RESEARCH HIGHLIGHT

Structural variation of G protein-coupled receptor in birds

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G protein-coupled receptors (GPCRs) are key regulators of various vital biological processes. Regardless of their physiological significance, GPCRs generally harbor a certain number of amino acid substitutions, as well as insertions/deletions, particularly in the loop and tail regions, even at the species level. We previously revealed that the C-terminal domain of avian GPCRs shows length variations at the intra-species level. The presence or absence of deletion in helix 8 of the arginine vasotocin receptor affected the pattern of putative palmitoylation sites in the zebra finch. Moreover, *Turdus* thrushes harbored 18-amino acid tandem duplications at the distal part of the C-terminal tails of the mesotocin receptor. These findings illustrated that avian neuropeptide receptors accommodate structural changes at the C-terminal tails as a source of genetic variation that may lead to phenotypic differences in natural populations as a consequence of natural selection.

Keywords: G protein-coupled receptor; polymorphism; C-terminus, tandem duplication; Passeriformes; palmitoylation

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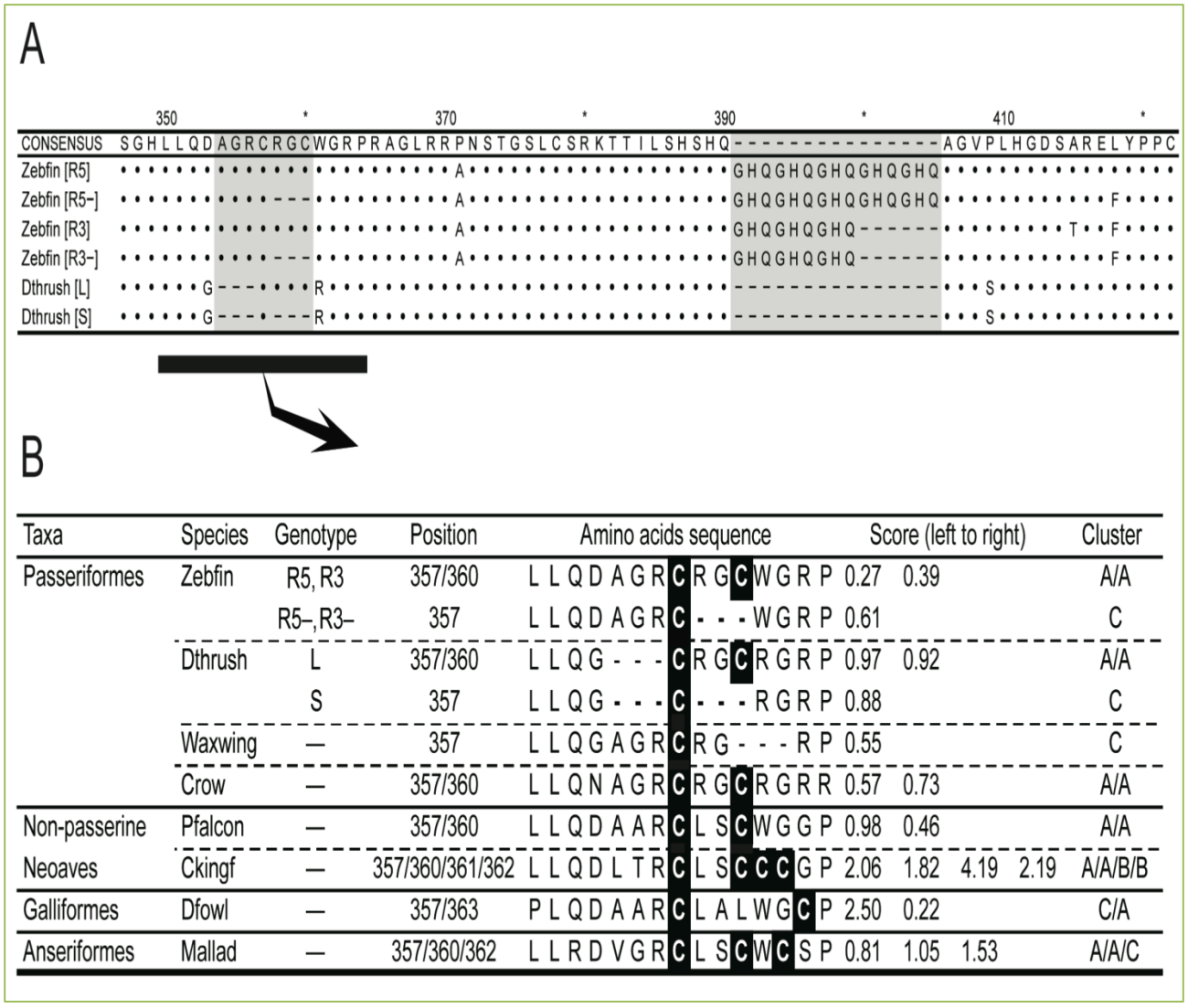
Introduction

G protein-coupled receptors (GPCRs) are known to constitute the largest family of cell surface receptors in eukaryotes. Because the physiological functions mediated by GPCRs play central roles in most of vital biological processes, numerous studies have been conducted to identify structural variations in the GPCR that lead to aberrant phenotypes, mainly in clinical cases [1, 2]. Indeed, candidate gene studies targeting human neurotransmitter-related genes have detected a significant level of genotype-phenotype associations in the *dopamine transporter* (*DAT*) [3], the *serotonin transporter* (*SLC6A4*) [4], and *monoamine oxidase A* (*MAOA*) [5]. Information on sequence variants within the family of human GPCRs is currently available in the public database [6] (e.g., GPCR Natural Variants database; http://nava.liacs.nl/) and utilized mainly for *in silico* drug design.

Recently, however, molecular genetic data have been accumulating on the comprehensive structural features of GPCR in non-human animals such as the chicken [7] and frog [8]. Several scientists have examined the phylogenetic relationships based on GPCR data, even including those isolated from non-model organisms [9, 10]. Suwa *et al*. have developed an integrated GPCR database (SEVENS; http://sevens.cbrc.jp/) that provides reliable information on GPCR structural variation identified from 59 eukaryote



Taxa	Species	Genotype	Position	Amino acids sequence	Score (left to right)	Cluster
Passeriformes	Zebfin	R5, R3	357/360	L L Q D A G R **C** R G **C** W G R P	0.27 0.39	A/A
		R5−, R3−	357	L L Q D A G R **C** - - - W G R P	0.61	C
	Dthrush	L	357/360	L L Q G - - - **C** R G **C** R G R P	0.97 0.92	A/A
		S	357	L L Q G - - - **C** - - - R G R P	0.88	C
	Waxwing	—	357	L L Q G A G R **C** R G - - - R P	0.55	C
	Crow	—	357/360	L L Q N A G R **C** R G **C** R G R R	0.57 0.73	A/A
Non-passerine	Pfalcon	—	357/360	L L Q D A A R **C** L S **C** W G G P	0.98 0.46	A/A
Neoaves	Ckingf	—	357/360/361/362	L L Q D L T R **C** L S **C C C** G P	2.06 1.82 4.19 2.19	A/A/B/B
Galliformes	Dfowl	—	357/363	P L Q D A A R **C** L A L W G **C** P	2.50 0.22	C/A
Anseriformes	Mallad	—	357/360/362	L L R D V G R **C** L S **C** W **C** S P	0.81 1.05 1.53	A/A/C

Figure 1. Amino acid sequences in the C-terminal tail of the arginine vasotocin V2 receptor (AVT2R) [17]. Dots and dashes indicate the identical and lack of amino acids to the consensus sequence, respectively. Two polymorphic sites are highlighted. Species names are abbreviated as "Zebfin" (zebra finch), and "Dthrush" (dusky thrush). Each genotype is shown within the brackets **(A)**. Comparison of the pattern of potential palmitoylation sites identified in the AVT2R. The potential palmitoylated cystein residues were predicted by CSS-Palm 2.0 software [41] and shown in reverse. The patterns of palmitoylated sites are indicated as A (-CXXC-), B (-CC-), and C (other types of combination). Species names are abbreviated as "Waxwing" (Japanese waxwing), "Crow" (jungle crow), "Pfalcon" (pygmy falcon), "Ckingf" (common kingfisher), and "Dfowl" (domestic fowl) (**B**). Reprinted with permission [17].

genome sequences [11, 12]. The accumulation of GPCR sequences from a broad range of organisms has enabled us to conduct comparative genetic approaches to identify key mutations that might influence phenotypic expression by altering physiological conditions.

Conformational modifications in some GPCRs, particularly in neuropeptide receptors can be a direct modulator of phenotypic variation, including behavioral and morphological heterogeneities. For example, oxytoxin (OXT) and arginine vasopressin (AVP) perform common physiological functions in a wide variety of species and show remarkable evolutionary conservation of structure and function. It is therefore important to collect data on GPCR polymorphisms from a wide range of organisms in order to explore and investigate the link between structural modifications on GPCRs and their neuroendocrinological influences on animal phenotypes. Animal models enable us to gain an understanding of neuropeptide function including its relationships with hormone release and its behavioral consequences [13].

Here, we outline the latest findings on the structural variations in avian GPCRs. We used a variety of avian species because information on genetic polymorphisms in avian GPCRs is limited. The melanocortin-1 receptor

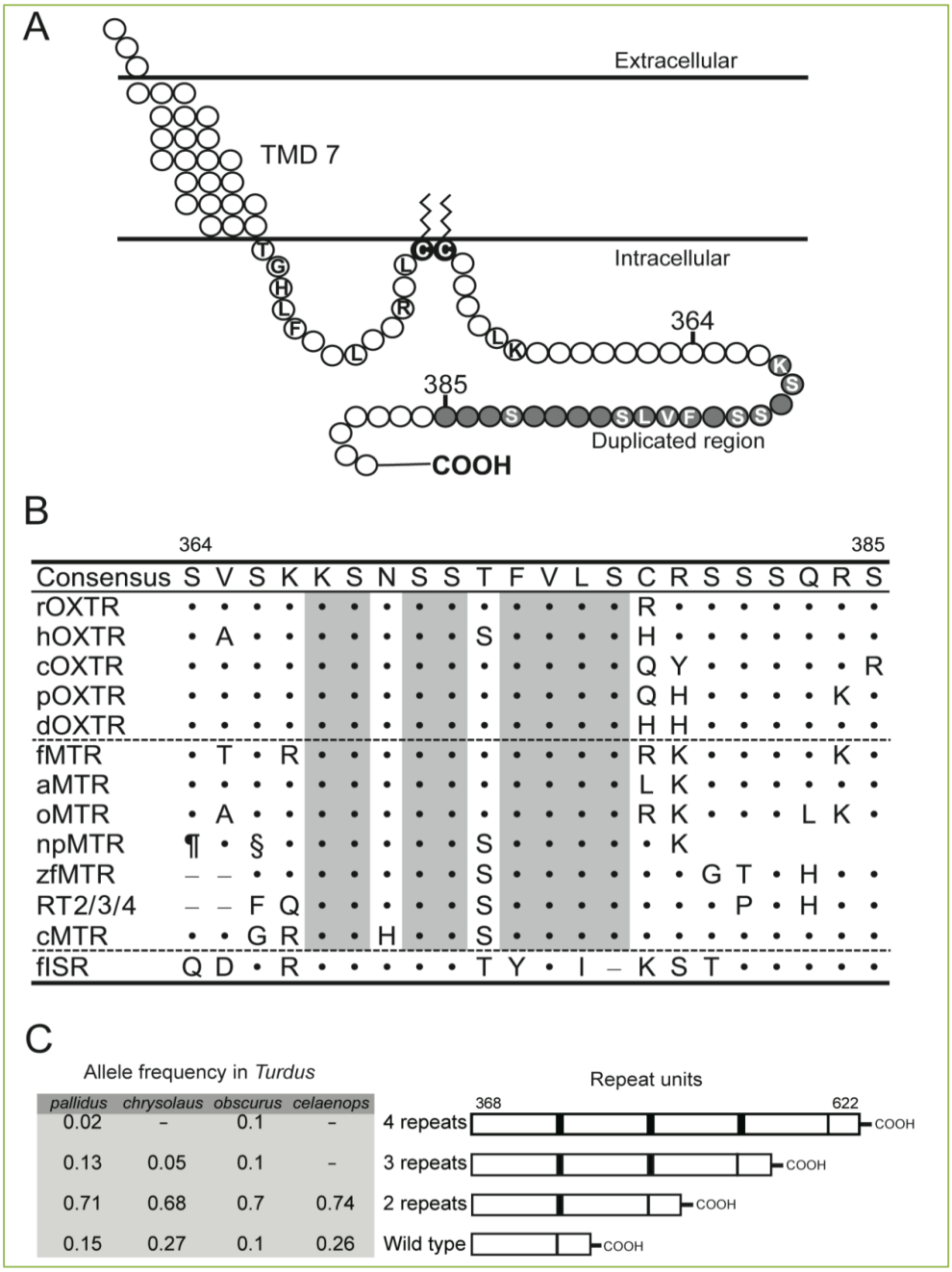


Figure 2. Structure of the C-terminal tail of the oxytocin/mesotocin receptor [18]. Schematic structure of the avian mesotocin receptor (MTR) is shown with palmitoylated double cysteins (black-white reversal). The amino acid residues highly conserved among vertebrates are indicated by capital letters. The region of the tandemly duplicated element in *Turdus* thrush is highlighted **(A)**. Comparison of the C-terminal structure in MTR with mammalian oxytocin receptors (OXTRs) and the fish isotocin receptor (ISR). The organisms and GenBank accession numbers are as follows: rat (rOXTR; NP037003.2), human (hOXTR; NP000907.2), cow (cOXTR; NP776559.1), porcine (pOXTR; NP999192.1), canine (dOXTR; NP001185588.1), western clawed frog (fMTR; XM002936297.1), gray short-tailed opossum (oMTR; XP001375059.1), green anole (aMTR; XP003224939.1), non-passerine Neoaves (npMTR; AB634795—AB634816), zebra finch (zfMTR; XM002188266.1), chicken (cMTR; NP001026740.1), and zebra fish (fISR; XP001341507.1). RT2, RT3, and RT4 are duplicated elements found in *Turdus* thrushes. Highly conserved motifs are shaded, and the symbols ¶ and § represent S/G/N/A/T and S/G, respectively. The sequence of npMTR is truncated because of internal primer design **(B)**. The pattern of tandem duplications at the C-terminal domain of MTR is shown with allele frequencies detected in east Eurasian thrushes **(C)**. Reprinted with permission [18].

(*MC1R*) is an exceptional GPCR that is well characterrized with respect to the genotype-phenotype correlation [14-16]. Likewise, given that dramatic phenoltypic differrences have been produced by relatively simple genetic changes,

the comparison of other GPCR sequences among closely related avian species provides a rare opportunity to examine the level of constraint on their secondary structures and to identify potential mutations responsible for phenotypic differences.

Polymorphisms in the C-terminal domain of GPCRs among avian species

Our recent studies have focused on polymorphisms at the C-terminal tails of arginine vasotocin [17] (AVT; homologous to mammalian AVP) and mesotocin [18] (MT; homologous to mammalian OXT) receptors of avian species due to the following reasons. First, the C-terminal tail of GPCRs displays extensive variations in length (17-106 amino acid residues in the GPCR family A) [19], as well as a moderate level of amino acid conservation as compared with those of the extracellular loops and the transmembrane helices [20]. Moreover, the C-terminal domain of GPCRs plays important functions other than ligand binding. For example, the C-terminal tail involves reversible phosphorylation, which has a significant role in signal desensitization and receptor internalization [21]. Thus, both amino acid substitutions and indels, which can change the physiochemical and conformational properties of the C-terminal tail, are candidate factors that affect the level of GPCR expression.

Chicken arginine vasotocin V2 receptor (AVT2R) is the second cloned vasotocin receptor subtype, which has an open reading frame of 425 amino acids [22]. Phylogenetic analysis of neurohypophysial hormone receptors among vertebrates showed that AVT2R shares a common receptor ancestor with the mammalian V1b-vasopressin [23]. Although our sequence alignment on avian AVT2R clearly showed a higher level of genetic diversity in the C-terminal domains compared to the avian mesotocin receptor (MTR), the C-terminal tails contained a considerable number of highly conserved amino acids among various avian orders [17]. For instance, all avian species have a Cys^{357} in the same position where double cysteines are found in mammals (Fig. 1A). In addition, Cys^{378} is also highly conserved in all avian species at the more distal part of the C-tails.

These C-terminal Cys residues that are conserved in a majority of the GPCRs are potential sites of palmitoylation, which is thought to mediate the interaction of the C-terminal tail with membranes, resulting in the formation of an additional cytoplasmic loop [24]. The potential palmitoylation sites in the C-terminus are highly conserved in mammals as well as among members of the OXT-like receptor superfamily [20]. However, deletion polymorphisms in the flanking regions of Cys^{357} were detected in 2 species of passerine (Passeriformes) birds, showing intra-specific polymerphisms regardless of the presence or absence of a 3- to 6-amino acid deletion adjacent to the palmitoylation site (Figs. 1A and B). The zebra finch (*Taeniopygia guttata*) can be divided into 2 amino acid types (AGRCRGC and AGRC) in the polymorphic site, whereas the dusky thrush (*Turdus eunomus*) also has 2 indel variations (CRGC and C) at the same site. In other words, each of these 2 birds can be sorted according to the conformation of potential palmitoylated cysteine(s), whether expressed as a single C or CXXC motif. From an evolutionary standpoint, both bird species are classified into the same avian order, Passeriformes, but these are not closely related in the avian phylogeny. It is thus tempting to speculate that triamino acid deletions may have independently occurred in each passerine lineage, and that the genomes of passerine birds may be tolerant to the conformational changes near the palmitoylation site.

Tandem duplication is another source of genetic variation in the C-terminal tail of vertebrate GPCRs. As far as we know, Mahlmann *et al.* first reported the existence of tandem repeats (SRKSSQCIPLDC) at the C-terminus of the teleost AVT receptor [25]. In our research, the zebra finch harbored short tandem repeats, either $(GHQ)_3$ or $(GHQ)_5$, at the distal part of the C-terminal tail of AVT2R [18] (Fig. 1A). Moreover, variable number of tandem repeat (VNTR) polymerphisms were exclusively identified at the C-terminal tail of the MTR of east Eurasian thrushes (Passeriformes, Turdidae; Fig. 2A). A duplicated unit consisted of 18 amino acids (KSNSSSFVLSCRSPSHRS), with the tandem repeat number varying from 1 to 4. Global alignment with other vertebrates revealed that the duplicated element was highly conserved not only among avian species but also among major vertebrates. In particular, the KSXSSXFVLS motif was perfectly conserved in all vertebrate OXT-like receptors, except for the fish isotocin receptor (Fig. 2B). Furthermore, 4 species of east Eurasian thrushes (*Turdus pallidus*, *T. chrysolaus*, *T. obscurus*, and *T. celaenops*) showed intra-specific polymorphisms at this VNTR locus, showing variations in the number of repeat units. Alleles with 2 repeats were the most predominant (68~74%) in these 4 thrush species (Fig. 2C).

Potential effects of indel mutations on post-translational modifications

Palmitoylation is one of the post-translational modifications that impart significant effects on GPCR expression [26]. Several studies have been conducted to test the effect of palmitoylation on GPCR functions, signaling, and trafficking [27-29]. Previous studies on the human AVP V2 receptor, showed that the elimination of palmitoylation sites by mutagenesis resulted in a 30% reduction of

binding sites on the cell surface, suggesting that intracellular trafficking was impaired [30]. A handful of mutagenetic studies on GPCRs also showed the vital roles of palmitoylation in the proximal C-terminal tails [31], even though the patterns and effects of palmitoylation are GPCR-dependent [21]. Palmitoylation-deficient mice carrying 2 Cys ($Cys^{322-323}$) to Thr and Ser mutations in the rhodopsin receptor resulted in profound changes of light-induced retinal degeneration [32]. Moreover, the $Cys^{328-329}$ to Ser mutant in the mouse 5-hydroxy- $tryptamine_{4a}$ receptor exhibited enhanced receptor phosphorylation and altered desensitization by affecting β-arrestin mediated signaling [33]. These observations raise the possibility that different patterns of palmitoylated cysteine detected in 2 passerine birds regulate GPCR expression by changing physiological conditions such as the formation of lipid raft and protein-protein interactions.

The truncation experiments conducted in OXT-like receptors provide us with important insights into the role of the C-terminal tail in their physiochemical properties. Hoare *et al*. revealed that the effect of C-terminal truncation on rat OXTR function is highly site-dependent, and that the portion between 39 and 51 residues from the C-terminus is required for interaction with G_q protein [34]. Together with the other outcomes from truncation experiments on several GPCRs, their findings support the idea that a cytoplasmic helical segment (H8) extending from the most proximal part of C-tail to vicinal palmitoylated cysteine residue(s) plays critical roles in GPCR functioning [35]. Considering that the C-terminal regions of the OXTR show a high conservation rate among various vertebrates, similar signal transduction mechanisms may also occur in avian MTR. It is interesting though that the distal part of the C-terminal tails of vertebrate OXT/MT receptors have highly conserved amino acids; the pattern of amino acid substitutions in this region is far from random. If only H8 and palmitoylated cysteine residues are critical factors that are needed to regulate GPCR signal transmission, the distal parts of C-tails may be responsible for the other GPCR functions. It should be stressed that the distal part of the C-terminal tail is a serine rich-region and Ser^{369}, $Ser^{371-372}$, and Ser^{382} are completely conserved among all vertebrate OXT-like receptors, including the fish isotocin receptor (Fig. 2B). Mammalian and chicken somatostatin receptor subtype 2A (sst2A) has also been reported to contain conserved serine residues at the C-terminus, suggesting a site-specific pattern of phosphorylation [36]. Thus, the high degree of sequence identity in the distal part of the C-terminal tails indicates that substantial evolutionary pressure maintains phosphorylation sites. In our previous research, we found that serine residues in each of the tandemly duplicated elements are completely conserved among east Eurasian thrushes. This raises the question of whether aberrant elongation on the C-terminal tail affects the MTR functions at that level, which in turn affects their social behaviors in the natural environment. The allele frequency of these duplicated elements indicates that the allele with 2 repeats (i.e., duplicated once) is predominant among all thrushes, suggesting that no damaging effect acts on the duplication events; however, this cannot serve as evidence for the upregulation of MTR expression by tandem duplications. Further investigation are needed to clarify the effect of tandem duplications as a potential regulator of GPCR expression.

Future directions

Here, we present our attempts to identify intra- and inter-specific polymorphisms in various types of GPCRs in several avian species. There is a need to overcome several difficulties in associating these GPCR polymorphisms with phenotypic heterogeneities. Firstly, the physiochemical effects of structural mutations on GPCR expression should be examined at each polymorphic locus identified in our studies, either by site-direct mutagenetic replacement or by truncation experiments. With regard to the interindividual variability in response to neuropeptide hormones, we advocate the need for further approaches to examine how genetic variations in target genes influence physiological expression. This is analogous to the phermacogenetic approach that attempts to identify genetic variations in human OXTR genes that contribute to individual differences in social behavior and cognition [37]. Finally, it is essential to understand the evolutionary meaning of structural and physiological modifications in GPCRs, particularly in relation to its contribution to behavioral divergence. Avian species may be suitable for this research purpose because some avian taxa are highly diversified in terms of their behavioral features (e.g., feeding style, migration, and courtship behavior) even among the closely related species. In fact, Goodman *et al*. illustrated that a projection of OT antagonist had significant effects on group-size preferences in a flock of zebra finches, and that group-size preferences (gregarious or territorial) among finch species were partly explained by different patterns of MTR distributions across brain areas [38-40]. We need to examine the association between quantitative data on behavioral experiments and the genetic and physiological features on various GPCRs using model avian species such as chicken and zebra finch. Such data will provide a new piece of puzzle in understanding complex gene-brain-behavior relations in non-human vertebrates.

Conclusions

Our investigations have demonstrated that some avian

GPCRs have undergone drastic changes in the structure of their C-terminal domain. These changes are not random but appear to be correlated with various physio-biochemical processes such as palmitoylation and phosphorylation. However, it may be difficult to directly link the mutations identified in the present study with phenotypic variations in natural populations. Other physiological and biochemical approaches will provide supportive evidence that some of the structural modifications in the C-terminal domain of GPCRs would have a potential to become genetic factors affecting phenotypic traits.

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Conflict of interest

The authors declare that they have no conflict of interest.

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