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Plasma concentration of dopamine varies depending on breed, sex, and the genotype of *DRD4* in horses

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Abstract

Dopamine (DA) is known to be a key modulator of animal behaviors. Thus, the plasma concentration of DA might be used as a biomarker for the behavioral characteristics of horses. The behavioral characteristics of horses vary depending on the breed, age, and sex. Moreover, the DA receptor genotypes are also related to horse behaviors. Thus, the aim of this study was to investigate the DA concentration variations of horse plasma by breed, age, sex, or genotype of its receptor. The horses were divided by breed into Thoroughbred (n = 13), Pony (n = 9), Warmblood (n = 4), and Haflinger (n = 5). The age variable was divided into three different groups: post-pubertal (2–5 years, n = 6), adult (6–13 years, n = 19), and aged horses (15–24 years, n = 6). The sex variable was divided into geldings (n = 8) and mares (n = 23). Approximately 10 mL of blood was collected, and an ELISA kit was used to measure the plasma concentration of DA. Polymerase chain reaction analysis was performed to identify the genetic variation in the DA D4 receptor gene (*DRD4*). SPSS statistical software was used for statistical analysis. The DA concentrations in geldings were significantly lower than those in mares. There was no significant difference in DA concentrations among breed and age groups. Horses with the GG and GA genotypes had significantly higher plasma concentrations of DA compared to horses with the AA genotype for the G292A gene. Briefly, the plasma concentration of DA varied depending on the sex and genotype of G292A. These factors should be considered when the concentration of DA is used as a biomarker for the behavioral characteristics of horses. In conclusion, the DA concentration or *DRD4* genotype of horse plasma has the potential to be used as a biomarker that can predict the behavioral characteristics of horses.

Keywords: Horse, Dopamine receptor, Behavior, Biomarker

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Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Yoon M.
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The Animal Experiment Ethics Committee of Kyungpook National University approved the protocol for animal use (2020-0131).

INTRODUCTION

Running a safe riding and breeding program requires knowledge of the behavioral characteristics of horses. Behavioral characteristics of animals are known to vary depending on the breed, age, and sex [1,2]. In addition, the genotypes of animals affect the behavioral characteristics of individual animals [3]. The behavioral characteristics of each animal can also be determined by the individual's physical, hormonal, and nervous systems [4]. Neurotransmitters, which are chemical messengers that transfer specific signals from a neuron through the synapse into the targeted cells, play a pivotal role in the operation of complex neural networks [5]. It was previously reported that neurotransmitters are related to the expression or inhibition of social behaviors, such as aggressive, sexual, and maternal behaviors [6].

Dopamine (DA) is one of the neurotransmitters known to have an important role in the nervous networks [7]. Primarily, it plays a functional role in regulating reward and pleasure centers in the brain [8]. According to the previous research results on humans and animals, when aversive events occur, the DA reward system induces stress reduction through appropriate DA secretion [9–11]. Furthermore, DA also plays an important role in eliciting motor function in animals, which is why DA-based drugs are widely used to reduce Parkinson's disease in humans [12]. In rats, maternal behaviors, such as licking and grooming, increased in the group with a higher DA-detected signal than in the lower signal group, highlighting the importance of DA activity in the maternal behavior of rats [13]. Based on the results of these studies, we hypothesized that DA is one of the biomarkers determining the behavioral characteristics of horses.

Interestingly, variations have been observed in the concentration of DA among dog breeds, suggesting a causal relationship between DA level and behavioral characteristics [14]. In monkeys, the concentration of DA differed by age group [15]. In several animals, it has been identified that the DA concentration in the brain is controlled by sex hormones [16], suggesting that there is a difference in DA concentration by sex of animals. A previous study reported that the allele frequency of the DA receptor D4 gene (*DRD4*) was associated with the variation of DA concentrations among dog breeds [17]. Thus, the objectives of this study were to evaluate (1) the effect of breed, age, or sex on the plasma concentrations of DA in horses and (2) the correlation between genotypes by the single nucleotide polymorphisms (SNP) in *DRD4* and the concentration of DA in horse plasma.

MATERIALS AND METHODS

Animals

This study was performed at the Horse Industry Complex Center of Jeonju Kijeon College in the Korea. The Animal Experiment Ethics Committee of Kyungpook National University approved the protocol for animal use (2020-0131). A total of 31 horses, divided into groups based on breed, age, and sex, were used in this study. There were four breed groups, including Haflinger (n = 5), Pony (n = 9), Thoroughbred (n = 13), and Warmblood (n = 4) and three age groups: post-pubertal (2–5 years; n = 6), adult (6–13 years; n = 19), and aged (15–24 years, n = 6). Sex was divided into geldings (n = 8) and mares (n = 23). The horses were individually stabled (3.5 × 3.5 m cages) and fed 1.5% of their body weight (BW) of Timothy hay (dry matter basis) with 0.5% BW of commercial feed per day. Additionally, the horses had *ad libitum* access to water throughout the experimental period. The horses were used for horseback riding and therapeutic riding and participated in competitions such as show jumping and dressage.

Blood collection

The period of blood collection was in May, the reproduction stage of horses. For the analysis of the plasma concentration DA, approximately 10 mL of blood was collected from the jugular vein of the horses using an 18-gauge needle connected to an EDTA tube (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA). Samples were collected between 09:00 and 10:00 h to avoid the riding and meal periods of the horses. After collection, the tubes containing blood were immediately stored in a 4℃ icebox. The residual blood samples were centrifuged at 1,500´g for 10 min at room temperature (℃) to separate the plasma and preserved at −20℃ until further analysis.

ELISA analyses

The frozen plasma samples were thawed in a 38℃ prewarmed water bath and resuspended using a 1000 μl capacity micropipette for sufficient mixing within the tube. The DA concentration of the plasma samples was analyzed using an ELISA kit (HREB0039, ELISA Genie, Dublin, Ireland). The sensitivity of the DA ELISA kit was 0.23 ng/mL. The loaded plasma samples were detected at 450 nm absorbance on a Sunrise absorbance microplate reader (Tecan, Männedorf, Switzerland). The inter-assay and intra-assay coefficients of variation for the ELISA of DA were 20.86% and 9.04%, respectively.

Genotyping

Blood samples were isolated to allow DNA extraction using a DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany), and 504 base pairs of the exon 3 region in the equine *DRD4* gene (GenBank) were amplified by polymerase chain reaction (PCR) analysis. The PCR analysis was performed as previously described [18], with minor modifications. Briefly, the extracted DNA from the blood samples was diluted to a total concentration of 100 ng/μL. The *DRD4* forward (5′-CCGCTCATGCTGCTGCTCTACTGG-3′) and reverse (5′-TGCG CTCCCGGCCGGTGATCTT-3′) primers (Cosmogenetech, Seoul, Korea) were diluted to a final concentration of 10 pmol with dH2O, respectively. The PCR was performed using a G:BOX Chemi XRQ (Syngene, Bangalore, India) with the following cycling conditions of six steps: initial denaturation at 94℃ for 5 min, denaturation at 94℃ for 1 min; annealing at 65℃ for 15 s, extension at 72℃ for 1 min, repetition of 35 cycles from annealing to extension at 72℃ for 5 min; and final extension at 72℃ for 10 min. The samples were electrophoresed on a 2% agarose gel at 100 V for 10 min. The expression bands of DA in each DNA sample were observed and captured with a Mastercycler X50s (Eppendorf, Enfield, CT, USA).

Statistical analyses

The DA concentration in each plasma sample was statistically analyzed using IBM SPSS Version 25 statistical software (IBM, Armonk, NY, USA). Differences in DA concentration by breed and age were analyzed using one-way ANOVA with least significant difference (LSD) post-hoc analysis. Differences in the DA concentration by sex were compared using Student's *t*-tests. Furthermore, ANCOVA analysis was conducted to identify the interaction between breed, age, and sex. The interquartile range (IQR) value was two, and the DA concentration data that exceeded two terms of the first and third quartiles of the raw data were determined as outliers. The allele frequencies according to each genotype were derived from an instrumented distribution and were used to calculate the percentage of the expected genotypes. The frequencies of genotypes and alleles were counted directly. The frequencies of observed and expected genotypes were compared by the χ^2 test using CERVUS 3.0.3 [19]. All the data were represented by mean ± SEM and the p-values < 0.05 were considered statistically significant.

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RESULTS

Plasma DA concentration by horse breed, age, and sex

The mean concentrations of DA in plasma by breed ranged from 7.3 ± 2.10 to 16.4 ± 3.65 pg/mL. There was no significant difference among the breed groups (Table 1). The mean concentrations of DA in plasma by age ranged from 7.3 ± 2.38 to 10.9 ± 2.31 pg/mL. There was no significant difference among the three age groups (Table 2). The mean concentrations of DA in geldings were significantly lower than that in mares (4.8 ± 0.71 vs. 10.5 ± 1.31 pg/mL, $p < 0.05$; Table 3). The significant difference according to sex was valid even when the covariance factors such as breed or age were removed.

Genetic analysis to identify genotype-related variations in DA concentration

The SNPs (G292A and C147T) were used as genetic markers to assess the correlation between genotypes and DA concentration. For the G292A SNP, genotypes GG, GA, and AA were detected with frequencies of 25.80%, 64.52%, and 9.68%, and the G and A allele frequencies were 58.06% and 41.94%, respectively (Table 4). For the C147T SNP, the frequencies of the CC, CT, and TT genotypes were 90.32%, 9.68%, and 0%, and the gene frequencies of the C and T alleles were 95.16% and 4.84%, respectively (Table 5). The DA concentrations (ng/mL) by genotypes GG, GA, and AA for the G292A SNP were 8.1 ± 1.65, 10.1 ± 1.50, and 4.8 ± 0.91, respectively, and 9.1 ± 1.19 and 8.5 ± 1.32 by genotypes CC and CT, respectively. Animals with the GG and GA genotypes had significantly higher DA concentrations compared to the AA genotype. For the C147T SNP, there was no significance between the DA concentrations of CC and CT genotypes (Table 6).

Table 1. Mean concentrations ± SEM of dopamine by horse breed

	Breed and number of horses			
	Thoroughbred (n = 13)	Pony (n = 9)	Warmblood (n = 4)	Haflinger (n = 5)
Dopamine (pg/mL)	8.3 ± 1.65	7.9 ± 1.51	16.4 ± 3.65	7.3 ± 2.10

Table 2. Mean concentration ± SEM of dopamine by horse age

	Age and number of horses		
	2 to 5 years (n = 6)	6 to 13 years (n = 19)	15 to 24 years (n = 6)
Dopamine (pg/mL)	7.3 ± 2.38	9.0 ± 1.45	10.9 ± 2.31

Table 3. Mean concentration ± SEM of the dopamine by horse sex

	Sex and number of horses	
	Geldings (n = 8)	Mares (n = 23)
Dopamine (pg/mL)	4.8 ± 0.71[a]	10.5 ± 1.31[b]

All data are presented as mean ± SE.

[a,b]Means with different superscript letters are significantly different ($p < 0.05$).

Table 4. Chi-square and Hardy-Weinberg equilibrium testing of SNP-type distributions for SNPs of G292A

SNPs	No.	Genotype frequency			Allele frequency		x^2 [1]	Diversity parameter		
		GG	GA	AA	G	A		Ho	He	PIC
G292A	31	0.258	0.645	0.097	0.581	0.419	$2.076^{0.150}$	0.645	0.495	0.368

[1]Degrees of freedom: 1.

SNP, single nucleotide polymorphism; Ho, observed heterozygosity; He, expected heterozygosity; PI, polymorphic information content.

Table 5. Chi-squared and Hardy-Weinberg equilibrium testing of SNP-type distributions for SNPs of the C147T

SNPs	No.	Genotype frequency			Allele frequency		x^2 [1]	Diversity parameter		
		CC	CT	TT	C	T		Ho	He	PIC
C147T	31	0.903	0.097	0	0.952	0.048	ND	0.097	0.094	0.088

[1]Degrees of freedom: 1.

SNP, single nucleotide polymorphism; ND, not done; Ho, observed heterozygosity; He, expected heterozygosity; PI, polymorphic information content.

Table 6. Genotypes of SNPs-related variation of dopamine concentration in DRD4

Genotype	SNP				
	G292A			C147T	
	GG (n = 8)	GA (n = 20)	AA (n = 3)	CC (n = 28)	CT (n = 3)
Dopamine (ng/mL)	8.1 ± 1.65^{b}	10.1 ± 1.50^{b}	4.8 ± 0.91^{a}	9.1 ± 1.19	8.5 ± 1.32

[a,b]Means with different superscript letters are significantly different ($p < 0.05$).

SNP, single nucleotide polymorphism; DRD4, dopamine receptor D4 gene.

DISCUSSION

The purpose of this study was to find out the internal factors affecting the plasma concentration of DA in horses. In our study, the plasma concentration of DA was varied depending on the sex and genotype of G292A in horses. First, we found that the sex of horses was one factor causing the variation of the plasma concentration of DA; geldings had a lower plasma concentration of DA compared with mares. This suggests that the dopaminergic pathway in horses is regulated by reproductive hormones, such as testosterone. This speculation is supported by a previous study showing that DA concentration in the cerebrospinal fluid (CSF) increased as the colts matured to stallions [20].

Melrose et al. [20] demonstrated that prepubertal mares had a significantly lower DA concentration in CSF compared to adult mares, showing that maturity contributed to determining the concentration of DA in horses. However, it is not known whether the DA concentration can increase post-puberty. In the present study, there was no significant difference in plasma concentration of DA among all the age groups, including post-pubertal (2–5 years; n = 6), adult (6–13 years; n = 19), and the aged group (15–24 years, n = 6). The reason for the lower concentration of DA in pre-pubertal mares compared to adult mares may be the very low levels of reproductive hormones before puberty. Based on these results, it is proposed that the age of horses after post-puberty may not be related to the plasma concentration of DA in the horses.

In this study, we aimed to identify the difference in plasma concentration of DA according to the horse breeds, but there was no significant difference. However, we verified that although there was no significant difference in the plasma concentration of DA by breeds ($p = 0.064$), post-analysis using LSD showed that Warmblood had significantly higher DA levels than the other three breeds. Unlike other breeds, Warmbloods have been bred as show horses for a long time [21]. Especially,

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the Warmbloods used in this study had participated in showjumping and dressage events in which the horses need to cooperate with trainers and riders. A previous study suggested that DA and serotonin amplify cooperation through social compliance and empathy [22]. According to our previous study, other neurotransmitters, oxytocin, and serotonin were significantly higher in Warmblood compared to Pony [23]. Stelly et al. [24] demonstrated enhanced DA levels in male rats exposed to acute and short-term stress. It suggested that animals exposed to acute or short-term stress selectively promoted reward-related DA release, causing changes in neural activity. In the present study, Warmblood horses had been selected and bred to conform and cooperate with humans to prepare for events. Thus, Warmbloods were exposed to frequent short-term stress during performance training and competition, causing a higher DA concentration compared to other breeds. However, the significant difference between the breeds may be due to the effect of sex, as there is a limitation that all four Warmbloods used in this study were mares. Therefore, it is considered that it can be possible to validate the difference in plasma concentration of DA between the Warmblood and other breeds through follow-up studies by securing additional samples.

In this study, we also attempted to identify whether the plasma DA concentration in horses differs depending on the *DRD4* genotype, which is known to be correlated with the behavioral characteristics of horses. The ratio of each genotype or allele frequency for *DRD4* was observed in two different SNPs, G292A and C147T. When the concentration of DA was classified according to the G292A genotype, animals with the GG and GA genotypes had significantly higher DA concentrations than those with the AA genotype. These findings suggest that GG and GA genotypes of G292A are associated with a high plasma concentration of DA in horses. Interestingly, all three horses with AA genotypes were identified as Thoroughbreds. This result is consistent with a previous report in which the highest frequency of the A allele gene of G292A was observed in Thoroughbred horses [25]. This may be because of the unique breeding history of Thoroughbred horses for racing performance. Another previous study also suggested that the A allele of G292A was associated with low curiosity and high vigilance in Thoroughbred horses [26]. These results suggest possible correlations among the plasma concentration of DA, the genotype of *DRD4*, and the behavioral traits of horses. Further study is warranted to clarify the *DRD4* polymorphism and behavioral characteristics of horses in association with the plasma concentration of DA in horses.

In this study, there was no T allele gene in the genotype classification according to C147T, which made it impossible to compare DA concentration according to the C147T genotype. Therefore, further research is needed to confirm the difference in DA concentration according to the C147T genotype of *DRD4* in horses. To conclude, we observed differences in the plasma concentration of DA in horses according to sex and the *DRD4* genotype.

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