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The Association of *BDNF* Gene Variants with Behaviour Traits in Sika Deer (*Cervus nippon*)

[1,4]Lv Shen-Jin, [2]Yang Yan, [3]Guo Jun and [4]Wei Wan-Hong
[1]College of Life Science, Linyi Normal University, Linyi, 276005 Shandong, China
[2]Shandong Linyi Agriculture Science Institute, Linyi, 276009 Shandong, China
[3]Jiangsu Institute of Poultry Science, Yangzhou, 225003 Jiangsu, China
[4]College of Bio-Science and Bio-Technology, Yangzhou University, Yangzhou, 225009 Jiangsu, China

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*). About 48 sika deer reared under Ping-Shan-Tang Farm (25 deers) and Zhu-Yu-Wan Park (23 deers), Yangzhou City, Jiangsu province, China were observed and blood samples 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, AA genotype (26 deer) rested significantly less than BB genotype (16 deers) ($p<0.05$). The AA genotype deer also performed significantly more locomotion behaviour ($p = 0.001$). At the primer P-5, deer of genotypes CC/DD/CD differed significantly in their watching behaviour. Deer of genotype CC performed significantly less resting and self-grooming behaviour than deer of genotypes CD or DD (both $p<0.05$). The findings suggest that polymorphisms in BDNF may be involved in some aspects of animal behaviour traits especially in the high sensitive sika deer reared for several years in China park.

Key words: Sika deer, BDNF polymorphisms, PCR-SSCP, animal behaviour, China park, genotypes

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; Yamada *et al*., 2002). Environmental factors such as physical exercise, dietary restriction and housing conditions to affect BDNF levels (Adlard *et al*., 2004; Cotman and Berchtold, 2002; Neeper *et al*., 1996). BDNF play the critical role in behaviour and its dependent mechanisms were involved in other aspects of behaviour (Gratacos *et al*., 2007; Yee *et al*., 2007; Zhu *et al*., 2009).

Several genetic studies have suggested that the Met variant of the BDNF Val66Met polymorphism is associated with poor performance on memory (Gong *et al*., 2009; Rybakowski *et al*., 2003). It has been reported that the val66met polymorphism has been associated with a phenotype of increased anxiety-related behaviours in stressful settings in animal studies (Hashimoto, 2007) and higher levels of trait anxiety/anxious temperament (Jiang *et al*., 2005; Lang *et al*., 2005) in human. The *BDNF* gene has been reported to be associated with various neuropsychiatric conditions such as obsessive-compulsive disorder (Hemmings *et al*., 2008), eating disorders (Monteleone *et al*., 2006) and substance dependence (Gratacos *et al*., 2007). Also, environmental factors such as physical exercise, dietary restriction and housing conditions have been shown to affect BDNF levels (Duan *et al*., 2001; Gobbo and O'Mara, 2005; Lambert *et al*., 2005; Zhu *et al*., 2009). A wealth of data reveal behavioural differences both due to genetic modification and environmental manipulation (Chan *et al*., 2006; 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

Corresponding Author: Wei Wan-Hong, College of Bio-Science and Bio-Technology, Yangzhou University, Yangzhou, 225009 Jiangsu, China

(Humphries *et al*., 1990). 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) or reduced fertility. During the observation, it is find that the animals show much difference in two conditions. The sika deer in the park take long time to watching and locomotion. Then it is important to check whether the long stress environment (park condition) may cause their behaviour change or not and whether the condition can cause their genetic transformation in group.

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

MATERIALS AND METHODS

Subjects: This study was carried on Ping-Shan-Tang farm (No = 25) and Zhu-Yu-Wan Park (No = 23), Yangzhou city, Jiangsu province, China. The Ping-Shan-Tang farm animals were housed in four 20×10 m paddocks with shelter of mantle. In Zhu-Yu-Wan Park, the animals roamed in a fenced paddock of grasses, shrubs and trees (120×80 m). All tested animals were aged between 3 and 7 years. All of which were all marked by ear-notches and collar-tags for easy identification. The animals roamed in a fenced paddock of grasses, shrubs and trees (120×80 m) at the Zhu-Yu-Wan park in Yangzhou city, Jiangsu province, China. Grass and fodder (maize, bean, bran, mineral, vitamin and salt) was supplied to the animals each day at 08:00 am and 15:00 pm; water was always available.

Data and samples collection: The study was conducted in two phases: from June 21-December 10, 2006 and again from Feb 21-July 10, 2007 during all weather conditions. Focal-animal sampling was used to determine the period of time spent on each behaviour (Altmann, 1974). Each sampling lasted for 8 h day^{-1} were spent collecting data including weekdays and weekends. About 4 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 08:30 am to 16:30 pm and subjects were sampled in a different order each day (using random numbers). Each individual was studied for a mean (±SE) of 33.3±0.09 h and a total of 50 weeks, 1600 h were spent observing these 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 (Whittington and Chamove, 1995; Webster and Matthews, 2006).

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

Genomic DNA preparation and primers sequences: Sika deer genomic DNA was extracted from the whole blood using the traditional phenol/chloroform method and dissolved in sterile water at a concentration of 100 ng uL^{-1} and then kept at -20°C until use. Approximately 50-100 ng of genomic DNA was subjected to polymerase chain reaction using specific primers synthesized by Shanghai Sangon Bio.Co (Shanghai, China). According to 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 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

Table 2: Information of primers for Sika deer with *BDNF* gene

Locus	Primer sequences (5'-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	54.0
P-5	CCAAGGTGGGTTCAAGAG TGCGGCATCCAGGTAA	224	53.5
P-6	TGGATGCCGCAAACAT GAACCGCCAGCCAATAC	341	55.6

DNA amplification and genotyping: PCR reaction was carried out in 20 μL of total volume, containing 0.20 μL (5U μL^{-1}) of Taq DNA polymerase, 2.20 μL of 10×buffer (including Mg^{2+}), 1.20 μL of 1 μM primers, 1.20 μL of 2.0 mM dNTPs, 1.40 μL of 50 ng L^{-1} 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 sec, annealing 54°C for 40 sec, extension 72°C for 30 sec and final extension 72°C for 5 min on Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). At completion, PCR products were stored at 4°C until electrophoresis analysis.

SSCP analysis and DNA sequencing: About 1 μL the PCR products were diluted with 5 μL of loading buffer (98% formamide, 10 Mm EDTA pH 8.0, 0.025% Xylene cyanol FF, 0.025% bromophenol blue and 2% Glycerol). After denature at 98°C for 10 min, the mixture was immediately placed on ice for 10 min before loading on a 15% acrylamide/bisacrykamide (arc:bis = 29:1) gel. After running at 5 v cm^{-1} 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.

Data analysis: The behaviour traits data were analyzed by one way ANOVA analysis through software SPSS 14.0. Effects of SNPs on behaviour traits were analyzed and the SNPs markers with significant correlation with behaviour traits of sika deer were further studied through Post Hoc multiple comparison (Duncan method). A fixed model was adopted according to the factors that affect behaviour traits by using the following model:

$$y_{ij} = u+I_i+e_{ij}$$

Where:

y_{ij} = The observed value of ith individual animal
u = The means of values
I_i = The effective value of genetype i
e_{ij} = The random error term

RESULTS AND DISCUSSION

PCR amplification and SSCP analysis were carried out for 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 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 AA genotype while that with A at 158 nt was defined as 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 identified as CC genotype while that with C at 138 nt was defined as DD genotype.

Allele and genotype frequencies: Among the 48 of sika deer, A allele was predominant in primer P-4 locus. The frequency of genotype AB was low in the samples (Table 3). For 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 both for the 2 primers.

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

With primer P-5, the resting behaviour of CC genotype was strongly significantly lower than that of CD and DD genotype ($p = 0.016$) and the watching behaviour of CC genotype was high 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 behaviours, the different among the three genotypes did not reach the significant level.

In this study it is employed direct observation to measure the behaviour traits in sika deer. Then researchers valued the relationship between SNPs of *BDNF* gene and behaviour traits to assess the effects of the two environmental conditions on behaviour. The finding is interesting because there are several pieces of evidence suggesting an association between BDNF polymorphisms and resting/watching behaviour.

The researchers of several earlier studies found that animals decreased/increased certain behaviours when exposed to park conditions (Hosey, 2000; Humphries *et al*., 1990). The long term stress environment will depress the animal's behaviour and welfare (Shen-Jin *et al*., 2010; Mallapur *et al*., 2005). Researchers hypothesized this stress conditions may

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

		Gene		Type of gene					Gene		Type of gene			
Primer	Detect	A	B	AA	BB	AB	Primer	Detect	C	D	CC	DD	CD	χ^2 value
P-4	No	-	-	26	16	6	P-5	No	-	-	9	32	7	12.50
Observed value	-	0.604	0.396	0.260	0.740	0.542	Observed value	-	0.333	0.125	0.188	0.667	0.146	24.13

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

Table 4: Comparison for behaviour traits of each genotype of BDNF in sika deer (min)

	Genotype of primer P-4			Genotype of primer P-5		
Behaviour traits	AA	BB	AB	CC	DD	CD
Foraging	243.926±12.011	231.251±9.9690	247.445±27.258	231.903±15.940	245.044±9.7200	228.319±25.527
Ruminating	38.763±3.9710	35.741±6.5970	39.742±8.1760	31.757±4.4110	39.547±4.4710	38.119±5.2450
Resting	101.724±11.059	151.558±15.518	126.478±32.485	83.073±11.495	120.798±10.935	173.631±28.145
Watching	56.360±6.5090	40.579±5.9710	37.268±11.340	99.389±7.4690^a	41.113±2.7470^b	18.291±2.9860^c
Locomotion	21.554±2.2630^a	9.066±1.3060^b	13.867±4.2490	17.896±4.0570	17.527±1.9940	9.531±2.8900
Self-grooming	7.223±1.3760	6.199±1.0670	4.978±1.9800	3.239±1.4020^a	7.799±1.1380^b	5.444±1.2600
Non-visible	10.415±1.6130	5.606±0.7840	10.222±4.8760	12.743±3.3500	8.140±1.2810	6.664±2.0320

With the different superscript a and b in the same line, significant at $p<0.05$

cause the animals genetic mutation. The findings were the result of decreased/increased locomotion, resting and watching behaviour.

As far as researchers know there is no published study exploring the polymorphism of the *BDNF* gene in sika deer with behaviour traits hence the results are not comparable to literature data. But the results are in line with those findings in the mouse model with BDNF (Chourbaji *et al*., 2008; Gobbo and O'Mara, 2005; Lambert *et al*., 2005). But the contradicting results have been reported that BDNF Val66Met variants (Beste *et al*., 2010; Chen *et al*., 2008). It is still unclear how the BDNF polymorphism affects neuronal functioning, brain structure and animal behaviour. In consideration of earlier findings pointing at differences between BDNF heterozygous animal and their littermates in terms of distinct emotionality-linked aspects i.e., difference aggressiveness, feeding behaviour (Govindarajan *et al*., 2006; Kernie *et al*., 2000; Lyons *et al*., 1999). Researchers were interested in extending the characterization of these mutants by examining whether specific housing conditions would modulate alteration particularly in normal behaviours. In this study, the different genotype had significant effect on resting behaviour and several types of genotype had significant effect on watching and locomotion behaviour in the park condition. The study demonstrated an interaction between enclosed environmental conditions and genetic factors in sika deer. There were able to show that mutations in animal significantly differ in several behaviours parameters when they have been housed in park, a poor quality and high stress rearing conditions for several years. The enclosed environmental conditions and the visitor may be kind of stress factors. As stress itself states a potential modulator of BDNF levels (Duman *et al*., 1997; Nibuya *et al*., 1995) and BDNF on the other hands, seems to be involved in the development of characteristic phenotypes of emotionality and depression (Ridder *et al*., 2005). For the sika deer, they spent much time watching or resting in a quiet corner. But for the farm condition, no visitor disturbs their normal living, except the feeder.

However, it should be noted the sample size (n = 48) of this study was too small. A direct consequence of the small sample size is the propensity to deviate from Hardy-Weinberg equilibrium (Lambert *et al*., 2005) as it was the case for the sample group thus. However, although the sample size is relatively small for a genetic association study, the findings are strengthened by at least three factors. First, all subjects came from the same locality and breed background. They have been reared for several years. Second, all animals were carefully observed for a long phase. Third, the subjects were also carefully assessed to their mutations.

CONCLUSION

In this study, findings suggest that the genetic mutation have some relationship with animal behaviour. The study had another limitation. Other main or interaction effects of genes on behaviour performance might have been overlooked because of insufficient statistical power. In addition, only two of polymorphisms in *BDNF* genes 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.

In conclusion, the present results suggest that BB and CD genotypes have a positive effect to do more resting. AA genotype deer performed more locomotion behaviour. Genotypes CC, DD and CD showed less and less time in their watching behaviour. This study may be a step toward defining the genetic contribution to

behaviour making. Further investigation is warranted to elucidate the biological mechanism of the BDNF polymorphism in behaviour decision and environmental functions.

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