Journal of Animal and Feed Sciences, 21, 2012, 468–476

The association of brain derived neurotrophic factor gene variants with behaviour traits in sika deer (*Cervus nippon*)*

S.-J. Lv[1,3,4], Y. Yan[2] and W.-H. Wei[3]

[1] Linyi University, College of Life Sciences
Linyi, 276005, Shandong, P.R. China
[2] Shandong Linyi Agriculture Science Institute
Linyi, 276009, Shandong, P.R. China
[3] Yangzhou University, College of Bio-science & Bio-technology
Yangzhou, 225009, Jiangsu, P.R. China

(Received 16 December 2011; revised version 21 May 2012; accepted 14 September 2012)

ABSTRACT

It is widely accepted that brain-derived neurotrophic factor (BDNF) is involved in modulating behaviour performance induced by environmental conditions. The aim of this study was to study polymorphisms of the BDNF gene and their relationship with animal behaviour in sika deer (*Cervus nippon*). Forty-eight sika deer reared at the Ping-Shan-Tang Farm (25 deer) and Zhu-Yu-Wan Park (23 deer), Yangzhou City, Jiangsu Province (China) were observed and blood samples were taken to identify BDNF genotypes. Data were subjected to ANOVA analysis to evaluate the link between genotype and animal behaviour traits. After PCR and electrophoresis, polymorphisms were found in two pairs of primers. At primer P-4, the AA genotype (26 deer) rested significantly less than the BB genotype (16 deer) ($P<0.05$). The AA genotype deer also exhibited significantly more locomotor behaviour ($P=0.001$). At primer P-5, deer of genotypes CC/DD/CD differed significantly in their watching behaviour. Deer of genotype CC exhibited significantly less resting and self-grooming behaviour than deer of genotypes CD or DD (both $P<0.05$). Our findings suggest that polymorphisms in BDNF may be involved in some aspects of animal behaviour traits, especially in the highly sensitive sika deer reared for several years in Chinese parks.

KEY WORDS: sika deer, BDNF polymorphisms, PCR-SSCP, animal behaviour

---

* Supported by the National Natural Science Foundation of China, Grants No. 31001027, 31272480

[4] Corresponding author: e-mail: lvshenjin_yang@sina.com

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family. It is abundantly expressed in the mammalian hippocampus and involved in a crucial role in various higher cognitive processes, including decision making, learning, and memory performance induced by environmental conditions (Gasic et al., 2009; Kang et al., 2010). Environmental factors such as physical exercise, dietary restriction, and housing conditions affect BDNF levels (Adlard et al., 2004). BDNF plays a critical role in behaviour, and its dependent mechanisms have been shown to be involved in other aspects of behaviour (Yee et al., 2007; Zhu et al., 2009).

Several genetic studies have suggested that the Met variant of the BDNF Val 66 Met polymorphism is associated with poor performance on memory tests (Gong et al., 2009). It has been reported that the Val 66 Met polymorphism has been associated with a phenotype of increased anxiety-related behaviours in stressful settings in animal studies (Hashimoto, 2007) and higher levels of the anxiety/anxious temperament trait (Lang et al., 2005) in humans. The BDNF gene has been reported to be associated with various neuropsychiatric conditions such as obsessive-compulsive disorder, eating disorders, and substance dependence. Also, environmental factors such as physical exercise, dietary restriction, and housing conditions have been shown to affect BDNF levels (Lambert et al., 2005; Zhu et al., 2009). A wealth of data reveal behavioural differences both due to genetic modification and environmental manipulation (Zhu et al., 2009).

Sika deer are highly nervous animals and can be easily excited or frightened. Although captive sika have been kept in farms or parks for decades and may be more tame than wild conspecifics, it is known that deer in general, even when reared and enclosed in deer farms, may not become as tractable as other species (Lu et al., 2010). The captive environment and/or human presence may result in undesirable stress to sika deer, expressed potentially as abnormal or stereotypic behaviour (Carlstead and Shepherdson, 1994). During our observation, we found that the animals spend much time watching and in locomotion. It was interesting to check, therefore, whether there is an association between the animals' behaviour and single nucleotide polymorphisms (SNP) in the BDNF gene.

Although many findings have been reported in the literature on the BDNF gene, no studies on the correlation between behaviour traits and genetic mutations on sika deer have been performed. Our study investigated the relationship between SNPs of the BDNF gene and behaviour traits of sika deer in a semi-housed environment. The purpose of this study was to characterize the relationship between behaviour and BDNF polymorphism among sika deer. The results of our study may offer some information to improve the welfare status of sika deer in China.

MATERIAL AND METHODS

Subjects

This study was carried on the Ping-Shan-Tang farm (n=25) and Zhu-Yu-Wan Park (n=23), Yangzhou city, Jiangsu province (China). The Ping-Shan-Tang farm animals were housed in four 20 m×10 m paddocks with a mantle shelter. In Zhu-Yu-Wan Park, the animals roamed in a fenced paddock of grasses, shrubs and trees (120 m × 80 m). All tested animals were males aged about 5 years and were marked by ear-notches and collar-tags for easy identification. Grass and fodder (maize, bean, bran, mineral, vitamins, and salt) were supplied to the animals each day at 8.00 a.m. and 3.00 p.m.; water was always available.

Data and sample collection

The study was conducted in two phases: from June 21 to December 10, 2006, and again from February 21 to July 10, 2007, under all weather conditions. Focal-animal sampling was used to determine the period of time spent on each behaviour (Altman, 1974). Eight hours were spent collecting data per day, including weekdays and weekends. Four days were used to observe every week. To reduce inter-observer variability, after 15 days of training, three observers collected all animal behaviour data. The observations were conducted at the same times each day from 8.30 a.m. to 4.30 p.m. and subjects were sampled in a different order each day (using random numbers). Each individual was studied for a mean (± S.E.) of 33.3±0.09 h. A total of 50 weeks, 1600 h were spent observing all animals across the two phases. Observations were made outside the paddock from a position that provided a good vantage but did not disturb the subjects. All behaviours were recorded and categorized as either actions or states (Table 1) according to an ethogram devised from existing work in this area (Webster and Matthews, 2006).

Table 1. Ethogram of sika deer behaviour

Behaviour	Description
Foraging	Head down foraging on grass or fodder; standing, chewing and swallowing grass or fodder
Ruminating	Standing or resting, regurgitating, then chewing cud
Resting	Lying with eyes open or closed
Watching	Head up and observing environment, other deer, or human visitors
Locomotion	Walking, running, trotting or cantering
Self-grooming	Grooming, licking of body, nibbling or scratching of skin
Non-visible	Animal could not be seen, thus state could not be determined

After observations, blood samples (10 ml) were collected from the jugular vein of each sika deer using vacuum tubes with the anticoagulant ACD, and stored at -20°C. All sampling procedures were permitted by the government and were in accordance with animal welfare standards.

Genomic DNA preparation and primer sequences

Sika deer genomic DNA was extracted from whole blood using the traditional phenol/chloroform method and dissolved in sterile water at a concentration of 100 ng/µl and then kept at -20°C until use. Approximately 50-100 ng of genomic DNA were subjected to the polymerase chain reaction using specific primers synthesized by Shanghai Sangon Bio.Co (Shanghai, China) according to the BDNF gene sequence (GenBank, accessions no. FD698038, NM007540 and BP460083). Six pairs of primers were designed. The primer sequences, location, and size of the amplified fragments are shown in Table 2.

Table 2. Information of primers for Sika deer with BDNF gene

Locus	Primer sequences (5' to 3')	PCR size bp	Annealing temperature °C
P-1	GTTATTTCATACTTCGGTTGC GGGAGTTCCAATGCCTC	604	55.4
P-2	GTTATTTCATACTTCGGTTGC AATACGCTTTTTGCTATCCATGGTT	663	55.4
P-3	TGAAAGAAGCCAACCTCC GAACCGCCAGCCAATAC	638	55.7
P-4	GGTTATTTCATACTTCGGTTGC TCCGCGTCTTTATTGTTTT	249	54
P-5	CCAAGGTGGGTTCAAGAG TGCGGCATCCAGGTAA	224	53.5
P-6	TGGATGCCGCAAACAT GAACCGCCAGCCAATAC	341	55.6

DNA amplification and genotyping

PCR was carried out in a total volume of 20 µl, containing 0.20 µl (5 U/µl) of Taq DNA polymerase, 2.20 µl of 10×buffer (including Mg2+), 1.20 µl of 1 µM primers, 1.20 µl of 2.0 mM dNTPs, 1.40 µl of 50 ng/l genomic DNA and sterile water to bring the total volume to 20 µl. PCR conditions were as follows: denaturation at 94°C for 5 min followed by 33 cycles shared in denaturation 94°C for 45 s, annealing 54°C for 40 s, extension 72°C for 30 s, and final extension 72°C for 5 min, on a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). At completion, PCR products were stored at 4°C until electrophoresis.

.

SSCP analysis and DNA sequencing

One microliter of the PCR products was diluted with 5 μl of loading buffer (98% formamide, 10 Mm EDTA pH 8.0, 0.025% xylene cyanol FF, 0.025% bromophenol blue, 2% glycerol). After denaturing at 98°C for 10 min, the mixture was immediately placed on ice for 10 min before loading on a 15% acrylamide/ bisacrykamide (acr:bis = 29:1) gel. After running at 5 v/cm for 16-20 h, the gel was stained using the silver staining method. For each homozygote, three PCR products were purified, recovered and sequenced by the ABI377 sequencer.

Statistical analysis

The behaviour traits data were analysed by one-way ANOVA using the SPSS 14.0 software package. The effects of SNPs on behaviour traits were analysed and the SNPs markers with a significant correlation with behaviour traits of sika deer were further studied through post hoc multiple comparison (the Duncan method).

A fixed model was adopted according to the factors that affect behaviour traits by using the following model:

$$yij=u+Ii+eij$$

where: *yij* - the observed value of ith individual animal, *u* - the means of values, *Ii* - the effective value of genetype I, *eij* - the random error term.

.

RESULTS

PCR amplification and SSCP analysis were carried out for the sika deer BDNF gene using six pairs of primers. The results showed that two pairs of primers (P-4 and P-5) resulted in polymorphisms. The primer pair P-4 yielded a 249 bp fragment and the P-5 yielded 224 bp. Three genotypes were identified by SSCP in both primer pairs. With the primer P-4, a C158A mutation, a synonymous mutation, was found. The homozygote with C at 158 nt was defined as the AA genotype, while that with an A at 158 nt was defined as the BB genotype. In the PCR fragments of primer pair P-5, one mutation, G138C, was found. This mutation also was a synonymous mutation. The homozygote with G at 138 nt was defined as the CC genotype, while that with C at 138 nt was defined as the DD genotype.

Allele and genotype frequencies

Among the 48 sika deer, allele A was predominant in the primer P-4 locus. The frequency of genotype AB was low in the samples (Table 3). For the primer P-5

locus, allele D was predominant in all sika deer. Genotype DD was predominant. The test results showed that the population was in Hardy-Weinberg disequilibrium for both primers.

Table 3. Gene and genotype frequency and equilibrium test of Hardy-Weinberg for BDNF gene

Primer P-4	Detect	Gene		Type of gene			χ^2 value
		A	B	AA	BB	AB	
	No			26	16	6	
	Observed value	0.604	0.396	0.542	0.333	0.125	12.50
P-5		C	D	CC	DD	CD	
	No			9	32	7	
	Observed value	0.260	0.740	0.188	0.667	0.145	24.13

note: df = 2, $\chi^2_{0.05(2)} = 5.99$, $\chi^2_{0.01(2)} = 9.21$

Association of SNPs with behaviour traits

Correlations between different genotypes and behaviour traits were analysed by the ANOVA procedure using SPSS 14.0 (Table 4). The results indicated that the resting behaviour in deer of the AA genotype was significantly lower than that of the BB genotype (P=0.046). The locomotion behaviour in deer of genotype AA was significantly higher than that in deer of genotype BB (P=0.001) in primer P-4. But for foraging, ruminating, watching, self-grooming, and non-visible behaviour, the difference among the three genotypes did not reach the level of significance.

Table 4. Comparison for behaviour traits of each genotype of brain derived neurotrophic factor in sika deer, min

Behaviour traits	Genotype of primer P-4		
	AA	BB	AB
Foraging	243.926 ± 12.011	231.251 ± 9.969	247.445 ± 27.258
Ruminating	38.763 ± 3.971	35.741 ± 6.597	39.742 ± 8.176
Resting	101.724 ± 11.059[a]	151.558 ± 15.518[b]	126.478 ± 32.485
Watching	56.360 ± 6.509	40.579 ± 5.971	37.268 ± 11.340
Locomotion	21.554 ± 2.263[a]	9.066 ± 1.306[b]	13.867 ± 4.249
Self-Grooming	7.223 ± 1.376	6.199 ± 1.067	4.978 ± 1.980
Non-visible	10.415 ± 1.613	5.606 ± 0.784	10.222 ± 4.876
Behaviour traits	Genotype of primer P-5		
	CC	DD	CD
Foraging	231.903 ± 15.945	245.044 ± 9.720	228.319 ± 25.527
Ruminating	31.757 ± 4.411	39.547 ± 4.471	38.119 ± 5.245
Resting	83.073 ± 11.495[a]	120.798 ± 10.935[b]	173.631 ± 28.145[b]
Watching	99.389 ± 7.469[a]	41.113 ± 2.747[b]	18.291 ± 2.986[c]
Locomotion	17.896 ± 4.057	17.527 ± 1.994	9.531 ± 2.890
Self-Grooming	3.239 ± 1.402[a]	7.799 ± 1.138[b]	5.444 ± 1.260
Non-visible	12.743 ± 3.350	8.140 ± 1.281	6.664 ± 2.032

note: with the different superscript a and b in the same line, significant at P<0.05

With primer P-5, the resting behaviour of the CC genotype was significantly lower than that of the CD and DD genotypes (P=0.016), and the watching behaviour of the CC genotype was significantly higher than that of DD and CD genotypes (P=0.000). Another result was that the self-grooming behaviour of CC genotype was significantly lower than that of CD genotype (P=0.048). For the non-visible behaviour, the differences among the three genotypes did not attain significance.

DISCUSSION

In this study we employed direct observation to measure behaviour traits in sika deer. We assessed the relationship between SNPs of the BDNF gene and behaviour traits. The findings are interesting because there are several pieces of evidence suggesting an association between BDNF polymorphisms and resting/watching behaviour.

The authors of several earlier studies found that animals decreased/increased certain behaviours when exposed to park conditions (Hosey, 2000). A long-term stress environment usually depresses an animal's behaviour and welfare (Lu et al., 2010). We hypothesized that stress conditions may cause genetic mutations in the animals. Our findings show associations with decreased/increased locomotion, resting and watching behaviour.

Our results confirmed this assumption and indicated that deer were affected by the environment or feeding conditions. As far as we know, there is no published study exploring the polymorphism of the BDNF gene in sika deer with behaviour traits; hence, our results are not comparable to literature data, but they are in line with findings in the mouse model with BDNF (Lambert et al., 2005; Chourbaji et al., 2008). Contradicting results have been reported, however, on BDNF Val 66 Met variants (Beste et al., 2010). It is still unclear how the BDNF polymorphism affects neuronal functioning, brain structure, and animal behaviour. In consideration of earlier findings pointing to differences between BDNF heterozygous animals and their littermates in terms of distinct emotionality-linked aspects, i.e. different aggressiveness, feeding behaviour (Govindarajan et al., 2006), we were interested in whether housing conditions would modulate alterations in normal behaviours. In this study, the different genotypes had significant effects on resting behaviour and several types of genotype had significant effects on watching and locomotion behaviour in sika deer. We were able to show that mutations in deer significantly differ in several behaviour parameters when animals have been reared in semi-household conditions. Enclosed environmental conditions may be a kind of stress factor. As stress itself is a potential modulator of BDNF levels (Duman et al., 1997) and BDNF, on the other hand, seems to be involved in the development of characteristic phenotypes of emotionality and depression (Ridder, et al., 2005) it is noteworthy that the sika deer spent much time watching or resting in a quiet corner.

It should be noted, however, that the sample size (n = 48) of this study was small. A direct consequence of the small sample size is the propensity to deviate from the Hardy-Weinberg equilibrium (Lambert et al., 2005), as was the case in our sample group; although our sample size is relatively small for a genetic association study, our findings are strengthened for such analysis by at least three factors. First, all subjects came from the same locality and breed background. They had been reared for several years. Second, all animals were carefully observed for a long time. Third, the subjects were also carefully assessed for mutations. In conclusion, the present findings suggest that genetic mutations in sika deer have some relationship with their behaviour. Our study had another limitation. Genetic interactions on behaviour might have been overlooked because of insufficient statistical power. In addition, only two polymorphisms in the BDNF gene were chosen for this study. Therefore, further investigation needs to be conducted on various polymorphisms of this gene to determine more definitively what influence the genes have on behaviour traits.

.

CONCLUSIONS

In conclusion, the present results suggest that the BB and CD genotypes have a positive effect increasing resting, AA genotype deer exhibited more locomotion behaviour. Genotypes CC, DD, and CD, less of this behavior and spent less time in watching behaviour. This study may be a step toward defining the genetic contribution to behaviour. Further investigation is warranted to elucidate the biological mechanism of brain-derived neurotrophic factor polymorphism in behaviour decisions and environmental functions.

ACKNOWLEDGEMENTS

We thank the staff at the Ping-Shan-Tang Farm and Zhu-Yu-Wan Park for allowing this research to be undertaken. Special thanks to Professor Christine for her helpful reviews and suggestions.

REFERENCES

Adlard P.A., Perreau V.M., Engesser-Cesar C., Cotman C.W., 2004. The time course of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. Neurosci. Lett. 363, 43-48

Beste C., Baune B.T., Domschke K., Falkenstein M., Konrad C., 2010. Paradoxical association of the brain-derived-neurotrophic-factor val66met genotype with response inhibition. Neuroscience 166, 178-184

Carlstead K., Shepherdson D., 1994. Effects of environmental enrichment on reproduction. Zoo Biol. 13, 447-458

Chourbaji S., Brandwein C., Vogt B.M., Dormann C., Hellweg R., Gass P., 2008. Nature vs. nurture: Can enrichment rescue the behavioural phenotype of BDNF heterozygous mice? Behav. Brain Res. 192, 254-258

Duman R.S., Heninger G.R., Nestler E.J., 1997. A molecular and cellular theory of depression. Arch. Gen. Psychiat. 54, 597-606

Gasic G.P., Smoller J.W., Perlis R.H. et al., 2009. BDNF, relative preference, and reward circuitry responses to emotional communication. Amer. J. Med. Genet. Pt B 150, 762-781

Gong P., Zheng A., Chen D. et al., 2009. Effect of BDNF Val66Met polymorphism on digital working memory and spatial localization in a healthy Chinese Han population. J. Mol. Neurosci. 38, 250-256

Govindarajan A., Rao B.S.S., Nair D., Trinh M., Mawjee N., Tonegawa S., Chattarji S., 2006. Transgenic brain-derived neurotrophic factor expression causes both anx-iogenic and antidepressant effects. Proc. Nat. Acad. Sci. USA 103, 13208-13213

Hashimoto K., 2007. BDNF variant linked to anxiety-related behaviours. Bioessays 29, 116-119

Hemmings S.M., Kinnear C.J., Van der Merwe L., Lochner C., Corfield V.A., Mool-man-Smook J.C., Stein D.J., 2008. Investigating the role of the brain-derived neurotrophic factor (BDNF) val66met variant in obsess-compulsive disorder (OCD). J. Biol. Psychiat. 9, 126-134

Hosey G.R., 2000. Zoo animals and their human audience: what is the visitor effect? Anim. Welfare 9, 343-357

Kang J.I., Namkoonga K., Haa R.Y., Jhunga K., Kimc Y.T., Kim S.J., 2010. Influence of BDNF and COMT polymorphisms on emotional decision making. Neuropharmacology 58, 1109-1113

Lambert T.J., Fernandez S.M., Frick K.M., 2005. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. Neurobiol. Learn. Memory 83, 206-216

Lang U.E., Hellweg R., Kalus P., Bajbouj M., Lenzen K.P., Sander T., Kunz D., Gallinat J., 2005. Association of a functional BDNF polymorphism and anxiety-related personality traits. Psychopharmacology 180, 95-99

Lu S.J., Todd P.A., Yang Y., Yang L., Fan H.M., Wei W.H., 2010. The effects of visitor density on sika deeer behaviour(Cervus nippon) in Zhu-Yu-Wan Park, China. Anim. Welfare 19, 61-65

Ridder S., Chourbaji S., Hellweg R., Urani A., Zacher C., Schmid W., Zink M., Hörtnagl H., Flor H., Henn F.A., Schütz G., Gass P., 2005. Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. J. Neurosci. 25, 6243-6250

Webster J.R., Matthews L.R., 2006. Behaviour of red deer following antler removal with two methods of analgesia. Livest. Sci. 100, 150-158

Yee B.K., Zhu S.W., Mohammed A.H., Feldon J., 2007. Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice. J. Neural Transm. 114, 431-444

Zhu S.W., Codita A., Bogdanovic N., Hjerling-Leffler J., Ernfors P., Winblad B., Dickins D.W., Mohammed A.H., 2009. Influence of environmental manipulation on exploratory behaviour in male BDNF knockout mice. Behav. Brain Res. 197, 339-346