The dopamine β-hydroxylase gene in Chinese goose (Anas cygnoides): cloning, characterization, and expression during the reproductive cycle

Qi Xu1, Yadong Song1, Ran Liu1, Yang Chen1, Yang Zhang1, Yang Li1, Wenming Zhao1, Guobin Chang1 and Guohong Chen1,2*

Abstract

Background: Dopamine β-hydroxylase (DBH) is a critical enzyme in the biosynthesis of catecholamines. This enzyme's role in neuroendocrine regulation is well known, but there are some indications that it may also modulate reproduction and endocrine in mammals and birds. We selected goose (Anas cygnoides) as an ideal model species for investigating the role of DBH in avian reproduction.

Results: Full-length cDNA encoding DBH was cloned from Zhedong goose using reverse transcription PCR and rapid amplification of cDNA ends. The cDNA consisted of a 126-base pair (bp) 5′-untranslated region (UTR), a 379-bp 3′-UTR, and an 1896-bp open reading frame encoding a polypeptide of 631 amino acids. The deduced amino acid sequence of gDBH shared high homology with an analogue from other birds and contained three conserved domains from a mono-oxygenase family including a DOMON domain and two Cu2_mono-oxygen domains. Real-time quantitative PCR analysis showed that gDBH mRNA was expressed in both reproductive and endocrine tissues of Zhedong goose, specifically in the hypothalamus, pituitary, ovary, and oviduct. More DBH mRNA of reproductive and endocrine tissues was detected at ovulation than at oviposition in Zhedong goose. Evidence of opposite trend of gDBH expression was found between the hypothalamus-pituitary and oviduct during the ovulation phase and the broody phase. In addition, we assessed DBH mRNA expression during ovulation in two breeds of geese that differ in egg production. The reproductive and endocrine tissues of Yangzhou geese with higher egg production had more gDBH expression than Zhedong geese. Finally, the five non-synonymous SNP(c.1739 C > T, c.1760G > T, c.1765A > G, c.1792 T > C and c.1861G > C) were identified in the coding region of DBH gene between Zhedong goose and Yangzhou goose.

Conclusions: We conclude that goose DBH mRNA show obvious periodically variation in reproductive and endocrine tissues during the reproductive cycle in geese.

Keywords: Goose, DBH, Gene expression, Reproduction

* Correspondence: ghchen@yzu.edu.cn
1Key Laboratory of Animal Genetics and Breeding and Molecular Design of Jiangsu Province, Yangzhou University, Yangzhou 225009, PR China
2School of Animal science and Technology, Yangzhou University, Yangzhou, PR China

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**Background**

Dopamine β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine in the biosynthesis of catecholamines [1–3]. The activity of DBH influences the levels of dopamine and the biosynthesis of norepinephrine and epinephrine. Dopamine β-hydroxylase’s importance in the nervous system is well established [4–9], but a few studies also point to its importance in reproduction. The evidence for this in mammals can be summarized as follows. DBH, regulated by the sympathetic nervous system, has major effects in the female reproductive system of pigs, where it influenced ovarian and oviductal function [10]. Injection of 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14,624), a DBH inhibitor, increased the number of progestin and estrogen receptors in female rat [11–13]. In research with other mammals, DBH regulated reproductive performance through modulating the concentration of catecholamines, as well as other physiological functions. Mice with targeted disruption of DBH had a high fetal mortality rate and altered maternal behavior [14, 15]. In pigs, polymorphism of DBH was related to reproduction and piglet survivability [16]. This background was intriguing and led us to speculate on the role of DBH in avian reproduction, about which little is known.

The goose (*Anas cygnoides*) is a commercially important food source that is widely cultivated in China. It is an ideal avian model for characterization of reproduction because of its obvious reproductive stages and strong broodiness [17]. In a previous study in which we used transcriptome profiling of ovaries from laying and brooding geese [18], we identified DBH as an important gene in the goose reproductive cycle. We have extended this study here by cloning the Zhedong goose DBH and characterized its spatio-temporal expression patterns by qPCR. Next, we undertook a correlative study of DBH expression and egg production by comparing DBH expression in the Yangzhou breed, which has high-egg production, with the Zhedong goose, a breed with low-egg production and strong broodiness behavior. The DBH expression profiles provide an invaluable information for understanding of the regulatory function of DBH in goose egg laying.

**Results**

**Zhedong Goose DBH cDNA cloning and sequence analyses**

The full-length cDNA of gDBH was acquired with RT-PCR and RACE. The DBH cDNA from Zhedong goose was 2399 nucleotides in length and consisted of a 126-nucleotide 5′ untranslated region (UTR), a 379-nucleotide 3′ UTR, and an 1896-nucleotide open reading frame (ORF) putatively encoding a single 631 amino acid protein (GenBank accession KU672379).

The other transcript variant was not detected in Zhedong goose in this study.

**Phylogenetic analysis of the putative DBH**

Alignment analysis of the DBH protein (Fig. 1) revealed that the putative goose DBH had high homology with analogues from the other four birds (chicken, duck, turkey and zebra finch). There was less homology with the non-bird species than the avian species.

The structural domain of the DBH protein of different species was compared with gDBH. It is relatively conservative and contains three potential domains (a DOMON domain, goose DBH 52-170AA; the two Cu2 mono-oxygen domains in the N-terminal and C-terminal, respectively, goose DBH 215-344AA, 360-524AA), which belonged to a mono-oxygenase family. The conserved domains in gDBH and the amino acid sequence similarity with other DBHs strongly suggested that it was a homologue of DBH from *Anas cygnoides*.

To determine the evolutionary relationship between gDBH and the other proteins, phylogenetic analysis was carried out by Clustal W and Mega 6.0. The amino acid sequences of gDBH and from another ten species were compared. Protein sequences were used for the rooted phylogenetic tree, which was constructed by the neighbor-joining method. The goose proteins from different species were divided into three major branches. *Gallus gallus, Anas platyrhynchos, Anas cygnoides, Meleagris gallopavo, Taeniopygia guttata* were grouped into a cluster. The second branch consisted of *Homo sapiens, Mus musculus, Sus scrofa, Bos taurus, and Capra hircus*. *Danio rerio* was separated and formed an independent branch (Fig. 2). The established evolutionary relationship tree was consistent with the real evolution of animals.

**Expression pattern of DBH in different tissues and reproductive cycle stages of Zhedong goose**

The qPCR demonstrated that DBH was differently expressed in fourteen tissues of Zhedong goose. High levels of DBH transcript were detected in hypothalamus, pituitary, ovary, oviduct, lung, cerebrum, and cerebellum tissues in Zhedong goose while levels were negligible in chest muscle tissue (Fig. 3).

We wished to examine the temporal expression patterns of gDBH expression in the hypothalamus, pituitary, ovary, and oviduct tissues during different stages of the goose reproductive cycle, specifically the pre-laying stage, ovulation, oviposition, and the broody phase. The results are shown in Fig. 4. Overall, there were differences in gDBH expression with respect to both time and tissue. Expression was lowest in all tissues during the oviposition phase. It was high in the hypothalamus, ovary, and pituitary in the pre-laying period. Expression...
| Species                  | Accession Number | Percentage Coverage |
|-------------------------|------------------|---------------------|
| Anas platyrhynchos      | DQ8531 Q1        | 100                 |
| Anas clypeata           | DQ8531 Q1        | 100                 |
| Taeniopygia guttata     | DQ8531 Q1        | 100                 |
| Gallus gallus           | DQ8531 Q1        | 100                 |
| Meleagris gallopavo     | DQ8531 Q1        | 100                 |
| Bos taurus              | DQ8531 Q1        | 100                 |
| Capra hircus            | DQ8531 Q1        | 100                 |
| Sus scrofa              | DQ8531 Q1        | 100                 |
| Homo sapiens            | DQ8531 Q1        | 100                 |
| Mus musculus            | DQ8531 Q1        | 100                 |
| Danio rerio             | DQ8531 Q1        | 100                 |

**Fig. 1** (See legend on next page.)
was higher in hypothalamus-pituitary than in the oviduct during the broody phase. In contrast, the expression of the \textit{DBH} gene was high in the oviduct during ovulation phase, but low in the hypothalamus-pituitary.

**Comparison of DBH expression of Zhedong goose and Yangzhou goose during the ovulation phase**

Two goose breeds, with markedly different egg performance, were selected to test the hypothesis that \textit{DBH} expression might be correlated with egg production. The comparison was made between the Zhedong goose, with low egg production, and the Yangzhou goose, with high egg production. The expression of \textit{DBH} was higher in reproductive and endocrine tissues of the high-egg-producing, Yangzhou goose than in the Zhedong goose during the ovulation phase. These differences were significant for the hypothalamus, pituitary, and ovary (\(P < 0.01\)), but not for the oviduct (Fig. 5).

**Identification of genetic variation on DBH in Zhedong goose and Yangzhou goose**

By sequence alignment, the five nsSNPs (non-synonymous SNP) including c.1739 C > T, c.1760G > T, c.1765A > G, c.1792 T > C and c.1861G > C were identified in the Exon 11 of goose DBH gene (Table 1). The allele frequency from c.1739 C > T and c.1792 T > C substitutions were significantly different between Zhedong goose and Yangzhou goose (\(P < 0.01\)).

**Discussion**

In this study, the goose \textit{DBH} gene was characterized from \textit{Anas cygnoides}. A DOMON domain and a Cu2 mono-oxygen domain were identified in the deduced amino acid sequence of gDBH. Both DOMON and Cu2 mono-oxygen domains were highly conserved in all the DBHs analyzed. The DOMON domain had been identified in the physiologically important enzymes including cellobiose dehydrogenase, extracellular fungal oxidoreductase and ethylbenzene dehydrogenase [19, 20]. Recent studies indicate that DOMON domains are responsible for heme or sugar recognition and binding at the cell surface [21] and it has been suggested to be a dopamine-binding domain in DBHs [22]. The Cu2 mono-oxygen domain may be the catalysis center of DBH and, as such, involved in the conversion of dopamine to norepinephrine. The conserved DOMON domain and Cu2 mono-oxygen domain identified in goose DBH in this study led us to speculate that it had the same function as other avian DBHs, namely, in the synthesis of catecholamines.

Quantitative PCR analysis revealed that \textit{DBH} was expressed in every tissue analyzed except chest muscle,
albeit to different degrees in different tissues. The information on the tissue distribution might provide clues about the role of DBH in various physiological functions in birds. The ubiquitous distribution of DBH that we observed in goose tissues had not been observed in mammalian tissues, where it was mainly present in the nervous system [5–7]. Of course, high expression of DBH was found in the cerebrum and cerebellum in goose, no doubt due to the involvement of norepinephrine and epinephrine in neuroendocrine processes. Interestingly, DBH mRNA was clearly present in both reproductive and endocrine tissues, specifically in the hypothalamus, pituitary, ovary, and oviduct. It may be, then, that DBH regulates hormone synthesis. DBH modulated the concentration of both progestin receptors and estrogen receptors [11–13], supportive of this suggestion. It is also reasonable that the lungs, which are regulated by the sympathetic nervous system, had high DBH expression in our results. This finding also agrees with the results [23, 24]. In human, analysis of DBH mRNA had been confirmed high expression in the brain, but also in sympathetically innervated organs, such as lung [23].

The expression of DBH, detected with mRNA accumulation, was assessed during the pre-laying, ovulation, oviposition, and broody phases. The expression level drastically fluctuated in reproductive tissues and endocrine tissues.
The DBH expression levels were high in the ovulation phase and among the lowest observed in the oviposition phase. Similar results were observed in catfish [25]. The fluctuations might be related to the different activity of norepinephrine during the reproductive cycle. Most research focused on DBH regulation of hypothalamo-pituitary-adrenal (HPA) responses [26, 27], but we also found that DBH played a role in hypothalamus-pituitary-gonadal (HPGA) axis. DBH catalyzed a key step in catecholamine biosynthesis, and catecholamine was believed to derive from the extrinsic innervation of the ovary and to participate in the regulation of ovarian development and mature gonadal function [28]. The levels of DBH in hypothalamus or ovary affected luteinizing hormone secretion [10, 29], leading us to speculate that an ovarian steroidogenesis surge, with increased noradrenaline release by the HPGA axis, occurs concomitant with increased DBH expression. Interestingly, DBH expression was higher in hypothalamus-pituitary than oviduct during the broody phase, while, in contrast, it was higher in the oviduct than the hypothalamus-pituitary during ovulation. We considered this to be evidence for a feedback mechanism that controlled either the enzyme or its gene expression, or both.

We also observed higher DBH expression in Yangzhou geese than in Zhedong geese during the ovulation phase. Yangzhou geese are known for excellent egg-production ability and no broodiness behavior, while Zhedong geese have low egg-production with broodiness behavior. Yangzhou geese might need to release more hormones to ovulate, so the observed higher DBH expression levels might be required to ensure sufficient hormone secretion. The similar results presented in the rat. Stoker TE et al. found DBH played an important role in the regulation of the acute effects on the hormonal control of ovulation [30]. Besides, we found the allele frequency from c.1739 C > T and c.1792 T > C substitutions were significantly different between Zhedong goose and Yangzhou goose. The two substitutions might be associate with egg-production or broodiness behavior.

Conclusion

In summary, we presented the molecular cloning and characterization of gDBH from Anas cygnoides and had analyzed its expression during the reproductive cycle. In Zhedong geese, DBH expression, as measured by mRNA accumulation, was higher at ovulation than at oviposition. We hypothesized feedback regulation of gDBH expression between hypothalamus-pituitary and the oviduct during ovulation and the broody phase. Expression of DBH during ovulation was higher in Yangzhou geese than Zhedong geese. Hence this finding provides correlative evidence that DBH expression is important in reproduction. Our findings reveal that the gDBH may regulate goose reproductive activity by the HPGA axis.

Methods

Animals

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Yangzhou University. Procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Yangzhou University, China, 2012) and the Standards for the Administration of Experimental Practices (Jiangsu, China, 2008). The two goose breeds used in this study, Zhedong goose and Yangzhou goose, were raised in the breeding farm of Jiangsu Lihua Animal Husbandry Co.,

| Breeds             | c1739C > T | c1760G > T | c1765A > G | c1792T > C | c1861G > C |
|--------------------|------------|------------|------------|------------|------------|
|                    | CC, CT, TT | GG, GT, TT | AA, AG, GG | TT, TC, CC | GG, GC, CC |
| Yangzhou goose (n = 29) | 12, 5, 2 | 4, 9, 16 | 0.008, 0.02 | 0, 9, 20 | 0.03, 0.01 |
| Zhedong goose (n = 22)  | 4, 5, 13 | 1, 1, 20 | 5, 5, 12 | 9, 3, 10 | 0, 22 |
Tissue sample collection
Geese were sacrificed by anesthetizing them with sodium pentobarbital. To investigate DBH expression patterns in different tissues, various tissues were removed, immediately frozen in liquid nitrogen, and stored at -80 °C for RNA isolation. These tissues were heart, liver, glandular stomach, lung, spleen, kidney, intestine, spleen, cecum, cecum, muscle, and infundibulum of the oviduct, pituitary, hypothalamus, and the stroma of the ovary. A group of Zhedong geese were sacrificed in the pre-laying stage, when they were 120 days old. Three groups of 380-days-old Zhedong geese (five geese/group) were selected: a laying group with an egg in the oviduct (ovulation, the release of an ovum from a ruptured follicle), a laying group without an egg in the oviduct (oviposition, the laying of the egg), and a brooding group (The goose sits in the nest and the distance between pubic bones is less than two finger widths). Another 5 laying Yangzhou geese with an egg in the oviduct (ovulation) was also selected for comparison with the Zhedong breed.

Zhedong goose DBH cDNA cloning and sequencing
Total RNA was extracted from collected tissue samples using TRIzol reagent according to the manufacturer's instruction (TaKaRa, China) and re-suspended in RNase-free water. The concentration and purity were determined with a NanoDrop Spectrophotometer (NanoDrop, USA). After purification, 2 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol. Primers were designed according the unigene (Xu et al., [18]; Additional file 1) and reverse transcription PCR (RT-PCR) was performed using ovarian cDNA from geese. The PCR product was purified, cloned into the pMD19-T vector (TaKaRa, China), and subjected to sequence analysis. The 5’- and 3’-ends of DBH were amplified via rapid amplification of cDNA ends (RACE) using the 5’-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, USA) and the 3’-Full RACE Kit (TaKaRa, China), respectively. RACE primers (Additional file 1) were designed using the partial DBH nucleotide sequence obtained from RT-PCR. Touchdown and nested PCRs were performed according to the manufacturer's instructions. Amplicons were then cloned into a plasmid vector for nucleotide sequencing by Sangon Biotech (Shanghai, China).

Bioinformatics analysis
The Zhedong goose cDNA and deduced DBH amino acid sequences were analyzed using DNASTar (version 7.1). Homology analyses were carried out using Clustal W (http://www.ebi.ac.uk/Tools/msa/). Conserved domains in the protein were identified by the conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). A rooted neighbor-joining tree was constructed to determine the phylogenetic relationship using MEGA 6.0 software with 1000 bootstrap replicates to establish the confidence level of each node.

DBH expression patterns in Zhedong goose and Yangzhou goose
To study expression of the cDNA encoding goose DBH (gDBH), we performed real-time quantitative PCR (qPCR) on total RNA isolated from the tissues. Assays were conducted in 20-μL reaction mixes using the SYBR Premix Ex Taq™ (TaKaRa, China) and performed on an ABI two-step RT-PCR system (Applied Biosystems 7500, USA) with diluted first-strand cDNA. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) served as an internal reference gene. Quantitative qPCR programs for DBH and GAPDH were: one cycle of 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 34 s of data collection, and one cycle for the melting curve analysis. All cDNA synthesis reactions were carried out using 100 ng of total RNA per reaction and assayed in three to four technical replicates for each set of biological samples. The same methods were used to determine the DBH mRNA expression profile during the reproductive cycle. For the DBH mRNA expression profile of the pre-laying Zhedong geese, the chest muscle tissue served as a calibrator. For the differential expression analysis during the reproductive cycle, the oviduct tissue from pre-laying Zhedong geese served as a calibrator. To compare the expression patterns between the Zhedong geese and Yangzhou geese, the mean ΔCt value of the hypothalamus tissue of Zhedong geese within each group was used as the calibrator. Relative expression of mRNA was calculated using the 2^(-ΔΔCt) method [31].

Identification of SNP on DBH in Zhedong goose and Yangzhou goose
According to the goose genome sequences (scaffold224136, scaffold224137), nine pairs of primers (shown in Additional file 1) were synthesized to identify the polymorphisms. PCR products were amplified from the DNA of Yangzhou geese and Zhedong geese, and sequenced directly by the GenScript Co., Ltd. (Nanjing, China). The obtained sequences were aligned by AlignIR(V2.0) software to screen the potential single nucleotide polymorphisms (SNPs) in the coding region.
Statistical analyses
Data analysis was performed by using SPSS17.0, then adopted one-way ANOVA analyses to compare the difference among the different tissues, periods and breeds, respectively. Comparisons of genotypes between the different breeds were evaluated by Chi-square ($\chi^2$) tests. $P < 0.01$ was considered statistically very significant in all.

Availability of supporting data
The data sets supporting the results of this article are included within the article and its additional files. The cDNA sequence of DBH from Zhedong goose been deposited in the GenBank of National Center for Biotechnology Information (NCBI) with accession number KU672379.

Additional file

Additional file 1: Primers used in this study. (DOCX 20 kb)

Abbreviations
DBH: dopamine $\beta$-hydroxylase; RACE: rapid amplification of cDNA ends; RT-PCR: reverse transcription PCR; UTR: untranslated region.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
GHC conceived of the study, and participated in its design and coordination. QX, YDS, RL, YCY, YZ and YL carried out the experiments. WMZ and GBC participated in the design of the study and performed the statistical analysis. QX and YDS contributed to the manuscript preparation. QX and GHC interpreted the results and contributed to edit the manuscript. All authors read and approved the final manuscript.

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