

USING ZEBRAFISH DISEASE MODEL IN MOLECULAR RESEARCH ON NEURODEVELOPMENTAL DISORDER GENERATION

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By
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We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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In this thesis, zebrafish were used as a model organism to study two different projects: issues stemming from the dopaminergic system during the neurodevelopmental period, specifically ADHD, and a Parkinson's disease model representing dopaminergic system disorders in old age. This approach allowed for an investigation of the lifelong effects of disorders caused by dopaminergic system dysfunction.

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent condition characterized by persistent inattention, hyperactivity, and impulsivity, often leading to significant impairments in daily functioning. Numerous medications have been developed to manage ADHD, with methylphenidate (commonly known as Ritalin or Concerta) being the most widely used active compound. While methylphenidate effectively improves attention, focus, and emotional regulation, its long-term use can lead to behavioral issues such as increased depression and anxiety, as well as physical side effects like sleep disturbances and decreased sensitivity to rewards. These side effects are more challenging to compensate for in adults compared to children, which may leave permanent effects. This study aims to investigate the long-term effects of methylphenidate use in adult women, with a focus on its role in sleep disorders, circadian rhythm disruption, and the potential implications for pregnancy, specifically on the susceptibility of offspring to accelerated brain aging. Our hypothesis is that methylphenidate use during pregnancy may contribute to changes in offspring telomere length and gene methylation patterns associated with brain aging, thereby increasing their vulnerability to neurodegeneration. To evaluate this, telomere length and methylation analyses were conducted on genes linked to brain aging in second-generation

offspring. Additionally, our hypothesis is that therapeutic interventions, such as melatonin for regulating sleep disturbances and oxytocin as an alternative to mitigate methylphenidate's side effects, may have protective effects. Zebrafish were used as the model organism in this study due to their high genetic similarity to humans and their ease of egg production, enabling multi-generational studies. As a result of this study, it was determined that neuroinflammation caused by circadian rhythm disruption and maternal stress was reduced through melatonin & methylphenidate and oxytocin & methylphenidate combinational treatments. In the offspring of the next generation, it was observed that the telomere length inherited at birth was shorter when treated with methylphenidate only.

In the second research project of this thesis, the origins of Parkinson's disease from two distinct locations—the brain and the gut—were investigated. Parkinson's disease is a neurodegenerative disorder characterized by the loss or reduction of dopaminergic neurons in the central nervous system. It is associated with aggregation of alpha-synuclein fibrils and resulting in motor function impairments. In the previous phase of this research, transgenic zebrafish models of Parkinson's disease were developed by integrating human alpha-synuclein gene into the zebrafish genome at the single-cell stage. In this phase of thesis, six-month-old transgenic zebrafish were used to test the hypothesis that Parkinson's disease can originate from either the brain or the gut. Alpha-synuclein protein was injected into the brain and gut, and its migration between these two organs was analyzed. The migration of alpha-synuclein fibrils was validated using immunohistochemistry techniques. Behavioral changes and motor function impairments were assessed using novel tank tests, swim endurance tests, and hyposmia tests. As a result of the study, it was validated that alpha-synuclein fibrils injected into the brain-to-gut and gut-to-brain migrate at different speeds.

Keywords: Methylphenidate, Circadian Rhythm, Epigenetic Transmission, Melatonin, Oxytocin, Parkinson's Disease, Transgenesis, Alpha-Synuclein, Zebrafish.

ÖZET

NÖROGELİŞİMSEL BOZUKLUK OLUŞUMUNA İLİKİN MOLEKÜLER ARAŞTIRMALARDA ZEBRA BALIĞI HASTALIĞI MODELİNİN KULLANILMASI

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Bu tezde zebrafish, iki farklı proje kapsamında model organizma olarak kullanılmıştır: nörogelişimsel dönemde dopaminerjik sistemden kaynaklanan sorunlar (özellikle DEHB) ve yaşlılıkta dopaminerjik sistem bozukluklarını temsil eden bir Parkinson hastalığı modeli. Bu yaklaşım, dopaminerjik sistem disfonksiyonunun neden olduğu bozuklukların yaşam boyu etkilerinin araştırılmasını sağlamıştır.

Dikkat eksikliği ve hiperaktivite bozukluğu (DEHB), sürekli dikkat eksikliği, hiperaktivite ve dürtüsellik ile karakterize edilen ve günlük işlevlerde önemli bozulmalara yol açan yaygın bir durumdur. DEHB'yi yönetmek için çok sayıda ilaç geliştirilmiş olup, metilfenidat (yaygın olarak Ritalin veya Concerta olarak bilinir) en yaygın kullanılan aktif bileşiktir. Metilfenidat, dikkat, odaklanma ve duygusal düzenlemeyi etkili bir şekilde iyileştirirken, uzun süreli kullanımı, artan depresyon ve anksiyete gibi davranışsal sorunlara ve uyku bozuklukları ile ödüllere karşı azalmış duyarlılık gibi fiziksel yan etkilere yol açabilir. Bu yan etkiler, yetişkinlerde çocuklara kıyasla telafi edilmesi daha zor olup, kalıcı etkiler bırakabilir. Bu çalışma, metilfenidat kullanımının uzun vadeli etkilerini yetişkin kadınlarda incelemeyi ve bu etkilerin uyku bozuklukları, sirkadiyen ritim bozulmaları ve özellikle gebelik sırasında çocukların hızlanmış beyin yaşlanmasına yatkınlığı üzerindeki potansiyel etkilerini araştırmayı amaçlamaktadır. Hipotezimiz, gebelik sırasında metilfenidat kullanımının, telomer uzunluğu ve beyin yaşlanmasıyla ilişkili gen metilasyon kalıplarında değişikliklere katkıda bulunarak, çocukların nörodejenerasyona yatkınlığını artırabileceğidir. Bunu değerlendirmek için ikinci nesil yavrularda telomer uzunluğu ve beyin yaşlanmasıyla ilişkili

genlerin metilasyon analizleri gerçekleştirilmiştir. Ayrıca, uyku bozukluklarını düzenlemek için melatonin ve metilfenidatın yan etkilerini azaltmak için alternatif olarak oksitosin gibi terapötik müdahalelerin koruyucu etkileri olabileceği hipotezimiz vardır. Bu çalışmada, insanlar ile yüksek genetik benzerlikleri ve çok nesilli çalışmalar yapmayı kolaylaştıran yüksek yumurta üretim kapasiteleri nedeniyle zebrafish model organizma olarak kullanılmıştır. Çalışmanın sonucunda, sirkadiyen ritim bozukluğu ve anne stresi nedeniyle oluşan nöroenflamasyonun melatonin ve metilfenidat ile oksitosin ve metilfenidat kombinasyon tedavileri ile azaltıldığı belirlenmiştir. Bir sonraki neslin yavrularında ise yalnızca metilfenidat ile tedavi edildiğinde doğumda miras alınan telomer uzunluğunun daha kısa olduğu gözlemlenmiştir.

Tezin ikinci araştırma projesinde ise Parkinson hastalığının beyinden ve bağırsaktan kaynaklanan iki farklı kökeni araştırılmıştır. Parkinson hastalığı, merkezi sinir sistemindeki dopaminerjik nöronların kaybı veya azalması ile karakterize edilen nörodejeneratif bir bozukluktur. Bu durum, alfa-sinüklein fibrillerinin birikimi ile ilişkilidir ve motor fonksiyon bozukluklarına neden olur. Araştırmanın önceki aşamasında, insan alfa-sinüklein geninin tek hücre aşamasında zebrafish genomuna entegre edilmesiyle transgenik zebrafish modelleri geliştirilmiştir. Tezin bu aşamasında, altı aylık transgenik zebrafishler, Parkinson hastalığının beyinden veya bağırsaktan kaynaklanabileceği hipotezini test etmek için kullanılmıştır. Alfa-sinüklein proteini beyne ve bağırsağa enjekte edilmiş ve bu iki organ arasındaki hareketi analiz edilmiştir. Alfa-sinüklein fibrillerinin hareketi immünohistokimya teknikleri kullanılarak doğrulanmıştır. Davranış değişiklikleri ve motor fonksiyon bozuklukları, yeni tank testleri, yüzme dayanıklılık testleri ve koku alma bozukluğu testleri kullanılarak değerlendirilmiştir. Çalışmanın sonucunda, beyin-bağırsak ve bağırsak-beyin yönlerinde enjekte edilen alfa-sinüklein fibrillerinin farklı hızlarda hareket ettiği doğrulanmıştır.

Anahtar sözcükler: Metilfenidat, Sirkadiyen Ritim, Epigenetik Aktarım, Melatonin, Oksitosin, Parkinson Hastalığı, Transgenez, Alfa-Sinüklein, Zebra Balığı



To My Family and My Husband...

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Chapter 1

Disruption of Circadian Rhythm and Its Epigenetic Transmission to Offspring in terms of Brain Aging Acceleration

1.1 Introduction

1.1.1 Attention-deficit/hyperactivity disorder and its Prevalence

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent condition marked by persistent inattention, hyperactivity, and impulsivity, often leading to significant impairments in daily functioning. While genetic factors are the primary contributors to ADHD, environmental influences also play a role, though many remain poorly understood[1][2]. Insights from extensive epidemiological, clinical, and longitudinal studies have highlighted the core symptoms of ADHD, its associated impairments—such as academic and occupational difficulties, poor social

interactions, and increased risk of accidental injuries—and its frequent comorbidities, including anxiety and mood disorders. ADHD is a diverse disorder with symptoms that vary between individuals and fluctuate over time within the same person [3][4]. Symptoms often worsen under conditions such as increased self-regulation demands, weak support systems, intensified comorbidities, or poor environmental fit. Recurrence is common, even after full remission. Severe ADHD typically has an earlier onset and a more stable course into adulthood compared to milder cases[5][6].

1.1.1.1 Molecular mechanisms related to ADHD

Multiple neurotransmitter circuits are implicated in ADHD. The dopaminergic system is critical for regulating movement, mood, attention, learning, memory, and reward and novelty processing. The noradrenergic system, on the other hand, is essential for attention, arousal, mood regulation, stress response, and signal-to-noise processing. While these two systems interact, they also exert distinct and independent effects[7].

The most extensive genome-wide association studies (GWAS) on ADHD identified 27 significant loci across the genome, implicating a total of 76 genes. Many of these genes are notably upregulated during early stages of brain development. The genetically based risk for ADHD shows enrichment in genes linked to specific neuronal subtypes in the brain and midbrain dopaminergic neurons and ADHD-associated genes are generally expressed in the neurons which are located in dopaminergic system in the midbrain[8].

ADHD risk has been associated with common genetic variants located in genes that are significantly expressed in the brain, particularly within the frontal cortex. An enrichment of ADHD-related variants has been identified in genes expressed in major brain cell types, including both excitatory and inhibitory neurons, as well as midbrain dopaminergic neurons. These findings are consistent with the motor, reward, and executive function impairments typically observed in ADHD. Executive functions such as attention and working memory are mediated by the

frontal cortex, while voluntary movement and reward processing are regulated by midbrain dopaminergic neurons[9][10][11].

In terms of levels of dopaminergic D2/D3 receptor availability in ADHD participants had lower D2/D3 receptor availability in the left hemisphere than the controls. The regions encompassed areas involved in the reward pathway, including the ventral caudate, nucleus accumbens, midbrain, and hypothalamus[12].

1.1.1.2 Genetic components of ADHD

Twin and family studies, including meta-analyses, indicate that ADHD has a high heritability of approximately 80%, highlighting the significant role of genetic factors in its development. This heritability remains consistent across sexes, age groups (childhood and adulthood), and symptom types (inattentive and hyperactive-impulsive). Research also shows that the same genetic factors contribute to both clinical ADHD and milder ADHD-like traits in the general population. Moreover, shared genetic influences partially explain the frequent co-occurrence of ADHD with other psychiatric disorders, such as anxiety, major depressive disorder, bipolar disorder, autism spectrum disorder, conduct disorder, and substance use disorders, as well as certain physical health conditions. Additionally, genetic links have been identified between ADHD and cognitive performance, EEG variability, and subtle structural brain differences[13][14][15][16].

Molecular genetic studies have reinforced findings from twin and family studies, highlighting a polygenic contribution to ADHD risk. Common genetic variants, such as single-nucleotide polymorphisms (SNPs), account for an estimated 14–20% of ADHD heritability. These studies reveal high genetic correlations across sexes and between childhood and adult ADHD diagnoses. SNP-based analyses also show strong genetic links between clinical ADHD and ADHD-like traits in the general population, as well as associations with various mental and physical conditions. Among these, educational attainment stands out, with higher ADHD polygenic scores linked to poorer cognitive performance and educational outcomes. Genetic variants associated with ADHD have also been connected to

smaller intracranial brain volumes and altered functional connectivity in subcortical brain regions[17][18][19].

The largest genome-wide association studies (GWAS) of ADHD identified 27 significant loci involving 76 genes, many of which are upregulated during early brain development. These genes are enriched in pathways associated with brain-specific neuronal subtypes and midbrain dopaminergic neurons. Key genes implicated include FOXP1 and FOXP2, which are linked to speech disorders and intellectual disabilities, as well as SORCS3, PTPRF, and MEF2C, which play critical roles in synaptic development and postsynaptic density membrane structure, further implicating synaptic dysfunction in ADHD pathology[20][21][22][23][24].

Research on rare genetic variants in ADHD have highlighted a higher prevalence of rare protein-truncating variants in genes that are evolutionarily conserved. Furthermore, several copy number variants (CNVs) associated with ADHD have been consistently linked to other conditions, such as autism spectrum disorder and schizophrenia[25][26].

Research has linked the polygenic risk for ADHD to negative health behaviors and psychosocial impairments, suggesting that some environmental factors associated with ADHD may partially reflect genetic influences. Studies adjusting for familial confounders, including genetic factors, have shown that certain environmental associations with ADHD weaken or disappear. For instance, while many epidemiological studies and meta-analyses have reported a link between maternal smoking during pregnancy and ADHD, sibling-control studies have found no such association. These findings are supported by evidence that a mother’s polygenic risk for ADHD strongly predicts whether she smokes during pregnancy. Additionally, studies have shown that maternal smoking does not predict ADHD in offspring when the pregnancy results from a donated oocyte, further emphasizing the role of genetic factors[27][28][29].

MRI mega-analyses have identified minor to moderate structural brain differences in children with ADHD compared to controls, though these differences

are not observed in adolescents or adults. Affected regions include a smaller total cortical surface area in late-developing cortical association areas, such as the frontal, cingulate, parietal, and temporal regions, which are crucial for executive functions. Additionally, reductions in cortical thickness have been noted in areas involved in emotion processing, including the fusiform gyrus and temporal pole[30][31].

1.1.1.3 ADHD and its effects on neurodevelopment

Current evidence supports the understanding of ADHD as a neurodevelopmental disorder characterized by delayed brain maturation. Studies of cortical thickness in individuals with ADHD compared to matched controls suggest that ADHD is not a deviation from typical development but rather a condition marked by delayed peak cortical thickness in key brain regions, including the prefrontal cortex[32][33]. This delay extends to cortical surface area maturation, in individuals with ADHD reaching peak area in the right prefrontal cortex at a median age of 14.6 years, compared to 12.7 years in healthy controls. Several studies further confirm neurodevelopmental abnormalities in ADHD, showing approximately 4% smaller volumes in cerebral and cerebellar regions, gray and white matter, and lobes compared to unaffected individuals. Volume reductions have been reported in the frontal lobes, premotor cortex, posterior cingulate, anterior and medial temporal lobes, cerebellar lobules, and basal ganglia structures. Additionally, children with ADHD who were not exposed to stimulant medication like concerta and ritalin demonstrated more rapid cortical thinning in the frontal-parietal areas between the ages of 2.5 and 16.4 years, indicating reduced cortical maturation [34] [35] [36] [37] [38].

Research on both common and rare genetic variants suggests that many ADHD-associated loci are involved in neurodevelopmental processes, supporting the connection between ADHD and changes in brain development, structure, and function. However, a significant limitation of these studies is that they predominantly rely on samples of European ancestry.

It is also shown that ADHD associated genes can have a role in neurodevelopment from different perspectives like neurogenesis, migration, synaptogenesis and selective cell death like BDNF, DRD5, FOXP2, GRM1, NOS, SLC6A4, SNAP25, PTPRF[39][40][41][42].

1.1.1.4 ADHD and sleep problems

Children with ADHD often experience a range of sleep difficulties, including resistance to bedtime, delayed sleep, frequent night and early morning awakenings, sleep-disarranged breathing, and excessive daytime sleepiness[43].

The pharmacological management of attention-deficit/hyperactivity disorder (ADHD) involves the use of both stimulant medications, such as methylphenidate (MPH) and amphetamine formulations, and non-stimulant options, including atomoxetine and alpha-2 agonists. These medications, available in various formulations (e.g., short-acting and long-acting, stimulant and non-stimulant), can significantly influence sleep patterns in individuals with ADHD, often with varying effects depending on the specific type and duration of the medication [44][45][46].

Children with sleep disorders and ADHD symptoms often show notable improvements in attention and hyperactivity when the underlying sleep disorder is treated. For instance, research on children with sleep-disordered breathing has demonstrated that surgical removal of tonsils and adenoids can lead to reduced ADHD symptoms, likely due to enhanced sleep quality post-surgery. Additionally, insufficient sleep or sleep restriction has been shown to worsen inattention and cognitive symptoms in both children with or without ADHD. In cases of ADHD accompanied by chronic sleep-onset insomnia, studies indicate that administering 3-6 mg of melatonin can shorten the time it takes to fall asleep and increase total sleep duration. However, despite these improvements in sleep, no significant effects were observed on ADHD symptoms, cognitive function, or quality of life. This suggests that while addressing primary insomnia (unrelated to stimulant medication) may improve sleep parameters, it does not necessarily lead

to the resolution of ADHD symptoms. This situation creates the need for alternative treatment methods like actualized in this thesis like oxytocin treatment besides the melatonin treatment[47].

1.1.1.5 Prevalence of ADHD in adults

The sex ratio of ADHD varies across the lifespan, reflecting differences in symptom presentation, recognition, and help-seeking behaviors. In youth, the male-to-female ratio is approximately 2.4:1 in the general population, increasing to 4:1 among those seeking treatment due to boys' more disruptive behavior. This ratio decreases to 2:1 in adolescence and narrows further in adulthood, ranging from 1.9:1 to 1.1:1 depending on the data source. These changes are influenced by factors such as delayed recognition of ADHD in females, a greater likelihood of women seeking help as adults, and the reduced emphasis on hyperactivity in adult ADHD diagnoses, which aligns more closely with female symptom patterns. Additionally, self-referral replaces parent referral in adulthood, and women are more likely than men to seek professional help for mental health concerns [48][49][50][51].

In adults, ADHD symptoms often differ from those in children, reflecting the unique demands of adult life. Common challenges include academic or workplace underperformance, difficulties in maintaining relationships, a higher risk of accidents, substance misuse (especially alcohol and stimulants), and mental health issues. Structural brain differences linked to ADHD persist into adulthood, suggesting that while symptoms may lessen with age, adults may still experience disorganization, impulsivity, restlessness, difficulty focusing, and procrastination, which can impair daily life, well-being, and productivity[52][53][54].

The question of whether adult ADHD is a continuation of childhood-onset neurodevelopmental disorder or a distinct condition was explored in a landmark longitudinal study published in the American Journal of Psychiatry. Tracking a cohort of 1,037 individuals over four decades, the study assessed ADHD diagnoses in childhood and adulthood through multiple data sources, including participant,

parent, and teacher reports, neuropsychological tests, and administrative records. It analyzed ADHD symptoms, clinical features, comorbidities, neuropsychological deficits, polygenic risks, and life impairment indicators. Key findings included: childhood ADHD, affecting 6% of individuals (primarily males), was linked to neurocognitive deficits, childhood comorbidities, and polygenic risks, with residual adult impairments. Another key finding for adult ADHD, with a lower prevalence of 3% and gender balance, was associated with substance dependence, life impairment, and treatment-seeking, but 90% of adult cases had no history of childhood ADHD. Moreover, adults with ADHD showed no childhood neuropsychological deficits or genetic risks associated with childhood ADHD, suggesting adult ADHD may be a distinct condition[55].

1.1.2 Treatment Options for Attention-Deficit/Hyperactivity Disorder

Medications approved for treating ADHD by regulatory agencies, including the FDA, fall into two categories: stimulants and non-stimulants. Stimulant medications, such as methylphenidate and amphetamine formulations, are commonly prescribed. Non-stimulants include noradrenaline reuptake inhibitors like atomoxetine and viloxazine extended release (ER), as well as α 2-adrenergic agonists such as clonidine ER and guanfacine ER. Outside of North America, methylphenidate is often the sole licensed stimulant medication available for ADHD treatment[56][57][58].

1.1.2.1 Methylphenidate, the most prevalent treatment for ADHD

Methylphenidate (MP), commonly known under the brand name Ritalin®[®], is the preferred medication for managing attention deficit hyperactivity disorder (ADHD). Although MP was initially synthesized in 1944 and patented a decade later in 1954, it wasn't until 2002 that the FDA formally approved its use for treating ADHD[59].

Methylphenidate, a widely used stimulant for treating ADHD, works by increasing the availability of dopamine and norepinephrine in the brain without directly increasing their overall production. Instead, it achieves this by blocking their reuptake through dopamine and norepinephrine transporters, preventing these neurotransmitters from being recycled back into the nerve cells. This mechanism enhances neurotransmitter signaling, particularly in the prefrontal cortex, a region critical for attention, impulse control, and executive functioning, as well as in the striatum, which is involved in reward processing and motor control. By targeting the underactivity of dopaminergic and noradrenergic systems often seen in individuals with ADHD, methylphenidate helps regulate behavior, improve focus, and enhance cognitive performance. The drug’s effects are immediate, but their duration depends on the formulation, such as immediate-release or extended-release variants, allowing for tailored symptom management. These therapeutic effects address the core symptoms of ADHD, making methylphenidate a cornerstone in the treatment of this neurodevelopmental disorder[60][61].

1.1.2.2 Animal models to study methylphenidate

There are studies conducted on animals to understand the effects of methylphenidate. Rodents are commonly utilized as models to study the behavioral consequences of methylphenidate (MP). Research has investigated both acute and chronic MP exposure in male rats, examining variables such as strain differences, dosing, and age. Acute studies revealed a dose-dependent relationship across three different doses, while chronic studies demonstrated similar dose-dependent effects, along with activity variations among different rat strains. Age was another critical factor, with adolescence defined as a developmental stage characterized by extensive neuroplastic changes in the central nervous system (CNS). Many studies focus sex-dependent responses to MP, with male rats being predominantly used, reflecting the higher prevalence of ADHD in boys compared to girls [62][63][64][65].

To study MP’s short- and long-term influences, various rat models have been developed. Spontaneously hypertensive rats (SHR), a strain bred to exhibit

ADHD-like impulsivity and hyperactivity, are widely utilized as the primary ADHD animal model. For instance, in one study using variable interval schedules, SHR rats demonstrated a more deferral of reinforcement compared to control rats, with shorter interresponse times, indicating pronounced impulsivity. These models enable researchers to explore the impact of MP on ADHD-related behaviors. However, not all MP studies employ SHR rats; some focus on healthy rats to assess the neuropharmacological and behavioral related influences of MP independent of ADHD pathology[66][67][68][69][70][71].

1.1.2.3 Mechanism of Action of Methylphenidate

Methylphenidate (MP) exerts its effects by inhibiting the reuptake transporters for key neurotransmitters, including dopamine (DAT), norepinephrine (NET), and serotonin (SERT). This inhibition prevents the reabsorption of these neurotransmitters, leading to elevated synaptic concentrations. The striatum has been identified as the primary site of MP uptake, with prior studies highlighting its significant role in MP's mechanism of action. Furthermore, the effects of MP vary depending on the type of dopamine receptor involved; dopamine 1 receptors (DRD1) exhibit heightened brain activity and excitation compared to dopamine 2 receptors (DRD2). As a result, MP functions as a stimulant, enhancing neurotransmitter levels in the synapse to deliver its psychostimulant, therapeutic, and potentially analgesic effects[62][72][73][74].

1.1.2.4 Effects of Methylphenidate on Female Adults

The meta-analysis is the first comprehensive study, to our knowledge, involving over 16 million pregnant women, that evaluates potential adverse outcomes in children born to mothers with ADHD who used specific ADHD medications (methylphenidate and atomoxetine) during pregnancy. The analysis compares these outcomes with those of mothers with ADHD who did not use prescription medications during pregnancy and with the general population. Data from ten

studies, covering 16,621,481 million pregnancies (30,830 of which involved mothers diagnosed with ADHD), were included. The studies were conducted across six countries from Denmark to Netherlands and to the US, and were all published after the year 2014, with half released after 2020. Results indicated a slightly higher frequency of congenital anomalies in offspring of mothers exposed to methylphenidate compared to both unexposed mothers and the general population. However, miscarriage rates were consistent across exposed and unexposed groups[75][76][77][78][79][80][81][82][83][84][85].

The most widely used and thoroughly researched treatments for ADHD include both immediate-release and extended-release types of methylphenidate and amphetamine-based drugs and stimulants. Notably, lisdexamfetamine and the methylphenidate patch are among the longest-acting options within their respective categories, offering symptom relief that can last over 12 hours. However, stimulant medications like methylphenidate are commonly associated with adverse effects on sleep, with insomnia or a delayed sleep onset latency (SOL) of more than 30 minutes being frequent issues. Another prevalent sleep-related problem, particularly in adolescents, is delayed sleep phase syndrome. This circadian rhythm disorder causes a shift in the sleep-wake cycle, resulting in late bedtimes and morning wake times, sometimes to the extent of reversing the day and night schedule. Such disruptions can severely impact academic performance and overall functioning[44][86][87][88].

In addition to these observations, the effects of stimulant medications on sleep in adults with ADHD have also been investigated. Recent studies have highlighted how stimulant treatments, such as methylphenidate (MPH), impact sleep patterns. For example, a polysomnographic study involving eight stimulant-naïve adults demonstrated that open-label MPH treatment improved sleep efficiency—the ratio of time spent asleep relative to time spent in bed—and provided a subjective sense of better restorative sleep. Despite these improvements with medication, ADHD adults still reported later bedtimes, greater difficulty falling and staying asleep, and more pronounced daytime sleepiness compared to non-ADHD controls [44][89][90][91][92][93][94].

The circadian rhythm in both humans and zebrafish regulates biological processes over a 24-hour cycle, aligning them with environmental cues like light and darkness. In humans, the central pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes peripheral clocks in various tissues. Light is the primary time cue, detected by photoreceptors in the retina, which transmit signals to the SCN. Core clock genes such as *CLOCK*, *BMAL1*, *PER*, and *CRY* drive oscillatory gene expression through transcriptional-translational feedback loops (TTFLs). Additionally, melatonin secretion by the pineal gland plays a crucial role in regulating sleep-wake cycles. In contrast, zebrafish, while sharing similar core clock genes and molecular mechanisms, have a unique circadian system. They lack a central pacemaker like the SCN, with peripheral clocks in tissues directly responding to light cues. Zebrafish cells also have photoreceptive elements, making them sensitive to light even outside the brain. While both species use light as the primary environmental cue, zebrafish exhibit a more decentralized circadian regulation compared to humans. Moreover, zebrafish display shorter, polyphasic sleep patterns, unlike the monophasic sleep cycle typical in humans. Despite these differences, zebrafish share conserved circadian mechanisms, such as light sensitivity and gene expression regulation, making them an excellent model for studying circadian biology and its implications for human health[95][96].

The systematic review of 42 studies examines the link between maternal circadian disruption during pregnancy and fetal outcomes, focusing on both short-term and long-term effects. Short-term outcomes, such as miscarriage, preterm birth, and low birth weight, showed inconsistent associations across studies, with significant findings for miscarriage in 89% of the studies but only 45% for preterm birth and low birth weight. For long-term outcomes, a stronger association was found between maternal circadian disruption and offspring development, including altered gene expression, metabolic dysfunction, and neurobehavioral issues such as anxiety and developmental delays. Mechanisms behind these effects include disrupted placental function, sleep deprivation, altered gene expression, and reduced melatonin production, which can negatively affect fetal growth and development[97].

1.1.3 Zebrafish Model Organism

The zebrafish is widely used as a model organism in research due to its numerous advantages. It is a cost-effective alternative to traditional model organisms like rats and mice, making it particularly appealing for large-scale studies. Zebrafish have a rapid reproductive cycle, capable of producing hundreds of offspring with 7-10 day intervals, and their eggs are big, robust, and easy to handle. Their fast neurodevelopmental and aging periods allow for efficient studies of developmental and degenerative processes. Additionally, the transparent nature of zebrafish embryos enables direct observation of external embryonic development, facilitating detailed analysis. Importantly, zebrafish share approximately 70% genomic similarity with humans, with 84% of genes associated with human diseases being highly conserved. Their circadian rhythm further enhances their utility in research involving sleep, behavioral studies, and neurodevelopment[98].

Zebrafish can also be utilized for behavioral assessments related to depression, anxiety, shoaling behavior, and motor dysfunctions. The parallels between human and animal behavior offer diverse opportunities to study these phenomena. By leveraging zebrafish, researchers can replicate and analyze complex human behaviors in a more straightforward and accessible manner, providing valuable insights into underlying mechanisms and potential therapeutic approaches[99].

1.1.4 Brain Aging

Brain aging is influenced by a combination of genetic, molecular, and environmental factors that impact cognitive function, neural integrity, and overall brain health. Key parameters include telomere length, which serves as a biomarker of cellular aging, with shorter telomeres linked to oxidative stress, reduced neurogenesis, and neurodegenerative diseases. Oxidative stress and the accumulation of free radicals damage neuronal cells over time, exacerbating aging-related decline. Inflammation, particularly chronic neuroinflammation, is another critical parameter, contributing to neuronal loss and synaptic dysfunction. Epigenetic

modifications, such as DNA methylation and histone changes, can regulate gene expression linked to aging processes and cognitive decline. Mitochondrial dysfunction, characterized by reduced energy production and increased production of reactive oxygen species, further accelerates neuronal aging. Environmental factors, including disrupted circadian rhythms, chronic stress, poor diet, and sleep disturbances, are also strongly associated with accelerated brain aging. These interconnected parameters collectively determine the trajectory of brain aging and susceptibility to neurodegenerative conditions[100][101].

Epigenetic clocks are among the most extensively studied biomarkers of aging, capturing molecular changes that often precede visible physiological and phenotypic signs of aging. These clocks estimate biological age by analyzing DNA methylation (DNAm) patterns at specific genomic sites. While diversified epigenetic clocks differ in the CpG sites they analyze, they consistently reflect overlapping aging-related processes, including metabolism, immune function, autophagy, cellular senescence, and mitochondrial activity, as demonstrated by numerous studies over the past decade[102].

In another study, it is discovered that increased exposure to maternal depressive cues and symptoms during pregnancy is linked to accelerated brain aging in offspring. Additionally, they found that both advanced and delayed structural brain aging were associated with heightened symptoms of anxiety and mood dysregulation in early adulthood, indicating that deviations in brain maturation—whether faster or slower—may serve as potential risk factors for mood disorders. Lastly, their findings revealed that accelerated brain aging acts as a mediator, indirectly connecting prenatal exposure to maternal depression with greater depressive symptoms in young adulthood[103].

Telomeres are repetitive DNA sequences at the ends of chromosomes that protect them from damage and degradation during cell division. They function like a protective cap, preventing the loss of essential genetic information. However, with each cell division, telomeres naturally shorten, eventually leading to cellular aging and dysfunction when they become critically short—a process known as replicative senescence. Telomere length is considered a biomarker of biological

aging, including in the brain. In the context of brain aging, shortened telomeres are associated with increased oxidative stress, inflammation, and decreased neurogenesis, which can contribute to neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Studies suggest that telomere shortening in neural cells may impair their ability to repair or regenerate, accelerating cognitive decline and brain aging. Environmental factors, such as stress, poor sleep, and exposure to neurotoxins, can exacerbate telomere shortening, linking it to circadian rhythm disruption and long-term brain health[104].

Also in other meta-analysis that is conducted for the relationship between telomere length and brain aging, it is found that telomere length showed a significant correlation with both brain volume and overall cognitive function, as evidenced by an effect size-based meta-analysis. It was also strongly linked to hippocampal volume and enhanced performance in cognitive domains related to attention, processing speed, and executive function[105].

In this study it is important that telomere length differences in newborns differ and it is an indicator of brain aging susceptibility coming with birth with corresponding treatments in the female parent. In terms of this point, there are some studies regarding the telomere length coming with birth. Telomere length is influenced significantly by genetic factors, with these effects potentially being established as early as the in utero stage. At birth, telomere length is notably consistent across various cell types, including white blood cells, umbilical artery cells, and skin cells. Women tend to have more white blood cells, a phenomenon potentially linked to the presence of estrogen. Estrogen plays a dual role by increasing telomerase activity and reducing oxidative stress. This suggests that the maternal genetic profile and uterine environment may shape telomere length during fetal development. However, there is currently no research examining the impact of methylphenidate use in adult females on the telomere length of their offspring[106][107][108][109].

Also in another study, longer maternal telomere length can contribute to the newborn telomere length positively. Research has found that certain maternal factors during pregnancy are associated with an increased risk of offspring having

shorter telomere lengths (TL). These factors include higher maternal anxiety levels, elevated fasting blood glucose, lower plasma levels of insulin-like growth factor-binding protein 3 and vitamin B12, as well as active smoking. Such findings highlight the potential impact of maternal health and lifestyle on the biological aging markers of offspring[110].

Biological aging at the organismal level is at least partially driven by molecular and cellular changes. It is a phenomenon observed across most animal species, with various epigenetic modifications occurring as part of the aging process. One well-documented change is DNA methylation at cytosine-phosphate-guanine (CpG) sites, which has been shown to correlate with age. Increasing evidence from studies suggests that alterations in DNA methylation at specific CpG sites can serve as predictors of chronological and biological age, leading to the development of "epigenetic clocks." These clocks are powerful tools for estimating age.

In this study, an epigenetic clock specific to the zebrafish genome was identified for age prediction, revealing 29 genes associated with aging. For this thesis project, six genes linked to brain aging were selected from this set, and primer sets were designed for their methylated and unmethylated sequences. The genes identified as associated with brain aging are *Reln*, *Scnb*, *Calb2a*, *Slc26a4*, *Mab2112*, and *FoxM1* [111][112][113][114][115][116][117][118].

However, there are no studies about the telomere length and methylation of brain aging related genes at birth in the offspring of the mother having disrupted circadian rhythm. In this study they are investigated.

1.1.5 Treatment Options

1.1.5.1 Role of Melatonin in Circadian Rhythm disruption and its relation with Dopaminergic System

Melatonin, often referred to as the "hormone of darkness," is a neuronal hormone primarily secreted by the pineal gland and other extra-pineal sites. It plays a vital role in regulating circadian rhythms and seasonal behaviors in vertebrates and mammals, while also influencing various physiological processes such as sleep regulation, body weight maintenance, and neuronal activity. Additionally, melatonin modulates pro-inflammatory and anti-inflammatory cytokines in pathological conditions, demonstrating therapeutic potential in treating neurodegenerative and psychiatric disorders, as well as cancer[119].

Sleep disorders are a significant comorbidity in individuals with ADHD. While the relationship between ADHD and sleep disturbances has been widely studied, research on effective management strategies for these sleep issues remains limited. In the literature, eight pharmacological treatments have been explored for addressing sleep problems in individuals with ADHD, with melatonin being one of the options. Of these, two treatments have shown promising results: immediate-release melatonin, particularly effective for adults, and L-dopa, especially beneficial for children. Melatonin, administered in doses of 3-6 mg daily, has been found to advance sleep onset. Thus, it is suggested that melatonin is effective for the short-term management of sleep-onset delay in children with ADHD. Based on this, melatonin was chosen as a treatment strategy in our research[120][121].

Another study investigating the effects of daily methylphenidate administration on melatonin and serotonin levels in ADHD patients revealed notable differences. ADHD patients exhibited reduced morning serotonin levels and a daily profile distinct from the control group, characterized by predominance during nocturnal periods. Prolonged-release methylphenidate did not cause significant changes in serotonin levels. Similarly, melatonin levels and their daily profiles were comparable between the ADHD and control groups, maintaining a diurnal

rhythm with elevated morning levels that diminished following prolonged-release methylphenidate use. However, melatonin levels were notably higher in children with the predominantly hyperactive-impulsive/conduct disorder subtype. Prolonged-release methylphenidate was associated with reduced melatonin excretion in both ADHD subtypes[122].

In other studies focusing on pregnant women, the balance and changes in melatonin levels within their bodies were explored to understand their role in regulating circadian rhythm, fetal health, and pregnancy quality. These studies revealed that melatonin is synthesized not only in the pineal gland in organ level but also within placental mitochondria in organel level in an individual manner. This is largely because the placenta can produce its own melatonin, eliminating reliance on the pineal gland. During pregnancy, melatonin serves as both an antioxidant and an anti-inflammatory agent, playing a vital role in maintaining a stable environment for the mother and fetus. In mammals, pineal melatonin production decreases with age, and a similar decline is presumed for the peripheral, non-releasable pool of melatonin. Recent findings suggest that reduced circulating melatonin levels in middle-aged females, along with a decline in mitochondria-generated melatonin, may contribute to the onset of ovarian failure and menopause. Both oocytes and granulosa cells of maturing follicles produce melatonin, with synthesis occurring in the mitochondria. Additionally, amniotic fluid surrounding the developing fetus in utero also contains melatonin, highlighting its role in fetal development[123][124][125][126][127][128][129][130][131][132][133].

Fetal programming involves alterations in biological functions and structures during fetal and early infancy development, shaped by specific factors that may influence health outcomes later in life. Because the fetus lacks direct interaction with the external environment, its developmental timing cues depend entirely on the mother during pregnancy. The daily rhythm of maternal plasma melatonin conveys the light-dark cycle information to the fetus, as melatonin easily crosses the placenta. A consistent maternal melatonin pattern is essential for optimal fetal development, functioning similarly to its role in regulating seasonal biological effects [123][134][135][136][137][138][139][140].

Another study investigated the neuroinflammation and glial damage associated with ADHD by applying three different treatments: methylphenidate (MPH), melatonin (aMT), and omega-3 fatty acids. The results demonstrated that when these three treatments were administered together, they significantly improved glial damage. Notably, the combined use of methylphenidate and melatonin has already been validated for its protective role. However, the combination of methylphenidate and oxytocin, which has not yet been validated, presents a novel alternative therapeutic approach in this context [141].

Numerous studies have explored the relationship between the circadian system and the dopaminergic system, particularly focusing on dopamine receptors' influence on clock gene and protein expression in the striatum. The striatum, receiving significant dopaminergic input, exhibits interactions between dopamine (DA) and clock genes. Research on cultured mouse striatal neurons has demonstrated the presence of DA receptors 1, 2, and 3, alongside clock genes such as *Per1*, *Clock*, *Bmal1*, and *NPAS2*. These DA receptors differentially arrange clock gene expression. Activation of the D1 receptor using the agonist SKF-38393 increased mRNA levels of these clock genes, whereas activation of the D2 receptor with the agonist quinpirole reduced *Clock* and *Per1* mRNA levels. Furthermore, quinpirole injections increased nighttime *PER1* expression but suppressed daytime levels, indicating the D2 receptor's essential role in maintaining proper *PER2* expression. Disruption of DA neurons through an injection of 6-hydroxydopamine significantly diminished *PER2* protein and mRNA expression, without evidence of recovery [142][143][144].

The suprachiasmatic nucleus (SCN), situated above the optic chiasm, is the central regulator of mammalian circadian rhythms, primarily synchronized by light signals. Within SCN neurons and peripheral tissues, 24-hour oscillations are generated through a transcriptional and translational feedback loop of clock genes. Dopamine (DA) plays a significant role in SCN function, as the primary SCN clock communicates timing information to other brain regions, influencing DA activity, while DA also exerts feedback effects on the SCN. Evidence supporting dopaminergic modulation of SCN-related circadian rhythms includes the presence of D1 and D5 receptors within the SCN. Furthermore, recent findings

suggest that methylphenidate alters electrical firing rates and clock gene expression in the SCN, indicating that drug-induced changes in DA neurotransmission may impact SCN clock activity[142][145][146][147][148][149][150].

The hypothalamus provides insight into the interconnected roles of dopamine, oxytocin, and melatonin. One notable area where dopamine exhibits circadian-like neuromodulatory effects is the arcuate nucleus. In this region, dopamine regulates the daily rhythm of prolactin (PRL), a hormone primarily associated with milk production in mammals, secreted by lactotrophs in the anterior pituitary gland. Beyond lactation, PRL's rhythmic release plays a critical role in maintaining the corpus luteum and supporting progesterone secretion during pregnancy in rodents. Thus, dopamine is intricately involved in the regulation of reproductive processes [142][151].

Although sleep improved, melatonin showed no impact on behavior, cognitive performance, or quality of life. These improvements were anticipated, as sleep issues and deprivation in children are linked to behavioral problems [152]. For this reason, there is a need for an alternative treatment method that can also lead to improvements at the behavioral level. At this point, the effects of oxytocin hormone therapy, known to regulate social behaviors, and the negative impacts of methylphenidate on circadian rhythm have been examined and compared with melatonin therapy.

1.1.5.2 Role of Oxytocin in Circadian Rhythm disruption and its relation with Dopaminergic System

Oxytocin (OT), a hypothalamic neuropeptide, stands out among neuropeptides due to its notable prosocial effects and potential therapeutic applications in addressing socioemotional deficits commonly seen in neurodevelopmental and psychiatric disorders such as autism spectrum disorders and schizophrenia. In vertebrates, all oxytocin-producing neurons originate from the proliferative neuroepithelium of the third ventricle and are generated within the hypothalamic region. Despite the absence of oxytocin production and release in the embryonic brain,

the expression of oxytocin receptors (OTR) during early development has no clear functional role, as OT and OTR knockout mice do not exhibit major anatomical abnormalities in nervous system development. This suggests that the role of OTR during early brain development is either subtle or compensated for by other peptide-receptor systems. Additionally, it is possible that immature precursor forms of OT may be released by developing hypothalamic neurons in early stages. This hypothesis of a compensatory mechanism between neuroendocrine hormones has been considered and applied in this thesis project[153][154][155][156][157].

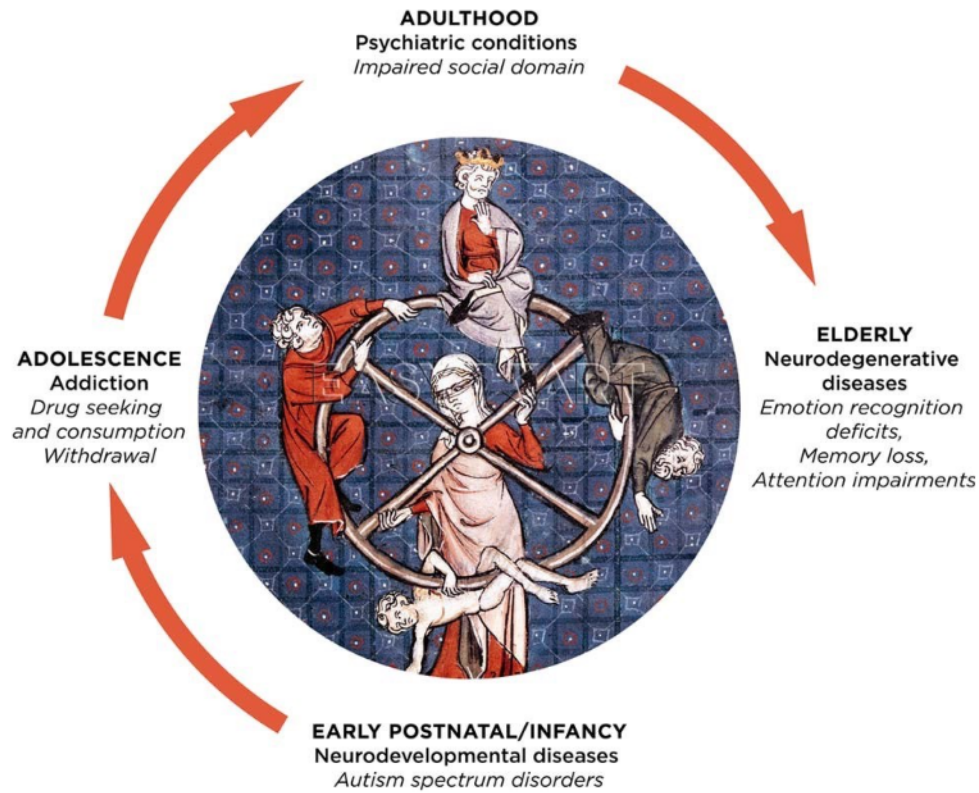


Figure 1.1: Consequences Deficiency in Oxytocin Hormone Through Different Life Stages [153]

As shown in Figure 1.1, oxytocin deficiency is associated with various problems at different stages of life. During the infancy period, when neurodevelopment is ongoing, oxytocin deficiency has been linked to impaired social skill development and an increased risk of autism. In early adulthood, low levels of oxytocin manifest as a predisposition to drug addiction and substance-seeking behavior. In

adulthood, oxytocin deficiency contributes to the development of psychiatric disorders. Furthermore, studies suggest that in old age, oxytocin deficiency may trigger or exacerbate neurodegenerative diseases[153].

The selection of oxytocin as an alternative therapeutic approach is supported by its documented relationship with dopamine and melatonin in various studies. Oxytocin is synthesized in two key hypothalamic nuclei: the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). Oxytocin neurons extend their projections to numerous regions containing dopaminergic neurons or areas influenced by them. Dopaminergic neurons play a role in modulating oxytocin release, and dopamine receptors have been identified on oxytocin neurons. Conversely, oxytocin has been shown to enhance dopamine levels in areas such as the medial preoptic area, the amygdala, and the ventral tegmental area (VTA), where oxytocin receptors are also present. This reciprocal relationship highlights the interconnected roles of these neuropeptides and neurotransmitters, making oxytocin a promising candidate for further investigation in therapeutic contexts[158][159][160][161].

Oxytocin and dopamine are released in response to activities such as feeding, sexual interactions, and social engagement in both humans and animals. Substances like amphetamines and cocaine directly influence dopamine release, and studies have also observed alterations in oxytocin levels following the use of these substances, as well as alcohol. Notably, oxytocin has even been proposed as a potential therapeutic option for addressing drug addiction[158][162][163][164].

Dopamine levels rise in response to various stressors, with the dopaminergic mesolimbic pathway playing a crucial role in stress-coping behaviors. However, prolonged exposure to chronic stress can lead to a reduction in dopamine levels. In contrast, oxytocin tends to exert a calming or sedative effect in such situations, underscoring the importance of determining the appropriate dosage of oxytocin for effective outcomes. Oxytocin can also diminish the influence of glucocorticoid-induced oxidative stress[158][165][166]. Another study found that the SNP in the oxytocin receptor sequence was highly prevalent in children with ADHD, which was linked to difficulties in recognizing facial expressions which is positively

correlated with mentioned earlier about diminishing in fusiform area [167].

In order to understand the relationship between oxytocin and melatonin, some studies have been actualized. A study reveals that in male rats, melatonin plays a role in regulating the activity of vasopressinergic and oxytocinergic neurons, with this influence being partially mediated by a cAMP-dependent pathway. Additionally, the impact of melatonin on these neurons is influenced by its concentration [168]. Also, it was found that the relationship of melatonin and oxytocin is resulted from the cAMP/PKA signalling pathway. It has been found that there is a negative correlation between melatonin and oxytocin. The application of 8-Br-cAMP enhanced the secretion of oxytocin (OT) and vasopressin (VP) when the hypothalamo-neurohypophyseal (H-NH) explants were preincubated. However, this stimulatory effect was completely suppressed by PKA inhibitors. At a concentration of 10^{-7} M, melatonin reduced the baseline release of OT and VP from the H-NH system and inhibited the cAMP analogue-induced secretion of these neurohormones under normal conditions. Interestingly, the suppressive effect of melatonin on OT and VP release was entirely blocked by cAMPS-Rp, which interferes with the cAMP/PKA pathway, whereas H-89 had no effect [168].

1.1.6 Objectives and Rationale of This Project

The importance of this project lies in addressing the significant health issue of Methylphenidate (MPH) overdose and its consequences, particularly sleep disturbances that can disrupt the circadian rhythm. This disruption can lead to brain abnormalities and neurobehavioral changes, which may be transmitted to subsequent generations. The aim of this project is to investigate the simultaneous treatment of MPH with oxytocin to mitigate the sleep problems associated with MPH overdose, as well as to explore the potential epigenetic transmission of brain aging acceleration to offspring.

The hypothesis of this study suggests that chronic exposure to MPH leads to persistent neurobehavioral adaptations in zebrafish, regardless of sex, indicating

that adolescent misuse of MPH could alter neurobehavioral plasticity, with long-lasting effects into adulthood. Moreover, it posits that the harmful effects of MPH use in adults are not compensable and may be passed on epigenetically through the eggs, potentially affecting offspring’s brain aging and development.

What distinguishes this project from previous studies is its focus on the combined compensatory effects of melatonin and oxytocin in counteracting the circadian rhythm disruption caused by MPH overdose. Furthermore, it uniquely explores how these disruptions may influence epigenetic transmission and accelerate brain aging in offspring in terms of telomere length at birth and methylation of epigenetic clock genes. This aspect of the research, examining both the immediate effects of treatment and the long-term impact on subsequent generations, has not been studied together before.

1.2 Materials & Methods

1.2.1 Materials

1.2.1.1 Zebrafish Strain

AB wild type 12 months old zebrafish.

1.2.1.2 Treatment Drugs

Methylphenidate	Ritalin and Concerta.
Melatonin	Ocean, Türkiye.
Oxytocin	O3251, Sigma Aldrich, USA.

Table 1.1: Drugs that are utilized in treatment groups.

1.2.1.3 Kits

DNeasy Blood&Tissue Kit	69506, Qiagen, Hilden, Germany
EZ Methylation Lightning Kit	D5030, Zymo Research, Irvine, USA

Table 1.2: Kits that are utilized in experiments.

1.2.1.4 RNA Isolation Reagents

RiboEX reagent	Trizol Reagent
Chloroform	Sigma Aldrich
Isopropanol	Merck 1.09634.2511
75% ethanol	Sigma Aldrich
DEPC treated water	Thermo Fisher

Table 1.3: RNA isolation Reagent for RiboEX Trizol Reagent Protocol.

1.2.1.5 Enzymes and Master Mixes

SensiFAST™ SYBR® No-ROX Kit	BIO-98020, Bioline, Luckenwalde, Germany
iScript Reverse Transcription Superscript for RT-qPCR	1708841, Bio-Rad

Table 1.4: Enzymes and Mater Mixes.

1.2.1.6 Primers

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
clock1a	AGCAGGGACAGAACCAGG	GTGTTGCGGTTGTGAATG
bmal1b	CCCTCTAGCTGTGGCTCAAG	TCCCGCCATTGGACATCTT
per1b	AGGAAGGCTGACAGATGATGAATG	CCAGAGTGGGCTAAAGCGAAGTA
cry1ba	TCGCCAAGTGCATAATTGGA	GTGTGTCTCCCGAGGAAGGA
tnf-a	GCGCTTTTCTGAATCCTACG	TGCCAGTCTGTCTCCTTCT
il-6	GCTATTCCCTGTCTGCTACACTGG	TGAGGAGAGGAGTGCTGATCC
oxtr	CCAACCTCTTCGTTCTTTACAC	ACAAAATCTCCCCAGCAATC
Nr3c1	ACAGCTTCTTCCAGCCTCAG	CCGGTGTTCTCCTGTTTGAT
drd2a	TGGTACTCCGGAAAAGACG	ATCGGGATGGGTGCATTTTC
drd3	ATCAGTATCGACAGGTATACAGC	CCAAACAGTAGAGGGCAGG

Table 1.5: qRT-PCR Primers for Adult cDNA

Methylated		Unmethylated		Gene Name
Forward	Reverse	Forward	Reverse	
AGGGTGTGTAGA GGAGTGCTGTC	TAATTCTCTATAC TCTAAAACCCGA	GTAGAGGAG TTGTGTTGGTT	AATTCTCTATACTC TAAAACCCAT	<i>Reln</i>
GATAAAAGGGGGTT AATACGTGT	TATAAAACCTAA AACAAAT- TAAATCG	AAGATAAAAGGG GGTTTAATAGT- GTA	AAAAATAACCTAAA ACAAATAATCTC	<i>Scnb</i>
GGGTTTTAATGG AGGAAGCGATT	CAAAAACCCA CT- CAACCTGAAT	GGGTTTTAAT GGAGGAAGATGAT	AAAAACACCTA AC- TATACCAATTC	<i>cabp2a</i>
AAACAAAATAA TGCTAAAAT- GCAGA	GTTATGAATTGA TGAGTGAGTGTT	AAAACAAAATAA TGCTAAAAT- CAATC	GTTATGAATTGA TGAGTGAGTGTT	<i>slc26a4</i>
GCGGTGTTTA AGAGGAGCGAC	GAAATCGCAA TTCACCTTGGC	GTGGTGTTTAA GGTTATGATG	CCAAATCACA AACTTCATCTC	<i>mab211l2</i>
TTGAATAAGA TGATTGATTGGCC	CTCTACTCCA CC- TAACCGA	TGAATAAGAGTA TTTGATTGTG	CTCTACTCCACT	<i>foxm1</i>

Table 1.6: Primer Sequences for Methylation and Unmethylation

1.2.1.7 Buffers

60X Embryo medium (E3):

- 17,2 grams of NaCl,
- 0,76 grams of KCl,
- 2,9 grams of CaCl₂.2H₂O,
- 4,9 grams of MgSO₄.7H₂O

1X Embryo medium (E3):

16 ml 60X E3 medium was poured into a bottle, and the volume was completed to 1L with distilled water. 10 μ l methylene blue was put in the final solution. This 1X embryo medium is utilized for zebrafish embryo.

1.2.1.8 Equipments

qRT-PCR machine	CFX96 Real-Time System, Bio-rad
Thermal Cyler	C1000 Thermal Cyler, Bio-rad
Magnetic Stir	IKA RCT Basic
4°C centrifuge	CT15RE, VWR
Mini Centrifuge	Centrifuge 5418, Eppendorf
Incubator	INCU-Line, VWR
Light Microscope	Optika Pro View
Autoclave	SteamArt OT 90L, Nüve
Nanodrop	NanoDrop 2000, Thermo Scientific
Behavior Tank Set Up	Custom made
Breeding Tank Set Up	It was custom-made in the factory.
Camera for Behavior Experiments	iPhone 11 Pro Max
Waterbath	Bath NB9, Nüve

Table 1.7: Equipments that are utilized in the experiments

1.2.1.9 Software

Phyton	Phyton software
Ethovision	Phyton software
ImageJ	Fiji - free
GraphPad Prism	GraphPad software

Table 1.8: Softwares

1.2.2 Methods

1.2.2.1 Zebrafish Maintenance

12-month-old AB (Wild Type) strain zebrafish were used in this study. In the Bilkent University ASBAM fish laboratory, the system water is maintained at a temperature of 28°C and a pH range of 7.0 to 7.5. The fish are kept under a 14-hour dark and 10-hour light cycle. They are fed twice daily with dry food and once with Artemia.

Ethics decision was dated 20/01/2025 (No:2021/18)

1.2.2.2 Experimental Workflow

To observe the effects of methylphenidate and to explore different treatment approaches, four distinct treatment groups were established. In the first group, methylphenidate was administered alone. In the second group, methylphenidate was combined with melatonin and given to the fish as a solution. In the third group, methylphenidate and oxytocin solutions were administered together. The fourth group, serving as the control, received no treatment. Each group contains 7 female zebrafish which are 12 months old when the drug administration was finished.

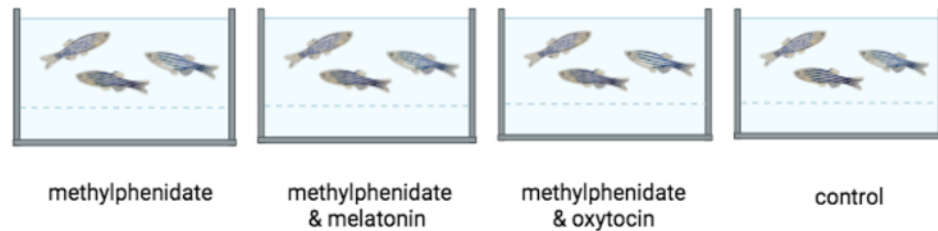


Figure 1.2: Treatment Groups for Female Adult Zebrafish. 28 days period, n=7 for each group.

To prepare the solutions, the drugs were dissolved in system water at specific concentrations per liter using a magnetic stirrer. Following drug administration, *Artemia* was provided as a meal to facilitate oral drug intake. Fish consuming the *Artemia* were observed to ingest and absorb the drugs through feeding. The administered doses were 6 mg/L of methylphenidate, 3 mg/L of melatonin, and 10 μ M of oxytocin daily. These treatments were applied for 28 days.

During the treatment period, zebrafish were subjected to spawning procedures twice to empty the eggs from their abdomens. This process stimulated the formation of new eggs, influenced by the administered drugs. After the 28-day treatment period, dissection was performed to study the effects of the treatments. Brain tissue and abdominal egg samples were collected from adult female zebrafish to analyze epigenetic changes in the second generation. This ensured that only maternally transmitted effects were examined, as the genetic contribution of male zebrafish sperm was excluded.

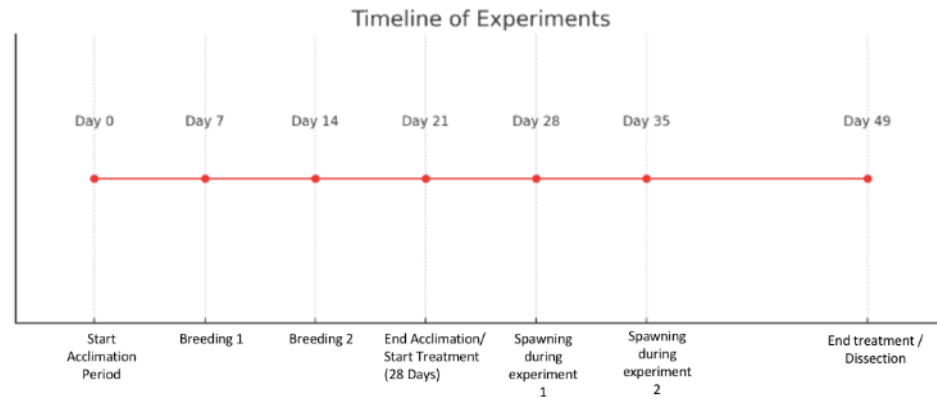


Figure 1.3: Timeline of Treatment and Experiment Procedure

The drug administration process was carried out in a quarantine room, separate from the main fish system, to ensure controlled conditions. The study was conducted in the ASBAM zebrafish laboratory at Bilkent University.

As shown in the figure above, this long-term experiment involved two generations. In the first generation, RNA was isolated from adult female zebrafish,

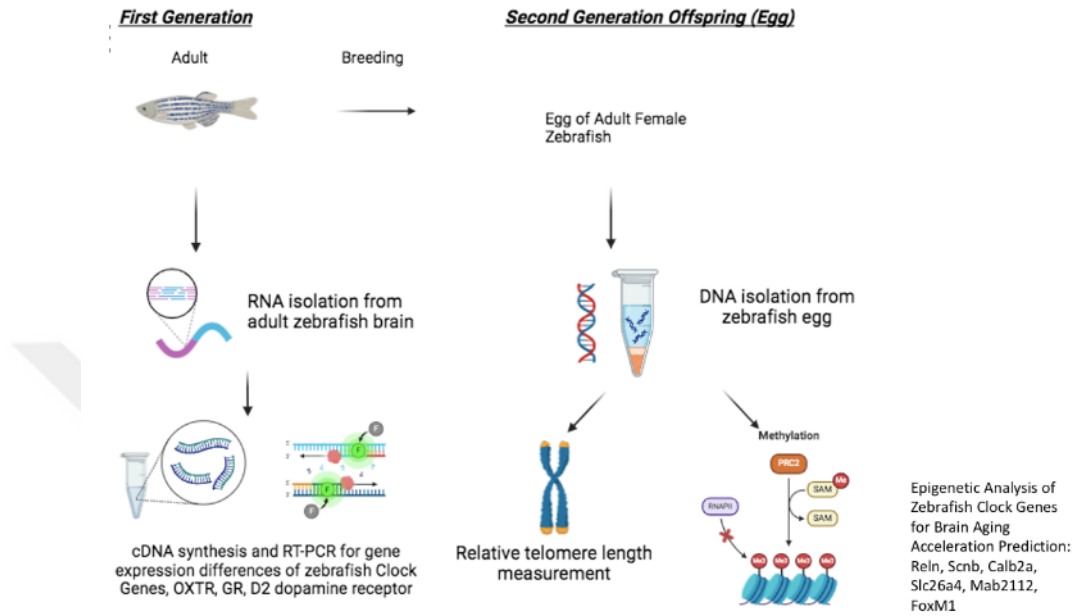


Figure 1.4: Molecular Biology Assays for Zebrafish Generations in Longitudinal Study.

followed by the synthesis of complementary DNA (cDNA). The results of the four different treatment groups were analyzed using qRT-PCR. Gene expression levels were measured for genes related to circadian rhythm, such as oxytocin receptor, dopamine receptor, and the glucocorticoid receptor gene associated with stress.

For the offspring collection of treatment groups, eggs collected from the abdomens of adult female zebrafish were subjected to DNA isolation. Analyses were conducted to compare telomere length in the eggs to determine whether they exhibited a predisposition to brain aging. Additionally, methylation levels in the zebrafish clock genes were examined to assess potential epigenetic transmission.

1.2.2.3 Experiments in the First Generation Adult Zebrafish

1.2.2.3.1 Behavioral Experiments in Adult Zebrafish: Novel Tank Test

To begin, an infrared light is placed behind the novel tank, with a white acrylic

sheet positioned between the tank and the light to diffuse it and eliminate glare. Treatments are administered, and a waiting period of 30 minutes is observed to allow for drug absorption before initiating the novel tank assay. The tank is then filled to 3/4 capacity with fish system water prepared through system water.

A single acclimatized fish is transferred from a holding tank to a beaker containing fish system water and left to rest for an additional 10 minutes. The fish, along with the water, is then gently introduced into the novel tank. Using a still-frame video image, the tank's top and bottom areas are identified by calculating its height and dividing it by two. The time spent in each area and the total distance traveled are measured using tracking software[169][170][171].

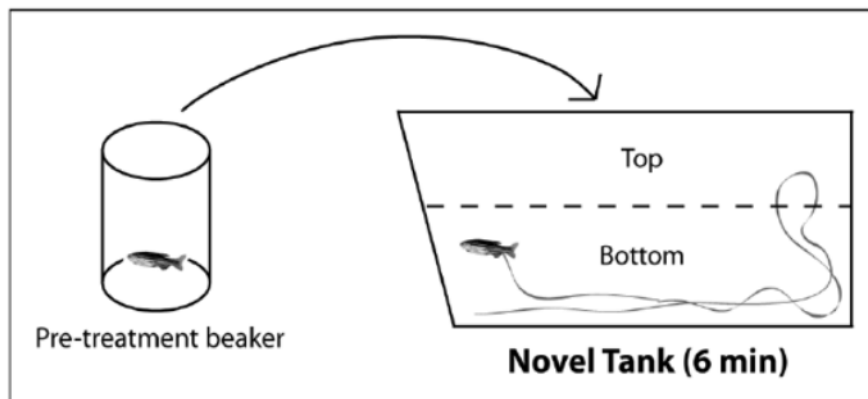


Figure 1.5: Representation of Novel Tank Test Set up

Adult zebrafish typically exhibit a preference for the bottom half of the tank when introduced to a novel environment due to stress. As the stress diminishes, they progressively spend more time in the top half. Three zebrafish per experimental group are monitored during the assay [172].

1.2.2.3.2 Behavioral Experiments in Adult Zebrafish: Shoaling Assay

The drugs were administered 30 minutes before the start of the experiment, allowing time for absorption before proceeding to the behavioral tests.

For the measurement of group behavior, four fish were placed simultaneously in

a test tank measuring 25 cm in length, 15 cm in height, and 6 cm in width. The control group was tested under identical conditions, except that only distilled water was added to the tank. Fish behavior was recorded during a 5-minute observation period. After each trial, the tank was cleaned, and the water was replaced with system water. All experiments were conducted with a sample size of $n = 8$ per group. In each monitoring, 4 zebrafish were put in the shoal tank and 2 trials were monitored.

The recorded videos were exported to Image J 1.49 software for Windows™ for further analysis. Shoaling behavior was assessed using screenshots taken every 15 seconds over the test period, totaling 20 screenshots per group. The proximity between the fish (inter-fish distance, nearest neighbor distance, and farthest neighbor distance) and group dispersion (shoal area) were calculated by calibrating the screenshots to the tank size and marking each fish for automated quantification.



Figure 1.6: Shoaling Assay Experimental Set Up

The vertical position of zebrafish was evaluated manually by counting the number of fish in the upper half of the tank every 15 seconds over the test period. Additionally, the latency for the group to enter the upper area was recorded using

a handheld stopwatch. All results were analyzed by a trained observer who was blind to the experimental conditions[173][174][175][176].

1.2.2.3.3 Molecular Biology Experiments in Adult Zebrafish: Tissue Collection

Fish were euthanized by immersion in ice-cold water for 10 minutes, ensuring the cessation of gill movement. Decapitation was then performed using a scalpel in ice-cold 1x PBS. The brain and eggs were carefully dissected, immediately flash-frozen in liquid nitrogen, and stored at -80°C until DNA extraction.

1.2.2.3.4 Obtaining Zebrafish Egg Directly from Adult Female Zebrafish

During the experiments, multiple breeding sessions were conducted to empty the eggs from the females' abdomens, and drug administration was continued. This approach ensured that the drug's effect on newly formed eggs could be studied more effectively as the drug dosage increased, while the older eggs were removed.

In the final stage, the eggs used for the experiments were directly collected as unfertilized during dissection. After harvesting brain tissue from the adult females, the abdominal region was dissected, and the eggs were removed from the abdomen.

The use of unfertilized eggs aimed to exclude the influence of male zebrafish. This allowed the study to focus solely on the effects of maternal drug administration on the offspring in the second generation.

	A (methlphenidate only) n=7	B (meth+melatonin) n=7	C (meth+oxt) n=7	D (control) n=7
embryos of the group	AE	BE	CE	DE
Brain of the adult group	AB1	BB1	CB1	DB1
	AB2	BB2	CB2	DB2
	AB3	BB3	CB3	DB3
	AB4	BB4	CB4	DB4
	AB5	BB5	CB5	DB5
	AB6	BB6	CB6	DB6
	AB7	BB7	CB7	DB7

Table 1.9: List of zebrafish utilized in tissue collection

1.2.2.3.5 Obtaining Zebrafish Egg Directly from Adult Female Zebrafish

Adult Zebrafish Brain Tissue

The RNA extraction protocol involves a series of precise steps to ensure high-quality RNA is obtained from tissue samples. Begin by homogenizing approximately 50-100 mg of tissue in 1 mL of RiboEX reagent, ensuring that tissue volume does not exceed 100 μ L. After homogenization, incubate the mixture at room temperature for 5 minutes to allow lysis, followed by centrifugation at 12,000 rpm for 10 minutes at 4°C. Carefully transfer the supernatant to a new tube and add 0.2 mL of chloroform per 1 mL of RiboEX, shaking vigorously for 15 seconds to facilitate phase separation. Incubate at room temperature for 2 minutes, then centrifuge at 12,000 rpm for 15 minutes at 4°C. Collect the aqueous phase, which contains RNA, and add 0.5 mL of isopropanol per 1 mL of RiboEX, gently mixing the solution by inverting the tube 3-5 times. After a 10-minute incubation at room temperature, centrifuge again at 12,000 rpm for 10 minutes at 4°C and discard the supernatant. To wash the RNA pellet, add 1 mL of 75% ethanol and centrifuge at 7,500 rpm for 5 minutes at 4°C. Remove the ethanol carefully, ensuring complete removal, and air-dry the pellet for 5 minutes, leaving it slightly damp. Finally, dissolve the RNA in DEPC-treated water (300-500 μ L

for tissue samples) and incubate at 56°C for 15 minutes. For immediate use, store the RNA at 4°C or -20°C, ready for use in cDNA synthesis.

1.2.2.3.6 Molecular Biology Experiments in Adult Zebrafish: cDNA Synthesis

The Bio-Rad iScript Reverse Transcription Supermix protocol is designed to efficiently synthesize cDNA for RT-qPCR analysis. Begin by preparing a master mix according to the number of samples, using a ratio of 5 µL of iScript RT Supermix to 10 µL of nuclease-free water per reaction. For example, for 10 samples, prepare 50 µL of Supermix and 100 µL of water. Add 15 µL of this prepared master mix to each 5 µL RNA sample to reach a final volume of 20 µL per reaction. Load the samples onto a Bio-Rad thermal cycler, setting it to run the following conditions: priming at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, and an RT inactivation step at 95°C for 1 minute. Once completed, store the synthesized cDNA at -20°C. If required, the cDNA can be diluted to a 1:3 or 1:5 ratio for further applications.

1.2.2.3.7 Molecular Biology Experiments in Adult Zebrafish: qRT-PCR for Adult Zebrafish Brain

The qPCR protocol using Sensifast SYBR No ROX 2x master mix (Bioline) begins with preparing a primer mix by combining 10 µL each of forward and reverse primers with 180 µL of nuclease-free water. After mixing, spin briefly and store at -20°C until use. Before setting up the reactions, thaw the enzyme and cDNA on ice, then spin briefly to collect the contents. For each reaction, assemble the mix on ice by adding 10 µL of Bioline SYBR enzyme, 2 µL of cDNA, 6 µL of nuclease-free water, and 2 µL of primer mix. For the negative control, substitute the cDNA with 2 µL of nuclease-free water. Seal the plate, and program the qPCR thermocycler as follows: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 57°C for 30 seconds for annealing, and 72°C for 30 seconds for extension. For the *per1b* and *Il6* genes, adjust the annealing temperature to 61°C, keeping all other settings the same. These degrees were put in a gradient qRT-PCR in one run with gradient temperatures

for annealing with 57°C and 61°C. This protocol ensures consistent amplification conditions across all samples, with special adjustments for specific genes when needed. The primers in the ‘Table 5’ were utilized.

1.2.2.4 Experiments in the Second Generation Offspring: Eggs of Adult Zebrafish

1.2.2.4.1 Total DNA Isolation

To isolate total DNA from eggs of female adult zebrafish from each group, start by placing the tissue in a 1.5 mL tube and breaking it down into small pieces. Add 180 µL of ATL buffer and further homogenize the tissue using a pipette tip. Use a rotator or vortex to ensure thorough mixing. Add 20 µL of Proteinase K directly into the liquid, given the small sample volume, and vortex briefly. Incubate at 56°C for 3-4 hours or overnight, using a thermomixer or shaking water bath if available, or vortex periodically. After incubation, vortex for 15 seconds or briefly centrifuge the mixture. Optionally, perform a quick centrifugation immediately after removing from the incubator.

Next, add 200 µL of AL buffer and incubate at 70°C for 10 minutes, followed by a brief centrifuge. Add 200 µL of 100% ethanol, briefly centrifuge, and vortex thoroughly. Transfer the mixture to a mini spin column, centrifuge for 1 minute at 8,000 rpm, and discard the flow-through. Place the column in a fresh collection tube and add 500 µL of AW1 buffer, centrifuging for 1 minute at 8,000 rpm. Discard the flow-through, then transfer the column to another new collection tube and add 500 µL of AW2 buffer. Centrifuge at 14,000 rpm for 3 minutes, then open the lid and air-dry the column for 2-3 minutes. Place the column in a clean, capped tube and add 100 µL of AE buffer directly onto the membrane. Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 8,000 rpm. Repeat the elution with another 100 µL of AE buffer, allowing the column to incubate at room temperature for 1 minute before a final 1-minute centrifuge at 8,000 rpm. Store the isolated DNA at -20°C for future use.

The isolated DNA was utilized in telomere length measurement and bisulfite conversion for two different experiments related to eggs.

1.2.2.4.2 Telomere Length Measurement

To measure telomere length in zebrafish tissues, the Sensifast SYBR No ROX 2x master mix (Bioline) is used in a 20 μ L reaction. For each reaction, combine 10 μ L of Sensifast SYBR No ROX 2x master mix (Bioline), 2 μ L of DNA, 2 μ L of primer mix, and 6 μ L of PCR-grade water. Briefly centrifuge the mixture to ensure thorough mixing. The telomere primer mix consists of 10 μ L each of forward and reverse primers, with 180 μ L of water added for a total volume of 200 μ L. For telomere length amplification, cycle conditions are set to 95°C for 10 minutes for initial denaturation, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative telomere length is then calculated using the formula $2^{-(C_{ttelo}-C_{tp3dio})}$, allowing for accurate quantification of telomere length across samples relatively.

Primer pair	Forward (5' to 3')	Reverse (5' to 3')
zebrafish- Telo	ATAGTAGGATAGG GTTTTGGTTTTG	CCTTCAATTCTTC AAAAAATTAAGCTC
zebrafish- P3dio	GGGGACGGCA GAAGAAATGA	AGGTCCACACTA AGCAAGCC

Table 1.10: Primers for zebrafish-telo and zebrafish-P3dio

1.2.2.4.3 Bisulfite Conversion

The EZ DNA Methylation Kit (ZYMO) is used for bisulfite conversion of methylated DNA, where unmethylated cytosine residues are converted to uracil while methylated cytosines remain unchanged. To ensure optimal results, DNA is divided into multiple tubes to increase the final yield of methylation data for PCR analysis. For each gene, 4 μ L of DNA (2 μ L for each primer set) is required, so 14 μ L of DNA with a concentration of 35-40 ng/ μ L (200-500 ng total) is used as the starting material.

To prepare the conversion reagent, 750 μ L of water and 210 μ L of M dilution buffer are added to the CT conversion reagent vial, followed by vortexing for 10

minutes. This solution is enough for 10 DNA treatments and can be stored at -20°C. For sample processing, 14 µL of DNA, 5 µL of M dilution buffer, and 31 µL of water are added to each tube, followed by a 15-minute incubation at 40°C. Then, 100 µL of the prepared conversion reagent is added, and the tubes are incubated in the dark at 50°C for 16 hours. After incubation, the samples are placed on ice for 10 minutes before continuing with the next steps.

For binding and washing, 400 µL of M binding buffer is added to a column in a collection tube. The DNA mixture is transferred into the column, mixed by inversion, and centrifuged at 10,000 rpm for 30 seconds. The flow-through is discarded, and the column is placed in a new collection tube. The column is washed with 100 µL of M wash buffer, centrifuged at full speed, and then washed twice with 200 µL of M wash buffer, each followed by a 30-second centrifuge. For elution, the column is placed into a 1.5 mL microcentrifuge tube, and 10 µL of M elution buffer is added directly onto the membrane. After a 30-second centrifuge, the final methylated DNA is collected and stored at -20°C for short-term use or -70°C for long-term storage.

1.2.2.4.4 Methylation Specific PCR

Methylation sensitive PCR msPCR primers were taken from Mayne 2020 article [111] as seen in ‘Table 6’ and they designed these primers with MethPrimer v2.0 that is producing two pairs of primers for when the DNA is methylated and unmethylated. msPCR was actualized with the final cycling conditions: Initialisation step 95° C/15 mins, denaturation step 95° C/30 seconds, annealing 55° C/40 seconds and extension 72° C/40 seconds, for 40 cycles. msPCR was performed using Sensifast SYBR No ROX 2x master mix (Bioline) in a Bio-Rad CFX96 Real Time PCR. The ΔC_t values for each primer pair were utilized as a quantitative analysis technique for methylation[111].

1.2.2.5 Data Analysis

For the analysis of behavioral experiments, movements in the novel tank test were examined using EthoVision software. Statistical heatmaps were generated using both EthoVision and Python software. For the shoaling assay, screenshots were scaled in ImageJ, and distances were measured. The analyses were performed using Python software.

Telomere length was calculated using the formula $2^{-(Ct_{telo}-Ct_{p3dio})}$, and normalization was carried out in Excel. Similarly, other qPCR results were normalized in Excel, with statistical analyses and graph plotting conducted in Python. For all experiments, a cutoff value of $p \leq 0.05$ was used to determine statistical significance.

1.3 Results

1.3.1 First Generation Adult Zebrafish Female qPCR Results

Firstly, in order to compare the gene expression profile in different treatment groups complementary DNAs for brain tissues were analyzed in qRT-PCR machine to see the differences for clock genes, validation of intake of drugs and neuroinflammation (Figure 1.7). For qPCR analysis, $\Delta\Delta Ct$ method is used. Values were initially normalized to actin via the formula $2^{-(Ct_{value\ of\ the\ gene}-Ct_{value\ of\ actin})}$. Then each value is normalized to the mean value of the control group, which set the control group to 1. Mean with SEM is utilized for statistics. p-values are assigned as $* < 0.05$, $** < 0.001$, $*** < 0.0001$. Nested one way ANOVA is utilized with posthoc analysis performed with Tukey's test. Error bars represent the $\pm$ SEM. By this way validation of positive feedback loop clock genes and negative feedback loop of clock genes are analyzed. (Figure 1.7)

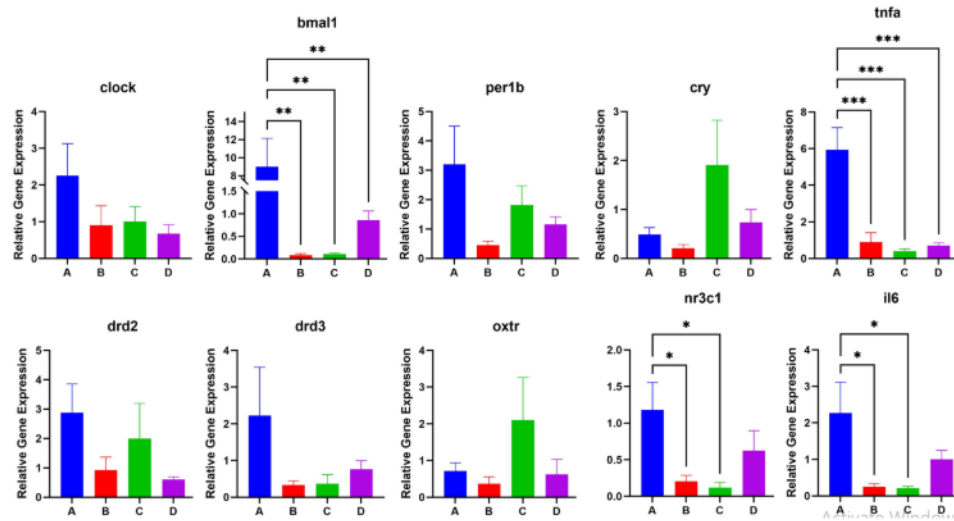


Figure 1.7: **Relative Gene Expression Results for First Generation Adult Zebrafish Female after treatment period:** (n=6 different individual zebrafish in each group). Y axis shows relative gene expression and X axis show treatment groups. (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment).

We observed significant changes in bmal1, tnfa, nr3c1, il6 genes significantly in methylphenidate treatment compared to other dual treatments which are meth & melatonin, meth & oxytocin treatment and control group.

1.3.2 First Generation Adult Female Zebrafish Behavior Experiment Analysis

1.3.2.1 Novel Tank Analysis

After the examination of treatment with different combination of drugs and validation of its effect with qPCR machine on gene expression level, behavioral analysis was done on treatment groups which are the female parental generation.

In order to understand the different combination of drug treatments two different behavioral analyses were done on adult zebrafish which are novel tank assay to understand the depression and anxiety; shoaling assay for inclination analysis to create shoals and socializing. In the novel tank test, spending more time in the bottom area signifies depression and anxiety. In the heat map analysis different colors can depict duration frequency in that area and reddish color can show higher frequency and duration in that area. (Figure 1.8)

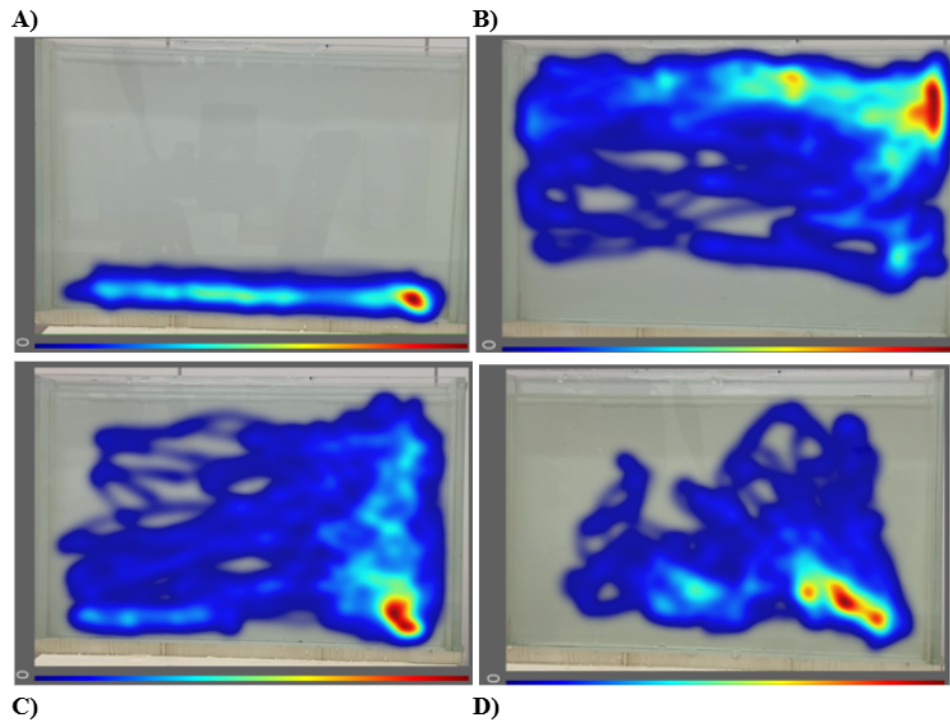


Figure 1.8: Novel Tank Analysis Heatmap for First Generation Adult Zebrafish Female after treatment period. (n=3 trial) (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment). Higher frequency and duration depicted with red, lower values depicted with cooler tones like blue or green showing lower duration. Created with EthoVision.

When compared with the control group, fish with the methylphenidate only treatment spent more time in the bottom area showing more depressive behaviors and less exploration inclination.

After this heat map analysis individual parameters obtained from novel tank

analysis can be seen in Figure 1.9. The tank is divided into two parts horizontally, for duration analysis, which is denoted as bottom and top area. Duration shows the total seconds spent in the top and bottom area separately. Velocity center point shows the velocity of individual fish during recording. Moving and not moving parameters show the time fish spent moving and freezing time, respectively. Distance moved center point shows total distance travelled in the tank by individual fish for exploration ability analysis. (Figure 1.9)

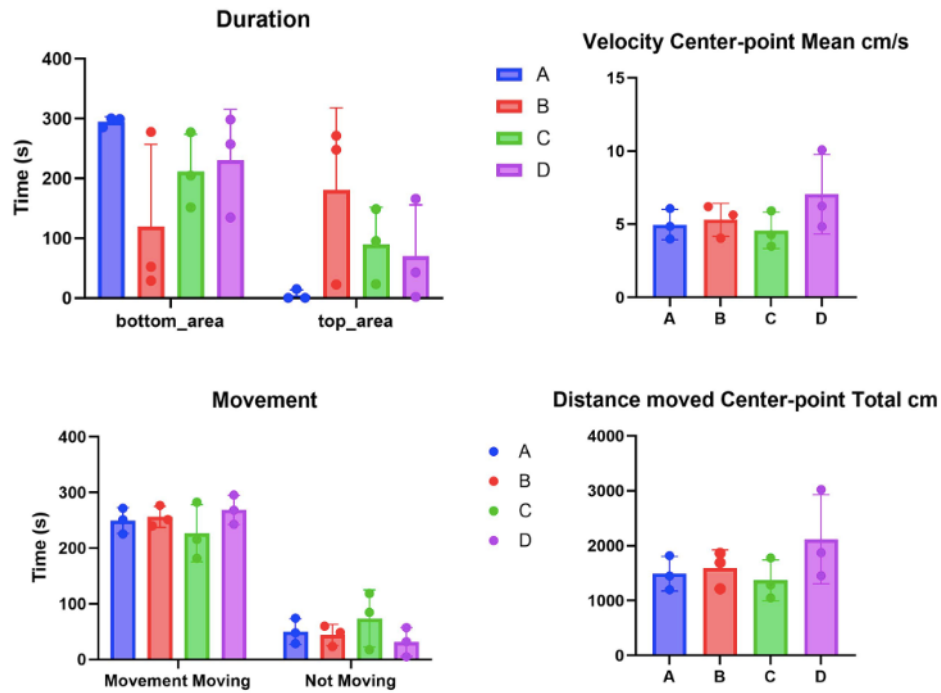


Figure 1.9: **Novel tank individual parameters statistical analysis (n=3 trial)** (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment) (For Cumulative Duration statistics, interaction p- value= 0.0231, row factor = 0.0024 indicating significance, mean with SEM).

Duration time shows significant differences for top and bottom area total duration time for methylphenidate only treatment. It shows that preference for the bottom area is higher for methylphenidate only compared to other compensatory drugs like melatonin and oxytocin. However, there is no significant change for not moving (freezing behavior) movement type among groups.

1.3.2.2 Shoaling Assay

The second behavioral analysis in adult fish was actualized with shoaling assay. Shoaling assay is performed with four different zebrafish in each trial to observe their grouping inclination in a smaller area. For each treatment group, two different trials were monitored. Center zebrafish is selected for neighbour zebrafish distances as nearest, interfish, farthest neighbor distances in cm. Total shoaling area is calculated as cm². Tank height is set as scale in ImageJ for measurement. Tank measurements were 25x15x6 cm. Mean with SEM is utilized for statistics. p-values are denoted as * < 0.05 , ** < 0.001 , *** < 0.0001 . Nested one way ANOVA is utilized. Error bars represent the +SEM.

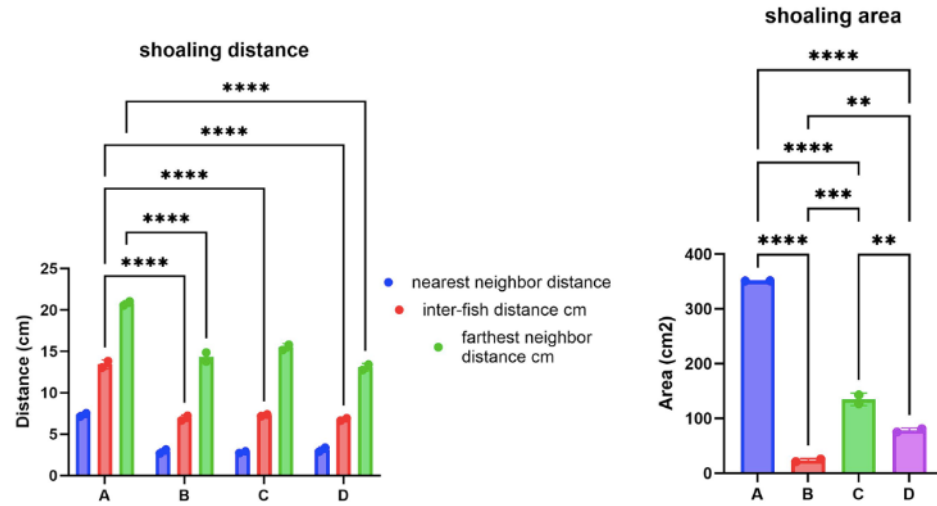


Figure 1.10: **Shoaling assay of adult zebrafish (n=8 zebrafish, n=2 trial for each experimental group having 4 zebrafish).** (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment) (Analysis is done with ImageJ and Graphs are generated in Graphpad Prism). The Y axis shows distance in cm for the first graph and area in cm² for second graph. X axis shows different treatment groups in both graphs.

As seen in Figure 1.10, there is a significant difference for the shoaling area for methylphenidate only treatment compared to other dual treatment groups and control. This shows that socializing inclination is decreased with methylphenidate

treatment and it can be compensated either with melatonin or oxytocin. However, oxytocin treatment showed more variation for shoaling among different time points and trials.

1.3.3 Second Generation Embryo Relative Telomere Length Results Across Treatment Groups

In order to analyze the sensitivity to brain aging coming with birth in the offspring of treatment groups as epigenetic transmission of effects, two different analyses were done with DNA of offsprings. Initially relative telomere length analysis was done with qPCR machine according to Ct values for telomere and p3dio primers. Initial normalization is done to p3dio gene. Second normalization done to average of control group set to 1. Mean with SEM is utilized for statistics. p-values are denoted as $* < 0.05$, $** < 0.001$, $*** < 0.0001$. Nested one way ANOVA is utilized. Error bars represent the +SEM. DNA was pooled for each group before qPCR. Graphs are drawn using GraphPad Prism according to Ct values.

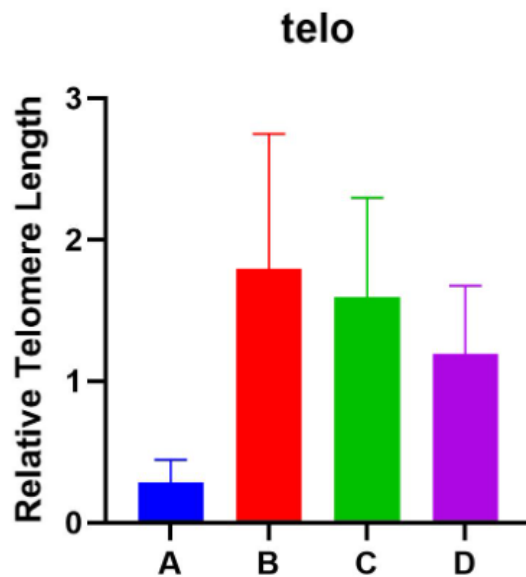


Figure 1.11: **Relative Telomere Length Values across treatment groups in the second generation zebrafish embryo.** The Y axis shows relative telomere expression and the X axis shows treatment groups. (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment). Graphs are drawn using GraphPad Prism according to Ct values.

There is a significant difference for relative telomere length expression in the offspring of adult treatment groups in the comparison with methylphenidate only treatment to other groups which are melatonin and oxytocin compensatory treatments and control group.

1.3.4 Second Generation Embryo Methylation Status of Zebrafish Aging Related Genes Results Across Treatment Groups

Bisulfite conversion was done to the DNA of offspring to analyze methylation status. primer sets are generated for methylated and unmethylated sequences separately. Bisulfite-treated DNA were put to qPCR machines for two primer sets for each treatment group. DNA was pooled for each group before qPCR. Nested one way ANOVA was utilized for statistics. Positive values showed methylation

in the corresponding treatment group. Graphs are drawn using GraphPad Prism according to Ct values.

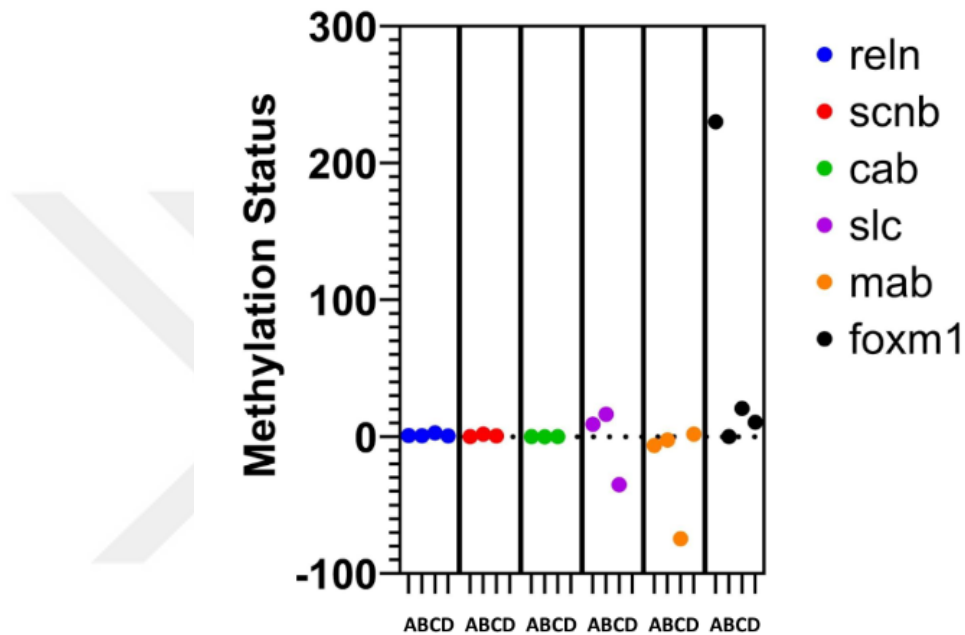


Figure 1.12: **Methylation Status Across the second generation embryos of different treatment groups.** The Y axis shows the methylation status as positive numbers indicating methylation. X axis shows the treatment group for each selected gene. (A = Methylphenidate treatment, B= Methylphenidate & Melatonin treatment, C= Methylphenidate & Oxytocin treatment, D= Control Group - No Treatment) Graphs are drawn using GraphPad Prism according to Ct values.

The use of methylphenidate alone resulted in methylation of the foxm1 gene, and both melatonin and oxytocin hormone resulted in unmethylation of the foxm1 gene.

1.4 Discussion

In the 24-hour cycle that forms the circadian rhythm, there is a molecular mechanism at play. The transcription factor called CLOCK dimerizes with another transcription factor, BMAL1, in the nucleus. This dimer (CLOCK/BMAL1) promotes the transcription of CRY and PER genes. Once the transcripts of PER and CRY are translated into the cytosol, the resulting proteins dimerize, return to the nucleus, and interact with the CLOCK/BMAL1 complex. This interaction inhibits the ability of BMAL1 and CLOCK to activate the transcription of PER and CRY genes, stopping the production of new transcripts that encode PER and CRY.

As PER and CRY proteins degrade, CLOCK and BMAL1 are freed to once again promote PER and CRY expression. This cycle repeats, forming a negative feedback loop that generates the 24-hour circadian rhythm.

The expression of circadian rhythm-related genes, as shown in Figure 1.7, was compared across four different treatment groups. In group A, which received only methylphenidate, a significant increase in the expression of CLOCK and BMAL1 genes was observed compared to the other groups.

Specifically, CLOCK gene expression increased fourfold in group A compared to the control group. Similarly, a twofold increase in BMAL1 gene expression was observed in the control group and the group that received only methylphenidate. This indicates that the positive feedback loop was induced, triggering an increase in PER-CRY synthesis exclusively when methylphenidate was administered.

This means that the 24-hour cycle is disrupted, and an increase in the positive feedback loop is observed before the negative feedback loop can take effect. This result indicates a disturbance in the circadian rhythm. Studies have shown that the overexpression of CLOCK-BMAL1 leads to the inhibition of the cell cycle[177]. Additionally, some studies have reported that the overexpression of BMAL1 could promote the invasion and metastasis of breast cancer cells[178].

Therefore, it can be concluded that the 24-hour circadian rhythm is disrupted exclusively in group A, which received only methylphenidate.

In Figure 1.7, the analysis of BMAL1 and CLOCK gene expression shows that in groups B and C, which received methylphenidate along with melatonin or oxytocin, there was an additional decrease compared to the control group. This suggests that both melatonin and oxytocin have therapeutic effects that counteract the circadian rhythm-disrupting effects of methylphenidate. However, the greater decrease compared to the control group indicates a suppression effect, which may be regulated through dosage adjustments. Therefore, further studies on dosage optimization are necessary.

When examining the expression levels of the CRY1 and PER1B genes across the four groups, an interesting observation was made. The CRY1 gene showed overexpression with oxytocin treatment compared to melatonin treatment, which displayed the opposite trend. Specifically, CRY1 expression was reduced by half in the melatonin-treated group compared to both the control group and the methylphenidate-only group.

The circadian cryptochrome CRY1 is a pro-tumorigenic factor that rhythmically modulates DNA repair. The observed increase in CRY1 expression in the presence of oxytocin suggests an enhancement of the negative feedback loop. CRY1 and CRY2 have redundant functions as transcriptional repressors in the cerebellum, and an increase in CRY1 is associated with a lengthening of the period of the SCN oscillator[179].

From this, the following conclusion can be drawn: the hormone oxytocin played a role in activating the negative feedback loop, which contributes to regulating the accelerated circadian rhythm caused by the increased transcription factors. When the PER1 gene was examined in Figure 1.7, a parallel increase in PER1 expression was observed, consistent with the previously analyzed increase in the CLOCK-BMAL1 dimer, as PER1 activation is driven by this dimer[180].

This validates that methylphenidate induces an acceleration of the circadian

rhythm, as shown in Figure 1.7.

Among the treatments in Figure 1.7, melatonin was the most effective in compensating for the additional increase, bringing *per1b* expression levels close to those of the control group. However, the hormone oxytocin also demonstrated efficacy in mitigating the effects. As a summary it can be said that negative feedback loop related genes were not affected that much which are *per1b* and *cry1*. However, positive feedback loops related to genes have significant results in the presence of methylphenidate treatment alone and these genes are *clock* and *bmal1*. This situation can indicate activation of circadian rhythm more than the normal control situation in a 24 hour cycle. One outlier value was excluded for each group.

To validate the absorption of the administered drugs by the fish, the expression levels of their receptors which are *drd2*, *drd3*, *oxtr*, and *nr3c1* in the brain were analyzed, as shown in Figure 1.7. The absorption of methylphenidate by the fish was confirmed through the increased expression of DRD2 and DRD3 receptors. Interestingly, with the intake of melatonin, decrease in the dopamine receptor expression was observed although methylphenidate was present simultaneously. Also, difference between dopamine receptor types was observed in the presence of oxytocin hormone and inhibition of *drd3* receptor was actualized more in the presence of oxytocin instead of *drd2* to compensate for the overexpression of dopamine receptors. However, it is observed that there is a problem with dosage of melatonin and oxytocin because the effect coming from methylphenidate drug is overly compensated with melatonin and oxytocin hormone and the actual desired effect for relating hyperactivity disorder is minimized due to the extra other treatments.

Interestingly, in the presence of oxytocin and melatonin, DRD2 receptor expression was observed at levels comparable to the control group. This suggests that in the presence of dopamine and melatonin, the direct expression of receptors is suppressed. As a result, the excessive dopamine present in the synaptic clefts due to methylphenidate cannot bind to receptors on adjacent neurons, effectively reducing the impact of methylphenidate.

When examining the oxytocin receptor, no significant difference was observed between the control group and the oxytocin-treated group, as shown in Figure 1.7. This indicates that the underlying suppression mechanisms require further in-depth investigation. The activity of the glucocorticoid receptor, which is known to be associated with stress, was reduced in the presence of both melatonin and oxytocin compared to the group that received only methylphenidate.

In Figure 1.7, the expression analysis of inflammation-related genes which are *tnfalpa* and *il6* was conducted on adult female zebrafish across the four treatment groups. The most significant finding is that methylphenidate usage induces inflammation in brain tissue. Another important result is that both melatonin and oxytocin, when administered simultaneously with methylphenidate, exhibited anti-inflammatory effects in brain tissue. Lastly, zebrafish individuals can act similar to human patients having diversified characteristics and this situation can increase variation as depicted in variation bars. In adult zebrafish analysis each zebrafish in an individual group was analyzed separately and pooling was not performed.

Behavioral experiments conducted on adult female zebrafish from the first generation treated with medications revealed significant differences in behavior across the four treatment groups, as shown in Figure 1.8 during the novel tank test. In this test, the tank is conceptually divided into two halves: the lower half represents increased depression, while movement in the upper half indicates reduced depression. The fish's swimming speed and exploratory movements between different areas of the tank provide insights into anxiety and depression levels. As seen in section A of Figure 1.8, zebrafish treated with only methylphenidate displayed increased time spent in the lower half of the tank, along with slower movements. This suggests that high doses of methylphenidate lead to an increase in depression. Additionally, the reduced exploratory behavior and avoidance of other tank areas further indicate elevated depression levels. In contrast, zebrafish treated with melatonin or oxytocin showed a more balanced movement pattern throughout the tank. These fish exhibited increased exploratory behavior, suggesting a reduction in depression tendencies. Furthermore, the increased time spent in the upper half of the tank supports the conclusion that melatonin and

oxytocin treatments alleviate depressive behaviors. Lastly, the behavioral patterns observed in Group D of Figure 1.8 (control group) were similar to those in the melatonin- and oxytocin-treated groups, demonstrating the compensatory effects of these hormones in reducing depression. In figure 1.8, heatmaps for four different groups in terms of novel tank tests are depicted which were designed according to the statistics of each zebrafish in the tank. These parameters are: Distance moved Center-point Total cm, Velocity Center-point Mean cm/s, In zone Arena / Center-point Frequency, In zone Arena / Center-point Cumulative Duration s, In zone bottomarea / Center-point Frequency, In zone bottomarea / Center-point Cumulative Duration s, In zone bottomarea / Center-point Latency to First s, In zone toparea / Center-point Frequency, In zone toparea / Center-point Cumulative Duration s, In zone toparea / Center-point Latency to First s, Zone transition Center-point / toparea Frequency, Zone transition Center-point / bottomarea Frequency, Movement Moving / Center-point Cumulative Duration s, Movement Not Moving / Center-point Cumulative Duration s.

In figure 1.9, individual parameters are depicted for the novel tank test. It has been observed that there is an interaction effect in the duration of top and bottom area when the comparison was done together for top and bottom area duration. Top and bottom area durations are not significant in itself. The results shown in Figure 1.10 pertain to the second behavioral experiment, the shoaling assay, which analyzed and compared the social tendencies of zebrafish across the four treatment groups. The parameters examined included nearest neighbor distance, inter-fish distance, farthest neighbor distance, and shoal area. The results revealed that methylphenidate usage significantly reduced social tendencies in zebrafish, as demonstrated by the behavioral assay. The best compensation effect was observed when melatonin was administered alongside methylphenidate. However, oxytocin also provided a significant level of compensation. The results shown in Figure 1.11 and Figure 1.12 were taken from second generation zebrafish eggs which are the offspring of treatment groups of adult female zebrafish. DNA was isolated from 4 different groups of offspring corresponding to the parental treatment group.

The results shown in Figure 1.11 are about the relative telomere length

comparison for offsprings of four different treatment groups. When comparing the total telomere length in the offspring eggs from the group treated with methylphenidate, it was found to be shorter than in the other group. This suggests that long-term use of Ritalin or Concerta could potentially lead to brain aging in the second-generation offspring, who may be born with shorter telomeres and experience accelerated aging in later life. The most compensatory effect was observed with melatonin treatment, which supports our hypothesis that children born with an increased susceptibility to aging due to circadian rhythm disruption could be compensated for by melatonin. However, when compared to the group treated solely with methylphenidate, a difference in telomere length was found between the eggs of adult fish that received oxytocin treatment. This indicates that oxytocin could serve as a compensatory treatment to counteract the effects of short telomere length and prevent the offspring from being born with this predisposition. In that situation in order to get more significant results there is the need for a longer treatment period to get epigenetically affected offspring.

The results shown in Figure 1.12 are about the methylation status of clock genes that are found to be related to brain aging and the results are depicted for comparison of offsprings of four different treatment groups. As seen here in figure 1.12, the use of methylphenidate alone resulted in methylation of foxm1 gene, and both melatonin and oxytocin hormone resulted in unmethylated foxm1 gene which is responsible for cell proliferation and cell cycle. foxm1 gene is also participating in the DNA damage checkpoint response [181]. That is a gene that is increased as the age increases as a marker of brain aging.

1.5 Conclusion and Future Perspectives

The main goal of this study was to find adjunctive treatments that could mitigate the negative effects of methylphenidate (Ritalin or Concerta) on circadian rhythm, which may impact the second-generation offspring. The first approach involved administering melatonin, a known circadian rhythm-regulating hormone, along with methylphenidate to see if it could reduce the negative effects. This

combination showed the greatest compensatory effect, alleviating the issue of brain aging susceptibility in the second-generation offspring, which could arise due to circadian rhythm disruption or other factors. An innovative result from this study was the potential use of oxytocin as an alternative treatment.

In this study, there is a need for dosage optimization of melatonin and oxytocin because some adult qRT-PCR results showed differences compared to the control group. Dosage adjustment is required to achieve a compensation effect closer to that of the control group. Additionally, to study transgenerational epigenetic effect transmission, it is necessary to allow the next generation to grow and obtain their offspring (grandchildren generation), which is feasible.

Furthermore, more detailed and comprehensive research is needed to translate these findings to humans. At this stage, combinational treatments could be applied to adult women using the medication before pregnancy, followed by controlled pregnancies after observing the compensation effect. Since oxytocin is a hormone that triggers labor, it cannot be used during pregnancy.

Due to time constraints, a longitudinal study could not be conducted. However, growing the offspring and subjecting them to behavioral assays is also necessary. Additionally, telomere-level changes could be analyzed using more specific methods beyond qPCR, such as chip-seq, to investigate heterochromatin structures. The IC50 method could also be employed to optimize dosages and better understand the effects of the drugs.

Chapter 2

Early indicators of nervous system dysfunction in gut-first and brain-first animal models of Parkinson's Disease

This section highlights the second research project I contributed to during my academic journey which is “Early indicators of nervous system dysfunction in gut-first and brain-first animal models of Parkinson's Disease”. In the first study, dopaminergic system malfunctioning was examined in the context of neurodevelopmental disorders, specifically ADHD, which emerges during childhood. In this study, we investigate the manifestation of dopaminergic system dysfunction in old age as a neurodegenerative disease, focusing on a Parkinson's disease model. Through this thesis, we have explored disorders arising from dopamine deficiency at different stages of life.

2.1 Introduction

In the earlier part of the study, a fellow researcher in our laboratory developed a transgenic zebrafish model of Parkinson’s disease. To achieve this, a plasmid designed for the production of alpha-synuclein (asyn) was injected into single-cell embryos using the Tol2 vector system. The fish that survived were raised until they reached six months of age. The experimental tasks involving these six-month-old fish were conducted as part of this thesis. Specifically, to investigate whether the pathological effects of Parkinson’s disease are more pronounced when originating in the brain or the gut, asyn fibrils were injected into these two distinct organs. The contribution of this section of the thesis to the overall project includes the analysis of behavioral experiments and the measurement of the migration capacity of asyn fibrils from the brain to the gut or vice versa.

Parkinson’s disease (PD) is marked by the abnormal misfolding of the alpha-synuclein (asyn) protein, which leads to progressive brain neurodegeneration and the development of motor symptoms. The bidirectional and trans-synaptic spread of pathogenic asyn along the body-brain axis is thought to play a significant role in PD progression. Beyond brain damage, PD is also associated with extensive peripheral nerve damage, particularly in organs like the gut and heart, which can lead to severe non-motor symptoms that may appear up to two decades before motor symptoms emerge. This early ”pre-motor” stage of the disease is highly variable among patients, with differing levels of involvement across neuronal systems. Such variability in the early disease presentation may be linked to differences in the site of disease initiation, whether in the body or the brain[182].

A recent imaging study in human patients proposed that Parkinson’s disease (PD) can be categorized into two subtypes: (1) a ”body-first” type, where damage to the cardiac and enteric nervous systems occurs before the brain is affected, and (2) a ”brain-first” type, where neuronal degeneration in the brain precedes nerve damage in peripheral organs. Currently, there is no cure for PD, and available treatments focus solely on managing motor symptoms. Therefore, developing animal models that replicate a distinct pre-motor phase (providing a therapeutic

window) and align with human PD subtypes is essential for testing targeted treatment strategies. In this study, we aim to replicate these two subtypes in transgenic zebrafish by introducing pathogenic alpha-synuclein (asyn) into the gut to mimic the body-first type and into the brain to simulate the brain-first type[183][184][185][186].

Parkinson’s disease can develop due to genetic factors, with some patients inheriting genetic variants from their families that increase the risk. Even with a single mutation, the regulation of the alpha-synuclein protein, encoded by the SNCA gene, can be altered. Mutations in this gene may lead to the aggregation of the alpha-synuclein protein, contributing to the onset of Parkinson’s disease[187][188].

Alpha-synuclein, produced in the central nervous system, is a small and soluble protein naturally expressed in its monomeric form throughout the body [189]. However, in its mutant form, even minor changes in the amino acid sequence can disrupt the protein’s proper conformation, leading to aggregation. These aggregates interfere with cellular function, and the mutant alpha-synuclein protein ultimately impairs motor functions. Over time, this aggregation in the central nervous system contributes to the development of Parkinson’s disease symptoms. The propagation of alpha-synuclein (asyn) aggregates through synaptically connected networks is considered a key factor in the progression of Parkinson’s disease (PD). This process leads to Lewy pathology and neurodegeneration not only in the central nervous system (CNS) but also in various peripheral organs. Notably, asyn aggregates have been identified in the gut tissue of PD patients, with gastrointestinal symptoms, such as constipation, often manifesting up to two decades before the appearance of motor symptoms[190]. These observations support the “gut-first” hypothesis of PD, which suggests that the pathological conversion of normal asyn into aggregated forms begins in the gut. From there, the aggregates are thought to spread to the brain through parasympathetic (vagal) and sympathetic nerve pathways[191].

Over time, both subtypes of Parkinson’s disease progress to a similar advanced stage, characterized by widespread involvement of the entire brain and multiple

peripheral organs. Figure 2.1 provides an overview of the distinct clinical features associated with the body-first and brain-first PD subtypes.

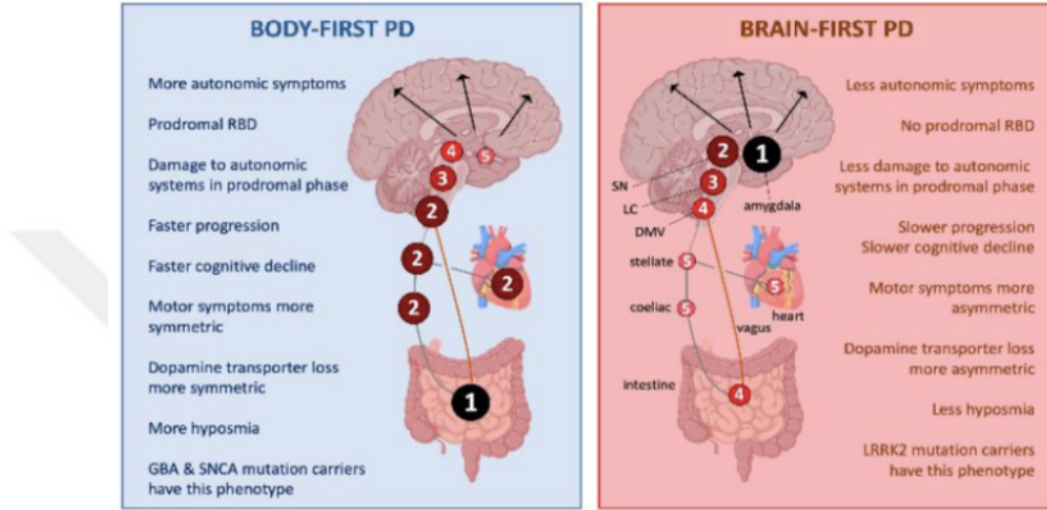


Figure 2.1: Comparison of body-first and brain-first PD subtypes [192].

Currently, no treatment exists to cure Parkinson’s disease (PD) or slow its progression[192]. The early or pre-motor phase is considered the optimal window for implementing personalized disease-modifying therapies (DMT). Therefore, it is essential to develop animal models that clearly exhibit a pre-motor phase and accurately reflect the subtypes of human PD[193][194][195]. In this study, a transgenic zebrafish model was created, and analyses were conducted on six-month-old zebrafish to investigate early-stage PD characteristics to analyze the effects of diversified initiation places of Parkinson which are brain or body (gut) via injection of synthetic pre-formed fibrils (PFF) of asyn through brain or gut into the transgenic Parkinson model zebrafish, which were generated by our laboratory. Then, observation of the migration ability of synthetic asyn PFF was actualized. By this way, we can observe the different pathologies in the adult transgenic Parkinson zebrafish model with immunohistochemistry analysis and behavioral analysis [196][197][198].

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Zebrafish Line

A 6 months old asyn-tg zebrafish which is generated as Parkinson transgenic model was utilized for injections. All zebrafish experiments were done in Bilkent University ASBAM Zebrafish laboratory. In the Bilkent University ASBAM zebrafish laboratory, the system water is maintained at a temperature of 28°C and a pH range of 7.0 to 7.5. The fish are kept under a 14-hour dark and 10-hour light cycle. They are fed twice daily with dry food and once with Artemia. Ethics decision was dated 22/12/2021 (No:2021/18).

2.2.1.2 Equipment and Tools

16 mm coverslip	Marienfeld
Confocal Microscope	SP8, Leica
Cryostat	CM1850, Leica
Histobond Adhesive Slides	0810001, Marienfeld
Incubator	INCU-Line
Oh Meter	SevenCompact, Mettler Toledo
PAP Pen	ab2601, Abcam
Vortex	Vortex Mixer, VWR
Zebrafish Breeding Tank	Custom Made
Insulin Syringe	BeyanLab
Hamilton 10 ul Microliter Syringe Model	80301, Hamilton
Glass Capillary Needle	Sutter Instruments
Pipettes	Rainin, Greiner, Eppendorf

Table 2.1: Equipment and tools for pdpam project

2.2.1.3 Chemicals and Reagents

Methylene Blue	947.D05, Interlab
Sodium Chloride NaCl	969.033.1000, Isolab
Nuclease Free Water	CBioline, Promega
Hydrochloric acid	Interlab
Tris Base	T1503-500G, Sigma Aldrich
Acetic Acid	901.013.2500, Merck
Formaldehyde 37%	1.04002.2500, Merck
Optimum Cutting Temperature (OCT)	4583, Tissue- Tek
PBS Tablet	Sigma Aldrich
Sucrose	S0389, Merck
Trizma Base	T1503-500G, Sigma Aldrich
Triton X-100	T8787, Sigma Aldrich
Alpha Synuclein PFF	From collaboration lab self-made
KCl	960.033, Interlab
CaCl ₂	102382, Merck

Table 2.2: Chemical and Reagents for pdpam project

2.2.1.4 Antibodies

Alpha synuclein (Rabbit mAb)	E4U2F, Cell Signaling Technologies, 1:500 dilution
Beta actin	ab170325, ABCAM. 1:500 dilution
DAPI	D9542-1MG, Sigma Aldrich

Table 2.3: Antibodies utilized in pdpam project

2.2.2 Methods

2.2.2.1 Cerebroventricular microinjection (CVMI) and peritoneal injections of - syn fibrils

The experimental transgenic zebrafish were fasted prior to the day of injection to clear the gut area.

For the preparation of alpha-synuclein pre-formed fibrils (PFF), a 5mg/ml stock solution was diluted with sterile PBS and loading dye to achieve a final

injection concentration of 1mg/ml in a 10 l volume. Synthetic alpha-synuclein was then injected into the brains of transgenic Parkinson's disease model zebrafish for the brain-first initiated model, using cerebroventricular microinjection (CVMI). The fish were gradually anesthetized with ice-cold water, after which they were carefully positioned on a wet sponge to ensure proper alignment and prevent hypoxia [199].

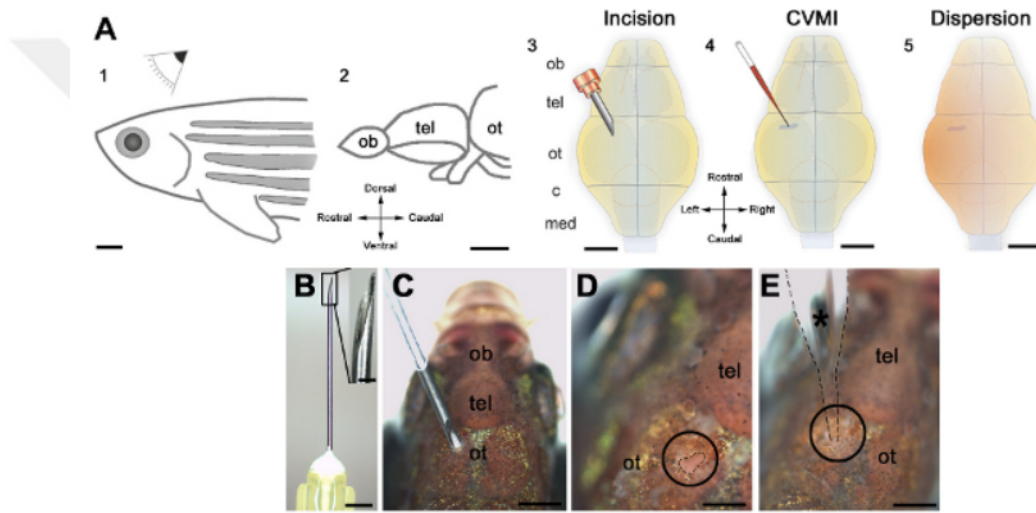


Figure 2.2: Cerebroventricular Microinjection Depiction for Adult Zebrafish Brain from Telencephalon Region [199]

For the cerebroventricular injections, the fish were oriented with their heads facing upwards, as shown in Figure 2.2. The skull was punctured using an insulin needle, and a Hamilton syringe with a fine needle was used to inject 10 μ l of the injection solution into the telencephalon region of the brain at a concentration of 0.1 mg/ml. Following the injection, the fish were quickly transferred to system water to recover. Any fish displaying neurological abnormalities were euthanized and dissected for further analysis[199].

For intraperitoneal injections designed for the body first Parkinson model, the fish were carefully positioned upside down to allow for the delivery of the solution into the abdominal cavity. The injections were carried out using a Hamilton syringe, directed into the area of the abdomen just behind the pelvic girdle. To ensure the gastrointestinal tract was empty and to maximize available space in the

abdomen, the fish were fasted for 24 hours prior to the procedure. On the day of the injection, the fish were gradually anesthetized with ice-cold water and placed on their ventral side in a slot within a wet sponge. The injection, consisting of 10 l of solution, was administered at a 45-degree angle using a Hamilton syringe[200].

Injection Material & Injection Organ	Brain	Gut
PBS	PBS Brain injection	PBS Gut injection
asyn fibrils	Asyn fibrils brain injection	Asyn fibrils gut injection

Table 2.4: Four Experimental Group: Injection Places and Injection Materials

2.2.2.2 Behavioral Analysis: Novel Tank Test

After the day of injection, the behavior tests were recorded with Iphone 11 Pro Max. It is done one day later because the synthetic alpha synuclein fibrils can be cleared by immune system of fish.

To begin, an infrared light is placed behind the novel tank, with a white acrylic sheet positioned between the tank and the light to diffuse it and eliminate glare. Treatments are administered, and a waiting period of 30 minutes is observed to allow for drug absorption before initiating the novel tank assay. The tank is then filled to 3/4 capacity with fish system water.

A single acclimatized fish is transferred from a holding tank to a beaker containing fish system water and left to rest for an additional 10 minutes. The fish, along with the water, is then gently introduced into the novel tank. Using a still-frame video image, the tank's top and bottom areas are identified by calculating its height and dividing it by two. The time spent in each area and the total distance traveled are measured using tracking software[170][171].

Adult zebrafish typically exhibit a preference for the bottom half of the tank when introduced to a novel environment due to stress. As the stress diminishes, they progressively spend more time in the top half. Three zebrafish per experimental group are monitored during the assay [172].

2.2.2.3 Behavioral Analysis: Swim Endurance Test

To compare motor dysfunction between the brain-first and body-first models, the fish were subjected to a swim endurance test the day after brain and gut injections. The test setup involved a tank with strong air currents, where a swim tunnel was created by fixing the fish to the middle area on the right side of the tank. Once the tank was filled with system water, the fish from each group were first placed in a holding tank for 10 minutes. After this, the fish were allowed to enter the experimental tank from the left side, and the motor was turned on, creating conditions that would make swimming more difficult by generating horizontal air bubbles that obstructed the fish's swimming direction. The parameters observed during the test included the amount of time spent in a designated area at the back left, the time it took for the fish to fall to the back of the tank after exhaustion, the ability and duration of swimming against the current, and swimming speed. Fish that reached exhaustion fell to the back of the tank, which is an important parameter for assessing swim endurance capacity. After the test, the fish were filmed for 5 minutes, removed from the experimental tank, rested in the holding tank, and then returned to the experimental tank[201].

2.2.2.4 Behavioral Analysis: Hyposmia Test

To compare hyposmia, or partial loss of smell, between the brain-first and body-first fish models, a protocol adapted from a previous study was applied [202]. An ultra-highly scented rotifer food was placed in the bottom right corner of the experimental tank, surrounded by decorative tank items that prevented the food from being seen but did not obstruct its scent. The experimental fish, which had been kept in the holding tank on the far left side for 10 minutes, were then introduced into the tank. During a 5-minute recording period, the time it took for the fish to locate the food and the total time spent in the food area were recorded and analyzed[202].

2.2.2.5 Buffers

The 10X TAE stock solution was prepared by dissolving 48.5 grams of Tris-base, 11.4 ml of acetic acid, and 20 ml of 0.5M EDTA in 800 ml of distilled water. The final volume was then adjusted to 1 liter. The 1X TAE solution was prepared by adding 100 ml of the 10X stock solution to 900 ml of distilled water. A 4% paraformaldehyde solution was prepared by dissolving 40 grams of paraformaldehyde in 800 ml of preheated sterile PBS. After the solution cooled, it was sterile-filtered and the volume was adjusted to 1 liter with PBS. Sucrose solutions were prepared for tissue dehydration at concentrations of 10%, 20%, and 30%. The 10% sucrose solution was made by dissolving 1 gram of sucrose in 10 ml of distilled water. The 20% sucrose solution was prepared by dissolving 2 grams of sucrose in 10 ml of distilled water. The 30% sucrose solution was created by dissolving 3 grams of sucrose in 10 ml of distilled water. The 10X TBS stock solution was prepared by dissolving 12 grams of Trizma Base and 80 grams of NaCl in 800 ml of distilled water using a magnetic stirrer. The pH was adjusted to 7.6 with HCl, and the final volume was adjusted to 1 liter. The 1X TBS solution was prepared by mixing 100 ml of the 10X TBS stock solution with 900 ml of distilled water. The TBS 0.025% Triton X-100 (TBS-T) solution was prepared by adding 250 μ l of Triton X-100 to 1 liter of 1X TBS. The blocking solution (5% BSA in TBS-T) was prepared by dissolving 0.5 grams of BSA in 10 ml of TBS-Triton solution.

2.2.2.6 Tissue preparation and cryosectioning

The fish undergo dissection the day after the behavior experiments to perform immunohistochemistry analysis. Brain and intestinal tissues from each group of fish are collected for migration analysis. The zebrafish, euthanized by prolonged exposure to ice water, have their tissues extracted in sterile PBS. To preserve the tissues in three dimensions, they are first immersed in a 4% paraformaldehyde solution overnight for fixation. After fixation, on the following day, the paraformaldehyde is removed by washing the tissues three times for 5 minutes

each in PBS, with partial drying between washes. The tissues were gradually dehydrated in 10%, 20%, and 30% sucrose solutions, with the duration of each step determined by the sinking of the tissue into the solution. However, all tissues were kept in the 30% sucrose solution overnight for cryoprotection. After this process, both brain and gut tissues were collected and embedded in O.C.T. compound, then stored at -80°C until sectioning the next day. Tissue sectioning was performed using a Leica cryostat, where the embedded tissues were carefully detached from the O.C.T. mold and secured onto the cryostat's sample holder with additional O.C.T. The cryostat was set to -30°C , and sections were sliced at a thickness of 15 to 25 μm , depending on tissue integrity. The tissue sections were carefully transferred onto HistoBond adhesive slides and dried by heating on a heat block at $56-60^{\circ}\text{C}$ for 15 minutes. After allowing the slides to cool and ensuring the tissue sections were securely adhered, the immunohistochemistry staining procedure was initiated.

2.2.2.7 Immunohistochemistry Analysis from Brain and Gut Sections

After drying of tissue sections washing steps should begin. The tissue sections were first washed three times for 5 minutes each with 1X TBS-Triton X-100 solution at room temperature to remove any remaining contaminants. After the washing, the slides were dried again before the blocking solution was applied. The slides were placed onto petri dishes, and a 5% BSA in TBS-Triton X-100 blocking solution was added to fully cover the tissue sections. The slides were incubated for 2 hours at room temperature to block nonspecific binding sites. Following incubation, the blocking solution was carefully discarded. Next, the primary antibody, diluted at a ratio of 1:1000 in the blocking buffer, was applied to the slides, which were then incubated overnight at 4°C .

After the primary antibody incubation, the slides were washed five times for 5 minutes with TBS-Triton X-100 and air-dried. The secondary antibody, diluted at 1:1000 in the blocking buffer, was applied to the sections, and the slides were incubated for 2 hours in the dark at room temperature. Two types of secondary antibodies were used, depending on the primary antibody: rabbit primary with

rabbit secondary antibody and anti-mouse secondary for the mouse primary. Following this incubation, the slides were washed with TBS and stained with DAPI for 10 minutes. After the final wash with TBS, the slides were air-dried under a dark chamber. To complete the process, coverslips were fixed onto the slides with nail polish, and images were captured using a Leica SP8 Confocal Microscope at 20X, 40X, and 63X magnifications.

2.2.2.8 Data Analysis of pdpam Project

The behavioral experiment analyses were performed using the EthoVision software, while the slides from the immunohistochemistry staining were analyzed under a confocal microscope. After the imaging, the slides were evaluated by two different researchers for further analysis.

2.3 Results

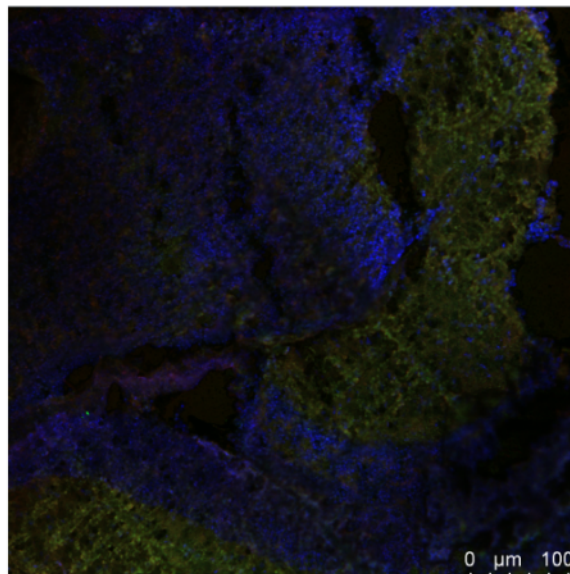


Figure 2.3: Immunostaining of brain tissue in asyn-tg fish injected with synuclein into the brain. Green: asyn antibody, Blue: DAPI, Red: Beta Actin.

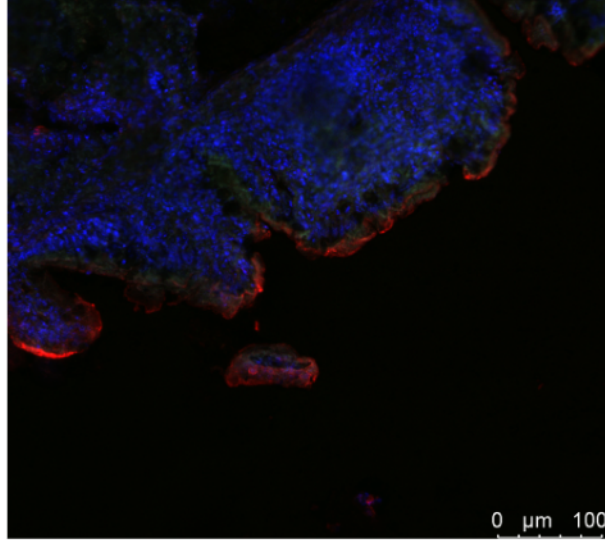


Figure 2.4: Immunostaining of intestinal tissue in asyn-tg fish injected with synuclein into the brain. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

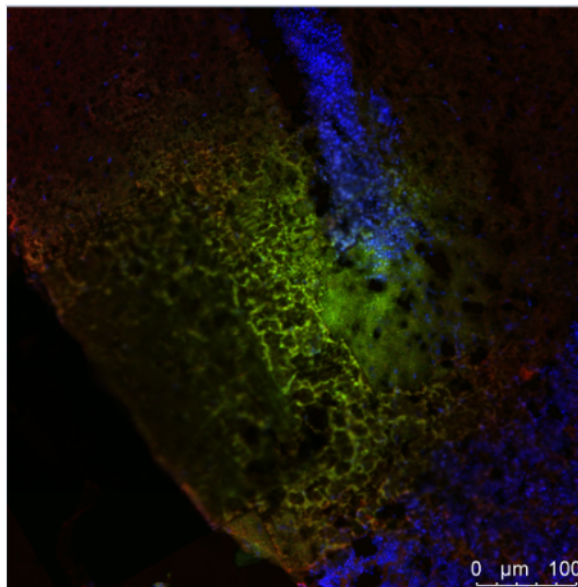


Figure 2.5: Immunostaining of brain tissue in asyn-tg fish injected with synuclein into the intestine. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

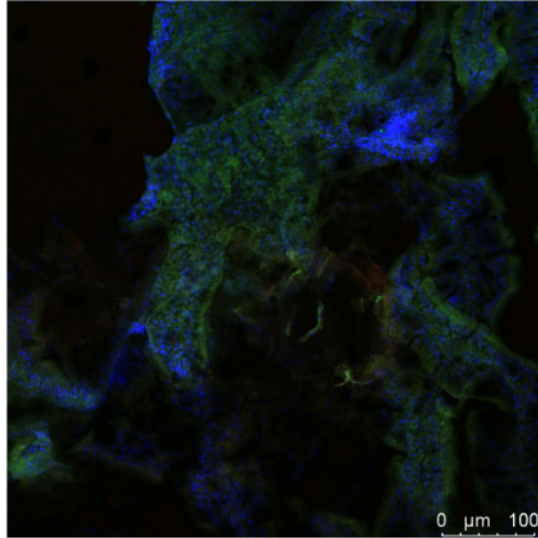


Figure 2.6: Immunostaining of intestinal tissue in asyn-tg fish injected with synuclein into the intestine. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

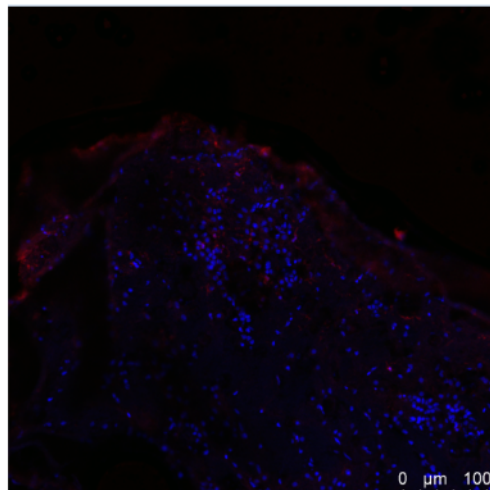


Figure 2.7: Immunostaining of brain tissue in asyn-tg fish injected with PBS into the brain. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

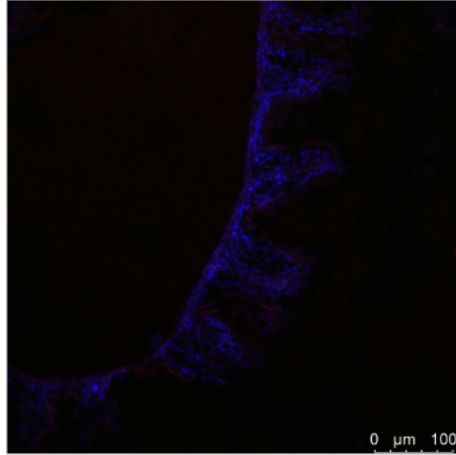


Figure 2.8: Immunostaining of intestinal tissue in asyn-tg fish injected with PBS into the brain. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

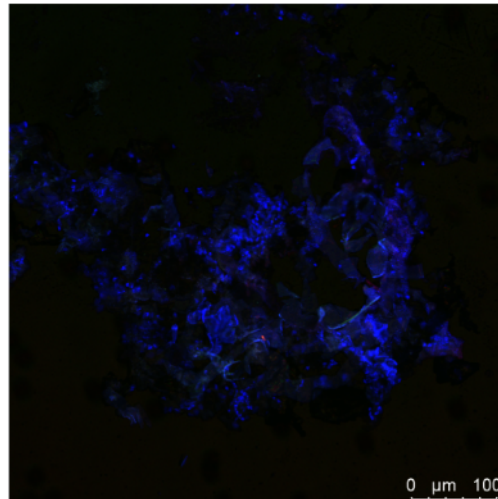


Figure 2.9: Immunostaining of brain tissue in asyn-tg fish injected with PBS into the intestine. Green: asyn antibody, Red: beta-actin antibody, Blue: DAPI.

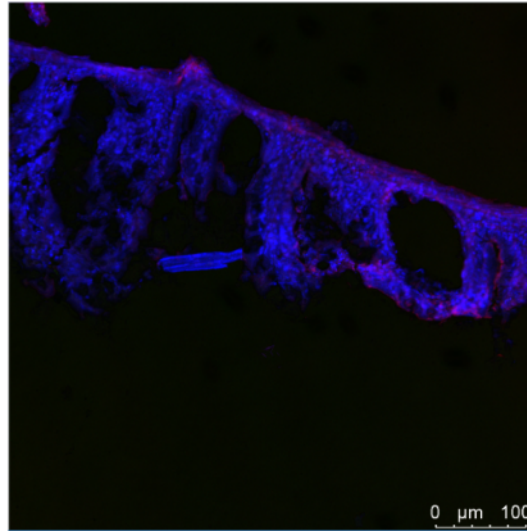


Figure 2.10: Immunostaining of intestinal tissue in asyn-tg fish injected with PBS into the intestine. Green: asyn antibody, Blue: DAPI.

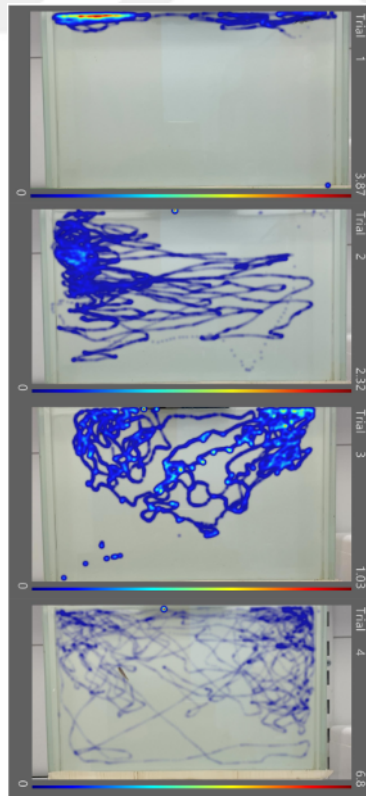


Figure 2.11: Visual results of the Novel Tank Test. Trial 1: PBS injected into the gut, Trial 2: PBS injected into the brain, Trial 3: asyn injected into the gut, Trial 4: asyn injected into the brain. Analyses were performed using EthoVision.

Trial	Total Distance from Central Point (cm)	Speed from Central Point (Average cm/s)	Total Time (s)	Time Spent Moving (s)	Time Spent Immobile (s)
pbs gut	1475.3	4.91849	243.41	253.62	46.413
pbs brain	1230.68	4.10684	270.704	136.103	163.83
asyn gut	1064.9	3.55212	236.403	221.72	78.2783
asyn brain	603.16	2.01054	289.25	95.962	204.037

Table 2.5: Statistical results of the Novel Tank Test. Analyses were performed using Ethovision

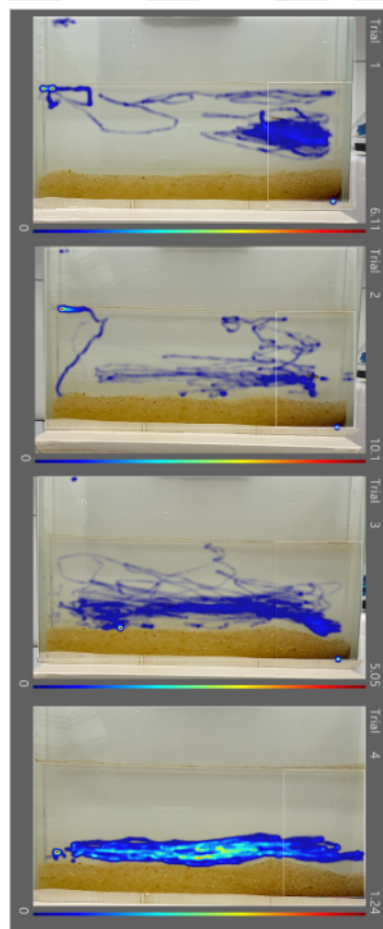


Figure 2.12: Visual results of the hyposmia test. Trial 1: PBS injected into the gut, Trial 2: PBS injected into the brain, Trial 3: asyn injected into the gut, Trial 4: asyn injected into the brain. Analyses were performed using Ethovision.

Trial	Total Distance from Central Point (cm)	Speed from Central Point (avg cm/s)	Movement / Total Time from Central Point (s)	Immobility / Total Time from Central Point (s)	Stop Frequency in Food Area	Total Time in Food Area
pbs gut	800.41	2.6692	165.73	134.234	37	158.22
pbs brain	883.16	2.9491	123.69	176.07	24	85.885
asyn gut	1341.2	4.47019	243.2	56.8235	24	89.489
asyn brain	572.533	1.91355	124.02	175.442	9	37.03

Table 2.6: Statistical results of the hyposmia test. Analyses were performed using Ethovision.

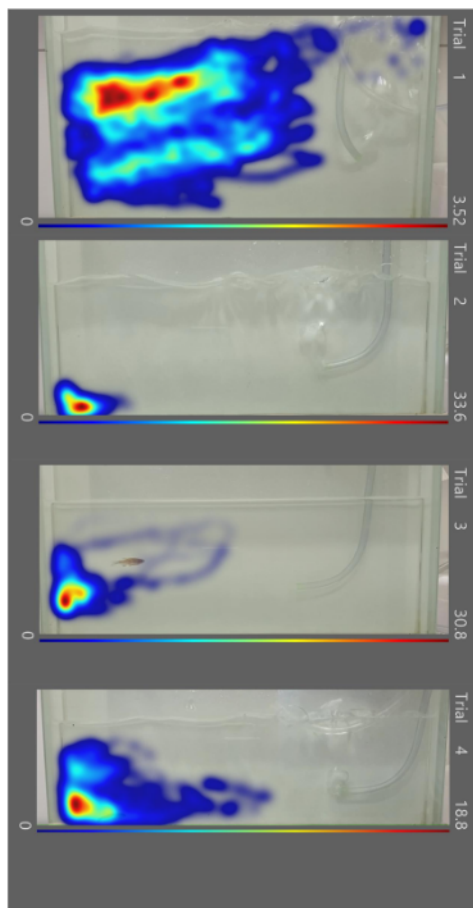


Figure 2.13: Visual results of the swim endurance test. Trial 1: PBS injected into the gut, Trial 2: PBS injected into the brain, Trial 3: Asyn injected into the gut, Trial 4: Asyn injected into the brain. Analyses were performed using Ethovision.

Trial	Mobility - Frequency	Mobility - Total Du- ration	Immobility - Fre- quency	Immobility - Total Duration
pbs gut	278	26.8268	417	38.7054
pbs brain	17	0.867534	24	164.53
asyn gut	24	1.5015	604	215.88
asyn brain	8	0.46713	382	254.72

Table 2.7: Statistical results of the Swim Endurance test. Analyses were performed using Ethovision.

2.4 Discussion

During this period, injections were performed on asyn transgenic fish, and immunostaining was carried out. In the previous period, asyn-tg fish were not yet adults, so adult fish without asyn-tg were used. In this phase, after the asyn-tg fish reached 6 months of age, they were injected with asyn fibrils, and immunostaining was conducted. In the fish injected with asyn into the brain, staining with asyn antibody is shown in Figure 2.3, where a widespread distribution of asyn is observed. Although these fish were 6 months old and considered adults, they were still relatively young, and due to the high regenerative ability of zebrafish at this age, staining was done 3 days after the injection.

When we performed immunostaining on the intestinal tissue of fish injected with asyn into the brain, we observed asyn staining overlapping with beta-actin staining towards the outer regions of the intestinal tissue (Figure 2.4). This result shows that asyn can infiltrate into the gut even just 3 days after injection into the brain.

For the intestinal injections, asyn fibrils were injected into the abdominal cavity of asyn-tg fish. The fish were anesthetized (with ice-cold water), held on their backs, and injections were performed into the abdominal cavity using a dental needle and Hamilton syringe. Three days after the abdominal injection, we observed the presence of fibrils in the brain (Figure 2.5). While we can observe direct infiltration into the brain from the abdominal cavity with small molecules like BrdU within 4 hours, we were able to see the spread of asyn fibrils, which are relatively larger molecules, into the brain.

When we injected asyn fibrils into the abdominal cavity, we observed spread in some areas (Figure 2.6) but not in others (Figure 2.6). Despite the spread from the abdomen to the brain, the heterogeneous regions in the intestines suggested that the fibrils did not spread rapidly in the gut or did not spread completely due to the heterogeneous nature of the intestinal tissue.

The fish used in these experiments were asyn-tg fish, which stably harbor the

human asyn gene. As we know from the literature, asyn-tg fish do not produce the human protein, but they must carry this gene in order to create a Parkinson's model [203]. The injection of alpha-synuclein fibrils alone, or the presence of asyn transgenic fish alone, is insufficient for the formation of synuclein aggregates. However, after injecting fibrils into asyn-tg fish, fibril aggregates were observed, which was consistent with our findings. To confirm this, we also injected PBS into asyn-tg fish. We performed immunostaining with asyn antibody on the brain (Figure 2.7) and intestinal (Figure 2.8) tissues after PBS injection into the brain. Similarly, after injecting into the intestine, we conducted asyn staining on the brain (Figure 2.9) and intestinal (Figure 2.10) tissues. In these experiments, no asyn fibrils were observed, confirming that asyn-tg fish do not form fibrils without injection.

In the tank test, the fish swimming in the lower areas of the tank indicates a tendency toward depression, while swimming in the upper areas suggests that the fish is not depressed. Exploring larger areas indicates the presence of exploratory behavior. A more pronounced blue color signifies more movement, whereas a faded blue indicates a more stationary state. As seen in Figure 2.11, the fish injected with PBS in the gut moved mostly on the surface, while the fish injected with PBS in the brain moved both near the surface and more extensively. The fish injected with asyn in the gut also showed high exploratory behavior, as it moved around the tank. The most dramatic differences were observed in the fish injected with asyn in the brain. As indicated by the pale blue color, these fish moved significantly less than the others. Looking at the analysis results provided in Table 2.5, we can see that the first three groups showed similar movement distances from the central point, while the group injected with asyn in the brain had significantly less movement. In terms of movement speed, the PBS-injected group showed the highest speed, while the asyn brain group had the lowest. We also observed a much higher immobility duration in the asyn brain group, which could be considered a marker for depression. Based on these results, when evaluating the Parkinson's-like model using the novel tank test, we can conclude that direct injection of asyn into the brain has a significant effect, whereas asyn injected into the gut is less effective.

The buried food test adapted for zebrafish involved placing a small food pellet beneath a thin layer of substrate in a 1 L tank. Zebrafish were introduced into the tank and allowed to explore for 10 minutes. The latency in finding the buried food pellet was recorded as a measure of food-seeking behavior guided by olfactory cues. It is expected that hyposmic zebrafish would show a longer latency compared to controls, indicating a diminished sense of smell.

In the hyposmia test, a highly scented food was placed in the bottom right corner of the tank. This area (marked by the white rectangle in Figure 2.12) was defined as the feeding area. The visibility of the food was obstructed by adding beads to the tank bottom. By impairing visibility, it was expected that fish would rely solely on their sense of smell to navigate towards the food area. It was anticipated that fish with impaired or weakened olfactory senses would spend less time in this area. According to Figure 2.12 and the results presented in Table 2.6, zebrafish injected with asyn in the brain exhibited a significant decrease in frequency of stopping and total time spent in the feeding area. In comparison to zebrafish injected with PBS in the brain, zebrafish injected with PBS in the gut showed fewer effects, suggesting that even PBS injections in the brain caused damage. Furthermore, zebrafish injected with asyn in the brain demonstrated a significant reduction in comparison to those injected with PBS, indicating that asyn injection had a notable impact. Although the effect of gut asyn injection was less pronounced than brain asyn injection, it was still considered effective. Consistent with the novel tank test, the total movement time and speed were significantly reduced in the brain asyn group. In contrast, the increase in total time and speed in the gut asyn injection group could suggest increased anxiety.

The motor function test involved examining parameters such as average speed, distance covered, and the fish's preferred position. Individual zebrafish were placed into a new tank containing fresh aquarium water, with a plastic tube positioned within the tank. One end of the tube was connected to a small air pump, which provided a constant airflow, creating a strong air current in the tank. The fish's swimming behavior was observed in response to this airflow.

The swim endurance test was conducted to measure the motor functions of

the fish. A tube placed at the right side of the tank (Figure 2.13) produced a strong airflow, with bubbles created to hinder the fish's swimming in that area. Fish that swam near the bubbles and had longer swim times were interpreted as having good motor functions, while those that stayed further away and showed reduced movement were considered to have diminished motor functions. In the gut PBS-injected control group, the fish displayed a widespread and active swimming pattern, as seen in Figure 2.13. In the brain PBS-injected group, experimental damage was suspected. Increasing the number of fish in this trial would help determine if this was an experimental error or if the PBS injection had the same effect. According to the analysis results in Table 2.7, the gut PBS-injected group had a significantly higher mobility frequency compared to the other three groups, while the brain asyn-injected group showed the lowest mobility frequency. Similar results were found when considering total mobility duration. In terms of immobility frequency and total duration, significant differences were observed in the asyn-injected groups.

2.5 Conclusion

As a result of the analyses conducted in the second work package of the PD-PAM project, it has been observed that the brain-first or body-first Parkinson's model is indeed accurate. Immunohistochemistry analysis revealed that α -synuclein fibrils migrate from the brain to the gut and from the gut to the brain. Additionally, significant findings emerged from the behavioral experiments, including increased susceptibility to depression and anxiety, partial loss of smell, and motor function impairment.

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