.037), indicating that there was left paw preference at population-level asymmetry in the stress group in the Collins test.

To statistically test whether individual-level asymmetries existed in the Collins Test, the ABS-LI was computed. The mean ABS-LI in the control group was 0.493 ± 0.05, whereas it was 0.506 ± 0.05 in the stress group, which was significantly distinct from zero for CG and SG [$t_{(41)}$=9.074; p<0.001; $t_{(37)}$=8.595; p<0.001, respectively]. These findings demonstrated that rats showed individual-level asymmetry in both experimental groups in Collins Test.

3.1.4.2. Relation Between LI and the First Paw Use

The relationship between LI and first-paw use was analysed using the Chi-square test to evaluate whether the first-paw intervention was a good indicator of overall paw preference in rats. The findings of this study demonstrated that the first paw use significantly predicts the general paw preference direction (X^2 (2, N = 80) = 36,3, p <0.001).

3.1.4.3. Effects of Prenatal Stress, Age and Sex on Paw Preferences

LIs were evaluated using a Three-way ANOVA between-subject factor group (Condition: SG/CG; Sex: F/M) and the within-subjects factor condition (Age: PD21, PD40, PD60, PD90).

3.1.4.3.1. Effect of Prenatal Stress on LI

The results of the ANOVA revealed a significant main effect of prenatal stress on LI ($F(1,64)=4,4$ $p=0.039$), indicating that rats showed a more leftward paw preference in the stress condition ($p<0.05$). The mean LI for the control group was 0.023, whereas the mean LI for the stress group was -0.277 in this test (Figure 3.18).

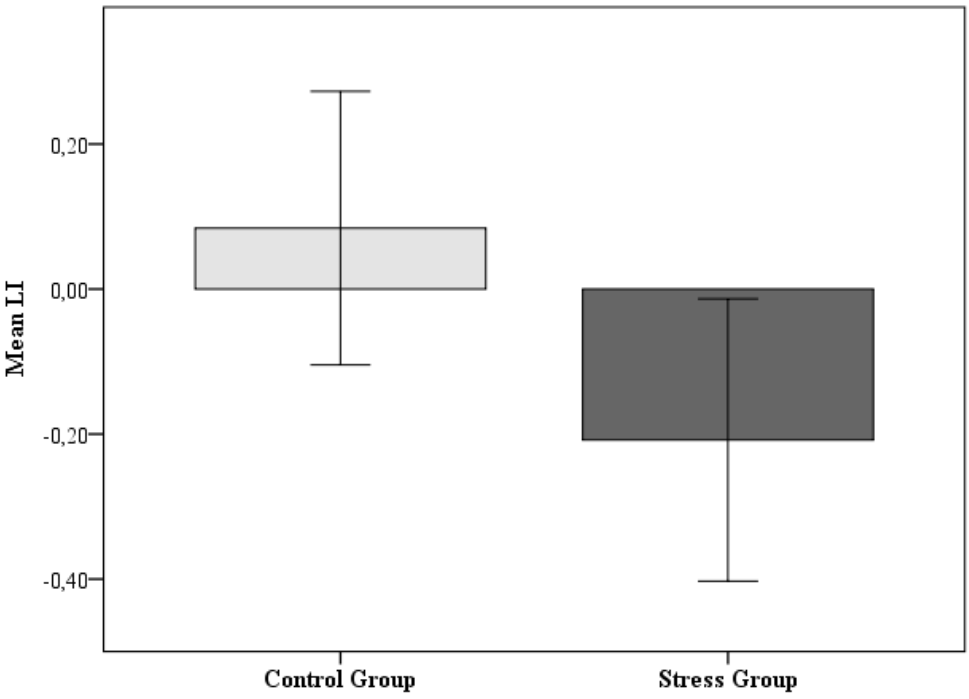

Figure 3.18. Representation of mean LI values for stress and control groups.
Compared to the control group, animals exposed to prenatal stress showed a more leftward bias for paw preference in the Collins test. Error bars represent 95% confidence intervals.

3.1.4.3.2. Age Effect on LI

The effect of age on LI was examined, as offspring were tested at different stages of postnatal development. The results of ANOVA demonstrated that the age effect on LI failed to reach significance ($F(3,64) = 1.312$; $p=0.278$). The mean LI was 0.39 for the PD21, -0.14 for the PD40, -0.36 for the PD60, and -0.37 for the PD90 groups in the Collins test (Figure 3.19).

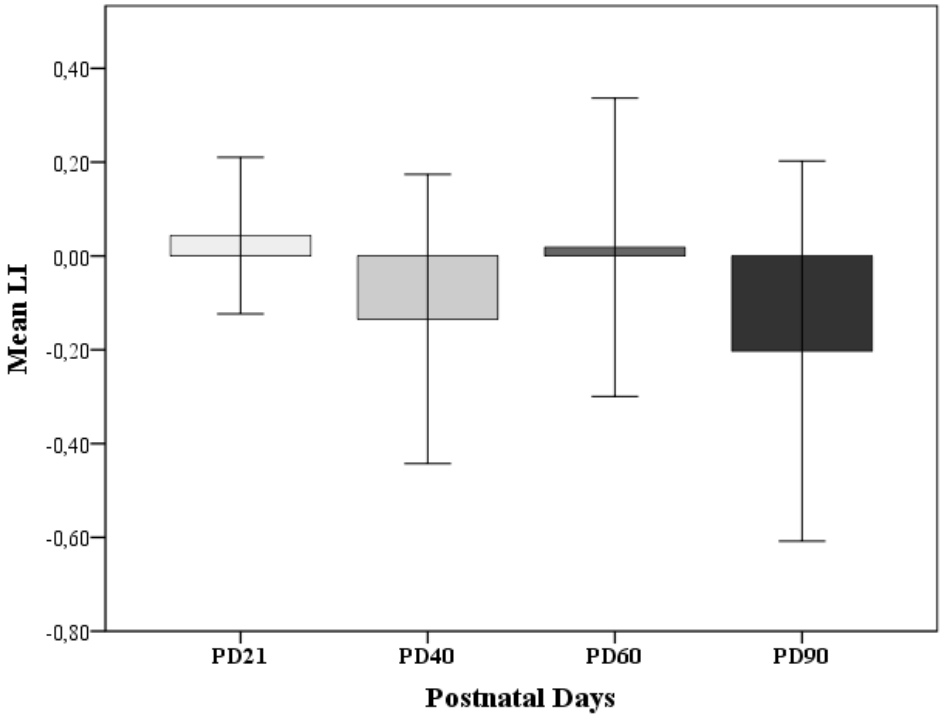

Figure 3.19. Representation of mean LI values for different postnatal developmental stages in the Collins test.

3.1.4.3.3. Sex Effect on LI

The effect of sex on LI was examined. The results of ANOVA reached significance for the sex effect on LI ($F(1,64) = 5.399$; $p=0.023$), indicating that males showed more leftward bias than the females ($p<0.05$). The mean LI for the female individuals was 0.038, whereas the mean LI for male individuals was -0.292 in the Collins test (Figure 3.20).

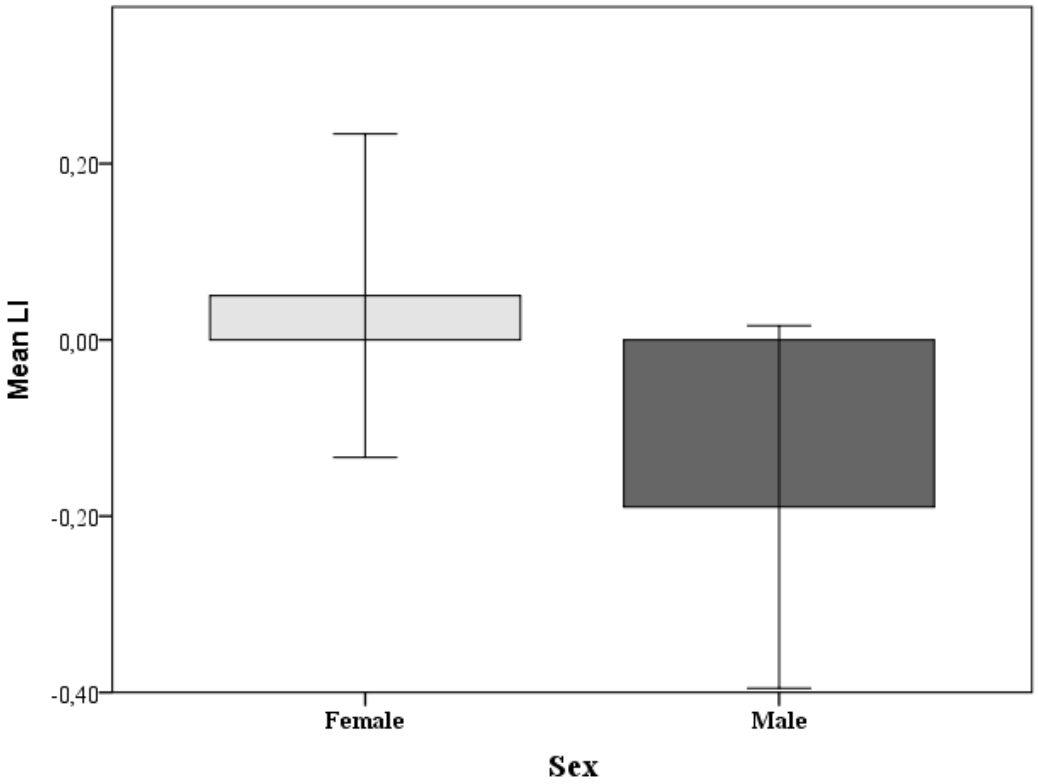

Figure 3.20. Representation of mean LI values for females and males in the Collins test. Error bars represent 95% confidence intervals.

3.1.4.3.4. Effect of Prenatal Stress on ABS-LI

Similar to analyses conducted for LI value, a Three-way ANOVA between-subject factor group (condition: SG/CG; Sex: F/M) and the within-subjects factor condition (Age: PD21, PD40, PD60, PD90) was also performed for ABS- LI to evaluate the strength of the laterality in rats.

The results showed that the effect of prenatal stress on ABS-LI failed to reach significance ($F(1,64)=0.399$ $p=0.530$). The mean ABS-LI for the control group was 0.504, whereas the mean LI for the stress group was 0.056 in this test (Figure 3.21).

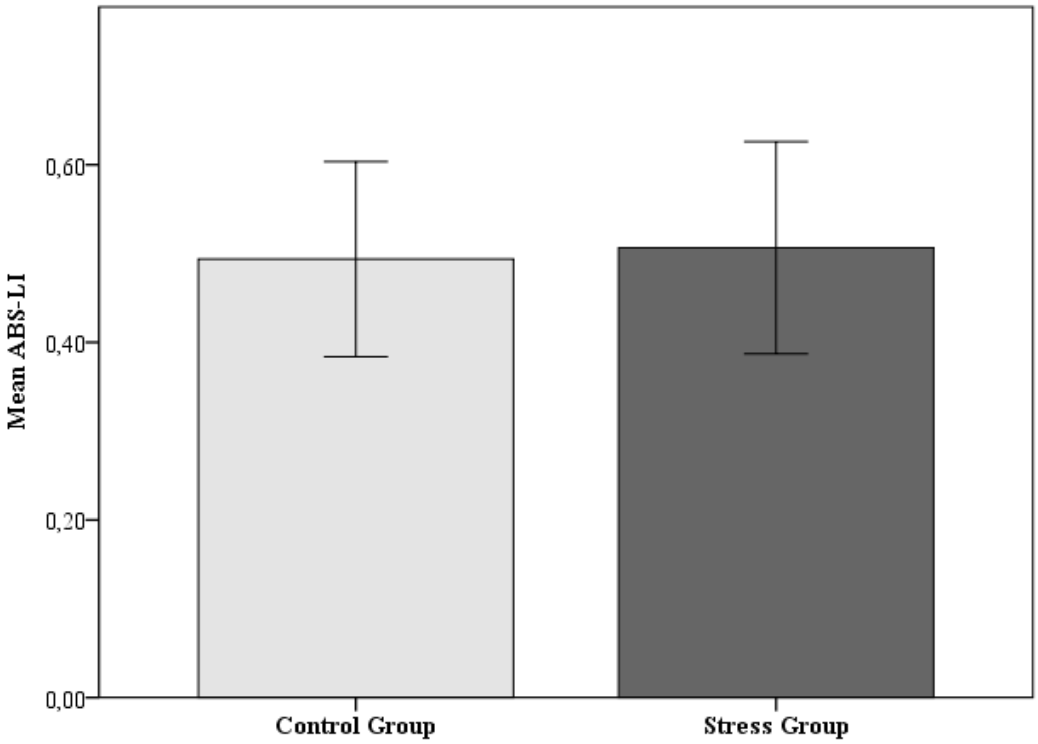

Figure 3.21. Representation of mean ABS-LI values stress and control group in the Collins test. Error bars represent 95% confidence intervals. The findings of this study indicated that there was no significant effect of prenatal stress on ABS-LI.

3.1.4.3.5. Age Effect on ABS-LI

The results of the ANOVA revealed a significant effect of age on ABS-LI ($F(3,73) = 5.221$; $p=0.003$), indicating that offspring at PD21 had less ABS-LI compared to other age groups. The mean ABS- LI was 0.28 for the PD21, 0.6 for the PD40, 0.58 for the PD60, and 0.62 for the PD90 groups in the Collins test.

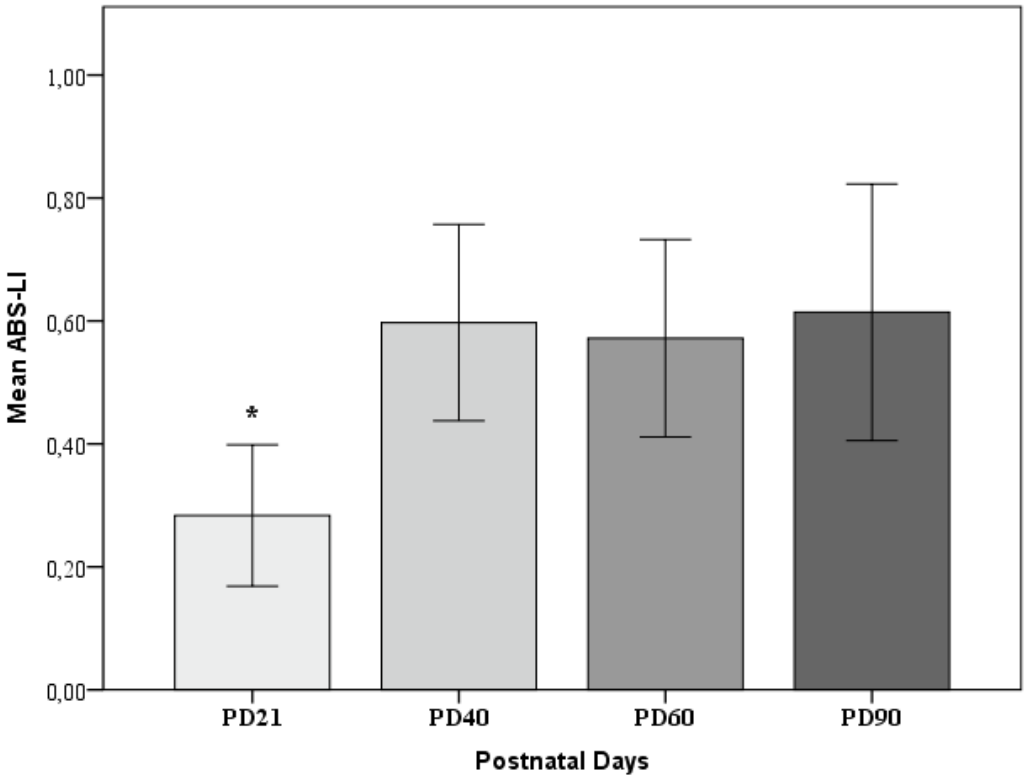

Figure 3.22. Representation of mean ABS-LI values for different postnatal developmental stages in the Collins test.
The asterisk (*) indicates statistical significance ($p<0.05$). PD21 group showed significantly lower ABS-LI value compared to other age groups. Error bars represent 95% confidence intervals.

3.1.4.3.6. Sex Effect on ABS-LI

The results of ANOVA failed to reach significance for the sex effect on ABS-LI ($F(1,64) = 1.764$; $p=0.189$). The mean ABS-LI for the females was 0.477, while it was 0.580 for males (Figure 3.23).

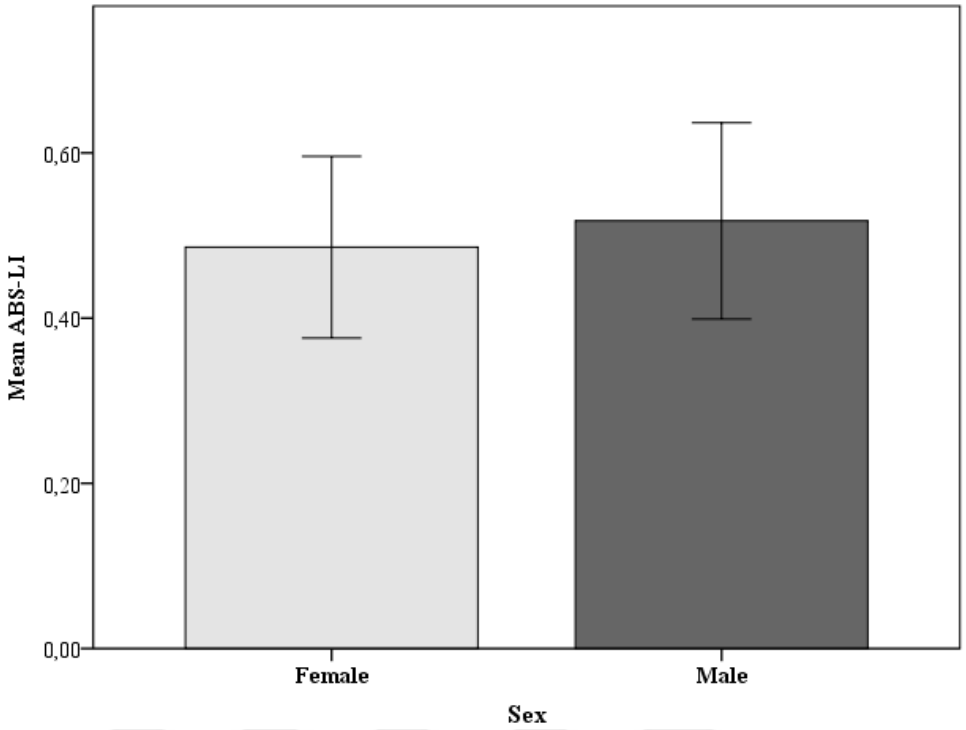

Figure 3.23. Representation of mean ABS-LI values for females and males in the Collins test. Error bars represent 95% confidence intervals.

3.1.4.3.7. Prenatal Stress and Sex Interaction for ABS-LI

A significant interaction was found between prenatal stress x sex ($F(1,64) = 5.707$; $p=0.020$). There was no significant difference between females and males in the control group ($p>0.05$). However, there was a significant difference between females and males in the stress group ($p=0.032$). It was found that the ABS-LI value in males was higher than in females in the stress group ($p=0.032$) (Figure 3.24).

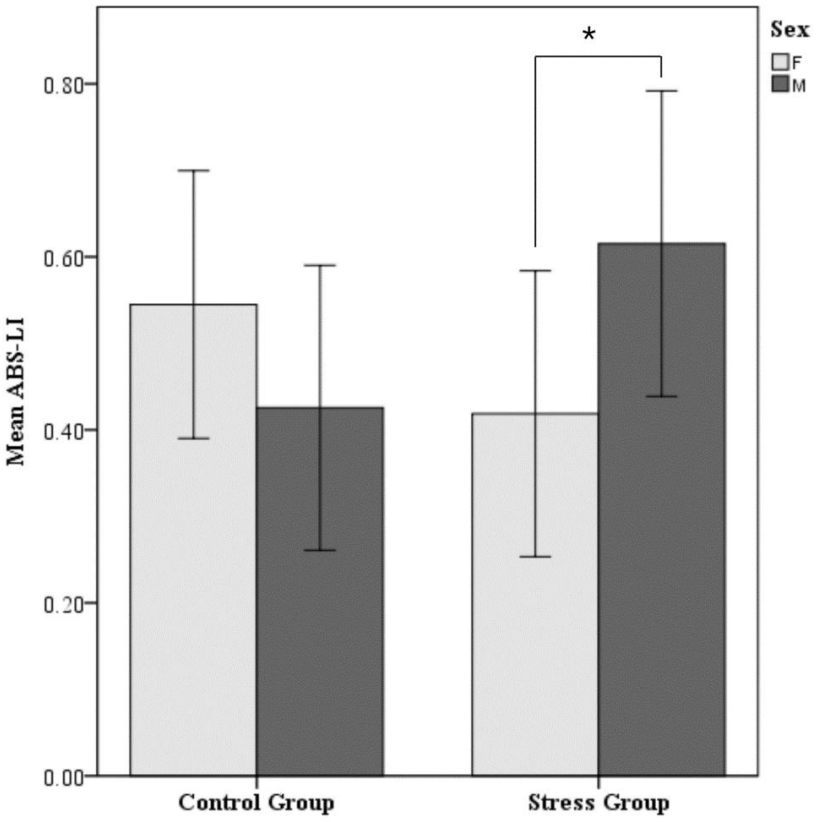

Figure 3.24. Comparison of the mean ABS-LI values of male and female individuals in the control and stress groups.
Asterisk (*) represent the significant difference ($p<0.05$). In the stress group, males had a higher mean ABS-LI than females. Error bars represent 95% confidence intervals. F: Female; M: Male.

A pairwise comparison between control and stress groups was conducted within each sex group using the Bonferroni correction procedure (Holm, 1979). The result of this comparison showed that there was almost a significant difference between stress and control groups in females PD60 age ($F(1,37)=3,449$, $p=0.071$), while there was no difference in males ($F(1,27)=1,468$, $p=0.236$). As the difference between the control and stress groups among females was near to being statistically significant, age groups were also analysed separately. Independent sample t-tests showed a significant difference between control and stress females in PD60 ($p=0.14$); while there was no other difference in other age groups between stress and control (all's $p>0.05$) (Figure 3.25).

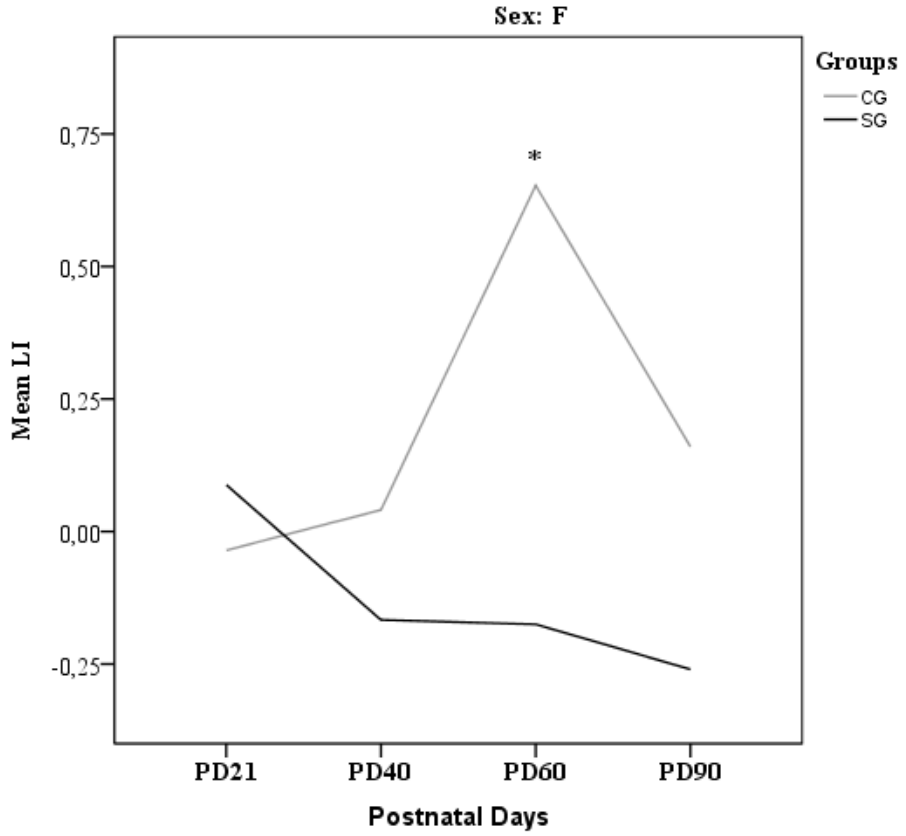

Figure 3.25. Representation of the relationship between stress and age in females according to the mean LI value.
The asterisk (*) indicates the significance

3.1.5. Pasta Matrix Reaching Test

3.1.5.1. Population-level and Individual-level Asymmetries

Thirty-one out of 34 animals in the PD60 group used their paws to reach the pasta pieces in the Pasta Matrix Reaching test. According to the results of this test, 56.25% of the rats were right-pawed, 37.5% were left-pawed, and 6.25% were ambilateral in the control group. In the stress group, 40% of the rats were right-pawed, 46.66% were left-pawed, and 13.33% were ambilateral. The distribution of paw preferences in the Pasta test based on prenatal stress condition, age groups, and sex is presented in Table 3.2.

Table 3.2. Individual paw preferences of the rats in PD60 in Pasta Matrix Reaching Test according to Binomial Z-score.
Groups: (SG: Stress group; CG: Control group); Sex: (F: female; M: male); Paw preference (R: right paw; L: left paw; A: ambilateral).

PD60 (n=31)											
F ♀ (n=14)						M ♂ (n=17)					
SG (n=7)			CG (n=7)			SG (n=8)			CG (n=9)		
L	R	A	L	R	A	L	R	A	L	R	A
3	3	1	2	5	0	4	3	1	4	4	1

The mean LI in the control group was 0.177 ± 0.16, while it was -0.075 ± 0.157 in the stress group. The results of one-sample t-tests showed that the LI was not significantly different from zero in CG and SG [t(15) = 1.103; p=0.287; t(14) = -0.479; p=0.639, respectively], indicating that the both of the experimental groups did not show population-level asymmetry towards one side in Pasta Test.

To statistically test whether individual-level asymmetries existed in the Pasta Test, the ABS-LI was computed. The mean ABS-LI in the control group was 0.597 ± 0.25, whereas it was 0.529 ± 0.28 in the stress group, which were significantly distinct from zero for CG and SG [t(15) = 9.235; p<0.001; t(14) = 7.247; p<0.001, respectively]. These findings demonstrated that rats showed individual-level asymmetry in both experimental groups in Pasta Test.

3.1.5.2. Relation Between LI and the First Paw Use

To evaluate whether the first paw is a good indicator of general paw preference, its relationship with the general paw preference direction was also tested in the Pasta Test. In line with the Collis results, the first paw significantly predicts the overall paw preference of the rats (X^2 (2, N = 31) = 14.9, p <0.001).

Stability of individual paw preferences:

Correlations coefficients between LIs of the PD60 age group, obtained from Collins and Pasta tests, were also examined to test whether individual rats showed stable side preferences. There were no significant correlations between LI obtained in the Collins test with that obtained in the Pasta test overall in the control group ($r=0.333$, $p=0.151$). In a similar manner, there was also no correlation between those LIs in the stress group ($r=0.404$, $p=0.078$). These findings suggested that individual side preferences in rats were not stable and task dependent.

3.1.5.3. Effect of Prenatal Stress and Sex on Paw Preference

LIs of the PD60 age group were evaluated using a Two-way ANOVA between-subject factor group (Condition: SG/CG; Sex: F/M). The results of the ANOVA failed to reach significant main effects of prenatal stress and sex on LI [$F(1,27)=1.603$ $p=0.216$ and $F(1,27)=0.808$ $p=0.377$, respectively]. The mean LI for the control group was 0.207, whereas the mean LI for the stress group was -0.078 in the Pasta Test. In addition, the mean LI for the female individuals was 0.166, whereas the mean LI for male individuals was -0.037 in this test. No significant interaction was found between prenatal stress x sex ($F(1,27) =1.488$; $p=0.233$).

Similar to analyses conducted for LI value, a Two-way ANOVA with the same subject factor groups was performed for ABS-LI. The results showed that the main effect of prenatal stress on ABS-LI failed to reach significance ($F(1,27)=0.705$ $p=0.409$). The mean ABS-LI for the control group was 0.604, whereas it was 0.522 for the stress group in this test. The results showed that the main effect of sex on ABS-LI also failed to reach significance ($F(1,27)=0.279$ $p=0.602$). The mean ABS-LI for the female individuals was 0.537, whereas it was 0.589 for the male individuals in this test. No significant interaction was found between prenatal stress x sex ($F(1,27) =2.336$; $p=0.138$).

3.1.6. Head and Body Turning Asymmetry Test

The accuracy of the created training data was evaluated (Figure 3.26), and they were found insufficient for reliable analysis due to the technical limitations in video quality, such as recording under dim red light and low resolution After the video analysis approach failed in DeepLabCut due to insufficient prediction likelihood and tracking consistency, the cost of further computing resources was not justified and manual analysis was imperative.

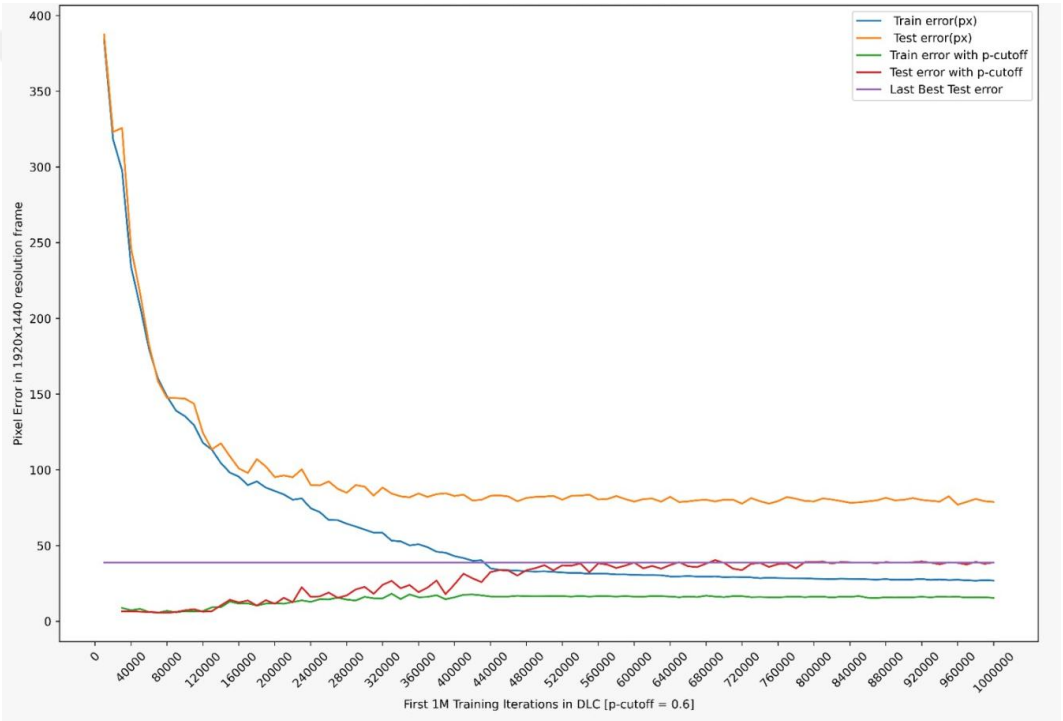

Figure 3.26. Evaluation of DLC Rat Model.
The train error rate (pixels) with p-cut-off evaluation results with 1.000.000 iterations in rats.

After some of the videos had to be excluded due to technical reasons, the remaining 137 videos for the first explorative approach and 123 videos for the first drinking behaviour per animal were scored by two independent raters. To assess inter-rater reliability in analyses for these behavioural parameters, Cronbach's alpha coefficient was calculated. The body position of the first explorative approach

showed a good internal consistency (Cronbach's α = 0.82) among raters. In contrast, the reliability of the body turning for this behaviour was not inadequate (Cronbach's α = 0.59). According to Koo et al. 2016, Cronbach's alpha coefficient did not show good internal consistency for the turning behaviour of the first explorative approach, therefore, this behavioural parameter was compulsorily excluded. For the first drinking behaviour, the body position showed a good internal consistency (Cronbach's α = 0.836). The body turn also showed non-statistically significant trend for good reliability (Cronbach's α = 0.708) (Table 3.3). Hence, further analyses of drinking behaviour were continued to perform. The relationship between body turning direction for the drinking behaviour and stress condition, age, and sex was analysed using chi-square. The relationship between body turning direction for the drinking behaviour and stress condition, age, and sex was analysed using Chi-square. None of these associations reached significance (all p's >0.05).

Table 3.3. Distribution of body-turning behaviours in rats for the first exploratory approach and the first drinking behaviour scored by two independent raters.
L indicates the leftward turning; R indicates the rightward turning; N indicates there is no turning behaviour, a neutral position.

Independent Raters	***First Explorative Approach***			***First Drinking Behaviour***		
	L	*R*	*N*	*L*	*R*	*N*
Rater 1	53	42	42	52	49	22
Rater 2	67	45	26	55	46	23
	Cronbach's α = 0.59			**Cronbach's α = 0.83**		

3.2. Molecular Analyses

3.2.1. RNA Quality and Quantity Control

Concentrations of RNA obtained from the motor cortex ranged between 325 and 600 ng/µl. The total RNA amount was in the range of 13 - 24 µg for each sample. Nanodrop measurements of RNAs obtained from the motor cortex of each

hemisphere of some animals are representatively presented in Table 3.4**.** These values indicated that the RNA isolation procedure was properly conducted and that highly pure RNAs were obtained.

Table 3.4. Spectrophotometric measurements of RNA samples of the motor cortices of both hemispheres for some representative animals in both groups.

#	**Name**	**Conc.** ng/µl	**A230 (10 mm)**	**A260 (10 mm)**	**A280 (10 mm)**
1	Blank 1	0,0000	0,000	0,000	0,000
2	4L_G3_M6F3	456,44	5,132	11,42	5,446
3	4R_G3_M6F3	399,80	4,564	9,999	4,762
4	5L_G4_M8F3	422,96	5,012	10,58	5,040
5	5R_G4_M8F3	403,96	4,584	10,11	4,800
6	6L_G5_M10F3	447,20	5,061	11,19	5,310
7	6R_G5_M10F3	441,76	4,968	11,05	5,254
8	7L_G6_M11F3	476,72	5,404	11,93	5,693
9	7R_G6_M11F3	496,28	5,848	12,42	5,900
10	11L_G7_M13F3	369,40	4,232	9,237	4,400
11	11R_G7_M13F3	325,52	3,720	8,143	3,872
12	12L_G8_M15F3	445,16	4,998	11,14	5,288
13	12R_G8_M15F3	488,76	5,567	12,23	5,831

In addition to the spectrophotometric quality control, the integrity and purity of the RNA were also verified by gel electrophoresis. 18S and 28S rRNA bands were clearly visible in each sample, while no gDNA bands were detected (Figure 3.27).

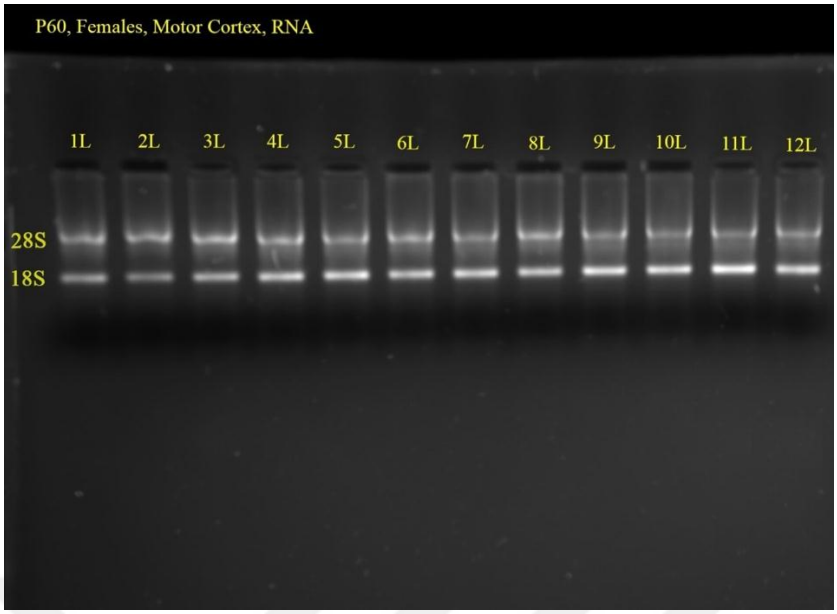

Figure 3.27. The RNA gel electrophoresis.
Gel image showing two rRNA bands (28S and 18S) of the left hemispheres of the females at PD60 in both groups used in microarray gene expression analysis.

Considering the importance of using intact RNA that was previously emphasized, total RNA was also examined utilizing capillary electrophoresis, the commercially available Agilent 2100 Bioanalyzer system in the present study. RIN values higher than 8 were observed for 10 of the representative samples, indicating high integrity of the RNA (Figure 3.28). Only for sample 9, the Agilent Expert Software failed to assign a RIN value, possibly due to a technical error in the capillary system. In the gel-like image generated by the software, 28S and 18S bands were observed, while no DNA contamination was detected, as in other samples.

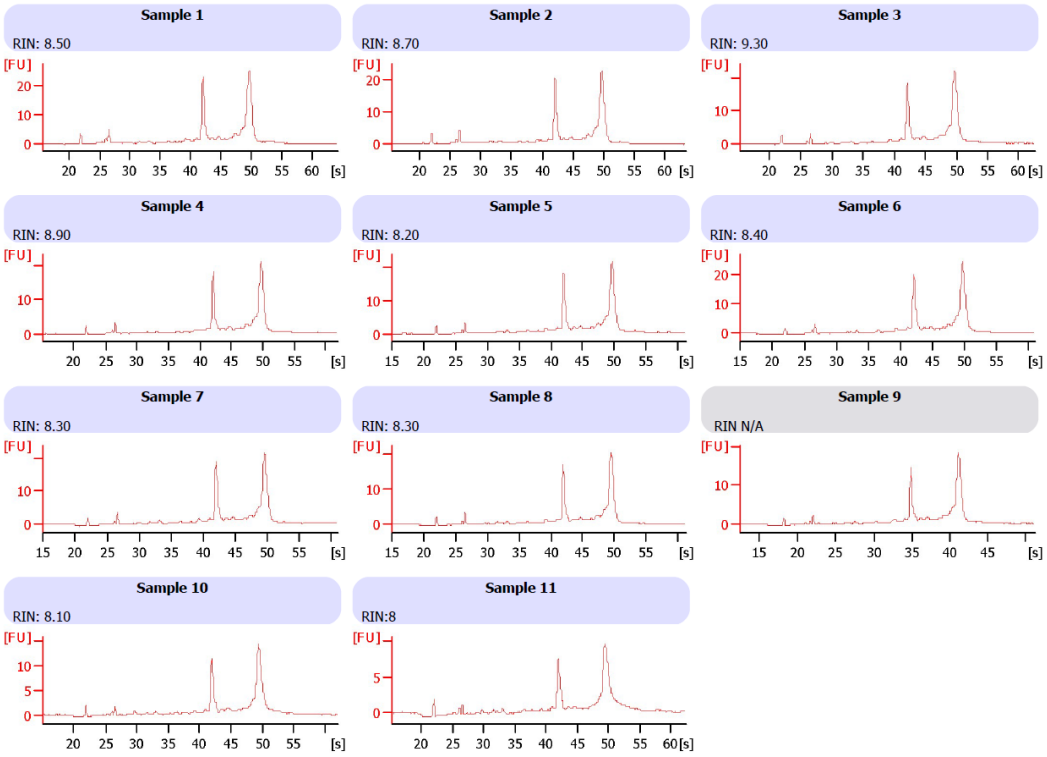

Figure 3.28. RIN values of the samples assigned by The Agilent Expert Software.
All electropherograms showed the characteristic 18S fragment peak at 41 s and 28S fragment peak at 48 s migration time. The marker peak is visible at 22 s migration time. All electropherograms also revealed a modest peak in the 5S region at a migration time of 26 s. Except for sample 9, a RIN value was assigned for all samples. The RIN value was higher than 8 for all samples, with sample 11 having the lowest (8.0) and sample 3 the highest (9.3), indicating largely intact RNA.

The gel-like image generated by Agilent Expert Software for the total RNAs extracted from the motor cortex of the brain in rats are also depicted in Figure 3.29. All gel images displayed two distinct bands with migration times of approximately 50 s and 42 s and sizes of approximately 1900 and 900 nucleotides (nt), respectively. The larger band represents the 28S rRNA, while the thinner band represents the 18S rRNA. Bioanalyzer results coincided with the results of the spectrophotometric and gel electrophoresis evaluations. These results confirmed that RNA samples were of high quality.

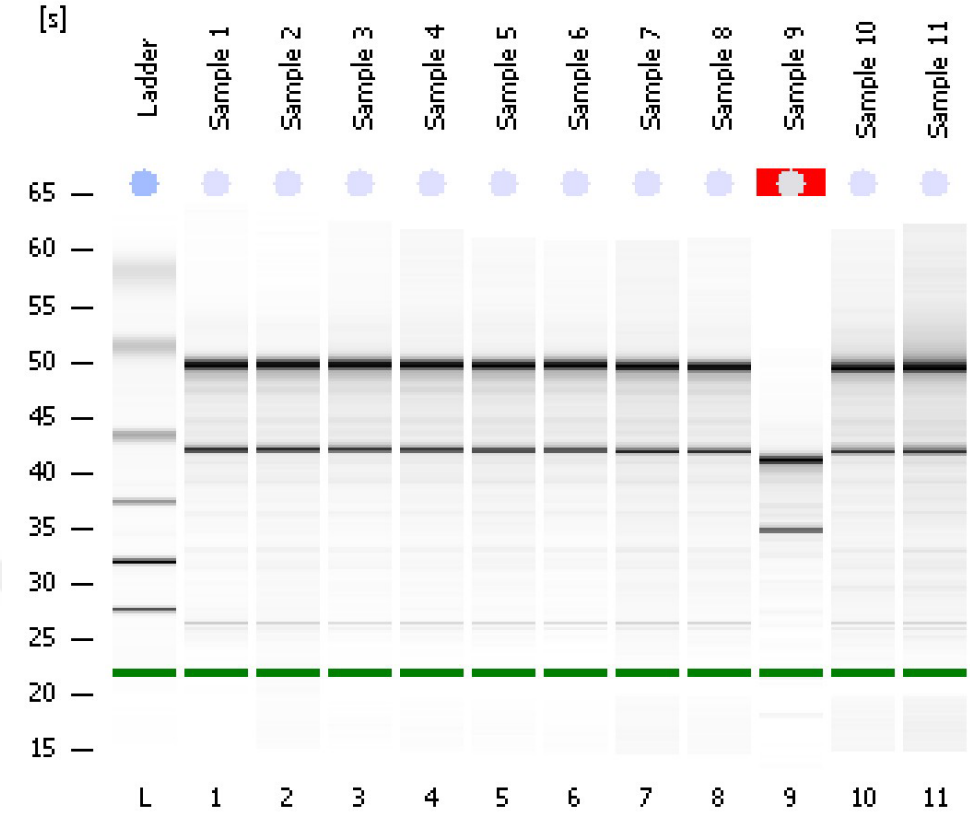

Figure 3.29. The gel-like image generated by Bioanalyzer (Agilent) for the total RNAs extracted from the motor cortex of the brain in rats.
A ladder (on the left side) with fragment sizes 25, 200, 500, 1000, 2000, and 4000 nt was used. Each sample displayed two distinct visible rRNA bands. The location of the bands for sample 9 was shifted due to the technical error. There were two distinct bands with the same distance between them as in the other samples, presumably the 28S and 18S fragments.

3.2.2. Microarray-based Gene Expression Analysis

To investigate the prenatal stress induced molecular alterations in the right and left hemispheres of the brain in rats, each pooled RNA sample obtained from females at PD60 was analysed by the Agilent Rat Gene Expression (4x 44K) Microarray consisting of 26.930 oligonucleotide probes. To evaluate the microarray performance, Metric Results of the QC Report generated by the Agilent Feature Extraction Software (v10.7) was used. In order to produce the QC report, the software processed the digital image generated by the scanner from the excited microarray (Figure 3.30) and transformed the image of each spot to a numerical reading. These reports showed that all metrics in the table were reported as "Good",

indicating that the data is in the expected range. In addition, the grid was found normal in all reports, and the net signal statistics which are an indication of the dynamic range of the signal for both non-control and spike-in probes, were also found good. These findings proved that the performance of the microarray was sufficient and efficient for the further analyses.

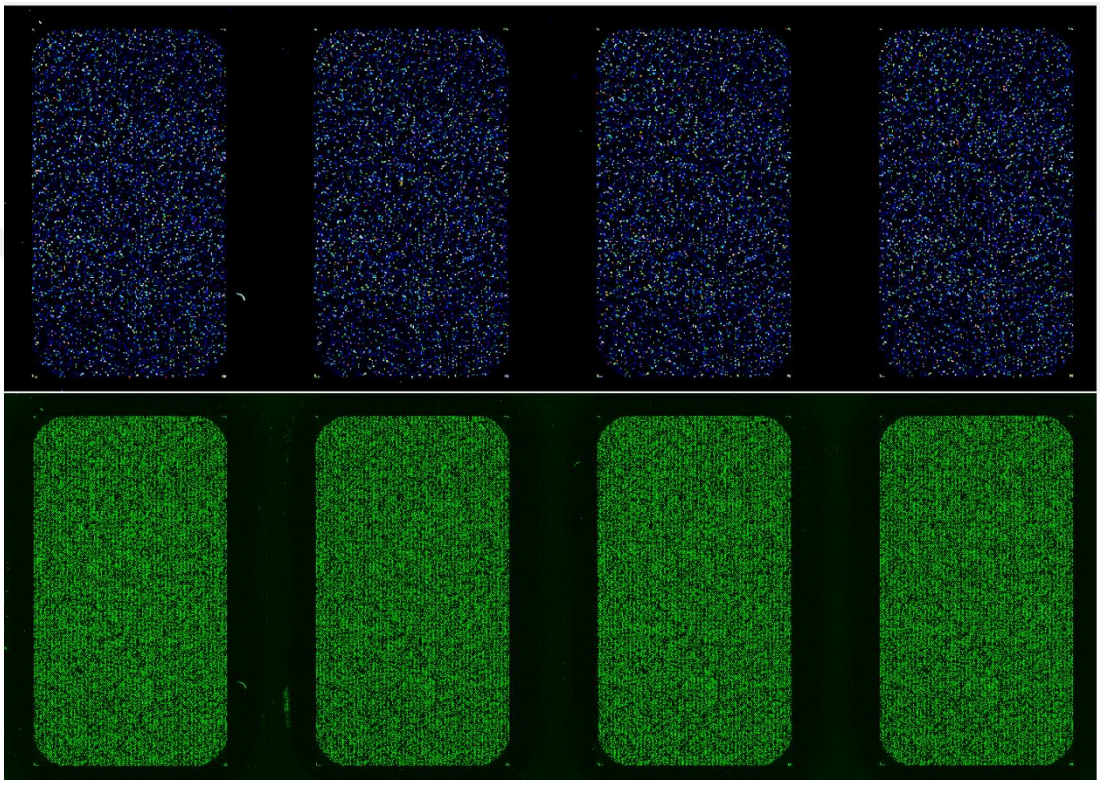

Figure 3.30. Typical microarray images generated by the Agilent Feature Extraction Software (v10.7).
The multicolor image at the top corresponds to the software-generated version, while the green version at the bottom corresponds to the program's output as a tiff file.

To assess differential expressions between Control-left and Control-right hemispheres, Stress-left and Control-left hemispheres, Stress-right and Control-right hemispheres, two datasets with and without the NormExp Background Correction were generated for each comparison. Background correction (BC) was followed by quantile normalization. For all comparisons, significant p value was adjusted to <0.05 with FDR control. A detailed explanation of this analysis was previously provided in the Section "Materials and methods: Microarray-based gene expression analysis". According to the results of the Control-left and Control-right hemispheres

comparisons, 1 mRNA was expressed significantly different in the analysis with BC, whereas 5 mRNAs were expressed significantly different in the analysis without BC. Between Stress-left and Control-left hemispheres, 5 mRNAs were found to be expressed significantly different when BC was applied to the analysis, while 24 mRNAs differently expressed when BC was not applied. Stress-right and Control-right hemispheres comparison showed that 70 mRNAs expressed significantly different when the BC was included the analysis whereas 280 mRNAs expressed significantly different when background correction was not included. Finally, the background corrected data was used in this study. Overall, the results of the stress and control group comparisons for the gene expression analysis demonstrated that there was a higher number of differentially expressed genes in the right hemisphere than in the left hemisphere. In between stress and control groups for right hemisphere, differentially expressed genes which has >1.7-fold changes, are depicted in Table 3.5.

Table 3.5. List of genes differentially expressed between right hemispheres of stress and control groups at >1.7-fold up/down regulation and adjusted-P values of <0.05.

ProbeName.x	Entrez ID.x	Symbol	logFC	AveExpr	t	P.Value	adj.P.Val	FC
A_64_P075928	192274	Foxe1	1,963585	11,87892	3,751653	0,005256	0,029539	3,9003
A_42_P489710	289757	Polm	1,590606	13,14602	4,911802	0,000862	0,010089	3,011758
A_64_P040994	266674	Cyp4a8	1,562033	11,4715	6,313643	0,000114	0,003449	2,952696
A_44_P196793	295297	Etv3	1,442546	9,570447	3,373247	0,009237	0,042115	2,718001
A_64_P061524	257652	Prb1	1,385601	10,43022	3,279511	0,010451	0,045538	2,612808
A_64_P051822	294245	Ly6g5c	1,344472	9,976562	4,784199	0,001021	0,011056	2,539373
A_64_P058266	286962	Cox6c-ps1	1,29227	11,72443	3,543204	0,006676	0,034362	2,449131
A_64_P048187	289560	Igfbp7	1,25156	9,30774	3,738137	0,00523	0,029447	2,380987
A_64_P111878	366979	LOC366979	1,207197	10,827	3,416257	0,008091	0,038599	2,308887
A_44_P121668	311387	Slc24a5	1,113902	10,55476	3,840733	0,003987	0,024756	2,164302
A_44_P296631	312437	Krcc1	1,023241	9,346526	4,781369	0,000969	0,010778	2,03248
A_64_P094855	690423	Jsrp1	1,018537	10,32091	4,554247	0,00119	0,012073	2,025864
A_64_P398958	310324	Dcun1d1	1,011141	12,68599	3,476563	0,006904	0,035139	2,015505
A_64_P143355	363024	Slc44a2	1,009281	10,24008	8,971403	6,77E-07	0,000452	2,012907
A_44_P314492	304109	Tiam1	1,001881	11,5345	6,96009	1,63E-05	0,001572	2,00261
A_64_P037938	308318	Zfp772	0,99525	10,92521	3,160904	0,011752	0,049138	1,993426
A_64_P317478	500882	RGD1564548	0,975053	11,66939	3,966017	0,002978	0,020792	1,965714
A_64_P012714	313231	Alg2	0,973856	10,41116	3,625665	0,005446	0,03018	1,964084
A_64_P157579	287559	Phb-ps1	0,971622	10,88187	3,788861	0,004068	0,025018	1,961044
A_44_P217383	300635	Olr1335	0,958709	11,5814	7,044388	1,25E-05	0,001445	1,94357
A_64_P127803	407756	Btla	0,956959	10,98916	3,241376	0,010169	0,044748	1,941214

Table 3.5. Continued. List of genes differentially expressed between right hemispheres of stress and control groups
at >1.7 fold up/down regulation and adjusted-P values of <0.05 (continued)

A_43_P12992	117018	Kcnh2	0,945614	10,0327	4,792083	0,000765	0,009397	1,926008
A_64_P049584	316384	Cavin2	0,930655	14,30381	3,210456	0,010447	0,045538	1,906141
A_64_P000271	294562	Pou5f1	0,928339	10,1685	3,460225	0,007127	0,03585	1,903084
A_64_P048126	499843	LOC499843	0,928279	11,02614	4,814675	0,000654	0,008669	1,903004
A_64_P017408	499219	LOC499219	0,927767	10,20649	4,798629	0,000723	0,009119	1,902329
A_64_P013908	84683	Cox4i2	0,917428	11,20101	3,201478	0,010749	0,046406	1,888744
A_44_P299598	24171	Add2	0,916855	12,71648	3,280148	0,009348	0,042382	1,887995
A_64_P156436	414784	RT1-T24-4	0,904282	9,961477	3,510603	0,006552	0,034065	1,871612
A_44_P133366	309900	Tulp1	0,903262	8,591062	3,851922	0,004557	0,026883	1,87029
A_44_P356962	309548	Shoc2	0,902098	10,2358	9,388652	2,86E-07	0,000275	1,868782
A_44_P452163	50599	Kcnj3	0,900388	13,07121	5,674158	0,000123	0,003567	1,866568
A_64_P363385	396552	Ugt1a9	0,892585	9,905161	3,247906	0,010119	0,044609	1,856499
A_64_P016549	84484	Slc4a4	0,876431	11,79113	5,402534	0,000202	0,004667	1,835828
A_64_P031906	500594	LOC500594	0,873693	11,35445	6,728289	1,76E-05	0,001612	1,832347
A_64_P149407	308393	Pnma8b	0,860426	11,7372	3,657991	0,00474	0,0276	1,815574
A_64_P156448	685157	LOC685157	0,856892	10,06077	3,170233	0,011376	0,048173	1,811132
A_44_P289132	300950	Spsb4	0,854989	13,00293	3,257776	0,009426	0,04258	1,808744
A_64_P030267	298065	Tex10	0,852512	9,183374	17,87418	8,34E-11	4,81E-07	1,805642
A_64_P059577	679898	Zmat2	0,843535	11,62461	4,181808	0,001825	0,015377	1,794442
A_64_P165726	498407	Purb	0,84042	12,92261	3,375178	0,007616	0,037268	1,790571
A_64_P075647	116685	Lmnb1	0,838118	10,38077	7,249615	7,05E-06	0,001221	1,787716
A_64_P102942	117183	Rgcc	0,836167	10,75162	5,72315	0,000109	0,003381	1,7853
A_64_P110018	303141	Rapgef6	0,835883	14,3822	3,944965	0,00265	0,01924	1,78495
A_64_P013900	295277	Hist2h4	0,834966	10,81179	4,206965	0,001766	0,0151	1,783815
A_64_P082540	113886	Kif1c	0,8272	10,25356	7,430465	5,18E-06	0,001046	1,774238
A_64_P058492	303163	Igtp	0,795019	15,40027	3,133122	0,011051	0,047266	1,7351
A_64_P101573	498695	Idnk	0,794295	13,27893	4,731798	0,00059	0,008185	1,734229
A_64_P036631	29236	Rpsa	0,788874	13,54332	5,627276	0,000103	0,003307	1,727726
A_64_P112008	64627	Hist1h4b	0,788098	10,53078	5,089741	0,000329	0,006026	1,726796
A_64_P120594	690680	Vom2r15	0,782518	12,07201	9,386272	2,69E-07	0,000267	1,720131
A_64_P030761	301020	Smarcc1	0,780629	10,22945	5,848265	8,21E-05	0,003013	1,717879
A_64_P122912	680498	Hist1h3b	0,774771	13,4819	3,524002	0,005546	0,03057	1,710918
A_64_P071353	685823	Xpnpep3	0,774344	10,39653	3,235084	0,00972	0,043408	1,710413
A_64_P014402	290648	Pgpep1	0,77219	12,3842	4,595381	0,000751	0,009342	1,707861
A_64_P142497	680844	Treml2	0,766304	11,09496	3,506555	0,005853	0,03163	1,700906
A_64_P047656	266733	Slc12a8	0,765851	10,99623	5,676901	9,81E-05	0,003248	1,700373

Subsequently, background corrected data obtained from microarray analysis was used for GSEA. As a result of stress and control groups right hemisphere comparison, no gene set with FDR q-value ≤0.05 was found (Table 3.6).

Table 3.6. Gene sets enriched in the right hemispheres of stress and control groups.
Enrichment score (ES); Normalized enrichment (NES); The nominal p-value (NOM p-val); False Discovery Rate (FDR q-value); Familywise-error rate (FWER); The peak ES score (Rank at max).

Gene Sets	Size	ES	NES	NOM p-val	FDR q-val	FWER p-val	Rank at max	Leading edge
HALLMARK_REACTIVE_OXIGEN _SPECIES_PATHWAY	43	0.57	1.32	0.026	0.242	0.215	4224	tags=53%, list=29%, signal=75%
HALLMARK_CHOLESTEROL _HOMEOSTASIS	69	0.53	1.27	0.019	0.282	0.428	3520	tags=45%, list=24%, signal=59%
HALLMARK_WNT_BETA_ CATENIN_SIGNALING	35	0.55	1.27	0.043	0.189	0.429	3536	tags=46%, list=25%, signal=60%
HALLMARK_MYOGENESIS	179	0.50	1.26	0.002	0.165	0.492	4350	tags=44%, list=30%, signal=62%
HALLMARK_HEME_METABOLISM	162	0.48	1.19	0.016	0.397	0.878	5079	tags=48%, list=35%, signal=73%
HALLMARK_HEDGEHOG_SIGNALING	29	0.51	1.15	0.206	0.602	0.976	1907	tags=31%, list=13%, signal=36%
HALLMARK_P53_PATHWAY	179	0.46	1.14	0.055	0.616	0.987	5480	tags=53%, list=38%, signal=85%
HALLMARK_XENOBIOTIC _METABOLISM	170	0.46	1.14	0.053	0.584	0.990	5186	tags=46%, list=36%, signal=72%
HALLMARK_ESTROGEN_RESPONSE _EARLY	162	0.45	1.14	0.062	0.527	0.991	3613	tags=33%, list=25%, signal=44%
HALLMARK_APICAL_JUNCTION	172	0.45	1.13	0.056	0.515	0.994	5004	tags=43%, list=35%, signal=65%
HALLMARK_BILE_ACID _METABOLISM	100	0.46	1.13	0.117	0.482	0.995	6180	tags=54%, list=43%, signal=94%
HALLMARK_MYC_TARGETS_V1	177	0.45	1.13	0.058	0.470	0.997	5916	tags=60%, list=41%, signal=100%
HALLMARK_UNFOLDED_PROTEIN _RESPONSE	97	0.46	1.12	0.125	0.462	0.997	5855	tags=59%, list=41%, signal=98%
HALLMARK_KRAS_SIGNALING_DN	142	0.45	1.12	0.088	0.437	0.997	4662	tags=36%, list=32%, signal=53%
HALLMARK_GLYCOLYSIS	179	0.44	1.10	0.113	0.582	1.000	4444	tags=42%, list=31%, signal=60%
HALLMARK_MTORC1_SIGNALING	191	0.43	1.09	0.121	0.585	1.000	5106	tags=51%, list=35%, signal=78%
HALLMARK_PI3K_AKT_MTOR_ SIGNALING	96	0.44	1.08	0.227	0.595	1.000	5080	tags=48%, list=35%, signal=74%
HALLMARK_HYPOXIA	181	0.43	1.08	0.173	0.611	1.000	5743	tags=52%, list=40%, signal=86%

Table 3.6. Continued. Gene sets enriched in the right hemispheres of stress and control groups.

HALLMARK_SPERMATOGENESIS	109	0.44	1.08	0.224	0.599	1.000	3910	tags=32%, list=27%, signal=44%
HALLMARK_PEROXISOME	96	0.43	1.06	0.298	0.712	1.000	3529	tags=31%, list=25%, signal=41%
HALLMARK_ADIPOGENESIS	183	0.42	1.05	0.276	0.734	1.000	4254	tags=37%, list=30%, signal=51%
HALLMARK_ANDROGEN_RESPONSE	83	0.43	1.05	0.338	0.714	1.000	5480	tags=53%, list=38%, signal=85%
HALLMARK_INTERFERON ALPHA_RESPONSE	82	0.43	1.04	0.336	0.721	1.000	5355	tags=43%, list=37%, signal=68%
HALLMARK_OXIDATIVE_ PHOSPHORYLATION	167	0.42	1.04	0.301	0.695	1.000	6035	tags=54%, list=42%, signal=92%
HALLMARK_KRAS_SIGNALING_UP	172	0.42	1.04	0.321	0.670	1.000	5197	tags=41%, list=36%, signal=63%
HALLMARK_PROTEIN_SECRETION	82	0.43	1.04	0.386	0.651	1.000	5432	tags=48%, list=38%, signal=76%
HALLMARK_APICAL_SURFACE	31	0.45	1.04	0.407	0.633	1.000	5427	tags=48%, list=38%, signal=78%
HALLMARK_TGF_BETA_SIGNALING	46	0.44	1.04	0.398	0.644	1.000	4757	tags=43%, list=33%, signal=65%
HALLMARK_FATTY_ACID_ METABOLISM	139	0.42	1.04	0.351	0.622	1.000	4917	tags=43%, list=34%, signal=65%
HALLMARK_ESTROGEN_RESPONSE LATE	167	0.41	1.03	0.374	0.664	1.000	5963	tags=47%, list=41%, signal=79%
HALLMARK_COMPLEMENT	164	0.40	1.01	0.458	0.753	1.000	5107	tags=39%, list=35%, signal=60%
HALLMARK_MITOTIC_SPINDLE	173	0.40	1.00	0.487	0.758	1.000	3838	tags=29%, list=27%, signal=40%
HALLMARK_UV_RESPONSE_UP	141	0.40	1.00	0.483	0.749	1.000	3588	tags=33%, list=25%, signal=43%
HALLMARK_INTERFERON_GAMMA RESPONSE	155	0.40	1.00	0.487	0.731	1.000	5400	tags=39%, list=38%, signal=61%
HALLMARK_COAGULATION	111	0.40	1.00	0.491	0.730	1.000	3345	tags=26%, list=23%, signal=34%
HALLMARK_APOPTOSIS	155	0.39	0.97	0.619	0.856	1.000	5552	tags=42%, list=39%, signal=68%
HALLMARK_IL2_STAT5_SIGNALING	173	0.38	0.95	0.733	0.948	1.000	5254	tags=35%, list=37%, signal=54%

Table 3.6. Continued. Gene sets enriched in the right hemispheres of stress and control groups

HALLMARK_NOTCH_SIGNALING	26	0.42	0.94	0.633	0.950	1.000	5569	tags=54%, list=39%, signal=88%
HALLMARK_MYC_TARGETS_V2	55	0.40	0.94	0.663	0.926	1.000	4719	tags=44%, list=33%, signal=65%
HALLMARK_TNFA_SIGNALING VIA_NFKB	173	0.38	0.94	0.758	0.909	1.000	5183	tags=39%, list=36%, signal=61%
HALLMARK_EPITHELIAL_ MESENCHYMAL_TRANSITION	169	0.37	0.92	0.841	0.965	1.000	7370	tags=59%, list=51%, signal=119%
HALLMARK_UV_RESPONSE_DN	133	0.37	0.92	0.809	0.942	1.000	5684	tags=44%, list=39%, signal=71%
HALLMARK_INFLAMMATORY RESPONSE	174	0.36	0.91	0.892	0.956	1.000	2570	tags=18%, list=18%, signal=21%
HALLMARK_ALLOGRAFT_REJECTION	145	0.36	0.90	0.854	0.939	1.000	4187	tags=25%, list=29%, signal=35%
HALLMARK_PANCREAS_BETA_CELLS	30	0.39	0.90	0.703	0.920	1.000	8046	tags=73%, list=56%, signal=166%
HALLMARK_DNA_REPAIR	130	0.36	0.89	0.863	0.921	1.000	7027	tags=55%, list=49%, signal=107%
HALLMARK_IL6_JAK_STAT3 SIGNALING	74	0.36	0.87	0.846	0.943	1.000	4849	tags=31%, list=34%, signal=47%
HALLMARK_G2M_CHECKPOINT	169	0.34	0.85	0.972	0.961	1.000	7520	tags=54%, list=52%, signal=111%
HALLMARK_E2F_TARGETS	181	0.32	0.81	0.989	0.977	1.000	7059	tags=47%, list=49%, signal=91%
HALLMARK_ANGIOGENESIS	33	0.31	0.72	0.963	0.991	1.000	7626	tags=55%, list=53%, signal=116%

The differentially expressed genes between stress and control right hemispheres, based on fold changes regardless of p-values, are presented in Figure 3.31.

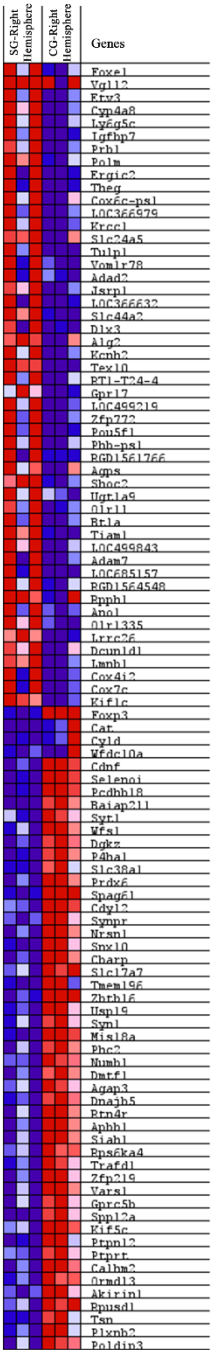

Figure 3.31. The heatmap of differentially expressed genes between the right hemispheres of SG and CG, according to their fold changes

The differential expression of up- (red) and down-regulated (blue) genes within each chip was scaled in accordance with the colour code shown in the heatmap produced by the GSEA software. SG: Stress group; CG: Control group.

According to bioinformatic results of the microarray analysis, *FOXE1* and *POLM* genes with the highest fold changes were selected as target genes for qRT-PCR analysis.

To choose the best reference genes, geometric mean of the ranks for the novel panel of candidate reference genes consisting of 11 genes, including some traditional ones such as *Gapdh* and *ACTB*, was calculated using the RefFinder web-based tool (Table 3.7). According to RefFinder results, the relative expression variability of the majority of the candidate genes on this panel was lower or equal to the traditional ones. *ITPR1* and *YWHAZ* genes were chosen as reference genes since they showed the lowest variability and high stability in expression in the analysis. In addition, their pre-designed optimized probe-based gene expression assays were also available and generated as specific to exon-exon junction region. The comprehensive stability rankings for candidate reference genes derived from RefFinder is presented in Figure 3.31.

Table 3.7. RefFinder results for the data were combined from 14 different microarray subarrays and utilized as input.
Geometric mean (GM); Stability Value (SV); Pearson's correlation coefficient {(r)}; Standard Deviation (SD).

RefFinder Comprehensive Ranking		**Delta CT**		**NormFinder**		**geNorm**		**BestKeeper** Ranking			
Genes	**GM**	**Genes**	**SV**	**Genes**	**SV**	**Genes**	**SV**	**Genes**	**[r]**	**Genes**	**SD**
Hsp90ab1 Itpr1	0|1	Hsp90ab1	0.28	Hsp90ab1	0.14	Hmbs	0.00	Hsp90ab1	0	Hsp90ab1	0
Itpr1	0|1|2	Itpr1	0.28	Itrp1	0.14	Rpl13a	0.00	Itpr1	0	Itpr1	0
Hmbs	2.83	Rpl13a	0.28	Rpl13a	0.14	Hsp90ab1 ITPR1	0.00	Hmbs	0	Rpl13a	0
Rpl13a	3.00	Hmbs	0.28	Hmbs	0.14	B2m	0.10	Rpl13a	0	Hmbs	0
Hprt1	5.69	Hprt1	0.37	Hprt1	0.22	Ywhaz	0.16	B2m	0.001	Ywhaz	0.133
Ywhaz	5.73	Ywhaz	0.38	Ywhaz	0.25	Hprt1	0.20	Ywhaz	0.435	Hprt1	0.133
B2m	6.44	B2m	0.43	B2m	0.34	Gapdh	0.27	Hprt1	0.525	B2m	0.133
Gapdh	8.00	Gapdh	0.46	Gapdh	0.36	ACTB	0.31	ACTB	0.716	Gapdh	0.408
Actb	9.00	Actb	0.48	Actb	0.38	Ppia (CycA)	0.35	Ppia (CycA)	0.738	Actb	0.408
Ppia (CycA)	10.00	Ppia (CycA)	0.52	Ppia (CycA)	0.43	Gusb	0.38	Gusb	0.801	Ppia (CycA)	0.5
Gusb	11.00	Gusb	0.53	Gusb	0.45					Gusb	0.5

Figure 3.32. The comprehensive stability rankings derived from RefFinder.
Results of analyzing the novel panel of candidate reference gene expression using the RefFinder web-based tool for normalizing gene expression for qRT-PCR.

3.2.3. qRT-PCR for *FOXE1* and *POLM* Genes

Efficiency and robustness evaluations of reverse transcription and PCR reactions during optimization showed that the Cq values for all wells containing NTCs (only nuclease-free water) were not determined. For the negative control (without the reverse transcriptase enzyme) samples in the *FOXE1* gene assay, 8-10 cycles higher Cq value than the observed Cq was determined, which was considered an acceptably high cycle range (Blick et al., 2013). These results prove the reliability of the of reverse transcription and PCR reactions. Moreover, as emphasized before in

the material and methods section, to avoid any between-plate variations, each plate was run with NTC (No Template Control), and IRC (Inter-Run Control) samples with their technical duplicates for each gene. The Cq values were not determined from the wells containing NTCs from all plates, indicating the good quality of the results. Representative amplification curves of the samples and NTC from one plate were shown in Figure 3.32. Average M values of the reference genes were calculated as 0.17, indicating the high stability under different conditions since it is less than 0.5.

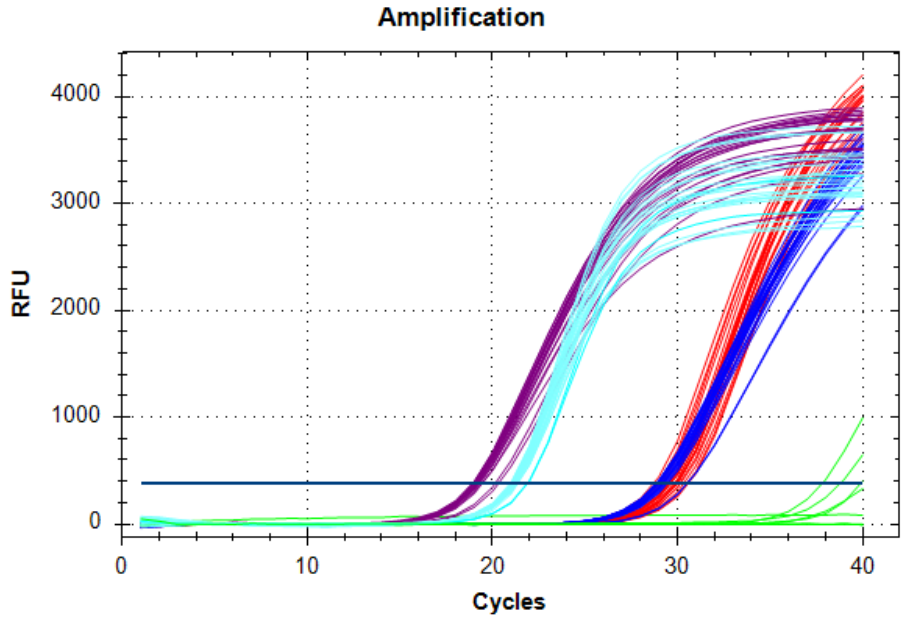

Figure 3.33. Amplification curves of the samples and NTCs obtained from a 96-well plate performing qRT-PCR
FOXE1 (red curve); *POLM* (blue curve); *YWHAZ* (purple); *ITPR1* (turquase); NTC (green), Relative fluorescence units (RFU).

The PCR primer efficiency for *FOXE1* was found to be 92.6%, for *POLM* 89%, for *YWHAZ* 90.1%, and for *ITPR1* 93%. These values were used to calculate the relative expression values for each gene according to the Pfafll method.

The p-values obtained for each group from the Shapiro-wilk normality test used for ΔCq data is presented in Table 3.8. The expression values of the *POLM*

gene were normally distributed for all experimental groups (all p's > 0.05). In contrast, the expression values of the *FOXE1* gene for the male samples deviated from the normal distribution for control groups in both the hemispheres and the left hemispheres of the stress group (all p's < 0.05).

Table 3.8. Shapiro-Wilk normality test results for each experimental group
p-value>0.05 was considered as normally distributed.

Target gene	Groups			Sample size	p-value	Normal distribution
FOXE1	Female	Control	Left	8	0.565	Yes
			Right	8	0.781	Yes
		Stress	Left	7	0.146	Yes
			Right	7	0.079	Yes
	Male	Control	Left	10	0.008	**No**
			Right	10	0.007	**No**
		Stress	Left	9	0.004	**No**
			Right	9	0.604	Yes
POLM	Female	Control	Left	8	0.486	Yes
			Right	8	0.291	Yes
		Stress	Left	7	0.652	Yes
			Right	7	0.358	Yes
	Male	Control	Left	10	0.054	Yes
			Right	10	0.941	Yes
		Stress	Left	9	0.691	Yes
			Right	9	0.855	Yes

When comparing the expression levels between the different groups, relevant sex differences were found:

- *FOXE1* gene was found to be significantly upregulated in the LH of the females in SG as compared to the LH of their male counterparts in SG (ΔΔCq=2.21, p=0.0045) (Figure 3.33). Similarly, the RH of females in SG also exhibited a significantly higher expression of *FOXE1* gene in comparison the RH of males in SG (ΔΔCq= 1.44, p=0.0047) (Figure 3.34).

In the CG, however, compared to the LH of males in CG, the *FOXE1* gene was found to be significantly upregulated in the LH of females in CG (ΔΔCq=1.54, p=0.005) (Figure 3.35).

-*POLM* gene was found to be significantly upregulated in the RH of the males in CG (ΔΔCq=1.18, p=0.0005) compared to the RH of the females in CG (Figure 3.36). *POLM* gene was also found to be expressed significantly downregulated in the RH of the females in SG (ΔΔCq=0.87, p=0.007) compared to the RH of the males in SG (Figure 3.34).

Significant differences between the hemispheres were only found in males in the control group:

- Compared to the LH of males in CG, the *FOXE1* gene was found to be significantly upregulated in the RH of males in CG (ΔΔCq=1.49, p=0.035) (Figure 3.35).

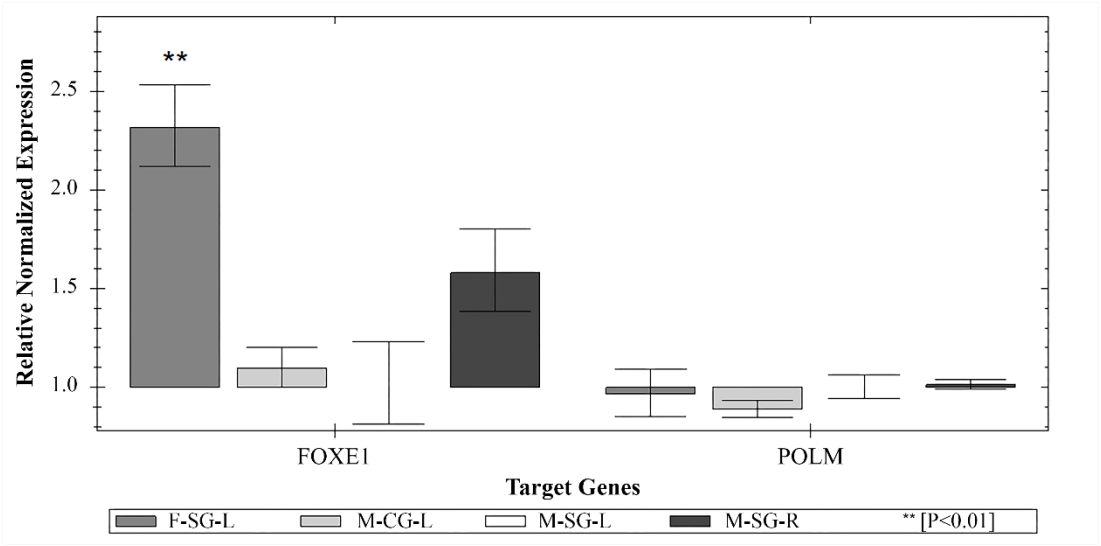

Figure 3.34. Normalized expressions compared to left hemispheres of the male in the stress group The results are shown as the mean ± SEM and asterisks (**) indicate significant differences (p<0.01).

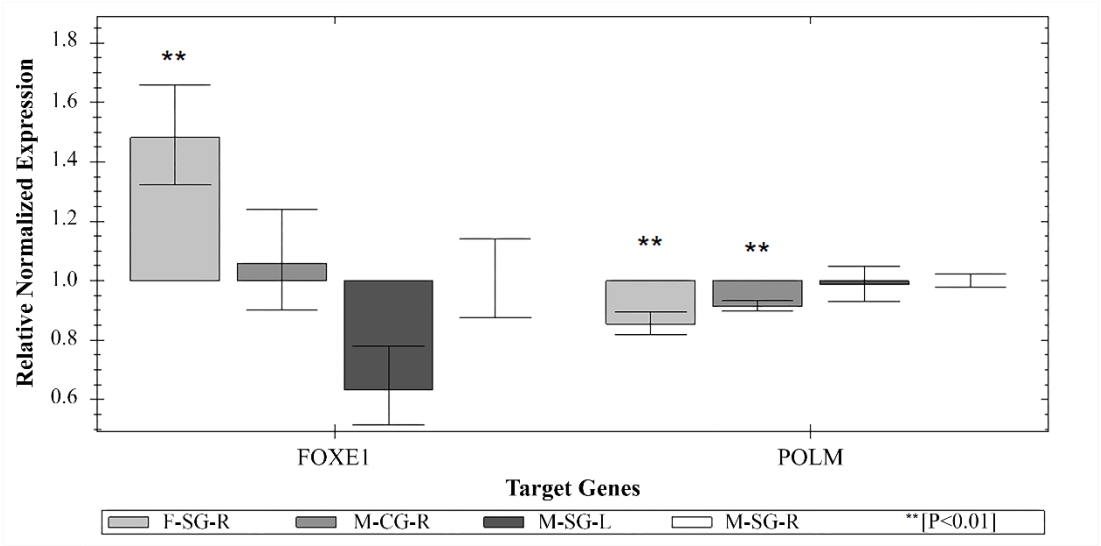

Figure 3.35. Normalized expressions compared to right hemispheres of the male in the stress group The results are shown as the mean ± SEM and asterisks (**) indicate significant differences (p<0.01).

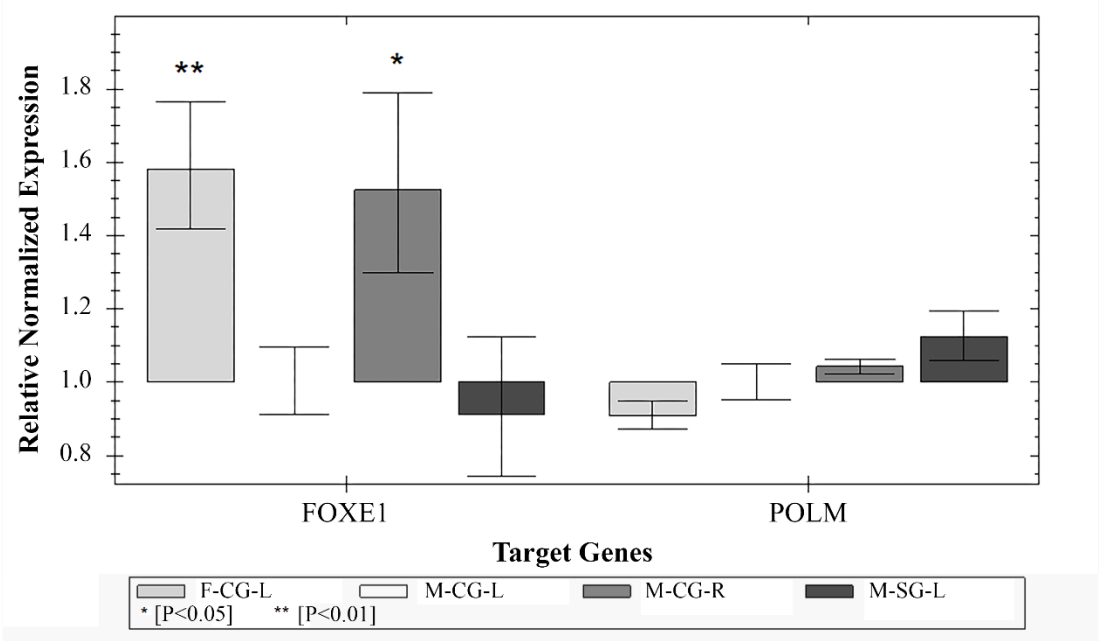

Figure 3.36. Normalized expressions compared to left hemispheres of the male in the control group The results are shown as the mean ± SEM. Asterisk (*) and asterisk (**) indicate statistical significances (p <0.05; p<0.01, respectively).

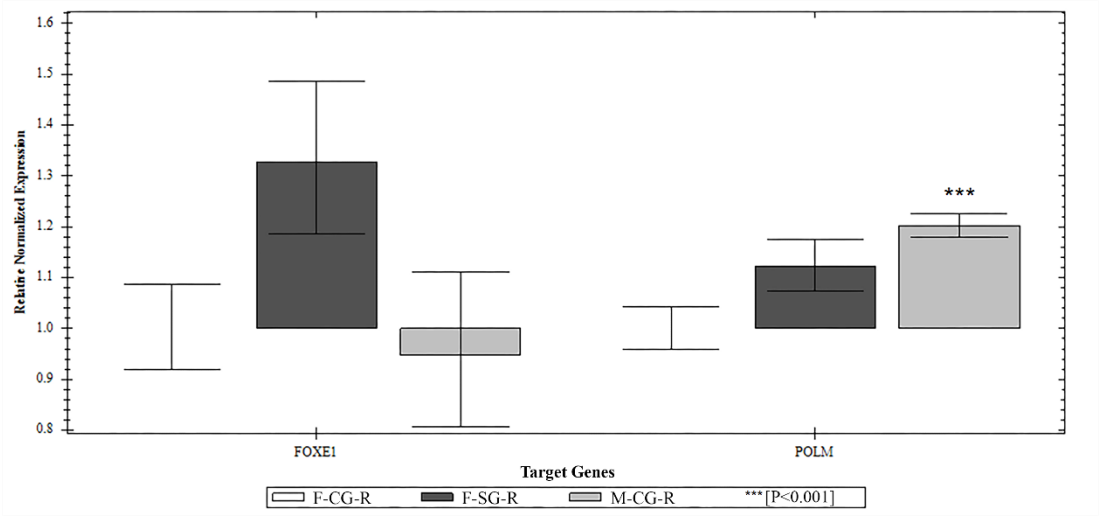

Figure 3.37. Normalized expressions compared to right hemispheres of the females in the control group The results are shown as the mean ± SEM and the asterisk (***) indicate significant differences ($p<0.001$).

In addition, in line with the results from the Microarray based gene expression analysis, a few effects of stress could be revealed:

- *FOXE1* gene was found to be slightly upregulated but not statistically significant in the RH of the females in SG ($\Delta\Delta Cq=1.31$, $p=0.061$) compared to the RH of the females in CG.

- *POLM* gene was found to be slightly upregulated but not statistically significant in the RH of the females in SG ($\Delta\Delta Cq=1.11$, $p=0.0735$), compared to the RH of the females in CG.

- *POLM* gene was found to be significantly differentially expressed in the RH of the males in SG ($\Delta\Delta Cq=1.08$, $p=0.009$) compared to the RH of the males in CG.

The results of the qPCR-RT analysis were found in line with the microarray-based gene expression analyses for the pooled RNA samples, indicating that *FOXE1* and *POLM* genes were upregulated in the right hemisphere of the SG ($\Delta\Delta Cq$ values 1.57 and 1.15, respectively) in comparison to CG in females (Figure 3.37). However,

to compare the groups using t-test was not able to be performed for the pooled RNA samples, since only the pooled RNA samples themselves and one technical replicate of them were used in the qRT-PCR due to insufficient concentrations for some of the individual samples after microarray analysis.

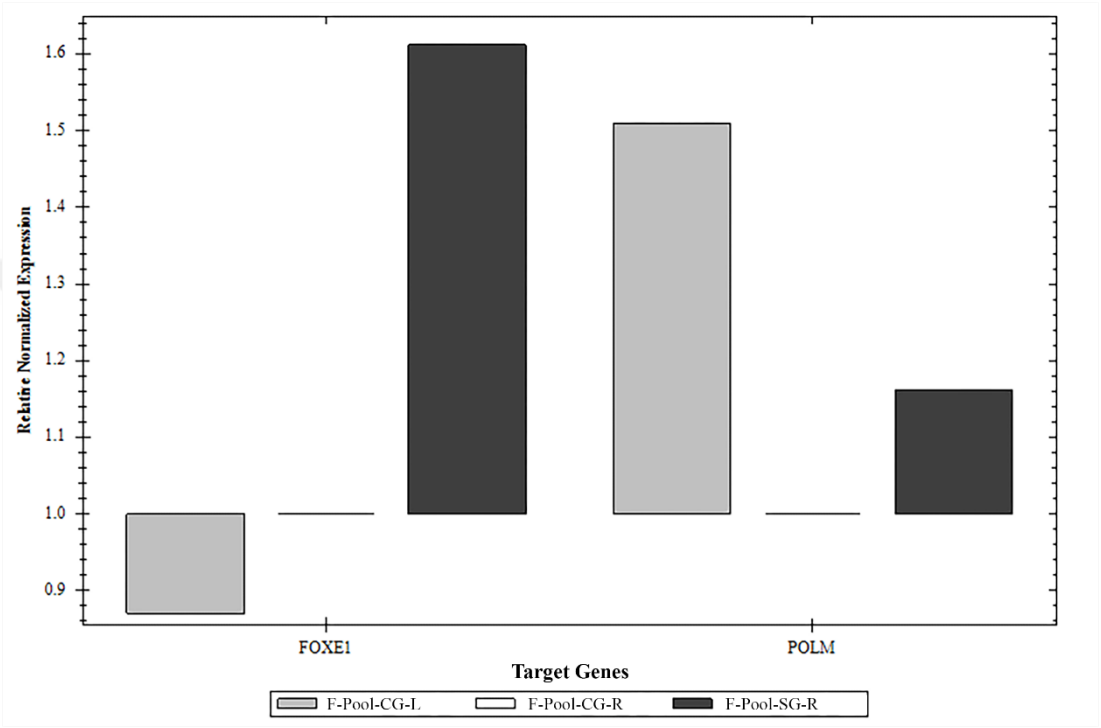

Figure 3.38. Normalized expressions of pooled RNA samples studied in microarray analysis Right hemisphere of the females in the control group was used as control group.

3.2.4. Correlation Between Expression Levels of *FOXE1* and *POLM* Genes and Paw Preference

The relationships between the strength and direction of lateralisation and the expression status of target genes in both hemispheres were determined using the Spearmen correlation coefficient analysis. To this end, LI, and ABS-LI values from both the Collins and Pasta test were used for this analysis.

The Collins results demonstrated a positive correlation between the expression level of the *POLM* gene and LI in the right hemisphere of the animals (r=0.476, p=0.034). In contrast, no correlation between the expression level of the *FOXE1* gene and LI was found (r=-0.036, p=0.881). In the left hemispheres of the animals, there was no significant correlation between the expression level of both genes and the LI (all p's >0.05). In both hemispheres, no significant correlation between the expression levels of both genes and the ABS-LI values was found (all p's >0.05) All correlations between the strength and direction of the lateralisation obtained from the Collins test and the expression levels of the target genes are presented in Table 3.9.

Table 3.9. Correlation between laterality and expression levels of target genes
Laterality Index (LI), Absolute Laterality Index (ABS-LI), Number of the animals (N)

Test	Hemisphere	Gene	Values	LI	ABS-LI
Collins Paw Preference Test	Right	*FOXE1 ΔCq*	Correlation Coefficient	-0,036	0,135
			Sig. (2-tailed)	0,881	0,569
			N	20	20
		POLM ΔCq	Correlation Coefficient	0,476	-0,082
			Sig. (2-tailed)	**0,034***	0,731
			N	20	20
	Left	*FOXE1 ΔCq*	Correlation Coefficient	-0,193	0,090
			Sig. (2-tailed)	0,415	0,707
			N	20	20
		POLM ΔCq	Correlation Coefficient	0,021	-0,296
			Sig. (2-tailed)	0,930	0,206
			N	20	20

The Pasta results demonstrated no correlations between the *FOXE1* and *POLM* gene expression level and LI in both hemispheres of the animals (all p's >0.05). A positive correlation was found between the expression level of *POLM* and ABS-LI in the left hemisphere (r=0.365, p=0.043), while not for the *FOXE1* gene (r=0.097, p=0.6). All correlations between the strength and direction of the lateralisation obtained from the Pasta test and the expression levels of the target genes are presented in Table 3.10.

Table 3.10. Correlation between laterality and expression levels of target genes
Laterality Index (LI), Absolute Laterality Index (ABS-LI), Number of the animals (N)

Test	Hemisphere	Gene	Values	LI	ABS-LI
Pasta Matrix Reaching Test	Right	*FOXE1 ΔCq*	Correlation Coefficient	0.097	0.040
			Sig. (2-tailed)	0.605	0.832
			N	31	31
		POLM ΔCq	Correlation Coefficient	0.132	0.019
			Sig. (2-tailed)	0.480	0.919
			N	31	31
	Left	*FOXE1 ΔCq*	Correlation Coefficient	-0.121	0.097
			Sig. (2-tailed)	0.518	0.602
			N	31	31
		POLM ΔCq	Correlation Coefficient	-0.109	0.365
			Sig. (2-tailed)	0.559	**0.043***
			N	31	31

4. DISCUSSION

Prenatal stress exposure and maternal separation (MS) are frequently employed in a multitude of studies investigating the consequences of early adversity and exhibit certain similarities in their respective impacts (Estanislau and Morato, 2005). A systematic review based on studies from 2008 to 2017 on gestational stress in rodents reported that restraint is the most frequently used methodology for prenatal stress protocol (Weinstock, 2017). This study aimed to investigate the effects of prenatal stress on functional cerebral asymmetries in rats using different behavioural tests and genetic analyses. The main findings of the study showed that prenatal stress has an impact on postnatal behavioural lateralisation and induces greater changes in gene expression levels in the right hemisphere of offspring than the left hemisphere.

Studies on humans (Rondo et al., 2003; Wadhwa et al., 1993) and animals showed that offspring of mothers exposed to stress during pregnancy have low fetal and birth weights (Collier et al., 1982; Schneider et al., 1999). The findings of this study also demonstrated that prenatal stress has an adverse effect on offspring's ability to gain weight during the postnatal period of development. The difference in weight between the control and stress groups on PD20 after the birth evident that the stress exposure worked successfully, and the pups were physiologically affected by this early development adverse experience.

In contrast to the previous findings that prenatal stress alters the maternal care of the dams (Bosch et al., 2007; Champagne and Meaney, 2006), the present study did not observe any significant effects of prenatal stress on mother-offspring interaction across the various behavioural parameters examined. These results also differed from the previous report of a systematic review on the effects of MS, which is the other most common paradigm to induce early life stress in animals, suggesting that MS induces the alterations in maternal behaviour of the dams (Alves et al.,

2020). For example, Hillerer et al. (2011) reported that the administration of restraint and overcrowding stress to pregnant rats between the 4^{th} and 16^{th} day of gestation inhibits lactation-induced anxiolysis and increases the duration of active lactation in rats, these findings were not supported by the results of nursing behaviour analysis of this study. Although some studies indicated that gestational stress causes a decrease in the licking/grooming frequency of the dams (Champagne and Meaney, 2006; Patin et al., 2002), in this study, there was no evidence that these maternal care behaviours are affected by restraint stress during the gestational time, in line with the results of Hillerer et al. (2011). The absence of alteration in maternal care behaviours resulting from prenatal stress implies that the duration and type of stress may also be significant factors to consider. One could speculate that the emergence of pathological processes takes place concomitantly with a decline in overall well-being throughout the process as a result of increased HPA axis activation (Bosch et al.2007), while the dam may be able to endure other stressors. These findings require further investigation to determine the impact of the type and duration of the stress exposure experience during pregnancy on maternal care behaviours of the dams. However, in this study, the lack of any differences in maternal care practices between the stress and control groups allowed for addressing the primary research question of whether prenatal stress impacts functional asymmetry in offspring. These findings gave an opportunity to only examine the impact of prenatal stress by eliminating the potential for increased maternal care to compensate for the adverse effects induced by prenatal stress protocol.

USVs have been shown to accurately and non-invasively represent the emotional state of rodents. Negative, stressful, and threatening situations in weaned young and adult rats initiate 22 kHz USV emission (Schwarting and Wöhr, 2012), while positive, appetitive states induce 50 kHz emission (Brudzynski, 2013; Burgdorf et al., 2011). Even though analysing USVs emitted by dams upon reunion with their offspring has been shown to be a simple and reliable method for determining the affective states of dams (Bölükbas. et al., 2020), the current study does have some limitations when employing USV analysis for a maternal care assessment. Major difficulties were encountered in distinguishing between dam and

offspring USVs in raw records obtained from simultaneous recordings, and more precise analysis tools were needed. In addition, there were technical glitches in filtering background noise. In addition to the aforementioned limitations, it is also crucial to note that the absence of any notable disparity in behavioural measures between the stress and control groups, as observed in the maternal care video analysis findings, is another factor that led to the exclusion of the ultrasonic vocalization (USV) analysis in the assessment of maternal behaviour.

To evaluate the efficacy and reliability of the prenatal stress protocol in the current investigation, dams were also examined in the elevated plus maze test to measure anxiety-related behaviours after the weaning. A significant prolongation of the time spent in the closed arm was observed in the dams that underwent a restriction stress protocol during the gestational period, as compared to those in the control group. Consistent with this finding, the time spent in open arms was significantly shorter in the dams in SG than in the CG. The findings of this study indicated that subjecting rats to movement restriction stress during the last trimester of their gestational period resulted in an increased tendency to avoid open arms. Considering that time spent in the open arms is a measure inversely related to anxiety (Cruz et al., 1994; Rodgers and Johnson, 1995), the results of this study clearly demonstrated that restriction stress protocol successfully induced stress in pregnant rats.

This study supports the previous reports on the role of prenatal stress exposure in developing anxiety-related behaviours in the later stages of life (Bosch et al., 2007; Lautarescu et al., 2020). The findings of this study showed that the animals in PD60 age group exposed to prenatal stress exhibit less explorative behaviours by spending less time in open arms but more time in the closed arms of EPM test comparing to their control counterparts. Although the most crucial time in postnatal development for brain development is adolescence (Schneider, 2013), interestingly, this study showed that PD60-early adulthood- may be a more sensitive postnatal developmental period in rats when the effects of prenatal stress are most strongly

manifested. Evidence from a review comparing different developmental time scales characterizing human and rodent brain development (Zeiss, 2021) has improved the understanding of the underlying reason for this finding by suggesting that rats reach the adult brain weight not shortly before puberty, unlike humans. Another interesting finding of this study is that although there was no difference between the experimental groups for the time spent in the open arm, in the PD90 age group, the control group spent more time in the closed arm compared to the stress group. While this finding is very surprising, it might be indicated that prenatal stress is followed by sensitive periods during development, and its consequences might change over the course of development. However, further investigations in rats are required to prove this claim and gain a deeper understanding of the effect of early life stress in the later stages of life, such as young adulthood and adulthood.

Recent studies have shown that stress-induced atypical turning asymmetry may be a mediator in the development of early life stress and psychiatric disorders (Mundorf et al., 2020; Mundorf et al., 2021). Therefore, the effect of prenatal stress on general turning behaviour exhibited during EPM has been investigated in this study. However, no effect on turning behaviour has been found. It can be suggested that prenatal stress may not directly affect the turning behaviour asymmetry in the later life span to the same extent as maternal separation. However, to the best of our knowledge, this is the first study to investigate the effect of prenatal stress on turning behaviour in rodents. Thus, further investigations with a larger sample size are required to clarify the validity of this conclusion.

Within the framework of this investigation, a diverse range of animals across various postnatal developmental stages and body sizes were subjected to distinct motor laterality tests. Consequently, it became necessary to develop a novel and unified apparatus that would correspond to these variations. The novel apparatus designed for rodents in this study provides key advantages as it integrates three distinct motor laterality tests and can be adjustable to accommodate variations in animal size enabling comparable conditions among age groups. Moreover, another

prominent advantage of this experimental apparatus was its ability to expedite the adaptation process for rats, considered neophobic animal species (Würbel et al., 2017), to the testing environment. Furthermore, this novel testing apparatus provides a simple, practical, and cost-effective method for the experimenter. Last but not least, this apparatus had another great importance in enhancing animal welfare. Food and beverage deprivation protocols are routinely applied to generate and sustain the motivational states required for a specific test condition (Dietze et al., 2016). Nevertheless, these protocols restrict animals from fulfilling their biological and physiological needs, leading to additional stressors, and rendering testing under neutral conditions impossible to achieve. This novel apparatus effectively prevented the unnecessary suffering of animals arising from their biological and physiological needs while also enabling the avoidance of unreliable data acquisition. According to the experimenter's observations, most of the animals voluntarily entered the test setup by jumping without any support from the experimenter as soon as the cage was opened and were motivated to reach the food rewards used during the test. This innovative approach holds promise to become a standard methodology and make valuable contributions to future laterality research in rats.

Collins Paw Preference test results of the present study confirmed the absence of population bias and the presence of asymmetry at the individual level in rats in the control group, in line with a recent meta-analyses study on rat paw preference in rodents (Manns et al., 2021). In line with this meta-analysis, rats in both experimental groups showed individual level asymmetry either being right- or left-preferred for the paw usage. Consistent with the findings of the meta-analysis, the offspring in the control group did not exhibit any population-level asymmetry in paw preference. Interestingly, rats in stress groups showed a leftward bias population-level asymmetry which can be considered atypical and altered behavioural laterality possibly induced by prenatal stress. A leftward bias population-level asymmetry in the stress group supported the main finding of this study showing that there is the main effect of prenatal stress on paw preferences in rats.

It has been previously proposed that the first paw intervention is a good indicator of the general paw preference for the direction in various species (Demirbas et al., 2023; Isparta et al., 2020). In line with these finding, first paw use of the rats predicts the overall paw preferences of the individuals as well. Exploring this correlation in further studies could be important for understanding paw preference stability in rats.

The key finding of this study is that prenatal stress alters the functional cerebral asymmetries in rats. Moreover, this finding is in line with the previous studies indicating that prenatal stress influences the behavioural laterality in the later stage of life (Alonso et al., 1991; Fride and Weinstock, 1988; Kofman, 2002). In addition to the main effect of stress, male individuals have a higher predisposition to be left-pawed than females, in line with the human studies (Castillo et al., 2020). Another interesting finding of this study is that PD21 age group showed significantly reduced asymmetry in comparison to other age groups. Moreover, the number of ambilateral individuals was higher than the left - and right-pawed ones in this age group. However, this difference between the age groups and the higher number of ambilateral individuals in the PD21 group may suggest that PD21 is an early postnatal time to test fine motor activity for food reaching, which might be not fully developed yet. The presence of a notable difference between the females in control and stress groups within the PD60 age cohort is a subject of considerable interest, no difference was found in males. In accordance with the previous finding of Alonso et al. (1991), the present data also suggest that these sex differences in behavioural asymmetry may be related to hormonal influences during the development. However, this interesting finding from females aligns with the results obtained from the EPM experiments, providing further evidence to support the hypothesis that the PD60 age group exhibits the most significant manifestations of prenatal stress. Based on this robust conclusion, to investigate the molecular background of prenatal stress on functional cerebral asymmetry, female individuals belonging to PD60 age group were selected as the sample group for further genetic analyses.

The results obtained from the Pasta test in the PD60 age group did not exhibit consistency with Collins's results that prenatal stress exposure alters functional cerebral asymmetry. It can be argued that paw preference in rats is task dependent as it has been previously reported in different species such as common marmosets (Piddington and Rogers, 2013), dogs (Yasemin Salgirli Demirbas et al., 2023) and otters (Manns et al., 2018). However, although no stability was observed between paw preference determination tests in this study, the initial paw usage predicts the general paw preference of the animals not only in the Collins test but also in the Pasta test. Similar to Collins test results, Pasta results were in accordance with the rat meta-analysis which indicates that the rats do not show population level asymmetry but show individual level asymmetry for one direction (Mann et al. 2020).

Soyman et al. (2018) showed that rats exhibit a tendency towards leftward head-turning asymmetry following exposure to a stress-inducing condition. Nevertheless, the findings obtained from the manual coding analysis in this study did not provide evidence to support this finding. Further analysis is required to verify that prenatal stress does not impact other observable motor laterality measurements, such as head-turning asymmetry, as indicated in both the method section and subsequent discussion of limitations.

Technical limitations of the head turning asymmetry test are listed below:

- dim red light caused underexposed frames with blurry noise,
- reflections in plexiglass walls as well as the fur of the animals, caused further exposure artifacts,
- frame size at > 1920 px might have been too large for the receptive field of the DLC model, typically trained on images <700 px,
-single camera position could not capture freely moving rats in the depth of the box, causing self-occlusion of body parts and large differences in scale (i.e. animal size near and far from the camera),

- multi-view approach would be needed to capture the freely moving behaviour of rats in a constrained box, however, see camera synchronization and triangulation issues in (https://gitlab.ruhr-uni-bochum.de/ikn/syncflir).

To assess the quality and quantity of the RNAs obtained from the motor cortices of the animals, the concentration of RNAs was measured by using a Spectrophotometer. The ratio of absorbance at 260 and 280 nm (the A260/280 ratio) and 260 and 230 nm (the A260/230 ratio) are commonly utilized to determine the purity of nucleic acid extractions. The ratio of A260/280 for pure RNA is considered as ~2.0 while the expected ratio of A260/230 for pure RNA is commonly in the range of 2.0-2.2 (Wilfinger et al., 1997) and all RNAs obtained from motor cortices of the animals were within this range of values. Considering the importance of utilizing intact RNA in obtaining precise gene expression data using modern biological methods, such as microarray and qRT-PCR analysis (Fleige and Pffafl, 2006; Pérez-Novo et al. 2005), the integrity and purity of the RNA samples were further verified using gel electrophoresis and capillary electrophoresis methods. The results of all these evaluations indicate that all steps from biological sampling to RNA isolation in the present study were carried out successfully.

Various studies reported that technical or financial commonly encountered in microarray and/or RNA-seq experiments include budget constraints, inadequate RNA input from one subject, and substantial biological variability within groups (Assefa et al., 2020; Peng et al., 2003). Consequently, the adequacy and experimental validation of the pooling of RNA samples have been extensively investigated, and pooling has become a great solution in such cases (Kendziorski et al., 2005; Peng et al., 2003; Zhang et al., 2005). Due to the small sample size and biological variations in the experimental groups of this study, the pooling of RNA samples that provide adequate statistical power, was chosen as the best way for the micro array analysis.

Another key finding of the present study is that microarray-based gene expression analysis comparing the stress and control groups indicated a greater

number of differentially expressed genes in the right hemisphere compared to the left hemisphere. This interesting finding aligns with previous research demonstrating that negative experiences and adverse stimuli are predominantly processed in the right hemisphere of the brain, and that exposure to such stimuli can lead to increased activation of the right hemisphere (Berretz et al., 2020; Cerqueira et al., 2008; Davidson and Fox, 1989; Bölükbas et al., 2020; Stomp et al., 2021). To the best of our knowledge, this is the first study to demonstrate that prenatal stress has a greater impact on the right hemisphere compared to the left at the gene expression level induced by prenatal stress exposure in rats.

Given the lack of a comprehensive investigation in the existing literature regarding the impact of prenatal stress on hemispheric asymmetries at the molecular level, microarray-based gene expression analysis, which enables the simultaneous determination of expression levels for thousands of genes, was performed instead of the candidate gene approach in this study. One of those identified genes, *LMO4*, was differentially expressed in the human fetal brains during the gestational period of 12 to 14 weeks. This differential expression between the two hemispheres gradually diminished over time and eventually became symmetrical by the 19th week of gestation. Another investigation of the same study on mouse brains revealed that *LMO4* expression in the mouse brain on postnatal day 1 was moderately asymmetrical; however, this asymmetry was not consistent in either the right or left direction (Sun et al., 2005). Since there is no study comparing the expression of the *LMO4* gene in the motor cortices between hemispheres in adult mice or rat brains, it was not possible to make a comparison with our results. Further research is necessary to attain a more profound comprehension of the potential differentiation in the expression of the *LMO4* gene between hemispheres as well as to ascertain the possible roles of *LMO4* gene in functional lateralisation.

In addition, *LRRTM1*, *COMT*, *APOE*, *AR*, *PCSK6* and *SETDB2* genes, which were found to be associated with handedness in humans (Francks et al., 2007; Savitz et al., 2007; Bloss et al., 2010; Arning et al., 2015; Arning et a., 2013; Ocklenburg et

al., 2016, respectively), did not exhibit differential expression in the motor cortical regions of the two hemispheres in the current investigation. Furthermore, FOXP2, CCKAR, and GRINB2 genes, which were found to be associated with language lateralization (Ocklenburg et al., 2013a; Ocklenburg et al., 2013b; Ocklenburg et al.,2011 respectively), were also found not to be expressed differently between the two hemispheres in both groups, similar to genes previously found to be associated with handedness in humans. Taken together, these divergent findings may be driven by the variations in species and the experimental framework, including differences in tissue samples and methodological approaches.

Gene set enrichment analysis was performed using microarray data to detect whether there was a differentially enriched gene set and/or whether these genes played a role in the condition-specific biological pathways between conditions. The findings of the present study indicate that the defined gene sets do not play a role in either lateralization or stress-related pathways in rats. The current understanding of the molecular underpinnings of stress and laterality remains incomplete, highlighting their complex nature. Thus, one may suggest that the gene sets identified in this study are required to evaluate using data derived from subsequent studies

POLM gene has been previously identified as one of the chronic psychological stress-responsive genes in peripheral blood cells in humans (Kawai et al., 2007). Some transcription factors of the forkhead/winged-helix (FOX) family, such as Forkhead box P2 (*Foxp2*) have been found to be related to language lateralisation and also associated with the strength of hand preference (Crespi et al., 2017). However, *FOXE1*, which is also a member of the same family of transcription factors, was investigated for its role in stress response and cerebral asymmetry for the first time within the scope of this study. The findings from the microarray-based gene expression analysis revealed that the *FOXE1* and *POLM* genes exhibited the greatest differential expression between the right hemisphere of female subjects in SG and CG. Based on an analysis of the microarray data and the mentioned characteristics of the *POLM* and *FOXE1* genes as described previously, these genes

were chosen as the most suitable candidates for further qRT-PCR investigation in the present study.

Selecting tissue-specific reference genes for normalization in qRT-PCR gene expression analyses is also widely accepted as the gold standard practice for ensuring accurate results (Jeon et al., 2020). Although, there has yet to be a consensus in the current literature on the most appropriate reference genes for the motor cortices of the brain; traditional *ACTB* and *GAPDH* genes have been utilized most as reference genes (Bonefeld et al., 2008). However, according to both microarray-based gene expression level analysis and qRT-PCR validations of this study, *YWHAZ* and *ITPR1* genes are suggested as the most stable and reliable reference genes for the stress related gene expression analysis of the samples obtained from the motor cortices of the rat brain.

qPCR-RT analysis for the pooled RNA samples showed that the results are compatible with the microarray results indicating increased right hemispheric activation at the gene expression level in the later stage of animals exposed to prenatal stress. However, it should be noted that it was not possible to conduct a statistical analysis to confirm this finding due to the technical limitations of the study since only insufficient concentrations for some of the individuals remained after the microarray analysis.

In this study, both *FOXE1* and *POLM* genes were significantly differentially expressed according to sex, while *FOXE1* exhibited differential expression based on hemispheres as well. The results showed that the *FOXE1* gene was expressed significantly higher in females than males in both hemispheres in the stress group. However, interestingly, in the control group comparison, the expression of the *FOXE1* gene was found to be significantly upregulated in only the left hemisphere. This study presents, in a nutshell, in the absence of prenatal stress exposure, there is no difference in the expression level of the *FOXE1* gene between females and males

in the right hemisphere, there is a significant difference between the right hemispheres of the females and males in the presence of stress exposure. One may assume that the right hemisphere's response to prenatal stress is more sexually dimorphic, as suggested before for HPA axis response (Schneider et al., 1998; Szuran et al., 2000)

Similar to *FOXE1* gene expression, significant differences were also identified for the expression level of the *POLM* gene between females and males in the right hemisphere. The expression level of the *POLM* gene was found to be more upregulated in the right hemispheres of both stress and control group males compared to the right hemispheres of females. These results demonstrated a sex-related differentiation in the right hemisphere for the *POLM* gene expression independent of stress exposure.

The results of the correlation analysis between the behavioural laterality and gene expression status showed a positive correlation between LI obtained from the Collins test and the expression level of the *POLM* gene in the right hemisphere, while no correlation was found in the left hemisphere. The differential expression of the *POLM* gene in response to prenatal stress and its association with behavioural laterality, specifically within the right hemisphere, suggests that the *POLM* gene holds promise as a candidate gene for future studies investigating the relationship between stress and lateralisation. However, the correlation analyses for the Pasta test were not found to be similar to the Collins results. The LI values obtained from the Pasta test were not correlated with the expression levels in both hemispheres, while a positive correlation was found between the ABS-LI value used to evaluate the strength of lateralisation and the expression level of the *POLM* gene in the left hemisphere. As previously mentioned, the findings of the current study indicate that the manifestation of behavioural laterality in rats is task dependent. Hence, this aspect can explain the fundamental cause for the observed disparity between these two motor laterality tests in the correlation analysis. These findings need to be investigated with larger sample size to reach a conclusion.

In accordance with the findings derived from the microarray results, the expression levels of both genes were slightly more upregulated in the hemispheres of the females in the stress group compared to the control group. However, these differences were non-statistically significant trends. These conclusions require further investigation to provide more extensive molecular insights into the field of stress and laterality research.

5. CONCLUSION

This study is the first to investigate the effect of prenatal stress on functional cerebral asymmetries at different postnatal developmental stages using different motor laterality tests and gene expression analyses in rats. The result of this study indicates that prenatal stress affects the capacity of offspring to gain weight during the postnatal period of development. No effect of prenatal stress on the maternal care behaviours of the dams was observed. However, no significant influence of prenatal stress on the maternal care behaviours of the dams was observed. The results obtained from the EPM experiment proved that the movement restriction protocol administered to pregnant rats during the last trimester of gestation effectively induced stress in pregnant rats. The findings from the EPM analysis reveal that animals in the PD60 age group subjected to prenatal stress demonstrated reduced exploratory behaviours and increased anxiety-related behaviours compared to their control counterparts. This study proposes that PD60 age, early adulthood, may represent a critical postnatal developmental phase in rats, during which the impacts of prenatal stress are particularly pronounced. The current study did not yield any evidence supporting the impact of prenatal stress on general turning behaviour. The novel testing apparatus designed by combining three different motor laterality tests in the current study has the potential to become a standard methodology and contribute to further studies in rodents. The primary results of the present study indicate that prenatal stress influences functional cerebral asymmetries at later stages of life. Moreover, another interesting finding of the study is that prenatal stress causes a leftward bias at the population level for the paw preference in rats, indicating that stress exposure causes the right hemispheric activation at the behavioural level. This study also reports that the direction of the initial paw usage indicates the rat individual's general paw preferences. When the effect of prenatal stress on motor lateralisation was examined in each age group separately, a significant difference between the experimental groups was only found in females in the PD60 age group. The present study showed that prenatal stress does not cause any alterations in head-

turning asymmetry in rats at the postnatal developmental stages. Another key finding of the present study is that prenatal stress induces greater alterations in gene expressions in the right hemisphere than in the left hemisphere of the rats. This study represents the initial evidence indicating that prenatal stress has a greater impact on the right hemisphere of the brain compared to the left at the gene expression level in rats. *YWHAZ* and *ITPR1* genes are recommended as the most stable and reliable reference genes for the stress-related gene expression analysis of the samples obtained from the motor cortices of the rat brain, according to both microarray-based gene expression level analysis and qRT-PCR validations of this study. According to the microarray-based gene expression analysis of the present study, *FOXE1* and *POLM* genes are the most differentially expressed genes between the right hemispheres of female individuals in SG and CG. The differential regulation of the *POLM* gene in response to prenatal stress and its correlation with behavioural laterality, particularly in the right hemisphere, indicate that the *POLM* gene exhibits potential as a candidate gene for future investigations exploring the relationship between stress and lateralisation.

SUMMARY

Behavioural and Genetic Evaluation of The Effect of Prenatal Stress on Functional Cerebral Asymmetries in Rats

Functional cerebral asymmetry (FCA) refers to the phenomenon in which the brain hemispheres specialize in regulating specific functions. In the past, it was assumed that FCAs are driven by genetic factors and remain constant throughout the lifespan once developed. Recent studies have demonstrated that FCAs have some degree of plasticity and are susceptible to environmental influences, potentially can be changed throughout an individual's lifespan. A growing body of evidence indicates the idea that environmental factors, such as stress, are associated with both functional and structural changes in cerebral asymmetries. Moreover, previous studies have demonstrated that applying prenatal stress induces enduring alterations in behavioural and neurochemical asymmetries in rat offspring, which persist into adulthood. The present study hypothesized that prenatal stress exposure alters the FCAs in rats. The primary aim of this study was to examine whether there are behavioural and molecular alterations induced by prenatal stress during various postnatal developmental phases in offspring. In this context, it was aimed to elucidate the genetic basis of cerebral lateralisation and to understand how prenatal stress affects brain organization at the gene level through microarray-based gene expression analysis. To this end, RNAs obtained from the primary and secondary motor cortices of the left and right hemispheres of the rat brains were used as a biological sample. Another object of this study was to examine sex-dependent differences in behavioural asymmetry and genetic regulation in offspring exposed to prenatal stress. This study also aimed to investigate the impact of prenatal stress on the maternal care behaviour of the dams. The subjects of the present study comprised 16 timed-pregnant Sprague-Dawley rats and their offspring (N= 131). A total of 16 pregnant rats were included in the study, with 8 rats assigned to the stress group (SG) and the remaining 8 to the control group (CG). The restriction stress protocol was applied to the dams for 7 days, covering gestation days 15-21 with a duration of 2 hours per day. Maternal care behaviours of the dams were assessed to evaluate the effect of stress exposure during gestation, and no differences were found between stress and control groups. The dams were administered the Elevated Plus Maze (EPM) test to evaluate the effectiveness of the movement restriction stress protocol. EPM test results showed that dams exposed to restriction stress showed fewer exploratory behaviours and more anxiety-related behaviours, indicating that the restriction stress protocol worked successfully. After weaning, the EPM test was applied to the offspring to measure anxiety-related behaviours, and different motor laterality tests were applied to measure FCAs. The results of the EPM test showed that in the PD60 age group, the offspring in the SG spent more time in the closed arms, while the pups in the CG spent more time in the open arms. This indicates that prenatal stress even has a long-lasting effect on offspring. The findings of this study revealed that prenatal stress exposure does not influence the general turning asymmetry or head-turning asymmetry in rats. In the present study, the Collins paw preference test and Pasta Matrix Reaching tests were used to assess the paw preference of the rats. The findings of these motor laterality tests showed that the direction of the first paw intervention of the rats is a good indicator of the overall paw preference of the individuals for the direction. The findings also showed that the paw preference of the rats is task dependent. The main finding of this study indicates that prenatal stress alters functional cerebral asymmetries in rats. Additionally, an intriguing finding from the Collins test in the present study is that prenatal stress induces a population-

level leftward bias in the paw preference of rats, suggesting that stress exposure cause to right hemispheric activation. Microarray gene expression analysis was conducted on female individuals belonging to the PD60 group, exhibiting a more pronounced impact of prenatal stress. According to microarray-based gene expression analysis, the number of differentially expressed genes in the right hemisphere was found much higher than in the left hemisphere, indicating that prenatal stress has a greater impact on the right hemisphere at the gene expression level. According to microarray results, the *FOXE1* and *POLM* genes are the most differently expressed genes with the greatest fold changes between the right hemispheres of female offspring in SG and CG. Considering the differential modulation of the *POLM* gene in reaction to prenatal stress and its association with behavioural laterality, specifically in the right hemisphere, suggests that the *POLM* gene holds promise as a potential candidate gene for future research studies investigating the link between stress and lateralisation. Overall, these results prove that prenatal stress exposure can change behavioral and hemispheric asymmetries in rats.

Keywords: Behavioural laterality, FOXE1, Microarray, POLM, Prenatal stress

ÖZET

Ratlarda Prenatal Stresin Fonksiyonel Serebral Asimetri Üzerindeki Etkisinin Davranışsal ve Genetik Açıdan Değerlendirilmesi

Serebral asimetri (serebral lateralizasyon) beynin sağ ve sol hemisferlerinin fonksiyonel ve anatomik olarak özelleşmesidir. Fonksiyonel serebral asimetri (FSA) ise, beyin yarım kürelerinin belirli fonksiyonların kontrolü açısından özelleşmesi olarak tanımlanmaktadır. Beyin hemisferlerinin anatomik ve fonksiyonel olarak asimetrik olduğu iyi bilinmesine karşın, hemisferik asimetrilerin moleküler ve genetik temelleri henüz tam olarak anlaşılamamıştır. El tercihi, insanlarda; pati tercihi ise hayvanlarda beyin lateralizasyonunun en belirgin fonksiyonel ifadesidir. Bu nedenle, hemisferik asimetrilerin ve el tercihlerinin ontogenezinin anlaşılması, bu tip hastalıkların patogenezi hakkında önemli bilgiler elde edilmesi açısından çok önemlidir.

Kısa bir zaman öncesine kadar fonksiyonel serebral asimetrilerin çoğunlukla genetik faktörlerce yönlendirildiği ve zaman içerisinde stabil olduğu varsayılmasına karşın; güncel çalışmalar ile FSA'ların bir dereceye kadar plastisiteye sahip olduğu ve bireyin ömrü boyunca değişebileceği gibi kısa zaman dilimlerinde de değişebileceği kanıtlanmıştır. Canlılarda, FSA ve stres arasındaki ilişkiyi araştıran çok sayıda çalışma mevcuttur. Yapılan çalışmalar, akut ve/veya kronik stresin FSA'yı etkilediğini, hatta serebral asimetrinin stres cevabında koruyucu roller üstlendiğini göstermektedir. Bununla birlikte, prenatal stresin beyin organizasyonu üzerindeki etkisi ve bu etkinin altında yatan moleküler mekanizmalar yeterince bilinmemektedir. İnsan ve deney hayvanı çalışmalarından elde edilen veriler ışığında kronik prenatal stresin, nöronal gelişim üzerinde uzun vadede etkili olabileceği ortaya konmuştur. Bu durumun, bebeklik döneminde tespit edilebilen ve erişkinlik döneminde de devam edebilecek farklı davranışsal patolojilere neden olabileceği bildirilmiştir. Örneğin yaşamın ilerleyen dönemlerinde anksiyete bozuklukları ve depresyon gibi çeşitli psikiyatrik hastalıklara sebep olduğu bilinmektedir. Prenatal dönemde maruz kalınan olumsuzluklar sebebiyle meydana gelen epigenetik değişikliklerin, yaşamın ilerleyen dönemlerinde görülen obezite, kardiyovasküler hastalıklar ve endokrin bozukluklar gibi yaygın kronik hastalık riskleriyle ilişkili olduğu varsayılmaktadır. Bunun altında yatan temel unsur, hücrelerin iç veya dış çevresel uyaranlara cevap olarak adapte olma kabiliyeti anlamına gelen "fenotipik plastisite" fikrine dayanmaktadır. Kronik prenatal stresin, gelişmekte olan embriyonun kortikal ve limbik sistem morfolojisini değiştirerek serebral lateralizasyonu azaltabileceği de bildirilmiştir.

Bu tez kapsamında, ratlarda prenatal stresin FSA üzerindeki etkisi çeşitli davranış testleri ile değerlendirilmiş ve beynin her iki hemisferine ait motor korteksteki gen ifade düzeylerinin belirlenerek davranışsal asimetri ile ilişkisi ve bu ilişkinin prenatal stresten nasıl etkilendiği araştırılmıştır. Mevcut literatürde canlılarda fonksiyonel serebral asimetri ile stres arasındaki ilişkiyi araştıran birçok çalışma olmasına karşın; bu çalışmaların büyük bir çoğunluğu akut ve kronik stresin FSA'yı nasıl etkilediğinin anlaşılmasına yöneliktir. Yapılan tez çalışması çeşitli davranış testleri ve beyin dokusunda gen ifade düzeyine ilişkin analizler içermekte ve prenatal stresin ratlarda FSA üzerine etkisini ve moleküler alt yapısını inceleyen ilk araştırma olma özelliği taşımaktadır.

Bu çalışmada gebe kalma zamanı bilinen Sprague Dawley ratlardan (n=16) 8 tanesi rastgele kontrol grubu olarak seçilmiş ve diğer 8 gebe rata ise stres protokolü uygulanmıştır. Deneylere hem anne ratlar hem de yavru ratlar (n=131) dahil edilmiştir. Stres grubundaki gebe ratlar (n=8) gebeliğin son trimesteri (15-21 günler arası), günde 2 saatlik hareket kısıtlama stresine maruz bırakılmışlardır. Stres protokolü 12 saatlik aydınlık/karanlık periyotlarında ratların en aktif olduğu dönem olan karanlık döngülerinde uygulanmıştır. Yavruların doğdukları gün postnatal gün (PG) 0 olarak kabul edilmiştir. PG2'de yavruların cinsiyetleri belirlenmiş ve her biri hassas terazide tartılarak kiloları kaydedilmiştir. Her bir anneden olabildiğince eşit sayıda erkek ve dişi yavru içeren ve en fazla 10 yavrudan oluşan örneklem grupları oluşturulmuştur.

Gebelik döneminde annelere uygulanan stresin annelerde yavru bakımı üzerine etkilerini araştırmak amacıyla PG2, PG6, PG14 ve PG20 günlerinde anne yavru bakımı değerlendirmesi yapılmıştır. Değerlendirmede, yavruları yalamak ve tımar etmek, emzirmek, yavruları geri almak, yuvayı yeniden inşa etmek, yuvada geçirilen süre, kendi kendini tımar etmek, yetiştirme ve yavru arama davranışı olmak üzere 7 parametre açısından değerlendirilmiştir. Ayrıca kamera kaydıyla eş zamanlı olarak ultrasonik vokalizasyonları da kayıt altına alınmıştır. Behavioural Observation Research Interactive Software (BORIS) (Davranışsal Gözlem Araştırması İnteraktif Yazılımı) kullanılarak anne bakım davranış değerlendirmelerinden elde edilen videolar analiz edilmiştir. Bu araştırmanın sonuçları gebelik döneminde anne ratlara uygulanan stresin yavru bakım davranışlarında bir etkisi olmadığını göstermektedir. Anne bakımı davranışlarında stres ve kontrol grupları arasında herhangi bir fark olmaması, prenatal stres protokolünün neden olduğu olumsuz etkileri telafi etmek için artabilecek anne bakımı potansiyelini ortadan kaldırarak yalnızca prenatal stresin etkisini inceleme fırsatı verdi. Böylelikle, yalnızca prenatal stresin yavrularda fonksiyonel asimetriyi etkileyip etkilemediği şeklindeki birincil araştırma sorusunun araştırılmasını mümkün kılmıştır.

PG21'de yavrular sütten kesilerek PG21, PG40, PG60 ve PG90 zamanlarında test edilmek üzere farklı yaş gruplarına ayrılmıştır. Kontrol ve stres grubundaki her bir anneden her bir yaş grubu için rastgele 1 erkek, 1 dişi yavru seçilmiştir ve yavrular kafeslere kafes başına aynı cinsiyetten 2 hayvan olacak şekilde yerleştirilmiştir. Kalan PG40, PG60 ve PG90 yavruları ise davranış testlerinden iki gün öncesine kadar her kafeste aynı cinsiyetten yavrular olacak şekilde tutulmuştur. Görme sistemleri düşük ışık koşullarına uyum sağlamış ratların tüm davranış değerlendirmeleri de yine benzer şekilde kırmızı ışık koşulları altında 12/12 saatlik bir aydınlık/karanlık döngüsünün karanlık aşamasında gerçekleştirilmiştir. Bütün postnatal uygulamalardan önce hayvanlar, testlerin uygulandığı davranış odasına testten en az 30 dakika öncesinde alınarak, odaya uyum sağlamalarına izin verilmiştir.

Rodentlerde kaygıyla ilgili davranışlarını değerlendirmek için yaygın olarak kullanılan Yükseltilmiş Artı Labirent Testi kullanılarak 16 anne ve 4 farklı gelişimsel yaş grubundaki her bir yavru olmak üzere toplam 131 yavruya anksiyete değerlendirilmesi yapılmıştır. Testin tamamı kamera ile kayıt altına alınmıştır ve elde edilen videolar, açık kaynaklı bir Python aracı olan DeepLabCut kullanılarak analiz edilmiştir. Bu çalışmanın bulguları, prenatal strese maruz kalan PD60 yaş grubundaki hayvanların, kontrol grubuna kıyasla EPM testinde açık kollarda daha az, kapalı kollarda ise daha fazla zaman geçirerek daha az keşfedici davranışlar sergilediklerini göstermiştir. Beyin gelişimi için doğum sonrası gelişimde en önemli dönem ergenlik olsa da ilginç bir şekilde, bu çalışma PD60'ın erken yetişkinlik döneminin, prenatal stresin etkilerinin en güçlü şekilde ortaya çıktığı, ratlarda doğum sonrası daha hassas bir gelişim dönemi olabileceğini göstermiştir. Bu çalışma, prenatal stres maruziyetinin yaşamın sonraki aşamalarında kaygı ile ilişkili davranışları geliştirmedeki rolüne ilişkin önceki çalışmaları desteklemektedir. Ayrıca, mevcut araştırmadaki prenatal stres protokolünün etkinliğini ve güvenilirliğini değerlendirmek için

sütten kesmeden sonra kaygı ile ilgili davranışları ölçmek için yükseltilmiş artı labirent testinde anne ratlar da incelenmiştir. Gebelik döneminde hareket kısıtlandırma stres protokolü uygulanan annelerde kontrol grubuna göre kapalı kolda geçirilen sürenin önemli ölçüde uzadığı gözlenmiştir. Bu bulguyla uyumlu olarak, açık kollarda geçirilen süre stres annelerinde kontrol annelerine göre anlamlı derecede daha kısa olması ve açık kollardan kaçınma eğiliminin daha fazla olduğu bulunmuştur. Açık kollarda geçirilen sürenin kaygı ile ters orantılı bir ölçü olduğu düşünüldüğünde, bu çalışmanın sonuçları, kısıtlama stres protokolünün hamile ratlarda stresi başarılı bir şekilde indüklediğini açıkça göstermiştir.

Bu tez kapsamında motor lateralite testlerinin gerçekleştirilmesi amacıyla; ratların vücut büyüklüklerine göre ayarlanabilir olan, Collins Pati Tercihi Belirleme Testi, Pasta Matrix Erişim Testi ve Baş Çevirme Asimetrisi Testlerini içeren yeni bir test cihazı geliştirilmiştir. Bu testler sırasında test edilen ratın hareketleri sürekli olarak video kaydı alınmıştır. Collins testi her bir oturum 30 dakika olacak şekilde uygulanmıştır. Test kapsamında, ratın patilerini kullanarak test aparatı içerisinde yer alan tüp içerisinden ödül mamasına erişmesi beklenmiştir. Ratların kullandıkları pati sayıları gerçek zamanlı olarak sayılmış oluıp, 50 patiye ulaştığında test sonlandırılmıştır. Ratların tek test oturumunda 50 pati yanıtına ulaşmaması durumunda ise, gerekli sayıya ulaşana kadar sonraki günlerde test uygulanmaya devam edilmiştir. Pasta testi, Collins testine alternatif bir başka pati tercihi belirleme testi olarak kullanılmıştır. Testin uygulanma prensibi Collins testine benzer şekilde olup, ratlar için cazip bir yiyecek olan fındık kremasına batırılmış makarna çubukları spagettiler aparata yerleştirilerek ratın bu ödül mamasına erişimi gözlemlenmiştir. Pasta testi, rat Collins testinde 50 pati yanıtını tamamladığında ya da Collins testine ve kullanılan mama ödülüne hayvanın hiç ilgi göstermediği durumlarda (genellikle 4-5 seanstan sonra) uygulanmıştır. Her test bir oturumu 15 dakika olacak şekilde hayvanlar test edilmiştir. Ayrıca, Collins testi bu çalışmada birincil ve standart pençe tercihi belirleme yöntemi olduğundan, mama ödülüne ve aparatın besleme tüpüne dikkat etmeye devam eden hayvanlara testin son gününe kadar Collins testi uygulanmaya devam edilmiştir. Video analizleri, deneyi gerçekleştiren kişi ve bağımsız gözlemci tarafından her iki test için de aynı şekilde değerlendirilmiştir. Sağ ve sol pati tercihinin belirlenmesinde binomial Z-değeri; lateralizasyonun gücünün belirlenmesinde ise lateralizasyon indeksi (LI) göz önünde bulundurulmuştur. Collins testi sonuçlarına göre kontrol grubunda ratların %35,7'si sağ, %23,8'si sol pati tercihi gösterirken, %40,4'ü ise ambilateral olarak belirlenmiştir. Stres grubunda ise ratların %21'i sağ, %47,3'ü sol pati tercihi göstermiş %31,5'i ise ambilateral olarak belirlenmiştir Çalışmanın sonuçları, yakın bir zaman önce ratlarda pati tercihi üzerine yapılan bir meta-analiz çalışmasının sonuçlarına paralel olarak, kontrol grubu ratların popülasyon düzeyinde bir asimetrinin göstermezken ve bireysel düzeyde asimetri gösterdiklerini doğrulanmıştır. İlginç bir şekilde, stres grubunda ise, prenatal stres tarafından indüklendiği düşünülen atipik ve değiştirilmiş davranışsal lateralizasyon olarak kabul edilebilecek bir eğilimle sola doğru popülasyon düzeyinde asimetri olduğu bulunmuştur. Stres grubundaki popülasyon seviyesindeki sola doğru koydumasimetri, bu çalışmanın ana hipotezini doğrulayarak ve ratlarda prenatal stresin pati tercihleri üzerinde ana etkisinin olduğunu göstermektedir. Test sırasında kullanılan ilk pati ve Z-Score arasındaki ilişki incelendiğinde ilk pati kullanımının genel pati tercihinin belirlenmesinde iyi bir gösterge olduğu bulunmuştur. Tez kapsamında, prenatal stresin lateralizasyon indeksi üzerine önemli bir etkisinin olduğu ortaya konulmakla beraber, ratların stres durumunda sol pati tercihinin arttığı görülmüştür. Ayrıca, istatistiksel analizler sonucunda, yaşın bireysel LI değeri üzerine etkisinin olduğu görülmüş ve PG21'deki yavruların diğer yaş gruplarına göre daha az LI değerine sahip olduğu yani lateralizasyon gücünün az olduğu görülmüştür. Cinsiyetin etkisine bakıldığında ise kontrol grubunda anlamlı bir değişiklik görülmezken stresli grupta bireysel LI değerinin erkeklerde dişilerden daha yüksek olduğu bulunmuştur.

Baş Çevirme Asimetrisi Testinde ratların baş ve vücut döndürme asimetrilerini belirlemek için motor öğrenme veya beceri gerektirmeyen basit içme davranışları kaydedilmiştir. Baş ve vücut döndürme asimetri testi, Collins ve Pasta Matrix Uzanma Testleri tamamlandıktan sonra 4 gün boyunca 30'ar dakikalık ardışık 4 seans halinde uygulanmıştır. Fakat sunulan doktora tezinde detaylı olarak açıklanan teknik limitasyonlardan ötürü bu davranış testinden güvenilir sonuçlar elde edilememiştir.

Davranış testlerinden sonra, derin anestezi uygulanmış olan ratlardan beyin dokusu eldesi için dekapitasyon işlemi gerçekleştirilmiştir. Çok hızlı bir şekilde çıkartılan beyinler sıvı nitrojen ile muamele edilerek şoklanmış ve hemen akabinde de sonraki genetik analizler için -80 °C'de saklanmıştır. Daha sonra beynin motor korteks bölümleri anatomik olarak dissekte edilmiştir. Ardından doku homojenizasyonu yapılarak RNA izolasyonu gerçekleştirilmiştir. RNA miktar ve kalite açısından spektrofotometrik olarak değerlendirilmiş, bütünlüğü ve saflığı denatüre jel üzerinde kontrol edilmiştir. Bunlara ek olarak stres ve kontrol gruplarından rastgele seçilen dişi ve erkek hayvanların RNA numuneleri de RNA bütünlüğünün kapiller elektroforez tekniğine ile doğrulanmıştır.

Collins Testi sonuçları dikkate alınarak, prenatal stresin neden olduğu nörobiyolojik değişikliklerin araştırılması için prenatal stres etkisinin daha belirgin olduğu Postnatal gün (PG) 60 grubuna (stres n=6, kontrol n=6) ait dişi bireyler mikrodizin temelli gen ifade analizinde kullanılmıştır. PG60'a ait kontrol ve stres grubundaki dişilerin sağ ve sol hemisferlerinin motor korteksinden elde edilen RNA'lardan toplamda 4 adet RNA gen havuzu (Stres-sağ, Stres-sol, Kontrol-sağ ve Kontrol-sol) oluşturulmuştur. 100 ng total RNA'lar üretici firmanın talimatları doğrultusunda Siyanin 3-CTP (Cy3) floresan boyasıyla etiketlenerek işaretli cRNA'lar elde edilmiştir. Bu işaretli cRNA'lar pürifiye edilmiş ve spektrofotometrik olarak değerlendirilmiştir. Etiketlenmiş cRNA'lar üreticinin talimatlarına göre mikrodizi çipi üzerine hibridize edilmiştir. Hibridizasyon sonrası diziler yıkanmış ve yıkama işlemi sonrasında mikrodizin çipi yüksek çözünürlüklü DNA Mikrodizin Tarayıcı'da taranmıştır.

Mikrodizin analizinde PG60'a ait kontrol ve stres grubundaki dişilerden elde edilen RNA'lardan oluşturulan her gen havuzu toplam 26.930 oligonükleotide özgü probdan oluşan Agilent Rat Gene Expression (4x 44K) Microarray kullanılarak analiz edilmiştir. Kontrol-sol ve Kontrol-sağ hemisferler, Stres-sol ve Kontrol-sol hemisferler ile Stres-sağ ve Kontrol-sağ hemisferler arasındaki ifade farklılıklarını değerlendirmek için arka plan düzeltmesi (BC) yapılan ve yapılmayan iki veri seti oluşturulmuştur. Arka plan düzeltmesinden sonra kuantil normalizasyon gerçekleştirilmiştir. Tüm karşılaştırmalar için $p<0,05$ olarak alınmıştır. Kontrol-sol ve Kontrol-sağ hemisfer karşılaştırmalarına göre, BC yapıldığında 1 gen, BC olmadan ise 5 genin ifadesinde farklılık olduğu tespit edilmiştir. Stres-sol ve Kontrol-sol hemisferler arasında, BC uygulandığında 5 genin; BC uygulanmadığında ise 24 genin farklı şekilde ifade edildiği belirlenmiştir. Stres-sağ ve Kontrol-sağ hemisfer karşılaştırmasında ise, BC uygulandığında 70 genin, BC uygulanmadığında ise 280 genin ifadesinde farklılık olduğu belirlenmiştir. Sonuç olarak, gen ifade analizinde stres ve kontrol grupları açasından karşılaştırma yapıldığında sağ hemisferde sol hemisferden daha fazla sayıda genin ifadesinde farklılık olduğu gösterilmiştir.

Mikrodizin analizinden elde verilen ışığında, stres ve kontrol sağ hemisferleri arasında en yüksek kat değişimine sahip olan iki gen olan *FOXE1* ve *POLM* genleri hedef gen olarak seçilerek, qRT-PCR analizi PG60 grubuna ait dişi ve erkek bireyler için gerçekleştirilmiştir. Normalizasyon için *YWHAZ* ve *ITPR1* genleri referans gen olarak kullanılmıştır. Seçilen her bir gen için iki tekrarlı qRT-PCR analizi gerçekleştirilmiştir. Ayrıca plateler arası varyasyonu önlemek adına her bir plate teknik kopyaları bulunan tek bir örnek kalibratör olarak kullanılmıştır. Her bir hedef gen için örneklerin ifade analizleri, plateler arası

kalibrasyon ve referans gen stabilite analizleri Bio-Rad CFX Maestro Software (Version 2.3) kullanılarak gerçekleştirilmiştir. İfade değişikliklerinin hesaplanması için ΔΔCq değerleri kullanılmış ve ifade verilerinin analizi için Student t-testi uygulanmıştır. $p<0,05$ istatistiksel olarak anlamlı kabul edilmiştir. Mikrodizin analizde kullanılan kontrol ve stres dişi örneklerinden elde edilen havuz RNA örnekleriyle yapılan qRT-PCR analiz sonuçları mikrodizin sonuçlarıyla örtüşmesine karşın yeterli miktarda havuz RNA örneği olmadığı için sadece birer teknik replikaları ile beraber analiz edilebilmişlerdir ve anlamlılık testi yapılamamıştır. Bireysel olarak analiz edilen örneklerin qRT-PCR sonuçları ise her iki genin de ekspresyon kat değişimleri cinsiyete ve hemisfere göre anlamlı kat değişimi gösterirken, *FOXE1* geni için ise daha anlamlı kat değişimleri gösterdiği bulunmuştur. Daha büyük örneklem grubuyla bu iki genin ilerleyen lateralizasyon çalışmalarında incelenmesi gerekmektedir.

Bu çalışma, kontrol grubu dişi ve erkeklerin sağ hemisferleri arasında *Foxe1* geninin ekspresyon düzeyinde bir fark olmadığını, stres maruziyetinde ise dişi ve erkeklerin sağ hemisferleri arasında anlamlı farklı olarak ifade edildiğini göstermektedir. *Foxe1* geni ifadesine benzer şekilde, *Polm* geninin sağ hemisferde dişiler ve erkekler arasında ifade düzeyi için de önemli farklılıklar belirlendi. *Polm* geninin ekspresyon seviyesinin, hem stres hem de kontrol grubu erkeklerin sağ hemisferlerinde, dişilerin sağ hemisferlerine kıyasla daha fazla yukarı doğru regüle olduğu bulundu. Bu sonuçlar, stres maruziyetinden bağımsız olarak *Polm* geninin ekspresyonu için sağ hemisferde cinsiyete bağlı bir farklılaşma olduğunu gösterdi.

Bu çalışma, ratlarda farklı motor lateralite testleri ve gen ekspresyon analizleri kullanılarak prenatal stresin, fonksiyonel serebral asimetriler üzerindeki etkisini, doğum sonrası farklı gelişim aşamalarında araştıran ilk çalışmadır. Çalışmanın ana sonuçları aşağıda özetlenmiştir. Prenatal stresin yavruların doğum sonrası gelişim döneminde kilo alma kapasitesini etkilediğini, prenatal stresin annelerin bakım davranışları üzerinde herhangi bir etkisi olmadığını göstermektedir. Bununla birlikte, prenatal stresin annenin yavru bakım davranışları üzerinde önemli bir etkisi gözlenmemiştir. EPM deneyinden elde edilen sonuçlar, gebeliğin son üçte birlik diliminde gebe ratlara uygulanan hareket kısıtlama protokolünün gebe ratlarda etkili bir şekilde strese neden olduğunu göstermektedir. EPM analizinden elde edilen bulgular, prenatal strese maruz kalan PD60 yaş grubundaki hayvanların, kontrol grubundakilere kıyasla keşif davranışlarında azalma ve kaygı ile ilgili davranışlarda artış gösterdiğini ortaya koymaktadır. Bu çalışma, erken yetişkinlik dönemi olan PG60 yaşının, ratlarda prenatal stresin etkilerinin özellikle belirgin olduğu kritik bir postnatal gelişim evresini temsil edebileceğini öne sürmektedir. Mevcut çalışma, prenatal stresin genel dönme davranışı üzerindeki etkisini destekleyen herhangi bir kanıt ortaya koymamıştır. Mevcut çalışmada üç farklı motor lateralite testinin birleştirilmesiyle tasarlanan yeni test aparatı, standart bir metodoloji haline gelme ve rodentlerde daha ileri çalışmalara katkıda bulunma potansiyeline sahiptir. Bu çalışmanın en önemli sonucu, prenatalın stresin yaşamın ilerleyen aşamalarında fonksiyonel serebral asimetrileri etkilediğini göstermesidir. Ayrıca, çalışmanın bir diğer ilginç bulgusu da prenatal stresin ratlarda pati tercihi için popülasyon düzeyinde sola doğru yanlılığa neden olmasıdır, bu da strese maruz kalmanın davranış düzeyinde değişikliklere sebep olan sağ hemisferik aktivasyona neden olduğunu göstermektedir. Bu çalışma aynı zamanda ilk pati kullanım yönünün ratların genel pençe tercihleri hakkında iyi bir gösterge olduğunu bildirmektedir. Bu çalışmanın bir diğer önemli bulgusu, prenatal stresin ratların sol yarım küresine kıyasla sağ yarım küresindeki gen ifadelerinde daha büyük değişikliklere neden olmasıdır. Bu çalışma, prenatal stresin ratlarda gen ifadesi düzeyinde beynin sağ yarım küresi üzerinde sola kıyasla daha büyük bir etkiye sahip olduğunu gösteren ilk kanıtı temsil etmektedir. *YWHAZ* ve *ITPR1* genleri, bu çalışmanın hem mikro dizi tabanlı gen ifade düzeyi analizine hem de qRT-PCR doğrulamalarına göre, rat beyninin motor korteklerinden elde edilen örneklerin

stresle ilişkili gen ifade analizi için en kararlı ve güvenilir referans genler olarak önerilmektedir. Bu çalışmanın mikrodizin tabanlı gen ifadesi analizine göre, *FOXE1* ve *POLM* genleri stres ve kontrol grubundaki dişi bireylerin sağ hemisferleri arasında en farklı şekilde ifade edilen genlerdir. *POLM* geninin prenatal strese yanıt olarak farklı şekilde regüle olması ve özellikle sağ hemisferde davranışsal lateralite ile korelasyonu, *POLM* geninin stres ve lateralizasyon arasındaki ilişkiyi araştıran gelecekteki araştırmalar için aday bir gen olma potansiyeli sergilediğini göstermektedir.

Anahtar Sözcükler: Davranışsal lateralizasyon, FOXE1, Mikrodizin, POLM, Prenatal stres

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