Cloning and characterization of two subunits of calcineurin cDNA in naked carp (*Gymnocypris przewalskii*) from Lake Qinghai, China

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Abstract: The naked carp (*Gymnocypris przewalskii*), a native teleost, plays an important role in maintenance of the ecological balance in the system of Lake Qinghai (altitude, 3.2 km) on the Qinghai-Tibet Plateau in China. Calcineurin (CN) is the only member of the serine/threonine phosphatase family that can be activated by both Ca²⁺ and calmodulin (CaM) and involved in many important physiological processes such as salt tolerance/adaptation. In this report, cDNAs of CN catalytic subunit paralogue isoforms: *GpCAa* (GenBank accession no. JQ407043), *GpCAg* (GenBank accession no. JQ407043), and CN regulatory subunit (*GpCB*) (GenBank accession no. JQ410473), were isolated from *Gymnocypris przewalskii* and their expression patterns in embryos development were characterized. Gene expression profile demonstrated that *GpCA* and *GpCB* mRNA was distributed ubiquitously in all embryonic stages and showed decline until final stage of development. Immunohistological analysis revealed CN localization in different tissues including kidney, heart, brain, spermary, and gill. Collectively, these results provide molecular basis and clues to further understand the role of CN during embryos development and its function in tissues for the adaptation mechanism of naked carp. *(Folia Histochemica et Cytobiologica 2014, Vol. 52, No. 3, 232–243)*

Key words: *Gymnocypris przewalskii*; calcineurin; embryogenesis; calcineurin subunits; cloning; cDNA; IHC; Qinghai Lake

Introduction

Lake Qinghai is a saltwater lake [pH 9.4, titratable alkalinity (30 mmol/l⁻¹), Mg²⁺-rich (18.7 mmol/l⁻¹), Ca²⁺-poor (0.30 mmol/l⁻¹) and saline (9 per thousand)] located within the Qiadam Basin of the Qinghai–Tibetan Plateau of western China at an elevation of 3200 m [1]. The naked carp, *Gymnocypris przewalskii*, mainly distributed in the Lake Qinghai, has always received much attention, not only because of its fishery importance but also its ecological significance in the fish-bird-grassland system around Lake Qinghai. Every year the migrant birds from other parts of the world breed in the spring in the Bird Island in Lake Qinghai and around its adjoining freshwater rivers. Approximately 3000 tons of naked carp were captured by birds as their feed. The commercial fishing of *Gymnocypris przewalskii* from the 1950s until the 1990s was forced to be stopped for the sharp decrease of fish stocks. In the past fifty years, the total amount reserves has decreased from 20 × 10⁴ tons in 1960 to less than 0.5 × 10⁴ tons now, especially because of decreasing water level and increasing salinity [2-4]. The naked carp has to face critical challenges such
as the increasing salinity levels of the lake, which is mainly caused by continuously decreased water levels at a rate of 10–12 cm per year over the last 50 years [5]. The increase of salinity began since 1978 and its rate is currently accelerating [6, 7]. Further, another challenge for the naked carp is the salinity changes during their annual migration from the saline lake to adjoining freshwater rivers including Quanjihe river, Shaliuhe river, Heimahe river and Buhaha river, where they spawn and then return to the lake [4]. At present, the basic biological adaptation mechanism of naked carp to the living condition, including low temperature, saline water, and hypoxia, has not been fully investigated. Therefore, understanding the embryonic developmental process of naked fish in freshwater and the adaptation mechanisms to saline lake water will be of great help for the artificial reproduction of naked carp, wild stocks recovery, and the ecosystem protection around Lake Qinghai.

Calcineurin (CN), which is composed of two subunits: catalytic subunit (Calcineurin A, CNA) and regulatory subunit (Calcineurin B, CNB), is the only member of the serine/threonine phosphatase family that can be activated by both Ca<sup>2+</sup> and calmodulin (CaM) [8–10]. Calcineurin has been proven to be a multifunctional enzyme and plays critical roles in regulating various cellular activities through direct dephosphorylation of the target proteins. After activation by both Ca<sup>2+</sup> and CaM, CN dephosphorylates its cytoplasmic substrate, the nuclear factor of activated T cells (NFAT). The activated NFATc translocates from the cytosol into the nucleus to control the expression of target genes. Previous studies showed that, in the sea urchin egg, a high intracellular calcium increase after egg fertilization has been observed [11]. It was also found that CN mRNAs are expressed maximally just before full maturation of the testis [12], and CNA was highly expressed in the scallop gonad [13]. Recently, it has been demonstrated that CN-NFAT signalling regulates the early lineage specification in mouse embryos [14]. Balanced Ca/NFAT signalling in progenitor cells of the developing embryo is essential, while inappropriate activation of NFAT is deleterious in embryonic development [15]. Many data suggest that CN is involved in the embryonic development; however, the possible mechanism of CN in controlling differentiation remains largely unknown.

In this study, cDNAs sequences of CN catalytic subunit and CN regulatory subunit were isolated from *Gymnocypris przewalskii*, and their expression patterns at different embryonic developmental stages and localization in different tissues were analysed. These results provide some important clues to further understand the diverse roles of CN in the development of naked carp.

**Material and methods**

**Animals.** Live individuals of artificially cultivated 2 years old naked carp (20–25 cm in length and 100–150 g in wet weight) were supplied by the Rescue Centre of *Gymnocypris przewalskii* (Qinghai, China) and cultured in an aquarium with flowing freshwater in the laboratory. In order to avoid stress reaction, the naked carp were kept 3–5 days for acclimatization before being used. These animals were used for cloning of subunits of CN cDNAs.

**Preparation of zygotes.** Germ cells were collected from wild adult in Quanjihe river (Qinghai, China) in June and July during spawning migration period. Zygotes were incubated in flowing water (the water temperature maintained 10–15°C) from Shaliuhe river (K ≤ 8 mg/L; Na<sup>+</sup>, 18 mg/L; Ca<sup>2+</sup>, 45 mg/L; Mg<sup>2+</sup>, 15 mg/L; Cl<sup>-</sup>, 15 mg/L; SO<sub>4</sub><sup>2−</sup>, 15 mg/L; HCO<sub>3</sub>−, 200 mg/L) after artificial fertilization in order to simulate the wild hatching condition. The zygotes at different embryonic stages were collected and stored in liquid nitrogen.

**RNA extraction and cDNA synthesis.** Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) agent from gill and zygotes. For calcineurin subunits fragments cloning, single strand cDNA was reverse transcribed from total RNA extracted from gill using PrimeScript RTase Kit (Takara, Kyoto, Japan).

**Isolation and sequencing of calcineurin subunits.** Total RNA from gill of was reverse transcribed to single strand cDNA, this cDNA was used for CN cDNA fragment cloning as template. In order to avoid the sequence errors and individual difference, we repeated the reverse transcription more than three times and the gills were from different individuals. *GpCaAz* cDNA fragment was amplified using the following primers: GpCAF53 (forward) and GpCAR53 (reverse) (Table 1), which were designed according to the conserved region of *Oncorhynchus mykiss* calcineurin catalytic subunits (CNA) cDNA. 5’ untranslated region of *GpCaAz* cDNA was isolated using Nested PCR method. The first PCR reaction was performed using gene specific primer GpCaAzAP1 (Table 1) and 5’ RACE (Rapid-Amplification of cDNA Ends) Outer Primer. The Nested PCR reaction was carried out using gene specific primer GpCaAzAP2 (Table 1) and 5’ RACE inner Primer following the introduction. For the 3’ untranslated region of *GpCaAz* cDNA, the first PCR reaction was performed using gene specific primer GpCaAzSP1 (Table 1) and 3’ RACE Outer Primer, then the Nested PCR Reaction was performed using gene specific primer GpCaAzSP2 (Table 1) and 3’ RACE inner primer.
following the instruction of 3’ Full RACE Core Set Ver.2.0 (Takara, Kyoto, Japan). Finally, primers GpCA\(\alpha\)CF1 and GpCA\(\alpha\)CR1 (Table 1) were used for confirmation of the GpCA\(\alpha\) coding sequence.

For GpCA\(\gamma\) cDNA isolation, GpCAF55 (forward) and GpCAR55 (reverse) were used for fragment amplification (Table 1). 5’ untranslated region of GpCA\(\gamma\) cDNA was isolated using gene specific primer GpCA\(\gamma\)F5 (Table 1) and nested PCR primer GpCA\(\gamma\)F5R (Table 1). For the 3’ untranslated regions of GpCA\(\gamma\) cDNA, the PCR reactions were performed using gene specific primer GpCA\(\gamma\)F5SP1 (Table 1) and nested PCR primer GpCA\(\gamma\)F5SP2 (Table 1). Con-
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The Green Supermix 10 μl, cDNA (1 μg/μl) 1 μl, reverse
promoters GpCBF17 (forward) and GpCBR17 (reverse) (Table 1) were used to obtain an expected product. 5′ untranslated region of GpCB cDNA was isolated using two primers GpCBAP1 and GpCBAP2 (Table 1). Meanwhile, GpCBSP1 and nested primer GpCBSP2 (Table 1) were used to obtain 3′ untranslated region. Finally, primers GpCBF1 and GpCBR1 (Table 1) were used to confirm the nucleotide sequence of GpCB.

In all the PCR reactions, the annealing temperature of the primers was 60°C, and the elongation time were set as 1 min per kilobase DNA.

Sequence analysis. All PCR products were sequenced in Sangon Biotech Company (Shanghai, China). Homology analysis of the nucleotide and protein sequence was conducted with the BLAST algorithm at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Vector NTI (InforMax, Frederick, USA) was used to perform multiple alignments and structure the phylogenetic tree with the NJ method.

Expression analysis of Calcineurin subunits during embryonic development. Real time PCR was carried out to determine the expression levels of calcineurin subunits in zygotides at different embryonic stages (hours post-fertilization, hpf): multicellular stage (3 hpf); blastula stage (13 hpf); gastrula stage (41 hpf); neurula stage (68 hpf); organogenesis (96 hpf).

Total RNA was extracted with Trizol agent (Invitrogen, Carlsbad, USA) from zygotes, and was reverse transcribed to single strand cDNA. The cDNA was used as the template of RT-PCR. Primers (Table 1) were as follows: GpCAsgqF3 and GpCAsgqR3 (Table 1) (for GpCAc); GpCAsqF3 and GpCAsqR3 (Table 1) (for GpCAy); GpCBRTF1 and GpCBRT1 (Table 1) (for GpCB). β-actin, used as the positive control [16], was amplified with the primer pairs of β-actinRTF1 and β-actinRTR1 (Table 1). For real time PCR, it is carried out on IQ5 Real-time Quantitative PCR instrument (Bio-Rad, Hercules, CA, USA). The real time PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix 10 μl, cDNA (1 μg/μl) 1 μl, reverse primer (10 μM) 0.5 μl, forward primer (10 μM) 0.5 μl and ddH O 8 μl.

Expression of GpCA in E. coli and polyclonal antibody preparation. Fragment of GpCAc cDNA was amplified with gene-specific primers GpCAexPF1 (forward) containing an NcoI site (underlined) and GpCAexPR1 (reverse) containing an XhoI site (underlined). The amplicon was inserted into expression vector pET-28b (Novagen, Darmstadt, Germany) to construct recombinant plasmid pET-28b/GpCA. The recombinant plasmids were amplified in E. coli strain DH5α, purified and sequenced to verify the validity of ORF before transformed into E. coli strain BL21 (DE3, Novagen). Protein expression was induced with 1 mM isopropyl-b-D-galactopyranoside (IPTG) at 37 degrees centigrade, until the optical density of the culture reached 0.6. After 3 hrs of induction, bacterial cells were harvested by centrifuging at 8000 g for 5 min. Most of the recombinant protein was expressed in form of inclusion body, which was subject to sonication on ice. The inclusion body was purified, dissolved in lysis buffer (containing 8 M urea, 0.02 M NaH₂PO₄, pH 7.2, 0.5 M NaCl) and filtrated with 0.45 μm filter before applied to a Hi-Trap HP Ni affinity column (Bio-Rad). After elution, recombinant protein was analysed on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then dialyzed against phosphate-buffered saline (PBS). Protein yields were measured by BCA assay kit (Pierce, Rockford, IL, USA). Purified polyclonal antibody against the purified recombinant proteins was obtained following the reported method [17–19]. The purified antibody was used for Western blotting analysis and immunohistochemistry.

Immunohistochemical analysis of GpCA in different tissues. Tissues were fixed in 4% paraformaldehyde, dehydrated in ethanol, cleared in xylene, and then embedded in paraffin blocks. 5-μm sections were cut and mounted on slides coated with polylysine. Immunohistochemical staining was performed as follows: (a) paraffin sections were deparaffinised and rehydrated to water; (b) incubated in 3% H₂O₂ for 10 min, then washed with PBS 3 times for 15 min; (c) slides were boiled in citric acid buffer solution for 20 min, then washed with PBS for 3 times after cooled; (d) blocked with goat serum (10% in PBS) for 15 min; (e) Rabbit anti-GpCA polyclonal antibody (1:250 in PBS) was incubated at 4°C for 12 hours, then slides were rinsed with PBS; (f) Histostain™Plus Kits (Invitrogen) and DAB kit (Boster, Wuhan, China) were used to detect the immune complex following instructions; (g) counterstained with haematoxylin.

Statistics. Data were expressed as means ± SE (Standard Error of Mean) of three independent experiments; statistical analyses were performed with Minitab 16 program (Minitab, University Park, PA, USA). Comparisons between groups were analysed via ANOVA followed by Duncan’s multiple range tests and p < 0.05 was considered as statistically significant.
Results and analysis

Cloning and sequence analyses of GpCα and GpCγ

A 298 bp cDNA fragment of GpCα was obtained after RT-PCR amplification using primers (GpCAF53 and GpCAR53). After rapid amplification of cDNA ends (RACE) with gene specific primers (GpCAzAP1 and GpCAzAP2 for 5’UTR, GpCAzSP1 and GpCAzSP2 for 3’UTR) and confirmation PCR with gene specific primers (GpCAzCF1 and GpCAzCR1), the complete cDNA sequence of GpCα was cloned (Figure 1A). The complete cDNA sequence contains a 726 bp 5’ untranslated sequence, an ORF consisting of 1503 bp, a TAA stop codon, a 456 bp 3’ untranslated sequence, and a poly (A) tail of 11 nucleotides. The deduced GpCα protein consists of 500 amino acids with a molecular weight of 56,316 Da and theoretical isoelectric point (pI) 5.60 according to ProtParam analysis [20]. In the process of GpCγ cDNA isolation, GpCAF55 and GpCAR55 were synthesized to amplify a 495 bp fragment. After 5’RACE with GpCγAP1 and GpCγAP2, 3’RACE with GpCγSP1 and GpCγSP2, and a confirmation PCR with GpCγCF1 and GpCγCR1, the complete 2685 bp cDNA sequence of GpCγ was isolated (Figure 1B). Structural analysis showed that it contains a 208 bp 5’ untranslated sequence, an ORF of 1536 bp, a TAA stop codon, a 378 bp 3’ untranslated sequence, and a poly (A) tail of 11 nucleotides. The deduced GpCγ protein consists of 511 amino acids with a molecular weight of 56,801.8 Da and theoretical pI 5.61.

After Conserved Domain Database search and ScanProsite annotation [21, 22], both GPCα and GpCγ show a conserved metallophosphatase domain of Calcineurin and a serine/threonine specific protein phosphatases signature (Figure 1).

In mammals, CNA is classified into three isoforms: α-, β-, and γ-type. CNAα isom is widely distributed; CNAβ isoform has a conserved feature of an N-terminal polyproline motif, whereas CNAγ is specifically expressed in the human testis [23]. Studies of the mRNAs encoding the different isoforms of the catalytic subunit (A subunit) of calcineurin revealed that they are expressed at different levels in rat thymus and kidney using in situ hybridization histochemistry with specific antisense oligonucleotide probes [24]. Immunocytochemical analysis revealed that both CNAα and CNAβ immunoreactivities differ in regional and cellular localizations in rat brain [25]. In addition, knockout mice lacking each of the isoforms had shown different dysfunctions, and the absence of CNAα resulted in impaired kidney function [26, 27].

All the results indicated that each isoform plays its own function.

In gene bank, only two paralogue isoforms, α- and γ-type, have been characterized in Danio rerio and Salmo salar respectively, the accession numbers of Danio rerio are CAM46977.1 and NP_001074063.2, ACI67288.1 and AC133493.1 for Salmo salar. The alignment between naked carp Gymnocypris przewalskii and Danio rerio shows that the homology of CNAα and CNAγ is 93% and 94%, respectively (Figure 2).

Notably, according to the phylogenetic tree analysis, the two CNA isoforms with the highest similarity in human is HsCαγ. It is speculated that the variation of GpCNAα and GpCNAγ formed after G. przewalskii species formation, and their functions need to be further investigated.

In naked carp, the two paralogue isoforms of CNA can be divided into 4 domains: catalytic core, CNB-binding domain, calmodulin-binding domain and inhibitory peptide. The paralogue alignment shows that the identity of the two protein sequences is 83%. The identity is 90% for catalytic core domain, 96% for the CNB-binding domain, 100% for the calmodulin-binding domain and 86% for the inhibitory peptide (Figure 3). In addition, the poly-proline sequence, which may play a role in substrate recognition, was not found in the isoforms as that in the mammalian CNA β isoform.

Cloning and sequence analyses of GpCB

To clone the complete GpCB cDNA sequence, GPCBF17 and GPCBR17 were utilized to gain a 243 bp fragment. Gene specific primers (GpCBAP1 and GpCBAP2) were used for 5’RACE, while gene specific primers (GpCBSP1 and GpCBSP2) for 3’RACE. In the end, a confirmation PCR was performed with gene specific primers (GpCBCF1 and GpCBCR1). The complete 1137 bp cDNA sequence of GpCB contains 513 bp coding sequence (CDS) flanked by 47 bp of 5’ untranslated sequence and 577 bp of 3’ untranslated sequence; a TAA stop codon, and a poly (A) tail of 11 nucleotides (Figure 4).

As showed in Figure 4, the deduced 170 amino acids GpCB protein contains four conserved EF-hand type calcium-binding motifs including EF-1 (aa 31–42), EF-2 (aa 63–74), EF-3 (aa 100–111), and EF-4 (aa 141–152) with a calculated molecular mass of 19.3 kDa and an isoelectric point of 4.64. EF-hand proteins display a multitude of unique conformational states, together constituting a conformational continuum [28]. Calcium binding could induce the conformational change in the EF-hand motif, then leading to the activation of calcineurin.

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Based on the reported data, CNB subunit shares high degree of conservation throughout evolution from yeast to mammalian, which makes it possible to functional interchange of CNB subunits between mammalian and *N. crassa* [29]. In this report, the sequence of GpCB shares various degree of similarity with those of CNB from other species (47% identity to zebrafish, 48% to salmon, 92% to mollusk and 98% to rainbow trout).

**Gene expression profiles of calcineurin subunits during embryonic development**

Changes of intracellular free calcium concentration serve as a key signal (the second messenger) to modulate various physiological activities. In most eggs, fertilization triggers a propagating calcium explosion [30], which is demonstrated as a pre-requisite for egg activation [11]. In zebrafish, the defect in egg activation is a consequence of failure in Ca^{2+} wave induction [31]. In *Xenopus* embryos, calcineurin is transiently activated immediately after Ca^{2+} addition to a concentration that induces exit from metaphase arrest in meiosis II. Both the activation and subsequent inactivation of CN in fertilized eggs are crucial for the commencement of vertebrate embryonic development [32]. Injecting CN inhibitors into dorsal side can block dorsal-side signalling that affect late-stage development of the heart, kidney, liver, gut and somatic tissue during embryogenesis [33].

In our report, real time RT-PCR assay (Figure 5) demonstrated that the expression patterns of GpCaA,
GpCA and GpCB share the same trend after fertilization. From multicellular stage to organogenesis, GpCA was expressed constitutively and declined from blastula stage, and reached its lowest level at neurula stage. In contrast, the expression level of GpCB.
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According to our results, CN three subunits are expressed constitutively during various developmental stages. All of them showed higher expression level at the beginning stage. In the later stage of development, their expression level declined and reached to the minimum expression level. Finally, at the organogenesis stage the three studied genes’ expression recovered quickly to the same level to that at the beginning stage of embryonic development.

**Figure 4.** cDNA sequences and deduced amino acid sequences of GpCB: The four EF-hand type calcium-binding sites from 1 to 4 in GpCB are lightly shaded, underlined, bold underlined and black shaded, respectively

**Figure 5.** Real-time RT-PCR assays of *G. przewalskii* Calcineurin mRNA in discrete stage of embryogenesis. Relative mRNA expressions were determined by the comparative Ct method considering PCR efficiency based on the normalization against the expression of β-actin in each sample. Fold increases (Mean ± SV based on triplicate assays) were represented in histograms (dense for GpCA, none for GpCAB and sparse for GpCB). Identical letters on the histograms indicate that the means were not statistically different based on ANOVA followed by Duncan’s multiple range tests at p = 0.05

*CA* showed the lowest level at blastula and gastrula stage, and GpCB showed minimum expression level at the neural stage.

In all animals, the earliest stages of embryonic development rely on maternal gene products, which are present in the egg at the time of fertilization. With the development of embryo in the later stage, transcription of the zygotic genome is gradually activated. It means that the maternal gene products and zygote transcriptional products coordinately regulate the embryonic development. According to our results, CN three subunits are expressed constitutively during various developmental stages. All of them showed higher expression level at the beginning stage. In the later stage of development, their expression level declined and reached to the minimum expression level. Finally, at the organogenesis stage the three studied genes’ expression recovered quickly to the same level to that at the beginning stage of embryonic development. The expression pattern of CN three
subunits demonstrated that CN is essentially needed in the whole process of embryonic development in naked carp, although their expression level showed a transient decrease probably due to the transitional period of maternal control of embryonic development and the onset of zygotic transcription.

Immunohistochemical characterization of GpCA presence in naked carp’s tissues

Calcineurin is widely distributed in mammalian tissues, including brain, bone osteoclasts, heart, kidney, liver, and others. In naked carp, we detected that CN localized in separate tissues including kidney, brain, heart, gill, and spermary.

In the kidney of naked carp, CN was specifically detected in the epithelial cells in renal tubule (Figure 6A) which are adjoining renal capsules and play important roles in ion reabsorption and waste products secretion. It was found that CN inhibitors can increase salt-sensitivity of the kidney after transplantation [34]. Distinctive distribution of CN demonstrates that it participates in the renal physiology.

In the heart of naked carp, immunopositive signals congregated in the middle of the organ and formed several branching regions as showed in Figure 6B. After dephosphorylated by CN, the member of NFAT family is translocated from the cytosol to the nucleus of cells where NFAT promotes gene transcription. Those transcribed genes play an important part in the development of cardiac hypertrophy [35–38]. In addition, cardiomyocytes can control their local neural milieu by expression of nerve growth factor (NGF) through calcineurin-NFAT pathway, which triggers sympathetic neural growth [39]. These results implied functions of CN specified heart areas also in naked carp.

In spermary of naked carp, the staining intensity distributed around the edge of seminiferous lobules occupied by spermatocytes (Figure 6C). In mammalian, testis-specific isoform of CN is expressed when meiosis begins, and increases in amount depending on the maturation of spermatogenesis [40]. In situ hybridization has shown this specific CN isoform was specifically localized to spermatocytes where meiosis occurs, but none or very few have been observed in spermatogonia, spermatids [41]. Immunohistochemical localization of CN in the mouse testis further confirms that it simply accumulates in spermatids nuclei and abundant increases during the initial stage of nuclear elongation, with almost no signal present in the cytoplasm [42]. All of the results suggest that CN in the testis is related to spermatogenesis.

In the brain of naked carp, positive signals appeared at the edge of the cerebral cortex as showed in Figure 6D. Calcineurin immunoreactivity was present in neurons, but a marked regional variation in strength of the immunoreactivity was observed; the caudate-putamen, hippocampal formation, and substantia nigra were intensely stained [43, 44]. Many studies have shown that CN takes part in the regulation of Ca\(^{2+}\) channel activity [45, 46], signal transduction at synapses and age-related changes in cognition during the aging process [47, 48], alteration of synaptic function and memory [49, 50]. Together, CN may be a useful target for treatment of age-related memory impairments and neurodegenerative disease.

In the case of gill, strong signals distributed in the epithelial cells of the branchial filaments and the base of gill filaments as showed in Figure 6E. Gills perform a variety of physiological functions in adult fish including respiratory gas exchange, ion and water balance, excretion of nitrogenous wastes and the maintenance of acid-base balance. In the gill, Ca\(^{2+}\)-ATPases modulate the inward flux of Ca\(^{2+}\) into gill epithelial cells [51, 52], and may play a part in gill acclimation responses to environmental Ca\(^{2+}\) changes and osmoregulation [53]. The presence of CN in naked carp’s gill suggests that also in this teleost fish CN may play a key role in calcium uptake and accumulation.

As mentioned above, CN is localized in various cells of different tissues. The function of CN especially in the immune system, osteoclast bone resorption, cardiac hypertrophy, skeletal muscle hypertrophy, spermatid motility, apoptosis, hormone secretion and other processes has been described in many studies. In light of the demonstrated in this study immunohistological localization of GpCA, it is implicated that the function of CN in naked carp from Lake Qinghai needs to be further investigated.

Conclusions

In this study, we have isolated cDNA sequences of paralogue isoforms of CN catalytic subunits and CN regulatory subunit from Gymnocypris przewalskii. Gene expression profiles and immunohistochemistry analysis demonstrate that CN distribute ubiquitously in early embryonic stages and in various tissues. Our findings provide some important clues for further understanding the physiological characteristics of naked carp during embryonic development and in adaptation to the environment changes.

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Figure 6. Immunohistochemical localization of CNA in diverse tissues of *G. przewalskii*. A. Kidney; B. Heart; C. Spermary; D. Brain; E. Gill. Immunostaining signals are indicated by arrow. Bars 100 μm in (B), (E); 50 μm in (A), (C) and (D).

RT — renal tubule; SL — seminiferous lobule; CC — cerebral cortex; GF — gill filament

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