Neuropeptide F and its receptor genes in the cephalopod *Sepiella japonica*: identification, characterization, expression and possible role in starvation resistance and food intake promotion

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Abstract

Neuropeptide F (NPF) is a homolog of vertebrate neuropeptide Y (NPY) and interacts with the G protein-coupled receptor (GPCR) NPFR. The molecular structure, distribution patterns, and biological functions of NPF and NPFR have been identified in several invertebrate studies; however, reports concerning mollusks remain relatively scarce. In this study, the NPF and NPFR genes were identified and characterized in cuttlefish (*Sepiella japonica*), hereafter called *Sj*NPF and *Sj*NPFR, respectively. Evidences from multiple alignments and phylogenetic analysis indicated that *Sj*NPF exhibits a high degree of homology with the NPF of *Idiosepius paradoxus*, and *Sj*NPFR demonstrates a high degree of homology with the NPFR of *Sepia pharaonis*. *In situ* hybridization and qPCR data reveal the spatiotemporal expression patterns of both genes in various functional lobes of the brain and other tissues. Furthermore, starvation stress experiments showed that the gene expression levels, gastric protease activity, and intestinal histology were positively correlated with starvation and negatively correlated with refeeding, suggesting their potential role in hunger resistance and the promotion of food intake. This study partially contributes to the understanding of the roles of *Sj*NPF and *Sj*NPFR during the developmental processes of cuttlefish, with the aim of providing a theoretical foundation for elucidating the functions of *Sj*NPF and *Sj*NPFR in cephalopods.

1 Cloning and identification of full-length cDNA sequence of NPF and NPFR from *S. japonica*

Figure 1. Full-length cDNA sequence and predicted amino acid sequence of *Sj*NPF (A) and *Sj*NPFR (B). The predicted amino acid sequence is shown below the base sequence. The predicted signal peptides marked with black horizontal lines. The predicted mature peptide is marked with red horizontal lines. The predicted phosphorylation sites are boxed with different color (green for the Tyrosin (Tyr), blue for the Threonine (Thr), and red for the Serine (Ser)). The predicted glycosylation sites are marked with triangle. The predicted start codon (ATG), and stop codon (TGA) are boxed with black. The gray shaded parts are transmembrane helix 1-7 on conserved domain.

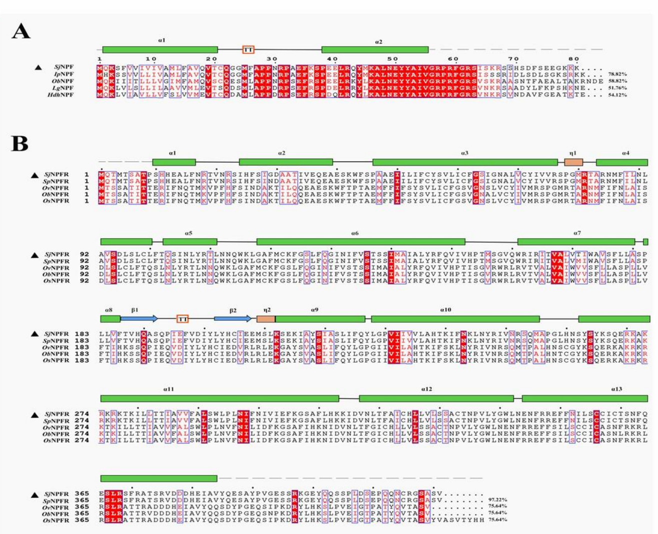

Figure 2. Alignments of amino acids of *Sj*NPF (A) and *Sj*NPFR (B) with NPF and NPFR sequences from other species. (A) The predicted *Sj*NPF amino acid sequence contains 2 α-helices (green box) and 1 Turn-Turn structure (TT). (B) The predicted *Sj*NPFR amino acid sequence contains 13 α-helices (green box), 2 β-sheets (blue box), 2 η-helices (orange box) and 1 Turn-Turn structure (TT). The residues displayed in all sequences (100%) are showed in red background, and highly conserved regions (≥75%) are displayed in red font.

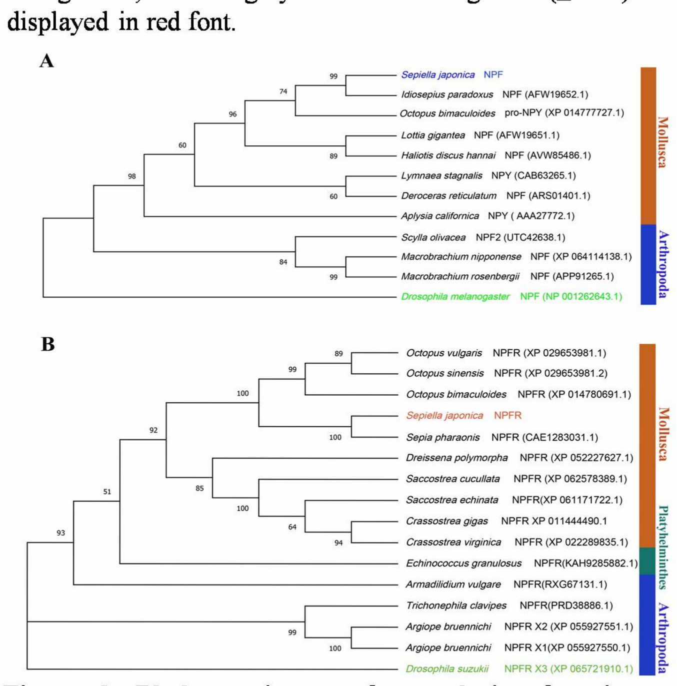

Figure 3. Phylogenetic tree for analysis of amino acid sequence (a) of NPF(Y) (A) and amino acid sequence of NPF (Y) R (B). *Sj*NPF and *Sj*NPFR are displayed in blue and red, respectively; The out group is noted by green.

2 Expression patterns of *SjNPF* and *SjNPFR* genes at different developmental stages

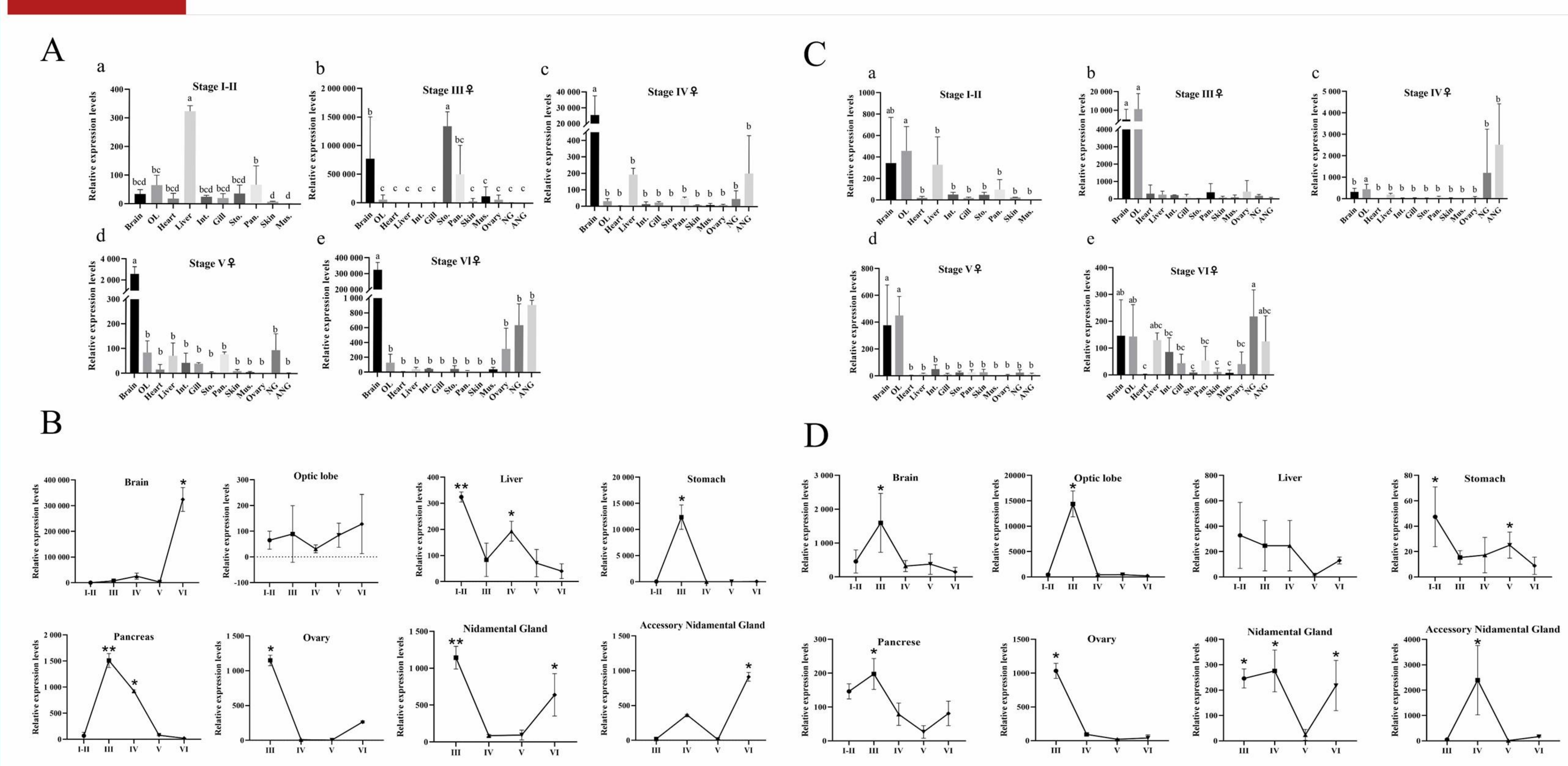

Figure 4 . The expression patterns of *SjNPF* and *SjNPFR* at different developmental stages in female cuttlefish. The expression patterns of *SjNPF* (A and B) and *SjNPFR* (C and D) in different tissues at each stage. Statistical analyses are conducted using one-way ANOVA. The data are presented as mean ± SD (n = 3). Bars labeled with different letters indicate statistically significant differences at the $p < 0.05$ level，with an asterisk above the error bars indicating a significant difference (* for $p < 0.05$, ** for $p < 0.01$). OL, Optic lobe; Int, intestine; Sto, Stomach; Mus, Muscle; NG, Nidamental gland; ANG, Accessory nidamental gland; Pan, pancreas.

3 Tissue distribution and localization of *Sj*NPF and *Sj*NPFR in *S. japonica*

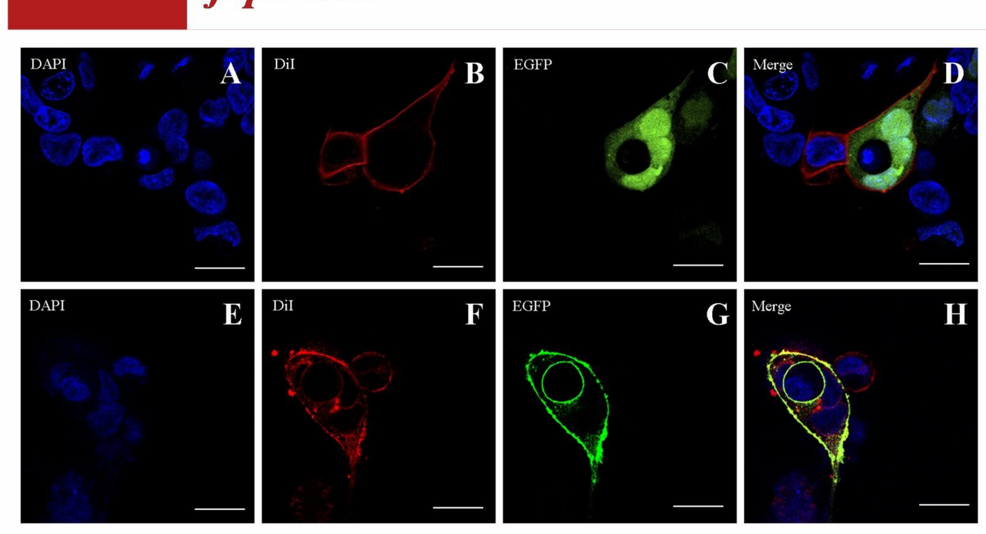

Figure 5. Localization of *Sj*NPF-EGFP(A-D) and *Sj*NPFR-EGFP(E-F) in HEK293 cells. DAPI: nuclear staining (blue) (A); DiI: cell membrane staining (red) (B); EGFP: *Sj*NPF-EGFP and *Sj*NPFR-EGFP fusion protein (green) (A); Merge (D). The scale is 25 μm.

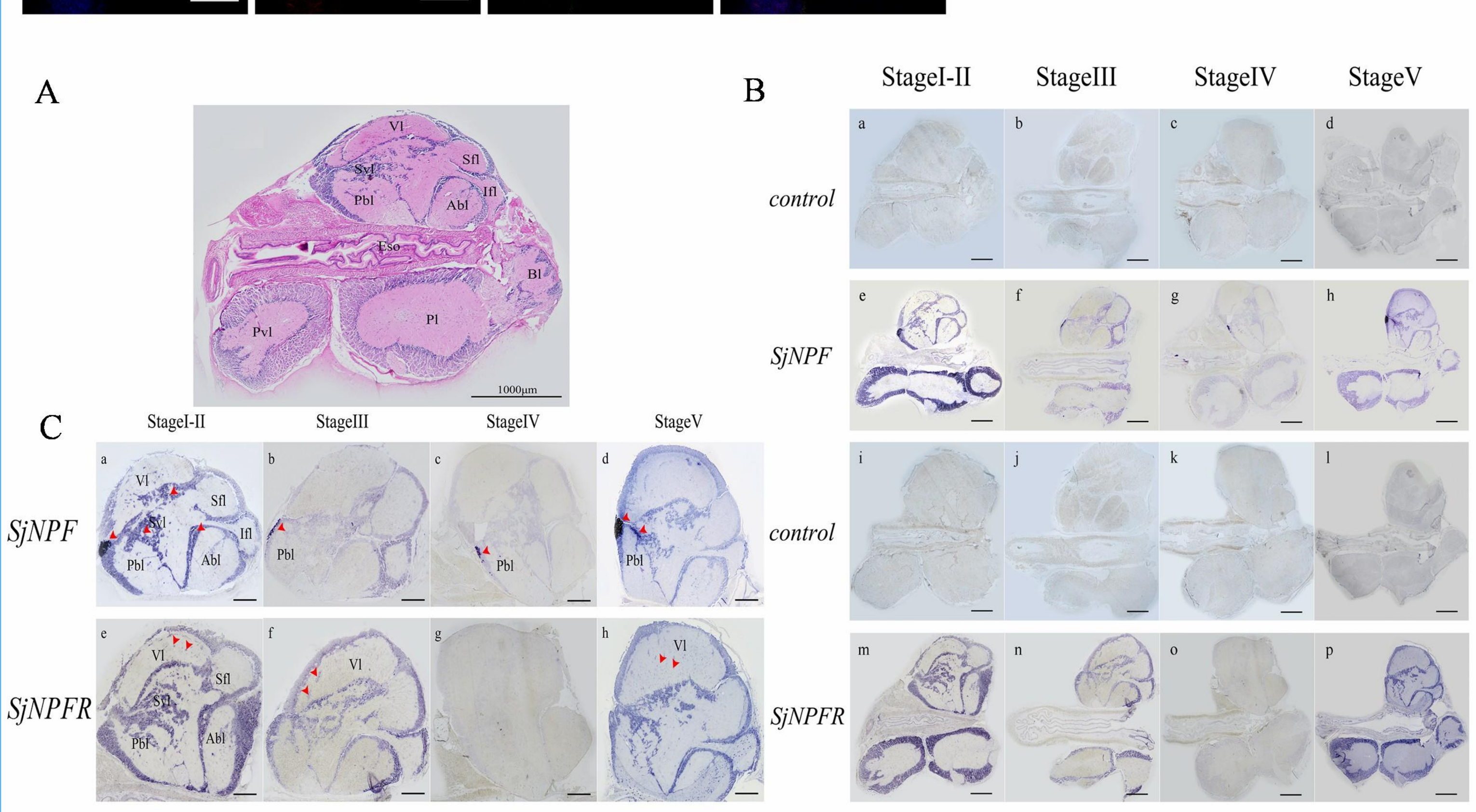

Figure 6. *SjNPF* and *SjNPFR* mRNA *in situ* hybridization in the brain of *S. japonica* at four developmental stages. Hematoxylin-eosin staining of the brain of *S. japonica* (A). The blue area is the nucleus and the red area is the cytoplasm. (B) *In situ* hybridization analysis of *SjNPF* and *SjNPFR* mRNA in brain at stage I-II (a, e, i, m), III (b, f, j, n), IV(c, g, k, o) and V(d, h, l, p). (C) *In situ* hybridization analysis of *SjNPF* and *SjNPFR* mRNA in supraesophageal ganglion at stage I-II (a, e), III (b, f), IV(c, g and V(d, h). Blue dots are positive signals; red arrows refer to the specific expression sites and the scale shown in the picture is 1000μm. The above pictures are all vertical slices of the brain of female cuttlefish without needles. Abl, Anterior basal lobes; Eso, Esophagus；Ifl, Inferior frontal lobe; Sfl, Superior frontal lobe; Vl, Vertical lobe；Svl, Subvertical lobe; Bl, Brachial lobe; Pbl, Posterior basal lobes; Pl, Pedal lobe；Pvl, palleovisceral lobes.

4 Preliminary study on the feeding function of *Sj*NPF and *Sj*NPFR in *S. japonica*

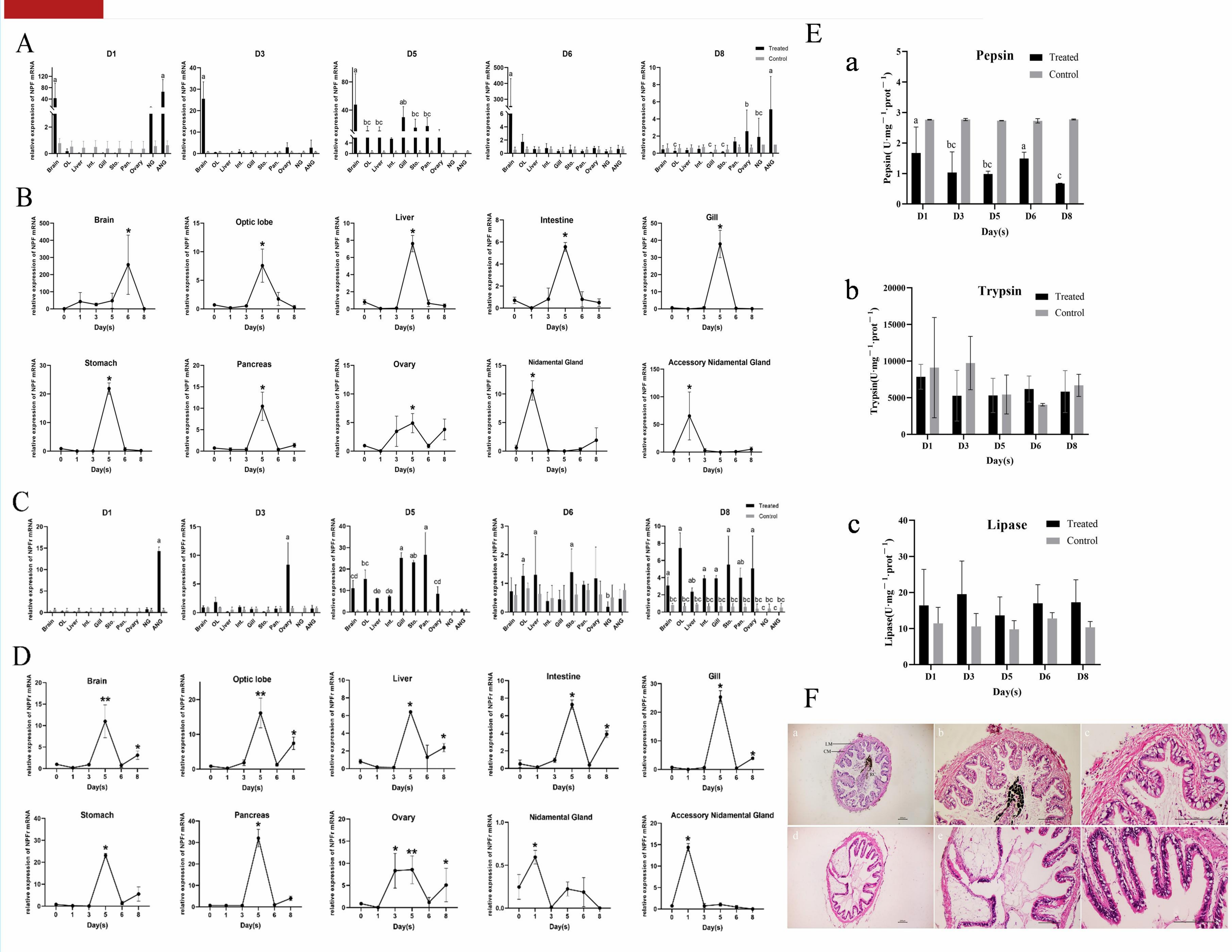

Figure 7. Under starvation stress, changes in *SjNPF* and *SjNPFR* mRNA expression and digestive capacity. (A-D) The expression patterns of *SjNPF* (A and B) and *SjNPFR* (C and D) in different tissues at different time points. Statistical analyses are conducted using one-way ANOVA. The data are presented as mean ± SD (n = 3). Bars labeled with different letters indicate statistically significant differences at the $p < 0.05$ level，with an asterisk above the error bars indicating a significant difference (* for $p < 0.05$, ** for $p < 0.01$). (E) Changes in enzyme activity of pepsin (a), trypsin (b), and lipase (c) under feeding regulation. (F) H.E staining of the intestine in *S. japonica* from control groups (a, b, c) and experimental group (d, e, f) under feeding regulation. The results are presented as mean ± standard deviation, and different letters above the error bars indicate significant differences ($p < 0.05$).CM, Circular muscle; LM, Longitudinal muscle.

Conclusion

In summary, the present study identified and characterized the NPF and NPFR in the *S. japonica* for the first time. Through multiple sequence alignments and phylogenetic analysis indicated *Sj*NPF was highly conserved across various species, and the four residues at the C-terminus was particularly conserved. According to the subcellular localization results, *Sj*NPF was located in the cytoplasm, while *Sj*NPFR was located in the cell membrane. Based on the results of *in situ* hybridization and qPCR, *SjNPF* was significantly expressed in the digestive organs during the early developmental stages. In later stages, *SjNPF* had high expression levels in the brain and gonadal tissues, suggesting a potential role in reproductive processes. Notably, *SjNPFR* was significantly expressed in the brains of females except stage VI. Starvation stress experiments demonstrated that *Sj*NPF and *Sj*NPFR can regulate feeding behavior in cuttlefish. The *NPF* and *NPFR* genes may exert its effects on the regulation of energy metabolism, growth, development, and reproductive performance directly or indirectly. Nevertheless, the precise molecular regulation mechanisms of these genes require further exploration.