

Revisiting the Gastrin-releasing Peptide/bombesin System: A Reverse-evolutionary Study Considering Xenopus

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Abstract

Bombesin is a putative antibacterial peptide isolated from the skin of the frog, *Bombina bombina*. Two related (bombesin-like) peptides, gastrin-releasing peptide (GRP) and neuromedin B (NMB) have been found in mammals. The history of GRP/bombesin discovery has caused little attention to be paid to the evolutionary relationship of GRP/bombesin and their receptors in vertebrates. We have classified the peptides and their receptors from the phylogenetic viewpoint using a newly established genetic database and bioinformatics. Here we show, by using a clawed frog (*Xenopus tropicalis*), that GRP is not a mammalian counterpart of bombesin and also that, whereas the GRP system is widely conserved among vertebrates, the NMB/bombesin system has diversified in certain lineages, in particular in frog species. To understand its derivation of GRP system in the ancestor of mammals, we have focused on the GRP system in *Xenopus*. Gene expression analyses combined with immunohistochemistry and Western blotting experiments demonstrated that GRP peptides and their receptors are distributed in the brain and stomach of *Xenopus*. We conclude that GRP peptides and their receptors have evolved from ancestral (GRP-like peptide) homologues to play multiple roles in both the gut and the brain as one of the 'gut-brain peptide' systems.

Introduction

The fourteen-amino acid peptide, bombesin, was initially described as a possible antibacterial peptide isolated from the skin of the European fire-bellied toad, *Bombina orientalis*, and was shown to have potent bioactivity in the mammalian nervous system^{1,2}. Subsequently, the mammalian bombesin-like peptides, gastrin-releasing peptide (GRP)² and neuromedin B (NMB)³, were isolated. GRP is a 27-amino acid peptide (29-amino acids in rodents) originally isolated from the porcine stomach as the mammalian equivalent of bombesin². Many studies have indicated that GRP is widely expressed in the central nervous system (CNS) in addition to the gastrointestinal tract in mammals^{4,5}. Because GRP could reproduce most of the biological effects of bombesin in many mammals, GRP had long been considered as the mammalian equivalent of amphibian bombesin⁵⁻⁷. Bombesin-like peptides appear to function *via* a family of three G protein-coupled receptors (GPCRs)⁸, namely the GRP-preferring receptor (GRPR or BB2 receptor)⁹, the NMB-preferring receptor (NMBR or BB1 receptor)¹⁰, and the potential orphan receptor, bombesin receptor subtype-3 (BRS-3 or BB3 receptor) in mammals¹¹. To date, in the mammalian CNS, it has been reported that the GRP system might be integral, through GRPR-mediated mechanisms⁹, in a variety of autonomic-related functions, including food intake^{12,13}, circadian rhythms¹⁴⁻¹⁶, fear memory consolidation¹⁷⁻¹⁹, male reproductive function²⁰, control of sighing²¹, and itch sensation^{22,23}.

In birds, the expression of orthologous genes for GRP and GRPR has been reported in the chicken CNS^{24,25}. To our knowledge, the only report of a central role of GRP in avian behavior is decreased feeding after the intracerebroventricular administration of GRP in chickens²⁶. In amphibians, however, little information is currently available on the function of the GRP/GRPR system in the CNS, because GRP has long been considered as the mammalian equivalent of bombesin⁵⁻⁷. Thus, the central role of GRP in

non-mammalian species remains unclear. However, it has been reported that frogs synthesize both GRP and bombesin, which are genetically distinct peptides, suggesting that GRP is not mammalian bombesin²⁷. In addition, molecular cloning analyses revealed that 3 classes of receptor subtypes were identified in the frog brain²⁸. Based on amino acid sequence, two of the classes were clearly the amphibian orthologous genes of the GRPR and NMBR, but not the BRS-3²⁸. Moreover, a fourth class (BB4) of receptor from *Bombina* was identified in amphibians and, interestingly, this had a higher binding affinity for bombesin than for either GRP or NMB²⁸.

As described above, understanding of the orthologous relationships among GRP/NMB/bombesin and their receptors in vertebrates is still in its infancy. Taking advantage of the recent availability of various non-model vertebrate genome information, in this study, we classified the peptides and their receptors from the viewpoint of phylogeny. In particular, to understand its derivation of GRP system from the ancestor of amniotes, we have focused on the GRP system that is widely conserved among vertebrates, and examined its expression in the anamniote clawed frog (*Xenopus tropicalis*) and contrasted it with bombesin, as amphibians represent an important place in the evolution of tetrapods and our understanding of bombesin and GRP.

Results

Sequence and structure of GRP and GRPR in *Xenopus*

To verify sequences of *GRP* and *GRPR* in *X. tropicalis*, we cloned cDNA encoding *GRP* and the *GRPR* in *X. tropicalis* and confirmed they are identical to the sequences registered in the GenBank (*GRP*: XM_018090834.1, *GRPR*: XP_002938295.1). The deduced amino acid sequence of *Xenopus* prepro-GRP reveals three major components: a signal peptide (31aa in *Xenopus*; 31aa in medaka fish; 27aa in chicks; 23aa in rats; 23aa in humans) (Fig. S1, gray box); this is followed by the bioactive GRP₁₋₂₉ (mature GRP, GRP₁₋₂₄ in medaka fish; GRP₁₋₂₇ in chicks; GRP₁₋₂₉ in rats; GRP₁₋₂₇ in humans) (Fig. S1, highlighted in pink), including a motif encoding a 10-amino acid-peptide called neuromedin C (NMC or GRP-10; GRP₁₅₋₂₄ in medaka fish; GRP₂₀₋₂₉ in chicks; GRP₂₀₋₂₉ in rats; GRP₁₈₋₂₇ in humans) at the carboxyl-terminus of mature GRP (Fig. S1, magenta box). The motif is highly conserved in vertebrates; and finally a carboxyl-terminal extension peptide termed pro-GRP₃₃₋₁₂₁ in *Xenopus* (pro-GRP₂₈₋₉₉ in medaka fish; pro-GRP₃₁₋₁₂₄ in chicks; pro-GRP₃₃₋₁₂₄ in rats; pro-GRP₃₁₋₁₂₅ in humans) (Fig. S1, black bar). The mature GRP in *Xenopus* shared high similarity, particularly at the identical [Ser²] form-NMC (GRP-10) region, with that in rodents and birds but not with the [Asn²] form-NMC in humans and fishes (Fig. S1). Peptide sequences of NMB are highly conserved among vertebrates, particularly 11 amino acids in the carboxyl-terminus are identical between human, rat, chick, medaka, and zebrafish (Fig. S1). Bombesin-like peptides have previously been identified in some frogs; *Rana catesbeiana*, *R. pipiens* (ranatensin)²⁹, *Alytes maurus* (alytesin)³⁰, *Phyllomedusa sauvagii* ([Leu⁸] form-phyllolitorin and [Phe⁸] form-phyllolitorin)³¹, *Bombina orientalis* ([Ser³, Arg⁹, Phe¹³] form-bombesin, [Phe¹³] form-bombesin, [Leu¹³] form-bombesin)

^{32,33} and *Bombina variegata* ([Phe¹³] form-bombesin, [Phe¹³] form-bombesin-like peptide, bombesin, and [His⁶] form-bombesin) ³⁴ (Fig. S1, highlighted in blue). Sequences of carboxyl-terminus regions of GRP, NMB and bombesin peptides are quite similar, 4 amino acids (W, A, G, M) are common between these peptides. On the other hands, those of signal peptide region and carboxyl-terminal extension peptide region were diversified between GRP, NMB, and bombesin, and also between the animals.

In terms of receptors for bombesin-like peptides, hydrophobicity analysis showed that *Xenopus* GRPR, NMBR, and BRS-3 contain seven transmembrane domains (TM1–7) as well as those in other vertebrates (e.g. human, rat, chicken, and zebrafish) (Fig. S2). Homology was highest in the hydrophobic domains, and the [Asp⁸⁵] in the second hydrophobic domain that has possibly been linked to ligand binding is well conserved in vertebrates ³⁵ (Fig. S2).

Phylogenetic analyses of GRP/NMB/bombesin and GRPR/NMBR/BRS-3

To clarify the relation among GRP/NMB/bombesin family, phylogenetic analysis of GRP/NMB/bombesin precursors (preprohormone) was performed (Fig. 1). Homologous genes for GRP/NMB/bombesin family peptides were found in gnathostomes (vertebrates except cyclostomes/agnathans). For the analysis, we used the deduced amino acid sequence in Mammalia *Homo sapiens* and *Rattus norvegicus*, Aves *Gallus gallus*, Reptilia *Gekko japonicus*, Amphibia *X. laevis*, *X. tropicalis*, *Nanorana parkeri* (frogs), *Rhinatrema bivittatum* and *Microcaecilia unicolor* (Caecilians), teleost fish *Danio rerio* and *Oryzias latipes*, cartilaginous fish *Callorhinchus milii* and *Rhincodon typus*, because their genomes have been decoded. In addition, the sequences of prepro-bombesin-like peptides, which have been reported for other frog species were also included (Fig. 1).

The prepro-GRP/NMB/bombesin sequences were divided into two major clades: GRP and NMB/bombesin clades (Fig. 1). A single GRP gene was found in almost all animals examined, although *X. laevis* which is allotetraploid has one gene on each of chromosome L and S (Fig. 1, pink box). The NMB/bombesin clade was further divided into the sub-clades: the NMB clade (Fig. 1, yellow box) and the bombesin clade (Fig. 1, blue box); the bombesin clade and NMB clade were found only in frogs; and all the other animals including caecilians, respectively. *X. tropicalis* and *Nanorana parkeri* have single bombesin gene, and *X. laevis* has the gene on each of chromosome L and S, but these frogs do not possess any genes of the NMB group (Fig. 1). In addition, all the precursors of bombesin-like peptides which have previously been identified in other frogs: one in *Rana catesbeiana*, *R. pipiens*, and *Alytes maurus*; two in *Phyllomedusa sauvagii*; three in *Bombina orientalis*; and four in *Bombina variegata* ³⁴ were also included in the bombesin clade (Fig. 1).

These results indicate two possibilities for the evolution of NMB/bombesin: one is the specialization of NMB into bombesin in the frog lineage; the other is the divergence into NMB and bombesin clades resulting, respectively, in the undetectability of frog NMB and the disappearance of bombesin in vertebrates other than frogs. We therefore examined synteny in genes surrounding the amphibian

NMB/bombesin locus (Fig. 2). Comparison of the genome of *X. tropicalis*, *Nanorana parkeri*, *Microcaecilia unicolor* and *Rhinatrema bivittatum*, indicates that the order of genes around the caecilian NMB genes and the frog bombesin genes were highly conserved, although an inversion of the *ZNF11 - SEC592A - bombesin - KT112* region of *X. tropicalis* genome has occurred. Thus, it can be concluded that bombesin and NMB are respective orthologues and that specialization of the NMB sequence in the frog lineage resulted in bombesin (Fig. 2).

We also performed phylogenetic analysis of the receptors for the GRP/NMB/bombesin family; GRPR, NMBR and BRS-3 (Supplemental Fig. 3). The results suggest that Gnathostomata basically have orthologues in each of the three groups with no specialization in the frog lineage as seen in NMB/bombesin. The BB4 receptor in *Bombina* also belongs to the mammalian BRS-3 group. In addition, the conservation of GRPR and NMB, BRS3 was not found in teleosts (e.g. *Danio rerio* and *Oryzias latipes*) and cartilaginous fish (e.g. *Callorhynchus milii* and *Rhincodon typus*), but only in archaic fish such as *Latimeria chalumnae* (coelacanth), *Erpetoichthys calabaricus* (reedfish), *Lepisosteus oculatus* (gar), and *Acipenser ruthenus* (sturgeon) (Supplemental Table 1).

Reverse transcription (RT)-PCR of GRP and GRPR mRNA in *Xenopus*

In contrast to the diversification of NMB/bombesin in the frog lineage and the loss of the BRS-3 gene in some fish lineages, GRP and the GRPR are widely conserved in vertebrates. In this study, we used frogs to investigate the principal (conserved, original) role of these bombesin-family systems. We confirmed the expression of *GRP* and *GRPR* mRNA in a variety of *Xenopus* tissues (brain, spinal cord, heart, lung, and stomach) by RT-PCR. Bands were detected at the expected sizes for *GRP* and *GRPR* genes in the brain (Fig. 3). *GRP* mRNA was highly expressed in the brain, spinal cord, stomach, and weakly expressed in the lung (Fig. 3; *upper panel*). Although *GRPR* mRNA was detected in all tissues, the expression level was low in the heart (Fig. 3; *middle panel*). As the internal control in *Xenopus*, nearly equivalent amounts of *GAPDH* cDNA were amplified from RNA preparations among these tissues, which showed that no significant RNA degradation had occurred and a proper RT was obtained (Fig. 3; *bottom panel*).

Real-time quantitative PCR (qPCR) of GRP and GRPR mRNA in *Xenopus* CNS

To quantify the *GRP* and *GRPR* expression at the transcription level, we performed real-time qPCR analyses for four parts of the CNS of males and females: the telencephalon; the diencephalon/mesencephalon/pons/cerebellum; the medulla oblongata; and the spinal cord. Although *GRP* and *GRPR* mRNA expression was detectable in these all tissues of both sexes, no sex differences and no interactions between sex and tissue were detected in any of the tissues we examined (black bars indicate means of males, magenta bars indicate means of females) (Fig. 4). Thus, in the comparisons of

expression level between tissues, sexes were combined. The expression of *GRP* mRNA was higher in the diencephalon/mesencephalon/pons/cerebellum and the medulla oblongata, than in the telencephalon and the spinal cord (Fig. 4a). In contrast, the expression of *GRPR* mRNA was the highest in the spinal cord, and the lowest in the telencephalon (Fig. 4b).

Distribution of GRP in *Xenopus* CNS

The expression of GRP was next localized in *Xenopus* CNS. The examination of transverse (from rostral to caudal) brain and spinal cord sections revealed the presence of many cell bodies and fibers of GRP-immunoreactive (+) neurons in *Xenopus* CNS. The overall neuroanatomical distribution of GRP⁺ neuronal cell bodies and their fiber projections is schematically summarized in Table 1. The specificity of the GRP antiserum reactivity was confirmed by control absorption experiments in which the primary rabbit antiserum against *Xenopus* GRP₂₀₋₂₉ was preabsorbed with an excess of *Xenopus* GRP₂₀₋₂₉ antigen peptide ([Ser²] form-NMC); in these experiments no immunostaining was seen (Supplemental Fig. 4). In the spinal cord, GRP⁺ fibers and numerous varicosities were found throughout the spinal grey matter area (Supplemental Fig. 4a, b, d, and e). A cluster of GRP⁺ cell bodies was located mainly in the dorsal field of spinal grey in the cervical spinal cord (df; Supplemental Fig. 4a, b, and e). In the thoracic and lumbosacral spinal cord, similar GRP⁺ fibers were frequently observed, but few GRP⁺ cell bodies could be detected.

Table 1

Localization of gastrin-releasing peptide (GRP)-immunoreactive cell bodies and fibers in *Xenopus* central nervous system.

Brain area		Cell bodies	Fibers
Acc	nucleus accumbens	++	+
Apl	amygdala pars laterails	++	+
Apm	amygdala pars medialis	++	+
Av	anteroventral tegmental nucleus	-	+
ca	commissura anterior	-	-
Cb	nucleus cerebelli	-	++
df	dorsal field of spinal grey	++	+++
Ea	nucleus entopeduncularis anterior	-	+
fr	fasciculus retroflexus	-	+
ftg	fasciculi tegmentales	-	++
Hbv	nucleus habenularis ventralis	-	+
lfm	nucleus interstitialis of flm	-	+
lf	lateral field of spinal grey	-	++
lfb	lateral forebrain bundle	-	++
ll	lemniscus lateralis	-	++
mfb	medial forebrain bundle	-	++
Mg	nucleus preopticus magnocellularis	-	++
mmf	medial motor field of spinal grey	-	++
Mp	Medial pallium	-	-
ola	tractus olfactorius accessorius	+	+
optb	tractus opticus basalis	-	++
optl	tractus opticus lateralis	-	++
Pb	nucleus parabrachialis	-	+
Poa	nucleus preopticus anterior	-	++
Pv	posteroventral tegmental nucleus	-	+
Ra	nucleus raphes	+	++

Brain area		Cell bodies	Fibers
Ri	nucleus reticularis inferior	+	-
Ris	nucleus reticularis isthmi	-	++
Rm	nucleus reticularis medius	-	++
SC	nucleus supraquiasmaticus	-	+
Strd	striatum, dorsal part	-	-
Strv	striatum, ventral part	-	+
TP	tuberculum posterius	+++	+
trVds	tractus descendens nervi trigemini	-	+
VH	nucleus hypothalamicus ventralis	-	-
VL	nucleus ventrolateralis thalami	-	++
vlf	ventrolateral field of spinal grey	-	++
VLs	superficial ventral nucleus	-	+
VM	nucleus ventromedialis thalami	++	++
vmf	ventromedial field of spinal grey	-	++
vt	ventriculus tertius	-	+
Vds	nucleus descendens nervi trigemini	-	++
VIIIv	nucleus ventralis nervi vestibulocochlearis	-	+
IXm	nucleus motorius nervi glossopharyngei	-	+
Xm	nucleus motorius nervi vagi	++	+
XII	nucleus motorius nervi hypoglossi	-	+
The relative density of labeling was classified as weak (+), moderate (++), and strong (+++). cc: central canal; LV: lateral ventricle; 3V: third ventricle.			

In the telencephalon, abundant but weakly GRP-immunoreactive cell bodies and fibers were located throughout the ventral telencephalic area, *e.g.* cell bodies and fibers in the nucleus accumbens (Acc) and thin fibers in the tractus olfactorius accessorius (ola) (Fig. 5b). Immunoreactive cell bodies were abundant in the amygdala pars medialis (Apm; Fig. 5c), and in the amygdala pars lateralis (Apl; Fig. 5d). In the diencephalon, a small number of labeled cells were located in the nucleus ventromedialis thalami (VM; Fig. 5e). In the hypothalamus, many intensely labeled cell bodies and fibers were found in the tuberculum posterius (TP; Fig. 5f). In the brainstem, large but only weakly immunoreactive cell bodies and fibers were found in the nucleus raphes (Ra; Fig. 5g).

GRP⁺ fibers were widely distributed throughout the CNS (Table 1). A fine network of GRP⁺ fibers was also observed in the Apm, in the Apl, and prominently in the striatum, pars ventralis (Strv; Fig. 6b). GRP⁺ fibers were also found in some diencephalic nuclei surrounding the third ventricle; e.g. VM (Fig. 5e), nucleus preopticus anterior (Poa; Fig. 6c), and nucleus preopticus magnocellularis (Mg; Fig. 6d). A weak distribution of GRP⁺ fibers was also found in the area near the tractus opticus lateralis (optl), the tractus opticus basalis (optb; Fig. 6e), and the nucleus reticularis isthmi (Ris; Fig. 6f). In the posterior part of the medulla oblongata, the network of GRP⁺ fibers spreading in the nucleus descendens nervi trigemini (Vds) was intensely immunoreactive and such fibers were also scattered in the tractus descendens nervi trigemini (trVds; Fig. 6g).

Expression of GRPR protein in *Xenopus* CNS

Western immunoblot analysis with the polyclonal antiserum against *Xenopus* GRPR was performed to determine the presence of GRPR protein in homogenates derived from the brain and spinal cord of adult male *Xenopus*. An intense protein band was observed in the brain and spinal cord extracts, and its electrophoretic mobility was located at ~ 43 kDa, which is the expected molecular weight of *Xenopus* GRPR (Fig. 7). Preabsorption of the antiserum with an excess of antigen peptides (50 µg/mL) prevented the immunostaining of the ~ 43-kDa protein band in the brain and spinal cord (Fig. 7). Immunoblot analyses were repeated independently three times by using different three frogs and gave similar results.

Discussion

GRP was first identified from the porcine stomach as a mammalian orthologue of the anuran bombesin². Thus, GRP has long been considered as the mammalian equivalents of bombesin⁵⁻⁷. It has also been reported that frogs have independent genes for both GRP and bombesin, and this raises the possibility that mammals have an as yet uncharacterized gene encoding a true mammalian bombesin²⁷. *Bombina* was shown to express the genes for bombesin in the brain, stomach and skin, and for GRP in the brain and stomach only, respectively²⁷. It suggests that bombesin and GRP are distinct peptides which could have different physiological functions in amphibians. However, due to the historical background of the GRP discovery^{1,2}, little attention has yet been paid to the evolutionary relationship of GRP/bombesin and of its receptors in vertebrates. Our phylogenetic analysis indicates that GRP/NMB/bombesin can be divided into two clades; GRP and NMB/bombesin clades (Fig. 1). We further found by using synteny analysis that bombesin and NMB are relative orthologues and that specialization of the NMB sequence only in the frog lineage resulted in bombesin (Fig. 2). In *Bombina*, a bombesin function has been suggested to be an antibacterial peptide or toxin secreted from the skin¹. Frogs may have started to produce bombesin in the skin as exocrine secretions to protect themselves from bacterial infection and/or predators in the terrestrial adaptation. To our knowledge, this is the first demonstration of the gene

divergence of bombesin and bombesin-like peptides in vertebrates, which appeared to be divided into the two families (*i.e.* GRP and NMB/bombesin) from the vertebrate ancestor (Fig. 8).

For bombesin-like peptide receptors, we found that most gnathostomes have an orthologous gene in each of the three groups (*GRPR/NMBR/BRS-3*), although BRS-3 was not found in Teleostei and Chondrichthyes (Supplemental Fig. 3). This suggests that *GRPR/NMBR/BRS-3* diverged into three branches in the ancestor of gnathostomes but that the BRS-3 genes have been lost in Teleostei and Chondrichthyes lineages. Otherwise, it is possible that there were two genes for GRPR and NMBR respectively at the divergence of cartilaginous fish, and that the BRS-3 gene then appeared by duplication of the GRPR gene or NMBR gene in the ancestor of Sarcopterygii while the BRS-3 gene was lost in teleost fish. If this is the case, the amino acid sequence of BRS-3 appears to be specialized, considered from the standpoint of the phylogenetic tree. In addition, no specialization for the receptors was observed in the frog lineage as seen in bombesin-like peptides. In mammals, despite the molecular characterization of BRS-3, determination of its function has been difficult as a result of its low affinity for GRP and NMB and its lack of an identified natural ligand^{36,37,38}. Because the natural ligand (corresponding to bombesin) for this receptor has never been identified in mammals, birds, or reptiles, BRS-3 is currently considered to be an orphan receptor³⁶⁻³⁸. BRS-3-deficient mice develop a mild obesity, associated with hypertension and impairment of glucose metabolism³⁹. These results indicate that BRS-3 is required for the regulation of endocrine processes responsible for energy balance in mammals³⁹. Knockout mouse studies have also demonstrated that neither *GRPR* nor *NMBR* is on its own essential⁹, suggesting that these three bombesin-like peptide receptors can compensate for each other's function in mammals. It is often the case that a number of GPCRs with different affinities can be coupled to just one neuropeptide. Recently, it was reported that, in placental mammals, BRS-3 has lost its binding affinity for NMB/GRP and is constitutively active in a ligand-independent manner, in contrast to BRS-3 in non-placental vertebrates including *Xenopus*, which has significant affinity for NMB/GRP³⁷. Particularly in *Bombina*, BRS-3 was suggested to be a 'bombesin-preferring receptor'²⁸. This rather promiscuous relationship between GPCR and ligand could be important in the diversity of cellular functions controlling different life phenomena.

Despite the diversification of NMB/bombesin in the frog lineage and the loss of the BRS-3 gene in some fish lineages, GRP and GRPR are conserved through vertebrates. Peptides synthesized in endocrine cells of the gastrointestinal tract and in neurons are traditionally considered not only as modulators of metabolism, energy balance, appetite, *etc.*, but also as neuromodulators (neuropeptides); so called '*gut-brain peptides*' in mammals⁴⁰. GRP appears to be one of gut-brain peptides, and the GRP system might play multiple roles in both the gut and the brain⁵. Indeed, Holmgren *et al.*⁴¹ reported immunohistochemical evidence that the myenteric plexus and circular muscle layer of the stomach of the mudpuppy (*Necturus maculosus*), a salamander, are richly innervated by GRP⁺ fibers, and Kim *et al.*⁴² reported that endogenous GRP potently stimulates the contraction of longitudinal muscle and relaxes circular muscle in *Xenopus* stomach, suggesting that GRP is important in the regulation of gastric motility in *Xenopus*. Furthermore, in *Xenopus*, we found expression of the mRNA for both *GRP* and *GRPR* in the brain and stomach, suggesting that *Xenopus* GRP systems play physiological roles locally in the

CNS as well as in the stomach. In addition, our qPCR analysis suggests that, in *Xenopus* CNS, the expression of *GRP* mRNA is highest in the brain, whereas *GRPR* mRNA expression is highest in the spinal cord. Our immunohistochemical analysis shows that GRP⁺ cell bodies are distributed in several telencephalic, diencephalic, and rhombencephalic regions and spinal cord of *Xenopus* (see Table 1). In particular, we observed many GRP⁺ cell bodies in the hypothalamus and putative limbic system in *Xenopus*, which corresponds well with the mammalian case⁵. GRP⁺ fibers were also distributed widely throughout the CNS of *Xenopus* (Table 1). Thus, GRP might play an important role in multiple physiological functions in *Xenopus* CNS. In mammals, it is reported that orofacial pruriceptive inputs are processed mainly in the superficial layers of the trigeminal sensory nucleus caudalis in the medulla oblongata, which is similar to the spinal dorsal horn⁴³. Therefore, GRP/GRPR signaling in the trigeminal ganglion-trigeminal sensory nuclei of the medulla oblongata appears to play an important role in orofacial itch sensation in mammals^{44,45}. We also found that, in *Xenopus*, abundant GRP⁺ fibers are distributed in the trVds and Vds areas, which appear to correspond to the trigeminal somatosensory system in mammals. Thus, GRP may modulate neurotransmission and integration of somatosensory information in *Xenopus*. Taken together, these results indicate that GRP functions not only as a gastrointestinal bioactive peptide but also as a neuropeptide in *Xenopus* CNS, and that GRP functions as a gut-brain peptide in both amphibians and mammals.

In conclusion, GRP has long been considered as the mammalian equivalents of bombesin^{6,7} (Fig. 8a). We now demonstrate, by phylogenetic and synteny analyses, that GRP is not a mammalian orthologue of bombesin, and that the GRP system is widely conserved throughout vertebrates, whereas the NMB/bombesin system diversified in some lineages (Fig. 8b). Furthermore, we demonstrate that the GRP system might play multiple roles both in the gut and in the brain of amphibians as one of the 'gut-brain peptide' systems.

Indeed, it has recently been reported that the expression of the common ancestral genes for *GRP/NMB/bombesin* (possibly *GRP* as an ancestral gene) in amphioxus (which belongs to the subphylum Cephalochordata, an extant representative of the most basal chordates) is abundant in the gut, and is also observed in the cerebral vesicle that has been proposed as the homologue of the vertebrate brain⁴⁶. However, the original roles of GRP as an ancestral gene remains unclear at the moment and further functional studies by using lower vertebrates are needed to draw a firm conclusion.

Methods

Animals

Male and female adult clawed frogs (*X. tropicalis*), the Nigerian BH (Golden) strain, were provided by Hiroshima University Amphibian Research Center through the National BioResource Project (NBRP) of the Japan Agency for Medical Research and Development (AMED). Frogs were maintained in same sex groups according to well established protocols. Sexually matured males (average ~ 4 cm in body length)

and females (average ~ 5 cm in body length) (0.5–2 years old in both sexes) were used in this study (Igawa et al., in preparation).

Phylogenetic analysis and gene synteny analysis

For a molecular phylogenetic analysis of precursors of GRP/NMB/bombesin family peptides and GRPR/NMBR/BRS3, protein sequences were obtained from NCBI protein database. Multiple alignments were produced with CLUSTALX (2.1) with gap trimming⁴⁸. Sequences of poor quality that did not well align were deleted using BioEdit⁴⁹. Phylogenetic analyses were performed using the Neighbor-Joining method⁵⁰ by CLUSTALX with the default parameters (1,000 bootstrap tests and 111 seeds).

Representative phylogenetic trees were drawn by using NJ plot⁵¹. Signal peptide site in GRP/NMB/bombesin and transmembrane domain in GRPR/NMB/BRS-3 were predicted by SignalP-5.0⁵² and TMHMM2.0 program⁵³.

For comparison of gene synteny around the NMB/bombesin gene in the amphibian genome, the genome regions upstream and downstream of NMB/bombesin genes were examined using assembled genome sequences of aMicUni1.1 (GCF_901765095.1) for *Microcaecilia unicolor*, aRhiBiv1.1 (GCF_901001135.1) for *Rhinatrema bivittatum*, ASM93562v1 (GCF_000935625.1) for *Nanorana parkeri*, *X. tropicalis_v9.1* (GCF_000004195.3) for *X. tropicalis*.

Genome and amino acid sequences of *Homo sapiens*, *Rattus norvegicus*⁵⁴, *Gallus gallus*⁵⁵, *Gekko japonicus*⁵⁶, *X. laevis*⁵⁷, *X. tropicalis*⁵⁸, *Nanorana parkeri*⁵⁹, *Rhinatrema bivittatum*^{60,61}, *Microcaecilia unicolor*, *Danio rerio*⁶², *Oryzias latipes*⁶³, *Callorhinchus milii*⁶⁴, *Rhincodon typus*⁶⁵, *Rana catesbeiana*, *Rana pipiens*²⁹, *Alytes maurus*³⁰, *Phyllomedusa sauvagii*³¹, *Bombina orientalis*^{32,33}, *Bombina variegata*³⁴, *Latimeria chalumnae*^{66,67}, *Lepisosteus oculatus*⁶⁹, *Branchiostoma floridae*⁷⁰, *Octopus bimaculoides*⁷¹ and *Drosophila melanogaster*⁷² used for the analysis were obtained from GenBank. Accession IDs were shown in Supplemental Table 1.

cDNA cloning of GRP and GRPR in Xenopus

Adult male frogs ($n = 2$) were anesthetized with 1% MS-222 (tricaine, Sigma-Aldrich, St. Louis, MO) and sacrificed by decapitation. Immediately, dissected tissues (hypothalamus, spinal cord, and stomach) from frogs were fixed with RNAlater solution (Ambion, Austin, TX) and stored at -30°C until RNA extraction. Total RNA was extracted from samples using the illustra RNAspin Mini RNA Isolation kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. The concentration of total RNA was measured using a Qubit RNA assay kit (ThermoFisher Scientific, Waltham, MA). In order to identify genomic sequences of *Xenopus* GRP and GRPR, the 1st cDNA was synthesized from 200 ng of total RNA of stomach origin by using oligo-dT primers and an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The sequences of primers for cDNA cloning were designed based on the gene sequences in GenBank (see Supplemental Table 1 and Supplemental Table 2). The resulting RT-PCR

amplicons of the stomach sample (full open reading frame sequence for *GRP* or partial sequence for *GRPR*) were subcloned into the pGEM-T easy vector (Promega, Madison, WI) followed by transfection into *Escherichia coli* DH5 α competent cells (Takara Bio, Shiga, Japan). Positive clones were identified by blue-white screening and at least three positive clones were sequenced. The primer sequences used in this study are shown in Supplemental Table 2.

RT-PCR

To determine the tissue distribution of *GRP* and *GRPR* mRNA in *X. tropicalis* ($n = 3$ of each sex), RT-PCR analysis was performed. Total RNA was extracted from various tissues (brain, spinal cord, heart, lung, stomach), using illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). First-strand cDNA was synthesized from 200 ng of total RNA in a 20- μ L reaction volume using oligo-dT primers and iScript RT (Bio-Rad Laboratories). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Primer pairs are shown in Supplemental Table 2. The resultant PCR amplicons were electrophoresed on 2% agarose gels. RT-PCR studies were repeated four times using independently extracted RNA samples from different animals. Consistent results were obtained from each run.

Real-time qPCR

The quantification of *GRP* and *GRPR* in *Xenopus* CNS was performed using real-time qPCR. In brief, whole brains and spinal cords of males or females were quickly removed and placed on ice ($n = 3$ of each sex). Brains were divided into 3 parts: 1 the telencephalon; 2 the diencephalon/mesencephalon/pons/cerebellum; and 3 the medulla oblongata. The preparations were carefully dissected under a dissecting microscope (Olympus, Tokyo, Japan). Total RNA was isolated from samples using illustra RNAspin Mini RNA Isolation Kit with RNase-free DNase I (GE Healthcare) according to the manufacturer's protocol. Samples were reverse transcribed from 500 ng of total RNA in a 20 μ L volume using iScript cDNA synthesis kit (Bio-Rad Laboratories). Real-time qPCR was carried out on a C1000™ Thermal Cycler (Bio-Rad Laboratories). Reactions were performed in 20 μ L solution, with 200 nM of each primers, 1 μ L of 5 ng cDNA samples and SYBER-green master mix (KAPA SYBER FAST qPCR kit, KAPA Bio-systems, Boston, MA, USA) according to the manufacturer's instructions. Assays (in triplicate) were repeated at least twice with the constitutive *Droscha* as a normalizing control. Primer pairs are shown in Supplemental Table 2. The data relative changes in mRNA expression were determined using the $2^{-\Delta\Delta CT}$ method. Statistical tests were performed using linear mixed model with Bonferroni correction for multiple comparisons by IBM SPSS ver. 24 for Windows (IBM, Armonk, NY). The fixed effects factors are sex and tissue, and the random effects factor is individual.

Immunohistochemistry

Adult male frogs ($n = 4$) were killed by decapitation under deep anesthesia (see above). Brains and spinal cords were immediately dissected out and immersion-fixed in Bouin's fixative solution [saturated picric acid:10% unbuffered formalin:acetic acid = 15:5:1 (v/v)] overnight at 4 °C. Subsequently, tissues were dehydrated and embedded in paraffin wax. Serial sections of each tissue were cut transversely on a

microtome at 10- μ m in thickness. We performed immunohistochemical analysis according to our established methods²⁰. In brief, endogenous peroxidase activity was eliminated from the sections by incubation in a 0.1% H₂O₂ in absolute methanol solution for 20 minutes followed by three 5-minute rinses with phosphate-buffered saline (PBS) (pH 7.4). After blocking nonspecific binding with 1% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100 for 30 minutes at room temperature, sections were then incubated in Can Get Signal A (TOYOBO) with a 1:2,000 dilution of primary rabbit antiserum against rat GRP₂₀₋₂₉, a 10-amino acid-peptide called NMC or GRP-10 (AssayPro, St. Charles, MO, RRID: AB_2571636) for 48 hours at 4°C. This GRP antiserum has previously been shown to be specific for rodent GRP^{20,45}. The amino acid sequence of NMC (epitope residues) is identical between frogs and rodents. Immunoreactive-products were detected with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine (DAB) development according to our previous method²⁰. The histology of *Xenopus* brain was studied by the Nissl (cresyl violet; Muto Pure Chemicals, Tokyo, Japan) staining. GRP-expressing cells in the CNS were observed under the Olympus FSX100 microscope. Nomenclature of brain areas and nuclei was based on the stereotaxic atlas of the CNS of anurans⁷³.

Control procedures for the DAB method were performed using pre-absorption of the working dilution (1:2,000) of the primary antiserum with saturating concentration of frog GRP₂₀₋₂₉ antigen peptide (GSHWAVGHLM; 50 μ g/mL: ²[Ser]-NMC, produced in AnaSpec; San Jose, CA) overnight at 4 °C before use. The information of GRP antiserum used in this study is shown in Supplemental Table 3.

Western immunoblotting

Western blotting was conducted according to our previously described methods⁷⁴. In brief, adult male frogs ($n = 3$) were sacrificed by decapitation under deep anesthesia (see above). Brains and spinal cords were quickly removed and placed on ice, and were snap-frozen immediately in liquid nitrogen. The preparations (100 μ g protein/lane) were boiled in 10 μ L sample buffer containing 62.5 mM trishydroxymethyl-aminomethane-HCl (Tris-HCl; pH. 6.8), 2% SDS, 25% glycerol, 10% 2-mercaptoethanol, and a small amount of bromophenol blue. Samples were run on a 4–20% SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) using a semidry blotting apparatus (Bio-Rad Laboratories). Membranes were blocked with PVDF Blocking Reagent for Can Get Signal (TOYOBO, Tokyo, Japan) for 30 minutes at room temperature and incubated for 1 hour at room temperature in Can Get Signal Solution 1 (TOYOBO) with a 1:100,000 dilution of rabbit polyclonal antiserum against GRPR (RRID: AB_2832951). The polyclonal antiserum was produced in a rabbit using a *Xenopus* GRPR fragment peptide. The sequence of antigen peptide was 195–208 amino acid residues (CAPYPHSNGLHPRI) of *Xenopus* GRPR (GenBank accession No. XP_002938295.1). Blotted membranes were washed three times with 0.05% Tween 20 in Tris-HCl buffered saline (TBST) and incubated with horseradish peroxidase-conjugated goat polyclonal antibody against rabbit IgG (Bio-Rad Laboratories) at 1:10,000 dilution in Can Get Signal Solution 2 (TOYOBO) for 1 hour at room temperature. After washing for three times with TBST, blots were visualized by Immun-Star WesternC Chemiluminescence Kit (Bio-Rad Laboratories). Images of the different immunoblots were slightly adjusted in brightness and contrast to provide a uniform background. Control procedures were performed by pre-absorption of the working

dilution (1:100,000) of the primary antiserum with a saturating concentration of *Xenopus* GRPR₁₉₅₋₂₀₈ antigen peptide, CAPYPHSNGLHPRI (50 µg/mL, produced in Sigma-Aldrich) overnight at 4 °C before use. The information of GRPR antiserum used in this study is shown in Supplemental Table 3.

Ethics statement

All experimental procedures were approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Okayama University (Okayama, Japan. <http://www.cc.okayama-u.ac.jp/~animal/committee.html>), although an individual ID of approval was not provided because we used only amphibians for the experiments in this study. All efforts were made to minimize animal suffering and reduce the number of animals used in this study. The study design including experimental animals (strain, sex, age and sample size), experimental procedures and statistical methods is described in compliance with the ARRIVE guidelines.

Declarations

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Author Contributions

A.H., D.F., K.T., T.O. and Y.K. performed immunohistochemical experiments. A.H. and K.T. performed Western blotting. A.H., M.H., Y.K., and T.O. performed molecular biology, phylogenetic, and gene synteny analyses. T.S. interpreted the data and provided the advice. M.H. and H.S. wrote the paper. A.H. and M.H. contributed equally to this study. H.S. supervised the whole study. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Competing interests

The authors declare no conflict of interest.

Data availability statement

All relevant data are within the manuscript and the data that support the findings of this study are available from the corresponding author upon request.

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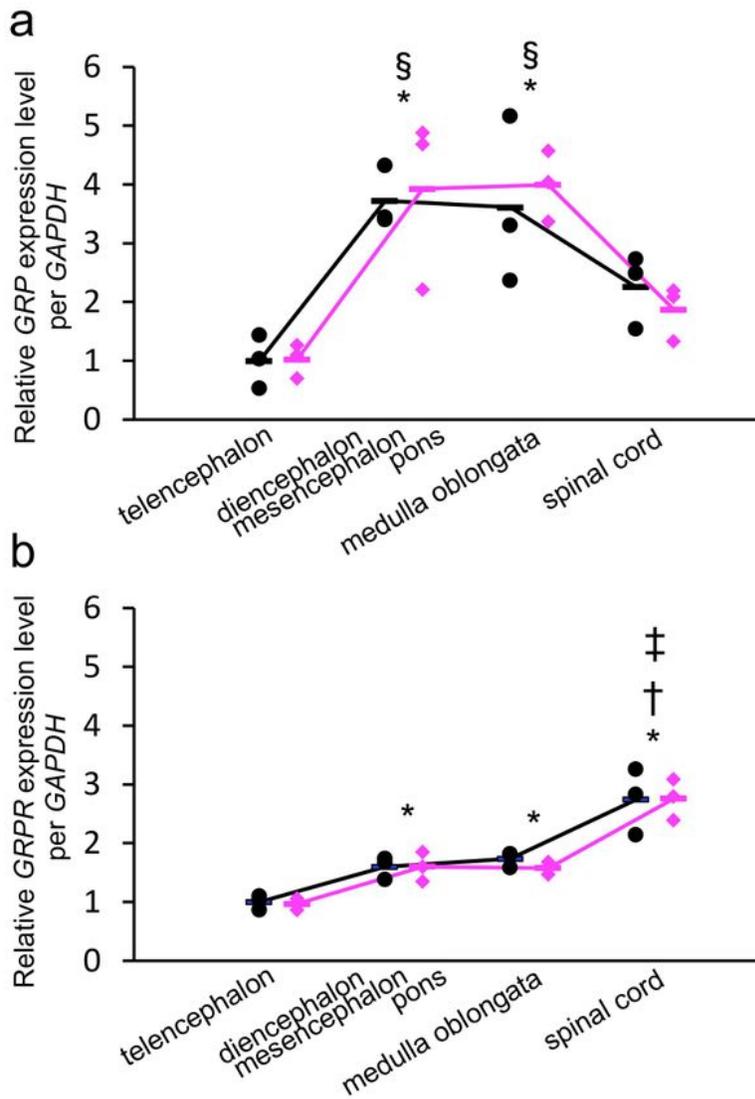
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Figures

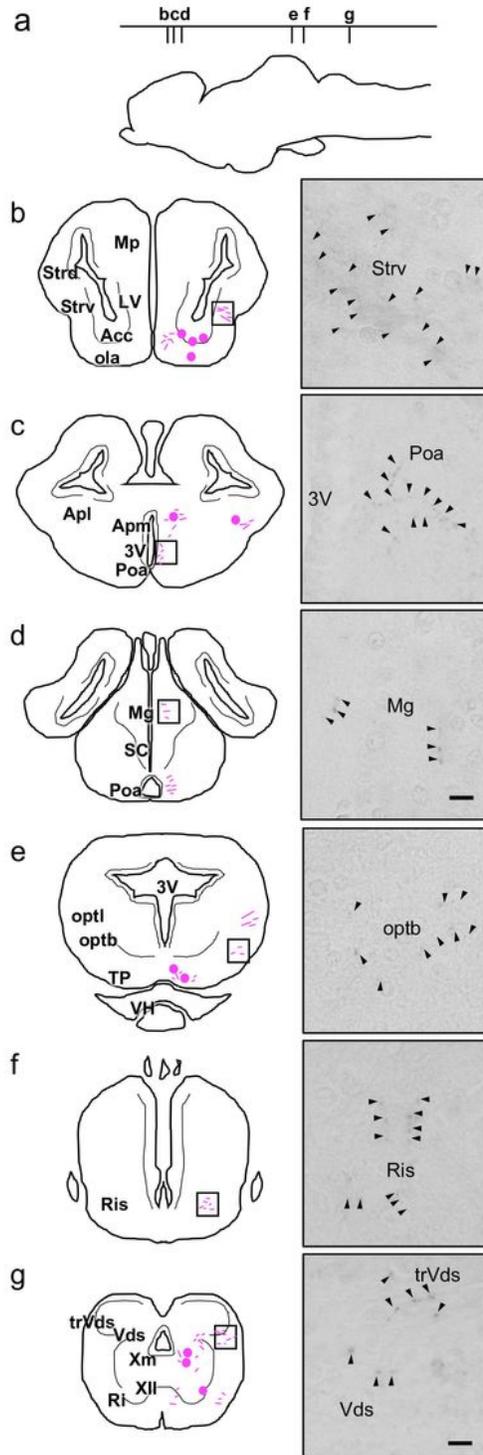


Hirooka *et al.*, Figure 4

Figure 4

The expression levels of gastrin-releasing peptide (GRP) and GRP receptor (GRPR) mRNA in *Xenopus* central nervous system were measured by the real-time quantitative PCR. Relative expression levels of GRP (a) and GRPR (b) in the central nervous system: the telencephalon, the diencephalon/mesencephalon/pons/cerebellum, the medulla oblongata, and the spinal cord of males and females were analyzed. P values indicate statistical tests using linear mixed model with Bonferroni

correction for multiple comparisons (vs. telencephalon, *P < 0.01; vs. diencephalon/mesencephalon/pons/cerebellum, †P < 0.01; vs. medulla oblongata, ‡P < 0.01; vs. spinal cord, §P < 0.05). No sex differences and no interactions between sex and tissue were detected. Dots and bars indicate values of each sample and means of the samples, respectively. Black: males, magenta: females.



Hirooka *et al.*, Figure 6

Figure 6

Fibers in *Xenopus* brain immunohistochemically labeled with the anti-gastrin-releasing peptide (GRP) antiserum. (a) Letters (b–g) in the schematic lateral profile of the brain indicate the rostrocaudal level of each transverse section. In the schematic sections (left panels), immunoreactive cell bodies and fibers in *Xenopus* brain are shown by magenta circles and magenta lines, respectively. In the micrographs of immunohistochemical staining for GRP (right panels), a fine network of GRP-immunoreactive fibers is observed in the striatum, ventral part (Strv; b). GRP-immunoreactive fibers are also observed in the preoptic area (Poa; c) and in the nucleus preopticus magnocellularis (Mg; d). A weak distribution of GRP-immunoreactive fibers is also found in the area near the tractus opticus basalis (optb; e), and the nucleus reticularis isthmi (Ris; f). In the posterior part of the medulla oblongata, GRP-immunoreactive fibers are observed in the nucleus descendens nervi trigemini (Vds) and in the tractus descendens nervi trigemini (trVds; g). Scale bars = 50 μ m, 10 μ m in enlarged images. Arrowheads indicate representative GRP-immunoreactive fibers. For abbreviations, see Table 1.