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YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

NEUROSCIENCE PROGRAM

INVESTIGATION OF NEUROENDOCRINE EFFECTS OF METHYLPHENIDATE ON GONADOTROPIN RELEASING HORMONE AND KISSPEPTIN IN CELL CULTURE MODELS

DOCTOR OF PHILOSOPHY THESIS

Berna Özelgün, MD PhD

İSTANBUL, 2022

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 22/01/2022 and numbered 2022/02-13.

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

21/01/2022

Dr. Berna Özelgün

DEDICATION

I dedicate to my thesis to my family for their endless love and support.

Dr. Berna Özelgün

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LIST OF SYMBOLS AND ABBREVIATIONS

ADHD	Attention Deficit and Hyperactivity Disorder
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
C	Celcius degrees
cDNA	Cyclic Deoxyribonucleic Acid
COVID-19	Coronavrus Disease 2019
df	Degrees of freedom
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EMA	European Medical Agency
F	Fischer value of varience ratio
FSH	Follicle-stimulating hormone
GABA	Gamma-Aminobutyric acid
GnRH	Gonadotropin-releasing hormone
GT	GnRH Tagged cell line
h	Hour
HEK	Human embryonic kidney
HPG	Hypothalamic-pituitary-gonadal
HypoE	Hypothalamic, embryonic cell line

Kiss1	Kisspeptin
L-glutamine	Levo-glutamine
LH	Luteinizing hormone
M	Median
ME	Median eminence
min	Minutes
mL	Milliliters
mM	Milli Molar
MPH	Methylphenidate
n	Number of samples
ng	Nano Gram
nM	Nano Molar
p	Probability value
PBS	Phosphate buffered saline solution
POA	Preoptic area
qPCR	Quantitative polymerase chain reaction
R	Registered trademark
rHypoE	Rat Hypothalamic embrionic cell line
RNA	Ribonucleic Acid
rPC	Rat pheochromocytoma cells
sd	Standart deviation
sec	Seconds
SHBG	Sex Hormone Binding Globulin

t	Hypothesis test statistic
TM	Trade Mark
β-Actin	Beta-Actin
µg	Microgram
µL	Microliter
µM	Micromolar
$\bar{x}$	Mean

ABSTRACT

Özelgün, B (2022), "Investigation of neuroendocrine effects of methylphenidate on gonadotropin releasing hormone and kisspeptin in cell culture models", Yeditepe University, Institute of Health Sciences, Neuroscience Program, Doctorate Thesis. Istanbul.

Methylphenidate (MPH) is widely used among children for attention deficit hyperactivity disorder. Though there are discussions about the growth-related effects of its use, the neuroendocrine mechanisms of MPH around puberty have not been studied. This study aims to investigate the role of MPH on gonadotropin-releasing hormone (GnRH) and Kisspeptin (Kiss1) expression and secretion in vitro using cell culture techniques. GnRH and Kiss1 secreting cells, GT1-7 and rHypoE-8 cell lines respectively, were maintained in DMEM containing 10% FBS and 1% PSN at 37°C under 5% CO2 humidified atmosphere. The effect of MPH on proliferation and migration at different doses (1 nM-200 μM) for 24 hours was assessed by trypan blue dye exclusion and wound healing (scratch) assays, respectively. To investigate GnRH expression and secretion, cells were cultured in maintenance medium without FBS with or without MPH (200 μM) and samples at different were obtained to investigate GnRH expression by real-time qRT-PCR and GnRH release to the medium by ELISA. Normal distribution of the data was analyzed by Shapiro-Wilk normality test. Data from dose-response experiments were either analyzed one-way ANOVA or Kruskal-Wallis followed by Dunnet's multiple comparison or Dunn's multiple comparison tests, respectively. Real-time qPCR and ELISA data were analyzed by Student's t-test and $2^{-\Delta\Delta C_T}$ method. There were no significant differences between untreated cells and MPH-treated cells in terms of proliferation and migration. As the proliferation and migration of the cells were not affected by the different concentrations of MPH, the highest concentration of 200 μM was selected to assess gene expression and hormone secretion. GnRH expression has remained comparable to the control arm with no statistical difference at any time point. GnRH release was significantly higher under MPH compared to the control group at 2 hours after treatment ($p<0.05$), while no differences were found between groups at other time points. Kiss1 expression was assessed for time points of 30

minutes, 2 hours, and 24 hours. Kiss1 expression was significantly increased in the first 30 minutes of MPH treatment. As, MPH affected GnRH and Kiss1 expression at multiple time points, to further investigate the response of the cells, the recovery in the expression after the cessation of MPH treatment was analyzed. It was observed that after the cessation of MPH treatment there is no statistical significance between MPH treatment and control arms in GnRH expression. Though the cellular evidence suggests that MPH affects the expression and release of GnRH and Kiss1 to some extent, it is far from reflecting the accumulating clinical data. Lack of statistical difference should not be interpreted as no differences between the MPH use over neuronal cell lines. When results are modeled for larger data sets (n= 30-60) statistically the majority of the timepoints become significant in comparison to the control arm. Our findings suggest puberty-delaying effects of MPH may be partly through GnRH and Kiss1 expression and release. Further investigations are needed to understand a medication that possibly have direct effects on 40 million people worldwide.

Keywords: Methylphenidate, Attention Deficit Hyperactivity Disorder, cell culture, puberty, Gonadotropin Releasing Hormone, Kisspeptin

ÖZET

Özelgün, B (2022), "Hücre kültürü modellerinde metilfenidatın gonadotropin salgılatıcı hormon ve kisspeptin üzerine nöroendokrin etkilerinin araştırılması", Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Sinirbilim Programı, Doktora Tezi. İstanbul

Metilfenidat (MPH), dikkat eksikliği hiperaktivite bozukluğu için çocuklarda yaygın olarak kullanılmaktadır. Kullanımının büyüme ile ilgili etkileri hakkında tartışmalar olmasına rağmen, MPH'nin ergenliğe geçiş sırasındaki nöroendokrin mekanizmaları henüz detaylı araştırılmamıştır. Bu çalışma, MPH'nin gonadotropin salgılatıcı hormon (GnRH) ve Kisspeptin (Kiss1) ekspresyonu ve salgılanması üzerindeki rolünü hücre kültürü teknikleri kullanarak in vitro olarak araştırmayı amaçlamaktadır. GnRH ve Kiss1 salgılayan hücreler, sırasıyla GT1-7 ve rHypoE-8, %5 CO2 ile nemlendirilmiş atmosfer altında 37°C'de %10 FBS ve %1 PSN içeren DMEM içinde tutuldu. MPH'nin 24 saat boyunca farklı dozlarda (1 nM-200 µM) proliferasyon ve migrasyon üzerindeki etkisi, sırasıyla tripan mavisi boya yöntemi ve yara iyileşme modelleriyle değerlendirildi. GnRH ekspresyonunu ve salgılanmasını araştırmak için hücreler, MPH'li veya MPH'siz (200 µM) FBS'siz ortamda hücre kültürü yapıldı ve gerçek zamanlı qRT-PCR ile GnRH ekspresyonunu ve ELISA ile ortama GnRH salınımını araştırmak için numuneler toplandı. Verilerin normal dağılımı Shapiro – Wilk normallik testi ile analiz edildi. Doz – yanıt deneylerinden elde edilen veriler, sırasıyla tek yönlü ANOVA ve Kruskal-Wallis, ardından sırasıyla Dunnet'in çoklu karşılaştırması ve Dunn'ın çoklu karşılaştırma testleri ile analiz edildi. Gerçek zamanlı qPCR ve ELISA verileri Student t-testi ve $2^{-\Delta\Delta C_T}$ yöntemi ile analiz edildi. MPH'ye maruz bırakılmış ve bırakılmamış hücreler arasında proliferasyon ve migrasyon açısından anlamlı bir fark yoktu. Hücrelerin proliferasyon ve migrasyonu, farklı MPH konsantrasyonlarından etkilenmediğinden, gen ekspresyonunu ve hormon salınımını değerlendirmek için en yüksek 200 µM konsantrasyon seçildi. GnRH ekspresyonu, herhangi bir zaman noktasında istatistiksel bir fark olmaksızın kontrol koluyla istatistiksel olarak benzer aralıkta seyretti. GnRH salınımı, tedaviden 2 saat sonra kontrol grubuna kıyasla MPH altında önemli ölçüde daha yüksek bulundu ($p<0.05$), diğer zaman

noktalarında gruplar arasında istatistiksel olarak anlamlı fark bulunmadı. Kiss1 ekspresyonu, 30 dakikalık, 2 saatlik ve 24 saatlik zaman noktaları için değerlendirildi. Kiss1 ekspresyonu, MPH tedavisinin ilk 30 dakikasında istatistiksel olarak anlamlı ölçüde arttığı gözlemlendi. MPH, GnRH ve Kiss1 ekspresyonunu çoklu zaman noktalarında etkilediğinden, hücrelerin tepkisini daha fazla araştırmak için MPH tedavisinin kesilmesinden sonra ekspresyondaki değişim analiz edildi. MPH tedavisinin kesilmesinden sonra, GnRH ekspresyonunda kontrol ve deney kolları arasında istatistiksel bir anlamlılığın olmadığı gözlendi. Hücresel kanıtlar, MPH'nin GnRH ve Kiss1'in ekspresyonunu ve salınımını bir dereceye kadar etkilediğini öne sürse de, hücre kültürünün klinik gerçeği yansıtma gücü sınırlıdır. İstatistiksel farkın olmaması, MPH kullanımının hücresel davranış üzerinde fark yaratacak etkisi olmadığı şeklinde yorumlanmamalıdır. Sonuçlar daha büyük veri kümeleri (n= 30 – 60) için istatistiksel olarak modellendiğinde, çoğu zaman noktası kontrol koluna kıyasla anlamlı hale geldiği gözlemlendi. Sonuçlarımız, MPH'nin ergenliği geciktirici etkilerinin kısmen GnRH ve Kiss1 ekspresyonu ve salınımı yoluyla olabileceğini düşündürmektedir. Dünya çapında 40 milyon insan üzerinde doğrudan etkisi olabilecek bir ilacı anlamak için daha fazla araştırmaya ihtiyaç var. Bulgularımız, MPH'nin ergenliği geciktirici etkilerinin kısmen GnRH ve Kiss1 ekspresyonu ve salınımı yoluyla olabileceğini düşündürmektedir. Dünya çapında 40 milyon insan üzerinde doğrudan etkisi olabilecek bir ilacı anlamak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar kelimeler: Metilfenidat, Dikkat Eksikliği Hiperaktivite Bozukluğu, hücre kültürü, ergenlik, Gonadotropin Salgılatıcı Hormon, Kisspeptin

1. INTRODUCTION AND PURPOSE

With the result of this study, we are aiming to investigate the effects of MPH on GnRH and Kisspeptin secretion and expression over cell culture so that we can shine more light on the use of MPH in children and adolescents during pubertal onset and possible effects on reproductive system.

Furthermore, the effect and the reversibility of these effects are investigated, by employing a recovery model in GT1-7 cells. This study will be the first to examine the effects of MPH on the onset of puberty and reproductive hormones and will shine light on the safer use during the transition to puberty and yield to new opportunities for the MPH treatment.

2. GENERAL INFORMATION:

2.1 Attention Deficit and Hyperactivity Disorder

Attention Deficit and Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder characterized by a persistent pattern of inattention, hyperactivity and impulsivity that interferes with functioning or development. The symptoms are usually, but not explicitly, present prior to age 12 years and exhibit in two or more settings(1).

The syndrome was first described by Weikard as lack of attention, and having the characteristics of unwariness and carelessness in 1775 (2, 3) and has been described in many cultures and references along the line of recent history (4).

The syndrome consists of two axis, inattention and hyperactivity-impulsivity. Inattention might manifest as disorganization or social and organizational malfunctioning like to being able to maintain focus on tasks and topics, difficulty in continued listening, and losing objects. Hyperactivity-impulsivity encompasses increased activity, inability to maintain seating, posture or stance. Both clusters of symptoms should be inconsistent with the expectations from the age group of the patient. Though considered as a pediatric developmental disorder, ADHD might continue into adulthood, yielding social, academic and occupational malfunctions. (1)

After syndrome's first depiction by Weikard in 1775 (2, 3), Crichton has published a book "On Attention and its Diseases" in 1798 and has spared a chapter on the attention and related deviations from normal functionality. He defines the attention as the external sense or thought that occupies the mind to a degree that the person does not receive another clear perception or external stimulus, of which normal range might vary among individuals and might alter in time with a strong emphasis that these alterations. ADHD might be an increase or a decrease from the range and might be innate or as a result of neurological dysfunction(5).

In 1846, a cartoon series "Struwwelpeter" was published by Dr. Heinrich Hoffmann, with the main character Zappel-Philipp, demonstrating the symptoms of

ADHD. This supports the view of ADHD is not cultural or sociological deviation from normal attention span but building on biological basis that carried out over centuries(6).

In 1902, Dr. George Still in his lecture series "On Some Abnormal Psychical Conditions in Children" at the Royal College of Physicians of London, defined the pediatric cases with a lack of moral control without intellectual impairment or physical disease. This establishes the differentiation of other neurodevelopmental and organic pathologies as later stated in the DSM criteria(7).

In 1932, Kramer and Pollnow described motor restlessness and lack of attention as "hyperkinetic disease of infancy" reflecting the symptoms as defined in DSM criteria of hyperactivity as the leading manifestation of ADHD(8).

After 1950s, researchers have found associations with minimal brain damage, namely conditions like asphyxia during birth, encephalitis and other lesions, have been associated with overactive behaviors and kinetic impulse disorder (9, 10)

Strauss and Lehtinen, analyzed brain injured and non-injured on behavioral symptoms and addressed the difference between the two groups, leading onto developing educational and rehabilitational interventions(11).

Clements, Peters and Douglas later studied the perceptual, motor, learning and behavioral patterns and shaped the characteristics of the hyperactivity syndrome similar to the stated in DSM criteria today (12, 13).

In 1980, American Psychiatry Association included "Attention Deficit and Hyperactivity Disorder (ADHD) in Diagnostic and Statistical Manual of Mental Disorders (DSM-III) as a syndrome characterized by hyperactivity and impulsivity providing a standardization to diagnosis used in clinical practice up to our day.(14).

2.2 Diagnosis of ADHD:

For the diagnosis of ADHD, there should be clear evidence that the symptoms interfere with, or reduce the functioning and the symptoms should not be pertaining to another psychotic or mental disorder (1, 15).

The diagnostic criteria of ADHD defined by American Psychiatric Association in 2013 (DSM 5) are widely accepted to date in the world and in Turkey. (1, 16)

ADHD is a syndrome characterized by;

- A persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development
- Symptoms present prior to age12 years
- Symptoms present in two or more settings
- Interference with the functioning
- The absence of any other psychotic or mental disorder

There might be combined presentation of inattention and hyperactivity-impulsivity, predominantly inattentive presentation, or predominantly hyperactive/impulsive presentation.

The severity of ADHD can be classified into mild, moderate or severe depending on the impairments in social or occupational functioning(1).

Besides the DSM 5 manual, validated scales, like Vanderbilt ADHD Diagnostic Rating Scales, Swanson-Nolan-Pelham Rating Scale and Strengths and Weaknesses of ADHD Symptoms and Normal Behavior Scale, can used by teachers, parents or caregivers besides the clinicians to further understand the behavior patterns and symptoms(4).

2.3 Prevalence of ADHD:

In DSM 5-2013, it is stated that, ADHD occurs widely in about 5% of children and about 2.5% of adults (1). The estimations around prevalence might vary on the geography and culture. Besides these, the calculations might also vary depending on the data being derived from a teacher, a parent or the child (17-19).

In more recent publications the prevalence has been estimated to be higher. In a systematic review and meta-analysis of 175 studies and 179 estimates, ADHD prevalence

estimated to be 7.2% among children and adolescents(20). In a community-based study in South Carolina and Oklahoma, in which teachers screened 10,427 children 5-13 years of age, the prevalence was 8.7% in South Carolina and 10.6% in Oklahoma(21). In another community-based study in North Carolina County, it is stated that, 15.5% of the sample met DSM criteria for ADHD (22).

Studies on prevalence of ADHD in Turkey revealed that rates are between 3% to 21.8% and display a male predominance(23-28). The largest among them, screening 5830 children, ages 6-13, in 30 cities have revealed 19.5% diagnosis rates of ADHD in Turkey (26).

In 2016 United States National Survey of Children's Health study, the estimated number of children, 2–17 years of age, was 6.1 million to be ever diagnosed by ADHD diagnosis, 62.0% of these children are thought to receive medication (29).

Turkish Statistical Institute data states the children and adolescents (<18 years old) to be 27.5% of the general population(30). This would carry the ADHD diagnosis rates to 686.029 and 4.985.142 for the prevalence of 3% to 21.8% respectively. Considering the population affected with the syndrome, ADHD deserves deeper understanding and research.

2.4 Pathophysiology of ADHD:

Neurocognitive, neuroimaging and genetic theories of ADHD have revealed that genetics, developmental variations and environmental factors play role in the etiology of ADHD and not one but multiple mechanism pool up to demonstrate the symptomology (31-35).

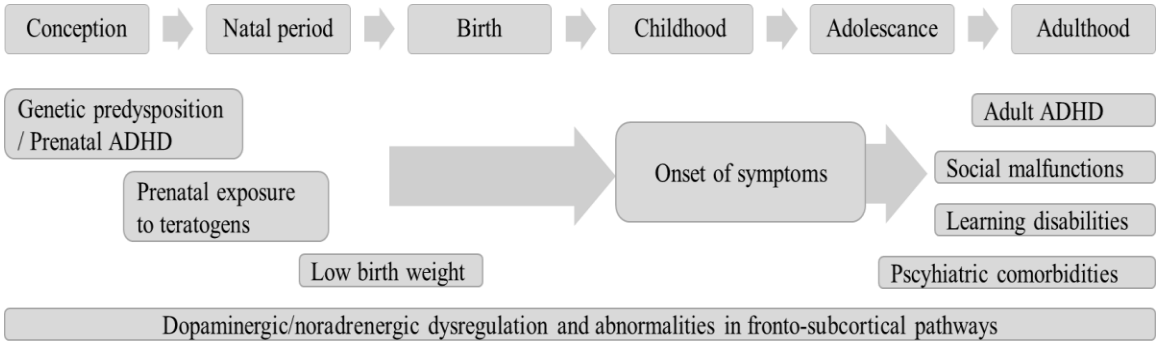

Figure 1. Etiology of ADHD, adapted from Markis et al. 2009(34)

Studies have shown that cortical regions contribute to the neurobiology of ADHD. The centers for working memory (dorsolateral prefrontal cortex) and executive thinking, decision and strategy (ventromedial prefrontal cortex) and attention orientation (parietal cortex) are reflecting the clinical characteristics of ADHD.

Subcortical structures of frontostriatal circuit play important role in affective and cognitive components of executive control. Structural and functional abnormalities have been reported in frontostriatal circuit consisting of, ventral and dorsal anterior cingulate cortex, basal ganglia extending to cerebellum and amygdala.

Besides the frontostriatal circuit, it has been suggested that neurotransmitter circuits might also be affected in ADHD. The dopamine system plays an important role in reward mechanisms, including planning and response initiation and reaction to novelty. The noradrenergic system regulates response to urgent stimuli and state-dependent cognitive processes. These systems consist of fibers in mesocortical, nigrostriatal, mesolimbic paths, locus coeruleus and substantia nigra. Both systems have been found to be involved in ADHD(4).

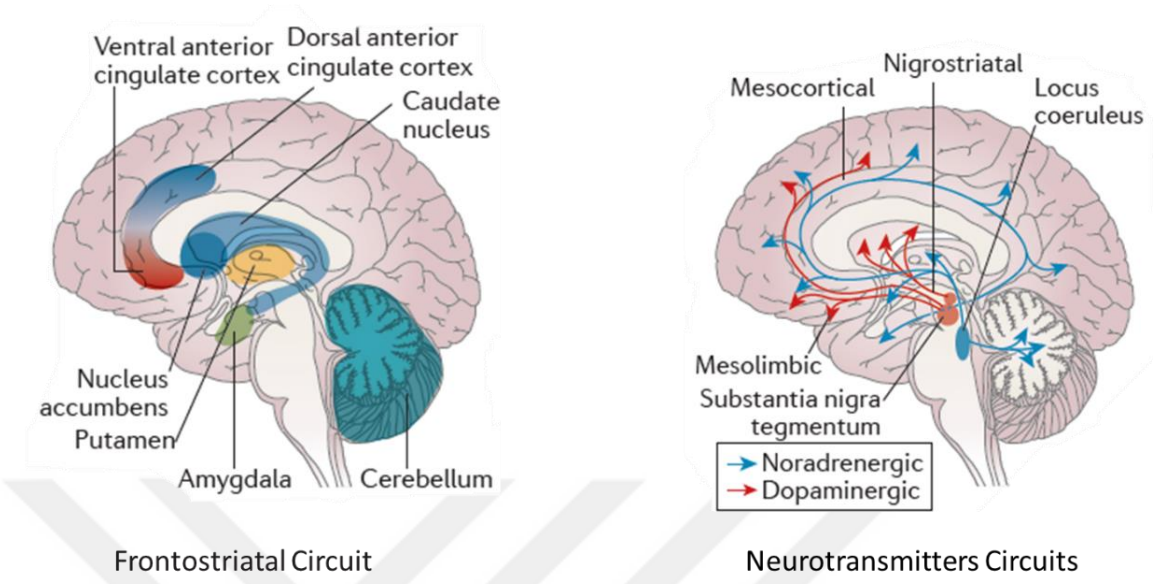

Figure 2: Neurobiology of ADHD, adapted from Farone et al. 2015(4)

In a study comparing 259 children and adolescent with control group of 222, the children with ADHD demonstrated executive functioning deficits, associated with frontostriatal circuit and functions of attention, reasoning and problem solving. (36).

Imaging studies have revealed morphological abnormalities among children and adults with ADHD. In a magnetic resonance imaging study of 24 adults with ADHD, a smaller anterior cingulate cortex and dorsolateral prefrontal cortex were found when compared with 18 healthy controls(37) . The dorsolateral prefrontal cortex has the main center for executive functions including working memory and selective attention and serves in cognitive selection of sensory information and responses.(38) These variations might be the neurobiology behind the inattention and malfunctions in goal-oriented tasks in ADHD. The anterior cingulate cortex is considered as the region coordinating salience detection, emotional reactivity, and social regulation and the variations in the anterior cingulate cortex might result in hyperactivity and impulsivity (37, 38). Not just the morphology but the signaling pathways of anterior cingulate cortex and dorsolateral prefrontal cortex under ADHD have been investigated(34, 39).

The functional magnetic imaging reflecting the symptoms can be reversed over successful treatment modalities, confirming the regions affected in ADHD. In a comparative study of 21 adults with ADHD, functional magnetic resonance imaging measures have been shown to normalize after 6 weeks of methylphenidate treatment but not with placebo(40).

Besides the morphological abnormalities, monoaminergic system has been widely investigated for the etiology of ADHD. It has been suggested that ADHD might be rooting from a dysfunction in the reward center, namely the dopaminergic system(41). In the literature there has been evidence that ventral tegmental area lesions have been related to hyperactivity (42) and animal models supported this, showing chemical destruction of frontal lobe dopaminergic neurons would match the patterns of ADHD and would be improved by stimulants (43). Furthermore, there has been studies relating attention and hyperactivity symptoms to low levels of homovalinic acid, the main metabolite of dopamine, in cerebrospinal fluid(44-46) .

There are other hypothesis on the monoaminergic system linking not only the dopamine metabolism of the infant but to the mother in gestational period. Lowered maternal serum dopamine beta hydroxylase, the enzyme that converts dopamine to norepinephrine, has been found to cause intrauterine imbalance on the neurotransmitter levels, decreasing norepinephrine relative to dopamine resulting in the ADHD symptoms in a familial pattern(47).

An animal model study has demonstrated that a noradrenaline reuptake inhibition might result in the improvement of inattentive behavior and serotonin reuptake inhibition might improve hyperactivity symptoms without effecting inattention (48).

A study of DNA methylation and serotoninergic signaling through the amygdala serotonin transporter gene network, using genome-wide platforms, has shown that serotonin transporter gene network plays an important role, providing an additive affect to the postnatal adversity and variations in cortical architecture in the attention deficit and hyperactivity symptoms(49).

The neurobiology of ADHD is also determined by genetic factors. The results of family, twin, and adoption studies suggested strong evidence that there is a genetic susceptibility in ADHD (50). In a study of 197 twins, monozygotic twins were found to be more likely to demonstrate ADHD characteristics (51). In another study of 576 twin boys, it was suggested that genetic factors play more important role in the symptoms of inattention and impulsivity-hyperactivity than environmental factors (52).

In a study of 813 030 children born between 1992 and 2000, maternal smoking during pregnancy was found to be associated with the development of ADHD (53). In an adoption study of 480 children the hostile parenting behavior and maternal ADHD symptoms were found to be associated with the ADHD diagnosis of the children (54).

Besides the maternal smoking and hostile parenting behavior, social and psychological deprivation, polychlorinated biphenyls and fetal exposure to alcohol have also been associated with ADHD (55).

Considering the current literature, ADHD is thought to have a multifactorial causality combining genetic predispositions overlapping with environmental contributors.

2.5 Treatment of ADHD:

The ADHD is managed over pharmacotherapy and behavioral therapy. The research suggests that the behavioral therapy combined with medical treatment options provides more favorable outcomes. Nevertheless, lack of supportive staff and specialists in the teams of child psychiatry centers, developmental-behavioral pediatricians and child neurologists with neurodevelopmental disabilities training hinders the behavioral therapy pillar of the combination treatment (56).

The initiation of the pharmacotherapy and behavior therapy is an important step and both the patient, and the caregiver should be engaged in the decision conversation. Coghill and Seth have developed an algorithm to facilitate the decision process. If the patient meets all diagnostic criteria for ADHD, the age of the patient should be considered.

For the patients younger than 6 years of age, behavioral therapy options are advised. For those older than 6, pharmacotherapy options can be considered. For cases that the response is less than desired, other pharmacotherapy options might be considered. If the patient does not meet all the diagnostic criteria for ADHD, the patient is to be guided towards behavioral interventions before the pharmacological options. If the response is less than adequate with the behavioral options, pharmacological treatment modalities can be assessed. (57)

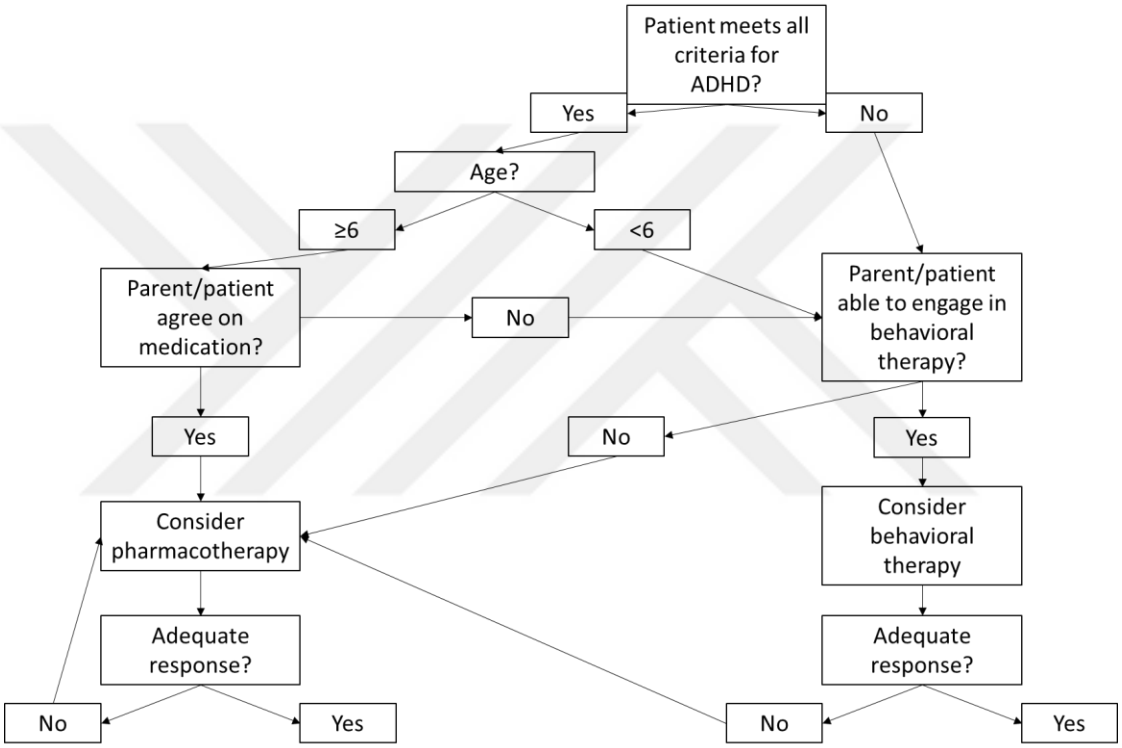

Figure 3: Treatment decision algorithm of ADHD.

Adapted from Coghill and Seth, Child & Adolescent Psychiatry & Mental Health, 2015 (57)

2.5.1 Behavioral Therapy

Behavioral therapies might vary on the nature, content and focus of the patients' needs and age group from improving and disciplining the socialization among peer group to organizational skills and school programs(58).

Although the varying nature of the behavioral therapies dilutes the measurable outcomes of the effects of behavioral therapy alone or in a combination with pharmacotherapy, it is still considered as the key component of the long-term ADHD treatment.

Behavioral therapy consists of two modalities, training of the adults in influence of the affected children and interventions directly targeting the children. Both options provide optimal outcomes when the intervention is followed upon a prolonged interval and constructive and timely feedback structures are in place to support the performance of the child (56).

2.5.2 Pharmacotherapy

The first pharmacotherapy for ADHD was discovered by Bradley in a study of 30 children with a variety of psychiatric symptoms, treated with benzedrine sulphate, a central nervous stimulant, resulting in significant increase in the school performance and improvement in the symptoms. It is discussed that stimulation of higher brain function, increases the voluntary control over impulsivity and hyperactivity(59).

Today, although recommendations around medications vary depending on the needs and the age of the patient, methylphenidate (MPH) is considered as the first line pharmacotherapy in international guidelines (56, 60, 61).

MPH, methyl a-phenyl-2-piperidineacetate hydrochloride, was first synthesized in 1944 as a similar molecule to amphetamine by Leandro Panizzon (62). Early uses of MPH have been on negative psychiatric symptoms like chronic fatigue, lethargy, depressive mood and psychosis associated with depression (60). After the licensing for the indication, MPH is established as the first line medical treatment for ADHD.

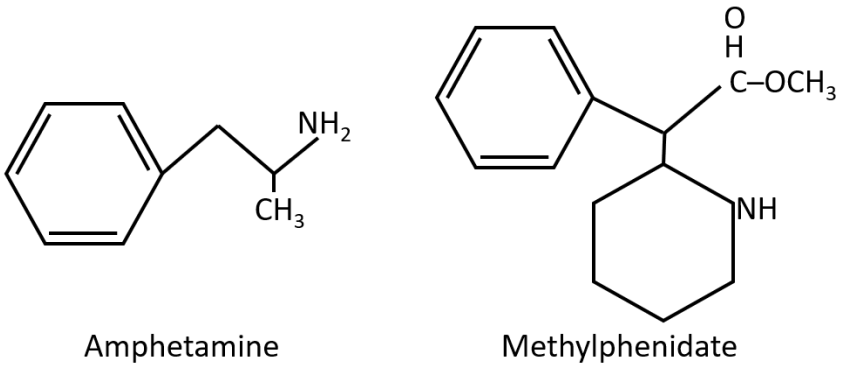

Figure 4: Chemical structure of amphetamine and MPH.

Adapted from Leonard et al. Human Psychopharmacology. 2004 (60)

It is hypothesized that MPH demonstrates its main effect over inhibiting the reuptake in dopamine transporter. When dopamine reuptake is blocked, extracellular levels of dopamine increases resulting in elevated resting level of dopamine on presynaptic dopamine receptors. These mechanisms modulate the impulse associated release that yield in lower differential rise between resting and excited neuron under the effect of MPH, generating less excitability and thus less motor activity, resulting in the improvement of clinical symptoms of attention deficit and hyperactivity (63).

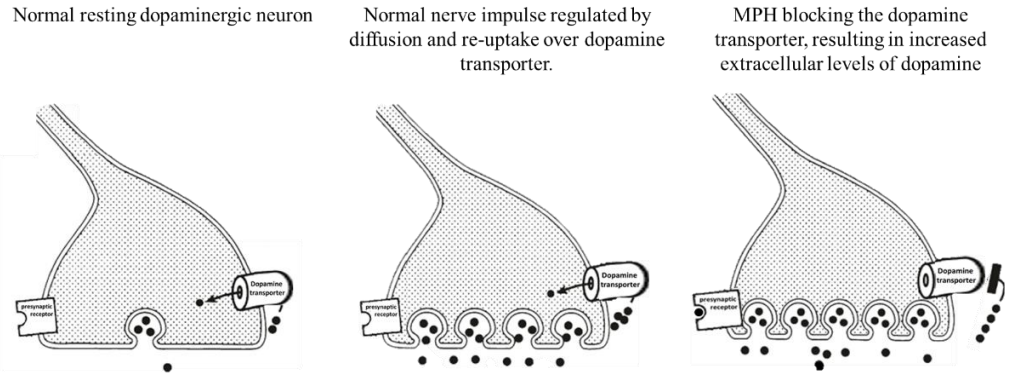

Figure 5: Cellular pharmacology of MPH mechanism of action.

Adapted from Seeman and Madras, 1998 (63)

MPH is freely soluble in water and in methanol, soluble in alcohol, and slightly soluble in chloroform and in acetone (64), reaches peak plasma concentrations following ingestion between 1and 3 hour and eliminated through renal route. The elimination half-life ranges from 2 to 3 hours. It is estimated that due to the chemical structure to allow high lipid solubility, MPH passes freely through blood brain barrier to penetrate central nervous system (65).

In National Survey of Children's Health study, it was estimated that almost two thirds (62.0%) of the children were taking medication and slightly less than half (46.7%) had received behavioral treatment for ADHD in United States (29).

The medication treatment is regulated with international guidelines of American Psychiatry Association (APA) and National Institute for Health and Care Excellence (NICE). In both, MPH is recommended as a first line treatment in combination with behavioral therapy and parental education. Although there are associated side effects and risks of medication use at early ages, considering the downfalls of ADHD being untreated, the benefits outweigh the risks and it is recommended as a relatively low risk option for treatment (56, 66). The literature reflects that MPH improves behavioral symptoms reported by patients, teachers, and parents, reducing hyperactivity and impulsivity (66, 67).

Pharmacotherapy has been shown to be protective on the functional neuroanatomy of the ADHD patients on, cerebellum, anterior cingulate cortex and basal ganglia providing the associated clinical benefits (68).

2.5.2.1 MPH use in ADHD Treatment

MPH is a widely used central nervous system stimulant and has "moderately reduced weight and height gain during prolonged use in children" noted as a common side effect in prescription information but in EMA annex it has been stated in the prescription information that the exact causal mechanism for the effects of methylphenidate on growth remains uncertain (69).

Contradicting to the general recommendation and drug license on discontinuing at puberty, MPH is used to treat attention disorders during puberty and adulthood.

In NICE guidelines, concerns around the impact of medications around growth have been discussed with the emphasis that untreated ADHD has the potential of more long term, penetrating influence on the life of the child, thus the benefits of the medications exceeds the risks (66). Aligned with the NICE guidelines EMA states that weight gain is moderately reduced and growth retardation have been reported with the long-term use of MPH and suggests the careful ongoing monitoring of growth and appetite along with other adverse events in children using long-term therapy (69).

2.5.2.2 The Effects MPH Use on Growth and Maturation:

MPH has been approved for the treatment of ADHD and has been in use over decades without any life threating side effects. Nevertheless, long-term use has been reported to cause a slowing of development in height and weight gain and the concerns around growth and maturation remain to be resolved.

The effect on growth, height and weight compared to the peers of the child, have been discussed widely in association to the effects over increased dopamine inhibiting growth hormone (GH) (70, 71)

In studies of case series a dose dependent inverse correlation was found between MPH dose and growth (72-76). Delays in height and weight velocity and the expected height and weight were observed in a dose dependent manner. Children receiving doses higher than 20 mg/day had the greatest impact (76). A longitudinal study of 5 years demonstrated with higher doses, not only the growth is affected more significantly but also the return to normal range is delayed, while it would take children under 1.5 mg/kg/day would demonstrate diminished wight gain after a year, the children under the dose higher than 2.5 mg/kg/day would still have lower weight gain after 4 years (77).

In a cohort study of 568 children, 6 – 12 years of age, receiving ADHD treatment the rates of low weight increased from 4.8% to 14.4% and low height from 3.3% to 8.3%, when followed up to 30 months (78).

In a study of 79 children, aged 6-12, annual follow up to 5 years have demonstrated that there is a significant and dose dependent impact of MPH on growth and weight(77). In a retrospective descriptive study of 96 children, height decreased at 6 months after initiation of ADHD medication, but growth matched the peer group after the discontinuation at puberty (79). In another study of 146 school children diagnosed with ADHD, MPH use was significantly associated with significant deceleration of height velocity and the magnitude of the deficit in height was associated with treatment duration with no significant influence on weight(80). Similar results were reflected in additional studies suggesting an association between growth retardation and MPH (81-84).

Although the correlation is well established, the clinical impact and the magnitude of the growth suppression and delay remains under discussion. Results suggest that clinicians should monitor the height and the weight of the children receiving medical treatment for ADHD (74, 75).

These findings have impacted the MPH license to include a warning on "Long-Term Suppression of Growth" and the evidence was found adequate to be included with a disclaimer to monitor growth during treatment and interrupt the medical treatment if the growth is considered to be impacted (64).

On the clinical practice not only height and weight but clinical experience shows that maturation and puberty initiation are also impacted on a variable level.

Puberty, the transition from childhood to adulthood, is regulated by hypothalamic-pituitary-gonadal (HPG) axis activity through a complex network of excitatory and inhibitory modulator factors. The axis starts developing in midgestational period and continue developing in the postnatal periods, staying dormant till the initiation of puberty (85-87).

Puberty is characterized by systemic, cognitive, and behavioral changes, including the development of secondary sexual characteristics, gonadal maturation, and gaining reproduction capacity. For the initiation of puberty pulsatile release of GnRH is needed to trigger the downstream of gonadal hormones (85). The pulsatile GnRH is produced in the preoptic area of the hypothalamus and released from axon terminals in the median

eminence in a pulsatile manner to stimulate the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which in turn act on the gonads to promote gametogenesis and the production of sex steroids (86). Sex steroids provide feedback loop to regulate GnRH, LH, and FSH release and Kisspeptin neurons have a key role as a regulator on GnRH secretion and release. High estrogens and progesterone levels trigger kisspeptin neurons to induce the surge of GnRH and LH in the anteroventral periventricular nucleus, whereas they inhibit kisspeptin expression in the arcuate nucleus in females. Through the kisspeptin neurons in arcuate nucleus, circulating testosterone, suppresses GnRH (88).

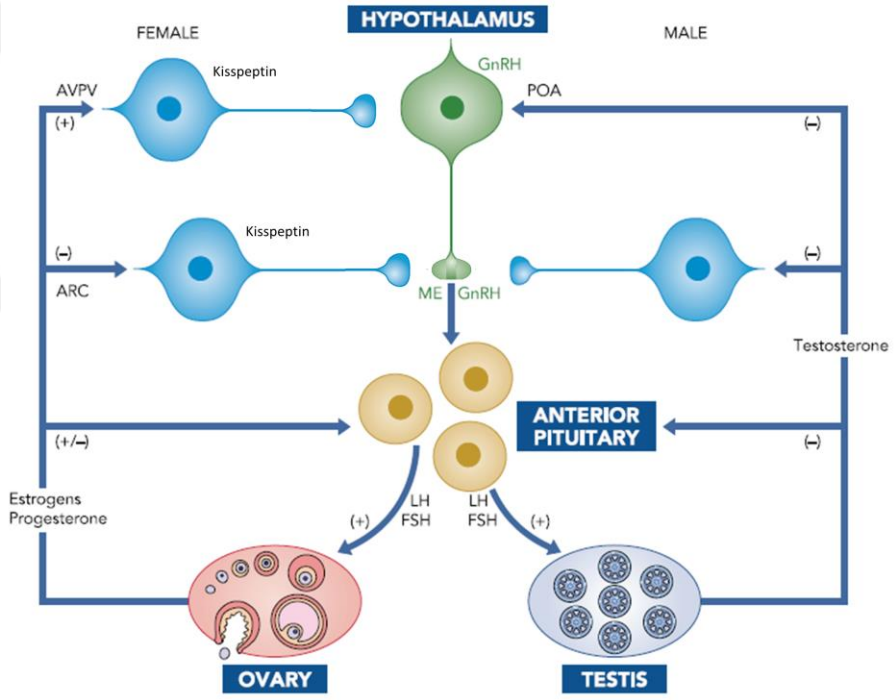

Figure 6: Hypothalamic–pituitary–gonadal axis

Adapted from d'Anglemont de Tassigny & Colledge Physiology 2010 (88)

Although the exact mechanism is still being investigated, there is strong evidence that alterations in the hypothalamic-pituitary-gonadal (HPG) axis would disrupt the timing and the physiology of puberty initiation.

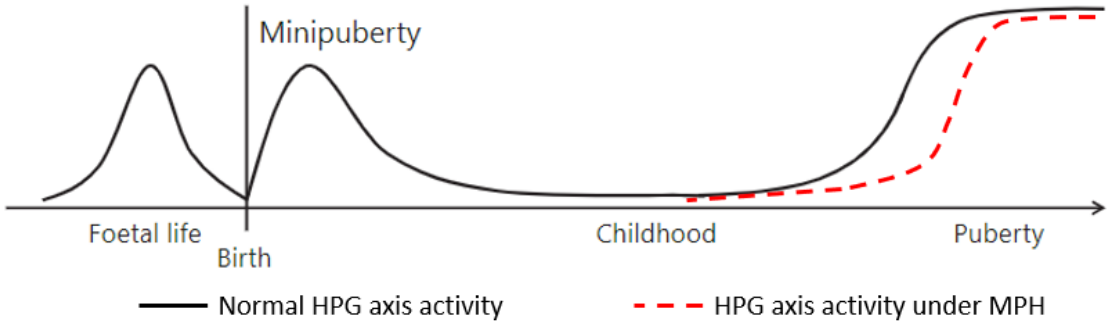

Figure 7: The estimated effects of MPH on HPG activity

Adapted from Kuiri-Hänninen et al. Hormone Research in Paediatrics 2014 (86)

Despite the fact that, the prescription information and EMA product information state that the effects of MPH is unknown (69), it has been shown that MPH causes a significantly increase in the extracellular dopamine levels resulting in stimulating the reward center and increasing the focus and motivation around the targets behaviors in humans (89) and the excitability of GnRH neurons were negatively correlated with dopamine, suggesting dopamine as a potent inhibitors of GnRH neuron in an animal model study (90).

These suggest, MPH might have a negative effect on the hypothalamic-pituitary-gonadal axis and can affect the development and pubertal initiation. The effects of MPH on maturation and puberty initiation are reflected in numerous case series in the literature.

The adverse effects of MPH have been widely studied on the growth perspective but on puberty onset is limited with several animal models, human studies, and case series. MPH and puberty have been studies in several animal models of male and female rats and one rhesus monkey study (91-95).

In a group of 36 female Long–Evans rats, it was found that chronic exposure to MPH around the onset of puberty alters endocrine functions, delayed onset of vaginal opening, more time spent with male stimulus, being less likely to leave the male after receiving a mount, which was interpreted as increased sensitivity to sexual stimuli (92).

In a study of thirty male rhesus monkeys, MPH treatment was found to be associated with delayed pubertal progression with decreased testosterone levels, impaired testicular development, delayed descent and reduced testicular volume (93).

In a study of Wistar rats, it was demonstrated that MPH treatment during childhood to early adulthood interfered on testicular function in rats at adult life, leading to increased interstitial tissue in seminiferous tubules and abnormal tail morphology but did not affect the onset of puberty (94). When the same study conducted in female Wistar rats by the same research team, there was no significant in sexual behavior or morphology (95).

Sprague Dawley female rats were evaluated for the effects of MPH use on endocrine parameters and behavior. When compared with the control group, MPH group demonstrated poor vaginal opening and unstable estrous cycle, impaired ovarian follicles and high levels of LH in the pituitary (91).

There have been longitudinal studies on the effects of MPH on the onset of puberty. In a study of sixty-five boys extended use of MPH resulted in lower height, weight and delayed pubertal development (96). In a case of a 20-year-old male patient that had 17 years of MPH treatment, delayed puberty, high-pitched voice, lack of body hair, impaired libido, inadequate erectile function, chronic fatigue, and low energy was reported. The patient's laboratory results demonstrated low FSH, LH and testosterone levels (97).

In another study of 49 boys with ADHD, it was found that the patients had significantly lower SHBG and higher testosterone percentages. MPH treatment resulted in normalization of sex hormones except free testosterone levels (98).

In a case report of a 14-year-old boy, the testosterone levels were found to be decreased after MPH initiation. After the cessation of MPH and changing to another ADHD medication, the testosterone levels returned to normal(99). Familial male puberty precoccus patient was reported to be treated with methylphenidate for sexual hyperactivity in a case study (100).

On contrast, there are studies that suggest no impact of MPH on pubertal development. In a study of 146 patience with ADHD and 70 healthy controls consisting of boys and girls no significant difference was found on testosterone, FSH, LH, estrogen,

progesterone or Sex Hormone Binding Globulin (SHBG) levels(101). In a case series of 7 children, levels of LH, FSH, estrogen and testosterone were found to be in the normal range (102).

2.6 GnRH and Kisspeptin

Gonadotropin-releasing hormone (GnRH) is produced and released from the hypothalamus in a pulsatile manner and plays important roles in the induction of the pubertal maturation in mammals. It is known that elevated pulsatile GnRH secretion in puberty induces the gonadotropin secretion that leads to elevated testosterone, FSH and LH secretion. Moreover, pulsatile GnRH during infancy leads to release of kisspeptin (Kiss1), an important neurohormone in sexual development, initiating the release of GnRH to the circulation leading to FSH and LH secretion. In the juvenile phase, GnRH and kisspeptin secretion to the systemic circulation is reduced by a neurobiological break, causing suspended FSH and LH secretion. However, at the onset of pubertal phase, this neurological brake is deactivated and pulsatile GnRH and Kisspeptin release is activated, this, in turn, leads to elevated release of FSH and LH(85, 103, 104).

GnRH system is not only impacted by Kisspeptin but also impacted by other monoaminergic neurotransmitters like melatonin, glutamate and GABA (105, 106). This would suggest some explanation on the changes in the attention and circadian rhythms in children with ADHD.

To understand the effects of MPH on puberty, the system can be investigated from the hypothalamic pituitary gonadal axis as well-regulated GnRH secretion from hypothalamus is essential for stimulation of gonadotropins and physiological maturation in both sexes (85, 104). To our knowledge the effects of MPH use on GnRH and Kisspeptin has not been studied to date.

Studying how different molecules and agents affect the modulation of neuronal cells have been an important aim in the neuroscience studies and neuronal cell lines have been developed to investigate these dynamics (107). To study the GnRH expression and secretion, hypothalamic neurons from transgenic mice was obtained and genetically

targeting tumorigenesis was performed to immortalize the neurons, the pure cell population was cloned with serial dilutions to obtain the cell lines GT1-1, GT1-3, and GT1-7. The doubling time, ease in culture and pulsatile pattern similar to that observed in vivo make the GT1-7 cell line a viable option to create a cell model for GnRH secretion (108, 109). Recent literature has suggested the regulation of GnRH neurons by Kisspeptin, as it has been demonstrated to regulate the GnRH release (105, 110). To study Kisspeptin expression, a specialized rat hypothalamic cell line, HypoE-8 was developed (107, 111).

3 MATERIALS AND METHODS

The effects of MPH on puberty onset were investigated in 2 cell lines, GnRH secreting GT1-7 cells and Kisspeptin secreting rHypoE-8 cells. GT1-7 cells were treated with different concentrations to assess the toxicity of MPH to cells.

Our study is the first use of MPH in neuronal cell cultures to our knowledge. On the literature there are studies on canine renal epithelial (cRE), rat pheochromocytoma (rPC-12) and human embryonic kidney (HEK-293) cell lines as seen in Table 1. The concentration range between 1 nM to 200 µM was selected for our experiments covering the existing range of experiments in clinical use and cell lines. (112-115)

Table 1. Cell culture experiments under different MPH doses in the literature

Cell line	Dose	Literature
Clinical use	20-60 ng/ml	Balcıoglu, 2009 (112)
cRE cell line	31.25 ng/ml	Salviano, 2015 (113)
rPC12 cell line	1, 10, 100 nM, 1, 10, 100 µM	Bartl, 2010 (114)
HEK-293 cell line	0.1, 0.3, 1 ng/mL	Wakamatsu, 2009 (115)

To obtain concentrations in increasing in a logarithmic manner the 200 µM was used as a stock and diluted to yield the concentrations seen in Figure 8.

Figure 8: Concentrations of MPH used in cell viability and wound healing experiments

It was observed that maximum dose of 200 µM did not to have a significant toxic effect on cell viability and migration. 200 µM was selected for further experiments on gene expression and hormone secretion.

For gene expression and hormone secretion all experiments were replicated in sets of three to provide the optimal randomization and demonstration power. The data was pooled for statistical analysis.

3.1 Cell Culture and Reagents

The mouse hypothalamic GnRH-producing cell line GT1-7 (108) was obtained from Dr. Pamela Mellon, University of California, San Francisco, Califormia as a gift to Prof. Dr. Bayram Yılmaz (116). The rat hypothalamic Kiss1-producing cell line rHypoE-8 (111) was purchased from Cedarlane Laboratories (France). Both cell lines were maintained in complete growth medium (DMEM (Gibco, 41966029) supplemented with 10% heat inactivated fetal bovine serum (Gibco, 10500) and penicillin (50 µg/mL)/streptomycin (50 µg/mL)/neomycin (100 µg/mL) mixture (Gibco, 15640055)) at 37 °C under 5% CO2 humidified atmosphere.

Figure 9: GT1-7 cells grown in DMEM medium under microscope

Methylphenidate hydrochloride (MPH; Ritalin®, Novartis) tablets (10 mg) were dissolved in sterile distilled water to a final stock concentration of 100 mM, centrifuged at 6,000 x g for 6 minutes and supernatant was collected. Next, the supernatant was centrifuged again at 6,000 x g for 6 minutes to remove all the undissolved particles, supernatant was collected, aliquoted and stored at -20ºC degrees until use.

3.2 Effect of MPH on Cell Viability on GT1-7 cells

Cells grown in T75 or T150 flasks up to 70-80% confluency were collected and the number of cells were assessed by trypan blue dye exclusion assay(117). A total of 50,000 cells/well were seeded on 24-well plates in complete growth medium. After overnight attachment, cells were treated with different concentrations of MPH (0 - 200 µM) in complete growth medium for 24 h. After treatment period, cells were washed with PBS without calcium and magnesium, trypsinized with 200 µL of trypsin-EDTA solution (0.25%; Thermo Fisher Scientific, 25050030) for 5-10 min. Trypsinization was terminated addition of 800 µL of complete growth medium and cells were resuspended and transferred to a clean 1.5 mL Eppendorf tube. Effect of cell viability of different concentrations of MPH was assessed by trypan blue dye exclusion assay(117).

3.3 In vitro Wound Healing Assay

Cell migration was assayed by using scratch wound healing assay *in vitro* (118). GT1-7 cells were seeded at a density of 800,000 cells/well in 6-well plates. After overnight attachment, a straight scratch was created by using a sterile pipette tip with a maximum volume of 200 µL. Then, microscope images of the starch at 10x magnification was obtained and the area that the image was obtained was marked with a circle. Subsequently, the cells were washed with PBS and were treated with 200 µM MPH for 24 hour. After treatment period, microscopy images of scratched area was obtained and the cell migration was assessed by using on image J as previously described (119).

3.4 Effect of MPH on GnRH Expression in GT1-7 cells

Effect of MPH on GnRH expression in GT1-7 cells were assessed by quantitative polymerase chain reaction (qPCR).

3.4.1 Treatment Protocol

GT1-7 cells were seeded at a density of 600,000 cells/well in 6-well plates. After overnight attachment, cells were washed with PBS and treated with 200 µM MPH in serum free medium (DMEM (Gibco, 31053) supplemented with L-glutamine (2 mM; Gibco, 25030-024), sodium pyruvate (1 mM, Pan Biotech, P04-431000) and penicillin (50 µg/mL)/streptomycin (50 µg/mL)/neomycin (100 µg/mL) mixture (Gibco, 15640055)) up to 24 hours (0.5, 1, 2, 4, 8 and 24 h). For recovery experiments, after 24 hours of treatment with 200 µM MPH, cells were washed with PBS and treated with serum free medium without MPH up to 24 h (0.5, 2 and 24 h). Respective control groups for all time points included in the experiments were treated in the same way without receiving MPH.

3.4.2 RNA Isolation, cDNA Synthesis and GnRH Expression Analysis

After treatment of the cells with MPH for respective period, RNA was collected in TRI reagent, stored at -80ºC until use and isolated according to the manufacturer's instructions (DirectZol™ RNA MiniPrep, Zymo Research, R2051). Isolated RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized from equal amounts of RNA (50 – 150 ng) for each set of experiments according to manufacturer's instructions (iScript™ cDNA Synthesis Kit, Bio-Rad, 1708891).

Primers for GnRH and β-Actin were designed by using the online primer designing tool Primer3web version 4.1.0 (https://primer3.ut.ee/). The primer list for GnRH and β-Actin are indicated in Table 2.

Table 2. Primers for GnRH and β-Actin used in qPCR.

Primer	Sequence (5´-3´)
m_Actin B-F	TGTTACCAACTGGGACGACA
m_Actin B-R	GGGGTGTTGAAGGTCTCAAA
m_GnRH -F	TGGGGGAAAGAGAAACACTG
m_GnRH -R	CCTGGCTTCCTCTTCAATCA

In order to analyze the effect of MPH on GnRH on different time point sequences were amplified by qPCR by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad, 1725270) on a CFX96 Touch Real-Time PCR Detection System (Bio Rad) with the thermal cycle conditions indicated in Table 3. Relative gene expression was assessed by using $2^{-\Delta\Delta CT}$.

Table 3. Thermal cycle conditions for GnRH and β-Actin primers in qPCR.

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95 ºC	3 min	1
Denaturation	95 ºC	15 sec	50
Annealing and extension	55 ºC	50 sec	

3.5 Effect of MPH on GnRH Secretion in GT1-7 cells

GT1-7 cells were seeded at a density of 600,000 cells/well in 6-well plates. After overnight attachment, cells were washed with PBS and treated with 200 µM MPH in serum free medium up to 24 hours (0.5, 1, 2, 4, 8 and 24 h). Respective control groups for all time points included in the experiments were treated in the same way without receiving MPH. For all time points, 500 µL of treatment medium was collected for GnRH secretion and stored at -80ºC until use.

Levels of GnRH secreted on respective time points were assessed by enzyme-linked immunosorbent assay according to manufacturer's instructions (Phoenix Pharmaceuticals, Inc., EK-040-02).

3.6 Effect of MPH on *Kiss1* Expression in rHypoE-8 cells

Effect of MPH on *Kiss1* expression in rHypoE-8 cells were assessed by qPCR.

3.6.1 Treatment Protocol

rHypoE-8 cells were seeded at a density of 800,000 cells/well in 6-well plates. After overnight attachment, cells were washed with PBS and treated with 200 µM MPH in serum free medium up to 24 hours (0.5, 1, 2, 4, 8 and 24 h). Respective control groups for all time points included in the experiments were treated in the same way without receiving MPH.

3.6.2 RNA Isolation, cDNA Synthesis and *Kiss1* Expression Analysis

After treatment of the cells with MPH for respective period, RNA was collected in TRI reagent, stored at -80ºC until use and isolated according to the manufacturer's instructions (DirectZol™ RNA MiniPrep, Zymo Research, R2051). Isolated RNA was quantified on a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized from equal amounts of RNA (100 ng) for each set of experiments according to manufacturer's instructions (iScript™ cDNA Synthesis Kit, Bio-Rad, 1708891).

Primers for Kiss1 and β-Actin were designed by using the online primer designing tool Primer3web version 4.1.0 (https://primer3.ut.ee/). The primer list for *Kiss1* and *β-Actin* are indicated in Table 4.

Table 4. Primers for Kiss1 and β-Actin used in qPCR.

Primer	Sequence (5´-3´)
r_Actin B-F	TCTTCCAGCCTTCCTTCCTG
r_Actin B-R	CACACAGAGTACTTGCGCTC
r_Kiss1_F	GGAACTCGTTAATGCCTGGC
r_Kiss1_R	AGGCTGACATGTCCTTCTCG

In order to analyze the effect of MPH on *GnRH* on different time point sequences were amplified by qPCR by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio Rad, 1725270) on a CFX96 Touch Real-Time PCR Detection System (Bio Rad) with the thermal cycle conditions indicated in Table 5. Relative gene expression was assessed by using $2^{-\Delta\Delta CT}$.

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95 ºC	3 min	1
Denaturation	95 ºC	15 sec	45
Annealing	49 ºC	20 sec	
Extension	52 ºC	30 sec	

Table 5. Thermal cycle conditions for Kiss1 and β-Actin primers in qPCR.

3.7 Statistical Analysis

The data collected through the experiments were analyzed using IBM Statistical Package for the Social Sciences (SPSS) for Windows 23.0 (IBM Corp., Armonk, NY). The normality of the data was assessed by Kolmogorov-Smirnov and Shapiro-Wilk tests. For comparisons between groups, “Independent Sample T-Test” was used for two group comparisons and “ANOVA Test” was used comparing more than two groups. Besides the classical statistical methods for absolute quantification, $2^{-\Delta\Delta C_T}$ method was used for the analysis of qPCR results as a relative quantification(120).

Considering the sample size and the distribution of the results, Mann Whitney U test was performed for GnRH expression, GnRH secretion, Kisspeptin expression and GnRH expression in recovery model as a confirmatory analysis.

A p-value lower than 0.05 was considered statistically significant.

3.8 Descriptive Analysis of the Experiment Results

Table 6. Distribution of cell viability and wound healing assays

Assays	n	$\bar{x}$	sd	M	Minimum	Maximum
Cell viability	72	62743,1	20607,7	62500,0	15000,0	145000,0
Wound healing	24	15,99750	5,52991	16,85890	4,34	28,26

Table 6 shows the descriptive statistics of the cell count and wound healing assays.

Table 7. Distribution of Actin and GnRH expression over time

Measurements		n	$\bar{x}$	sd	M	Minimum	Maximum
Actin	30 min.	12	19,207497	4,101524	17,378124	15,551771	29,965772
	1 hour	12	17,799361	1,791099	17,738963	15,031533	22,241829
	2 hours	12	16,952971	0,863725	17,170005	15,236314	18,110920
	4 hours	12	17,735507	0,618826	17,613863	16,769560	18,930830
	8 hours	12	18,254455	0,974232	18,471088	16,085899	19,260899
	24 hours	12	19,890842	0,534568	19,800176	19,226777	20,891858
GnRH	30 min.	12	16,888268	0,619127	16,852008	16,038107	18,213841
	1 hour	12	16,528701	0,869831	16,836529	14,400233	17,521951
	2 hours	12	16,543998	1,057251	16,536377	14,907788	18,208579

	4 hours	12	16,622953	0,757651	16,690632	15,420557	17,482052
	8 hours	12	16,468680	0,603957	16,576408	15,424169	17,200587
	24 hours	12	15,976758	0,347207	15,910442	15,448868	16,604188

Table 7 shows the descriptive statistics of Actin and GnRH expression over time by qPCR under the effect of MPH.

Table 8. Distribution of GnRH secretion over time

Measurements		**n**	$\bar{x}$	**sd**	**M**	**Minimum**	**Maximum**
GnRH	30 minutes	11	0,144085	0,095794	0,124452	0,020497	0,323089
	1 hour	11	0,099250	0,064608	0,089948	0,008910	0,202859
	2 hours	8	0,154703	0,140790	0,111596	0,003613	0,432743
	4 hours	12	0,092204	0,076430	0,075182	0,004106	0,245556
	8 hours	10	0,135729	0,110620	0,115646	0,001840	0,337776
	24 hours	8	0,180743	0,140794	0,175126	0,004409	0,448742

Table 8 shows the descriptive statistics of GnRH secretion over time by ELISA under the effect of MPH.

Table 9. Distribution of Actin and GnRH expression over time in recovery model

Measurements		**n**	$\bar{x}$	**sd**	**M**	**Minimum**	**Maximum**
Actin under MPH	30 min.	12	18,300400	1,422110	18,590588	15,798550	20,094690
	1 hour	12	19,005412	1,563553	18,946094	17,130150	21,742980
	2 hours	12	19,359444	2,120991	20,108326	16,176430	22,264660

	4 hours	12	21,304058	4,170930	20,758250	16,602200	26,930010
	8 hours	12	21,561429	4,722344	20,795158	16,978900	31,184050
	24 hours	12	25,844083	5,319530	24,126335	19,819350	34,257260
Actin under recovery	30 min.	12	22,041522	3,423510	21,583500	17,739450	27,905900
	2 hours	12	23,177991	3,906846	23,497917	17,072110	27,987530
	24 hours	12	25,977316	4,192878	25,668480	20,882570	32,146300
GnRH under MPH	30 min.	12	18,731586	1,914812	17,597956	16,656860	21,652860
	1 hour	11	18,712246	1,744639	18,483001	16,598900	21,647320
	2 hours	12	18,808072	2,030992	18,197330	16,296890	21,803850
	4 hours	12	20,334357	2,561188	21,201819	16,891580	23,097600
	8 hours	12	20,378177	3,895359	19,608278	16,150140	27,193740
	24 hours	10	23,210745	6,907616	19,323156	18,616190	36,004320
GnRH under recovery	30 min.	12	18,852323	1,782229	19,267610	16,388620	21,048720
	2 hours	12	22,270869	5,204176	22,212037	16,495220	32,454410
	24 hours	12	23,846398	4,693488	23,816882	17,274900	30,570510

Table 9 shows the descriptive statistics of expression over time in the recovery model.

Table 10. Distribution of Actin ve Kisspeptin expression over time

Measurements		n	$\bar{x}$	sd	M	Minimum	Maximum
Actin	30 min	11	17,469169	0,477415	17,719768	16,822020	18,160710
	2 h	12	17,128954	0,607085	17,117508	16,293510	18,187470
	24 h	12	21,311323	3,082506	19,607309	19,028300	27,694420
Kisspeptin	30 min	12	34,205115	0,646852	34,197494	32,954350	35,286380
	2 h	12	33,844671	0,540784	33,760026	33,203540	34,823430
	24 h	12	34,894406	1,192472	34,355621	33,923350	37,082310

Table 10 shows the descriptive statistics of Actin ve Kisspeptin expression over time by qPCR under the effect of MPH.

Table 11. Normality tests for cell viability and wound healing assays

Assays	Statistics	df	p
Cell viability	0,085	72	0,200
Wound healing	0,982	24	0,931

Kolomogorov-Smirnov and Shapiro-Wilk normality rest results for cell viability and wound healing assays are shown in Table 11, $p>0,05$ for all measurements in the experiements. It is determined that, cell viability and wound healing assays demonstrate a normal distribution.

Table 12. Normality test for GnRH secretion under MPH

Measurements		Statistics	df	p
GnRH	30 minutes	0,924	11	0,354
	1 hour	0,951	11	0,661
	2 hours	0,861	8	0,122

4 hours	0,892	12	0,123
8 hours	0,866	10	0,089
24 hours	0,954	8	0,752

Shapiro-Wilk normality rest results for GnRH secretion under MPH are shown in Table 12, p>0,05 for all measurements in the experiements. It is determined that, GnRH secretion under MPH demonstrates a normal distribution.

Table 13. Normality test for Actin and GnGHexpression over time

Measurements		**Statistics**	**df**	**p**
Actin	30 minutes	0,740	12	0,052
	1 hour	0,871	12	0,067
	2 hours	0,812	12	0,053
	4 hours	0,973	12	0,942
	8 hours	0,889	12	0,115
	24 hours	0,926	12	0,341
GnRH	30 minutes	0,918	12	0,267
	1 hour	0,880	12	0,088
	2 hours	0,954	12	0,689
	4 hours	0,900	12	0,160
	8 hours	0,912	12	0,225
	24 hours	0,972	12	0,934

Shapiro-Wilk normality rest results for Actin and GnGHexpression over time under MPH are shown in Table 13, p>0,05 for all measurements in the experiements. It is determined that, Actin and GnGHexpression over time under MPH demonstrates a normal distribution.

Table 14. Normality test for Actin and GnGHexpression under MPH and in the recovery period over time

Mesurements		**Statistics**	**df**	**p**
Actin under MPH	30 minutes	0,941	12	0,515
	1 hour	0,928	12	0,389
	2 hours	0,904	11	0,177
	4 hours	0,860	11	0,057
	8 hours	0,860	12	0,057
	24 hours	0,860	12	0,057
Actin under recovery	30 minutes	0,925	12	0,327
	2 hours	0,910	12	0,216
	24 hours	0,903	12	0,172
GnRH under MPH	30 minutes	0,866	12	0,058
	1 hour	0,926	10	0,373
	2 hours	0,867	10	0,059
	4 hours	0,867	12	0,059
	8 hours	0,871	12	0,066
	24 hours	0,730	12	0,051
GnRH under recovery	30 minutes	0,863	12	0,064
	2 hours	0,815	12	0,054
	24 hours	0,936	12	0,445

Table 14 shows the Shapiro-Wilk normality test results for Actin and GnGHexpression over time in recovery model. As $p>0,05$ for all measurements, it is determined that, Actin and GnGHexpressions under MPH and the recovery period over time demonstrate a normal distribution.

Table 15. Normality test for Actin ve Kisspeptin expression over time

Measurements		**Statistics**	**df**	**p**
Actin	30 minutes	0,901	11	0,189
	2 hours	0,933	11	0,447

	24 hours	0,938	12	0,469
Kisspeptin	30 minutes	0,929	12	0,367
	2 hours	0,730	12	0,051
	24 hours	0,740	12	0,052

Table 15 shows the Shapiro-Wilk normality test results for Actin ve Kisspeptin expression over time under MPH. As p>0,05 for all measurements, it is determined that, Actin ve Kisspeptin expressions over time under MPH demonstrate a normal distribution.

4 RESULTS

4.1 GT1-7 Cell Proliferation Analysis under Different MPH Concentrations

Cell growth was assessed by trypan blue dye exclusion assay. 50 000 cells were seeded on 24-well plates in complete growth medium for treatment with different concentrations of MPH (0 - 200 µM). The plates were allowed to rest for 24 hours for the cells to attach to the plate for 24 hours after the treatment. Then the cells were stained and counted. There was no statistical significance ($p>0,05$) between the control group and the different doses of MPH (Graph 1). It was observed that different concentrations of MPH did not affect the cell proliferation to a significance level.

Table 16. Cell counts under different MPH concentrations

Assay	Group	n	$\bar{x}$	sd	F	p
Cell count	Control	9	65833,3	34505,4	0,410	0,893
	0,001 µM	9	60555,5	14565,4		
	0,01 µM	9	64444,4	16287,3		
	0,1 µM	9	58055,5	19715,8		
	1 µM	9	58333,3	15612,5		
	10 µM	9	68888,8	21473,5		
	100 µM	9	58055,5	22836,9		
	200 µM	9	67777,7	18003,6		

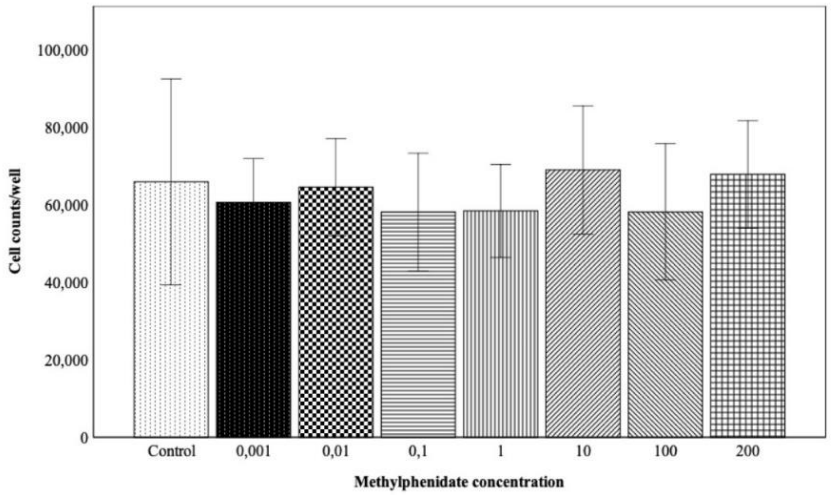

Figure 10: Cell proliferation under different methylphenidate concentrations

4.2 GT1-7 Cell Migration under Different MPH Concentrations

Migration analysis was performed by in vitro wound healing assay. After seeding GT1-7 cells, providing overnight attachment time under different doses of MPH, the cell monolayer was scratched and allowed to heal for 24 hours. The images were captured and analyzed for wound healing using image J software (Figure 11).

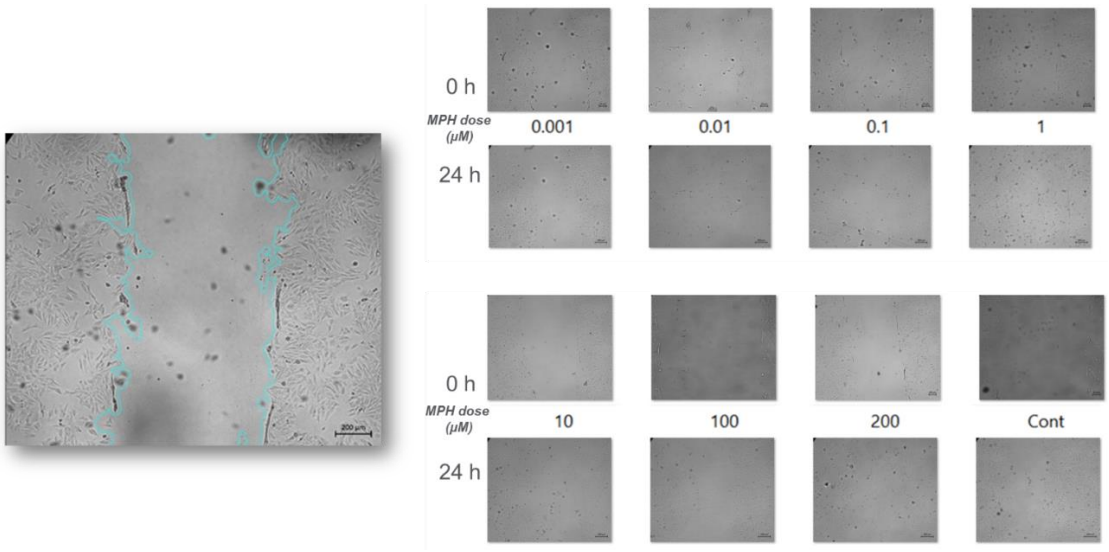

Figure 11: Image J assessment of the wound healing assay under different methylphenidate concentrations

Comparison of baseline (0 hours) and 24 hours demonstrated no statistical significance between MPH doses (Figure 12). This indicates the different concentrations of MPH does not affect GT1-7 cell migration and structural behavior.

Table 17. Wound healing under different MPH concentrations

Assay	Group	n	$\bar{x}$	sd	F	p
Wound healing	Control	3	22,5	6,47	1,438	0,258
	0,001 µM	3	15,7	1,55		
	0,01 µM	3	12,1	5,30		
	0,1 µM	3	18,4	1,74		
	1 µM	3	11,7	6,74		
	10 µM	3	18,3	4,84		
	100 µM	3	14,1	5,00		
	200 µM	3	15,3	6,81		

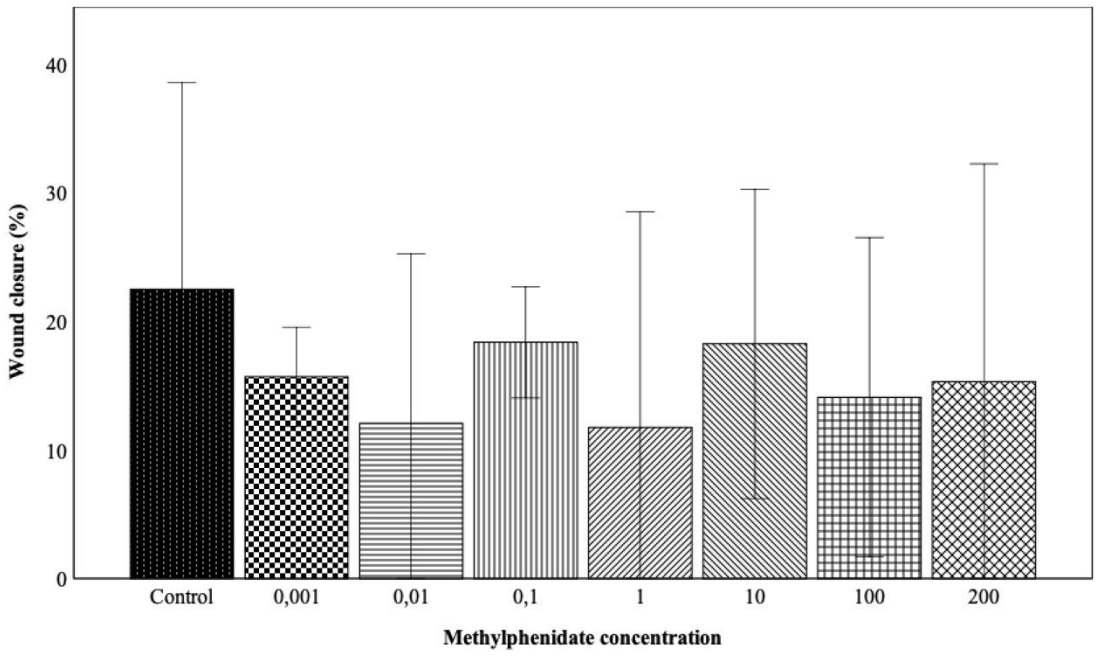

Figure 12: Wound healing under different methylphenidate concentrations

4.3 Effect of MPH on GnRH Expression in GT1-7 Cells

As the proliferation and migration of the GT1-7 cells were not affected by the different concentrations of MPH, the highest concentration of 200 µM was selected for GnRH expression analysis. To provide solid data, the experiment was repeated for 3 sets and the statistical analysis were carried for the whole data set. GNRH expression has remained comparable to the control arm with no statistical difference at any time point. (Figure 13 – 14).

Table 18. GnRH expression under 200 µM MPH and in control group over time

Measurements	Group	n	$\bar{x}$	sd	t	p
30 minutes	Control	6	17,183	0,670	1,809	0,101
	MPH	6	16,594	0,432		
1 hour	Control	6	16,941	0,565	1,801	0,102
	MPH	6	16,117	0,968		
2 hours	Control	6	17,074	0,806	1,946	0,080
	MPH	6	16,014	1,065		
4 hours	Control	6	16,480	0,825	-0,635	0,540
	MPH	6	16,766	0,731		
8 hours	Control	6	16,749	0,411	1,752	0,110
	MPH	6	16,188	0,667		
24 hours	Control	6	15,889	0,457	-0,868	0,406
	MPH	6	16,065	0,195		

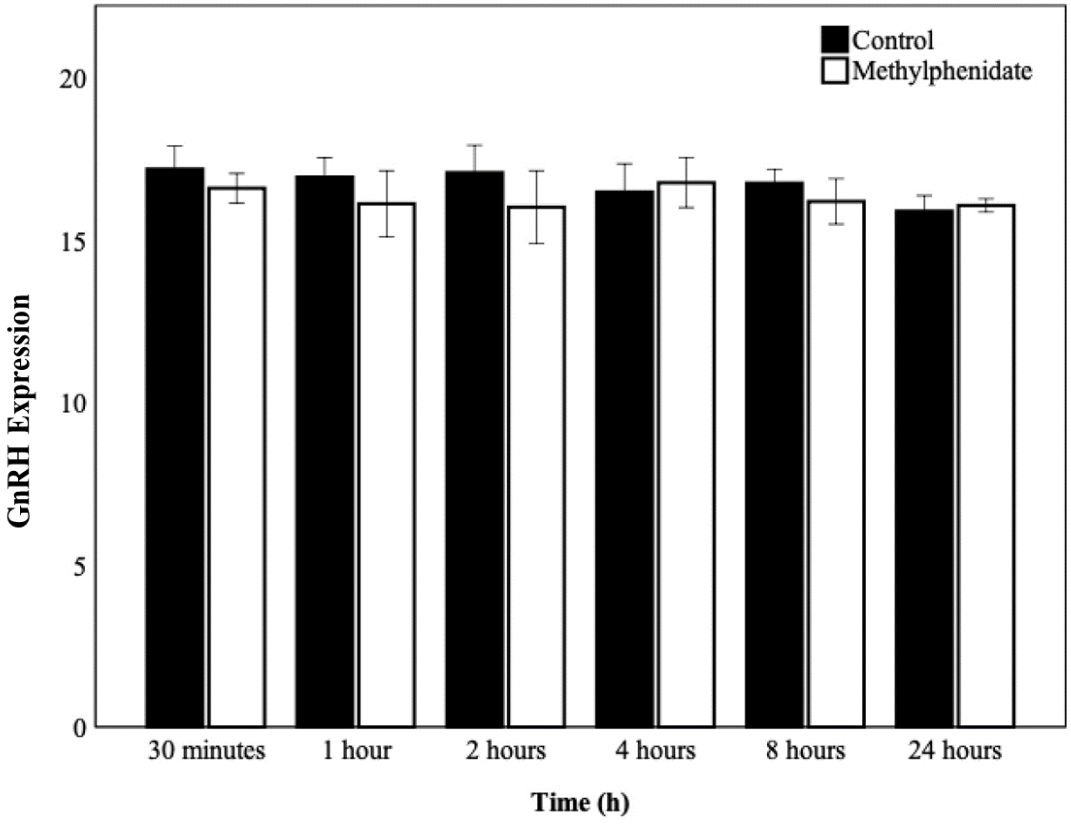

Figure 13: GnRH expression under 200 µM MPH and in control group over time

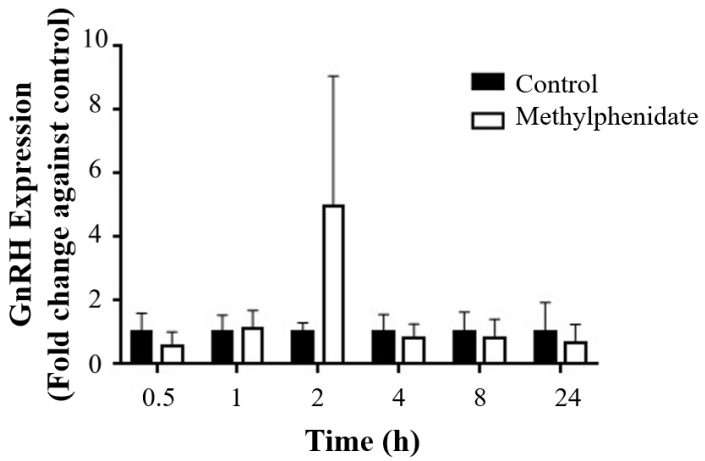

Figure 14: GnRH expression under 200 µM MPH and in control group over time, using $2^{-\Delta\Delta C_T}$ method

Table 19. GnRH expression under 200 µM MPH and in control group over time, confirmation with Mann Whitney – U

Measurements	Group	n	Median	Minimum	Maximum	Z	p
30 minutes	Control	6	16,955	16,522	18,213	-1,281	0,200
	MPH	6	16,690	16,038	17,023		
1 hour	Control	6	17,035	15,912	17,521	-1,761	0,078
	MPH	6	16,378	14,400	17,001		
2 hours	Control	6	17,106	16,126	18,208	-1,922	0,055
	MPH	6	15,734	14,907	17,438		
4 hours	Control	6	16,682	15,420	17,460	-0,801	0,423
	MPH	6	16,926	15,697	17,482		
8 hours	Control	6	16,817	16,113	17,200	-1,761	0,078
	MPH	6	16,131	15,424	16,931		
24 hours	Control	6	15,692	15,448	16,604	-1,121	0,262
	MPH	6	16,021	15,885	16,362		

4.4 Effect of MPH on GnRH Secretion in GT1-7 cells

As the proliferation and migration of the GT1-7 cells were not affected by the different concentrations of MPH, the highest concentration of 200 µM was selected to assess the GnRH release from GT1-7 cells.

Though provided numerical differences and demonstrated a trend of decrease at most of the time points, GnRH release under MPH from GT1-7 cells was statistically significant only at 2nd hour.

Table 20. GnRH secretion under 200 µM MPH and in control group over time

Measurements	Group	n	$\bar{x}$	sd	t	p
30 minutes	Control	5	0,117	0,059	-0,822	0,433
	MPH	6	0,166	0,199		
1 hour	Control	5	0,079	0,044	-0,941	0,371
	MPH	6	0,116	0,077		
2 hours	Control	5	**0,074**	**0,049**	**-3,147**	**0,020**
	MPH	3	**0,288**	**0,146**		
4 hours	Control	6	0,079	0,034	-0,555	0,599
	MPH	6	0,104	0,106		
8 hours	Control	4	0,097	0,024	-1,085	0,323
	MPH	6	0,161	0,140		
24 hours	Control	4	0,135	0,109	-0,901	0,402
	MPH	4	0,226	0,169		

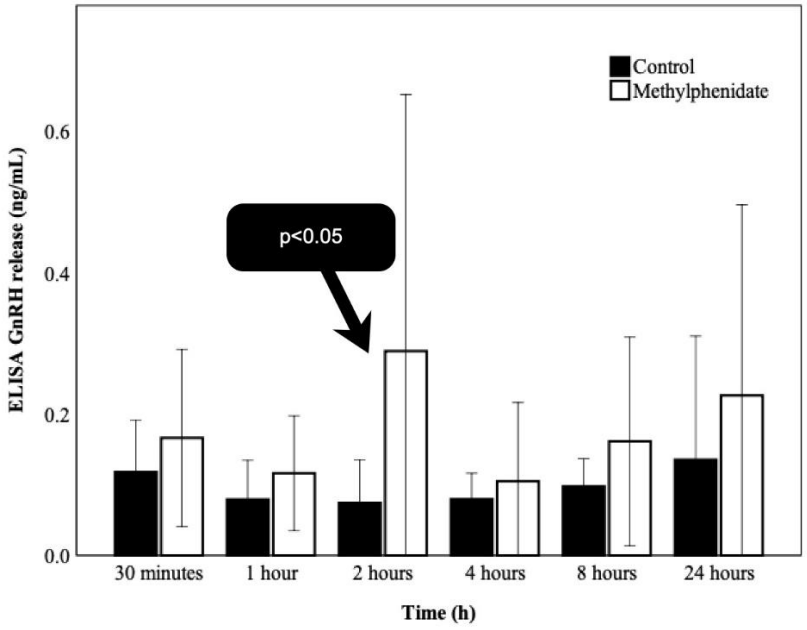

Figure 15: GnRH secretion under 200 µM MPH and in control group over time

Table 21. GnRH secretion under 200 µM MPH and in control group over time, confirmation with Mann Whitney – U

Measurements	Group	n	Median	Minimum	Maximum	Z	p
30 minutes	Control	5	0,124	0,058	0,205	-0,548	0,584
	MPH	6	0,138	0,020	0,323		
1 hour	Control	5	0,085	0,009	0,133	-1,095	0,273
	MPH	6	0,137	0,010	0,203		
2 hours	Control	5	**0,073**	**0,003**	**0,141**	**-1,938**	**0,050**
	MPH	3	**0,293**	**0,140**	**0,432**		
4 hours	Control	6	0,075	0,042	0,124	-0,320	0,749
	MPH	6	0,069	0,004	0,245		
8 hours	Control	4	0,097	0,074	0,122	-0,640	0,522
	MPH	6	0,137	0,002	0,338		
24 hours	Control	4	0,133	0,004	0,271	-0,866	0,386
	MPH	4	0,209	0,037	0,448		

4.5 Effect of MPH on *Kiss1* Expression in rHypoE-8 cells

The concentration of 200 µM MPH was selected as described above, to assess Kiss1 expression in rHypoE-8 cells for time points of 30 minutes, 2 hours and 24 hours. Kiss1 expression was significantly increased in the first 30 minutes of MPH treatment. (Figure 16 – 17)

Table 22. Kisspeptin expression under 200 µM MPH and in control group over time

Measurements	Group	n	$\bar{x}$	sd	t	p
30 minutes	Control	6	**33,782**	**0,499**	**-2,957**	**0,014**
	MPH	6	**34,628**	**0,492**		
2 hours	Control	6	33,845	0,619	0,005	0,996
	MPH	6	33,844	0,510		
24 hours	Control	6	35,112	1,525	0,614	0,557
	MPH	6	34,677	0,830		

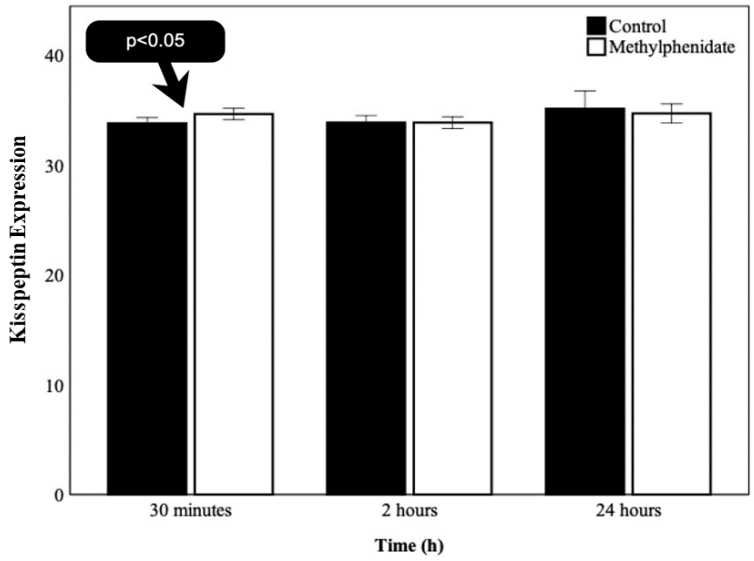

Figure 16: Kisspeptin expression under 200 µM MPH and in control group over time

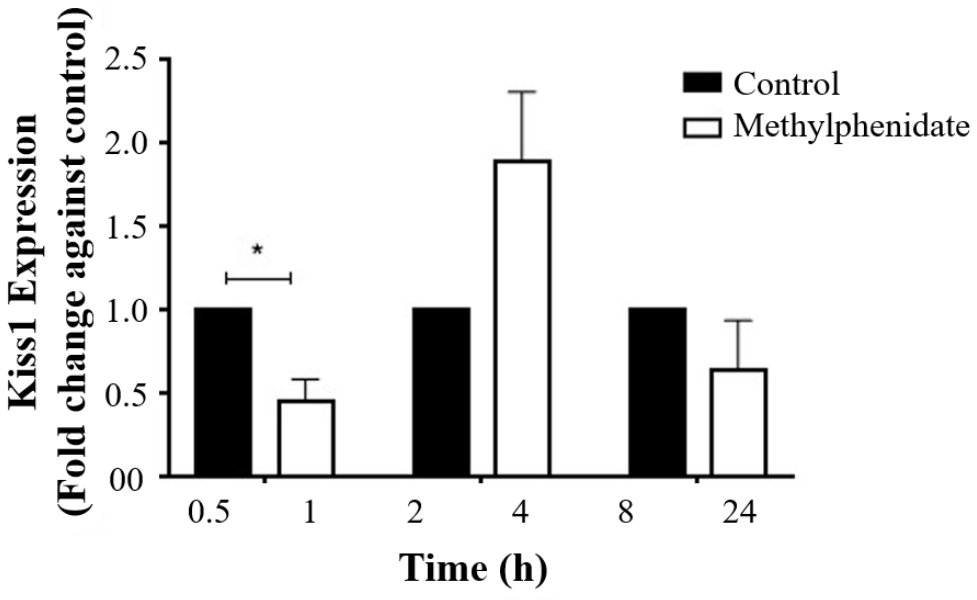

Figure 17: Kisspeptin expression under 200 µM MPH and in control group over time, using $2^{-\Delta\Delta C_T}$ method

Table 23. Kisspeptin expression under 200 µM MPH and in control group over time, confirmation with Mann Whitney – U

Measurements	Group	n	Median	Minimum	Maximum	Z	p
30 minutes	Control	6	**33,897**	**32,954**	**34,286**	**-2,242**	**0,025**
	MPH	6	**34,545**	**34,088**	**35,286**		
2 hours	Control	6	33,807	33,203	34,823	-0,160	0,873
	MPH	6	33,671	33,385	34,599		
24 hours	Control	6	34,247	34,002	37,082	0,000	1,000
	MPH	6	34,393	33,923	36,253		

4.6 GnRH Expression under Recovery Analysis

As it was observed that, MPH effects GnRH expression, we decided to study the recovery in the expression after the cessation of MPH treatment. To provide solid data, the experiment was repeated for 3 sets and the statistical analysis were carried for the whole data set. It was observed that the changes is GnRH expression was not statistically significant for any timepoints. After the cessation of MPH treatment there is no statistical significance between MPH treatment and control arms in GnRH expression. (Figure 18 – 19)

Table 24. GnRH expression under 200 µM MPH and after cessation of treatment

Measurements	Group	n	$\bar{x}$	sd	t	p
30 minutes	Control	6	19,055	2,179	0,567	0,583
	MPH	6	18,408	1,752		
1 hour	Control	6	19,581	1,875	1,627	0,138
	MPH	6	17,988	1,376		
2 hours	Control	6	18,165	2,016	-1,107	0,294
	MPH	6	19,451	2,005		
4 hours	Control	6	20,362	2,700	0,035	0,973
	MPH	6	20,307	2,672		
8 hours	Control	6	21,561	4,827	1,058	0,315

24 hours	MPH	6	19,195	2,593		
	Control	6	25,878	8,033	2,033	0,098
30 minutes after cessation	MPH	6	19,210	0,142		
	Control	6	19,151	2,142	0,563	0,586
2 hours after cessation	MPH	6	18,553	1,478		
	Control	6	20,310	2,905	-1,354	0,218
24 hours after cessation	MPH	6	24,232	6,474		
	Control	6	22,729	4,608	-0,812	0,436
	MPH	6	24,964	4,923		

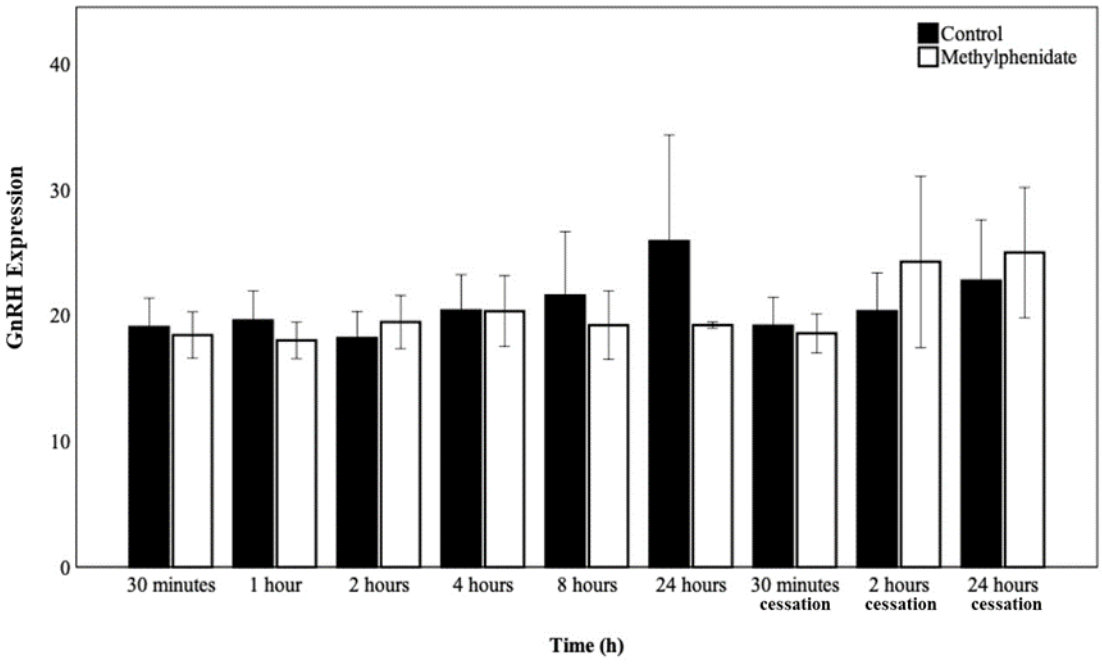

Figure 18: GnRH expression under 200 µM MPH and after cessation of treatment

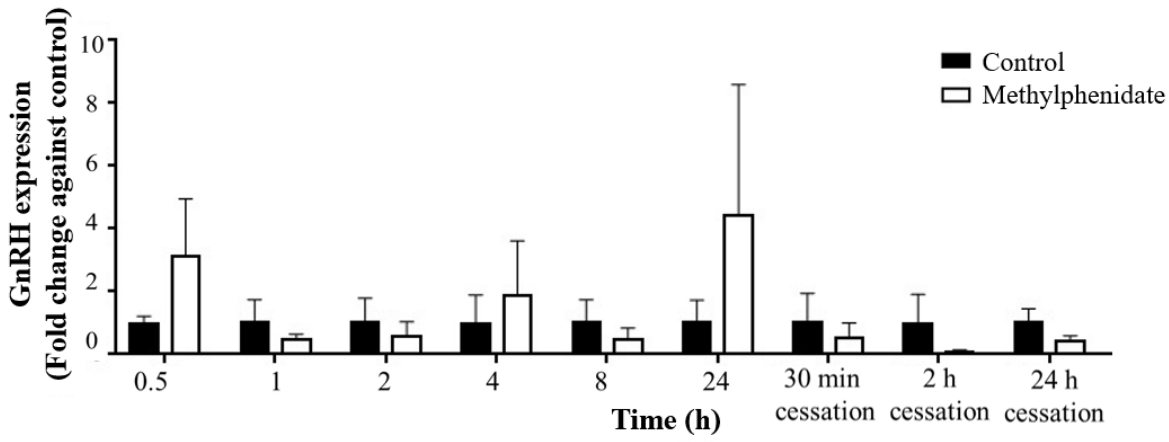

Figure 19: GnRH expression under 200 µM MPH and after cessation of treatment, using $2^{-\Delta\Delta C_T}$ method

Table 25. GnRH expression under 200 µM MPH and after cessation of treatment, confirmation with Mann Whitney – U

Measurements	Group	n	Median	Minimum	Maximum	Z	p
30 minutes	Control	6	18,617	17,062	21,652	-0,160	0,873
	MPH	6	17,597	16,656	20,891		
1 hour	Control	6	19,085	17,316	21,647	-1,461	0,144
	MPH	6	17,615	16,598	19,780		
2 hours	Control	6	17,146	16,296	20,756	-1,441	0,150
	MPH	6	19,430	17,178	21,803		
4 hours	Control	6	21,326	16,891	22,793	-0,160	0,873
	MPH	6	21,074	16,906	23,097		
8 hours	Control	6	21,268	16,150	27,193	-0,320	0,749
	MPH	6	17,787	17,216	22,545		
24 hours	Control	6	23,053	18,616	36,004	-0,853	0,394
	MPH	6	19,186	19,077	19,389		
30 minutes after cessation	Control	6	20,171	16,388	21,048	-0,480	0,631
	MPH	6	18,185	17,076	20,509		

2 hours after	Control	6	22,016	16,495	22,381	-1,121	0,262
cessation	MPH	6	22,328	17,802	32,454		
24 hours after	Control	6	22,875	17,274	28,189	-0,961	0,337
cessation	MPH	6	24,830	19,497	30,570		

5 DISCUSSION AND CONCLUSION

5.1 Discussion

ADHD is one of the most prevalent neuropsychiatric conditions among children and adolescents. Though prevalence varies with geography and culture in the literature, it is a prominent public health issue with estimated diagnosis rates of 5% to 21.8% of children and adolescents worldwide (1, 30). ADHD is recommended to be managed by behavioral and pharmacological interventions and among those MPH has the highest recommendation by the guidelines(56, 58, 66, 67, 121).

In a United States national model, it is estimated that 8.4% of children 2 to 17 years of age currently has ADHD, representing 5.4 million children in United States(29). This reflects to 63 million children and adolescents worldwide between the ages 5 – 19 (29, 122). Of the children living with ADHD, almost two-thirds are estimated to be treated with medication, mostly MPH, well into adolescence. With these estimations adding up to around 40 million children and adolescents under MPH worldwide, the dimensions of the effects of ADHD treatment on growth and puberty can be better perceived.

MPH was found as a chemical compound in CIBA laboratories in 1944 and has been used as a medication since 1954(62, 123) but to date the prescription information states that *" The mode of therapeutic action in Attention Deficit Hyperactivity Disorder (ADHD) is not known."* (124). For an unknown mechanism, being used by 40 million children and adolescents is a critical matter.

On the prescription information, growth retardation has been listed as a side effect and this is well reflected among clinicians and experts(75, 124) but the effects around the maturation and puberty have not been widely studied. There are limited animal studies and case series that provide some evidence that MPH might have a negative effect on the HPG axis and can affect the development and pubertal initiation.

In animal models, MPH use has been suggested to increase sensitivity to sexual stimuli in female rats(92), delay pubertal progression with impaired testicular descent and reduce testicular volume in male rhesus monkeys (93), increase in abnormal tail

morphology and interfere with testicular function among male rats (94), delay pubertal onset and adversely affect maturation of the female reproductive axis by retarding pituitary LH release in female rats (91).

In humans case series; MPH use presents to decrease sexual activity and slow the rate of physical development during puberty in boys (96), lower FSH, LH and testosterone levels and prevent the development of sexual phenotypical characteristics in a male patient (97). On contrast in a case series of 4 female and 3 male children, MPH use was associated with accelerated puberty (102) Familial male puberty precoccus is reported to be treated with methylphenidate for sexual hyperactivity in a case study (100)

The ambiguous evidence on the literature strikes confusing, thus the aim of our study is to shine light on the mechanisms of around the neuroendocrine effects of MPH.

Although the exact mechanism is not known MPH is thought to inhibit dopamine and increase the monoaminergic activity in the synaptic space(124). Dopamine is considered one of the most potent inhibitors of GnRH in central nervous system (90). This might explain the effects of MPH on GnRH expression and secretion.

Pulsatile secretion of GnRH crucial for healthy reproductive physiology(125). Differences in the amplitude and continuity of GnRH release might shift the FSH-LH balance(126, 127). In a study of ovariectomized female rhesus monkeys, it has been demonstrated that high-frequency GnRH pulses increases LH levels and low frequency shifts to FSH(128).

Kisspeptin potently activates GnRH release and GnRH serve as the ultimate central pathway to controlling the initiation of puberty, facilitating the LH and FSH release to act on the gonads to stimulate production of sex steroids for sexual maturation (88).

MPH might affect the pattern of GnRH release, resulting in the differences in the reported changes above in sexual behavior, maturation and secondary sex characteristics. This mechanistic effect around continuity might also explain ambiguity around the literature.

In our study, the oscillations in GnRH and Kisspeptin expressions demonstrate a heterogenous variations. This might be since GT1-7 and rHypoE-8 cells run in a circadian rhythm and the timing and the light conditions might have played a greater effect than anticipated standing as a limitation in our study(129).

Our study showed statistically significant differences in comparison to control groups in the second hour of GnRH release and 30 minutes of Kisspeptin expression. Considering the MPH peak reaching around 2 hours, the results are suggesting that the neuroendocrine function altering effects of MPH would be most prevalent in higher doses or when the concentration is at maximum in plasma.

Another limitation was the access to pure MPH as active ingredient. The access to pure MPH was not possible over multiple tries thought Sigma Aldrich and Novartis, as MPH is a controlled substance, and the transfer and shipping is heavily affected by COVID-19 pandemic during the experiment period. Considering the adversities, we decided on using MPH tablet. Although we dissolved and purified as suggested by the literature, the purification process stands as a limitation compared to acquiring and using the pure substance. Nevertheless, we believe that using tablet form provided a better representation of clinical use considering the high hydro solubility and diffusion rates through blood brain barrier.

MPH is used in oral daily tablet form. Through oral administration, the plasma concentration of methylphenidate peaks between 1 to 3 hours between 20 – 30 nM at the dopamine transporter in humans(63). This well correlates our time points of 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours for capturing the clinical use. MPH is metabolized and cleared out through renal route, in our experiment we did not model the clarification, thus the model reflected a stable constant concertation to reflect timepoints as snapshots.

The experiments showed that; there were no significant differences between the untreated cells and MPH-treated cells in terms of proliferation and migration, GNRH expression has remained comparable to the control arm with no statistical difference through the observed time points, GNRH expression has remained comparable to the control arm with no statistical difference at any time points, GnRH release was

significantly higher than the control at 2 hours after treatment, no statistical differences found between groups at other timepoints, Kisspetin expression was significantly increased in the first 30 minutes of MPH treatment compared to control group and after the cessation of MPH treatment there is no statistical significance between MPH treatment and control arms in GnRH expression.

GnRH release from GT1-7 cells under 200 µM MPH was statistically significant only at 2nd hour over six measurements, when the significance is calculated for 30 measurements, we observed that the differences become significant for all time points.

This is reflected also in GnRH expression. With the limited measurements, GnRH expressions do not demonstrate statistical difference between MPH use and control group but when the same calculations are expanded to reflect 30 measurements the differences became significant for all time points.

Considering the sample size and the distribution of the results, Mann Whitney U test was performed for GnRH expression, GnRH secretion, Kisspeptin expression and GnRH expression in recovery model as a confirmatory analysis. ANOVA and Mann Whitney – U tests yielded to statistical difference at the same timepoints which gives us confidence in the results.

Through the recovery model, the statistics become significant only after modelling 60 measurements, excluding the 4-hour timepoint at which the measurements have very close numerical value.

Kisspeptin expression is significant at 30 minutes timepoint. The differences in measurements for 24 hours become significant after modelling the statistics for 54 measurements and 2-hour measurement stay not significant as the groups have very close numerical value.

Having no statistical difference, does not prove there are no differences between the MPH use over neuronal sell lines. It just proves that in the group we observed in selected cell populations and sets, no statistical significance could be achieved, and further investigation is needed to understand a medication that possibly have direct effects on 40 million people worldwide.

5.2 Conclusion

There were no significant differences between the untreated and MPH-treated GT1-7 cells in terms of proliferation and migration. MPH creates oscillations in the GnRH expression and the effects of MPH is rapidly reversed after cessation of MPH treatment. Kisspeptin expression is impacted at the early minutes of MPH treatment but returns to normal rapidly at the second hour and maintains at comparable levels to control group levels at 24^{th} hour. These findings suggest more research is needed to understand the effects of MPH on the HPT axis and its effects around puberty.

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7. CURRICULUM VITAE

Personal Informations

Name	Berna	**Surname**	Özelgün
Place of Birth		**Date of Birth**	

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate	Neuroscience	Institute of Health Sciences, Yeditepe University	2015-2021
University	Medicine	Yeditepe University	2006-2012
	Genetics and Bioengineering	Yeditepe University	2006-2010
	Biomedical Engineering	Yeditepe University	2005-2006 (Transferred to Medicine)
High school	Science and Mathematics	Kadıköy Anadolu Lisesi	1997-2005

[#] All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Languages	Grades ([#])
English	ALES: 88.750
German	B1-Goethe Zertifikat

Work Experience (Sort from present to past)

Position	Institute	Duration (Year - Year)
Medical Manager	Mustafa Nevzat	03/12/2012 – 05/07/2013
Regional Medical Manager	Novartis Turkey	08/07/2013 – 06/02/2015
Medical Affairs Manager	Gilead Sciences Turkey	08/07/2013 – 06/02/2015
Senior Medical Affairs Manager	Gilead Sciences Turkey	28/10/2018 – 01/03/2020
Associate Director of Medical Affairs	Gilead Sciences Turkey	01/03/2020 – present
Associate Director, Marketing	Gilead Sciences, European Distributor Markets	15/03/2021 – present

Computer Skills

Program	Level
SPSS	Average
Microsoft Office	Excellent

***Excellent , good, average or basic**

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Biçer S, Ercan Sariçoban H, Özen AO, Saf C, Ergenekon Ulutaş P, Gürol Y, Yilmaz G, Vitrinel A, Özelgün B. Experience of influenza A H1N1 in a paediatric emergency unit. Infez Med. 2015 Jun;23(2):125-33
Bicer S, Col D, Erdag GC, Giray T, Gurol Y, Yilmaz G, Vitrinel A, Ozelgun B. A retrospective analysis of acute gastroenteritis agents in children admitted to a university hospital pediatric emergency unit. Jundishapur J Microbiol. 2014 Apr;7(4):e9148. doi: 10.5812/jjm.9148. Epub 2014 Apr 1
Tug N, Sandal S, Ozelgun B, Yilmaz B. Correlation of spermiogram profiles with DNA damage in sperm cells of infertile men: a comet assay study. Gynecol Endocrinol. 2011 Jan;27(1):49-54. doi: 10.3109/09513590.2010.487598. Epub 2010 May 26.

Proceedings presented in international scientific meetings and published in proceedings book.

Yaylalı ve ark. "COVID-19 döneminde HIV yayılımını etkileyen davranışsal faktörlerin değişiminin araştırılması", HIV/AIDS 2021, P-17
Ozelgun et al. Effects of Methylphenidate on GnRH Expression and Release in GT1-7 Cell Line, TNED 2021, OC-06
Yaylalı et al. Türkiye'de HIV'in Geleceğinin Modellemesi: Koruma, tanı ve tedavinin geliştirilmesinin hastalığa etkileri, HIV/AIDS 2020, P0104
Yaylalı et al. HIV Pozitif Hastalarda Hızlı Antiretroviral Terapi Uygulanması ve Etkileri: Bir modelleme çalışması, HIV/AIDS 2020, P0105
Yaylalı et al. Modeling the Future of HIV in Turkey: Disease Implications of Improving Prevention, Diagnosis, and Treatment, Glasgow 2020, P114
Sayan et al. ECFTAF 48. Hafta Ilaç Direnç Analizi, Poster EKMUD 2016
Wohl et al. Böbrek Hastalığı Riski Yüksek olan Hastalarda TAF Renal Güvenliliği, Poster EKMUD 2016
Post et al. Böbrek Yetmezliğinde TAF'ın Daha Uzun Süreli Güvenliliği, Poster EKMUD 2016
Tatar et Al. Direct Cost of HIV/AIDS in Turkey, Poster ISPOR 2016
Tatar et al. Indirect Cost of HIV/AIDS: Results of a Survey from a Turkish Research Center, Poster ISPOR 2016
Özçelik et al. "Ventilation and capacity in relation with increasing weight effort test and determination of respiratory efficacy in aerobic and anaerobic regions", EP-049, 31stAnnual Congress, TÜSAD, Turkish Respiratory Society, Izmir, Turkey,
Tug et al. "Correlation of spermiogram profiles with DNA damage in sperm cells of infertile men: a comet assay study", P-0266, 13th World Congress on Human Reproduction, 2009•
"A case of medical ethics", Oral Presentation, Yeditepe University Medical Student's Congress on Oncology and Hematology 2009
"Comet analysis on sperm cells of infertile men", Poster presentation, Pamukkale University Medical Student's Congress on Psychiatry and Scientific Researches, 2009
"Infertility and its effects on psychology", Poster presentation, Pamukkale University Medical Student's Congress on Psychiatry and Scientific Researches, 2009
Declaration ofYouth, Youth WaterForum, World WaterForum, Turkey, 2009
ISMOPH (International Students' Meeting on Public Health) Declaration, Turkey, 2009•