Original Article

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Website : www.jecajournal.com

DOI: 10.4103/jeca.jeca_3_18

# Presence of GFSKLYFamide-like neuropeptide in the nervous tissue of *Holothuria scabra*: Immunohistochemical evidence

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**Abstract:**

**BACKGROUND:** Physiological activities in animals and other biological systems are often regulated by neuropeptides. GFSKLYFamide neuropeptide, an echinoderm SALMFamide, was first isolated from the sea cucumber, Holothuria glaberrima, an echinoderm, in 1992 (Díaz-Miranda et al., 1992). Since this discovery, there have been unresolved questions regarding the interphyletic and intraphyletic distribution of GFSKLYFamide neuropeptide.

**AIM:** The study was done in an attempt to answer these questions.

**MATERIALS AND METHODS:** An immunohistochemical study was conducted on the radial nerve cord of Holothuria scabra utilizing an antibody specifically raised against GFSKLYFamide.

**RESULTS:** Results show strong and widespread localization of GFSKLYFamide immunoreactivity, including the ectoneural and hyponeural regions of the radial nerve cord.

**CONCLUSION:** This, to the best of our knowledge, is the fi rst report that provides evidence for the presence of GFSKLYFamide-like neuropeptide in the nervous tissue of this species.

**Keywords:**

GFSKLYFamide, holothuria scabra, immunohistochemistry, radial nerve cord, SALMFamides, sea cucumber

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## Introduction

Neuropeptides are regulatory molecules known to be involved in some physiological activities in biological systems (DeWied, 1969; Strand, 1999). They act as neurotransmitters and neuromodulators in animals, wherein a considerable amount of information has already been accumulated on neuropeptide distribution and functions (Kastin *et al.*, 1979; Raffa, 1988).

GFSKLYFamide neuropeptide, a heptapeptide with the amino acid sequence Gly-Phe-Ser-Lys-Leu-Tyr-Phe-$NH_2$, was first discovered in the sea cucumber, *Holothuria glaberrima*, an echinoderm in 1992 (Díaz-Miranda *et al.*, 1992). GFSKLYFamide belongs to a group of peptides called SALMFamide peptides. SALMFamide peptides or SALMFamides are the first set of neuropeptides to be fully characterized and sequenced from any echinoderm and they share some similarities with FMRFamide neuropeptide in that they were initially discovered based on radioimmunoassay using antibodies against FMRFamide (Elphick *et al.*, 1991a; Elphick *et al.*, 1991b; Díaz-Miranda *et al.*, 1992), and because of the presence of a Famide (-Phe-$NH_2$) at their carboxyl-terminal (Price and Greenberg, 1989; Walker, 1992). Although the presence of GFSKLYFamide has so far been demonstrated



**How to cite this article:** Ajayi A, Withyachumnarnkul B. Presence of GFSKLYF amide-like neuropeptide in the nervous tissue of *Holothuria scabra*: Immunohistochemical evidence. J Exp Clin Anat 2018;17(2):66-69.

only in the tissues of *H. glaberrima* (Díaz-Miranda *et al.*, 1995), a study on its pharmacological actions indicate that GFSKLYFamide act as a muscle relaxant (Díaz-Miranda and García-Arrarás, 1995).

Since the discovery of GFSKLYFamide, one of the major unresolved issues has been the question of the existence or otherwise of this neuropeptide in other phyla apart from echinoderms. Another is whether or not the neuropeptide could be found in other species within the echinoderm phylum (Ajayi and Withyachumnarnkul, 2013). In an attempt to answer these questions, we present in this study an immunohistological evidence for the presence of GFSKLYFamide-like neuropeptide in the nervous tissue of the sea cucumber, *Holothuria scabra*.

## Materials and Methods

### Animals

Adult sea cucumber, *H. scabra*, weighing about 125 g was used in this study [Figures 1 and 2]. They were maintained in filtered natural seawater within a temperature range of 28°C–31°C and salinity of about 32 ppt before being transferred to the laboratory in oxygenated sealed plastic bags.

### Antibody

Polyclonal antibody against GFSKLYFamide was generously provided by Professor García-Arrarás (University of Puerto Rico, USA). The antibody was raised, as described by Díaz-Miranda *et al.*, 1995, using 63 µg of synthetic GFSKLYFamide coupled to 15 mg bovine serum albumin (BSA) with 0.3% glutaraldehyde. The reaction was stopped by the addition of 1M glycine, and the mixture was dialyzed. Aliquot of the dialysate, BSA-GFSKLYFamide conjugate was emulsified with complete Freund's adjuvant and injected into two rabbits with half of the emulsion each, subcutaneously and intraperitoneally. Two boosters of the aliquot mixed with incomplete Freund's adjuvant were given after the initial injection, and sera were collected 7 and 14 days after each injection, preabsorbed with BSA, and assayed by immunohistochemical reactivity on sections of sea cucumber intestine and by dot blot. Section of the sea cucumber intestine was used for immunohistochemical specificity test because it has been shown to stain positive for GFSKLYFamide neuropeptide (Díaz-Miranda *et al.*, 1995). Moreover, GFSKLYFamide neuropeptide was initially isolated from the intestines of the sea cucumber, *H. glaberrima*.

### Hematoxylin and Eosin

Sections of radial nerve cord tissues were processed for hematoxylin and eosin (H and E) staining to appreciate the general tissue organization of the nervous tissue of *H. scabra*. Dissected tissues were fixed in Bouin's solution for about 5 h. Tissues were then dehydrated in graded series of ethanol, cleared in xylene, and infiltrated with paraffin using an automatic tissue processor (LEICA TP 1020). Tissues were embedded in paraffin blocks and sections of 7 µm thickness cut with microtome, mounted on poly-l-lysine-coated or Histogrip™-coated slides and dried overnight at 42°C. Sections were then deparaffi nized by passing through three changes of xylene for 5 min each, then rehydrated by passing through decreasing concentration of ethanol (in the following order: 100%, 95%, and 50%) 5 min each and finally in phosphate-buffered saline (PBS) before staining in H and E.

### Immunohistochemistry

Indirect immunofluorescence method was used to localize GFSKLYFamide-like immunoreactivity in the radial nerve cord of *H. scabra* using frozen sections. The radial nerve cord was processed for immunohistochemical analysis according to a previously described method (Ajayi and Withyachumnarnkul, 2013) [Figures 1 and 2]. Briefly, animals were anesthetized in ice for about 30 min before dissection. Dissected radial nerve cord tissues were fixed immediately in 4% paraformaldehyde for 5–24 h at 4°C, washed three times in PBS for 10 min each, and cryoprotected in 30% sucrose overnight. Sections of 7 µm thickness were cut with a cryostat (LIECA CM 1850), mounted on poly-L-lysine-coated slides and permeabilized in PBS-Triton X-100 (0.1%) for 5 min before blocking with normal goat serum (1:50 in PBS) for 1 h. Sections were incubated overnight at room temperature with a polyclonal antibody against GFSKLYFamide (1:1000 in PBS) followed by three washes in PBS–Tween 20 (0.05%) for 10 min each. This was followed by 1–2 h incubation in Alexa 488-conjugated goat anti-rabbit IgG (1:500 in PBS) at room temperature. Sections were then washed 3 times, 10 min each, in PBS–Tween 20. Slides were incubated in TOPO-3 (1:500 in PBS) at room temperature for 1 h and then rinsed in PBS–Tween 20 (0.05%) and mounted in buffered glycerol (pH 8.6). Tissues were examined and photographs were taken with an Olympus confocal laser scanning microscope (FV1000). Images were processed using OLYMPUS FLOVIEW 1.7b viewer and Adobe Photoshop CS3. Preabsorption control was done by substituting the primary antibody with PBS or preabsorbed antibody. Working dilutions of GFSKLYFamide antibody (1: 1000 in PBS) were preabsorbed overnight at 4°C with 100, 50, and 10 ng/µl of synthetic GFSKLYFamide peptide.

## Results

The nervous system of the sea cucumber, *H. scabra*, is simple in organization, and its tissues consist of five radial nerve cords, which run longitudinally on the

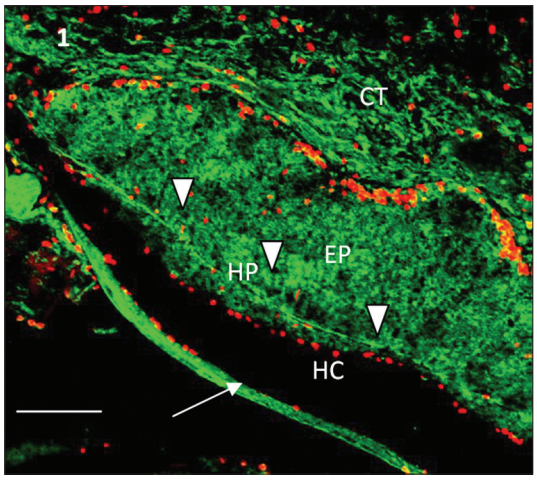


**Figure 1:** GFSKLYFamide-like expression in the radial nerve cord of *Holothuria scabra* showing abundant GFSKLYFamide-like immunoreactivity (green) in the ectoneural and hyponeural plexuses as well as the epithelial roof covering of the hyponeural canal of *Holothuria scabra*. Nuclei were counterstained with TOPO-3 (red)

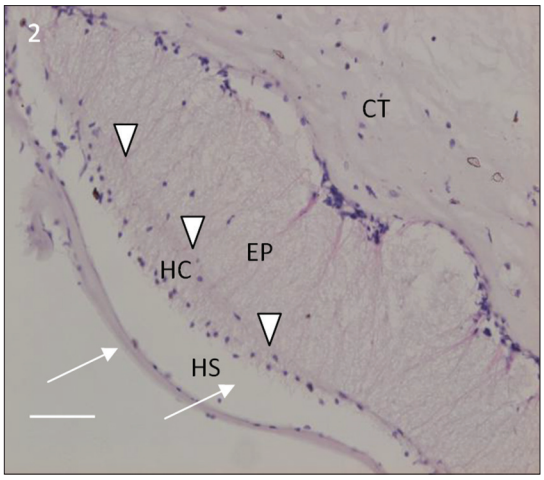


**Figure 2:** H and E staining of the radial nerve cord of *Holothuria scabra*. EP - Ectoneural plexus, HP - Hyponeural plexus, HC - Hyponeural canal, CT - Connective tissue (of the body wall), arrowheads = connective tissue partition between ectoneural and hyponeural plexuses, arrow = epithelial roof covering of the hyponeural canal. Scale = 50 μm

body wall, and unite anteriorly to form the circumoral nerve ring. The radial nerve cord consists of nerve fibers which are partitioned by a band of connective tissue into a hyponeural and ectoneural components [Figures 1 and 2]. Examination of the radial nerve cord for immunoreactivity to GFSKLYFamide antiserum showed strong and abundant GFSKLYFamide-like immunoreactivity in the tissues and nerve fibers within the ectoneural plexus of *H. scabra*. The hyponeural plexus, connective tissue partition between the hyponeural and ectoneural plexuses, and epithelial roof covering of the hyponeural canal also showed strong immunoreactivity to GFSKLYFamide antiserum [Figure 1].

## Discussion

The nervous system of *H. scabra* consists of the radial nerve cords and circumoral nerve ring (Hyman, 1955; Bai, 1971). In this study, the radial nerve cord of *H. scabra* was examined and found to be positive for GFSKLYFamide-like immunoreactivity. The ectoneural and hyponeural divisions of the radial nerve cord of *H. scabra* showed strong immunoreactivity to GFSKLYFamide polyclonal antibody. Presence of GFSKLYFamide-like immunereactivity in the radial nerve cord of the sea cucumber *H. scabra* suggests that GFSKLYFamide neuropeptide might be present in the nervous system of this species. It also provides some evidence in support of the possible functions of GFSKLYFamide neuropeptide. For example, the detection of GFSKLYFamide-like immunoreactivity in both compartments of the radial nerve cord suggests it might be involved in both motor and sensory functions in this species since the ectoneural compartment is said to have both motor and sensory function while the hyponeural compartment is said to be primarily motor in function (San Miguel-Ruiz *et al.*, 2009). Furthermore, it has been shown that the action of GFSKLYFamide neuropeptide and other SALMFamides is as muscle relaxants in sea cucumbers (Díaz-Miranda *et al.*, 1992; Díaz-Miranda and Garcia-Arraras, 1995; Elphick and Melarange, 2001; Melarange and Elphick, 2003).

Positive labeling of the radial nerve cord of *H. scabra* with an antibody against GFSKLYFamide neuropeptide, as demonstrated in this study, is consistent with an earlier report in which GFSKLYFamide-like immunoreactivity was shown to be present in the nervous tissues of the sea cucumber *H. glaberrima* (Diaz-Miranda *et al.*, 1995). Previous work from our laboratory also showed that GFSKLYFamide-like immunoreactivity might be found in other *H. scabra* tissues, such as the digestive organs (Ajayi and Withyachumnarnkul, 2015). This is an indication of the important role GFSKLYFamide neuropeptide might be playing in the sea cucumber.

This, to the best of our knowledge, is the first report that provides evidence for the presence of GFSKLYFamide-like neuropeptide in the nervous tissue of the sea cucumber, *H. scabra*. Although it is possible that the immunoreactivity reported in this study might be due to other similar peptides, it is recommended that the structural identity of the GFSKLYFamide-like immunoreactive element in *H. scabra* be determined.

## Conclusion

We have been able to demonstrate in this study the presence of GFSKLYFamide-like neuropeptide in the nervous tissue of the sea cucumber, *H. scabra*. Further

studies are warranted to determine the structural identity of the GFSKLYFamide-like immunoreactive element in this species.

## Financial support and sponsorship
Nil.

## Conflicts of interest
There are no conflicts of interest.

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