

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: *GpCA α* (GenBank accession no. JQ407043), *GpCA γ* (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, titraTab 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

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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 Quanjie river, Shaliuhe river, Heimahe river and Buhuhe 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^{2+} 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^{2+} 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 un-

derstand 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 carps 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 Quanjie 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^+ , 18 mg/L; Ca^{2+} , 45 mg/L; Mg^{2+} , 15 mg/L; Cl^- , 15 mg/L; SO_4^{2-} , 15 mg/L; HCO_3^- , 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. *GpCA α* 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 *GpCA α* cDNA was isolated using Nested PCR method. The first PCR reaction was performed using gene specific primer *GpCA α* AP1 (Table 1) and 5' RACE (Rapid-Amplification of cDNA Ends) Outer Primer. The Nested PCR reaction was carried out using gene specific primer *GpCA α* AP2 (Table 1) and 5' RACE inner Primer following the introduction. For the 3' untranslated region of *GpCA α* cDNA, the first PCR reaction was performed using gene specific primer *GpCA α* SP1 (Table 1) and 3' RACE Outer Primer, then the Nested PCR Reaction was performed using gene specific primer *GpCA α* SP2 (Table 1) and 3' RACE inner primer

Table 1. Primer names and primer sequences used for PCR

Primer names	Sequences	Application	Efficiency
GpCAF53	ATCAACCATCCCACCAC		
GpCAR53	TCTTCTCAGAGCCGTAGTC		
GpCA α AP1	GACCACCGTGTACGCAGAGGAACT	5'RACE	
GpCA α AP2	GGAAGAGGGTGGTGGGATGGTTGA	5'RACE	
GpCA α SP1	CCATCCCACCACCCTCTTCCTCCTC	3'RACE	
GpCA α SP2	CCTCTGCGTACACGGTGGTCTC	3'RACE	
GpCA α CF1	TGGCTAAGAAACCCTGACTGAACTG	Confirm PCR	
GpCA α CR1	TGCAGCATCTTAAACCCACATCTC	Confirm PCR	
GpCAF55	GTGTATGATGCGTGTATGG		
GpCAR55	GTTGGGCAGCCAGTAG		
GpCA γ AP1	TCCTGGGCTTCATGTGCTCTTAT	5'RACE	
GpCA γ AP2	AACGCACAGGAACTGCTGGTTGAG	5'RACE	
GpCA γ SP1	ATAAGAGCACATGAAGCCAGGAT	3'RACE	
GpCA γ SP2	AGTCAGACGACAGGCTTCCC	3'RACE	
GpCA γ CF1	TTTCCGTTTCTCCGCTCTAACA	Confirm PCR	
GpCA γ CR1	CACCACCCAATGCTAATGTCTAT	Confirm PCR	
GpCBF17	CGAGGGAGTCTCACAGTTC		
GpCBR17	CGTCCACCACCATCTTTT		
GpCBAP1	CTGGGTGTCTTCAGGTTATTCC	5'RACE	
GpCBAP2	TGTCATAAATCCTGAACGCAAAGC	5'RACE	
GpCBSP1	CGAGGGAGTCTCACAGTTC	3'RACE	
GpCBSP2	GCTCCGCTTTGCGTTCAGGATTTATG	3'RACE	
GpCBCF1	CAAGGCGAAGACCAGCCAGAAT	Confirm PCR	
GpCBCR1	TGAAAAGAGGGTGCTCGGTGAA	Confirm PCR	
GpCA α sqpF3	GCCCCGTGGTCTGGATT	Real time PCR	91%
GpCA α sqpR3	CACAGTTCAGTCAGGGTTTCTT	Real time PCR	
GpCA γ sqpF3	GCGGGCTAACGCGACTA	Real time PCR	99%
GpCA γ sqpR3	TGGGAGGGTACGGCACA	Real time PCR	
GpCBRTF1	GGGAATAACCTGAAGGACACC	Real time PCR	92%
GpCBRTR1	GTCTAAGCCACCAACCACAA	Real time PCR	
β -actinRTF1	ATGGGAACCGCTGCCTCTT	Real time PCR	99%
β -actinRTR1	GGTCAGCAATGCCAGGGTA	Real time PCR	
GpCAexPF1	CATGCCATGGGCACTAAAGAGCCGAAAG	Recombinant protein	
GpCAexPR1	CCGCTCGAGCTCGTTGGCAGCGTTAA	Recombinant protein	
GpCBexPF1	CATGCCATGGGAAATGAGGCGAGTTATCC	Recombinant protein	
GpCBexPR1	CCGCTCGAGCACGTCCACCACCATC	Recombinant protein	

following the instruction of 3' Full RACE Core Set Ver.2.0 (Takara, Kyoto, Japan). Finally, primers GpCA α CF1 and GpCA α CR1 (Table 1) were used for confirmation of the GpCA α coding sequence.

For GpCA γ cDNA isolation, GpCAF55 (forward) and GpCAR55 (reverse) were used for fragment amplification

(Table 1). 5' untranslated region of GpCA γ cDNA was isolated using gene specific primer GpCA γ AP1 (Table 1) and nested PCR primer GpCA γ AP2 (Table 1). For the 3' untranslated regions of GpCA γ cDNA, the PCR reactions were performed using gene specific primer GpCA γ SP1 (Table 1) and nested PCR primer GpCA γ SP2 (Table 1). Con-

firmation PCR was performed using primers GpCA γ CF1 and GpCA γ CR1 (Table 1).

For GpCB cDNA fragment, degenerate oligonucleotide primers 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 GpCBCF1 and GpCBCR1 (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 zygotes 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: GpCA α sqpF3 and GpCA α sqpR3 (Table 1) (for GpCA α); GpCA γ sqpF3 and GpCA γ sqpR3 (Table 1) (for GpCA γ); GpCBRTF1 and GpCBRTR1 (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 performed using iQTM SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA) and the level of expression was analysed with Livak's method. The thermocycler was programmed for 94°C denaturation for 2 min followed by 40 cycles of 94°C denaturation for 20 s, 56°C annealing for 20 s, and 72°C for 20 s. The reaction system including: iQTM SYBR[®] Green Supermix 10 μ l, cDNA (1 μ g/ μ l) 1 μ l, reverse primer (10 μ M) 0.5 μ l, forward primer (10 μ M) 0.5 μ l and ddH₂O 8 μ l.

Expression of GpCA in *E. coli* and polyclonal antibody preparation. Fragment of GpCA α 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 DH5 α , 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 isopropylthiogalactopyranoside (IPTG) at 37 degrees centigrade, until the optical density of the culture at 600 nm 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 μ m 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- μ m 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) HistostainTM-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 \pm 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 *GpCA α* and *GpCA γ*

A 298 bp cDNA fragment of *GpCA α* was obtained after RT-PCR amplification using primers (GpCAF53 and GpCAR53). After rapid amplification of cDNA ends (RACE) with gene specific primers (GpCA α AP1 and GpCA α AP2 for 5'UTR, GpCA α SP1 and GpCA α SP2 for 3'UTR) and confirmation PCR with gene specific primers (GpCA α CF1 and GpCA α CR1), the complete cDNA sequence of *GpCA α* 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 *GpCA α* 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 *GpCA γ* cDNA isolation, GpCAF55 and GpCAR55 were synthesized to amplify a 495 bp fragment. After 5'RACE with GpCA γ AP1 and GpCA γ AP2, 3'RACE with GpCA γ SP1 and GpCA γ SP2, and a confirmation PCR with GpCA γ CF1 and GpCA γ CR1, the complete 2685 bp cDNA sequence of *GpCA γ* 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 *GpCA γ* protein consists of 511 amino acids with a molecular weight of 57,801.8 Da and theoretical pI 5.61.

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

In mammals, CNA is classified into three isoforms: α -, β -, and γ -type. CNA α isoform is widely distributed; CNA β isoform has a conserved feature of an N-terminal polyproline motif, whereas CNA γ 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 CNA α and CNA β 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 CNA α 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 sala* respectively, the accession numbers of *Danio rerio* are CAM46977.1 and NP_001074063.2, ACI67288.1 and ACI33493.1 for *Salmo sala*. The alignment between naked carp *Gymnocypris przewalskii* and *Danio rerio* shows that the homology of CNA α and CNA γ 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 HsCA γ . It is speculated that the variation of *GpCNA α* and *GpCNA γ* 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 1137 bp complete 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.

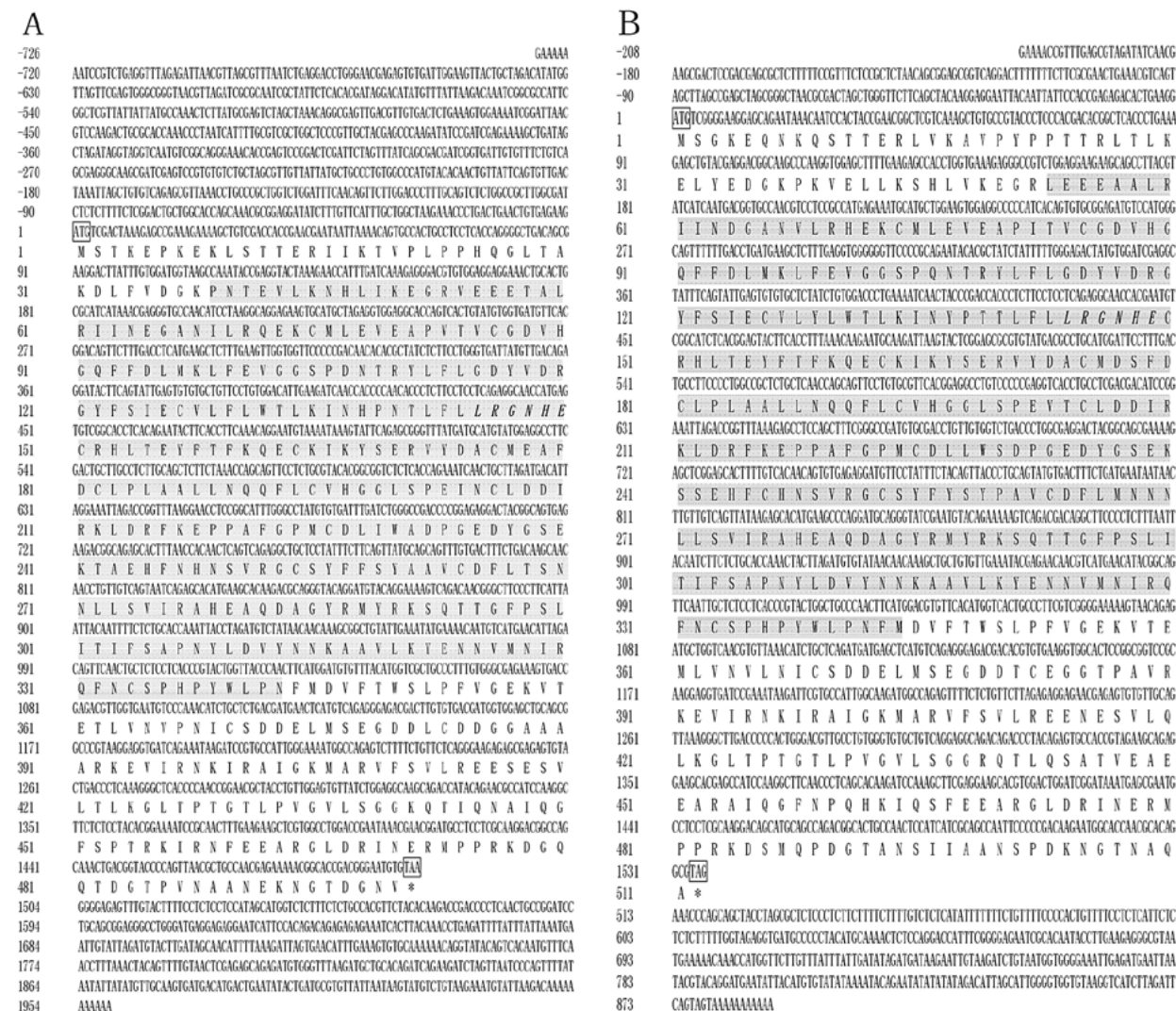


Figure 1. cDNA sequences and deduced amino acid sequences of *G. przewalskii*. Calcineurin A alpha isoform and gamma isoform: *GpCAα* (A) and *GpCAγ* (B). *GpCAα* and *GpCAγ* have putative metallophosphoesterase domains (shaded) including serine/threonine specific protein phosphatases signatures (in bold and italic)

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, ferti-

zation 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 Ca²⁺ wave induction [31]. In *Xenopus* embryos, calcineurin is transiently activated immediately after Ca²⁺ 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 *GpCAα*,

	CnCA	DdCA	TgCA	DmCA	PfCA	SkCA	DrCA α	DrCA γ	SsCA γ	HsCA γ	MsCA γ	HsCA β	MsCA β	HsCA α	MmCA α	SsCA α	GpCA α	GpCA γ
CnCA	100	42	38	44	50	49	50	51	51	51	51	52	52	52	52	28	50	51
DdCA		100	40	45	48	48	48	49	48	48	48	49	49	50	50	28	48	49
TgCA			100	46	60	59	57	56	57	54	53	54	54	54	42	57	56	
DmCA				100	72	72	70	68	70	67	67	69	69	69	43	70	69	
PfCA					100	84	80	79	79	77	77	78	78	80	80	53	80	79
SkCA						100	79	79	79	77	75	80	80	81	81	53	79	79
DrCA α							100	88	90	82	82	81	81	82	82	51	93	87
DrCA γ								100	91	84	83	83	83	84	84	50	85	94
SsCA γ									100	81	81	82	81	82	82	51	87	89
HsCA γ										100	90	82	82	83	83	50	81	82
MsCA γ											100	80	80	80	49	81	81	
HsCA β												100	99	84	84	49	81	83
MsCA β													100	84	84	49	81	83
HsCA α														100	100	53	81	83
MmCA α															100	53	81	83
SsCA α																100	52	49
GpCA α																	100	85
GpCA γ																		100

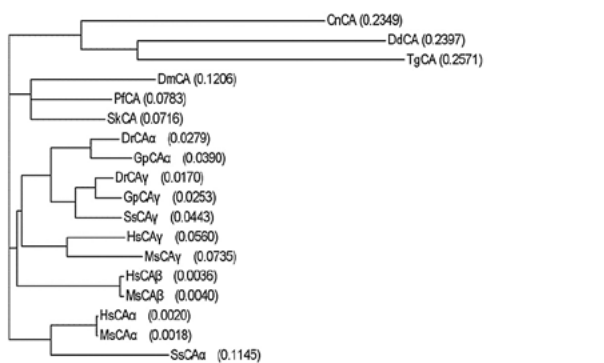


Figure 2. Phylogenetic tree analysis of *G. przewalskii* Calcineurin A with those of other species. The phylogenetic tree was generated by using NJ method. GenBank accession numbers for these amino acid sequences of Calcineurin A are as follows: CnCA, CNA [*Cryptococcus neoformans* var. *grubii*] (AAB97372.1); DdCA, CNA [*Dictyostelium discoideum AX4*] (XP_642811.1); TgCA, CNA [*Toxoplasma gondii*] (AAM97278.1); DmCA, CNA [*Drosophila melanogaster*] (CAA54807.1); PfCA, CNA [*Pinctada fucata*] (EU797510); SkCA, CNA [*Saccoglossus kowalevskii*] (NP_001158479.1); DrCA α , CNA [*Danio rerio*, alpha isoform] (CAM46977.1); DrCA γ , CNA [*Danio rerio*, gamma isoform] (NP_001074063.2); SsCA γ , CNA [*Salmo salar*, gamma isoform] (ACI33493.1); HsCA γ , CNA [*Homo sapiens*, gamma isoform] (EAW63677.1); MsCA γ , CNA [*Mus musculus*, gamma isoform] (P48455.1); HsCA β , CNA [*Homo sapiens*, beta isoform] (EAW54497.1); MsCA β , CNA [*Mus musculus*, beta isoform] (P48453.2); HsCA α , CNA [*Homo sapiens*, alpha isoform] (EAX06124.1); MsCA α , CNA [*Mus musculus*, alpha isoform] (NP_032939.1); SsCA α , CNA [*Salmo salar*, alpha isoform] (ACI67288.1); GpCA α , CNA [*Gymnocypris przewalskii*, alpha isoform] (JQ407043); GpCA γ , CNA [*Gymnocypris przewalskii*, gamma isoform] (JQ407043)

	BbCB	CiCB	DmCB	PfCB	SkCB	XiCB	GpCB	OmCB	IfcNB	DrCB	HsCB
BbCB	100	68	70	72	69	70	69	68	69	70	70
CiCB		100	44	46	46	44	44	44	44	45	45
DmCB			100	91	91	89	88	89	88	89	89
PfCB				100	93	92	91	91	91	92	92
SkCB					100	94	93	94	94	94	94
XiCB						100	98	97	98	99	99
GpCB							100	98	98	99	99
OmCB								100	97	98	98
IfcNB									100	99	99
DrCB										100	100
HsCB											100

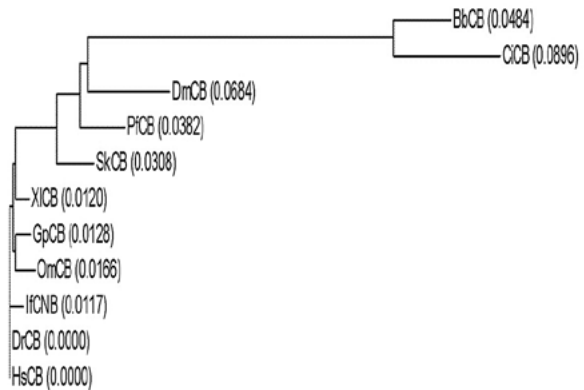


Figure 3. Phylogenetic tree analysis of *G. przewalskii* Calcineurin B with those of other species. GenBank accession numbers for these amino acid sequences of Calcineurin B are as follows: BbCB, CNB [*Beauveria bassiana*] (ACU46638.1); CiCB, CNB [*Coccidioides immitis* RS] (XP_001248933.1); DrCB, CNB [*Danio rerio*] (NP_001004553.1); SsCB, CNB [*Salmo salar*] (NP_001134594.1); HsCB α , CNB [*Homo sapiens*, alpha isoform] (EAW58961.1); MmCB α , CNB [*Mus musculus*] (EDL02306.1); DmCB, CNB [*Drosophila melanogaster*] (NP_524741.1); PfCB, CNB [*Pinctada fucata*] (ACI96107.1); SkCB, CNB [*Saccoglossus kowalevskii*] (NP_001161511.1); HsCB β , CNB [*Homo sapiens*, beta isoform] (EAW99878.1); GpCB, CNB [*Gymnocypris przewalskii*] (JQ410473); OmCB, CNB [*Oncorhynchus mykiss*] (NP_001154040.1)

GpCA γ and *GpCB* share the same trend after fertilization. From multicellular stage to organogenesis, *GpCA α* was expressed constitutively and declined from blastula stage, and reached its lowest level at neurula stage. In contrast, the expression level of *Gp-*

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-47                                     GAAGGTCAGTTCGCGAGTCTCTGCACAAGGCGAAGACCAGCCAGA
1   ATGGGAAATGAGCGGAGTTATCCCTTGGAGATGTGCACACACTTTCACCGTGATGAGATTAAGACTAGGGAAGCGGTTAAGAAACTC
1   M G N E A S Y P L E M C T H F D A D E I K R L G K R F K K L
91  GACCTGGATAACTCGGGTCTCTCAGCGTGGAGGAGTTTATGTCTCTACCCGAGTTGCAGCAGAATCCGCTGGTGCAGCGAGTCATCGAT
31  D L D N S G S L S V E E F M S L P E L Q Q N P L V Q R V I D
181 ATACTCGACACAGACGGAAATGGAGAAGTGGACTTTAAAGAGTTCATTGAAGCGCTCTCAGTTCAGTGTCAAGGGCGACAAGGAGCAG
61  I L D T D G N G E V D F K E F I E G V S Q F S V K G D K E Q
271 AAGTCCGCTTTGCGTTCAGGATTTATGACATGGATAAGGATGGCTACATATCCAACGGTGAGCTGTTCCAGGTGCTGAAGATGATGGTA
91  K L R F A F R I Y D M D K D G Y I S N G E L F Q V L K M M V
361 GGGATAACCTGAAGGACACCAGCTGCAGCAGATCGTCGACAAAACCATCATCAACGCAGACAAGGACGGGACGGGAGGATATCTTTC
121 G N N L K D T Q L Q Q I V D K T I I N A D K D G D G R I S F
451 GAGGAGTTCGCCTTGTGGTGGCTTAGACATTACAAAAAGATGGTGGTGACGTGTGA
151 E E F C L V V G G L D I H K K M V V D V *
514 ATGGGAAATGAGCGGAGTTATCCCTTGGAGATGTGCACACACTTTCACCGTGATGAGATTAAGACTAGGGAAGCGGTTAAGAAACTC
604 GACCTGGATAACTCGGGTCTCTCAGCGTGGAGGAGTTTATGTCTCTACCCGAGTTGCAGCAGAATCCGCTGGTGCAGCGAGTCATCGAT
694 ATACTCGACACAGACGGAAATGGAGAAGTGGACTTTAAAGAGTTCATTGAAGCGCTCTCAGTTCAGTGTCAAGGGCGACAAGGAGCAG
784 AAGTCCGCTTTGCGTTCAGGATTTATGACATGGATAAGGATGGCTACATATCCAACGGTGAGCTGTTCCAGGTGCTGAAGATGATGGTA
874 GGGATAACCTGAAGGACACCAGCTGCAGCAGATCGTCGACAAAACCATCATCAACGCAGACAAGGACGGGACGGGAGGATATCTTTC
964 GAGGAGTTCGCCTTGTGGTGGCTTAGACATTACAAAAAGATGGTGGTGACGTGTA
    
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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

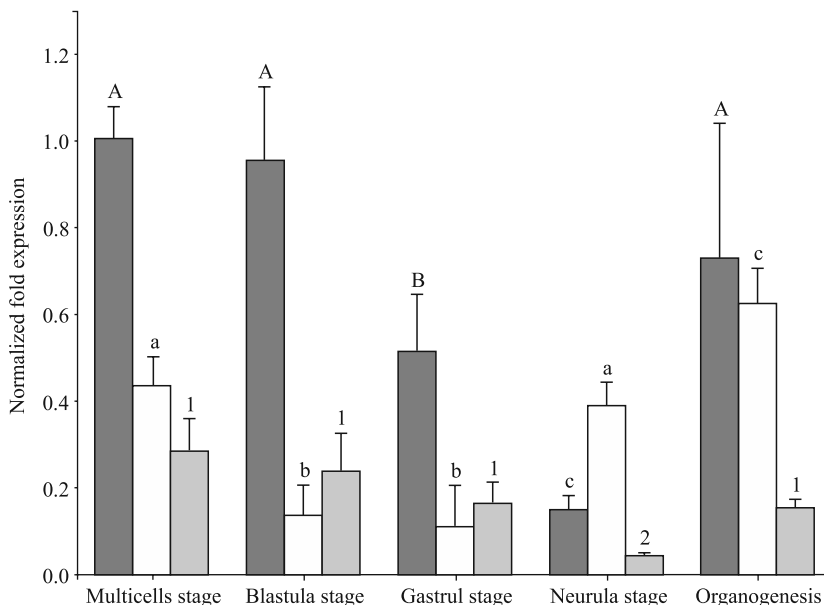


Figure 5. Real-time RT-PCR assays of *G. przewalskii* Calcineurin mRNA in discrete stage of embryogenesis. Relative mRNA expressions were determined by the comparative Ct method considering PCR efficiency based on the normalization against the expression of β -actin in each sample. Fold increases (Mean \pm SV based on triplicate assays) were represented in histograms (dense for *GpCA α* , none for *GpCA β* 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 γ reached 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 caudatoputamen, 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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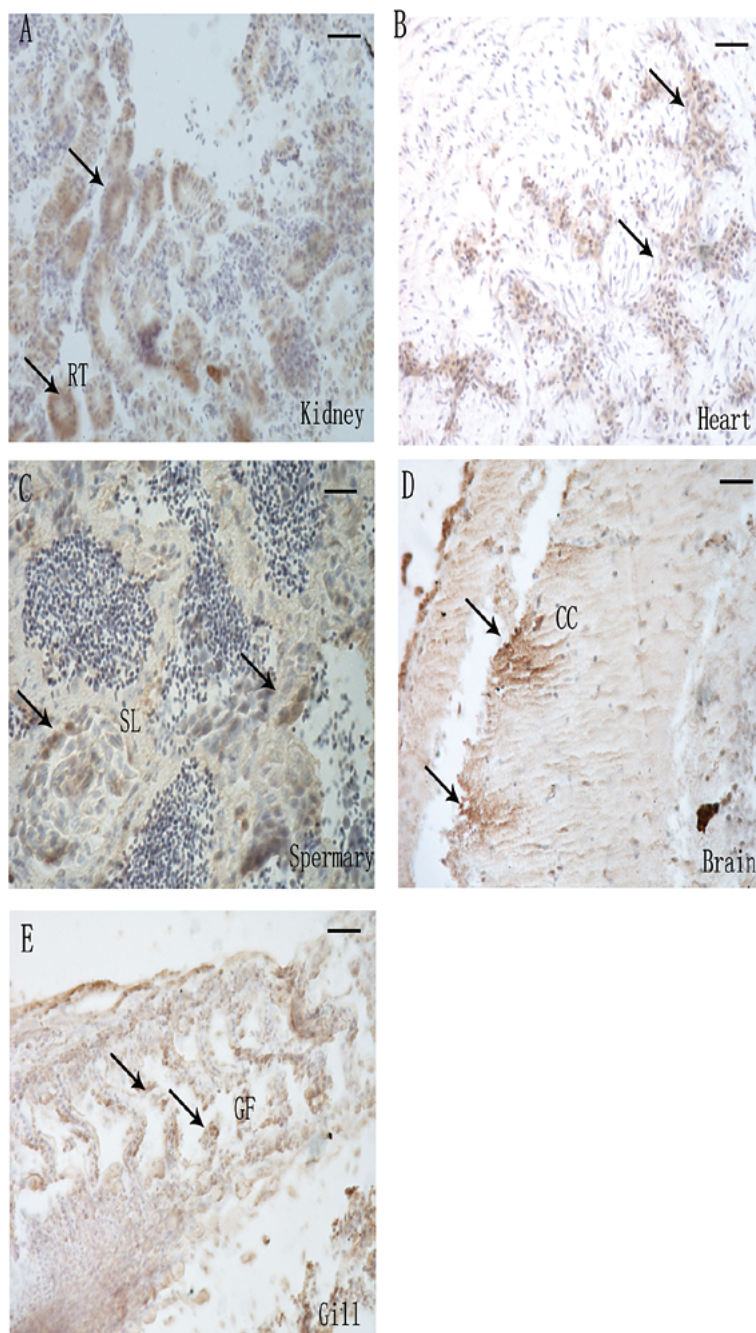


Figure 6. Immunohistochemical localization of CNA in diverse tissues of *G. przewalskii*. **A.** Kidney; **B.** Heart; **C.** Spermery; **D.** Brain; **E.** Gill. Immunostaining signals are indicated by arrow. Bars 100 μm in (B), (E); 50 μm in (A), (C) and (D). RT — renal tubule; SL — seminiferous lobule; CC — cerebral cortex; GF — gill filament

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