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Afr. J. Biomed. Res. Vol. 23 (January, 2020); 25- 28

Research Article

Presence of GFSKLYFamide-like Neuropeptide in the Tentacles, Body Wall and Tube Foot of *Holothuria scabra*: Immunohistochemical Evidence

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ABSTRACT

The neuropeptide GFSKLYFamide is a heptapeptide first isolated from the sea cucumber, *Holothuria glaberimma*. The holothurian body wall, tentacles and tube foot are important organs for locomotion, feeding and maintenance of homeostasis. This study was aimed at determining the localization of GFSKLYFamide in the tentacles, body wall and tube foot of *Holothuria scabra*. Ten male *H. scabra* weighing between 62g and 175g were used for this study. Using GFSKLYFamide polyclonal antibody and Alexa 488-conjugated goat anti-rabbit IgG as primary and secondary antibodies, respectively, indirect immunofluorescence method with confocal microscope imaging were used to localise GFSKLYFamide immunoreactivity in the tentacles, body wall and tube foot of *H. scabra*. The results indicate the presence of GFSKLYFamide-like neuropeptide in the epithelial layers of the tentacles, body wall and tube foot. GFSKLYFamide-like immunoreactivity was also detected within the connective tissue layer of the tentacles and tube foot.

Keywords: GFSKLYFamide, immunohistochemistry, *Holothuria scabra*, tube foot, tentacles, body wall

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Received: February, 2019; Accepted: November, 2019

Abstracted by:

Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

INTRODUCTION

Neuropeptides are small protein-like molecules that play crucial role in mediating physiological processes as well as maintaining homeostasis (Ajayi and Withyachumnarnkul, 2015; Semmens and Elphick, 2017; Suwansa-ard et al., 2018). Neurons synthesize neuropeptides which are derived from neuropeptide precursors (Nässel and Larhammar, 2013; Suwansa-ard et al., 2018). Neuropeptide GFSKLYFamide is a heptapeptide first isolated from the sea cucumber, Holothuria glaberimma (Diaz-Miranda et al., 1992). It belongs to the SALMFamide group of peptides (Elphick, 2014; Semmens and Elphick, 2017).

The sea cucumber *Holothuria scabra* is a premium detritus feeder, found in benthic areas of marine environments and belongs to the phylum Echinodermata, class Holothuroidea, order Aspidochirotida and the family of Holothuriidae (Wu et al., 2009; Mitu et al., 2017; Bahrami et al., 2018). *H. scabra* is characterized by dark-grey to black colouration with fine transverse white streaks and transverse wrinkles on the arched dorsal surface. The ventral surface is flat and lighter than dorsal surface (Yaghmour and Whittington-Jones, 2018).

The body wall, tentacles and tube feet of *H. scabra* are important for locomotion, feeding and response to external stimulus in this species. Exchange of gases also takes place through the tube feet (Hamel et al., 2001). The body wall is about 10–15 mm thick and is covered by a thin cuticle composed of dying cells from the epidermis (Dabbagh et al., 2012). From the external surface, the successive layers of tissue that compose the body wall are a cuticle, an epithelium, a layer of glandular cells, a dermis containing the pigments and connective tissues, muscle strands, haemal lacunae, muscle layer and coelomic epithelium (Hamel et al., 2001). Microscopic endoskeletal particles called ossicles are embedded in the body wall. The ossicles of both dorsal and ventral body wall are made up of tables and buttons which varies in length, diameter and number of nodulous (Yaghmour and Whittington-Jones, 2018; Dabbagh et al., 2012).

The tentacles of *H. scabra* are covered by a thin cuticle, below which lies a dermis. Below the dermis is the connective tissue that contains sensory fibres. The lumen of the tentacle is tentacular canal (Hamel et al., 2001). Glandular cells with secretory granules are visible among the epithelial cells. The

tentacles of *H. scabra* have rods ranging from 160 to 450µm in length. The tube feet are arranged irregularly on the ventral surface. They contain buttons and perforated rods; the buttons are 50µm long; while the perforated rods are 100–260µm long (Dabbagh et al., 2012).

Although GFSKLYFamide-immunoreactivity was reported in neuronal somata and fibres of appendages associated with the body wall, tentacles and tube foot of the sea cucumber *H. glaberrima*, nothing is known about the presence of GFSKLYFamide in the appendages of *H. scabra*. The objective of this study therefore is to provide immunohistochemical evidence for the presence of GFSKLYFamide neuropeptide in the tentacles, body wall and tube foot of *H. scabra.*

MATERIALS AND METHODS

Animals: Male *Holothuria scabra* weighing between 62g to 175g were used in this study. They were maintained in filtered natural sea water within a temperature range of 28°C to 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 63g of synthetic GFSKLYFamide coupled to 15mg BSA with 0.3% glutaraldehyde. The reaction was stopped by the addition of 1M Glycine, and the mixture 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 was 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.

Immunohistochemistry: Indirect immunofluorescence method was used to localise GFSKLYFamide-like immunoreactivity in the tentacles, body wall and tube foot of *H. scabra* using frozen sections. Tissues were processed for immunohistochemical analysis thus; animals were anaesthetized in ice for about 30 min before dissection. Dissected tissues were fixed immediately in 4% paraformaldehyde for 5 to 24 h at 4°C, washed three times in PBS for 10 min each and cyoprotected in 30% sucrose overnight. Sections of 7µm 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 primary antibody (1:1000 in PBS) followed by three washes in PBS-Tween 20 (0.05%) for 10 min each. This was followed by 1 to 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 then rinsed in PBS-Tween 20 (0.05%) and mounted in buffered glycerol (pH 8.6). Tissues were examined and photographs taken with an Olympus Confocal laser scanning microscope (FV 1000). 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 10ng/µl of synthetic GFSKLYFamide peptide.

RESULTS

The polyclonal antibody used in this has been characterized and used extensively in previous studies. It was reported to show high specificity for GFSKLYFamide neuropeptide and does not react against CCK, galanin, proctolin, and CARP.

In figure 2, result show epithelial layers of tentacles, body wall and tube foot express positive GFSKLYFamide-like immunoreactivity (green). GFSKLYFamide-like immunoreactivity is also dispersed within connective tissue layer of the tentacles (Fig. 2a) and tube foot (Fig. 2e) as well as in the luminal epithelial lining of the podial water canal.

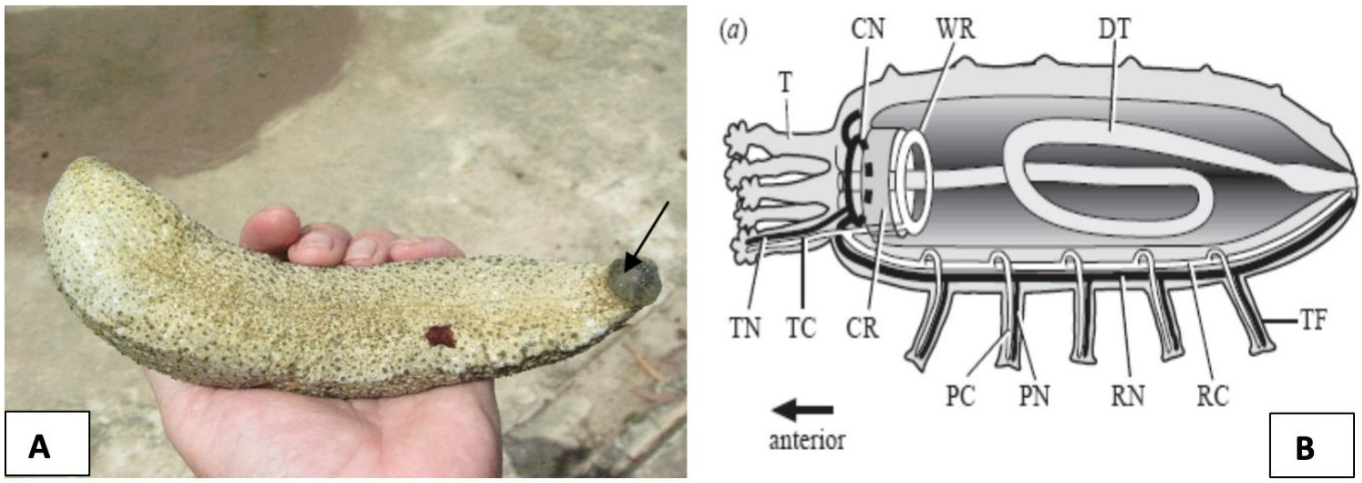

Plate 1
A. Ventral surface of *Holothuria scabra* showing the tentacles (arrow) and tube feet appearing as black dots on the body wall. **Figure 1B**. Schematic representation of the sea cucumber showing the tentacles (T) and tube feet (TF) (from Inoue et al., 1999).

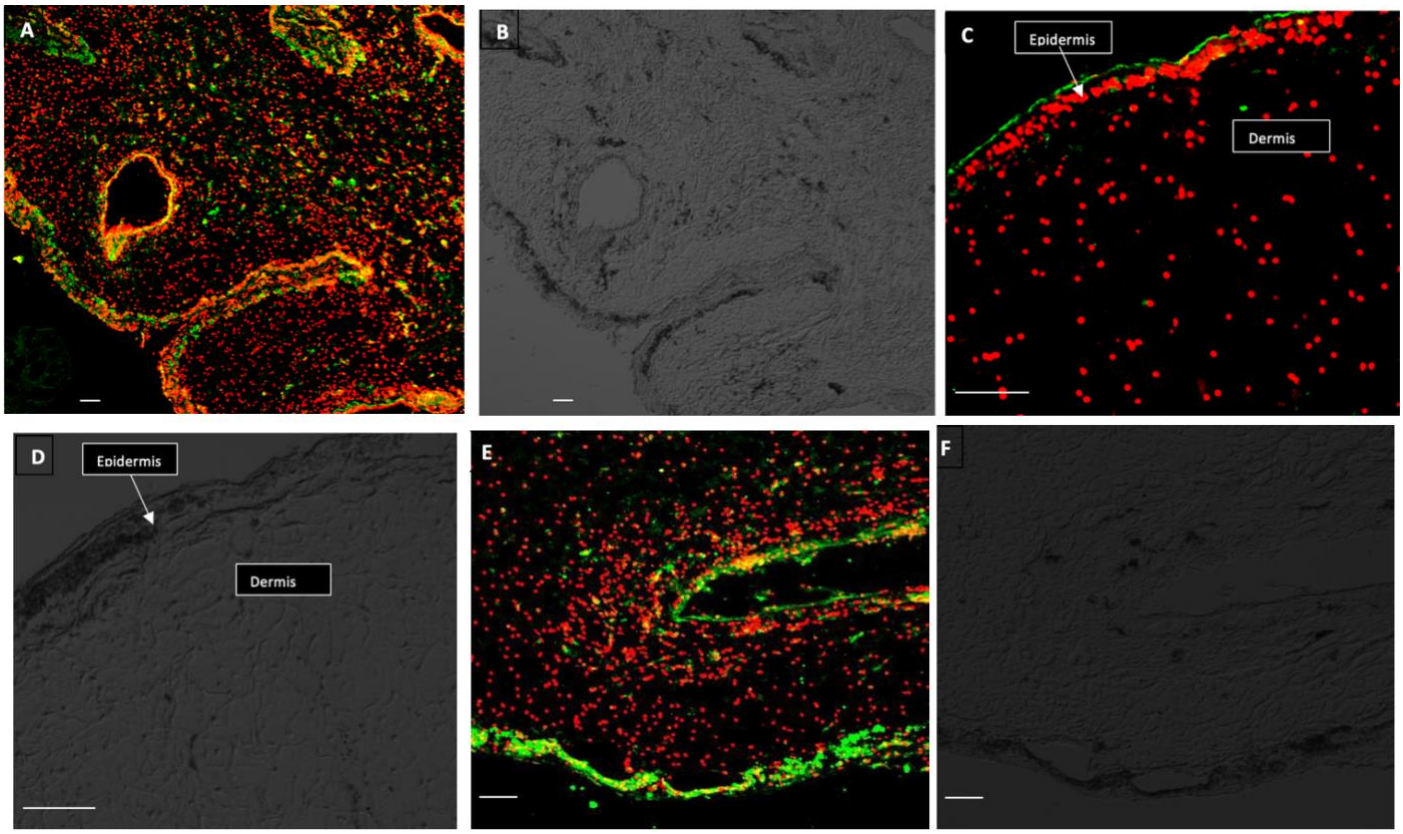

Plate 2.
GFSKLYFamide-like immunoreactivity in the (A) tentacles, (C) body wall and (E) tube foot. Nuclei were counterstained with TOPO-3 (red). Phase contrast images of the tentacles, body wall and tube foot shown in (B), (D) and (F) respectively. Scale bar = 50μm

DISCUSSION

Earlier reports describe GFSKLYFamide as a neuropeptide of heptapeptide nature that was first isolated from the sea cucumber, *Holothuria glaberimma* (Diaz- Miranda et al., 1992). GFSKLYFamide functions like other neuropeptides is believed to be important in mediating several physiological processes including neuromuscular transmitter or neuromodulation (Díaz-Miranda et al., 1995). Previous report on the expression of GFSKLYFamide-like immunoreactivity in the sea cucumber *H. scabra* was limited to the digestive, haemal, respiratory and reproductive systems (Díaz-Miranda et al., 1995; Ajayi and Withyachumnarnkul, 2015; Ajayi and Amedu, 2018). In the present study, the tentacles show GFSKLYFamide-like immunoreactivity distributed mainly around the apical surface of the epidermal layer. Immunoreactivity was also observe to be dispersed within the connective tissue layer of the tentacles. Earlier study reported, GFSKLYFamide-like immunoreactivity expression in the tentacles however it was in *H. glaberimma* (Elphick, 2014; Díaz-Miranda et al., 1995). Also, in a similar study carried out earlier, FMRFamide neuropeptide immunoreactivity was reported on the apical part of the epidermal layer of tentacles in *H. scabra* (Ajayi & Withyachumnarnkul, 2013).

GFSKLYFamide-like immunoreactivity in the body wall for this study was strongly express in the epithelial lining of the epidermal layer with weak expression seen in the dermal layer. Although Díaz-Miranda et al., 1995 reported GFSKYLFamide-like immunoreactive in nerve fibres of the longitudinal and circular muscle layers of the body wall, their study did not specify the level of GFSKLYFamide expression intensity in epidermis or dermis like the present study did.

The primary responsibility of tube feet in holothurians is for locomotion and exchange of gas (Hamel et al., 2001). This study show GFSKLYFamide-like immunoreactivity in the connective tissue of the tube foot as well as in the luminal epithelial lining. This observation is similar to the report of Ajayi & Withyachumnarnkul, 2013 on the expression of FMRFamide in the superficial layer of the epidermis as well as in the internal connective tissue layer of the tube foot surrounding the mesothelium. The difference between their report and the current observation was that neuropeptide FMRFamide and not GFSKYLFamide was investigated in the tube foot. Díaz-Miranda et al., 1995 reported the presence of GFSKLYFamide-like immunoreactivity in the tube foot. However their report is different from the present observation because in their report, GFSKLYFamide-like immunoreactivity was associated with the neuronal somata and fibres of the tube foot and not the epithelial layer and connective tissue as observed in the present study. Also, in their report, *H. glaberimma* was the model used for the study and not *H. scabra* as in the present study.

This study has shown immunohistochemical evidence for the presence of GFSKLYFamide-like neuropeptide in the tentacles, body wall and tube foot of *H. scabra*. It is however suggested that further studies be carried out to determine the existence of GFSKLYFamide-like immunoreactivity in the tentacles, body wall and tube foot of other holothurians that are yet to be investigated.

Acknowledgement
Authors appreciate support from the Center of Excellence for Shrimp Molecular Biology and Biotechnology (CENTEX Shrimp), Faculty of Science, Mahidol University, Thailand as well as by Professor García-Arrarás (University of Puerto Rico, USA) for the generosity in providing antibody..

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