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Original Article

Paternal exposure to methylphenidate induces ADHD-like behavioral phenotypes and altered gene expressions in mouse offspring

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ABSTRACT — Methylphenidate (MPH) is used as a first-line treatment for attention-deficit/hyperactivity disorder (ADHD). Because the onset of ADHD appears in early childhood and the incidence number is increasing, more patients could become adults with long-term use of MPH. In addition, few men would discontinue the medication during a fertile period. Recently, environmental factors such as diet and drug abuse have been reported to produce changes in the sperm epigenome and affect the health of the next generation. However, the effects of long-term administration of a psychostimulant such as MPH on the next generation is unknown. In this study, we examined the effects of paternal administration of MPH on the growth, behavior, and gene expression in offspring using a mouse model. Sires were subcutaneously administered MPH for 21 days and mated with naive dams. Upon reaching 6–7 weeks of age, offspring were subjected to spontaneous locomotor, elevated plus-maze, and passive avoidance tests. Additionally, RNA-seq and RT-qPCR were performed on the striatum. Paternal MPH exposure induced increased atomoxetine-sensitive impulsivity and decreased long-term memory function in the offspring. Enrichment analysis following RNA-seq revealed significant enrichment of terms involved in the nervous system. Gene expression levels of *Snap25*, *Syt1*, *Drd2*, *Maoa*, and *Comt*, which are associated with ADHD pathology, are altered in the striatum. These results suggest that continuous administration of MPH to male mice induces ADHD-like behavior and changes in the expression of genes involved in the nervous system in the brain of the next generation.

Key words: Methylphenidate, Paternal, ADHD, Behavior, Next generation, POHaD

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a clinically heterogeneous neurodevelopmental disorder with impaired attention, impulsivity, and hyperactivity that interfere with social and academic functioning (Biederman, 2005). It affects 8–12% of children worldwide (Biederman and Faraone, 2005; Faraone *et al*., 2003), and the incidence in adults is estimated at 4–5% (Kessler *et al*., 2006; Willcutt, 2012). The heritability of ADHD is reported to be about 76%, indicating that ADHD is a highly heritable disorder (Faraone *et al*., 2005). Although the causative genes for ADHD have not been identified, dopamine-related genes (*DRD1*, *DRD2*, *DRD4*, *DRD5*, and *SLC6A3*), serotonin-related genes (*HTR1B*, *HTR2A,* and *SLC6A4*), monoamine metabolizing enzyme-related genes *(MAOA*, *MAOB*, and *COMT*), and an exocytosis-related gene (*SNAP25*) have been reported to be associated with ADHD (Faraone *et al.*, 2005; Li *et al*., 2014; Yadav *et al.*, 2021). In addition to these genetic factors, various environmental and neuro-

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logical factors are thought to be intricately involved in the development of ADHD, though the exact etiological factors of the disease remain unknown (Banerjee *et al.*, 2007; Yadav *et al*., 2021).

Brain regions associated with ADHD, such as the prefrontal cortex, basal ganglia, and cerebellum, are interconnected by a network of neurons that control attention, emotion, and behavior (Arnsten and Pliszka, 2011; Sharma and Couture, 2014). Network activity between these regions is maintained by the interaction of neurotransmitters (NTs) via multiple pre- or post-synaptic receptors (Sharma and Couture, 2014). ADHD symptoms are known to be closely related to impaired dopamine (DA) and norepinephrine (NE) function (Prince, 2008). ADHD medications include central nervous system (CNS) stimulants and non-stimulants, but stimulants such as amphetamines and methylphenidate (MPH) are the preferred first-line agents. Therefore, as the number of prescriptions for ADHD patients increases (Anderson *et al.*, 2018; Renoux *et al*., 2016), so does the prescription of CNS stimulants for adults at sexual maturity. Because of the disadvantages of discontinuing treatment for ADHD, patients generally continue medication at sexual maturity. Several reports indicate that parental amphetamine exposure produces behavioral changes, and stunted growth in rat offspring (Adams *et al.*, 1982; Monder, 1981). In addition, because fetal exposure to MPH carries little risk of teratogenicity (Bolea-Alamanac *et al*., 2014; Huybrechts *et al.*, 2018), its prescription is continued in women willing to or carrying a baby. However, limited information is available on the effects on the child's behavior, growth, and learning and memory functions, when MPH is used for treatment during the perinatal period. Furthermore, there are no reports on the risk of reproductive toxicity when MPH is administered to men; therefore, discontinuation of MPH prescription for men who wish to have children is not recommended.

Recently, the developmental origins of health and disease (DOHaD) theory has been attracting attention as a concept that environmental factors during embryonic and developmental stages shape health and disease in adulthood and old age. We have investigated the effects of MPH intake during pregnancy on the next generation in mice, finding that fetal exposure to MPH caused ADHD-like behavioral changes and decreased expression of ADHD-related genes such as *Drd2* and *Slc6a3* in the offspring (Aoki *et al*., 2021). In recent years, the concept of paternal origins of health and disease (POHaD), which proposes that the paternal environment prior to conception alters the epigenome of sperm cells and affects the developmental program of the embryo and the health of the next generation, has also begun to attract attention. Several studies using rodent models have shown that metabolic disturbances appear in the offspring of sire fed low-protein or high-fat diets, which are directly related to changes in sperm epigenetics (Carone *et al*., 2010; Champroux *et al*., 2018; Fullston *et al*., 2013; Ng *et al*., 2010). The epigenetic changes detected in the offspring were related to changes in DNA methylation in the regulatory sequences of genes involved in lipid and cholesterol biosynthesis, as well as changes in microRNA expression in the testis (Carone *et al.*, 2010; Champroux *et al*., 2018; Fullston *et al.*, 2013). Thus, POHaD studies have been conducted with respect to diet and drugs of abuse, and the sperm epigenome has been reported to be affected by environmental factors such as nutritional stress and chemicals, affecting the health status of the next generation. Although, few studies have examined the effects of pharmaceuticals, the possibility that ADHD medications taken over a long period of time, from childhood to sexual maturity, may produce epigenetic changes cannot be ruled out. In addition, since the number of prescriptions of ADHD medications for males is more than twice that for females (Renoux *et al*., 2016), there is a need to clarify the effects of ADHD medication intake by males during sexual maturity on the next generation. Therefore, this study was conducted in mice to determine whether paternal MPH exposure alters the behavior and gene expression of the offspring.

MATERIALS AND METHODS

Reagents

MPH hydrochloride was synthesized by esterification of ritalinic acid with trimethylsilyl (TMS)-diazomethane as described previously (Aoki *et al*., 2021; Shioiri and Aoyama, 1986). The synthesized compound was confirmed to be MPH hydrochloride by 1H-NMR. No impurity peaks were observed on the NMR spectrum.

Animals

Male and female ICR mice (6 weeks old) were purchased from Sankyo Lab Service Co. (Tokyo, Japan). Mice were housed in plastic cages in a temperature-controlled room (22 ± 1°C) and maintained at a 12-hr light-dark cycle, with free access to food and water. All animal care and experimental procedures were conducted per the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Showa University (approved #23023 and #24025). Every effort was made to minimize the number of animals used and their suffering. Six-week-old male ICR mice (F0) were administered

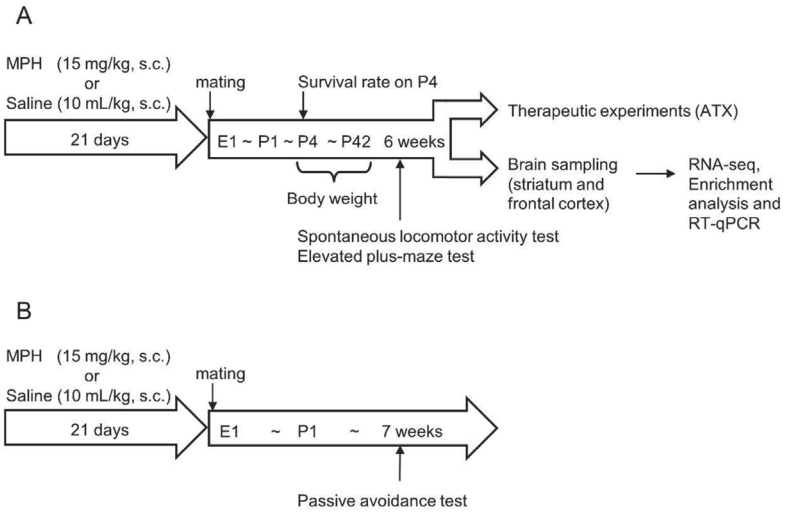

Fig. 1. Experimental design of this study. (A) Sires were administered MPH (15 mg/kg) or saline (10 mL/kg) subcutaneously for 21 consecutive days. After treatment, the sires were mated with drug-untreated female mice, and offspring were obtained. The survival rate of offspring was determined at P4, and offspring were weighed from P4 to P42. Offspring were subjected to spontaneous locomotor activity and elevated plus-maze tests at 6 weeks of age. The next day, half of the offspring were subjected to the therapeutic experiments. Brains (striatum and frontal cortex) were collected from the other half of the offspring. Gene expression analysis was performed using total RNA extracted from the brains. (B) MPH or saline were administered to sires, and offspring were obtained by the same procedure as in (A) and subjected to a passive avoidance test at 7 weeks of age.

MPH (15 mg/kg) or saline (10 mL/kg) subcutaneously for 21 consecutive days. The dose of MPH was determined with reference to our previous study (Aoki *et al.*, 2021). A drug-treated male mouse was mated with a drug-untreated female mouse during its estrous cycle. Female mice were kept alone from the time of vaginal plug confirmation until delivery.

Experimental design

The day of birth was designated as postnatal day 1 (P1). The survival rate was evaluated at P4. Body weights were measured at P4 and P7, and then every 7 days thereafter until P42 (Fig. 1A). When F1 mice were 6 weeks old, six F1 (hereafter, offspring born from saline-treated sire are referred to as the control group and those born from MPH-treated sire are referred to as the MPH group) were randomly selected from each F0 mother (control group (n = 54) consisting of 30 males and 24 females, and MPH group (n = 48) consisting of 24 males and 24 females) for spontaneous locomotor activity and elevated plus-maze tests. The next day, half of the F1 mice (control group (n = 27) consisting of 15 males and 12 females, and MPH group (n = 24) consisting of 12 males and 12 females) were subjected to therapeutic experiments with atomoxetine (ATX) (Tokyo Chemical Industry Co., Tokyo, Japan). Brains were collected and RNA was extracted from striatal and cortical regions. RNA from the striatum was subjected to RNA-seq analysis followed by enrichment analysis. RNA from the striatum and frontal cortex was subjected to RT-qPCR for the quantification of target genes (Fig. 1A). A passive avoidance test (control group (n = 12) consisting of 7 males and 5 females, and MPH group (n = 24) consisting of 12 males and 12 females) was conducted at 7 weeks of age (Fig. 1B). Details of the mice used in this study are shown in Table S1.

Spontaneous locomotor activity test

A spontaneous locomotor activity test was performed to assess hyperactivity. Mice were placed in activity chambers (W20 cm × D20 cm × H25 cm) and their activity was monitored using a video camera mounted on the ceiling for analysis. For the test, mice were placed in activity chambers and allowed to habituate to the environment for 10 min, after which the total distance traveled and mean travel speed were measured for 30 min. Locomotor activity was measured using the ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA).

Elevated plus-maze test

The elevated plus-maze test, which is used to assess anxiety behavior and impulsivity (Agmo and Belzung, 1998; Schmitt *et al*., 2002; Ueno *et al*., 2002), was performed as previously described with modifications (Wang *et al*., 2013). The plus-maze consists of two open arms

(30 cm × 6 cm) and two closed arms of the same size with 20 cm high walls emanating from a common central platform (6 cm × 6 cm) to form a plus shape. The entire apparatus was placed at a height of 40 cm above the floor. A video camera and illumination lamps were mounted on the ceiling. Anxiety-related behaviors for each mouse were recorded for a period of 10 min. At the beginning of the test, the mouse was placed on the central platform with its head facing an open arm. Recorded parameters included the numbers of open arms or closed arms entries (arm entry was defined as 80% of the body entering the arm; if the body did not enter open arm or closed arm, it was defined as entering the central platform), the total time that each mouse spent in open arms and closed arms, and the distance that each mouse traveled in open arms and closed arms. The data were analyzed with the ANY-maze video tracking software.

Therapeutic experiments

Mice were subjected to therapeutic experiments on the day after the spontaneous locomotor activity and elevated plus-maze tests, using ATX as a therapeutic drug. Thirty min after subcutaneous (*s.c.*) administration of ATX (3 mg/kg), mice were again subjected to the spontaneous locomotor activity test and the elevated plus-maze test.

Passive avoidance test

Passive avoidance involves the learned inhibition of natural response and provides information on learning and memory capabilities (Hermans *et al*., 1992; Olton, 1973; Rodier, 1977). The passive avoidance procedure consists of two sessions including the learning and the memory trials (Aoki *et al*., 2021; Tanaka *et al.*, 2006; Tanaka *et al*., 2011). The apparatus used in the step-through type of passive avoidance protocol consisted of two compartments (one lit compartment (W19 cm × D24 cm × H20 cm) and one dark compartment (W19.5 cm × D14 cm × H19 cm)), connected via a door. In the learning trial, each mouse was placed in the lit compartment and allowed to freely enter the dark compartment through the door. Once the mouse entered the dark compartment, an electrical foot shock was immediately delivered (1 s, 0.6 mA) using Passive Avoidance Control (O'Hara Co., Tokyo, Japan). Although mice repeatedly stepped through the door, they eventually remained in the lit compartment. The number of electrical foot shocks required to hold the mouse in the lit compartment for 5 min was recorded as a measure of the acquisition of passive avoidance. The memory trial was carried out 24 hr after the learning trial. The mouse was placed in the lit compartment. The dark compartment was not connected to the electric shock generator. As a measure of passive avoidance retention, we recorded whether each mouse entered the dark compartment during 5 min.

RNA-seq analysis

Total RNA samples collected from the striatum of 6 weeks old offspring (control group (n = 3) consisting of 1 male and 2 females, and MPH group (n = 3) consisting of 1 male and 2 females) were submitted to Macrogen Japan (Tokyo, Japan) for bioanalyzer quality control analysis, Illumina next-generation sequencing, and differential expressed gene (DEG) analysis. All submitted samples had an RNA Integrity Number > 9 (on a scale of 1–10, with 10 meaning the best quality sample with the least advanced degradation) and proceeded for library construction. Sequencing libraries were prepared from poly-A selected RNA of each sample using the TruSeq Stranded mRNA Library Prep Kit (Illumina Inc., CA, USA). Transcriptome sequencing (100 bp paired-end sequencing) was performed on a NovaSeq 6000 System (Illumina Inc.). Adapter sequences and low-quality bases in paired-end reads were removed with Trimmomatic (version 0.38). Filtered paired-end reads were mapped to the mouse reference genome (mm10) by HISAT2 (version 2.1.0) and expression levels were quantified by StringTie (version 2.1.3b). Statistical analysis was performed by DESeq2 to identify DEGs. DEGs were extracted by using the following criteria; fold-change (FC) ≥ 1.2, p-value < 0.05, and average read counts ≥ 100 for either the control or MPH group. Raw and processed data are available in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE211982. Identified DEGs were illustrated as a clustered heat map using Heatmapper (http://www.heatmapper.ca/).

Bioinformatics analysis

Metascape (https://metascape.org/gp/index.html) was used for enrichment analysis (Zhou *et al*., 2019). The DEG list containing 922 DEGs was entered into the Metascape platform and its key functional terms were analyzed. The process of converting the DEG list to the corresponding Entrez gene ID of *M. musculus* and the list of annotations was the latest version of the database at the time of analysis (last update: 04/22/2022).

Reverse transcription-quantitative PCR (RT-qPCR)

Brain samples (striatum and frontal cortex) collected from 6 weeks old offspring (control group (n = 6) consisting of 3 males and 3 females, and MPH group (n = 6) consisting of 3 males and 3 females) after the behavior test were flash-frozen in liquid nitrogen and stored at −80°C until total

RNA was extracted. Total RNA was isolated from the tissue using RNeasy Mini Kit (Qiagen Inc., CA, USA), and cDNA was synthesized with PrimeScript RT Master Mix (Takara Bio Inc., Siga, Japan). Real-time PCR with a FAM-labeled probe for the target cDNA and a VIC-labeled probe for the housekeeping gene was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, CA, USA) on a StepOne real-time PCR system (Applied Biosystems) or QuantStudio 3 (Applied Biosystems) according to the manufacturer's protocol. PCR was performed in two steps. Amplification conditions were 95°C for 20 sec in the hold stage, followed by 40 cycles of 95°C for 1 sec and 60°C for 20 sec in the PCR stage. mRNA levels were measured as the relative ratio to β-actin mRNA. All predesigned PCR primers and Taq Man MGB probes were purchased from Applied Biosystems. The assay IDs of the probes used in this study were as follows: mouse *Drd1*, Mm02620146_s1; mouse *Drd2*, Mm00438545_m1; mouse *Slc6a3*, Mm00438388_m1; mouse *Maoa*, Mm00558004_m1, mouse *Maob*, Mm00555412_m1; mouse *Th*, Mm00447557_m1; mouse *Snap25*, Mm01276449_m1, mouse *Stx1a*, Mm00444008_m1; mouse *Syt1*, Mm00436858_m1; mouse *Nrxn1*, Mm00660298_m1.

Statistical analysis

Data of average numbers of offspring, average numbers of survivors, gene expressions, and spontaneous locomotor activity test of sire were examined with analysis of Student's *t*-test. Data of body weight, spontaneous locomotor activity test, elevated plus-maze test, and therapeutic experiments with ATX were analyzed with mixed effect linear factorial ANOVA (Zoubovsky *et al*., 2022). In this model, to ensure that litter effects were controlled, litter was treated as a random factor (block) and for body weight analyses, group (control and MPH groups) was used as a between factor; for other analyses, group and sex were used as between factors within each block factor (Golub and Sobin, 2020). Data from the learning trial of passive avoidance test were examined with an analysis of Wilcoxon test. Data from the memory trial of the passive avoidance test were analyzed with a chi-square test. Statistical analyses were performed with JMP Pro 16.0.0 (SAS Institute, Cary, NC, USA).

RESULTS

Effects of MPH administration on sire and growth of the offspring

On day 20 during a total of 21 days of MPH administration, the spontaneous locomotor activity of the sire was significantly increased (Fig. S1). No significant toxicity including deaths were observed in sires during the MPH administration period. The number of births and survival rates at P4 of the offspring from MPH-treated sire (MPH group) were similar to those of the control group (Table S2). No malformations were observed in the MPH group (data not shown). Although the weight of the MPH group at P4 was equivalent to that of the control group, the weight of the MPH group at P7 and P14 before weaning was significantly lower than that of the control group (Fig. 2). The MPH group weighed at a similar level as the control group after P21 or later (Fig. 2).

Effect of MPH administration to sire on spontaneous locomotor activity of offspring

To evaluate hyperactivity, one of the typical phenotypes of ADHD, spontaneous locomotor activity was measured in 6 weeks old offspring. The total distance traveled and mean travel speed of the MPH group were equivalent to those of the control group (Fig. 3A). The next day, spontaneous locomotor activity was measured

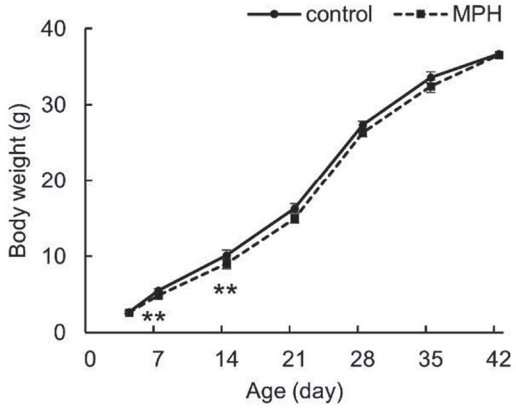

Fig. 2. Effects of MPH administration to sires on body weight of the offspring. Body weights were measured at P4 and P7, and then every 7 days thereafter until P42. Solid and dashed lines indicate the weights of offspring in the control and MPH groups, respectively. About 9 offspring were randomly selected at P4, and then 6 offspring were randomly selected at P21 and thereafter reared until P42. Data from control and MPH groups are shown as mean ± S.E.M. and were compared using mixed ANOVA with group model and litter as a random factor. (**$p < 0.01$, P4 control group, n = 128; P4 MPH group, n = 108; P7 control group, n = 79; P7 MPH group, n = 71; P14 control group, n = 79; P14 MPH group, n = 71; P21 control group, n = 54; P21 MPH group, n = 48; P28 control group, n = 54; P28 MPH group, n = 48; P35 control group, n = 54; P35 MPH group, n = 48; P42 control group, n = 54; P42 MPH group, n = 48).

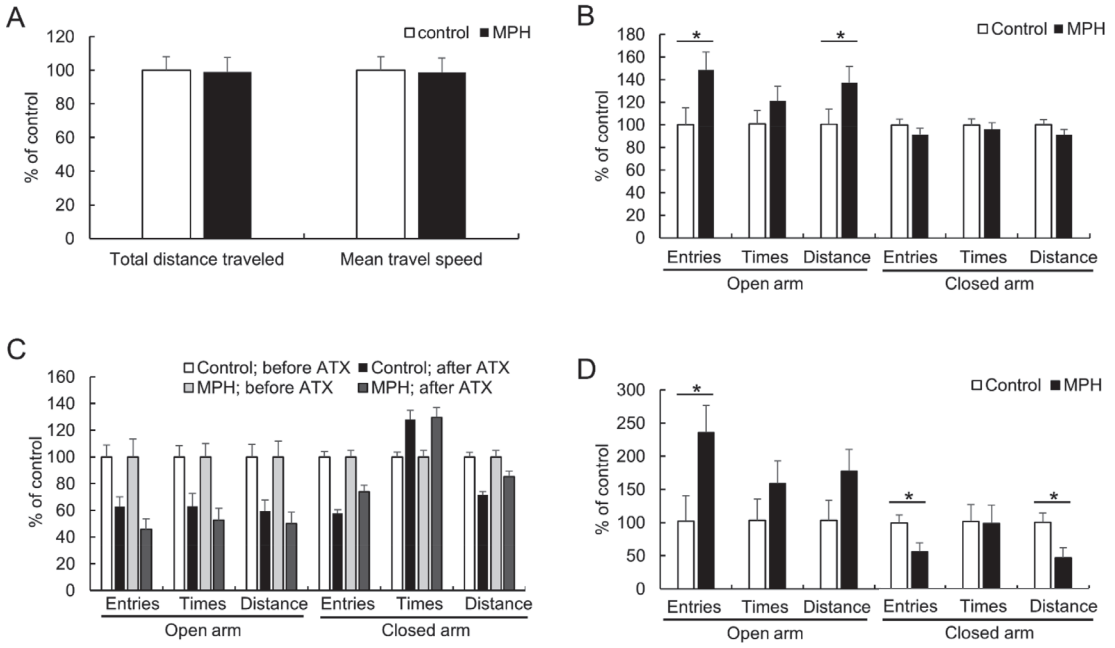

Fig. 3. Effects of paternal MPH treatment on the locomotor activity and impulsivity in the offspring. Behavioral assessment through a spontaneous locomotor activity test (A) and an elevated plus-maze test (B), (C), and (D) were carried out at 6 weeks of age. (A) The spontaneous locomotor activity test was conducted using a video tracking system to analyze the total distance traveled and the mean travel speed in the activity chambers during a 30-min period. Data from the control and MPH groups are shown as mean ± S.E.M. and were compared using mixed ANOVA with group × sex model and litter as a random factor. (control group, n = 54 (M30/F24) and MPH group, n = 48 (M24/F24)). (B) The elevated plus-maze test was analyzed using a video tracking system for the number of entries, time spent, and distance traveled for each open arm and closed arm. Data from the control and MPH groups are shown as mean ± S.E.M. and were compared using mixed ANOVA with group × sex model and litter as a random factor. (*$p < 0.05$, control group, n = 53 (M29/F24) and MPH group, n = 47 (M24/F23). (C) The day after the elevated plus-maze test, half of the mice were subjected to a therapeutic experiment with ATX (3 mg/kg, *s.c.*). Thirty min after administration of ATX, mice were again subjected to the elevated plus-maze test. The same items as in (B) were measured. Data are shown as mean ± S.E.M. (control group, n = 27 (M15/F12) and MPH group, n = 23 (M12/F11)). (D) Differences in data before and after ATX administration were calculated and compared for the control and MPH groups, respectively. Data are shown as mean ± S.E.M. and analyzed with mixed ANOVA with group × sex model and litter as a random factor. (*$p < 0.05$; control group, n = 27 (M15/F12) and MPH group, n = 23 (M12/F11)). (M, male; F, female)

again 30 min after administration of ATX, an ADHD medication. Because of acclimation, the total distance traveled and mean travel speed decreased in the control and MPH groups compared to those before ATX administration (Fig. S2A). Differences in each item before and after ATX administration were calculated and compared between control and MPH groups, and no significant differences were observed between the control and MPH groups (Fig. S2B).

Effects of MPH administration to sire on anxiety-like behaviors in offspring

Rodent models of ADHD show decreased anxiety-like behaviors (Söderpalm, 1989; Yen *et al*., 2013). Therefore, we assessed anxiety-like behavior in 6 weeks old offspring through the elevated plus-maze test. The number of entries and distance traveled in the open arms were significantly increased compared to the control group (Fig. 3B). The next day, the elevated plus-maze test was performed again 30 min after ATX administration. Similar to the spontaneous locomotor activity test, the elevated plus-maze test also showed an effect of acclimation. All items except for time spent in the closed arms decreased after ATX administration compared to before (Fig. 3C). The difference between before and after ATX administration in the number of entries into the open arms was significantly larger in the MPH group and the number of entries and distance traveled in the closed arms was significantly smaller in the MPH group (Fig. 3D).

Table 1. Effects of paternal MPH treatment on learning and memory in the offspring.

Learning trial				
Sex	Group	n	Median of electrical foot shocks (min–max)	*p*-value
Male and Female	Control	12	3 (2–7)	0.337
	MPH	24	3 (2–5)	
Male	Control	7	3 (2–7)	0.245
	MPH	12	2.5 (2–4)	
Female	Control	5	3 (2–5)	1.000
	MPH	12	3 (2–5)	
Memory trial				
Sex	Group	Entry (-)	Entry (+)	*p*-value
Male and Female	Control	11 (44.0)	1 (9.1)	0.059
	MPH	14 (56.0)	10 (90.9)	
Male	Control	7 (58.3)	0 (0)	0.017
	MPH	5 (41.7)	7 (100)	
Female	Control	4 (31)	1 (25)	1.000
	MPH	9 (69)	3 (75)	

A passive avoidance test was performed on offspring at 7 weeks of age. In the learning trial (upper table), the number of electrical foot shocks required to hold the mouse in the lit compartment for 5 min was evaluated as a measure of passive avoidance acquisition. Data from control and MPH groups are shown as median (min–max) and were compared using Wilcoxon test. In the memory trial (lower table), the mouse was placed in the lit compartment. As a measure of passive avoidance retention, we recorded whether each mouse entered the dark compartment within 5 min. The number of offspring that did not ("Entry (-)") or did ("Entry (+)") enter the dark compartment is shown in the table. Entry (-) and (+) values were compared between the control and MPH groups using chi-square test and p-values < 0.05 were considered statistically significative.

Effects of MPH administration to sire on learning and memory in offspring

To evaluate learning and memory deficits, which occur frequently in ADHD patients, a passive avoidance test was conducted in 7 weeks old offspring. In the learning trial, the number of electrical foot shocks required to hold in the lit compartment for 5 min was not significantly different between the control and MPH groups (Table 1). However, in the memory trial, the MPH group of male and female offspring combined was more likely to enter the dark compartment within 5 min than the control (Table 1, $p = 0.059$). When limited to male offspring, the MPH group shows a significant preference to enter the dark compartment (Table 1, $p = 0.017$).

Identification of DEGs in the striatum of offspring

As observed in ADHD patients, the MPH group showed decreased anxiety-like behavior and poorer memory function (Fig. 3B, Fig. 3D and Table 1). ADHD pathology is thought to involve the modulation of dopaminergic function (Tripp and Wickens, 2009). Accordingly, the striatum, which expresses high levels of dopamine receptors (DRD1, DRD2) (Beaulieu and Gainetdinov, 2011; Bouthenet *et al*., 1991), is one of the target regions for ADHD treatment. Indeed, MPH has been reported to be involved in dopamine release and reuptake in the striatum and to increase extracellular dopamine levels (Kimko *et al.*, 1999; Volkow *et al*., 2001). Therefore, transcriptome analysis was conducted to investigate changes of gene expression in the striatum of offspring by RNA-seq, a comprehensive analysis method. The results showed that the expression of 922 genes was altered, of which 564 genes were up-regulated and 358 genes were down-regulated (Fig. 4A). A clustering heat map of DEGs generated by Heatmapper is also shown in Fig. 4B. Enrichment analysis with Metascape showed significant enrichment of pathways and gene ontologies (GOs) related to neuronal function, such as "neuronal system" and "chemical synaptic transmission" (Fig. 4C). Each cluster is further subdivided into nodes, which form a network between related nodes (Fig. 4D). The node with the lowest p-value among all the nodes was the "neuronal system", which was composed of the genes shown in Table 2. Among the component genes, FC values for all genes associated with exocytosis were elevated (Table S3).

Changes in gene expression in the striatum and the frontal cortex of offspring

Functional brain studies of ADHD patients have shown abnormalities in the frontal, basal ganglia, and cerebellar

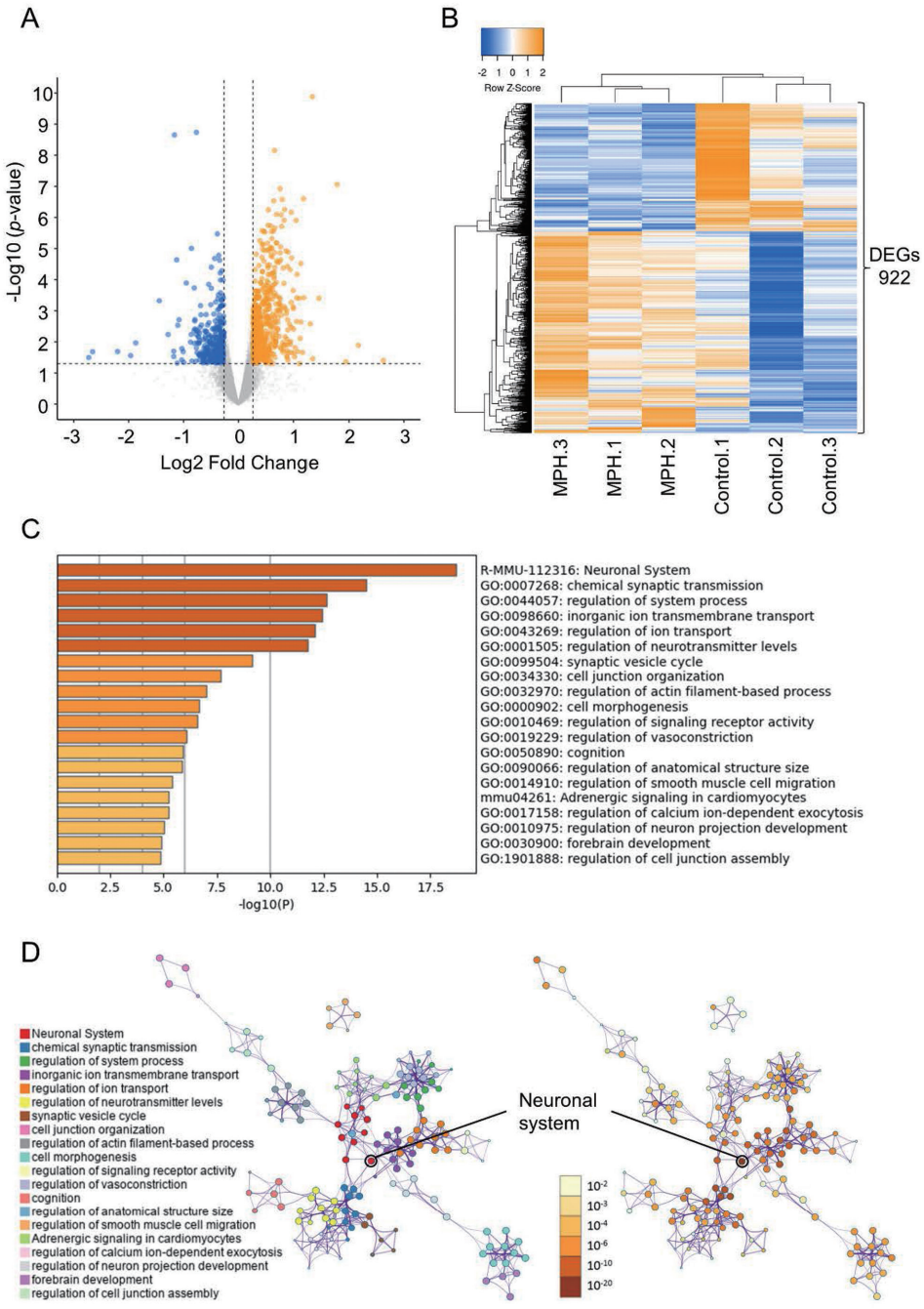

Fig. 4. RNA-seq of total RNA collected from the striatum and subsequent enrichment analysis. Total RNA collected from the striatum of offspring at 6 weeks of age was subjected to RNA-seq to identify DEGs (control group, n = 3 (M1/F2) and MPH group, n = 3 (M1/F2)). DEGs were subjected to enrichment analysis using Metascape. (A) Volcano plot of 13122 genes with average read counts ≥ 100 for either the control or MPH group. Genes that pass a threshold of fold change ≧ 1.2, p-value < 0.05 are highlighted by orange (up-regulated genes) and blue (down-regulated genes), containing 564 and 358 DEGs, respectively. (B) Clustering heat map of 922 DEGs. The vertical and horizontal axes represent the samples and the DEGs, respectively. (C) The top 20 enriched ontology clusters were created by enrichment analysis using Metascape and color-coded by p-value. The horizontal axis represents the $-\log10(p)$. Names of gene ontology and Reactome terms are listed on the right panel. (D) A network of the top 20 ontology clusters, created by Metascape and color-coded by cluster ID (left) and a network of ontology clusters color-coded by p-values (right). (M, male; F, female).

Table 2. List of genes constituting the "neuronal system", the node with the lowest *p*-value.

Source	Term ID	Term name	Log10P	Number of genes	Gene symbol
Reactome Gene Sets	R-MMU-112316	Neuronal System	−18.698	52	*Actn2, Adcy1, Cacnb3, Camk2d, Camk2g, Chrna4, Dlgap1, Flot1, Gabbr2, Gabra1, Gabra3, Gabrg2, Gng11, Gng2, Gng3, Grik4, Grin2a, Grin3a, Hcn1, Homer2, Il1rapl2, Kcna5, Kcnb2, Kcnc2, Kcnc4, Kcnf1, Kcng1, Kcnh4, Kcnh7, Kcnj3, Kcnj6, Kcnj9, Kcnk4, Kcnk9, Kcnma1, Kcnn3, Kcnv1, Lin7a, Lrfn1, Ncald, Nrxn1, Prkar1b, Prkcg, Rims1, Shank1, Slc17a7, Slitrk1, Snap25, Stx1a, Syn1, Syn2, Syt1.*

Gene list for "neuronal system", the nodes with the lowest *p*-values in all clusters are shown.

circuits (Durston *et al*., 2011). Among these circuits, the striatum-frontal circuit is considered to be more closely related to impulsivity (Bonelli and Cummings, 2007; Cho *et al*., 2013). Therefore, gene expression levels were quantified in the striatum and the frontal cortex by RT-qPCR. We focused on genes related to exocytosis that have been reported to be involved in ADHD (*Snap25*, *Stx1a*, *Syt1*, *Nrxn1*) based on the results of enrichment analysis (Table 2), and candidate genes that have been reported to be involved in the pathogenesis of ADHD (*Drd1, Drd2, Slc6a3, Maoa, Maob, Comt, Th*) (Li *et al.*, 2014; Yadav *et al.*, 2021). In terms of genes related to exocytosis, *Snap25* and *Syt1* were significantly upregulated in the striatum of the MPH group compared to the control group, while no significant differences were observed in the frontal cortex (Fig. 5). No significant differences in *Stx1*a and *Nrxn1* gene expressions were detected in either the striatum or the frontal cortex (Fig. 5). Regarding candidate genes thought to be associated with ADHD pathogenesis, *Drd2* expression was significantly decreased in the striatum in the MPH group compared to the control group, while no significant differences were detected in the frontal cortex (Fig. 5). Moreover, it was observed a significantly increased expression of *Maoa* and a decreased expression of *Comt* in both the striatum and the frontal cortex of the MPH group (Fig. 5). No significant differences in *Slc6a3*, *Maob*, and *Th* gene expressions were detected between MPH and control groups in either the striatum or the frontal cortex (Fig. 5). Data from the sex-separated analysis are shown in Supplementary Figs. 3 and 4. The data showed a similar trend as the combined male and female data.

DISCUSSION

This study sought to determine whether paternal MPH exposure alters the behavior of offspring and gene expression in the brain using a mouse model. To this end, we evaluated offspring growth, conducted behavioral tests, and analyzed gene expression levels comprehensively and quantitatively.

It has been reported that paternal exposure to MPH in fish has no effect on the number of offspring born and mortality (De Serrano *et al.*, 2021). In line with this, we have found in this study that paternal exposure to MPH had no effect on the number of births and survival rates of the offspring in mice. The weight of the MPH group was similar to that of the control group at P4, suggesting that paternal exposure to MPH did not affect fetus growth. Although the weight of the MPH group was significantly lower during the early lactation period in P7–14, after day 21 their weights were similar to the control group, suggesting that exposure of the sire to MPH had no significant effect on offspring growth.

Hyperactivity and impulsivity are typically observed symptoms in animal models of ADHD (Regan *et al*., 2022). We have previously shown that MPH exposure to pregnant mice produces ADHD-like hyperactivity and impulsivity in the offspring (Aoki *et al.*, 2021). However, the present study demonstrated that exposure of the sire to MPH did not affect spontaneous locomotor activity in the offspring. Conversely, in the elevated plus-maze test, the number of entries and distance traveled in open arms were parameters significantly increased in the MPH group. Although the elevated plus-maze test was initially developed to evaluate the effects of anxiolytic drugs in rats, it was subsequently applied to evaluate pathological behavior in mice (Lister, 1987; Pellow *et al.*, 1985). Indeed, it has since been used in the evaluation of impulsivity in ADHD models (Ueno *et al.*, 2002). Therefore, increases in the number of entries and distance traveled in open arms are interpreted as correlates of impulsivity. Therefore, it is suggested that sire exposure to MPH increases impulsivity in the offspring. ATX has been reported to suppress impulsivity in ADHD model mice (Pillidge *et al.*, 2014). In this study, although ATX administration decreased the number of entries into the open arms in both the control and MPH groups, the difference

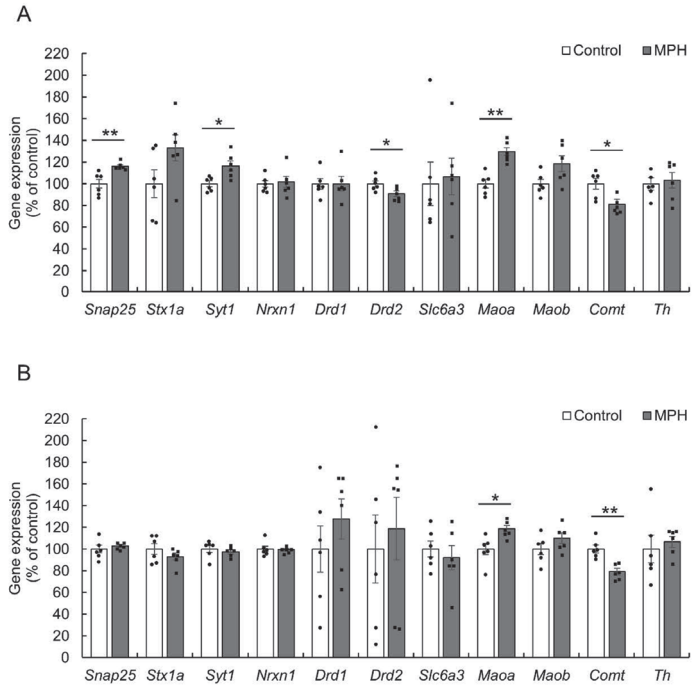

Fig. 5. Gene expression of ADHD-related genes in the striatum and the frontal cortex of offspring from MPH-administered sire. Brain samples from offspring were obtained at 6 weeks of age. (A) shows results for the striatum and (B) for the frontal cortex. Expressions of genes involved in exocytosis and reportedly associated with ADHD (*Snap25, Stx1a, Syt1, Nrxn1*) and candidate genes for ADHD pathogenesis (*Drd1, Drd2, Slc6a3, Maoa, Maob, Comt, Th*) were determined by RT-qPCR. Data from control and MPH groups are shown as mean ± S.E.M. and were compared using Student's *t*-test (*$p < 0.05$ and **$p < 0.01$, control group, n = 6 (M3/F3) and MPH group, n = 6 (M3/F3)). (M, male; F, female)

between before and after ATX administration in the number of entries into the open arms was significantly larger in the MPH group. A report evaluates impulsivity in the elevated plus-maze test by the number of entries into the open arms as a danger zone (Ueno *et al.*, 2002). These results indicate that impulsivity in the MPH group is suppressed by ATX, suggesting it is an ADHD-like symptom.

In addition to hyperactivity and impulsivity, impaired learning and memory functions are also common phenotypes in ADHD (Biederman *et al.*, 1991; Coghill *et al.*, 2014). Impairments in working memory and long-term memory have been reported in ADHD patients (Kofler *et al*., 2018; Rhodes *et al.*, 2012; Skodzik *et al.*, 2017). It has also been reported that working memory is impaired in spontaneously hypertensive rats, a rodent model of ADHD (Kantak *et al.*, 2008; Regan *et al*., 2022), and that spatial learning and memory are impaired in mice deficient in dopamine transporter and latrophilin 3 (Mortimer *et al.*, 2019; Regan *et al*., 2022). In the learning trials assessing short-term memory, the number of electrical foot shocks in the MPH group was not significantly different from that of the control group; in the memory trial to assess long-term memory, male offspring of the MPH group enter the dark compartment more frequently than the control group. These findings suggest that paternal MPH exposure, while not affecting the offspring's short-term memory, may impair their long-term memory. It is interesting to note that there were no sex differences in the increase in impulsivity seen in the MPH group. Moreover, and interestingly, the effects on long-term memory appeared to be male-specific. There are gender differences in the frequency of ADHD, with boys more frequently developing it than girls (Renoux *et al.*, 2016). Meanwhile, sex differences in the F1 phenotype are occasionally observed in POHaD studies using experimental animals. Whether the sex difference in impaired long-term memory observed in this study is due to paternal genetic transmission of the trial will require further analysis, including on the influence of imprinting genes.

RNA-seq and subsequent Metascape analysis of striatal samples of the 6 weeks old offspring revealed significant enrichment terms related to the nervous system and

NTs in the MPH group. This suggests that paternal MPH exposure may affect the neurological function of the next generation. Fusion, secretion, retrieval, and recycling of synaptic vesicles are essential for neurotransmission, and mutations in genes involved in these processes are known to be associated with neurodevelopmental disorders, including ADHD, autism, intellectual disability, and epilepsy (Bonnycastle *et al*., 2021; Li *et al.*, 2014). In this study, all genes associated with exocytosis were upregulated. Therefore, we performed RT-qPCR on *Snap25*, *Stx1a*, *Syt1*, and *Nrxn1*, genes associated with exocytosis and suggested to be related to ADHD (Cupertino *et al.*, 2017; Liu *et al*., 2017; Wang *et al*., 2019; Zhang *et al.*, 2021). Results showed that *Snap25* and *Syt1* expression levels were significantly increased and *Stx1a* expression levels tended to increase in the striatum of the MPH group. SNAP25 and STX1A proteins bind to each other to form the SNARE complex at the plasma membrane, which is involved in the fusion of synaptic vesicles (Südhof, 2013). SYT1 responds to calcium influx and binds to SNARE proteins, causing NT release (Südhof, 2013). It has been reported that the addition of BDNF to cultured cortical neurons increases the regulated release of NTs such as glutamate and GABA through increased levels of exocytosis-associated proteins including SNAP25, STX1A, and SYT1 (Takei *et al*., 1997). In this study, there was also an increase in the expression level of genes related to the secretion of synaptic vesicles in the striatum of the MPH group, suggesting that increased exocytosis may have caused an increase in NTs in the synaptic cleft.

In the MPH group, there were significant changes in the expression of *Drd2*, *Maoa*, and *Comt*, candidates of disease-responsible genes for ADHD. It has been reported that high sucrose intake by pregnant mice caused ADHD-like symptoms including increased impulsivity in the offspring, and that expression of *Drd2*, one of the receptors for dopamine that inhibits adenylyl cyclase by coupling to the Gi family protein, was reduced in the striatum (Choi *et al.*, 2015). Similarly, we have also reported that exposure of pregnant mice to MPH produces ADHD-like symptoms, including increased impulsivity in the offspring, and decreased expression of *Drd2* in the striatum (Aoki *et al.*, 2021). Since ADHD-like impulsivity was also observed in the present study and *Drd2* expression was simultaneously reduced, it is plausible that reduced expression of this gene is involved in ADHD-like impulsivity in experimental animals. Additionally, the expression of *Maoa*, an enzyme that catalyzes oxidative deamination and catabolizes monoamines, was increased in the MPH group, and the expression of *Comt*, an enzyme that degrades catecholamines by transferring methyl groups to 3,4-dihydroxybenzene groups (catechol), was decreased in the MPH group in both the striatum and the frontal cortex. *Maoa* knock-out mice have been reported to exhibit reduced anxiety-like behavior and aggressive phenotype (Cases *et al*., 1995). Moreover, a polymorphism of COMT with low activity (Val158Met) has been associated with several psychiatric disorders, including ADHD (Pálmason *et al*., 2010), and increased anxiety-like behavior and learning ability have been reported in *Comt* knock-out mice (Gogos *et al.*, 1998; Babovic *et al.*, 2008). Although some of these reports are inconsistent with the results of this study, it is possible that fluctuations in *Maoa* and *Comt* expressions affect the metabolic balance of those substrates, dopamine and norepinephrine. It has been reported that cognitive and memory functions are influenced by dopamine levels in the brain in an inverted U-shape manner (Cools *et al*., 2019). In other words, too much or too little dopamine in the synaptic cleft may impair the performance, leading to behavioral disturbances. The behavioral phenotype may be due in part to an imbalance of NTs in the synaptic cleft caused by increased exocytosis and altered expression of monoamine-metabolizing enzymes. In this regard, future studies should examine the extracellular concentration of dopamine and other monoamines in the brain.

Whether the altered expression of these genes is the cause of the abnormal ADHD-like behavior observed in this study or a biological response based on other potential causes is currently unclear and requires further analysis. A recent study reported that MPH administration to prepubertal male rats causes extensive sperm DNA damage, which was reflected in poor embryo quality (da Costa Nunes Gomes *et al*., 2022). Other pathways that affect the next generation when drugs are administered to males would be a direct effect on the ovum through semen transfer of the treatment, effects on the sperm epigenome, or both. Detailed analysis of the effects of MPH on semen transfer rates and ovum, as well as changes in DNA methylation profiles, histone modifications, and ncRNAs in the sperm genome due to MPH administration, is necessary to elucidate on the causative factors that contribute to changes in gene expression.

Altogether, we show that paternal MPH exposure induces functional deficits in the offspring in mice, including increased ADHD-like impulsivity and impaired long-term memory function, and that it alters brain expression of several genes known to be associated with ADHD. These results suggest that continued use of MPH in men during their reproductive period could impair the behavioral and memory functions in the next generation, and more cau-

tion should be paid when administering it to patients who wish to have children.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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