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PRELIMINARY IMMUNOHISTOCHEMISTRY STUDY ASCERTAINED THE EXPRESSION OF FMRFAMIDE-RELATED PEPTIDES IN THE INTESTINE AND DORSAL ROOT GANGLIA IN MICE

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ABSTRACT

FMRFamide-related peptides are widely disseminated in the mammalian central nervous system. They are involved in abroad pattern biological roles like pain modulation, cardiovascular, and neuroendocrine functions. Although, they have a wide range of functions their source and distribution in different mammalian organs are still not well-known. Numinous studies concerned with FMRFamide-related peptides distribution and biological role in insects and nematodes while the data in mammals are scarce. In the present study using immunohistochemistry, we detected FMRFamide-related peptides in the ileum, colon, and dorsal root ganglia of adult mice.

Keywords: Immunohistochemistry, Anti- FMRFamide, Mice, Ileum, Colon

INTRODUCTION

FMRFamide-related peptides are a class of amidated peptides including, Neuropeptides FF (NPFF), AF (NPAF), and SF (NPSF) (Price and Greenberg 1977, Yang *et al.*, 1985, Yang and Martin 1995 and Panula *et al.*, 1996). They are expressed and widely distributed throughout the mammalian central nervous system and implicated in a wide range of functions (Panula *et al.*, 1996). Although an anti-opiate effect (Tang *et al.*, 1984 and Malin *et al.*, 1990) and related pain modulation (Yang *et al.*, 1985 and Gouardères *et al.*, 1993) is the most prominent effects of FMRFamide-related peptides, they are also implicated in cardiovascular regulation (Panula *et al.*, 1996) and neuroendocrine functions (Majane and Yang 1990,

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Majane *et al.,* 1993). Interestingly, the precursor protein NPFF gene (ProNPFF), which encodes for NPFF has been cloned and shown to encode not only NPFF, but also NPAF and NPSF in human, murine, bovine, and rat tissues (Vilim *et al.,* 1999). Even though the biological roles of NPFF, NPAF, and related FMRFamides are well identified, their source and distribution in mammals are not well-defined. They represent the main identified family of neuropeptides in invertebrates (Peymen *et al.,* 2014). In insects, FMRFamide-related peptides have been isolated and shown to be distributed extensively throughout the nervous system, salivary glands, accessory glands, and muscle (Orchard *et al.,* 1997). In nematodes, they considered the chief regulators of energy balance, feeding behavior, reproduction, and sensory modulation (Peymen *et al.,* 2014).

MATERIALS AND METHODS

Animals

Ten adult male mice BL/6 (wild black mice) were sacrificed for sample collection. Animal housing and handling procedures were conducted in accordance with the European Directive 86/609/EEC.

Tissue processing for immunohistochemistry

Male BL/6 mice were sacrificed using cervical dislocation and then dissected. Tissues of interest were fixed in 4% paraformaldehyde solution for 2 hrs. For paraffin sections, after fixation, the tissues were placed in a cassette at the correct orientation. Next, the cassettes were placed in a Histokinette (24 hours-programm) to dehydrate the tissues in ascending series of ethanol solutions, subsequently to be cleared in methyl-benzoate and xylene solutions, and finally infiltrated with melted paraffin in order to become embedded in paraffin blocks. Next, 5-µm-thick paraffin sections were cut using a LEICA RM 2245 microtome.

Immunohistochemistry for paraffin sections

The paraffin sections were deparaffinized using xylene then rehydrated by gradient ethanol dilutions (100%-90%-70%) and finally rinsed in distilled water. Next, microwave antigen retrieval was performed by heating the sections in 0.1M sodium citrate/0.1 M citrate buffer (pH 6.0) for 5x3 min each; subsequently, the slides were left to cool down for 45 min. Blocking of endogenous peroxidase was carried out with 0.3 % hydrogen peroxide (H_2O_2) solution for 20 min. To block unspecific binding, incubation with (1:5) normal horse serum in PBS with 1% bovine serum albumin (BSA) was performed. Subsequently, sections were incubated with primary anti-FMRFamide (1:500) (Merck-Millipore, AB15348) diluted in 0.1 M PBS (phosphate buffer solution) with 1% BSA overnight at 4°C. Sections then were incubated with secondary biotinylated donkey anti-rabbit (1:200) in PBS with 1% BSA for 2 hrs, followed by 3 washes with PBS and incubation with Extravidin peroxidase (1:200) (Sigma) in PBS with 1% BSA for 30 min. After a washing step, DAB solution (Dako

K3468) was added until color developed and then the reaction was stopped by washing the section with PBS. Sections were then washed with tap water and distilled water, for 5 min each, followed by counterstaining with haematoxyline. All incubations were performed at RT (Room temperature), except for those with primary antibody which took place at 4°C.

RESULTS

We evaluated the presence of RF-amides in DRG (dorsal root ganglia) and in the ileal and colonic wall immunohistochemically. Negative controls in which the primary antibody was omitted or staining was done with IgG control did not yield any immunoreactivity. Staining of paraffin-embedded ileum, colon, and dorsal root ganglia (DRG) sections revealed the presence of FMRF-amides (Fig.1). In DRG FMRF-amide peptides were expressed within the nerve cells and nerve fibers (Fig.1A). In the ileum and colon, FMRF-amide immunoreactivity was detected in enteroendocrine cells and in intramuscular nerve fibers (Fig.1 C-F).

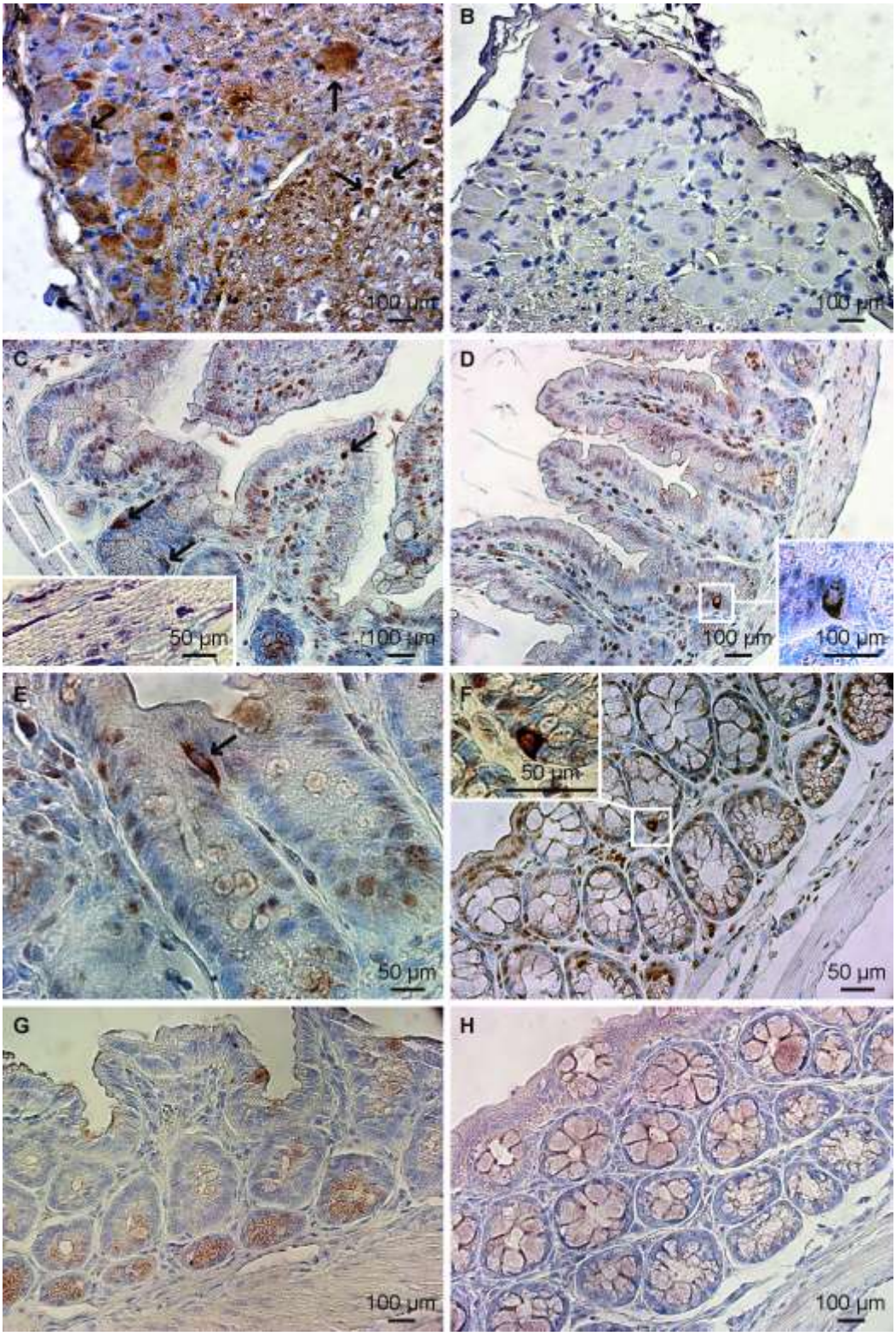

Fig. 1: Paraffin embedded sections, immunohistochemically stained for FMRFamide. (A) FMRFamides were expressed in nerve cells and nerve fibers in DRG (Arrows). (B) Omission of primary antibody led to elimination of immunostaining in DRG. (C) FMRFamides were detected in nerve fibers (square) and in enteroendocrine cells (arrows) in the ileum. (D-E) Enteroendocrine cells showed immunoreactive granules (square, arrow). (F) Enteroendocrine cells were also immunoreactive in the colon (square). (G-H) Staining with IgG control revealed no immunoreactivity in ileum or colon.

DISCUSSION

We used the anti-FMRFamide antibody to detect the potential sources of NPAF and NPFF *in situ*, which revealed the presence of FMRFamide peptides in the enteroendocrine cells and nerve fibers located in the enteric plexuses of the ileum and colon. We had proposed the cells were enteroendocrine cells according to their location and the solitary distribution. Although nothing is known before about the expression and function of RF-amides in the mammalian GI tract it is known that the FMRFamide is the most abundant neuropeptide in endocrine cells of insect alimentary tract (Oetken *et al.*, 2004 and Haselton *et al.*, 2008). Also, we detected the sources of NPAF and NPFF in dorsal root ganglia. Although, early studies proposed that there may be an NPFF-Iike peptide in the sympathetic ganglia and adrenal medulla and not detected NPFF in the spinal or sympathetic ganglia (Panula *et al.*, 1987 and Lee *et al.*, 1993). Later, they are identified to be highly condensed in the posterior pituitary, spinal cord, hypothalamus, and medulla in mouse, rat, the bovine, and human brain (Panula, Aarnisalo *et al.*, 1996).

CONCLUSION

The current study represents the first evidence of the expression of FMRFamide peptides in the mammalian gastrointestinal tract. Also, we are suggesting that enteroendocrine cells are potential sources. Further investigations and characterizations are recommended.

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دراسه نسيجوكيميائيه مناعية مبدئية كشفت عن وجود الببتيد FMRFamide في الامعاء وفي العقد الجذرية الظهرية في الفئران

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FMRFamide related-peptides عبارة عن مجموعة من الببتيدات منتشرة على نطاق واسع في الجهاز العصبي المركزى لها العديد من الوظائف من أهمها تنظيم الإحساس بالألم. تضم هذة المجموعه كلا من نيروببتيد NPAF, NPFF و NPSF وعلى الرغم من الدرايه الجيدة لدورها الحيوي مازال الكشف عن اثرها خارج الجهاز العصبي في الثدييات محدود للغايه. بالرغم انها تعتبر من اشهر واهم الببتيدات المعروفه في اللافقاريات وتلعب دور اساسي في جميع الوظائف الحيوية. لذلك في هذه الدراسه باستخدام الصبغات الكيميائية النسيجه المناعية قمنا بتفقد اثرها في الامعاء وفي العقد الجذرية الظهرية. من اجل ذلك تم عزل جزا من الامعاء الدقيقة (Ileum) وجزا من الامعاء الغليظة (Colon) وعزل ايضا العقد الجذرية الظهرية من الفئران السوداء البرية Bl/6. و باستخدام الاجسام المضادة Anti-FMRFamide تم الكشف عن الببتيدات FMRFamide الامعاء والتي من المحتمل ان تكون في كلا من خلايا الغدد الصماء المعوية والأليف العصبية الموجودة في الضفيرة المعوية. وبذلك تكون هذه الدراسة اول دراسة تكشف عن وجود **FMRFamide related-peptides** في الامعاء في الثدييات. ونوصي بمذيد من التحقق لتأكيد الاماكن المحتملة.