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Immunohistochemical Localization of RF-Amide and GABAA Receptors in the Hypostome and Tentacles of *Hydra vulgaris*

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IMMUNOHISTOCHEMICAL LOCALIZATION OF
RF-AMIDE AND GABA_A RECEPTORS IN THE
HYPOSTOME AND TENTACLES OF *HYDRA VULGARIS*

BY
JESSICA EASON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE
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UNIVERSITY OF RHODE ISLAND

2016

MASTER OF SCIENCE THESIS

OF

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2016

ABSTRACT

This study examined the distribution and localization of the neuropeptide arginine phenylalanine-amide (RF-amide) and GABA_A receptors in the hypostomal and tentacular nerve net of the cnidarian polyp *Hydra vulgaris*. Immunohistochemical techniques were used to visualize two distinct anti-GABA_A α -1 subunit positive-receptor ring structures, nerve cell bodies, neurites and epithelial cells, as well as an anti-RF-amide positive nerve ring, nerve cell bodies and neurites throughout the ectoderm of the hypostome and tentacles of ablated hypostomes. One proximal anti-GABA_A positive-receptor ring, exclusively labeling ectodermal effector cells, coinciding with an anti- α -tubulin- positive proximal nerve ring labeling nerve fibers is described. Another anti-GABA_A receptor- positive ring, labeling nerve cell bodies and fibers that contributed to the anti- α -tubulin- positive distal nerve ring is also reported. Anti-GABA_A receptors were also found on ectodermal effector and epithelial cells in the apex of the hypostome, in battery cell components in tentacles and at tentacle insertions, suggesting involvement of GABA_A receptors in both tentacle and contraction pulse control. Anti-RF-amide positive nerve fibers and nerve cell bodies of the proximal nerve ring and ubiquitously in bi-polar and tri-polar nerve cells and nerve fibers throughout the hypostome as well as in ganglion cells, sensory cells and neurites of the tentacle were identified. This study provides the first evidence of the localized distribution of anti-GABA_A α -1 subunit receptors in the nerves and effector cells of the hypostome of *H. vulgaris*. It also provides the first evidence of an intimate association between anti-GABA_A receptor and anti-RF-amide among the effector and neuronal cells, suggesting a mutual interaction in the neuro-effector systems controlling hydra's behavior.

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PREFACE

This thesis is being submitted in manuscript format. It is composed of one manuscript and three appendices. The title of the manuscript is “Immunohistochemical localization of RF-amide molecules and GABA_A receptors in the hypostome and tentacles of *Hydra vulgaris*”. This manuscript will be submitted to Tissue & Cell, with co-authors Bailey Munro, Linda Hufnagel and Gabriele Kass-Simon.

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MANUSCRIPT

**“IMMUNOHISTOCHEMICAL LOCALIZATION OF RF-AMIDE MOLECULES
AND GABA_A RECEPTORS IN THE HYPOSTOME AND TENTACLES OF
HYDRA VULGARIS”**

BY

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ABSTRACT

This study examined the distribution and localization of the neuropeptide arginine phenylalanine-amide (RF-amide) and GABA_A receptors in the hypostomal and tentacular nerve net of the cnidarian polyp *Hydra vulgaris*. Immunohistochemical techniques were used to visualize two distinct anti-GABA_A α -1 subunit positive-receptor ring structures, nerve cell bodies, neurites and epithelial cells, as well as an anti-RF-amide positive nerve ring, nerve cell bodies and neurites throughout the ectoderm of the hypostome and tentacles of ablated hypostomes. One proximal anti-GABA_A positive- receptor ring, exclusively labeling ectodermal effector cells, coinciding with an anti- α -tubulin- positive proximal nerve ring labeling nerve fibers is described. Another anti-GABA_A receptor- positive ring, labeling nerve cell bodies and fibers that contributed to the anti- α -tubulin- positive distal nerve ring is also reported. Anti-GABA_A receptors were also found on ectodermal effector and epithelial cells in the apex of the hypostome, in battery cell components in tentacles and at tentacle insertions, suggesting involvement of GABA_A receptors in both tentacle and contraction pulse control. Anti-RF-amide positive nerve fibers and nerve cell bodies of the proximal nerve ring and ubiquitously in bi-polar and tri-polar nerve cells and nerve fibers throughout the hypostome as well as in ganglion cells, sensory cells and neurites of the tentacle were identified. This study provides the first evidence of the localized distribution of anti-GABA_A α -1 subunit receptors in the nerves and effector cells of the hypostome of *H. vulgaris*. It also provides the first evidence of an intimate association between anti-GABA_A receptor and anti-RF-amide among the effector and neuronal

cells, suggesting a mutual interaction in the neuro-effector systems controlling hydra's behavior.

INTRODUCTION

Hydra vulgaris, a hydrozoan polyp, belongs to the phylum Cnidaria, the earliest phylum to possess a recognizable nervous system. The centralized yet simple nerve net of the hydrozoan nervous system maintains and facilitates complex behaviors similar to those of more evolved animals. This nerve net is organized into pharmacologically differentiable regions (Koizumi 2002), which contain all the classical neurotransmitters of more complex systems (Kass-Simon and Pierobon 2007). The diversity of neurotransmitters and their pharmacological properties, nerve cell types and neuronal connections of hydra makes the hydra nervous system an early version of a condensed complex nervous system.

Hydra has a long tube-shaped body column with two tissue layers, ectoderm and endoderm, that are separated by a gel-like mesoglea. The two tissue layers meet at the apex of the body to form a mouth (during feeding) at a dome-like hypostome that is surrounded by tentacles. The ectoderm of this nervous system has widely distributed interconnecting, synapsing neurons (Lentz and Barnett, 1965; Westfall, 1973; Kinnamon and Westfall, 1981; Koizumi, 2007). In the ectoderm of the hypostomal region of polyps and on the edge of the bell in hydrazoan medusae, the nerve net coalesces to form circumferential tracts, called nerve rings, comprised of the neurites of sensory and ganglion cells, capable of electrically integrating a variety of sensory, chemical and mechanical inputs (Davis et al., 1968; Matsuno and Kageyama, 1984; Dunne, et al., 1985; Grimmelikhuijzen, 1985, Kass-Simon and Pierobon, 2007, Hufnagel and Kass-Simon 2016).

It has been hypothesized that a neuropeptide-positive nerve ring in the hypostome of hydra reflects the common origin of the central nervous system of bilateral animals (Koizumi 2007). However, two distinct nerve rings were recently found within the hypostome of *Hydra vulgaris*, a proximal nerve ring running around the base of the hypostome and between each tentacle, as well as a distal ring closer to the apex -of the hypostome. The two rings are connected to one another by radial anastomosing neurons (Hufnagel and Kass-Simon, 2016). The proximal nerve ring is presumed to initiate body contraction pacemaker impulses as well as concerted tentacle contraction pacemaker impulses (Passano and McCullough, 1964; Rushforth and Burke, 1971; Kass- Simon, 1972, 1973, Kass-Simon and Passano, 1978; Hufnagel and Kass-Simon 2016), although this role of the contraction burst pacemaker site is not exclusive and other parts of the body column may initiate contraction pulses (Kass-Simon, 1973, Takagu et al., 2013). Like the proximal ring, the distal nerve ring is a loose organization of concentric, interconnecting neurons which is hypothesized to coordinate hydra's feeding response (Hufnagel and Kass-Simon, 2016). Axons of sensory neurons around the apex and the hypostome contribute to the distal ring and connect with the proximal ring (Hufnagel and Kass-Simon 2016). Either and/or both of these rings may correspond to the single denser nerve ring described by Koizumi (2007) and Grimmelikhuijzen (1985) in other species.

RF-amides, peptides characterized by a common carboxy-terminal arginine and an amidated phenylalanine motif, have been implicated as both fast neurotransmitters as well as neuromodulators in molluscs (Moulis and Huddart 2006). For many years, RF-amide has been considered to be the primary neurotransmitter in hydra's neuro-effector systems. Early studies showed that in hydra, RF-amide is

localized in dense-cored vesicles (Koizumi et al., 1989), and that distinct subsets of neurons with specific spatial distribution can be identified by immunohistochemical staining methods using antibodies made against RF-amides and tubulin (Grimmelikhuijzen 1985). Recent studies have shown that hydra RF-amide can gate cloned sodium channels expressed in *Xenopus* membranes (Golubvic, et al, 2007) and blockers of these sodium channels inhibit the reduced- glutathione (GSH) induced feeding behavior in *H. magnipapillata* (Assmann, et al., 2014). However, it is not at all clear from this evidence that RF-amide is acting as the primary fast neurotransmitter. In *H. vulgaris*, both hypostomal rings, the tentacular nervous system, and major portions of the ectodermal nerve net, label positively with anti-RF-amide antibody (Grimmelikhuijzen, 1985; Koizumi et al., 1992; Munro MS Thesis 2014). This suggests that RF-amide could act as a ubiquitous neuromodulator rather than a site-specific neurotransmitter.

Hydra are known to possess all of the neurotransmitters identified in humans and other mammals. These include catecholamines, serotonin and “fast” neurotransmitters glutamate, GABA, and acetylcholine (Kass-Simon and Pierobon, 2007 for review). In complex organisms, such as mammals and the moth *Manduca sexta*, GABA and the excitatory transmitter glutamate have been shown to co-localize with RF-amide. In the brain and sub-esophageal ganglion of these animals it has been suggested that RF-amide is co-transmitted with both GABA and glutamate (Homberg et al., 1990). Prior to the present study, evidence for the co-transmission and co-localization of RF-amide neuropeptides with “fast” neurotransmitters had yet to be obtained in Hydra.

Receptors for GABA have been described and/or characterized in many

species of different phyla: nematodes, mollusks, insects, crustaceans, tunicates, as well as in bacteria (Dent 2006). Biochemical and electrophysiological evidence has demonstrated that there is specific binding of GABA in hydra and that GABA affects the electrical impulses underlying the GSH induced mouth opening behavior (i.e. the feeding response) of the hypostome. It appears to do so by acting upon both its ionotropic GABA_A receptors and metabotropic GABA_B receptors (Pierobon et al., 1995; Kass-Simon et al., 2003; Lauro 2015). Therefore, GABA should be considered to be a part of the neuro-effector circuitry that mediates the feeding response in hydra.

This study uses immunohistochemical techniques to investigate where the ionotropic GABA_A receptors are located in *H. vulgaris* and to consider if their distribution is consistent with the known electrophysiological effect of GABA_A ligands in the hypostome and tentacles. The study is also aimed at determining the association of RF-amide positive nerve cells and cells containing GABA_A receptors.

MATERIALS AND METHODS

Animal Cultures: All experiments were carried out on *Hydra vulgaris*, incubated at 19 $\pm$ 2 °C in glass culture dishes containing bicarbonate versene culture medium (BVC; 0.1mM NaHCO₃, 1.0 mM CaCl₂, and 0.01 mM EDTA in distilled water, pH 7.0 $\pm$ 0.2; Kass-Simon et al. 2003). Animals were fed with *Artemia salina* nauplii on alternate days. The culture solution was changed 45 minutes after feeding.

Experimental hydras were chosen from 48 hour starved animals.

Immunohistochemistry: Individual hydra were transferred to the middle of a PAP-pen square on a slide. Each animal was transected directly underneath the tentacle/hypostome region using a scalpel (# 11 blade); the body was discarded, leaving only the tentacle and hypostome on the slide. The tissue was treated with dissociation medium (1 part glycerol, 1 part acetic acid, 7 parts deionized water), followed by Zamboni's fixative (Zamboni et al. 1967) and left overnight at 4°C in moist chambers. The following day, the slide-mounted preparations were washed with phosphate buffered saline (PBS), treated with 0.4 M glycine for 2 hours, washed with modified PBS solution containing 1% bovine serum albumin (MPBS/BSA; 1% bovine serum albumin, 0.2% Tween 80, 0.05% sodium azide, 0.1% polyethylene glycol 20 and 0.02% KCl; Erskine 1989) and subsequently incubated in primary antibody (Anti-RF-amide mouse monoclonal antibody received from O. Koizumi, Fukuoka Women's University, Japan or polyclonal anti-GABA_A receptor α 1 subunit produced in rabbit, affinity isolated antibody, Sigma-Aldrich), in moist chambers at room temperature (RT) overnight. The next day, the hydra were washed twice with MPBS/BSA and incubated overnight at RT in either 1/500 dilution of goat-anti-mouse secondary

antibody tagged with Alexa488 or a 1/400 dilution of goat-anti-rabbit secondary antibody tagged with Alexa488 (Molecular Probes/Invitrogen, Eugene, OR). The slides were washed twice in MPBS/BSA; then Prolong Gold mounting medium (Molecular Probes/Invitrogen, Eugene, OR) was added to each slide, and cover slips were applied. Once dry, the coverslips were sealed with nail polish and stored at -18°C until examined. Negative controls, in which the primary antibody was omitted, were run in all experiments. The RF-amide mouse monoclonal antibodies were produced by Dr. Osamu Koizumi and specificity was confirmed using ELISA. Protein alignments were performed using NCBI BLAST and Clustal Omega amino acid alignment tools. To ensure compatibility of the anti-GABA_A receptor α 1 subunit antibody (Sigma-Aldrich) with GABA receptors found in *Hydra vulgaris* (Appendix Figures 1-10). To compare to tubulin containing nerve structures, double labeling experiments were performed with anti- α -tubulin (Sigma Clone B512) primary antibodies and anti-GABA_A receptor. Anti-RF-amide labelled nerve structures were differentiated from anti-tubulin positive nerve structures by differences in the type of labeling; anti-RF-amide labeling was punctate whereas anti-tubulin labeling was continuous (Fig.1 and Fig. 4 a").

Controls: Specific binding of primary antibodies was demonstrated by the fact that negative control slides, lacking primary antibody, revealed only very low-level background fluorescence, which was not detectable under the imaging conditions used for the experimental slides. Blocking experiments were performed on anti-RF-amide positive macerated cells. All labeling by anti-RF-amide antibody was successfully blocked (results not shown). All results are the results of repeated experiments.

Imaging and Analysis: Slides were evaluated and digital photographs taken using a

Carl Zeiss LSM700 confocal system equipped with multiple lasers and excitation and emission fluorescence filters and ZENBlack imaging software. Further analysis of images was done using ImageJ/ FIJI software. The AlexaFluor488 labeled samples were imaged using FITC filters. The AlexaFluor635 tubulin labeled samples (double labeled with GABA_A receptor tagged with AlexaFluor488) were imaged using Rhodamine filters. Photographs were taken using Plan Neofluar 20X and 40X objectives. Z-stack series and tile scans were acquired. Z-stack series were done by obtaining multiple images sequentially at different planes of focus.

For the analysis of the results, brightness and contrast adjustments were applied equally to control and experimental images with Fiji (ImageJ Systems). All images selected showed fluorescence labeling above background.

Nerve cell bodies and their processes were identified by typical morphology and their strong labeling with anti- α -tubulin antibody. In tentacles nerve cell bodies and their processes were identified as fibers that extend over the entire tentacle, bisect numerous battery cells and/or fibers that connect cnidocytes. Although, in our whole-mount preparations, we were unable to observe cilia on/in the various cells, we identified putative ganglion and sensory cells by their locations and typical morphologies. Thus, ganglion cells are typically multi-polar and located deep within the ectoderm. In contrast, the bodies of sensory cells project to the surface of the ectoderm and are either mono-polar or have a triangular cell body whose distal apex is oriented toward the tissue surface (Westfall 1973). We also relied on the many depictions of nerve cells in hydra by other workers (e.g., Hadzi 1909; Burnett and Diehl 1964; Grimmelikhuijzen 1985; Bode et al. 1988).

Cell bodies were verified with the use of DAPI (4',6-diamidino-2-

phenylindole) a nuclear and chromosome counterstain that labels the nuclei of cells (images not included), which was included in the chemical composition of the mounting medium (Molecular Probes/Invitrogen, Eugene, OR) used in all experiments in this study.

RESULTS

Gene Sequence Analysis: Genes coding for six predicted GABA_A R subunit proteins were found by inspection of the published genome of *H. vulgaris*, each with at least two different isoforms. Amino acid alignments acquired using the National Center for Biotechnology Information's (NCBI) BLAST alignment tool showed that the predicted amino acid sequence for the GABA_A R antibody used in these experiments, originally made in rabbit, in recognition of the α 1 subunit in mouse, humans, bovine and chicken, strongly aligns with seventeen recognized GABA subunits in *H. vulgaris*. Among query coverage between the rat GABA_A R α 1 subunit and the GABA receptor subunits found in *H. vulgaris* ranged between 21% and 94% whereas Expect-values ranged from 1e-37 (GABA receptor subunit π -like isoform X1; XP_012558539) to 7e-42 (GABA receptor subunit β -2-like; XP_0125568381) (Appendix Fig. 1).

Amino acid alignments acquired using the more specific alignment tool, Clustal Omega, shows that the highly purified peptide immunogen corresponding to amino acids 28-43 of the rat GABA_A R α 1 subunit was strongly aligned with the six primary subunits of the recognized *H. vulgaris* GABA receptor subunits (β , β 1, β 2, β 4, π and ρ) (Appendix Fig. 3). The isoforms of these six subunits were found to be almost identical to one another (Fig. 2 of the appendix); therefore, only the six primary subunits were aligned with the immunogen of interest. Fig. 3 of the appendix shows that all six of the GABA subunits align in large consecutive amino acid sequences with the immunogen. This indicates that the GABA_A R α 1 subunit antibody from Sigma-Aldrich was compatible enough to use on *H. vulgaris* for the

immunohistochemical experiments performed in this study.

No amino acid alignments were done for the monoclonal RF-amide antibody used in these experiments, for they were specifically made for hydra by Dr. Osamu Koizumi (Fukuoka Women's University, Japan).

Immunohistochemistry: Hypostomal and tentacular nerve cells, condensed nerve rings and associated epithelial and effector cells in whole mounts of ablated heads labeled with anti-RF-amide, anti-GABA_A α -1 subunit receptor and/or double-labeled with anti- α -tubulin were examined by immunohistochemistry.

Proximal Nerve Ring and Associated Effector Cells

RF-amide is present in the neurites and cell bodies of the proximal nerve ring. Fig 1 shows punctate labeling with anti-RF-amide antibodies in the neurites of bi-polar and tri-polar ganglion cells throughout the hypostome, including those that contribute to the proximal nerve ring. All RF-amide positive structures in these experiments were neuronal. Some preparations display an optical 'edge-effect' composed of the anastomosing ectodermal nerve net and creates the appearance of an additional nerve ring (Fig 1a, 1b').

In contrast to the specific neuronal labeling by RF-amide antibody, GABA_A receptor labeling was patchily distributed on a ring of epithelial cells that was in coincidence with the tubulin positive proximal nerve ring. The mesoglea was identified as a dark, often black, often continuous, line within the hypostome that separates the endoderm from the ectoderm. This enabled the determination that GABA_A R labeling was primarily ectodermal (Fig 2). Fig 2 shows that in more distal optical sections GABA_AR labels both a proximal ring as well as pieces of the tentacle insertions (Fig 2 b-b'). Both of which are composed of epithelial cells. Without

labeling macerated cells of the hypostome it is unclear specifically which epithelial cells these receptors are on in this region. However, double labeling with anti-GABA_A R and anti- α -tubulin confirmed that the GABA_A R positive ring is separate from the loose tubulin containing fibers that make up the proximal nerve ring (Fig 3).

Distal Nerve Ring and Associated Effector Cells

These experiments did not show RF-amide distributed in the distal nerve ring; however, Munro 2014 has found RF-amide distributed in sensory cells and neurites in the apex that lead into the distal nerve ring.

Double labeling with anti-GABA_A R antibody and anti- α -tubulin antibody revealed that anti-GABA_A R labeled the nuclei of most of the cells found in the apex region including those of ectodermal epithelial cells, sensory cells and the nerve cells that make up the distal nerve ring (Fig 4). It is also clear that anti-tubulin (abundant in nerve cells) did not label nuclei (verifying specific binding of the anti-tubulin antibody). Yellow areas in Figs 4b-4c'' show that anti-GABA_A R and anti-tubulin co-labelled sensory cells and their contributing neurites that make up the distal nerve ring. In the apical region of the hypostome, anti-GABA_A R antibody also labeled ectodermal epithelial cells (maceration of the tissue is necessary to identify specific cell types), stenotele cnidocytes and the nuclei of each cell type whereas anti-tubulin labeled neurites, sensory cells and stenotele cnidocytes. Double labeling of GABA_A R and tubulin on sensory cells and neurites of the apex is in agreement with anti-RF-amide positive sensory cells and neurites in the apex (Fig 4d-4d'). The localization of anti-RF-amide positive sensory cells and neurites in the apex is in agreement with that described by others in *H. vulgaris*, *H. oligactis* and *H. attenuata* (Grimmelikhuijzen et al., 1985, 1996; Koizumi 1992, 2004; Munro Thesis 2014).

Tentacles

Anti-RF-amide antibody labeling was punctate and distributed in bi-polar and tri-polar ganglion cells, neurites and sensory cells throughout the tentacles. Many of the ganglion cells and neurites that positively labeled with anti-RF-amide antibody radiated from the tentacle attachment site near the proximal nerve ring throughout the entire tentacle (Fig 5a').

Anti-GABA_A receptor antibodies labeled nerve fibers, epithelial muscle fibers and myonemes that are associated with tentacle batteries cells (Fig 6). These receptors were also found in the nuclei of battery cells and numerous cnidocytes found in the tentacle and batteries cells of the tentacle. Three or more of the GABA_AR labeled battery cells clustered to form complete and partial donut-like rings along the tentacle, as described by Hufnagel et al., 1985. Because the tentacles used in these experiments were not completely relaxed, the battery cell complex bands are closer together than those described in the Hufnagel experiments (Fig 6).

GABA_ARs also appeared to be present at and around tentacle insertions (Fig 7 a-a'). Double labeling with anti-GABA_AR and anti- α -tubulin showed that GABA_AR labeling at tentacle insertions is not on tubulin labeled neurites and nerve cell bodies, but rather on associated effector cells (Fig 7b-b'). This is consistent with previously mentioned anti-GABA_AR labeling in the tentacle.

DISCUSSION

This study provides evidence for the localized distribution of GABA_ARs in the nerves and effector cells of the hypostome and tentacles of hydra and the wide distribution of RF-amide receptors in the neurites and cell bodies. It also provides the first evidence of an intimate association between RF-amide containing nerves and GABA_A receptor-containing effector and neuronal cells.

Protein Alignments

As in our previous experiments with GABA_B R antibodies (Hufnagel et al 2016), our protein alignments provided strong evidence that the antibody could recognize *H. vulgaris* GABA_A R epitopes.

Distribution of GABA_A Rs in the Hypostome.

In our analysis GABA_A Rs were exclusively localized on effector cells in a ring that coincided with the proximal nerve ring. Double labeling with anti-GABA_A R antibody and anti- α -tubulin antibody demonstrated that the GABA_A R positive ring made up of ectodermal epithelial cells was in fact different from but coincides with the tubulin positive nerve fibers that make up the proximal nerve ring. In contrast, GABA_A receptors were directly distributed on anti-tubulin labeled neurites and sensory cells in the apex and distal nerve ring, as well as on epithelial effector cells of the apex.

However, a recent study (Concas et al, personal communication) indicates that there are different subpopulations of GABA_A Rs present throughout the hydra, comprised of the α -, β -, and γ - or δ -like subunits. Although the study focused on the distribution of $\alpha 3$ subunits and did not address the distribution of the α R1 subunits used in our study, the suggestion that the molecular composition of different GABA_A Rs are differentially

distributed throughout the hydra is not inconsistent with our present study.

The results of these experiments are in agreement with biochemical and electrophysiological experiments concerning the presence and affinity of GABA. Pierobon et al., 1995 have shown that GABA and its allosteric modulators affect mouth opening and hypostome behavior. It appears to do so by acting upon ionotropic GABA_A receptors and metabotropic GABA_B receptors, with evidence that through these receptors GABA had an inhibitory effect on the ectodermal and endodermal pacemaker systems. It has also been shown that it is through GABA_A receptors that mouth closing is inhibited during the GSH-induced feeding behavior (Kass-Simon et al., 2003; Lauro et al., 2015).

Immunohistochemical studies performed by Hufnagel and Kass-Simon (2016) have shown that an ectodermal ring associated with the tubulin- containing proximal nerve ring labels with anti-GABA_B receptor antibody. The location of this ring is consistent with the physiology of hydra's hypostomal pacemaker systems. However, the same study did not identify GABA_B Rs in or in coincidence with the tubulin- containing distal nerve ring. The study also reported abundant, broadly distributed GABA_B R antibody in the endoderm of the hypostome whereas the experiments described in the present paper do not localize GABA_A R in the hypostomal endoderm. These findings suggest that endodermal metabotropic, not ionotropic, GABA receptors are involved in the feeding behavior.

GABA_A receptor presence at tentacle and around the base of the tentacle insertions may indicate the role of GABA_A receptors in the concerted body contractions (or contraction bursts) that are mediated and initiated at the proximal nerve ring (Kass-Simon 1972, 1973).

Distribution of RF-amide in the Hypostome.

In this study RF-amide was exclusively found in nerve cells and nerve fibers throughout the hypostome, including in the nerve fibers and nerve cells that make up the proximal nerve ring. Many sensory cells, bi-polar and tri-polar ganglion cells also positively labeled with RF-amide in the hypostome. These findings are in agreement with experiments described by Koizumi (1992), Grimmelikhuijzen (1985) and Munro (MS Thesis 2014) that show the ubiquitous distribution of RF-amide in the nerves of the hypostome of *H. vulgaris*, *H. oligactis* and *H. attenuata*. Hypostomal labeling described by Koizumi and Grimmelikhuijzen revealed sensory cells, ganglion cells, nerve fibers and a nerve ring comparable to the proximal nerve ring positively labeled with anti-RF-amide. Their studies did not find a RF-amide positive distal nerve ring in any species investigated. However, the tubulin positive distal nerve ring described by Hufnagel and Kass-Simon (2016) coincides with the RF-amide positive distal nerve ring described by Munro (MS Thesis 2014).

There have been experiments that have shown that RF-amides act as a ligand for some DEG/ENaC's (epithelial amiloride- sensitive sodium channels) (Assmann et al., 2014; Golubovic et al., 2007). Gene expression for different subunits of these hydra sodium channels were located at tentacle formation sites during development as well as differentially distributed at the tentacle base of adults. The hydra sodium channels were expressed in *Xenopus* oocytes where electrophysiological experiments were performed. Results of these experiments showed that two of the four described Hydra RF-amide neuropeptides elicited positive and sustained electrical currents. Sodium channel blocker (amiloride) affected feeding behavior in *Hydra magnipapillata*. The

administration of amiloride with reduced glutathione, which induces feeding behavior, resulted in the lack of tentacle coordination during the feeding response (Assmann et al., 2014; Golubovic et al., 2007). However, the electrophysiology of RF-amide neuropeptides in the hydra nervous system has yet to be performed directly in the hydra. Therefore, it remains unclear whether or not RF-amide neuropeptides are the primary ligands for any channel or if they are co-transmitted or act as a neuromodulator for other ligands such as GABA.

Distribution of GABA_ARs and RF-amide in the Tentacles

Due to the ubiquitous distribution of anti-RF-amide in nerve cell bodies and neural processes of the tentacle and lack of sufficient electrophysiological experiments addressing RF-amide function in the tentacle of hydra polyps, it is unclear what the specific function of RF-amide is in tentacle behavior. A similar situation exists for other cnidarians: Anderson (2004) reports that tentacles from representatives of all four classes of the phylum Cnidaria were examined using antibodies against the neuropeptides FMRFamide and RFamide to show the organization of neurons and nerve nets associated with cnidocytes. The study further describes that FMRFamide immunoreactive neurons formed plexuses at the base of the cnidocyte assemblages and that immunoreactive sensory cells connected peptidergic nerve nets to the surface of the tentacle. The authors suggest that while there is no direct physiological or pharmacological evidence that peptides are involved in the cnidocyte response, synaptic activity that can be recorded from the cnidocytes of various Cnidarian species (*Physalia* and *Cladonema*) may arise, directly or indirectly, from an RF-amide nerve net.

Electrophysiological studies described by Kass-Simon et al., (2003) show that GABA_A receptor blockers effect the tentacle pacemaker system by inhibiting tentacle burst firing whereas Scappaticci and Kass-Simon (2008) found that GABA_A receptor agonist, muscimol, and the antagonist, bicuculline, did not effect nematocyst discharge. They also report that metabotropic GABA_B agonists and antagonists significantly altered discharge probabilities although the results of experiments performed in this paper show extensive labeling of GABA_A receptors in the tentacle, including nematocysts.

The experiments performed in this study demonstrate the presence of RF-amide-containing nerve fibers that are directly associated with GABA_A receptors on nerve fibers and battery cells in the tentacle. Since neuropeptides and neurotransmitters have been found to co-localize in other species, it is likely that the RF-amide neuropeptide and GABA_A act in concert to control the neuro-effector system of the tentacles as well as the hypostome in *Hydra vulgaris*.

SUMMARY

The patterns of localization of anti-GABA_A α -1 subunit receptors and anti-RF-amide described in this study suggest that neuropeptides and neurotransmitters interact to mutually effect underlying neuronal control of behavior in the hypostome and tentacles of *H. vulgaris*. The distribution and close association of anti-RF-amide positive nerve cells and nerve fibers with anti-GABA_A R positive nerve fibers, sensory cells, battery cells and ectodermal epithelial cells, together with evidence of RF-amides co-localizing and co-transmitting with classical neurotransmitters in other organisms, makes it conceivable that RF-amide modulates the action of GABA in the tentacle and hypostome, or conversely that GABA sculpts a general RF-amide generated excitation. This question can only be answered by further physiological analysis.

FIGURES

Figure 1

Figure 1. The proximal nerve ring and hypostome labeled with anti-RF-amide antibody. a. Whole mount of the hypostome. PNR= proximal nerve ring. AEN= anastomosing ectodermal nerve net. M= mesoglea. ECT= ectoderm. END= endoderm. N= RF-amide positive neurites. BGC= RF-amide positive bi-polar ganglion cells of the hypostome. TGC= RF-amide positive tri-polar ganglion cells of the hypostome. a' and a'' denote the areas that are enlarged in a at 40x. Arrow (a'') denotes neurites that make up the PNR. b. RF-amide positive ganglion cells within the hypostome. b'. RF-amide positive neurites of the apex. b''. Control; primary antibodies omitted. Scale bars= 50 μ m.

Figure 2

Figure 2. The proximal ring labeled with anti-GABA_A R α 1 subunit antibody. a, b are non- sequential whole mount optical sections of a z-stack composed of 19 sections with each section in intervals of 2 and the entire z-stack ranging up to 36 μ m. PR=the GABA_A R positive proximal ring. M=the mesoglea. EC=the GABA_A R positive epithelial cells. ECT=the ectoderm. END=the endoderm. T=tentacles. INs indicate the

GABA_A R positive areas of the PNR in close proximity to the insertion sites of the tentacle. a. Whole mount of the hypostome. b'. Is an enlargement of b that depicts an optical section in which parts of insertion sites of the tentacles are evident. b'' is both an enlargement of b and a more proximal optical section of b' revealing the labeling of the GABA_A R proximal ring close to the mesoglea. b'''. Control; primary antibodies omitted. Scale bars=50µm.

Figure 3

Figure 3. The proximal ring double labeled with anti-GABA_A R α 1 subunit antibody and anti- α -tubulin antibody. a. Double labeled whole mount of the hypostome. Green labeling=anti-GABA_A R. Red labeling=anti- α -tubulin. PR=the proximal ring. M=the mesoglea. ECT=ectoderm. END=endoderm. N=the tubulin positive neurites. PNR=the proximal nerve ring. a'. GABA_A R positive proximal ring. a''. Tubulin positive proximal nerve ring. b. Enlarged double labeled image of the proximal ring from image a. b. shows that the tubulin positive nerves

are associated with the GABA_A R positive effector cells that make up a proximal ring. b'. GABA_A R positive proximal ring. b". Tubulin positive proximal nerve ring. b"". Control; primary antibodies omitted. Scale bars=50μm.

Figure 4

Figure 4. The distal nerve ring double labeled with anti-GABA_A R α 1 subunit antibody and anti- α -tubulin antibody. a. Double labeled whole mount of the hypostome. DNR=the distal nerve ring at the apex. Green labeling=the anti-GABA_A R and red labeling is anti- α -tubulin. N=the neurites of the sensory cells leading into the distal nerve ring. EC=the epithelial cells. Nu=the nucleus of the epithelial cells. EFC=effector cells. ST=stenotele cnidocytes. SC=the sensory cells. Yellow areas indicate where the GABA_A R positive structures co-localize with the tubulin positive structures that include sensory cells, neurites and effector cells. a'. The GABA_A R positive distal nerve ring. a''. The tubulin positive distal nerve ring. b and c are enlarged images enclosed in the white boxes labeled in a. b. The double labeled section of the distal nerve ring and the neurites that contribute to it. b'. The GABA_A R positive structures. b''. The tubulin positive structures. c. The double labeled image of different cell types and nerve structures found at the apex including the cells that contribute to the distal nerve ring. c'. The GABA_A R positive cell types and nerve structures. c''. The tubulin positive cell types and nerve structures. d-d'' show the different cell types in the apical region of the hypostome double labeled with anti-GABA_A R and tubulin. Unlabeled

arrows in d-d" indicate a cluster of epithelial cells, effector cells, sensory cells and neurites. b". Control; primary antibodies omitted. Scale bars =50 μ m.

Figure 5

Figure 5. Tentacles labeled with anti-RF-amide antibody. N=punctate RF-amide positive neurites. GC=ganglion cells. TGC=tri-polar ganglion cells. SC=sensory cells. a'. The tentacle at the base/attachment site. a". Control; primary antibodies omitted. Scale bars=50 μ m

Figure 6

Figure 6. Tentacles labeled with anti-GABA_A R α 1 subunit antibody.

Nu=the nucleus. CN=cnidocytes; here they are desmonemes. N=neurites.

My=myonemes. BC=battery cell complexes. IB=the in between area

between battery cell complex bands. DES=desmoneme cnidocytes.

ST=stenotele cnidocytes. a. Shows two tentacles at 40x. b. Is an image of

a tentacle at its base. The unlabeled arrow delineates a GABA_A R positive

discharged cnidocyte. c-c" are non-sequential optical sections of a z-stack

composed of 15 sections, with each section in intervals of 2 and the entire

z-stack ranging up to 40 μ m. d-d'" are non-sequential optical sections of a

z-stack composed of 15 sections, with each section in intervals of 2 and

the entire z-stack ranging up to 40 μ m. d. White box encloses the battery

cell complex bands. d"". Control; primary antibodies omitted. Scale

bars=50 μ m.

Figure 7

Figure 7. Tentacle insertions labeled with anti-GABA_A R α 1 subunit

antibody. a and a' are non-sequential whole mount optical sections of a z-stack composed of 19 sections, with each section in intervals of 2 and the entire z-stack ranging up to 36 μm . Arrows in a-a'' delineate the tentacle insertion sites. CN=cnidocytes. b. Is a double labeled whole mount of the hypostome. Green labeling=anti-GABA_A R. Red labeling= anti- α -tubulin. N= neurites. In=insertion sites. M=mesoglea. ECT= ectoderm. END=endoderm. b'. GABA_A R positive tentacle insertion site. b''. Tubulin positive proximal nerve ring. b''' and c are controls; primary antibody omitted. Scale bars=50 μm .

APPENDICES

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like isoform X2 [Hydra vulgaris]	214	214	88%	2e-63	31%	XP_012560050.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like isoform X1 [Hydra vulgaris]	214	214	88%	2e-63	31%	XP_012560049.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like isoform X1 [Hydra vulgaris]	187	187	94%	2e-53	29%	XP_012560779.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like isoform X2 [Hydra vulgaris]	186	186	88%	2e-53	29%	XP_012560780.1
PREDICTED: gamma-aminobutyric acid receptor subunit pi-like [Hydra vulgaris]	177	177	87%	5e-50	28%	XP_012560993.1
PREDICTED: glycine receptor subunit alpha-2-like [Hydra vulgaris]	166	166	86%	4e-46	28%	XP_012563985.1
PREDICTED: glycine receptor subunit alpha-2-like [Hydra vulgaris]	162	162	86%	1e-44	30%	XP_012561154.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like [Hydra vulgaris]	155	155	71%	7e-42	31%	XP_012556838.1
PREDICTED: gamma-aminobutyric acid receptor subunit pi-like isoform X1 [Hydra vulgaris]	150	150	88%	9e-40	27%	XP_012561012.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-1-like [Hydra vulgaris]	149	149	88%	1e-39	25%	XP_002161960.3
PREDICTED: gamma-aminobutyric acid receptor subunit pi-like isoform X3 [Hydra vulgaris]	149	149	87%	2e-39	25%	XP_012558541.1
PREDICTED: gamma-aminobutyric acid receptor subunit pi-like isoform X2 [Hydra vulgaris]	143	143	87%	1e-37	25%	XP_012558540.1
PREDICTED: gamma-aminobutyric acid receptor subunit pi-like isoform X1 [Hydra vulgaris]	143	143	87%	1e-37	25%	XP_012558539.1
PREDICTED: gamma-aminobutyric acid receptor subunit rho-2-like [Hydra vulgaris]	142	142	77%	2e-37	27%	XP_012560003.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-1-like isoform X2 [Hydra vulgaris]	135	135	65%	2e-35	27%	XP_012561013.1
PREDICTED: glutamate-gated chloride channel-like [Hydra vulgaris]	130	130	76%	8e-34	27%	XP_012560412.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-2-like [Hydra vulgaris]	100	100	39%	4e-24	32%	XP_012555765.1
PREDICTED: gamma-aminobutyric acid receptor subunit rho-2-like [Hydra vulgaris]	96.7	96.7	59%	3e-22	25%	XP_012561016.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-like [Hydra vulgaris]	87.4	87.4	21%	2e-19	40%	XP_012565237.1
PREDICTED: gamma-aminobutyric acid receptor subunit beta-4-like [Hydra vulgaris]	76.3	76.3	24%	3e-16	37%	XP_004205956.1

Figure 1. Amino Acid alignments of the GABA_A R α 1 subunit antibody and the seventeen recognized GABA subunits in *H. vulgaris* acquired using the National Center for Biotechnology Information's (NCBI) BLAST alignment tool show. Green highlighted lines are non-GABA subunits and thus can be ignored. Yellow highlighted regions are the

maximum and minimum of either the Query Cover or Expect value (E-value) further indicated by the red highlighted regions. For the purposes of this study the Max Score and Total Score categories can be ignored.

Figure 2. β 2 X1 a vs β 2 X1 b isoform

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|828217837|ref|XP_012560779.1|      MRKVIVLVTFVCFVAKGNLLEIKC--DLNKAESCQRTDDERAQESDKMVTQIKQLLKNYN
gi|828215630|ref|XP_012560049.1|      ---MI-----FF-IRTHALPHILVVLNIIPGNICINHEK---DLRDEDVDKTKVWLMNGYD
      * . . . * : . : . : . : . : . : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      RKLRLPHYIGKPVMLVDATIIISFSLNDAIDMMYITDMFFQRQWWDHRLAHNLSEPTIAI
gi|828215630|ref|XP_012560049.1|      KAIRPNYTGDAIMINLDMTVMRFGKLEVNMMPTLDLFLRQWIDYRLRNHLPEILTPNL
      : : * : * . . : : * : : * : * : : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      GTSHSPNIIWTPDPTVMFNSVTSALHHVIVENHKVDIYPNGTVFWGTRITVSPSCTLDLKA
gi|828215630|ref|XP_012560049.1|      GHDSPPDFIWTPTDVFNLNAQKASSHSVTVKSKLDIYPDGKVFVWGLRVSVSEANCLFDLRN
      * . . : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      YPMDVQICGFEIVSYTYDSRHLKYDWWKPGIEIKEESMLEFKLTDHKTIGEERYFVAGN
gi|828215630|ref|XP_012560049.1|      YPMDTQACDLGIVSYGYTIDHLLYLRWRNDPI-QILNQNLQSYTLTGIENTKIVEQFSMGK
      * * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      YTVLEGRFRFRKRLMGFAITQVYLPSTVIVTVSVWSVLWKRTVIPARVSLCITTVLTIISTI
gi|828215630|ref|XP_012560049.1|      FALLKAEFTFKRIAFSILQIFFPCVAIVCVSWISLWLHKHCSPARVGIGVTTLLTIISTI
      : : : : . . * * * : * : * : : : . . . * * : * : * : : : * * : : * : * : * :
gi|828217837|ref|XP_012560779.1|      WGNVNAQLPRVSYVKAIDVYLMTSFICIIILTLEYTIVLNIPDIMKKVR--NNPLFRK-RK
gi|828215630|ref|XP_012560049.1|      WGSVNRRLPNVSYVKAVDIYFMASFIFMTLIEYTVLNLGNLKNFKKKFVMKNAYKEISK
      * * . * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      MVKDTSE-----NNIQLLP---RAVNEESLSKE-----
gi|828215630|ref|XP_012560049.1|      LSKRVSISLSIDKVKPMNMRYSSEPCRSSTSIARYQRKSENSFLDDANLSCNQISQKF
      * : * . * : * : * : * : * : * : * : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      --AEKKKEQK-----IQQENIDICNRIEFLSLRFFPLAYTIFNIFYWS
gi|828215630|ref|XP_012560049.1|      YEEEFSEEDILLSSKKEVAEKHPAFMKLYLELRKASIVDKISRLFPLLFICFNIFYWL
      * . : : . . : : : : : : : : : : * : * : * : * : * : * : * :
gi|828217837|ref|XP_012560779.1|      IYLKEDLKTLE
gi|828215630|ref|XP_012560049.1|      KYNDNASPNKN
      * . . . .

```

Figure 3. $\beta 2$ X1 vs $\beta 2$ X2 isoform

CLUSTAL O(1.2.1) multiple sequence alignment

[illegible]

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|828218583|ref|XP_012561012.1|MDY-----QRAFKWTYNSCMVLIIILCARFTNASRFESTSDLEYSKTWSRIRQAYNPFL
gi|828211096|ref|XP_012558540.1|MLCKRRKFPVVLICFLYPWIT-----CVLSSEKESLGDKKVSEAWSKIQAYNPLL
gi|828211098|ref|XP_012558541.1|-----MAFKNLVQKLKSFYNNQGLGPIRFDPNQNLDGKKVSEAWSKIQAYNPLL
                                     *           . : . . * : *:***:*:*****:

gi|828218583|ref|XP_012561012.1|RPNFT---GNVTSVVMSLTILQFDSLNDDEKSFKTDMYLRLQLWDVYRAAHNLSTVLIFP
gi|828211096|ref|XP_012558540.1|RPNFTVSFLGNVTDVIIVSLTILQFDSVNDKDKSFKTDMYFRQRWVDYRAQHNLTTVLTPG
gi|828211098|ref|XP_012558541.1|RPNF----TGNVTDVIIVSLTILQFDSVNDKDKSFKTDMYFRQRWVDYRAQHNLTTVLTPG
      ****          *****.:*****:**.:*****:* ** ***** **:*** *

gi|828218583|ref|XP_012561012.1|IGKKHPSSYIWPVPTVFSGMLGGHIHTMTVGNHKLDIYPNGTIFWGCRITLKSXYCLFNLR
gi|828211096|ref|XP_012558540.1|FGKNHPSAFIWPVPTVFRVMTGGYIHNSNSTASHKVVDIYPNGTVSWGCRVTLVSHCTFDLH
gi|828211098|ref|XP_012558541.1|FGKNHPSAFIWPVPTVFRVMTGGYIHNSNSTASHKVVDIYPNGTVSWGCRVTLVSHCTFDLH
      :*:***:*:***** * ***: * :..**.*:*****: ***** ** * *:*:

gi|828218583|ref|XP_012561012.1|NYPMDTQRCEVGIESYAYDNHRHINYIWDKNHPVILHLELPQHTVQVENTSRYLKYYSFS
gi|828211096|ref|XP_012558540.1|NYPMDTQRCEIGVLSFAHDKTHINYVWIEDNPVSLILNHLPHQHSVHIENATNLSEHSFS
gi|828211098|ref|XP_012558541.1|NYPMDTQRCEIGVLSFAHDKTHINYVWIEDNPVSLILNHLPHQHSVHIENATNLSEHSFS
      *****: * :*: * :*: * :*. * * *.****:*:***:. : :****

gi|828218583|ref|XP_012561012.1|SNKEGEYTFLKFDAVFIIRRVQAYILQAYFPSASLVVMSWISFWFHTNCVAVRMSFGIVSL
gi|828211096|ref|XP_012558540.1|SNKEGEYSIIKFDVIFERRQAYIFYAQFFPSASLVVMSWISFWLHVNCVSARLGIGIVSL
gi|828211098|ref|XP_012558541.1|SNKEGEYSIIKFDVIFERRQAYIFYAQFFPSASLVVMSWISFWLHVNCVSARLGIGIVSL
      *****:***:. : * * *****:*:*****:*****:*****:*. * . * :.*****

gi|828218583|ref|XP_012561012.1|LTYYRLWEVVIKSTPKINYYTALDIYMLGCITTFICSLLEYGLATNHFHSRWKKKRSTHL
gi|828211096|ref|XP_012558540.1|LTYYRLWEVINAMPKINYYTALDIYMLGCTTFICSLLEYGLATNIHFTLWKQEKANYT
gi|828211098|ref|XP_012558541.1|LTYYRLWEVINAMPKINYYTALDIYMLGCTTFICSLLEYGLATNIHFTLWKQEKANYT
      *****: * :*****:*****:*****:*****:*****:*****: * :*:...:

gi|828218583|ref|XP_012561012.1|LNSLNKRRETLNQVDLAVSFSNSLNKNKQPLFY--LESSDTKATHPEGVDLYNNLGK-ANIR
gi|828211096|ref|XP_012558540.1|WQTIKKKQETRKSIDFTNVNT--LRTKNALNQMKQIYDTKQINDTDLVLFDNHGRVQSFR
gi|828211098|ref|XP_012558541.1|WQTIKKKQETRKSIDFTNVNT--LRTKNALNQMKQIYDTKQINDTDLVLFDNHGRVQSFR
      ::::*:** :.:* . *.:* * : *** . :*:** * :.**

gi|828218583|ref|XP_012561012.1|KMSXQEQKLKLKEYLSMWKSDPPKKRPYFPEMMVTGKPASLDIASRFLPFPGFLCFNV
gi|828211096|ref|XP_012558540.1|KTSIREQKLKLKEYLSMWKSDPLGNRPYFPEMMVTGKPASLDIVSRFLPFPGFLCFNI
gi|828211098|ref|XP_012558541.1|KTSIREQKLKLKEYLSMWKSDPLGNRPYFPEMMVTGKPASLDIVSRFLPFPGFLCFNI
      * * :*****:*****:*****:*****:*****:*****:*****:

gi|828218583|ref|XP_012561012.1|VYWAYFLQKT
gi|828211096|ref|XP_012558540.1|IYWASFL---
gi|828211098|ref|XP_012558541.1|IYWASFL---
      :*** **
```

57

Figure 5. β like GABA subunit against the immunogen

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43                -----QP-----      2
gi|828231662|ref|XP_012565237.1| MHARKSLIFNNDEAWIKRGGLFVDTMGAFDGAEVCELIVAQSELYVNLKWMKPIDRKT 60
                                     :*

gi|34328532:28-43                ----SQDE-----LKDNTTVFTR-----      16
gi|828231662|ref|XP_012565237.1| DLNVPDYSNVKIRTNSSTRLGDFGLNFTTLEVKFTLHRDFTLHFLRDFPFCILTVMLSWV 120
                                     . .:      * *      **

gi|34328532:28-43                -----      16
gi|828231662|ref|XP_012565237.1| SFYLSYKSTPARSTFGITTVLTIVTMSNNIRSTAPSSKTFRSLDYMLICMIFVFGALVE 180

gi|34328532:28-43                -----      16
gi|828231662|ref|XP_012565237.1| FAVVGITDPDFSPRWKNKGKRTKKNYDIVSVRFFHFLKILFVQKLEEIQSILVVSQEL 238

```

Figure 6. β 1 like GABA subunit against the immunogen.

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43                -----      0
gi|828191235|ref|XP_002161960.3| MMKLLIFQFLLCNIKMVVLKRELMYDNEIYALFENLFLNYNRMFRPNYYGKPVVLVDA 60

gi|34328532:28-43                -----      0
gi|828191235|ref|XP_002161960.3| SILSFSKVDASKMIYADMFRLQRWNDYRLRHNMSEKLTIVGNTNYPDSIIWTPDTPVFIN 120

gi|34328532:28-43                -----QPSQD-----      5
gi|828191235|ref|XP_002161960.3| SVNGLLHSVTVNNNKLDIHPNGDVFWGTRVTVSPACDLDLQDYPKDVQKCTFSMKSYSLS 180
                                     :*. *

gi|34328532:28-43                -----ELKDNTTVFTR-----      16
gi|828191235|ref|XP_002161960.3| SRHISYKWMKDPGIFIPKMPMAQFKLIKYSTERYVIGDATSNFTQLYSEFRLKRLAGFAI 240
                                     : * *: **:

gi|34328532:28-43                -----      16
gi|828191235|ref|XP_002161960.3| FHIFIPTTSIVITSWISMWLPLSASAARIGLSGTAMVTIANIWMIIDNQLPAVNYLKKID 300

gi|34328532:28-43                -----      16
gi|828191235|ref|XP_002161960.3| VYLIFSLVNIVLTMLEYAVVLNISVIAERFRKKKVKKYSRNDRLTTNIELEQIRQSSE 360

gi|34328532:28-43                -----      16
gi|828191235|ref|XP_002161960.3| MKNIQWGHKRRKQCDRNIEKENVRKLAQKIEKNAKIARKIEMTAQVFIPLTFIIPNIFY 420

gi|34328532:28-43                -----      16
gi|828191235|ref|XP_002161960.3| WVFYMKSDGEIGEIQTADVY 439

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Figure 7. β 2 like GABA subunit against the immunogen.

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43          -----QPSQDE-----          6
gi|828217837|ref|XP_012560779.1| MRKVIVLVTFPCVFAKGNLLEIKCDLNKAESCQRTDDERAQESDKMVTEQIKQLLKYNRK 60
                                     * : **

gi|34328532:28-43          -----          6
gi|828217837|ref|XP_012560779.1| LRPHYIGKPVVMVLVDATIISFNLDAIDMMYTIDMFFRQRWWDHRLAHLNLEPITIAIGT 120

gi|34328532:28-43          -----LKDNTTVF-----          14
gi|828217837|ref|XP_012560779.1| SHPSNIIWTPDTPVFMNSVTSALHHVIVENHKVDIYPNGTVFWGTRITVSPSCTLDLKAYP 180
                                     : * ***

gi|34328532:28-43          -----          14
gi|828217837|ref|XP_012560779.1| MDVQICGFIVSYTYDSRHLKYDWYWKPGIEIKEESMLEFKLTDHKTIGEERYFVAGNYT 240

gi|34328532:28-43          -----          14
gi|828217837|ref|XP_012560779.1| VLEGRFRFKRLMGFAITQVYLPSTVIVTVSWVSLWIKRTVIPARVSLCITTVLTISTIWG 300

gi|34328532:28-43          -----          14
gi|828217837|ref|XP_012560779.1| NVNAQLPRVSYVKAIDVYLMTSFICIIILTLEYTIVLNIPIIDMKVRNNPLFRKRKMVKD 360

gi|34328532:28-43          -----TR-----          16
gi|828217837|ref|XP_012560779.1| TSNNIQLLPRAVNNEESLKEAEKKKEQKIQENIDICNRIEFLSRLPFFPLAYTIFNIFYW 420
                                     . *

gi|34328532:28-43          -----          16
gi|828217837|ref|XP_012560779.1| SIYLKEDLKTLE          432

```

Figure 8. β 4 like GABA subunit against the immunogen.

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43          -----          0
gi|449664590|ref|XP_004205956.1| MKKVLLIILLSSYAYCTISLRNADRDVRLVYEELLKNYNKNLRPDYLGDSVNVLDGTIL 60

gi|34328532:28-43          -----          0
gi|449664590|ref|XP_004205956.1| SFGKVDPTNMKYTVDMYFRQRWNDYRLRHNLTAKLTIIISGSDYPSSIIWTPDTFFLNSVS 120

gi|34328532:28-43          -----QPSQDELKDN-----          10
gi|449664590|ref|XP_004205956.1| SIFHHVTVNNHKIDIYPNGFIFWGT-PREDICTEdgamma-aminobutyricacidrec 178
                                     * : * . : *

gi|34328532:28-43          -----TTVFTR-----          16
gi|449664590|ref|XP_004205956.1| eptorsubunitpi-likeisoformXHydravulgarisNCBIReferenceSequenc 237
                                     * : * *

gi|34328532:28-43          -----          16
gi|449664590|ref|XP_004205956.1| eXP-GenPeptIdenticalProteinsGraphics          272
                                     *

```

Figure 9. π like GABA subunit against the immunogen.

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43      ----- 0
gi|828218583|ref|XP_012561012.1| MDYQRAFKWTYNSCMVLIIILCARFTNASRFESTSDLEYSKTWSRIRQAYNPFLRPNFTGN 60

gi|34328532:28-43      -----QPSQDELKDNTTVFT-----R----- 16
gi|828218583|ref|XP_012561012.1| VTSVVMSTLILQFDSLNDDEKSFKTDMYLRQLWVDYRAAHNLSTVLIPVIGKKHPSSYIW 120
      . *.*:* . * . *

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| VPDTVFSGMLGGHIHTMTVGNHKLDIYPNGTIFWGCRITLKSICYCLFNLRNYPMDTQRCEV 180

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| GIESYAYDNRHINYIWDKNHPVILHLELPQHTVQVENTSRYLKYYSFSSNKEGEYTFLK 240

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| FDAVFIRRVQAYILQAYFPSASLVVVSISFWFHTNCAVARMSFGIVSLLTTYRLWEVVI 300

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| KSTPKINYTTALDIYMLGCITPICFSLLEYGLATNFHFSRWKKRSTHLLNSLNKRRETL 360

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| NQVDLRVSFSNSLKNKQPLFYLESSDTKATHEGVDLYNNLGKANIRKMSKQEQKLKLE 420

gi|34328532:28-43      ----- 16
gi|828218583|ref|XP_012561012.1| EYLSMWKSDPPKKRPYYFEPMMVTGKPASLDIASRFLFPFGFLCFNVVYWAYFLQKT 477

```

Figure 10. ρ like GABA subunit against the immunogen.

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CLUSTAL O(1.2.1) multiple sequence alignment

gi|34328532:28-43      ----- 0
gi|828215496|ref|XP_012560003.1| MDFYFSQYDYDERLKIPEIPDCSVNIVGKSVEDFWMPDVFVNASKATNFHFTVENRFT 60

gi|34328532:28-43      ----- 0
gi|828215496|ref|XP_012560003.1| TVNLSNGMILHHARITTTASCRMDFKNYPFDEQTCYLAIESYGHQDSYIVYKWLNNLT 120

gi|34328532:28-43      ----- 0
gi|828215496|ref|XP_012560003.1| DEKYIAVHDKEMANYDVTSAFKSYEKTPHHETSFSSATAKFTFRRRREYFILQIYLPNAV 180

gi|34328532:28-43      ----- 0
gi|828215496|ref|XP_012560003.1| IVAVTWVSFWITPAAVPARCGILVTSLLTLITMLSITNASMPKVGYIKALDLYLFASLIF 240

gi|34328532:28-43      ----- 0
gi|828215496|ref|XP_012560003.1| VFSLIEYILVLNTHSHKKRTSTYKEGNLFIGSQLKSDYKFSDIVDFSKNIQEKADLIND 300

gi|34328532:28-43      -----QPSQDELKD-----NTTVFT----- 15
gi|828215496|ref|XP_012560003.1| FIDYSKDATLLYQQLHTKKISQNERPEEIIHKCESRLLNEKLIKFKNRNSTFFLENEI 360
      :*.: .:* *:*.*

gi|34328532:28-43      -----R----- 16
gi|828215496|ref|XP_012560003.1| VEVEIAPKDENISFSAIDYFARVYFPFLFISFNVFYWLFPYYRN 403
      *

```

Figures 5-10. Amino Acid alignments of the six primary GABA subunits against the immunogen sequence corresponding to amino acids 28-43 of the GABA_A R α 1 subunit antibody used in these experiments. These alignments were acquired using Clustal Omega. 5. Alignment of the β like GABA subunit against the immunogen. 6. Alignment of the β 1 like GABA subunit against the immunogen. 7. Alignment of the β 2 like GABA subunit against the immunogen. 8. Alignment of the β 4 like GABA subunit against the immunogen. 9. Alignment of the π like GABA subunit against the immunogen. 10. Alignment of the ρ like GABA subunit against the immunogen. * = identical amino acids. : = similarity in amino acids.

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