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Characterization and Distribution of FMRFamide Immunoreactivity in the Rat Central Nervous System

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O'DONOHUE, T. L., J. F. BISHOP, B. M. CHRONWALL, J. GROOME AND W. H. WATSON, III. *Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system.* PEPTIDES 5(3) 563–568, 1984.— FMRFamide immunoreactive material (irFMRFamide) was studied in rat brain and gastrointestinal tract. Highest irFMRFamide concentrations were found in tissues of the gastrointestinal tract and, in the brain, highest concentrations were found in the hippocampus, midbrain, brainstem and hypothalamus. High pressure liquid chromatographic characterization of irFMRFamide demonstrated that the immunoreactive material in brain, pancreas and duodenum was different from molluscan FMRFamide but it was also distinct from any known neuropeptide.

FMRFamide immunoreactivity Central nervous system Gastrointestinal tract Rat brain

A molluscan cardioactive tetrapeptide, Phe-Met-Arg-Phe-NH_2 or FMRFamide, was isolated from the ganglia of the clam, *Macrocallista nimbosa* [30]. The peptide has positive ionotropic, positive chronotropic and antiarrhythmic actions on the clam heart [31]. It also acts on the hearts of a variety of other species including the horseshoe crab, *Limulus polyphemus* [41]. The peptide may, in fact, be involved in cardiovascular regulation in mammals as intravenous injection of FMRFamide in rats causes increased blood pressure, heart rate, and respiration rate [26]. However, the actions of FMRFamide are not limited to muscles. Iontophoretic application of FMRFamide excites rat medullary reticular formation neurons [16] and cultured mouse spinal neurons [11]. In *Helix*, the ionic mechanisms underlying the effects of FMRFamide on identifiable cells have been described [10].

A number of peptides related in structure to FMRFamide have been identified throughout the animal kingdom (Fig. 1). Another cardioexcitatory peptide, p-Glu-Asn-Phe-Ile-Arg-Phe-NH_2, has been isolated from the snail, *Helix Aspersa*. It has a similar bioactivity to FMRFamide, but appears to be more potent than FMRFamide on the *Helix* heart [29]. Peptides with similar amino acid sequences to FMRFamide have also been identified in mammals. As shown in Fig. 1, an identical FMRF sequence appears in proenkephalin [27]. The peptide Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH (Met-Enk-RF) is at the C-terminus of proenkephalin where it is preceded by paired basic amino acids which apparently serve as a signal for a trypsin-like enzyme which releases the peptide from the precursor. Although the C-terminal tetrapeptide of Met-Enk-RF is identical to FMRFamide it has only been found in the free acid form. Met-Enk-RF exists in high concentration in the mammalian brain and peripheral tissues [3, 33, 36, 37, 38]; is a biologically active opiate peptide [33, 36, 37]; but does not possess FMRFamide bioactivity because it lacks the C-terminal amide [18]. Structural similarities to FMRFamide also exist in the amino acid sequences of cholecystokinin (CCK), γ_1 melanocyte stimulating hormone (γ_1-MSH; 23), pancreatic polypeptide (PP; 7,22), peptide YY (PYY; 39) and neuropeptide Y (NPY; 40) (Fig. 1).

In addition to these known peptides with sequences similar to FMRFamide, an irFMRFamide has also been identified in mammalian brain [13, 14, 15, 42]. The peptide is clearly distinct from FMRFamide as it is larger and less basic than FMRFamide [15]. The fact that FMRFamide is similar in structure to the C-terminal regions of PP and NPY has led to the suggestion that FMRFamide immunoreactivity in brain may actually be due to PP or NPY [17] which have both been identified in mammalian brain by immunocytochemistry in the case of PP [20, 21, 24, 25, 28] or isolation and sequencing in the case of NPY [40]. The purpose of this paper and the next [9] was to determine if mammalian FMRFamide is a distinct peptide from NPY or PP and to describe the chromatographic characteristics and anatomical distribution of this FMRFamide-like peptide. In this paper, the immunological and chromatographic characteristics and gross distribution of FMRFamide are discussed and compared to NPY, PP and related peptides. In the next paper, the anatomy of the FMRFamide system as determined by immunocytochemistry is discussed and compared with that of NPY and PP containing neurons. In the third paper in the series [41], the anatomy and chromatographic properties of another FMRFamide-like peptide, in an invertebrate, *Limulus polyphemus*, are discussed and compared to FMRFamide in other invertebrates and mammals.

FMRFamide	Phe - **Met** - **Arg** - **Phe** - NH_2
pQNFIRFamide	pGlu - Asn - **Phe** - Ile - **Arg** - **Phe** - NH_2
Met-enkephalin-Arg-Phe	Tyr - Gly - Gly - **Phe** - **Met** - **Arg** - **Phe** - OH^2
γ_1-MSH	Tyr - Val - Met - Gly - His - **Phe** - **Arg** - Trp - Asp - **Arg** - **Phe** - NH_2
CCK-octapeptide	Asp - Tyr - Met - Gly - Trp - **Met**- Asp - **Phe** - NH_2
Pancreatic polypetide, NPY, PYY	**Arg** - Tyr - NH_2

FIG. 1. Amino acid sequences or partial sequences of peptides structurally related to FMRFamide. Homologous sequences are in bold faced letters.

METHOD

Subjects

For all experiments, subjects were male Sprague-Dawley rats (Taconic Farms) weighing between 250 and 300 grams, receiving ad lib water and rat chow in a climate controlled 12 hour light/12 hour dark colony room.

Radioimmunoassay

The peptide used for iodination was Tyr-Met-Art-Phe-NH_2 (TMRFamide, Peninsula Labs), prepared by a modified chloramine T method [19] using Na^{125} I (Amersham). Iodinated FMRFamide was purified on a Sephadex QAE column (Pharmacia) eluted with 0.02 M acetic acid containing 0.1% BSA.

FMRFamide-coupled antigen was prepared by conjugating FMRFamide (Peninsula) to succinylated thyroglobulin via the N-terminus of FMRFamide. Bovine thyroglobulin (Sigma) was succinylated with succinic anhydride (Sigma). New Zealand albino rabbits (NIH colony) were injected intradermally once a month for three months with the FMRFamide (100 μg) conjugate emulsified with Freund's complete adjuvant (Calbiochem). Rabbits were bled one week after each of the last two boosts. All three rabbits produced antisera of relatively high titer—the final antisera dilution required to bind 30% of the tracer ranged from 1:5,000 to 1:100,000.

Characterization of the specificity of the antisera was accomplished by determining the degree of cross-reactivity of various peptides with synthetic FMRFamide in radioimmunoassay (RIA). Bovine pancreatic polypeptide (BPP), NPY, the C-terminal hexapeptide of BPP (CTHP-NH_2), Met-Enk-RF, γ_1-MSH, BAM 22P, TMRFamide, and FMRF-OH were included in FMRFamide RIAs in concentrations ranging from 10^{-12} to 10^{-3} molar. All peptides investigated were purchased from Peninsula Labs except for BPP which was a generous gift from Dr. Ron Chance (Lilly).

In order to further characterize the antisera, inhibition of tracer binding induced by increasing concentrations of synthetic FMRFamide was compared to inhibition of tracer binding induced by increasing volumes of rat brain homogenate supernatants. This test for degree of parallel inhibition of tracer binding was performed for all three antisera.

RIA Procedure

On the first day of the assay, standards and sample aliquots were resuspended in a total volume of 800 μl of 0.05 M Tris-HCl (Sigma) containing 0.1% bovine serum albumin (Fraction V from Sigma), with 0.02% sodium azide added as a preservative. The final pH of the RIA buffer was 7.8 at 4°C. Next, 100 μl of the diluted antiserum (final dilution 1:10,000 for antisera 212, 222 and 1:150,000 for 232) and 100 μl of tracer (approximately 5000 cpm) were added to the assay tubes so that the total assay volume per tube was 1 ml. All tubes were then vortexed and incubated at 4°C for approximately 48 hours. On Day 3 of the assay, the antibody ligand complexes were precipitated by the addition of 200 μl of 30% sheep anti-rabbit antiserum in RIA buffer followed by the addition of 200 μl of a 19% solution of polyethylene glycol (Carbowax 6000, Fisher) in RIA buffer. All tubes were then vortexed, incubated for 30 minutes at 4°C, and centrifuged (6000×g for 30 minutes). After decanting the supernatants, the precipitates were counted in a Micromedic 4/600 Gamma Counter. Counts were automatically converted to picograms by a linear calculation program.

Reverse Phase High Pressure Liquid Chromatography

Elution characteristics of the FMRFamide-like substance in the rat brain and gastrointestinal tract were investigated by reversed phase HPLC. Rat forebrain, duodenum and pancreas were dissected from rats killed by decapitation. Samples were homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) in about 5 ml of ice cold 0.1 M HCl per brain. HCl was chosen for extraction because preliminary studies showed that the yield of irFMRFamide was slightly greater when rat brain tissue was homogenized in 0.1 M HCl as compared to 1 M acetic acid, and much greater than when extractions were done in water. Preliminary experiments also indicated that peptide yields from boiled tissue and from unboiled tissue were not significantly different, so boiling was omitted in subsequent extractions. After extraction, homogenates were ultracentrifuged (30,000×g for 60 minutes), and the supernatants were decanted and dried in a vacuum centrifuge (Savant Instruments, Hicksville, NY). The samples were resuspended in 3 ml of water and particulate matter was removed by centrifugation at 30,000×g for 60 minutes and filtration using a Millipore prefilter in a series with a Millipore 0.45 micron sample filter. The filtrate was injected onto the HPLC and run using a 10 minute isocratic de-salting step followed by a linear gradient of 15 to 42% acetonitrile in 90 minutes at a flow rate of 1 ml per minute.

The HPLC system consisted of: a U6K injector (Waters Associates, Milford, MA), two Waters model 6000A solvent delivery systems, a Waters Model 441 UV/VIS absorbance detector, a Waters extended wavelength monitor, a Houston Instruments Omni Scribe recorder, an LKB Bromma 2211 Superrac. and a Waters Model 720 Systems Controller. A 4 mm×30 cm Waters Bondapak C_{18} column was used. The mobile phase consisted of a linear gradient comprised of triethylammonium formate buffer (TEAF) [32] and an acetonitrile-TEAF mixture. TEAF was prepared by adjusting the pH of 0.25 M formic acid (J. T. Baker Chemical Co.) to pH 3.0–3.5 with triethylamine (Pierce Chemical Co.). Acetonitrile-TEAF was prepared by filtering acetonitrile (Burdick and Jackson) through a Millipore FH filter, degassing briefly, and mixing with TEAF which was also filtered (Millipore HA filter) and degassed just before use. This mixture was comprised of 60% acetonitrile and 40% TEAF. TEAF was prepared with water purified on a Millipore Milli-RO4 system employing reverse osmosis followed by a Millipore Milli-Q ion exchange-charcoal filtration system (Millipore Corp., Bedford, MA).

The elution of irFMRFamide was compared to the elution of 5 micrograms of synthetic FMRFamide (Peninsula) as monitored by RIA and by absorbance at 254 nm. For all injections, 1 ml fractions were collected for RIA. Brain and digestive tissue fractions were dried in a

TABLE 1

REGIONAL DISTRIBUTION OF irFMRFamide IN RAT BRAIN AND GASTROINTESTINAL SYSTEM

Region	Fmole/Region	Fmole/mg Wet Weight
Forebrain		
Olfactory Bulb	0	0
Septum	0	0
Striatum	0	0
Hypothalamus	6.6 ± 3.0	0.16 ± 0.07
Thalamus	2.3 ± 1.7	0.06 ± 0.04
Hippocampus	44.6 ± 11.8	0.38 ± 0.10
Cerebral Cortex	37.8 ± 12.5	0.05 ± 0.02
Midbrain	27.9 ± 5.8	0.26 ± 0.05
Cerebellum	3.4 ± 3.0	0.02 ± 0.02
Hindbrain		
Pons	14.5 ± 7.9	0.14 ± 0.08
Medulla	18.9 ± 6.7	0.18 ± 0.06
Cervical Spinal Cord	0	
Pituitary Gland		
Anterior Lobe	0	
Neurointermediate Lobe	21.7 ± 9.9	10.85 ± 4.95
Pineal Gland	0	
Gastrointestinal Tissues		
Pancreas	2886.0 ± 811.8	2.05 ± 0.58
Duodenum	562.5 ± 219.0	0.63 ± 0.25
Stomach	8020.0 ± 3370.2	4.91 ± 2.06
Large Intestine	343.3 ± 178.9	0.80 ± 0.42

Mean ± S.E.M.
N=6.

vacuum centrifuge (Savant), resuspended in 300 μl of RIA buffer, and duplicate 100 μl aliquots were taken from each fraction. Duplicate 1 μl aliquots were taken directly from standard fractions and the irFMRFamide was measured for all samples by RIA.

Distribution of irFMRFamide

A total of four experiments were performed to study the distribution of irFRMFamide. For each of the first three experiments, 6 rats were killed by decapitation, and dissected into the regions shown in Table 1. All tissues were individually frozen on dry ice immediately after dissection, weighed, and individually homogenized with a Polytron in 5 volumes of ice cold 0.1 M HCl. Following homogenization, samples were centrifuged (12,000×g, 45 minutes) and duplicate aliquots of the supernatants were added to 12×75 borosilicate test tubes. Tubes were then vacuum centrifuged to dryness and saved for RIA. In some experiments, supernatants were injected into SepPaks which were rinsed with deionized water and peptides were eluted with 50% acetonitrile. Aliquots were taken and samples dried by vacuum centrifugation. The fourth regional distribution study was carried out in exactly the same manner as the preceding three studies except that brain samples consisted of the pooled tissues of 2 rats, and that aliquots of supernatants were added to borosilicate tubes pre-filled with 1 ml of a 0.1% BSA (fraction V from Sigma) prior to vacuum centrifugation. Although the results of the four distribution studies were similar, the addition of BSA to the assay tubes in the fourth run led to more consistent results in the RIA.

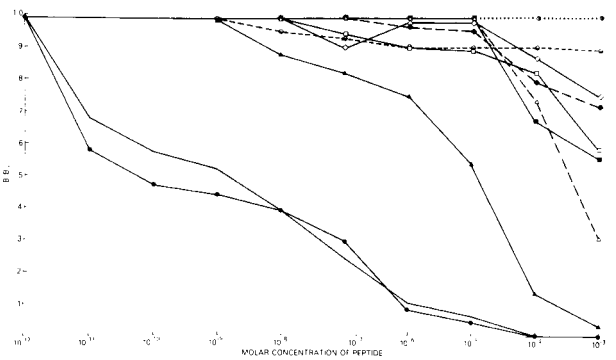

FIG. 2. Specificity of FMRFamide antisera 212; (●) FMRFamide, (−)-TMRF-NH_2, ■-FMRF-OH, ◑-CCK tetrapeptide, △-BPP, ◇-Met-Enk-RF, ◑-BAM 22, ▲-γ_1-MSH, -NPY, ◆-CTHP-NH_2.

RESULTS

Radioimmunoassay

All three antisera characterized showed similar cross-reactivity with the peptides examined. Data obtained using one of these antisera (No. 212) is presented in Fig. 2. Of the nine peptides investigated, only γ_1-MSH, BPP and TMRFamide showed cross-reactivity greater than 0.000001% as determined by comparing the ED50 of each peptide with that of standard FMRFamide. γ_1-MSH showed approximately 0.0003% cross-reactivity, BPP 0.00001%

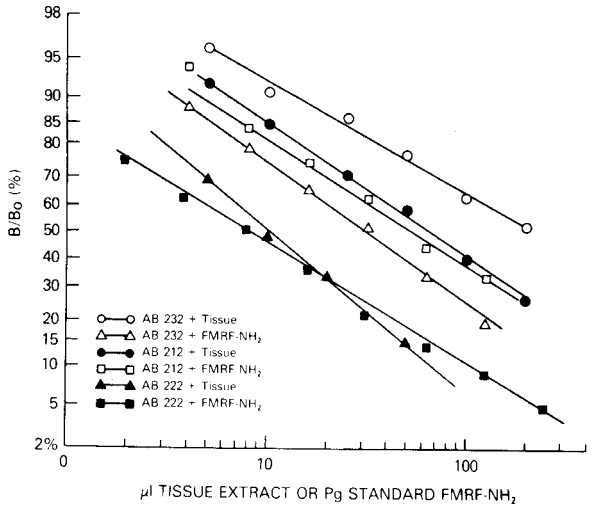

FIG. 3. Comparison of parallel inhibition of tracer binding by FMRFamide and by rat brain extracts in the FMRFamide RIA using different antisera.

cross-reactivity and, as expected, TMRFamide showed essentially 100% cross-reactivity. All three antisera appear to be directed at the C-terminus of FMRFamide.

All three antisera also measured approximately linear increases in FMRFamide equivalent immunoreactivity with increasing aliquots of rat brain tissue extract (Fig. 3). However, antiserum No. 212 appeared to recognize rat brain irFMRFamide better than antiserum No. 232 (by a factor of approximately 2:1) and use of this antiserum resulted in slightly better parallel inhibitor of tracer binding than when the other two antisera were used. Consequently, antiserum No. 212 was used exclusively for subsequent characterization and distribution experiments. Once characterized for rat brain homogenates, antiserum No. 212 was used to measure irFMRFamide in gastrointestinal tract, but attempts to generate parallel inhibition of tracer binding with digestive tissue extracts led to inconsistent results. Increasing aliquots of both duodenum (proximal 5 cm) and pancreas always produced increasing yields of irFMRFamide, but the magnitude of the increases was inconsistent. However, when extracts were run through the SepPaks before assay, better parallel inhibition was observed.

Reverse Phase HPLC

Results of multiple HPLC runs revealed three distinct peaks of irFMRFamide obtained from extracts of rat brain, duodenum and pancreas. Since all three immunoreactive peaks of each type of tissue extract eluted significantly later from the reverse phase column than did synthetic FMRFamide, the endogenous substance in the rat must be more hydrophobic than synthetic FMRFamide. As depicted in Fig. 4, immunoreactive peaks occurred in essentially the same fractions for duodenum and for pancreas. However, immunoreactive peaks obtained from brain appeared to be consistently shifted slightly to the right so that each individual peak eluted slightly later than the corresponding peak obtained for duodenum and for pancreas.

Distribution of irFMRFamide

The distribution of irFMRFamide in rat brain is presented

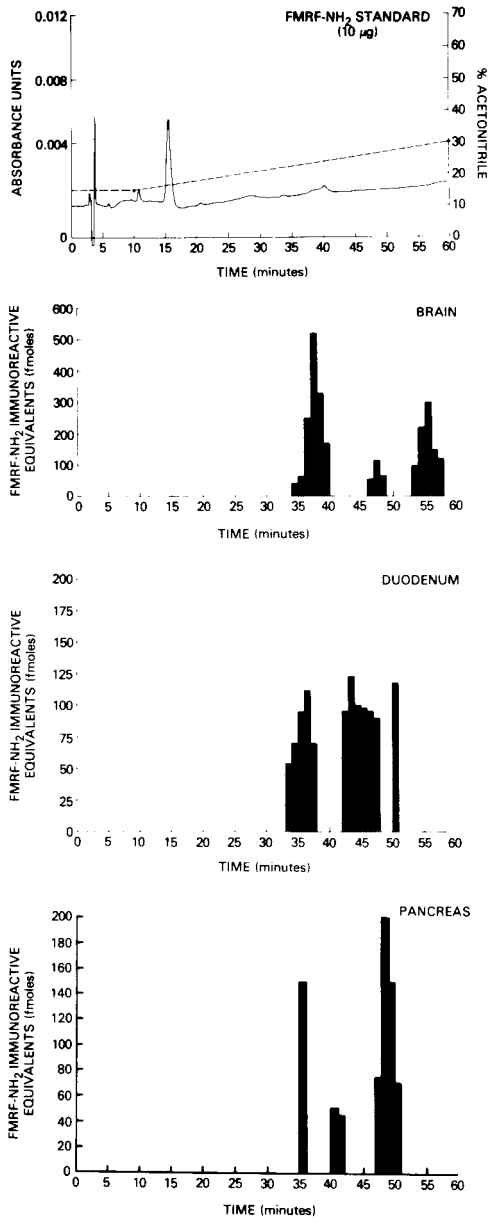

FIG. 4. Reversed phase high pressure liquid chromatographic fractionation of extracts of brain, duodenum, and pancreas compared to the elution of synthetic FMRFamide.

in Table 1. Concentrations of irFMRFamide in the rat brain were highest in the hippocampus, midbrain, brainstem, and hypothalamus, and lowest in the olfactory bulb, septum and striatum. Extracts of spinal cord contained measurable levels of irFMRFamide in only one distribution study in which relatively large sections of spinal cord were used.

The concentration of irFMRFamide in gastrointestinal tissues measured after the SepPak purification step is also presented in Table 1. Note that relatively high concentrations were found in the distal half of the stomach and in the pancreas.

DISCUSSION

The FMRFamide antisera developed and used for this study were quite sensitive and appeared to be directed against the C-terminus of FMRFamide and did not significantly crossreact with related peptides such as FMRF-OH, CCK, and Met-Enk-RF but crossreacted slightly with peptides that had similar C-terminal structures such as BPP (0.00001%) and γ_1-MSH (0.0003%). Although all three antisera showed similar cross reactivity to these known peptides, the antisera varied in their ability to recognize mammalian FMRFamide and antiserum 232 recognized only one-sixth the amount of irFMRFamide in rat brain as did antiserum 212. It also seems that the antisera used in these studies did not recognize rat brain FMRFamide as well as the antiserum used by Dockray and Williams [15] which quantitatively measured one to two orders of magnitude higher irFMRFamide levels in brain than does antiserum 212. The fact that different antisera crossreact with mammalian FMRFamide to such different extents suggests that the peptide is different in structure from synthetic or molluscan FMRFamide.

The results of HPLC experiments further demonstrate that mammalian FMRFamdie is structurally distinct from molluscan FMRFamide. Rat brain, duodenum and pancreas all contained three peaks of irFMRFamide that eluted significantly later than synthetic FMRFamide from the reversed phase column and are therefore more hydrophobic than molluscan FMRFamide. The structure of these peptides is, as yet, unknown. The increased hydrophobicity of mammalian FMRFamide may be due to the fact that they are larger or less charged than FMRFamide as has been suggested by Dockray and Williams [15]. If mammalian FMRFamide contains a methionine, as does the molluscan form, one of the peaks observed may be the methionine sulfoxide form of mammalian FMRFamide. It is interesting to note that irFMRFamide of both the duodenum and pancreas elute before that in brain. Perhaps a minor structural change due to synthesis of FMRFamide by a modified gene or a difference in post-translational processing is responsible for the chromatographic distinction of brain and gut immunoreactivity.

The results of these studies clearly demonstrate that irFMRFamide in rat brain as measured by our antibody is not identical to porcine NPY or BPP as had been hypothesized previously [17]. First, the cross reactivity of NPY and BPP with the FMRFamide antisera used in this study is minimal (0.00001%). Second, the assay detects a relatively small amount of irFMRFamide in pancreas which would not be expected if the assay recognized PP or PYY—the pancreatic form of NPY, which are both in extremely high concentrations in pancreas [7,39]. Third, HPLC results also indicate that irFMRFamide is distinct from porcine NPY or BPP as these peptides elute much later (at 35 and 37% acetonitrite, respectively). It is not known where rat NPY or PP elute as the structures of these peptides have not yet been described. Fourth, an antiserum raised against the C-terminal hexapeptide (CTH) of BPP, a structure that appears to be conserved in the PP sequence of most species, detects an immunoreactive peptide in rat brain [12] that can be chromatographically distinguished from FMRFamide (results not shown). Finally, as demonstrated in the next paper, FMRFamide antisera stain different neurons in brain than do PP, CTH, or NPY antisera which all stain identical regions [9].

Concentrations of FMRFamide were relatively high in the gastrointestinal tract and lower in the brain. Within the brain, highest irFMRFamide concentrations were observed in the hypothalmus, brainstem, midbrain and hippocampus. The neurointermediate lobe of the pituitary had particularly high irFMRFamide concentrations. However, it is possible that irFMRFamide in the pituitary may actually be due to γ-MSH which may be present in the intermediate lobe [1, 2, 4, 6, 34, 35]. Gamma MSH in the brain is in too low concentrations [2] to significantly interfere with the determination of brain irFMRFamide.

The presence of this extensive irFMRFamide neuronal system in mammalian brain suggests the involvement of this peptide system in numerous diverse actions. The fact that the peptide has been shown to be a potent excitatory agent on mammalian brain neurons [10,16] supports an important neuronal role for this peptide. It must be cautioned, though, that it was molluscan FMRFamide that was tested for physiological actions in mammals. It is possible that mammalian FMRFamide may have different actions from the molluscan form. If, however, mammalian FMRFamide is evolutionarily derived from molluscan FMRFamide, biological activity will likely be conserved. In fact, preliminary results indicate that mammalian brain may contain bioactive FMRFamide-like material. Nevertheless, to study and understand the role of FMRFamide in mammals, the structure of the mammalian peptide must be elucidated.

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