Characterisation of neuropeptide GPCRs NPR-4 and NPR-5 from the parasite *Brugia malayi*

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January 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Abstract

Parasitic infections of humans and animals are often associated with high morbidity and socioeconomic burden. Highly prevalent in developing countries, they contribute to the vicious cycle of poverty. Current drug treatments have suboptimal efficacy and can cause severe adverse reactions. Therefore, new potential targets are needed to discover novel anthelmintics. FMRFamide-like peptide (FLP) receptors, G-protein coupled receptors (GPCR) activated by the FLP family of neuropeptides, are appealing drug targets, due to their widely conserved and important roles in invertebrates, while being essentially absent from vertebrates.

We investigated two FLP receptors, *Bma*-NPR-4 and *Bma*-NPR-5, from the filarial parasite *Brugia malayi*, a cause of human lymphatic filariasis (elephantiasis). The two receptors were heterologously expressed in both Chinese hamster ovary (CHO) cells and the yeast *Saccharomyces cerevisiae*. Both receptors were activated by neuropeptides encoded on the *flp-18* precursor gene from *Caenorhabditis elegans*. The extent of receptor activation was quantified using an aequorin-based Ca^{2+} bioluminescence assay in CHO cells and an Alamar Blue-based yeast proliferation assay. Their structure-activity profiles were determined using truncated analogues and an alanine scan series of one of the FLP-18 neuropeptides (DVPGVLRF-amide).

We demonstrated the important role of the VLRF-amide motif for receptor agonism. *Bma*-NPR-4 and *Bma*-NPR-5 exhibited activity profiles comparable to each other and to their *C. elegans* orthologues, suggesting an evolutionary conservation of FLP receptors across the phylum Nematoda and validating *C. elegans* as a model nematode for research. The

insights gained can contribute to future efforts to discover non-peptide ligands as anthelmintics.

Abrégé

Les infections parasitaires chez les humains et les animaux entraînent fréquemment une morbidité élevée et un lourd fardeau socioéconomique. Particulièrement courantes dans les pays en voie de développement, elles contribuent au cercle vicieux de la pauvreté. L'efficacité des traitements existants est sous-optimale, et les médicaments peuvent provoquer de graves effets secondaires. Par conséquent, il est nécessaire d'identifier de nouvelles cibles potentielles pour la découverte d'anthelmintiques. Les FMRFamide-like peptides (FLPs) sont une famille de neuropeptides, très conservés chez les animaux vertébrés, et au rôle essentiel. En revanche, ils sont pratiquement absents chez les invertébrés. Ceci rend les récepteurs des FLPs, qui sont des récepteurs couplés aux protéines G (RCPG), des cibles médicamenteuses attrayantes.

Nous avons étudié deux récepteurs de FLP, *Bma*-NPR-4 et *Bma*-NPR-5, présents dans le *Brugia malayi*, un des nématodes filariens qui causent les filarioses lymphatiques (l'éléphantiasis). Nous avons exprimé chaque récepteur dans deux systèmes hétérologues, les cellules d'ovaire d'hamsters chinois (cellules CHO) et la levure *Saccharomyces cerevisiae*. Les deux récepteurs sont activés par les neuropeptides codés sur *flp-18*, le gène du précurseur du nématode libre *Caenorhabditis elegans*. Dans les cellules CHO, nous avons mesuré l'intensité d'activation du récepteur, quantifiée par un dosage de l'aequorine, une protéine luminescente qui détecte les augmentations de Ca^{2+} intracellulaire. Dans l'épreuve fonctionnelle à la levure, l'intensité d'activation du récepteur était proportionnelle à la prolifération de la levure, mesurée au moyen d'indicateur Alamar Blue. La détermination des relations structure-activité était effectuée à l'aide des analogues peptidiques de FLP-18f (DVPGVLRF-amide), parmi lesquels les

peptides ont été fragmentés ou les acides aminés ont été séquentiellement remplacés par l'alanine.

Nos résultats indiquent que le motif peptidique VLRF-amide joue un rôle important en termes d'activation des récepteurs. *Bma*-NPR-4 et *Bma*-NPR-5 présentent des relations structure-activité similaires, comparables à leurs orthologues de *C. elegans*. Ceci suggère que les récepteurs de FLPs sont conservés dans l'embranchement de nématodes lors de leur évolution, et valide le nématode *C. elegans* comme système-modèle de recherche. Les informations acquises de cette étude peuvent contribuer aux efforts en cours pour découvrir des ligands non peptidiques susceptibles de devenir des anthelmintiques.

Acknowledgements

The pursuit of my master's studies has been a memorable journey. I am grateful for all the support I received along the way, which has helped me overcome the challenges and accomplish my objectives.

First of all, I would like to thank my supervisor, Dr. Timothy G. Geary. He has always mentored me with great patience and kindness. Not only has he been constantly sharing his knowledge in science, but also providing continuous support and encouragement.

Moreover, I thank all the current and former members of the Geary/Beech lab: Vicki Muise for introducing me to various laboratory techniques and to this research project; Dr. Elizabeth Ruiz Lancheros for letting me continue her project; Serge Dernovici and Hilary Byrne for training me on microscopy; Mark Kaji for giving me invaluable advices and being a supportive friend; Drs. Christina Ballesteros and Thomas Duguet for teaching me cDNA synthesis and nested-PCR; and rest of the lab, Charles Viau, Damian Clarke, Jenn Noonan, Maude Dagenais and Peter Lee, for being great lab mates. I would like to extend my gratitude to all other labs at the Institute of Parasitology, for providing a collaborative environment for research. Special thanks to Dr. Salavati's lab for helping me with Western blotting.

I would also like to thank Dr. Liliane Schoofs at the Functional Genomics and Proteomics Research Group of Katholieke Universiteit Leuven, for collaborating with us on the mammalian cell works. Special thanks for Dr. Sven Zels and Ms. Elke Vandewyer for teaching me mammalian cell cultures and aequorin luminescence-based Ca^{2+} assay.

Furthermore, I thank Dr. Tim Geary for editing my thesis, and my friend Chloé Anastassiadis for editing my abstract in French. I thank the funding resources that contributed to this research project: the grants awarded to Dr. Tim Geary from NSERC and the Canada Research Chair program; the Department of Pharmacology and the Faculty of Medicine for granting me the Graduate Mobility Award, which allowed me to carry out part of the research project with our collaborators at KU Leuven.

Last but not least, I am very grateful for my family and friends. Their tremendous support has been a great motivation that keeps me going forward.

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List of abbreviations

DAG: diacylglycerol

ER: endoplasmic reticulum

FLP: FMRFamide (Phe-Met-Arg-Phe-NH_2) like peptide

GDP: guanosine diphosphate

GluCl: glutamate-gated chloride channel

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

INS: insulin-like peptide

IP_3: inositol 1,4,5-trisphosphate

LF: lymphatic filariasis

MDA: mass drug administration

NLP: neuropeptide-like protein

NPR: neuropeptide receptor

Oncho: onchocerciasis

PIP_2: phosphatidylinositol 4,5-bisphosphate

PLC-β: phospholipase C-β

SCH: schistosomiasis

STH: soil-transmitted helminthiases

WHO: World Health Organization

I. Introduction

All living organisms can be affected by parasites, which sustain themselves at the expense of the host. Infections caused by parasitic worms in humans and other animals, known as helminthiases, pose great health and socioeconomic burdens on endemic regions. Current anthelmintics, such as macrocyclic lactones, benzimidazoles, and diethylcarbamazine, are in general broad-spectrum, effective and well-tolerated. Mass drug administration programs have made the eradication of several parasitic diseases a feasible objective. However, infections caused by filarial nematodes pose challenges to treatment and control. The drugs used for this indication in humans primarily target the microfilariae, which are the first stage larvae secreted into the blood by adult filarial nematodes. This limits their utility, and also gives rise to complications and side effects. In addition, increasing concerns over drug resistance have been reported (Bockarie et al., 2009; Cupp et al., 2011; Geary and Mackenzie, 2011; Martin and Robertson, 2010).

The neuropeptidergic signalling system has received much recent attention as a source of potential drug targets, due to its widely conserved and essential roles in nematodes, including in feeding behaviours, metabolism, reproduction and neuromuscular functions. Their vertebrate counterparts, on the other hand, exhibit significant differences in sequence. No current drug targets the helminth neuropeptidergic system, although ample precedent for the value of drugs that affect neuropeptide receptors is found in the opiate class of analgesics. It is thus appealing to develop novel anthelmintics that can target this system and be used for treatment or co-treatment of helminth infections. In the free-living model nematode *Caenorhabditis elegans*, extensive studies have been carried out to

deorphanize and characterise G-protein coupled receptors (GPCRs) that recognise neuropeptides of the FMRFamide family, which are uniquely found in invertebrates. However, studies investigating FMRFamide-like peptides (FLPs) and their receptors in a parasitic nematode context have been lacking (Geary, 2010; Marks and Maule, 2010; Mousley et al., 2004).

In *C. elegans* and parasitic nematodes, FLP-18 are potent and myoactive neuropeptides. They activate two structurally distinct GPCRs, NPR-4 and NPR-5, which in turn modulate different functions. Moreover, FLP-18 neuropeptides are conserved across all nematodes examined (Cohen et al., 2009; Frooninckx et al., 2012; Peymen et al., 2014). It is thus of interest to characterise FLP-18 receptors from a parasitic species, such as the filarial nematode *Brugia malayi*, which is quite evolutionarily distinct from *C. elegans*. By doing so, we will be able to compare their structure-activity relationship (SAR) profiles with their orthologues from *C. elegans*, validating *C. elegans* as a model and providing a better understanding of FLP receptors in a parasitic nematode context. In addition, we can investigate if SAR profiling can distinguish the two different receptors activated by the same neuropeptides, suggesting different molecular architecture of the ligand-binding site. We hypothesize that the FLP receptors are conserved across the phylum Nematoda, and that FLP-18 receptors from *B. malayi* will exhibit comparable structure-activity profiles to each other and to their *C. elegans* orthologues.

The study will not only contribute to current knowledge about FLP receptor pharmacology, it will also provide insights into potential drug discovery. Using high-throughput screening and *in silico* techniques, future studies can search for non-peptide ligands that target the pertinent FLP receptors, which can potentially be developed into novel anthelmintics.

II. Literature review

2.1. Helminth infections

Helminthiases are endemic in many developing countries, namely regions of sub-Saharan Africa, central and east Asia, Latin America, and the Caribbean (Figure 1). The most prevalent helminthiases include: 1. Soil-transmitted helminthiases, such as ascariasis, hook worm infection and trichuriasis. 2. Trematode infections such as schistosomiasis. 3. Filarial nematode infections such as lymphatic filariasis and onchocerciasis (Lustigman et al., 2012).

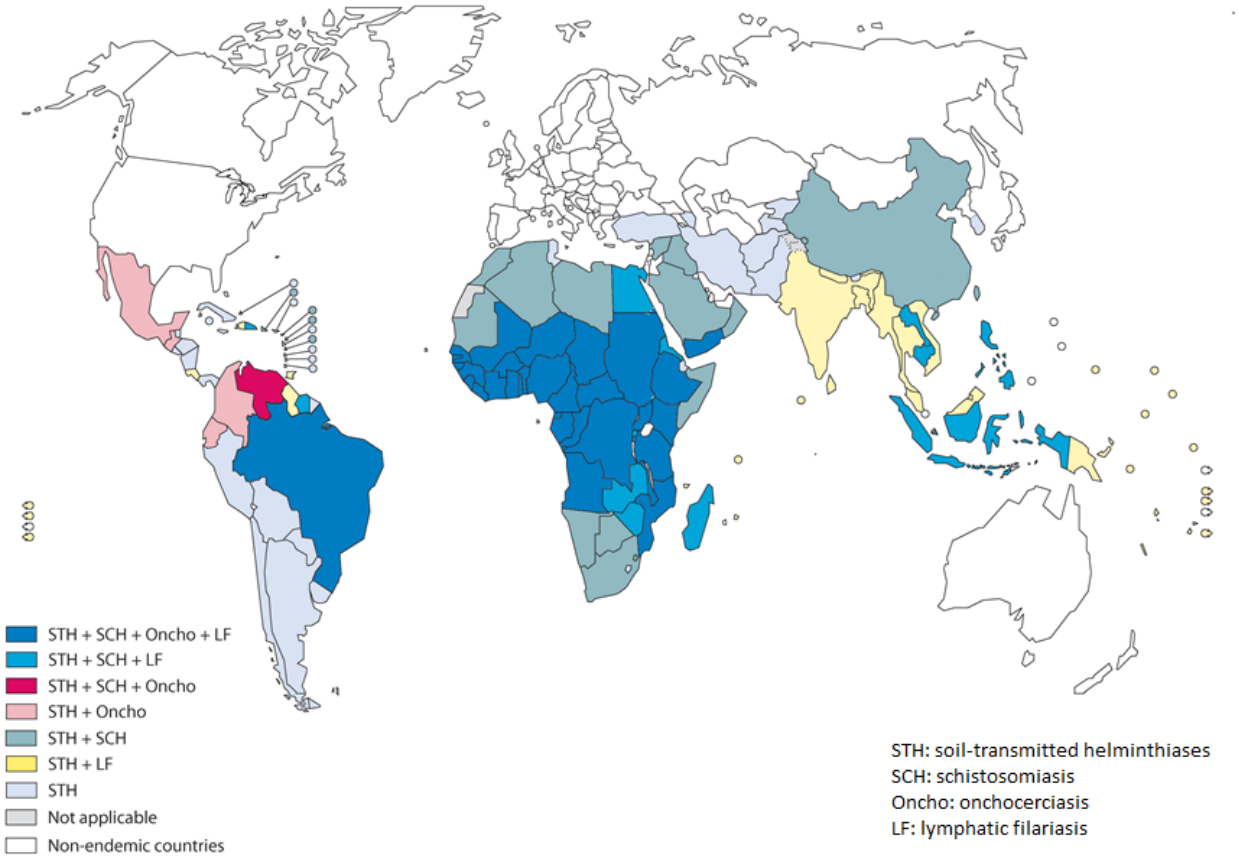


Figure 1. Global distribution of helminth infections (Lustigman et al., 2012)

These diseases can be debilitating and are often associated with severe symptoms including pain, disfiguration and various inflammatory responses triggered in the hosts. Furthermore, subtle morbidities are associated with lowered life quality and satisfaction, including absence from school or work, social stigma and rejection, which may all lead to depression and other psychological distress (Crompton and Savioli, 2007; Obindo et al., 2017). In addition to the negative impact on physical and psychological well-being in affected individuals, these parasitic infections are a socioeconomic burden to the afflicted societies.

Worldwide, approximately 120 million people are infected with lymphatic filariasis, 37 million people are infected with onchocerciasis, at least 207 million people are infected with schistosomiasis, and 1.5 billion people are infected with soil-transmitted helminth infections (Fenwick, 2012; Pullan et al., 2014; WHO, 2018). People are often infected with more than one parasite, further aggravating the symptoms and complicating treatment strategy. It was estimated that 883 million children require preventive chemotherapy for soil-transmitted helminth infections, and the number of people in need of preventive chemotherapy for lymphatic filariasis, onchocerciasis and schistosomiasis is 1.3 billion, 120 million and 239 million, respectively. (WHO, 2012). As a result, most endemic countries have implemented mass drug administration (MDA) programs, which deliver chemotherapeutic interventions at very low cost. Even though the cost can be as low as US$4.89 for screening and treating an individual, due to the large number of people who are infected or are at risk of being infected, the total annual cost of control is estimated to be US $2 billion, in addition to the donations of medicines from pharmaceutical industries (Bundy et al., 2018; WHO, 2012).

While most people in developed countries are free from pathogenic parasitic infections, parasites are nonetheless a burden to companion animals and to agriculture in these regions. Parasitic infections in companion animals and in livestock are one of the major driving forces that motivate research in search of better anti-parasitic drugs in developed countries (Geary et al., 2003; Geary et al., 2004). A recent sampling at animal shelters across Canada suggested that around 34% of dogs and 32% of cats were infected by at least one parasite (Villeneuve et al., 2015). The prevalence of parasitic infections is supposedly lower among house pets, given their regular veterinary visits and better-monitored living conditions. Nevertheless, infected animals pose a risk to other domestic companion animals as well as to their human owners. In livestock, parasitic diseases have always been a constraint to optimal productivity, even in developed countries such as the UK, Australia, New Zealand and the US, etc. Not only can these infections raise concerns about food safety, they also lead to environmental burden and economic loss (Learmount et al., 2015). Infections caused by liver fluke and gastrointestinal nematodes such as *Haemonchus contortus* and *H. placei*, among other related species, are the major cause of morbidity and mortality in sheep, goats and cattle. While livestock diseases are clearly understudied compared to diseases of humans, it is estimated that the world production of animal source foods is reduced by 20-50% due to disease. In the UK, gastrointestinal parasitic nematodes alone were estimated to cost the sheep industry more than US$100 million each year. In Europe, more than US$460 million was spent annually on the purchase of anthelmintic drugs. (Morgan et al., 2013; Nieuwhof and Bishop, 2005; Rushton and Bruce, 2017; Thumbi et al., 2014). In addition to the burden parasites have on animal husbandry, parasites also negatively impact plant agriculture in

most countries. Plant parasites can damage the quality and quantity of crops, decreasing the yield of various grains, fruits and vegetables. In the United States alone, damage caused by plant-parasitic nematodes has been estimated to cost at least US$80 billion per year (Jones et al., 2013; Nicol et al., 2011).

Unfortunately, many scientists have predicted an exacerbation in the prevalence of parasitic diseases around the world due to climate change and increased global travel. Parasitic diseases are most common and significant in tropical and subtropical regions, as those climates promote a rich reservoir of hosts and vectors for parasites and optimal conditions for larval survival in the environment. However, because of the changing climate, certain regions that did not previously have a favourable environment for parasites have now become potentially habitable (Short et al., 2017). As increased temperature, rainfall and humidity enhance the development and survival of some helminths, scientists have observed several parasitic diseases at extended altitude and latitude. For instance, *Angiostrongylus vasorum*, commonly known as the French lungworm, was originally native to the southern part of Western Europe and parts of South America, but the parasite has been expanding northward beyond its native territory over the years. It came to scientists' attention in 2009, when *A. vasorum* was reported to have infected a dog in Scotland for the first time; since then, more infected dogs and foxes were found in regions such as northern England, Scotland, Sweden and parts of the U.S. (Sohn, 2017). Other examples include the reemergence and expansion of various helminth species in the Arctic (Galaktionov, 2017; Kuchta et al., 2017; Okulewicz, 2017), the increase in parasitic infection frequency and intensity among sheep and cattle (Kenyon et al., 2009; Short et al., 2017), and the range extension of several human

helminthiases from tropical and subtropical regions into North America, East Asia and Europe (Hotez, 2017; Hotez, 2018; Zhu et al., 2017). In addition, a warm climate with high humidity also facilitates the development and abundance of arthropod vectors such as mosquitoes, which transmit some parasites from one host to another. These vectors can in turn be infectious at an expanded range over a longer period of time throughout the year, further intensifying the transmission of parasitic diseases. These rising parasitological challenges call for continuous monitoring and devising intervention strategies when necessary – since humans and other organisms in the extended territories lack acquired immunity against these new pathogens, they will likely suffer from more severe clinical symptoms once infected (Haines et al., 2006; Short et al., 2017).

2.2. Control interventions of helminthiases

Common strategies employed to combat helminth infections include chemotherapy and vector control. Many endemic regions have launched MDA programs, often in combination with vector control; this is a very cost-effective solution and renders many helminthiases eradicable (Croke et al., 2017; Kastner et al., 2017; Luroni et al., 2017; Rao et al., 2017). Current anthelmintics are generally effective, with few or no side effects, as they selectively target receptors in parasitic worms with less potency for the mammalian homologues. Most anthelmintics act on the neuromuscular system, which has essential roles in maintaining normal parasite biology, including feeding, reproduction and locomotion. Disrupting these fundamental behaviours impairs parasite propagation and/or survival inside hosts. Moreover, these drug targets are typically highly conserved across various helminth phyla, resulting in broad-spectrum anthelmintics that can treat

infections caused by many different species of parasitic worms (Martin and Robertson, 2010). Anthelmintics are categorized into a few classes as follows.

- ***Anthelmintics***

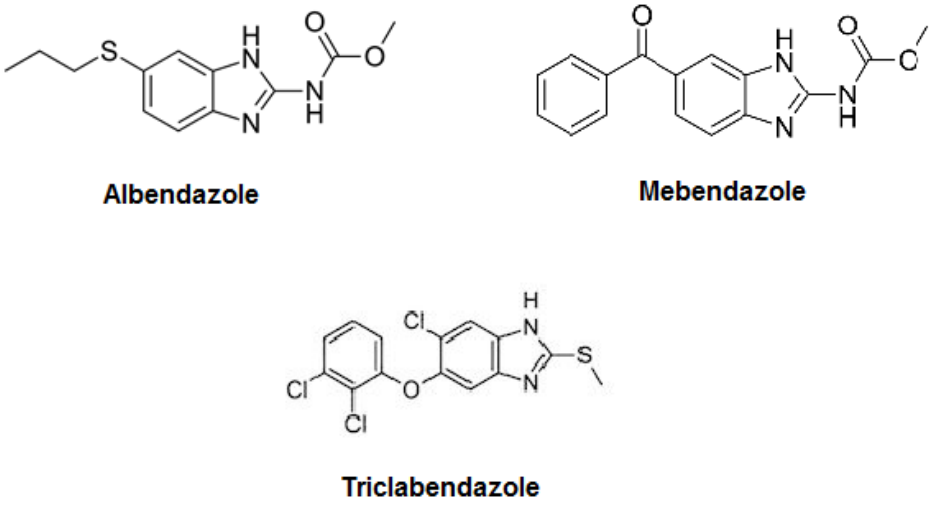


Figure 2. Benzimidazoles

Benzimidazoles (Figure 2) are a class of anthelmintics that include albendazole, mebendazole, triclabendazole and others. Benzimidazoles act by binding to β-tubulins, inhibiting polymerization and microtubule formation. The lack of functional microtubules impairs cell division, intracellular and vesicular transport and synapse formation (Conder, 2010; Martin and Robertson, 2010). The spectrum of action of benzimidazoles is quite broad. Benzimidazoles are widely used for the treatment of lymphatic filariasis and soil-transmitted helminthiases, infections caused by parasitic nematodes, and are also used to treat pinworm infections (enterobiasis). Along with triclabendazole, they are one of the very few anthelmintic treatments effective against foodborne trematode infections. (Keiser and Utzinger, 2005; Ronald, 2007).

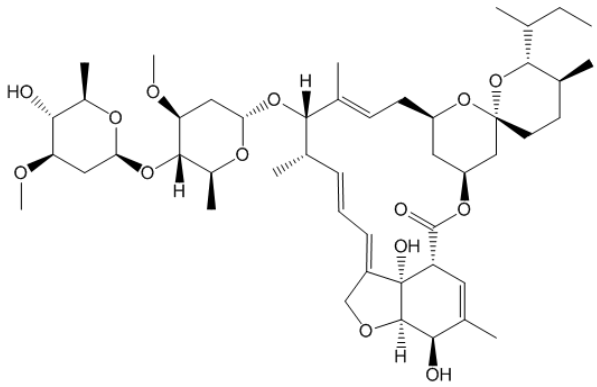


Figure 3. Ivermectin

Avermectins such as ivermectin (Figure 3) are effective treatments for lymphatic filariasis and onchocerciasis, diseases caused by filarial nematodes. Ivermectin binds to glutamate-gated chloride channels (GluCls), which are present in several types of invertebrates but not in vertebrates. By binding to GluCls on muscle cells, ivermectin cause increased permeability to Cl^- ions, leading to hyperpolarization and paralysis. It is suggested that ivermectin can interfere with the ability of microfilariae to secrete proteins that help them evade host immune responses (Moreno et al., 2010). As a result, ivermectin treatment drastically reduces microfilariae abundance and output, impairs parasite larval development and blocks fertility (Ballesteros et al., 2016; Geary, 2005).

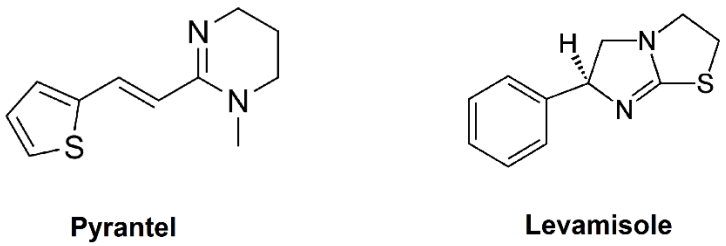


Figure 4. Nicotinic agonists

Pyrantel (Figure 4 left) and levamisole (Figure 4 right) are nicotinic acetylcholine receptor (nAChR) agonists commonly used for treating ascariasis and hookworm infections. By activating nAChRs, they cause sustained muscle contraction, leading to spastic paralysis and reduced egg-laying (Keiser and Utzinger, 2008).

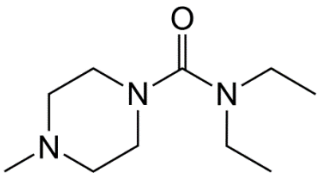


Figure 5. Diethylcarbamazine

Diethylcarbamazine (Figure 5) is used in combination with albendazole as part of the MDA program to eliminate lymphatic filariasis. It induces rapid clearance of microfilariae and prolonged sterility of *Wuchereria bancrofti*. Though very little is known about its mechanism of action, evidence suggested that diethylcarbamazine interferes with the arachidonic acid metabolism pathway, inhibiting the production of prostaglandins, which may render microfilariae more susceptible to immune attack (Sankari et al., 2013).

- ***Limitations of current anthelmintics***

Current anthelmintics have generally tolerable side effects in humans thanks to their reasonably high selectivity; adverse effects usually range from gastrointestinal discomfort, nausea and abdominal pain to mild neurological symptoms. However, this requires treating individuals with the correct regimens, which is not always easily achievable in underdeveloped regions where MDA and presumptive therapy are applied, and people are often co-infected with more than one parasite. The adverse effects can become very severe and even life-threatening if patients receive the wrong anthelmintic treatment. For

example, if patients infected with onchocerciasis are treated with diethylcarbamazine, severe adverse effects called Mazzotti reactions may occur, characterized by itching, rash, fever, tachycardia, edema, inflammation of the lymph nodes accompanied by swelling and pain, as well as eye lesions in some severe cases. It is postulated that the Mazzotti reaction is due to the microfilaricidal effect of diethylcarbamazine causing a rapid cell death, releasing parasite-specific antigens and bacterial endosymbionts, which in turn triggers immune responses of the host, leading to inflammation and allergy-like symptoms (Awadzi et al., 2015; Mazzotti, 1948; Olson and Domachowske, 2006). Treatment with diethylcarbamazine is also contraindicated in loaisis patients with a high microfilariae load because of the risk of drug-induced meningoencephalitis, which is thought to be caused by the accumulation of dead microfilariae in the cerebral capillaries after diethylcarbamazine administration (Blanton, 2007).

Mild Mazzotti reactions and other adverse effects have also been observed in onchocerciasis patients who are treated with ivermectin. Although the incidence is much lower and the symptoms are less severe in general, serious reactions and occasionally fatal outcomes have been reported in patients who harbor high loads of *Loa loa* microfilariae (Conder, 2010; Gardon et al., 1997; Greene, 1985). Comparable to the adverse effects of diethylcarbamazine treatment in loaisis patients with high microfilariae counts, some patients co-infected with onchocerciasis and loaisis also showed encephalopathy after receiving ivermectin treatment, and scientists postulated that similar mechanisms underlie the *Loa loa*-associated adverse reactions, in which microfilaricidal effects cause inflammatory responses in the central nervous system (Boussinesq et al., 1998; Mackenzie et al., 2003). These adverse reactions have limited the use of

diethylcarbamazine and ivermectin in Africa for MDA, due to the high prevalence of co-endemic onchocerciasis and loaisis.

Drug resistance is another growing concern, especially because intensive anthelmintics distribution in endemic regions and the implementation of MDA programs have generated great pressure to select resistant parasites. The development of anthelmintic resistance is widespread among livestock. Some species of parasitic nematodes have developed resistance to benzimidazoles and ivermectin in sheep, goats and cattle. Resistance to levamisole and pyrantel has also been reported (Bentounsi et al., 2012; Canton et al., 2017; Kaplan, 2004; Mickiewicz et al., 2017). In dogs, canine heartworm preventive drugs showed a decrease in efficacy, suggesting emerging resistance (Hampshire, 2005). Ballesteros et al. (2018) recently identified molecular markers in heartworms resistant to macrocyclic lactone endectocides, the common prophylactic treatment. In humans, even though there is no documented anthelmintic resistance, there have been suspicions of ivermectin resistance in onchocerciasis patients. In patients who had persistent skin microfilaria despite multiple treatments, researchers found adult female worms that were non-responsive to the antifertility effect of ivermectin (Awadzi et al., 2004; Geary, 2005; Osei-Atweneboana et al., 2011). Alleles of beta-tubulin associated with benzimidazole resistance have been found in some soil-transmitted parasitic nematodes that infect humans (Rashwan et al., 2017). Praziquantel, the single anthelmintic employed in MDA programs for the treatment of schistosomiasis, has also shown suspected emergence of drug resistance (Botros and Bennett, 2007; Cioli et al., 2014). Since MDA programs will take years before eradication of certain parasitic diseases can be achieved, scientists predict that drug resistance in human helminths may eventually develop.

Last but not least, many of the current anthelmintics have suboptimal efficacy in humans, and some are not suitable for use in children (Geary, 2012). The limitations of existing anthelmintics provide incentives for the discovery and development of new anthelmintics.

- ***Anthelmintic drug discovery and development***

Current antifilarial drugs mostly target microfilariae, lacking or having only minimal macrofilaricidal effects. Thus, the idea of searching for novel drug targets and developing novel anthelmintics that include macrofilaricidal efficacy is appealing. They can be good alternatives for single-use or co-treatment, not only enhancing treatment efficacy, but also greatly shortening the time to eradication and reducing the risk of drug resistance development (Geary and Mackenzie, 2011).

Almost all available anthelmintics were empirically discovered and developed for veterinary use, as parasitic infections have detrimental impacts on the health and productivity of livestock. (O'Dempsey, 2010; Conder, 2010). Traditional anthelmintic discovery and development methods include 1) testing a large library of compounds, such as the products obtained from fermentation of fungi or bacteria, through the utilisation of high-throughput screening; 2) searching for antiparasitic properties in existing compounds and their derivatives; 3) repurposing dropped compounds from other drug discovery projects, such as from anticancer drug research (Geary and Thompson, 2003; Klinkert and Heussler, 2006). These methods are based on forward pharmacology, in which compounds are screened to look for a desirable phenotypic or therapeutic effect. The pharmacodynamics and the mechanism of action are only deduced after the hit is found.

However, as knowledge and technology advance, and as more and more biological techniques and tools have become available, drug research has been shifting towards reverse pharmacology and rational drug design. Reverse pharmacology uses target-based drug discovery, in which a target with known physiological significance is first selected, followed by mechanism-based screening, to look for compounds that can interact with the target and consequently exert a therapeutic effect (Geary et al., 2015). Mechanism-based screening often employs more automated and robotised approaches with high or ultrahigh throughput, reducing the labour intensity and turnaround time. Rational drug design makes use of computational chemistry and enables researchers to generate derivatives with enhanced efficacy or better pharmacological properties, based on either lead compounds or existing anthelmintics (Geary, 2012; Geary et al., 2009).

Reverse pharmacology and rational drug design provide the advantage of exploiting novel drug targets. This can feed into the development of novel anthelmintics with macrofilaricidal properties, and it may ultimately be one of the solutions to overcoming current drug resistance.

2.3. Neuropeptidergic system as novel drug target

The empirically developed anthelmintics have proven the neuromuscular system of helminths to be critical for worm physiology, and that disrupting the neuromuscular system works effectively for parasite control. It is thus promising to search for novel drug targets from the same system, especially the neuropeptidergic system. The neuropeptidergic system possesses several characteristics that make it a good candidate: 1) Neuropeptide signaling has key roles in modulating fundamental helminth functions, ranging from locomotion, reproduction, mechano- and chemosensation, to feeding

behaviours, learning and memory. The numerous known nematode neuropeptides act like neurotransmitters, neuromodulators and hormones. Disrupting neuropeptide signaling will thus be a very effective anthelmintic strategy. 2) The families of neuropeptides are conserved across several invertebrate phyla, encompassing parasitic and non-parasitic worms from both the Nematoda and Platyhelminthes phyla, as well as Arthropoda. Importantly, the neuropeptides present in vertebrates are very distinct, allowing potential anthelmintics to have broad-spectrum actions while minimizing side effects in mammals. 3) Neuropeptides act on G protein-coupled receptors (GPCRs); since one-third of drugs in the market target GPCRs, nematode neuropeptide receptors will likely be druggable (Hauser et al., 2018; Mousley et al., 2004). Although peptides themselves do not have good drug-like properties (Lipinski, 2000), studies have focused on the search for nonpeptide compounds that can interfere with the neuropeptidergic signaling system, potentially by targeting posttranslational modifications or the degradation of neuropeptides, or by acting as agonists or antagonists of neuropeptide GPCRs (Geary, 2010). There are three large families of nematode neuropeptides: FMRFamide-like or FMRFamide-related peptides (FaRPs or FLPs), insulin-like peptides (INPs) and neuropeptide-like proteins (NLPs).

- ***FLPs and FLP receptors***

Peptides from the FLP family have received particular interest since the 1980s, due to their roles in modulating various physiological functions, including locomotion, feeding, metabolism and reproduction, in diverse helminth species across the Nematoda and Platyhelminthes phyla (McVeigh et al., 2009). FLPs are commonly characterised by a C-terminal tetrapeptide motif comprised of an aromatic residue, a hydrophobic residue,

followed by an Arg-Phe-amide. While the RFamide is highly conserved in all FLPs, many FLPs only have one of the other two features (Day and Maule, 1999; Geary et al., 1999; Mousley et al, 2004a; Mousley et al, 2004b). Existing studies on FLPs and FLP receptors have been mostly carried out in the non-parasitic nematode *C. elegans*, a well-characterised model organism.

Like all neuropeptides, a precursor gene (*flp*) encodes multiple FLPs with the same or different sequences. From the precursor gene to the final products, neuropeptide maturation involves several steps of posttranslational processing (Figure 6), including the cleavage of prepropeptide into propeptide; the propeptide is alternatively spliced into several peptides, which are then modified. The common posttranslational modification is C-terminal amidation, which protects neuropeptides from degradation. The peptide maturation process in invertebrates is distinct from vertebrates, making neuropeptide processing enzymes amenable drug targets (Li and Kim, 2008; Marks and Maule, 2010).

Being the largest neuropeptide family, 32 *flp* genes have been identified in *C. elegans*, which are predicted to encode more than 70 FLPs. The expression of FLPs among several nematode species was determined using immunocytochemical localization. Though the expression patterns differ depending on the species, they were shown to be widely distributed in the nervous system in all nematode species tested. It was also demonstrated that *flp* genes are expressed in more than half the total neurons, including interneurons, sensory and motor neurons. In addition, FLPs are also expressed in non-neuronal tissues. An overlap has been noticed, in which many cells express more than one *flp*, and most *flps* are expressed in multiple neurons (Kim and Li, 2004; Peymen et al., 2014).

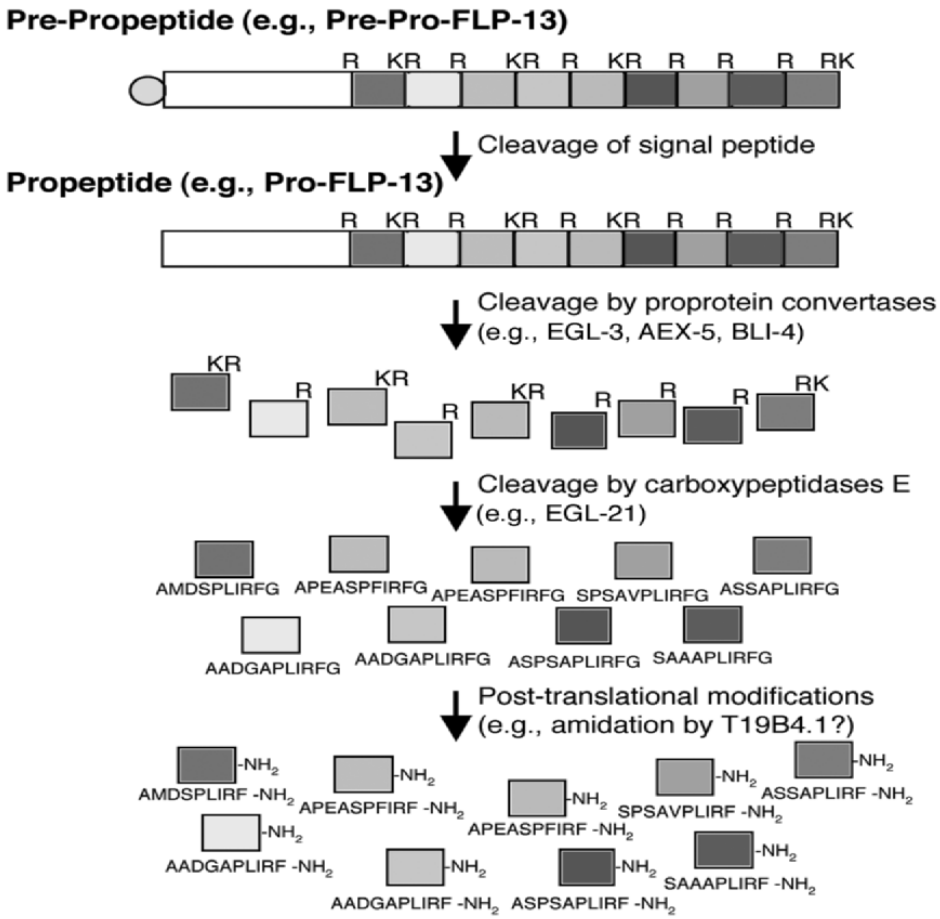


Figure 6. Posttranslational processing of neuropeptide gene product (Li and Kim, 2010)

FLPs are stored in large dense-core vesicles at the nerve terminal, and are released into synapses in response to Ca^{2+} influx. FLPs then act on their receptors. The deorphanization process has matched many *C. elegans* FLPs to their neuropeptide receptors (NPRs), usually by screening a large number of putative neuropeptide ligands against receptors that are heterologously expressed in Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, *Xenopus* oocytes or yeast. It was observed that a FLP often can activate more than one FLP receptor, and at the same time a FLP receptor can be activated by multiple FLPs (Frooninckx et al., 2012; Geary and Kubiak, 2005; McVeigh et al., 2006; Peymen et al., 2014). FLP signalling is terminated when the

FLPs are degraded by hydrolytic enzymes. FLP receptors and the hydrolytic enzymes are distinctly different from their homologs in vertebrates, making them potential drug targets (Geary, 2010; Stawicki et al., 2013).

The RFamide peptides found in vertebrates were shown to be evolutionarily related to FLPs. However, they have profoundly evolved and diverged from the ancestral FMRFamide peptides; other than the C-terminal RFamide sequence, very weak sequence similarities were observed between vertebrate RFamide peptides and FLPs (Ubuka and Tsutsui, 2018).

- ***NPR-4 and NPR-5***

NPR-4 and NPR-5 are neuropeptide GPCRs. Deorphanization of the receptors in *C. elegans* have confirmed that both are activated by FLP-18 neuropeptides, which are encoded on the *flp-18* precursor gene (Figure 7).

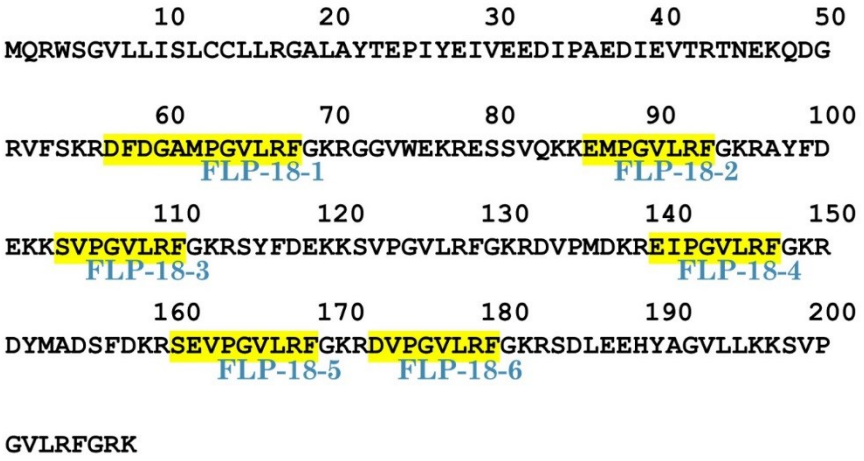


Figure 7. C. elegans flp-18 gene encoding six neuropeptides (Adapted from http://www.uniprot.org/uniprot/Q9N4V0)

In the study by Cohen et al. (2009), it was postulated that the AIY interneurons receive and integrate inputs from olfactory, gustatory and thermosensory neurons, then release FLP-18 neuropeptides in response. FLP-18 peptides act on different cell types that express NPR-4 or NPR-5 (Figure 8).

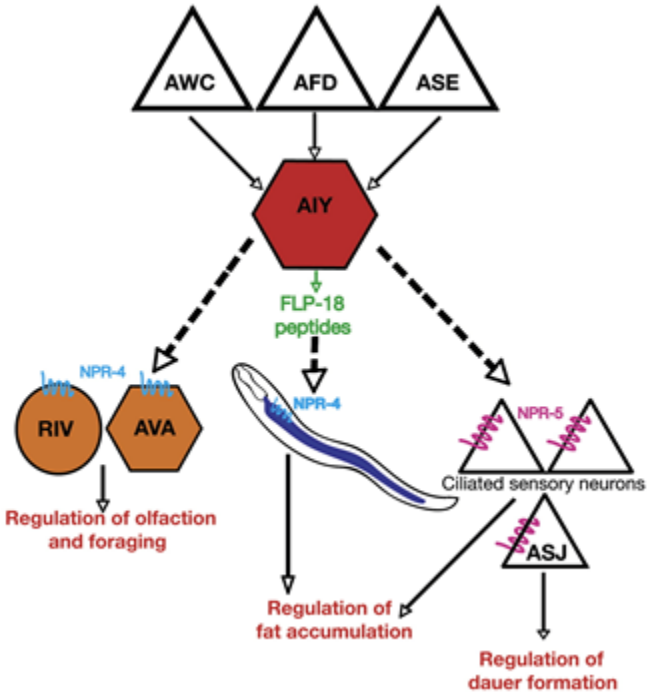


Figure 8. FLP-18 neuropeptides released by AIY interneurons regulate olfaction, foraging, dauer formation and fat accumulation by acting on NPR-4 and NPR-5 (Cohen et al., 2009)

NPR-5 expressing cells include a subset of amphid neurons, inner labial neurons, certain interneurons, in the phasmids, head, neck and body muscles. FLP-18 neuropeptide action through these cells suppresses dauer formation and intestinal fat storage, which contribute to maintaining homeostasis in *C. elegans*. NPR-4 is expressed on several specific neurons as well as on coelomocytes, parts of the intestine and rectal gland cells. Together, NPR-4 expressing cells help *C. elegans* find near-threshold odour sources and

promote the change in foraging strategy from local search to dispersal after prolonged food deprivation. In addition, a recent study also showed the effect of FLP-18 peptides on locomotion behaviour, more specifically the modulation of reversal length, through their action on NPR-4 (Bhardwaj et al., 2018). The activation of NPR-4 and NPR-5 by FLP-18 peptides have been also characterised and verified using various heterologous expression systems, including yeast and mammalian cells (Kubiak et al., 2008; Cohen et al., 2009; Li and Kim, 2014).

Although much has been learnt about the NPR-4 and NPR-5 from *C. elegans*, few studies have been done in parasite species. The use of *C. elegans* is favoured due to its well mapped neural networks and the availability of many tools for gene manipulation for functional genomics studies. Scientists thus often extrapolate results from *C. elegans* to parasitic helminths. However, there are essential differences between free-living nematodes and parasitic nematodes. Because they have distinct living environments, they often have different body temperature and feeding behaviour. An additional component for parasitic species is their interaction with the host, and evading the host immune system is necessary for their survival and propagation; this aspect is missing in *C. elegans*. Thus, it is also important to work on helminths of medical relevance in parallel.

In the parasitic nematode *Ascaris suum*, three neuropeptides share the same C-terminal -PGVLRF-NH_2 motif as FLP-18 peptides – AVPGVLRF-NH_2 (AF3), GDVPGVLRF-NH_2 (AF4) and GMPGVLRF-NH_2 (AF20). These *A. suum* neuropeptides showed comparable potency in activating NPR-5 (Kubiak et al., 2008) and are highly myoactive in *A. suum*. According to BLAST searches, both NPR-4 and NPR-5 have orthologues in many parasitic nematode species. The NPR-4 orthologue from the filarial parasite *Brugia malayi*

was heterologously expressed in HEK cells and was activated by *C. elegans* FLP-18 peptides (Anderson et al., 2014). These studies suggest a conservation of FLP-18 neuropeptides as well as their receptors across nematode species. Another study carried out in the plant parasite *Meloidogyne incognita* found that, silencing *flp-18* using RNA interference (RNAi) reduced migration and penetration into roots (Papolu et al., 2013). This has further supported the role of FLP-18 peptides in modulating chemosensation and olfaction-mediated foraging. Furthermore, it has demonstrated the possibility of parasite control by disrupting the neuropeptide signalling system.

III. Methods

3.1. Materials

A set of four yeast strains (*Saccharomyces cerevisiae*) was a gift from James Broach (Cadus Pharmaceutical Corp; Evans et al., 2009). These recombinant yeast strains are designed for the expression of heterologous, including invertebrate, GPCRs, by modifying and exploiting the endogenous yeast mating pheromone response pathway. Several key genes encoding proteins in this pathway have been deleted, including genes encoding the pheromone alpha factor GPCR (*STE2*), the Gα subunit (*GPA1*), the Gβγ-responsive promoter (*FUS1*), the enzyme imidazoleglycerol-phosphate dehydratase (*HIS3*) in the histidine biosynthesis pathway, the factor arresting cell growth in response to pheromone (*FAR1*), and the factor regulating desensitization to pheromone response (*SST2*). In addition, a recombinant gene encoding HIS3 downstream of the FUS1 promoter was introduced, coupling the expression of the HIS3 enzyme to Gβγ levels. The Gα subunit was modified at the C-terminus, at which the last five amino acids were replaced with various pentapeptides present in invertebrate Gα proteins. The Gα protein chimeras enhance coupling to heterologous invertebrate GPCRs. The four yeast strains differ in the Gα subunit chimeras: strain CY13393 contains a $G\alpha_i$ subunit, strain CY13395 contains a $G\alpha_{12}$ subunit, strain CY13397 contains a $G\alpha_q$ subunit, and strain CY13399 contains a $G\alpha_s$ subunit. To use this heterologous expression system, an invertebrate GPCR is transformed and expressed at the cell surface. When the GPCR is activated by an agonist, it triggers dissociation of the Gβγ complex from the Gα subunit. Gβγ in turn activates the FUS1 promoter via the MAP kinase signalling cascade, inducing the

expression of the *HIS3* reporter gene. This eliminates the histidine auxotrophy phenotype, allowing yeast survival in histidine drop-out medium.

The plasmid vector Cp4258 for the expression of invertebrate GPCRs was also obtained from Cadus. The vector contains a leader sequence at the N-terminus of the GPCR, which enhances targeting to the yeast surface. The vector also encodes the *LEU2* gene, which serves as a selection marker, allowing transformed cells to grow in leucine drop-out medium.

The *C. elegans* peptide DVPGVLRFa (FLP-18f), its N-terminally and C-terminally truncated analogues, and an alanine scan series, were synthesized by GenScript (Piscataway, New Jersey). Stock solutions were prepared in double-distilled water at 1 mM and stored at -20^{o}C. An additional series of FLP-18 analogues and FLP-18 derived peptides were kindly provided by Dr. Liliane Schoofs' laboratory at Katholieke Universiteit Leuven. A list of the peptides utilized in this study is provided in Table 1.

Table 1. C. elegans FLP-18 peptides and FLP-18f analogues

Peptide Name	Sequence
FLP-18a	GAMPGVLRF-amide
FLP-18b1	EMPGVLRF-amide
FLP-18b2	pGlu-MPGVLRF-amide
FLP-18c	SVPGVLRF-amide
FLP-18d1	EIPGVLRF-amide
FLP-18d2	pGlu-IPGVLRF-amide
FLP-18e	SEVPGVLRF-amide
FLP-18f	DVPGVLRF-amide
FLP-18g	DFDGAMPGVLRF-amide
FLP-18h	SYFDEKKSVPGVLRF-amide

FLP-18i1		ESSVQKKEMPGVLRF-amide
FLP-18i2		pGlu-SSVQKKEMPGVLRF-amide
FLP-18j2		SDLEEHYAGVLLKKSVPGVLRF-amide
FLP-18f analogue	M1	DVPGFLRF-amide
FLP-18f truncated analogues	M2	DVPGVLRF-OH
	D1	PGVLRF-amide
	D2	GVLRF-amide
	D3	VLRF-amide
FLP-18f alanine scanning series	A1	AVPGVLRF-amide
	A2	DAPGVLRF-amide
	A3	DVAGVLRF-amide
	A4	DVPAVLRF-amide
	A5	DVPGALRF-amide
	A6	DVPGVARF-amide
	A7	DVPGVLAF-amide
	A8	DVPGVLRA-amide

3.2. *In vitro* heterologous expression in *Saccharomyces cerevisiae*

Total RNA of *B. malayi* was provided by Dr. Christina Ballesteros. First strand cDNA was synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, Waltham, Massachusetts, #K1681). All experiments followed the protocols provided by the manufacturer.

The *Bma-npr-4* ORF has two potential coding sequences, one suggested by the *Brugia malayi* genome database (http://www.wormbase.org/species/b_malayi/transcript/Bm2254#06--10), and the other published by Anderson et al. (2014). The two sequences only differ at the 3' end, at which the sequence published by Anderson et al. contained 63 more nucleotides immediately before the stop codon. Based on the two coding

sequences, two sets of PCR primers were synthesized (Life Technologies, Pleasanton, California). The PCR products were expected to amplify both complete ORFs, with a BsaI restriction site added to the 5' end and a BamHI restriction site added to the 3' end. This enabled restriction digestion and ligation steps needed to sub-clone the *bma-npr-4* ORF into the Cp4258 vector. The DNA plasmid was transformed into DH5α competent *E. coli* using heat shock method as described by Froger and Hall (2007). The Cp4258 vector contains an ampicillin resistance gene, which allows the *E. coli* containing the plasmid construct to propagate on LB agar plates with ampicillin (100 µg/mL).

Bma-npr-5 had two coding sequences corresponding to two isoforms (http://www.wormbase.org/species/b_malayi/gene/WBGene00227183#0-9g-3). An additional step of rapid amplification of cDNA ends was carried out at the 5' end (5' RACE) using PCR, to confirm the 5' coding sequence. PCR primers were then designed for the amplification of complete *bma-npr-5* ORF, with BsaI and BamHI restriction sites added to the 5' and 3' ends, respectively. The *Bma-npr-5* ORF was also sub-cloned into the Cp4258 yeast expression vector, and the plasmid was transformed into *E. coli* for propagation.

Colony PCR using primers flanking the multiple cloning sites followed by Sanger sequencing at Genome Quebec confirmed the successful insertion of genes of interests in the Cp4258 vector. The purified plasmids were then transformed into the four yeast strains using electroporation as described by Thompson et al. (1998). Transformed yeast expressed the *LEU2* selectable marker encoded on the vector and grew in complete minimal medium lacking leucine (CM/Leu$^-$). As a result, *Bma*-NPR-4 and *Bma*-NPR-5 were expressed on the membrane in yeast strains containing different Gα subunits, as demonstrated in the diagram below (Figure 9).

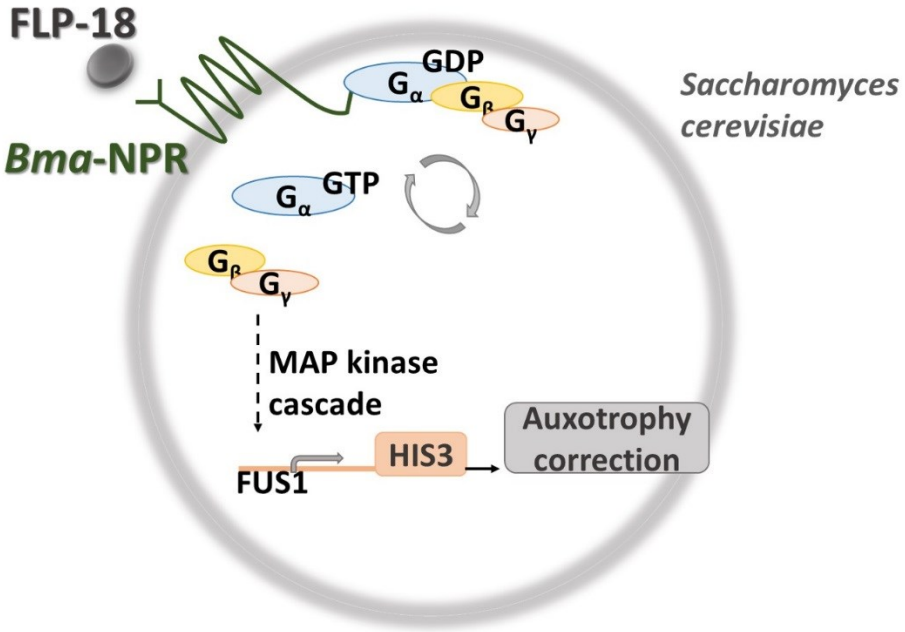


Figure 9. G protein signalling pathways in Saccharomyces cerevisiae

The NPR is coupled to the G_α chimera, which is in a heterotrimeric G protein complex with G_β and G_γ. Upon receptor activation by agonist, the GDP-bound G_α dissociated after GDP was replaced with GTP. The $G_{\beta\gamma}$ complex initiated the MAP kinase signalling cascade, in turn activating the FUS1 promoter. Activation of the FUS1 promoter led to the expression of HIS3, enabling growth in complete minimal medium lacking histidine (CM/Leu$^-$/His$^-$), in an agonist concentration-dependent manner (Dowell and Brown, 2009; Evans et al., 2009; Larsen et al., 2012).

The yeast functional assay followed the protocol adapted from Larsen et al. (2012). Briefly, yeast expressing Bma-NPR-4 or Bma-NPR-5 were grown overnight in CM/Leu$^-$. Cells were harvested the next day and washed multiple times using CM/Leu$^-$/His$^-$ to remove residual histidine. Cells were resuspended in CM/Leu$^-$/His$^-$ supplemented with 0.05 M MOPS, pH 6.8; multiple dilutions were done to reach a final concentration of 3000 cells/well in 96-well plates (Costar). Cells were incubated at 30^{o}C with different peptides

for approximately 44 hr. Cell growth was quantified using Alamar Blue (Thermo Fisher Scientific), which is converted from non-fluorescent resazurin to fluorescent resorufin by the reducing power of viable cells. The level of fluorescence was linearly correlated with cell growth and was measured using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, Vermont). Yeast cell growth in response to different peptides was tested at various concentrations, and each treatment was run four times in duplicate. Cells without any treatment served as negative control, and cells with histidine-supplemented medium (100 μM) served as positive control for yeast cell viability.

3.3. Heterologous expression in Chinese Hamster Ovary (CHO) cells

Bma-npr-4 and *bma-npr-5* cDNAs were amplified using PCR. PCR primers were synthesized with a Kozak translation initiation sequence added before the ATG start codon of both cDNAs to ensure proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). Additionally, the PCR primers added a BamHI restriction site to the 5' end and a XbaI restriction site to the 3' end of *bma-npr-5*; BamHI restriction sites were added to both 5' and 3' ends of *bma-npr-4*. Following restriction digestion and ligation, the amplified ORFs of *bma-npr-4* and *bma-npr-5* were subcloned into the mammalian cell expression vector pcDNA3.1(+) (Thermo Fisher Scientific). The DNA plasmids were propagated in *E. coli*, and the correct insertion of *bma-npr-4* and *bma-npr-5* was confirmed by Sanger sequencing at Genome Quebec.

For heterologous expression of the two receptors in mammalian cells and the subsequent aequorin-based Ca^{2+} bioluminescence assay, we followed protocols adapted from Lu et al. (2011). Briefly, the plasmids encoding *bma-npr-4* or *bma-npr-5* ORF were transiently

transfected into the A24 cell line of Chinese Hamster Ovary (CHO) cells, two days before the aequorin-based Ca^{2+} bioluminescence assay. The A24 cell line stably expresses aequorin and human $G_{\alpha16}$. The promiscuous human $G_{\alpha16}$ can couple to most GPCRs regardless of their endogenous Gα partner. Transfected cells were incubated for proliferation (37^{o}C, 5% CO_2, humidified). 16 hr prior to the aequorin-based Ca^{2+} bioluminescence assay, transfected cells were transferred to a 28^{o}C, 5% CO_2, humidified incubator. This was done because previous studies have reported better expression of these GPCRs in mammalian cells incubated at 28^{o}C overnight (Kubiak et al., 2003; Mertens et al., 2005).

To perform the aequorin-based Ca^{2+} bioluminescence assay, cells were collected by centrifugation at room temperature. 100 µL of the cell suspension was used to count the cells using the TC20 automated cell counter (Bio-Rad). Cell pellets were resuspended at $5x10^6$ cells/mL, in BSA medium (DMEM/F12 without phenol red, supplemented with 0.1% bovine serum albumin). Coelenterazine H (Invitrogen) was added to the tubes to a final concentration of 5 µM, which were then incubated for 4 hr at room temperature in the dark. Coelenterazine H is the cofactor that forms complexes with aequorin, which is stably expressed by the cell line. After 4 hr incubation, the cells were diluted to $5x10^5$ cells/mL with 0.1% BSA medium, followed by an additional 30 min incubation to equilibrate.

A dilution series of FLP-18 derived peptides and FLP-18f analogues were prepared in BSA medium for determining concentration-response relationships for the ligands with the two NPRs. Peptides were added in 96-well clear-bottom plates. Wells containing only BSA medium served as negative control. The plates were kept at 4^{o}C and were brought

to room temperature shortly before the Ca^{2+} bioluminescence assay. The scheme of the assay is shown in Figure 10.

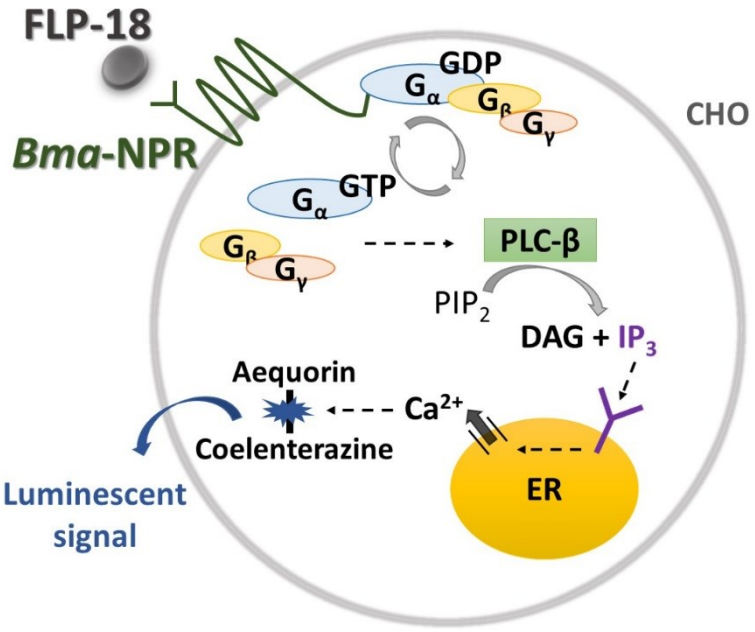


Figure 10. G protein signalling pathways in CHO cells

When the NPR is activated by a ligand, $G_{\alpha 16}$ coupled to the GPCR will bind to GTP instead of GDP, and the GTP-bound $G_{\alpha 16}$ dissociates from the $G_{\beta\gamma}$ complex. Both $G_{\alpha 16}$ and the $G_{\beta\gamma}$ complex can activate phospholipase C-β (PLC-β), which in turn catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 then activates IP_3 receptors on the endoplasmic reticulum (ER), triggering the release of Ca^{2+} from the ER into the cytosol. The intracellular Ca^{2+} breaks the bond between aequorin and coelenterazine H, emitting luminescence signals that can be recorded. The intensity of the luminescence is indicative of the intracellular Ca^{2+} level, which is related to the extent of receptor activation by the ligand. The effect of the peptides is thus directly correlated with the luminescence signal intensity (Caers et al. 2014; Lu et al., 2011; Mertens et al., 2004; Van Sinay et al., 2017).

Liquid handling and luminescence signal measurements were done using MicroBeta^2 LumiJET Microplate Counters (PerkinElmer). The compound plates were loaded in the microplate counter system. The system was programmed to first dispense the cells into a 96-well plate, followed by a series of measurements for luminescence signals; one recording was taken per second for 30 sec. The injectors then dispensed equal volumes of 0.2% Triton X-100 (in 0.1% BSA medium) into the wells, followed by another 30 sec of luminescence signal measurements. The cells were then lysed with Triton X-100 (0.2% Triton X-100 in 0.1% BSA medium), releasing all the cellular Ca^{2+}, including Ca^{2+} in the cytosol and Ca^{2+} inside the ER.

Data analysis was carried out using Microsoft Excel. The luminescence signal intensity was adjusted to minimize errors caused by variations in cell number per well and to exclude background levels of luminescence. Adjusted luminescence level thus directly reflects the extent of receptor activation by the peptide. The calculation is shown below:

$$Luminescence_{adjusted} = \frac{Peptide}{Peptide + Triton\ X} - \frac{BSA}{BSA + Triton\ X}$$

Peptide: the sum of luminescence measurements for the first 30 sec. The luminescence signal was emitted due to Ca^{2+} released from ER, which was downstream of the G protein cascade as a result of peptide activating the receptor.

Peptide + *Triton X*: the sum of luminescence measurements for 1 min. The luminescence signal was emitted due to Ca^{2+} released from ER after peptide activation of the receptor as well as Ca^{2+} released by Triton X lysis of the cells.

Dividing the luminescence signal emitted due to cytosolic Ca^{2+} by the luminescence signal emitted due to total cellular Ca^{2+} allowed us to normalize the data against the difference in cell count in each well.

A minor luminescence signal was collected from BSA medium-only negative control. This background luminescence corresponds to non-receptor-mediated signal, as there was no ligand to interact with the receptor. It was likely caused by disrupted lipid bilayer leading to a small amount of Ca^{2+} leak during cell injection (Caers et al., 2014). Thus, we could obtain the receptor-mediated signal by subtracting the non-receptor-mediated signal from the total luminescence signal.

For each peptide, luminescence measurements corresponding to the dilution series were adjusted according to the equation above. The data were then further transformed to define the level of receptor activation:

$$Activation = \frac{Luminescence_{Adjusted}}{Max[Luminescence_{Adjusted}\ at\ all\ concentrations]} \times 100\%$$

Activation level was expressed as % maximal luminescence response, i.e., % of adjusted luminescence over the highest adjusted luminescence for that specific peptide throughout the dilution series. Hence, for any peptide, the strongest luminescence signal collected at a specific concentration would always be 100%, and luminescence signals collected at other concentrations would be relative to the maximum luminescence.

Concentration-response relationships were generated using GraphPad. Peptide concentrations were logarithmically transformed, and each % activation level was plotted against its corresponding Log[concentration].

IV. Results

4.1. *Bma*-NPR-4 heterologous expression in CHO cells

Thirteen peptides encoded on the *C. elegans flp-18* precursor gene activated *Bma*-NPR-4 in the aequorin-based Ca^{2+} bioluminescence assay. *Bma*-NPR-4 responded to all 13 peptides in a concentration-dependent manner, and the concentration-response curves showed similar and parallel sigmoidal shapes (Figure 12). SEVPGVLRF-amide (FLP-18e) had a particularly potent action on *Bma*-NPR-4, with an EC_{50} value of 4.2 nM. DFDGAMPGVLRF-amide (FLP-18g), GAMPGVLRF-amide (FLP-18a), pGlu-MPGVLRF-amide (FLP-18b2) and SVPGVLRF-amide (FLP-18c), in descending order of potency, had EC_{50} values of 21 – 76 nM. The rest of the peptides, DVPGVLRF-amide (FLP-18f), pGlu-IPGVLRF-amide (FLP-18d2), EMPGVLRF-amide (FLP-18b1), EIPGVLRF-amide (FLP-18d1) and SYFDEKKSVPGVLRF0-amide (FLP-18h), had EC50 values of 130 – 320 nM.

Efficacy of FLP-18 peptides was determined by comparing the maximum activation of each to the maximum activation observed with FLP-18f. FLP-18f was chosen as the reference because subsequent structure-activity relationship (SAR) studies were done using truncated analogues and an alanine scan series derived from it. All peptides showed similar efficacy for *Bma*-NPR-4 (Figure 11), with the exception of FLP-18a, which had 75% efficacy compared to FLP-18f. Other peptides had efficacy ranging from 101-112%, not significantly different from FLP-18f. Efficacy and EC_{50} values for each peptide are presented in Table 2.

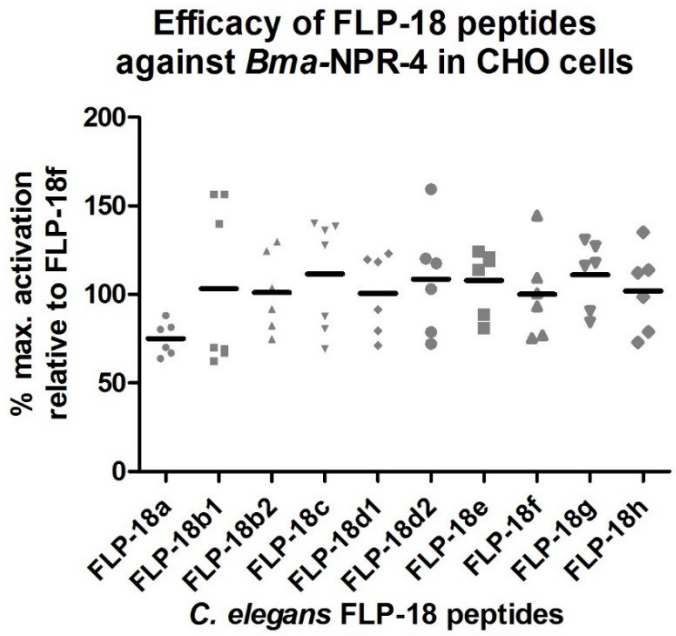
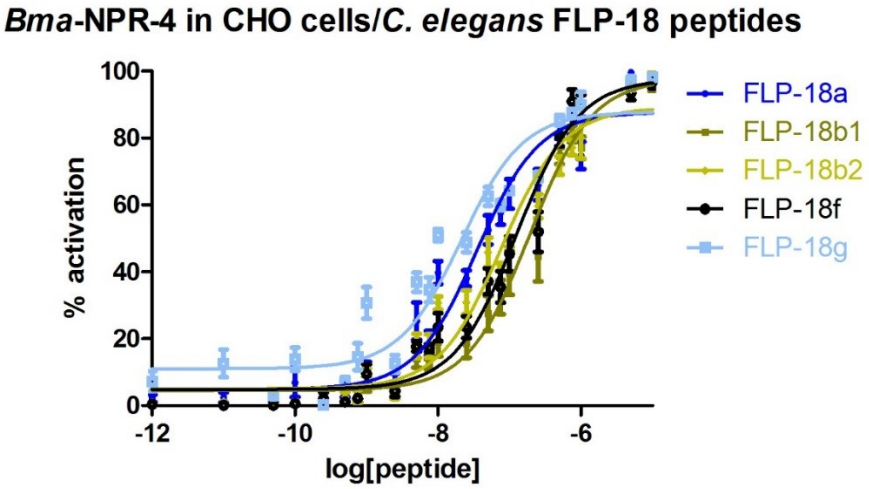


Figure 11. Efficacy of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in CHO cells



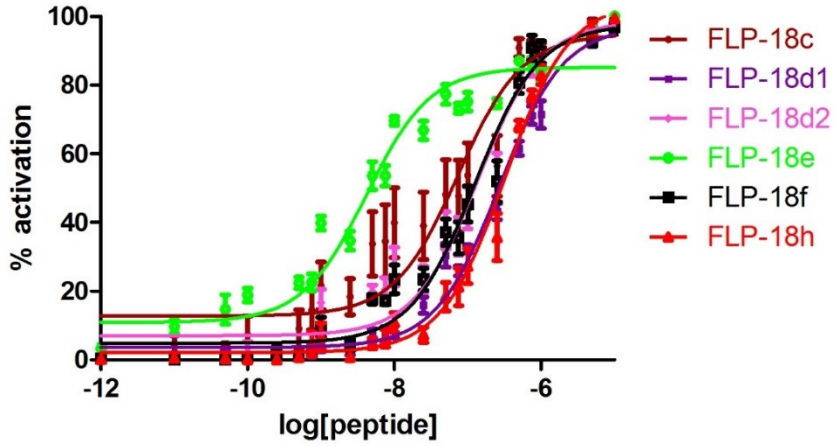


Figure 12. Concentration-response relationships of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in CHO cells

Table 2. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in CHO cells

***C. elegans* FLP-18 peptides**		***Bma*-NPR-4 in CHO cells**		
		EC_{50} (M)	Max. activation (Adjusted luminescence)	% relative to FLP-18f
FLP-18a	GAMPGVLRF-amide	3.487e-008	0.161252	75.05
FLP-18b1	EMPGVLRF-amide	1.914e-007	0.221838	103.25
FLP-18b2	pGlu-MPGVLRF-amide	7.639e-008	0.217251	101.11
FLP-18c	SVPGVLRF-amide	7.379e-008	0.239616	111.52
FLP-18d1	EIPGVLRF-amide	2.685e-007	0.215969	100.52
FLP-18d2	pGlu-IPGVLRF-amide	1.394e-007	0.233126	108.50
FLP-18e	SEVPGVLRF-amide	4.162e-009	0.231722	107.85
FLP-18f	DVPGVLRF-amide	1.254e-007	0.21486	100
FLP-18g	DFDGAMPGVLRF-amide	2.111e-008	0.238645	111.07
FLP-18h	SYFDEKKSVPGVLRF-amide	3.229e-007	0.218928	101.89

To further investigate the role of each amino acid and potentially significant motifs, the SAR studies included an alanine scan series and truncated analogues of FLP-18f. The truncated analogue, DVPGVLRF-OH (M2), for which the C-terminal amide was eliminated,

did not activate *Bma*-NPR-4 at the concentrations tested. Analogues with N-terminal truncations, PGVLRF-amide (D1), GVLRF-amide (D2) and VLRF-amide (D3), showed progressively descending potency while maintaining efficiency similar to the parent FLP-18f. Additionally, DVPGFLRF-amide (M1) replaced the VLRF-amide motif with FLRF-amide motif; F at this position is commonly observed in other FLPs. Its concentration-response curve was shifted to the right, indicating lower potency (Figure 14 top). Its efficacy was not significantly different from FLP-18f (Figure 13).

The alanine scan series replaced each amino acid of $D^1V^2P^3G^4V^5L^6R^7F^8$-amide (FLP-18f) with alanine, one at a time. When R^7 and F^8 were modified, no activation was observed for *Bma*-NPR-4. D^1 and V^2 modifications resulted in higher efficacy and very similar EC_{50} values compared to FLP-18f, with the concentration-response curve of AVPGVLRF-amide (A1) shifted slightly to the left, and DAPGVLRF-amide (A2) slightly to the right of FLP-18f (Figure 14 bottom). Replacing P^3 and G^4 had similar consequences for potency and efficacy. Compared to FLP-18f, both peptides had similar efficacy, but decreased potency, with DVPAVLRF-amide (A4) being less potent than DVAGVLRF-amide (A3). A further decrease in potency and efficacy was observed for V^5 and L^6 modifications, for which EC_{50} values were 19 µM and 41 µM, respectively. Their efficacy levels were 86% and 77% of the parent peptide (Figure 13). Values for efficacy and potency are summarized in Table 3.

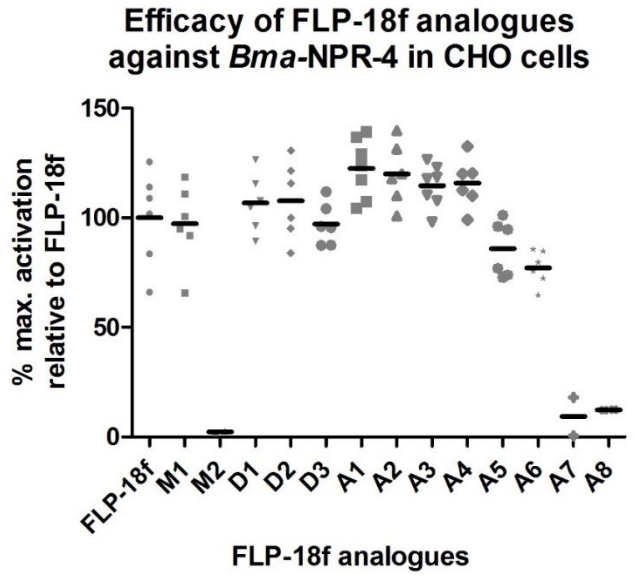
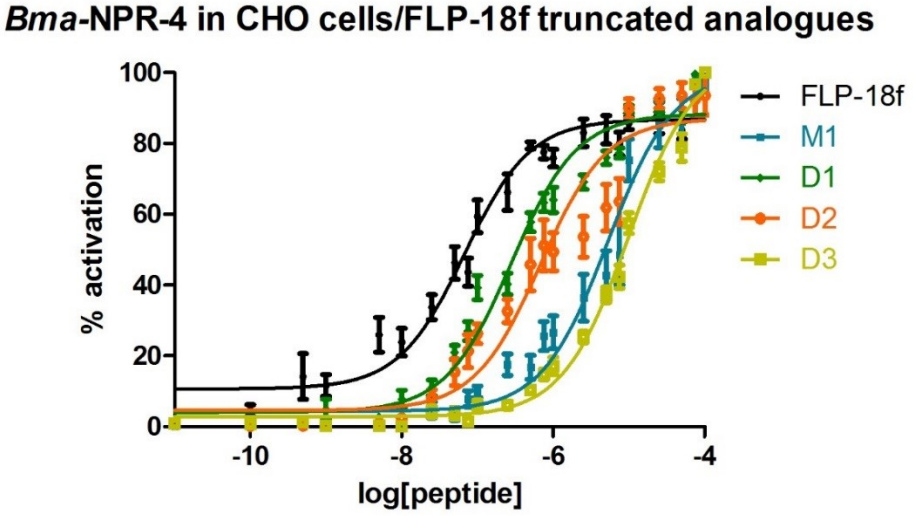


Figure 13. Efficacy of FLP-18f truncated analogues and alanine scan series for Bma-NPR-4 expressed in CHO cells



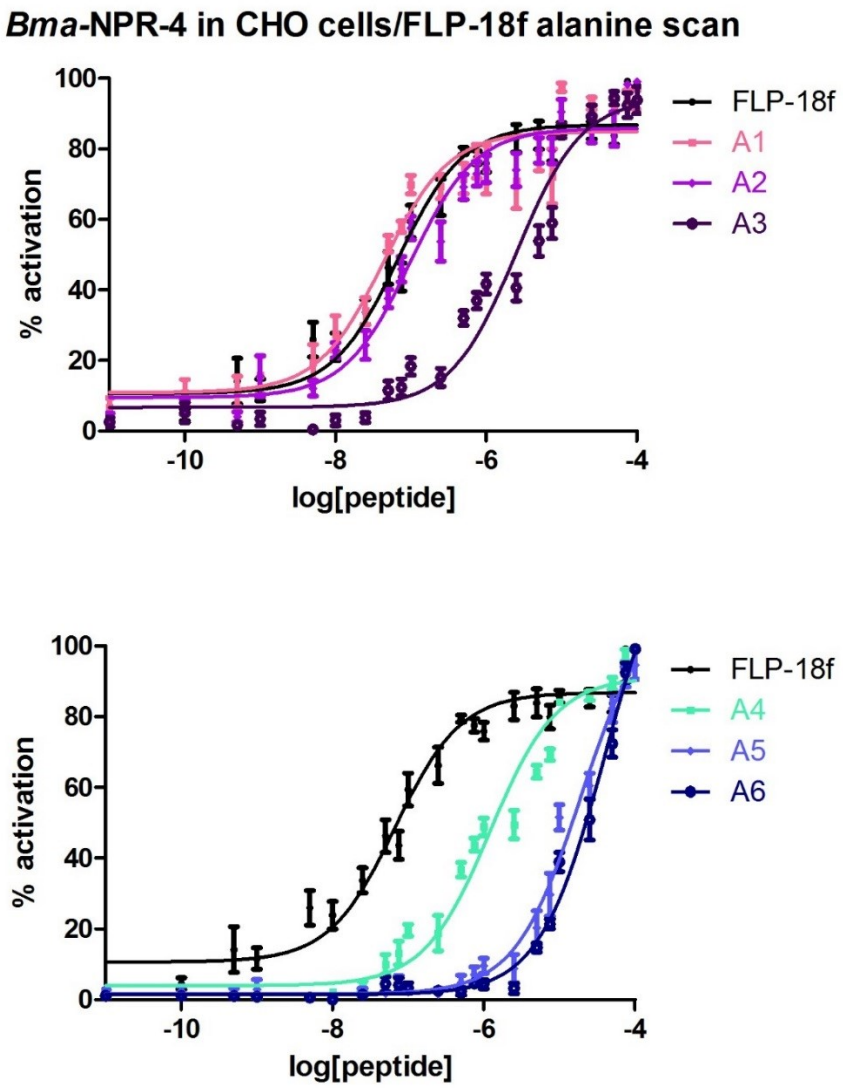


Figure 14. Concentration-response relationships of FLP-18f truncated analogues and alanine scan series for Bma-NPR-4 expressed in CHO cells

Table 3. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of FLP-18f analogues for Bma-NPR-4 expressed in CHO cells

FLP-18f analogues			***Bma*-NPR-4 in CHO cells**		
			EC_{50} (M)	Max. activation (Adjusted luminescence)	% relative to FLP-18f
FLP-18f parent compound		DVPGVLRF-amide	6.744E-08	0.252459	100
Analogue	M1	DVPGFLRF-amide	5.271E-06	0.245553	97.26
C-truncation	M2	DVPGVLRF-OH	-	0.005745	2.28
N-truncations	D1	PGVLRF-amide	2.798E-07	0.269562	106.77
	D2	GVLRF-amide	6.760E-07	0.272178	107.81
	D3	VLRF-amide	9.797E-06	0.245252	97.15
Alanine scan series	A1	AVPGVLRF-amide	4.355E-08	0.30951	122.60
	A2	DAPGVLRF-amide	9.749E-08	0.303053	120.04
	A3	DVAGVLRF-amide	2.515E-06	0.289523	114.68
	A4	DVPAVLRF-amide	1.212E-06	0.292374	115.81
	A5	DVPGALRF-amide	1.921E-05	0.217021	85.96
	A6	DVPGVARF-amide	4.052E-05	0.194833	77.17
	A7	DVPGVLAF-amide	-	0.023561	9.33
	A8	DVPGVLRA-amide	-	0.031114	12.32

4.2. *Bma*-NPR-5 heterologous expression in CHO cells

Bma-NPR-5 was functionally expressed in CHO cells. In parallel to *Bma*-NPR-4, the same aequorin-based Ca^{2+} bioluminescence assay was carried out with *Bma*-NPR-5, which also was activated by the 13 *C. elegans* FLP-18 peptides. However, lower luminescence levels were observed in general. The concentration-response curves displayed comparable sigmoidal shapes among all peptides (Figure 16). In descending order of potency, peptides FLP-18d2, FLP-18g, FLP-18e, FLP-18a and FLP-18c had EC_{50} values of 340 – 640 nM; peptides FLP-18b2, FLP-18b1, FLP-18d1, FLP-18f and FLP-18h had EC_{50} values of 1.2 – 1.9 µM. Furthermore, all peptides showed higher efficacy against

Bma-NPR-5 relative to FLP-18f, ranging from 117% – 142% (Figure 15). Values for each peptide are shown in Table 4.

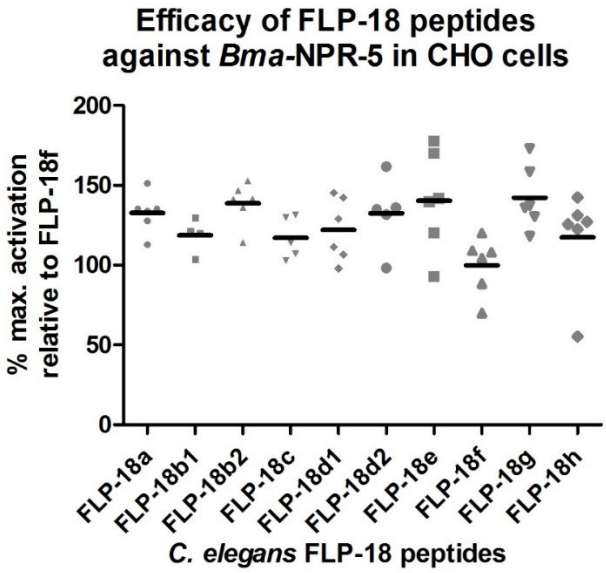
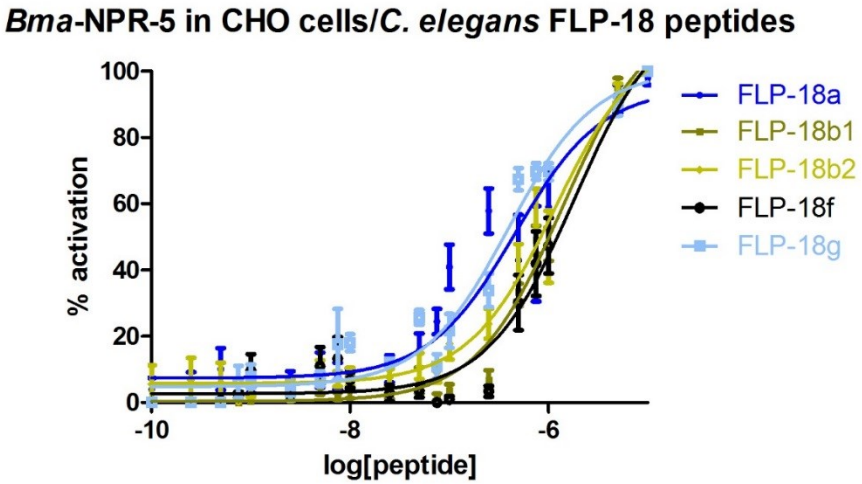


Figure 15. Efficacy of C. elegans FLP-18 peptides for Bma-NPR-5 expressed in CHO cells



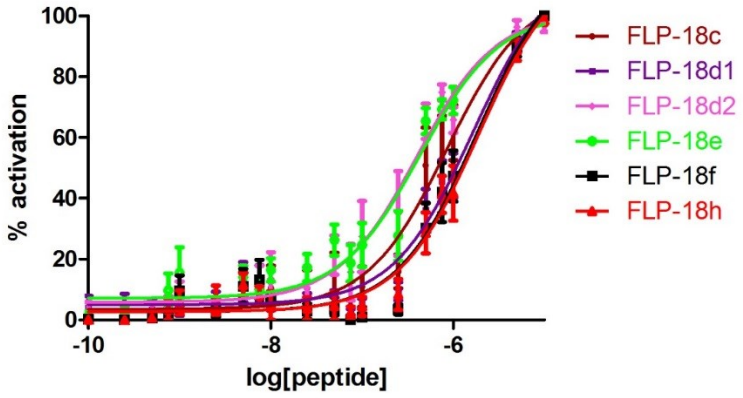


Figure 16. Concentration-response relationships of C. elegans FLP-18 peptides for Bma-NPR-5 expressed in CHO cells

Table 4. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of C. elegans FLP-18 peptides for Bma-NPR-5 expressed in CHO cells

***C. elegans* FLP-18 peptides**		***Bma*-NPR-5 in CHO cells**		
		EC_{50} (M)	Max. activation (Adjusted luminescence)	% relative to FLP-18f
FLP-18a	GAMPGVLRF-amide	4.640E-07	0.09097	132.73
FLP-18b1	EMPGVLRF-amide	1.273E-06	0.08135	118.69
FLP-18b2	pGlu-MPGVLRF-amide	1.163E-06	0.095149	138.82
FLP-18c	SVPGVLRF-amide	6.353E-07	0.080268	117.11
FLP-18d1	EIPGVLRF-amide	1.621E-06	0.083669	122.07
FLP-18d2	pGlu-IPGVLRF-amide	3.356E-07	0.090829	132.52
FLP-18e	SEVPGVLRF-amide	4.547E-07	0.096286	140.48
FLP-18f	DVPGVLRF-amide	1.814E-06	0.068539	100
FLP-18g	DFDGAMPGVLRF-amide	3.967E-07	0.097485	142.23
FLP-18h	SYFDEKKSVPGVLRF-amide	1.873E-06	0.080436	117.36

In SAR studies (Figure 17 and Figure 18), *Bma*-NPR-5 showed no activation when the C-terminal amide was removed (M2) or F^8 was modified (A8). C-terminal modifications of V^5, L^6, R^7 (A5, A6, A7) and the truncated VLRF-amide (D3) resulted in minimal luminescence signals, eliciting <25% of the maximum response relative to FLP-18f.

Respectively, VLRF-amide (D3) and DVPGALRF-amide (A5) had EC_{50} values of 1.1 M and 250 μM. The EC_{50} values of DVPGVLRF-OH (M2), DVPGVARF-amide (A6), DVPGVLAF-amide (A7) and DVPGVLRA-amide (A8) could not be determined, as they did not generate valid concentration-response curves. Replacing the VLRF-amide motif with FLRF-amide lowered the EC_{50} from 1.5 μM to 27 μM, and its efficacy also decreased to 61%. The N-terminally truncated analogue PGVLRF-amide (D1) had reduced potency and efficacy, while the further truncated GVLRF-amide (D2) had potency similar to PGVLRF-amide (D1), but its efficacy decreased to 41%. Alanine replacement of the D^1 residue (AVPGVLRF-amide) did not affect efficacy, while potency was slightly reduced. Modifications of V^2, P^3 and G^4, on the other hand, showed reduced potency, with EC_{50}s of 60 – 330 μM; efficacy decreased to 62 – 86%. Efficacy and EC_{50} values are shown in Table 5.

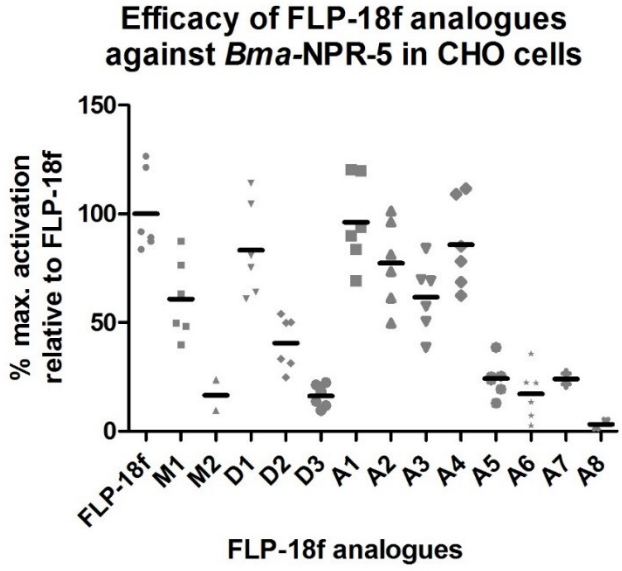


Figure 17. Efficacy of FLP-18f truncated analogues and alanine scan series for Bma-NPR-5 expressed in CHO cells

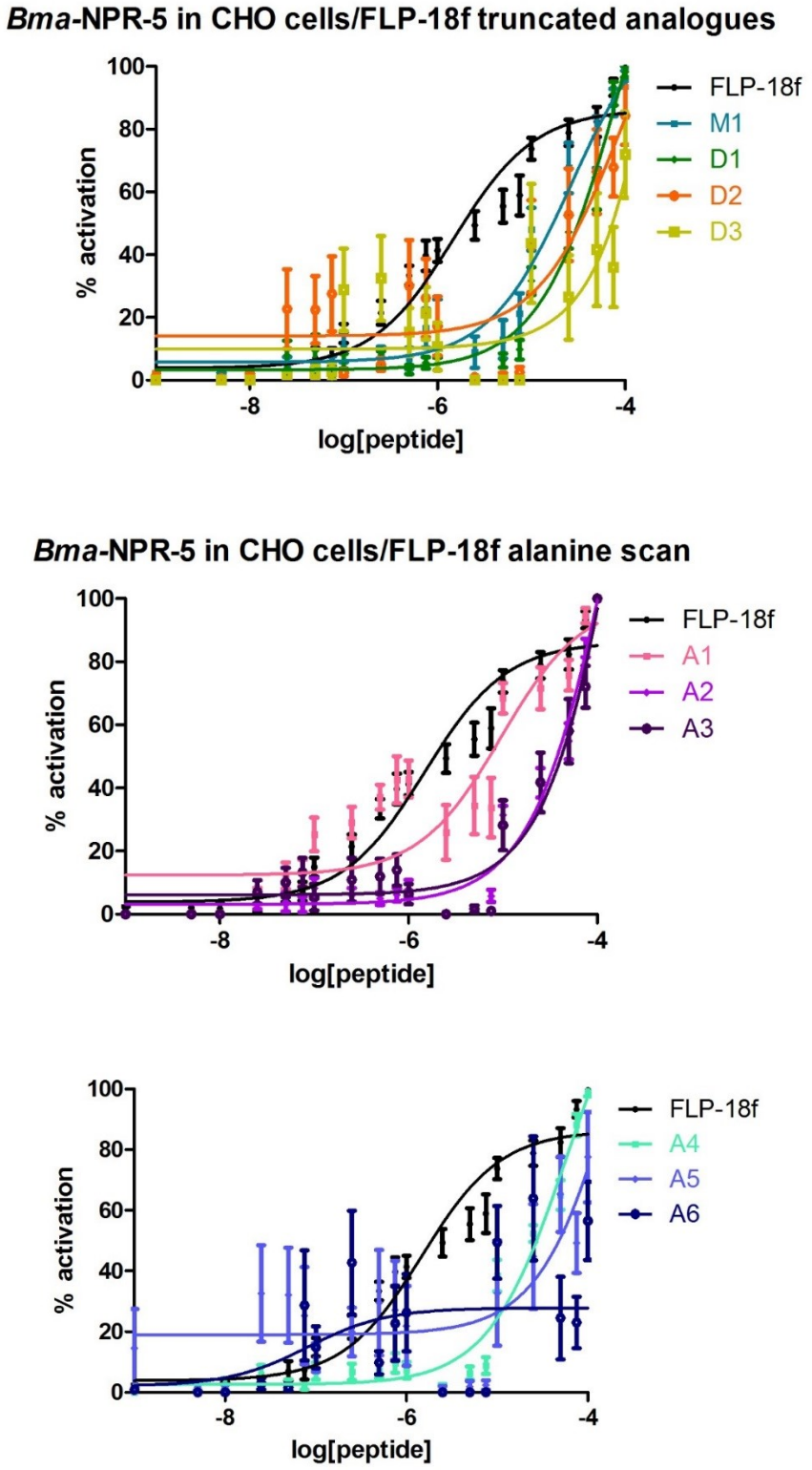


Figure 18. Concentration-response relationships of FLP-18f truncated analogues and alanine scan series for Bma-NPR-5 expressed in CHO cells

Table 5. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of FLP-18f analogues for Bma-NPR-5 expressed in CHO cells

FLP-18f analogues			***Bma*-NPR-5 in CHO cells**		
			EC_{50} (M)	Max. activation (Adjusted luminescence)	% relative to FLP-18f
FLP-18f parent compound		DVPGVLRF-amide	1.488E-06	0.124035	100
Analogue	M1	DVPGFLRF-amide	2.672E-05	0.075484	60.86
C-truncation	M2	DVPGVLRF-OH	-	0.020545	16.56
N-truncations	D1	PGVLRF-amide	9.804E-05	0.10338	83.35
	D2	GVLRF-amide	8.748E-05	0.050271	40.53
	D3	VLRF-amide	1.143	0.020117	16.22
Alanine scan series	A1	AVPGVLRF-amide	9.564E-06	0.119269	96.16
	A2	DAPGVLRF-amide	1.767E-04	0.095845	77.27
	A3	DVAGVLRF-amide	3.294E-04	0.076461	61.64
	A4	DVPAVLRF-amide	6.005E-05	0.106507	85.87
	A5	DVPGALRF-amide	2.523E-04	0.029928	24.13
	A6	DVPGVARF-amide	-	0.021387	17.24
	A7	DVPGVLAF-amide	-	0.029868	24.08
	A8	DVPGVLRA-amide	-	0.003966	3.20

4.3. *Bma*-NPR-4 heterologous expression in *S. cerevisiae*

Bma-NPR-4 was transformed in yeast strains expressing different Gα subunit chimeras. Only *Bma*-NPR-4 expressed in yeast strain CY13393 was activated by FLP-18 peptides, while other yeast strains expressing *Bma*-NPR-4 with different Gα subunit chimeras showed no activation, suggesting that *Bma*-NPR-4 is strictly coupled with $G\alpha_i$ in yeast (not shown). Except for FLP-18d2, all *C. elegans* FLP-18 peptides activated *Bma*-NPR-4 in the yeast system. FLP-18g had low efficacy (43%) relative to FLP-18f. In ascending order of efficacy, FLP-18i2, FLP-18j2, FLP-18h, FLP-18e and FLP-18c had intermediate efficacy (69 – 78%); and FLP-18i1, FLP-18b1 and FLP-18a had 85 – 99% efficacy compared to FLP-18f (Figure 19).

FLP-18a, FLP-18f and FLP-18c were the most potent of the 11 peptides tested, with EC_{50} values of 16 – 87 μM. The other peptides were much less potent, with EC_{50} values from 7.9 – 96 mM (Figure 20). Efficacy and EC_{50} values for each peptide are listed in Table 6.

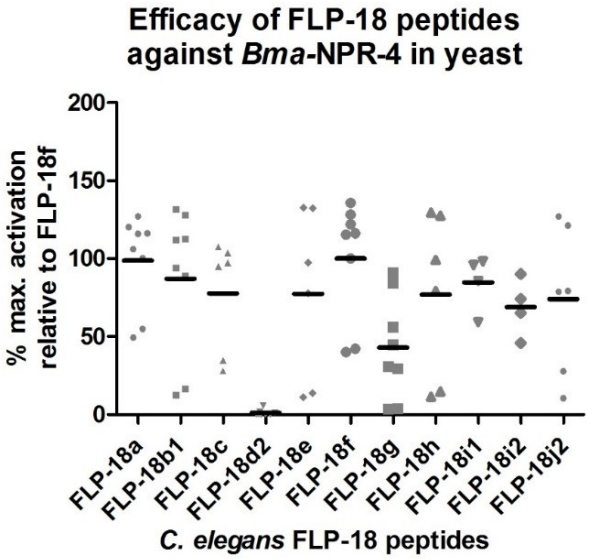
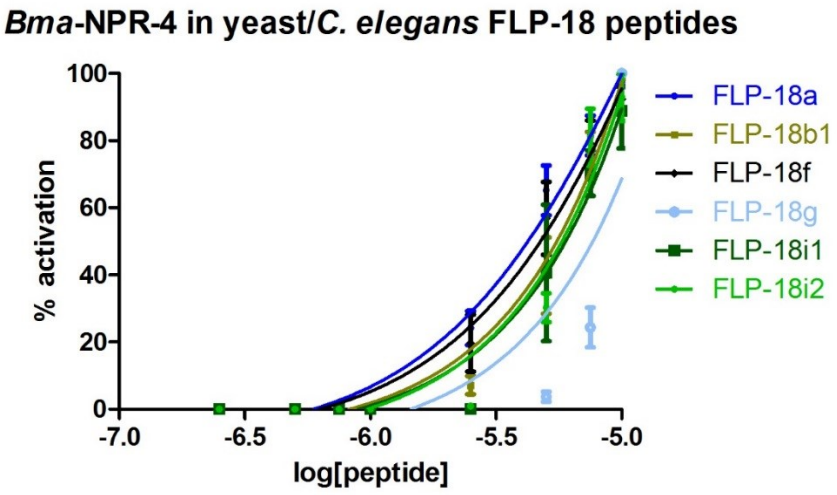


Figure 19. Efficacy of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in S. cerevisiae.



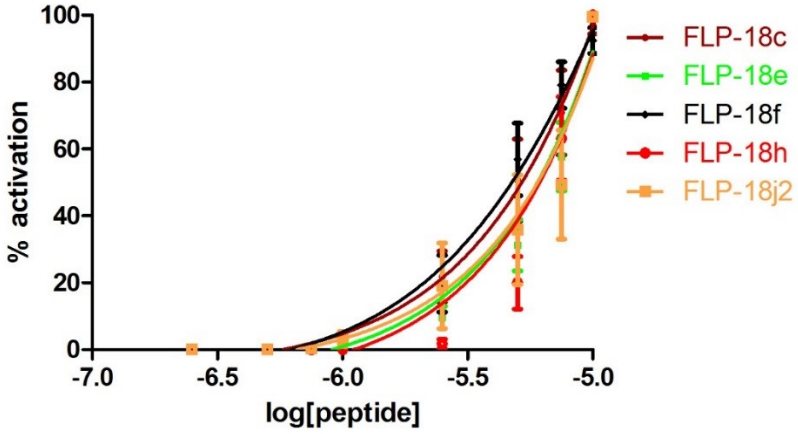


Figure 20. Concentration-response relationships of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in S. cerevisiae

Table 6. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of C. elegans FLP-18 peptides for Bma-NPR-4 expressed in S. cerevisiae

***C. elegans* FLP-18 peptides**		***Bma*-NPR-4 in yeast CY13393**		
		EC_{50} (M)	Max. activation (Fluorescence)	% relative to FLP-18f
FLP-18a	GAMPGVLRF-amide	1.550E-05	34089.63	98.76
FLP-18b1	EMPGVLRF-amide	~ 0.01215	30060.00	87.09
FLP-18c	SVPGVLRF-amide	8.689E-05	26809.17	77.67
FLP-18d2	pGlu-IPGVLRF-amide	-	387.00	1.12
FLP-18e	SEVPGVLRF-amide	~ 0.09621	26728.17	77.44
FLP-18f	DVPGVLRF-amide	2.289E-05	34516.50	100
FLP-18g	DFDGAMPGVLRF-amide	~ 0.05070	14796.63	42.87
FLP-18h	SYFDEKKSVPGVLRF-amide	~ 0.03369	26556.00	76.94
FLP-18i1	ESSVQKKEMPGVLRF-amide	~ 0.007864	29260.00	84.77
FLP-18i2	pGlu-SSVQKKEMPGVLRF-amide	~ 0.06450	23767.50	68.86
FLP-18j2	SDLEEHYAGVLLKKSVPGVLRF-amide	~ 0.01298	25580.83	74.11

No activation was observed when *Bma*-NPR-4 was exposed to FLP-18f analogues with C-terminal amide deletion, G^4, V^5, L^6, R^7 or F^8 modifications, and truncated VLRF-amide fragment (D3) (Figure 21). Changing the C-terminal tetrapeptide VLRF-amide to FLRF-

amide significantly reduced the efficacy and EC_{50} to 13% and 0.6 M, respectively. Truncating the first two N-terminal residues as in PGVLRF-amide (D1) and replacing the D^1 residue with alanine increased efficacy to 110%. PGVLRF-amide (D1) showed a minor decrease in potency, whereas AVPGVLRF-amide (A1) had a slight increase in potency. GVLRF-amide (D2) elicited the maximum response, while its concentration-response curve shifted to the right (Figure 22). Replacing the second residue V^2 with alanine did not change potency, but efficacy was lowered to 73%. With P^3 modification, efficacy and potency further decreased. Efficacy and EC_{50} values are listed in Table 7.

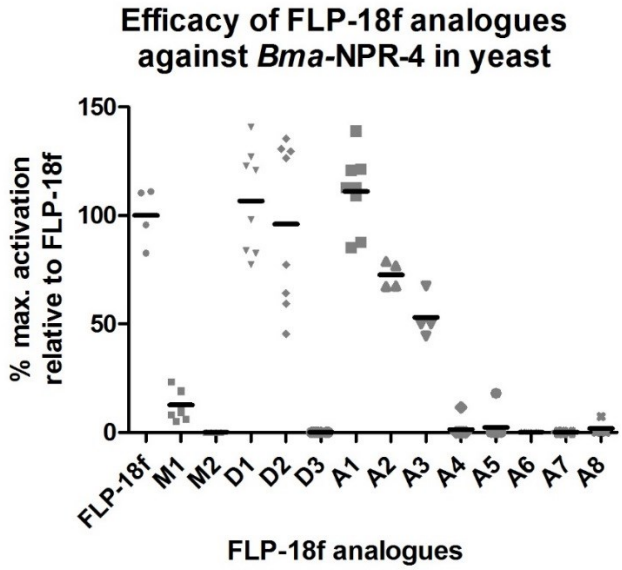


Figure 21. Efficacy of FLP-18f truncated analogues and alanine scan series for Bma-NPR-4 expressed in S. cerevisiae

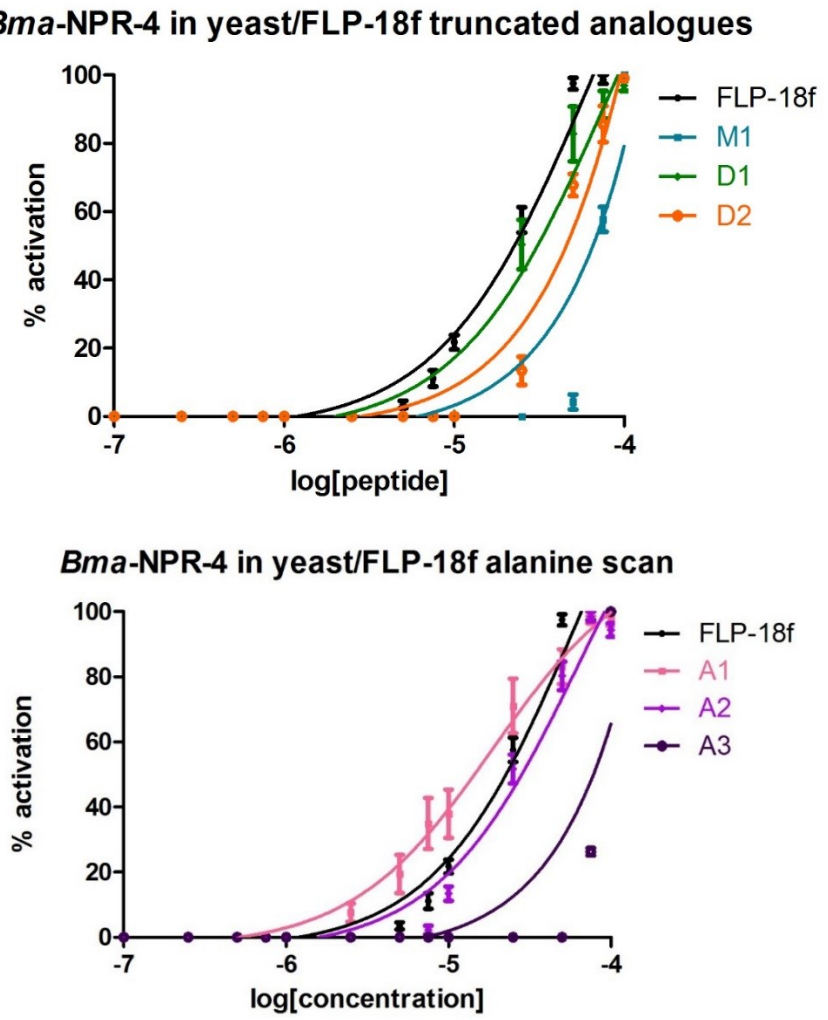


Figure 22. Concentration-response relationships of FLP-18f truncated analogues and alanine scan series for Bma-NPR-4 expressed in S. cerevisiae

Table 7. EC_{50}s, maximal activation levels and % max. activation relative to FLP-18f of FLP-18f analogues for Bma-NPR-4 expressed in S. cerevisiae.

FLP-18f analogues			***Bma*-NPR-4 in yeast CY13393**		
			EC_{50} (M)	Max. activation (Fluorescence)	% relative to FLP-18f
FLP-18f parent compound		DVPGVLRF-amide	6.240E-05	31100.75	100
Analogue	M1	DVPGFLRF-amide	~ 0.5972	3934.625	12.65
C-truncation	M2	DVPGVLRF-OH	-	0	0
N-truncations	D1	PGVLRF-amide	7.890E-05	33180	106.69
	D2	GVLRF-amide	5.994E-04	29874.13	96.06
	D3	VLRF-amide	-	0	0
Alanine scan series	A1	AVPGVLRF-amide	1.870E-05	34543.63	111.07
	A2	DAPGVLRF-amide	6.415E-05	22593.25	72.65
	A3	DVAGVLRF-amide	~ 0.6210	16492	53.03
	A4	DVPAVLRF-amide	-	448.75	1.44
	A5	DVPGALRF-amide	-	702	2.26
	A6	DVPGVARF-amide	-	0	0
	A7	DVPGVLAF-amide	-	0	0
	A8	DVPGVLRA-amide	-	567.5	1.82

4.4. *Bma*-NPR-5 heterologous expression in *S. cerevisiae*

Bma-NPR-5 was transformed into yeast strains expressing $G\alpha_i$, $G\alpha_{12}$, $G\alpha_q$ or $G\alpha_s$. However, functional expression of *Bma*-NPR-5 was not detected in any strain, as measured by activation in response to FLP-18 peptides (Figure 23).

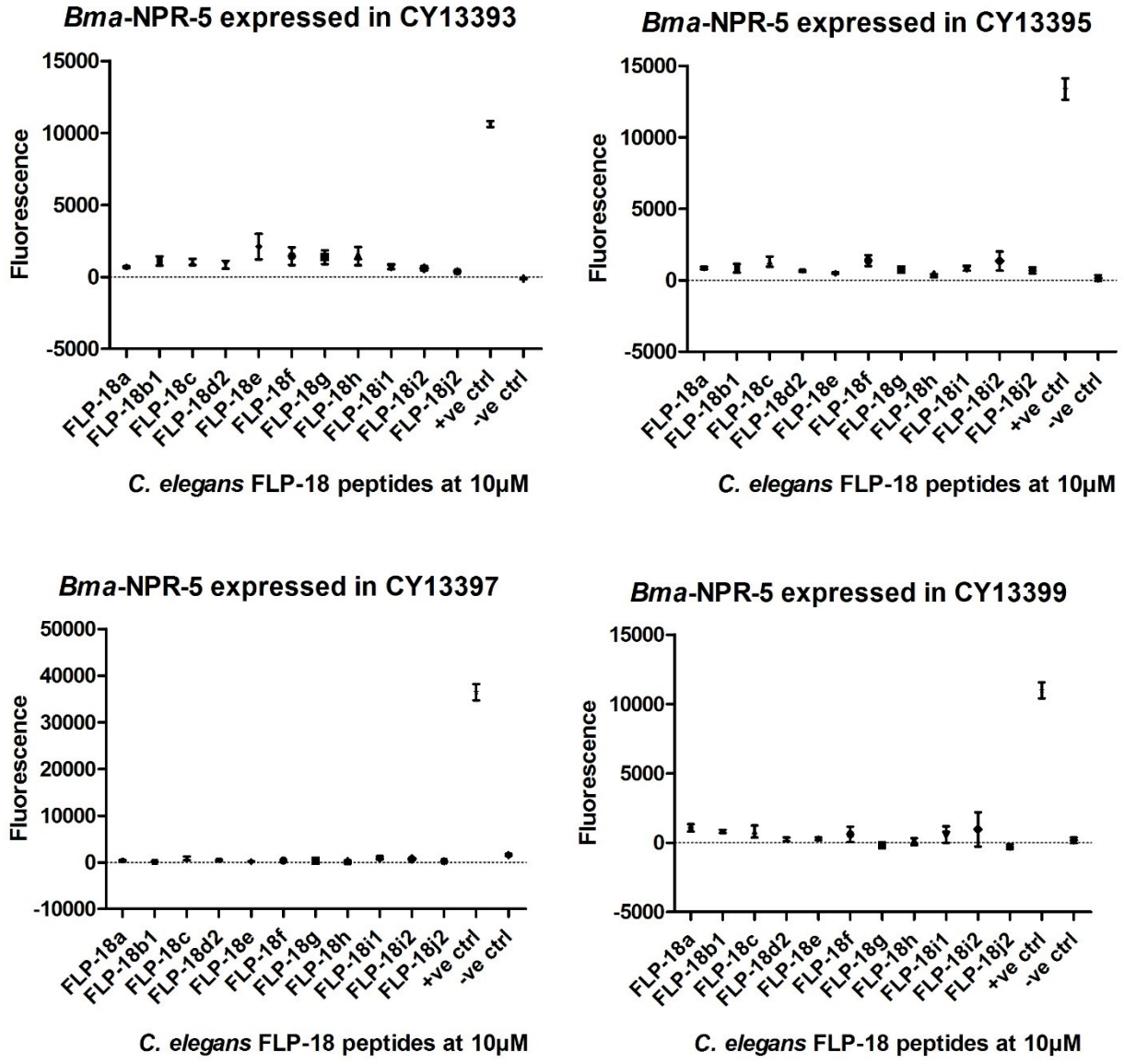


Figure 23. Efficacy of FLP-18 peptides for Bma-NPR-5 expressed in different strains of S. cerevisiae containing Gαi, Gα12, Gαq or Gαs.

V. Discussion

5.1. *Bma*-NPR-4 and *Bma*-NPR-5 have comparable structure-activity profiles

Bma-NPR-4 and *Bma*-NPR-5 functionally expressed in CHO cells were both activated by *C. elegans* FLP-18 peptides, suggesting that the ligand-receptor recognition features of these receptors are conserved among nematodes (Blaxter et al., 1998; Geary et al., 1999). Only minor differences in potency and efficacy were observed among peptides, with EC_{50} values of 4 nM – 0.3 µM for *Bma*-NPR-4 and 0.3 µM – 2 µM for *Bma*-NPR-5. All peptides share a common PGVLRF-amide motif at the C-terminus, suggesting that variations in amino acids at the N-terminus preceding PGVLRFamide only have minor roles in interacting with the peptide-binding pockets of the receptors. The results from *Bma*-NPR-4 are comparable to previous findings by Anderson et al. (2014), in which *Bma*-NPR-4 was heterologously expressed in the mammalian cell line HEK293-T. In their system, receptor activation leads to the inhibition of cAMP production, which was quantified using dual-luciferase assays. More specifically, they reported that both SEVPGVLRF-amide (FLP-18e) and EMPGVLRF-amide (FLP-18b1) elicited maximal responses with EC_{50} values of 6 nM and 2 nM. We found a similar EC_{50} for SEVPGVLRF-amide (FLP-18e), though the potency of EMPGVLRF-amide (FLP-18b1) was lower in our assay (EC_{50} = 0.2 µM). Variations may be due to the use of different expression systems and reporters for the readout. They also reported that *Bma*-NPR-4 activation signals via $G\alpha_i$, which was confirmed in our studies using the yeast system.

In the pharmacological characterization of the receptors, analogues derived from $D^1V^2P^3G^4V^5L^6R^7F^8$-amide, including truncated peptides and an alanine scan series, we observed that the tetrapeptide motif VLRF-amide is the shortest sequence that can

activate *Bma*-NPR-4 with full efficacy. Replacing V^5 or L^6 with alanine decreased efficacy and potency drastically; R^7 or F^8 modification or C-terminal amide deletion abolished peptide activation of the receptor, indicating that the VLRF-amide motif is essential for agonism. Similar observations were made with *Bma*-NPR-5, and in addition to VLRF-amide, the P^3 and G^4 residues are also important for activation of this receptor. While alanine substitution or deletion at P^3 and G^4 did not alter *Bma*-NPR-4 activation, modifying P^3 lowered efficacy in activating *Bma*-NPR-5. Moreover, PGVLRF-amide is the minimal peptide fragment that generated full efficacy in activating *Bma*-NPR-5, and shorter sequences had significantly lower efficacy and potency. Compared to C-terminal modification, which greatly reduced the response of *Bma*-NPR-4 and *Bma*-NPR-5, N-terminal modifications had only minor effects. Replacing D^1 with alanine did not alter *Bma*-NPR-5 activity, and slightly increased peptide potency against *Bma*-NPR-4, possibly due to the elimination of the charged side carboxyl group, resulting in easier access to the peptide-binding pocket on the receptor. Electrostatic interactions between D^1 and R^7 can form a transient loop, which may also affect peptide activity (Dossey et al., 2006). The conservation of the PGVLRF-amide fragment among all FLP-18 peptides is consistent with its essential role in receptor activation (Bowman et al., 2002).

The extent of *Bma*-NPR-5 activation in response to FLP-18 peptides, reflected by the level of emitted bioluminescence, was lower compared to *Bma*-NPR-4 activation. A possible explanation is that the FLP-18 peptides are intrinsically more efficacious against *Bma*-NPR-4 than against *Bma*-NPR-5. However, it is worth noting that bioluminescence levels depend on transfection efficiency, i.e., the number of receptors expressed on the cell surface, as well as the efficiency of receptor-Gα coupling. This is an alternative

explanation for why *Bma*-NPR-5 generated lower bioluminescence signals in response to FLP-18 peptides, compared to *Bma*-NPR-4.

These observations highlight the similarities between *Bma*-NPR-4 and *Bma*-NPR-5. Their peptide-binding pockets likely share similar conformational and biochemical properties, allowing both receptors to recognise the (PG)VLRF-amide fragment of FLP-18 peptides. On the other hand, differences in how the two receptors interact with FLP-18 peptides and their derivatives were demonstrated, which may contribute to differential recognition of neuropeptide ligands and subsequent functional differences.

5.2. The structure-activity relationship profiles of *Bma*-NPR-4 and *Bma*-NPR-5 are comparable to their *C. elegans* homologues

The deorphanization of *C. elegans* GPCRs has shown that both NPR-4 and NPR-5 are activated by FLP-18 peptides (Cohen et al., 2009; Kubiak et al., 2008). Cohen et al. heterologously expressed NPR-4 and NPR-5 in *Xenopus laevis* oocytes. They observed that EMPGVLRF-amide and SEVPGVLRF-amide were the most potent peptides against NPR-4, while EIPGVLRF-amide was the least potent. On the other hand, NPR-5 was activated by EIPGVLRF-amide and DVPGVLRF-amide with the highest potency, while DFDGAMPGVLRF-amide was the least potent. Kubiak et al. (2008) studied FLP-18 peptides and NPR-5 using a CHO cell expression system, and reported similar results with minor differences among the EC_{50} values.

In our study of *Bma*-NPR-4 activation, we observed that SEVPGVLRF-amide and EIPGVLRF-amide were the most and least potent peptides, respectively, suggesting high conservation of ligand recognition features between *C. elegans* NPR-4 and *Bma*-NPR-4. However, unlike *C. elegans* NPR-5, *Bma*-NPR-5 is activated by DFDGAMPGVLRF-

amide with the highest potency, while EIPGVLRF-amide and DVPGVLRF-amide were the least potent.

Previous SAR studies of FLP-18 analogues with *C. elegans* NPR-4 and NPR-5 were carried out by Dr. Ruiz-Lancheros (unpublished results), who heterologously expressed the two receptors in *S. cerevisiae*. Though EC_{50} values vary between *C. elegans* NPRs and *Bma*-NPRs, a comparable rank order of potency is maintained. In both *C. elegans* NPR-4 and NPR-5, N-terminal truncations and amino acid modifications from the N terminus towards the C terminus of DVPGVLRF-amide resulted in progressive decreases in potency. Alanine replacement of D^1 resulted in a slight increase in potency with both NPR-4 and NPR-5. VLRF and the C-terminal amide are essential for the activation of both *Bma*-NPR-4/-5 and *C. elegans* NPR-4/-5. The importance of C-terminal residues and the C-terminal amide is likely conserved among all FMRFamide-like peptides, not only across the phylum Nematoda, but also among many other invertebrates, suggested by similar observations with other neuropeptides from *C. elegans*, several parasitic worms and insects (Bowman et al., 2002; Geary et al., 1999; Marks et al., 1997; Omar et al., 2007; Orchard et al., 2001).

Conservation of SAR profiles of FLP-18 peptides in *Bma*-NPR-4/-5 and *C. elegans* NPR-4-/-5 validates the use of *C. elegans* as a model organism for discovery of broad-spectrum anthelmintics targeting the neuropeptidergic system. Since *C. elegans* has been well-characterised and screening studies are more feasible using free-living *C. elegans* than using parasitic species, it is important to confirm that knowledge gained from *C. elegans* studies is transferable.

Table 8 Comparison of the potency of FLP-18 peptides and FLP-18 analogues in NPR-4 and NPR-5 from B. malayi and C. elegans

Peptides		NPR-4 EC_{50} (M)		NPR-5 EC_{50}(M)	
		Bma-NPR-4	*C. elegans* NPR-4	*Bma*-NPR-5	*C. elegans* NPR-5
EMPGVLRF-amide		1.914E-007	1.2E-06 *	1.273E-06	1.88E-07 *
DFDGAMPGVLRF-amide		2.111E-008	-	3.967E-07	8.19E-07 *
SVPGVLRF-amide		7.379E-008	-	6.353E-07	1.60E-07 *
EIPGVLRF-amide		2.685E-007	-	1.621E-06	3.1E-08 *
SEVPGVLRF-amide		4.162E-009	1.1E-06 *	4.547E-07	2.99E-07 *
DVPGVLRF-amide		1.254E-007	-	1.814E-06	9.5E-08 *
FLP-18f	DVPGVLRF-amide	6.744E-08	4.8E-07 ‡	1.488E-06	1.93E-06 ‡
M1	DVPGFLRF-amide	5.271E-06	2.57E-06 ‡	2.672E-05	6.64E-06 ‡
M2	DVPGVLRF-OH	-	-	-	-
D1	PGVLRF-amide	2.798E-07	2.89E-06 ‡	9.804E-05	1.1E-05 ‡
D2	GVLRF-amide	6.760E-07	1.0E-05 ‡	8.748E-05	-
D3	VLRF-amide	9.797E-06	3.3E-06 ‡	1.143	-
A1	AVPGVLRF-amide	4.355E-08	3.2E-07 ‡	9.564E-06	1.0E-06 ‡
A2	DAPGVLRF-amide	9.749E-08	6.4E-07 ‡	1.767E-04	1.1E-05 ‡
A3	DVAGVLRF-amide	2.515E-06	8.1E-06 ‡	3.294E-04	2.0E-05 ‡
A4	DVPAVLRF-amide	1.212E-06	4.03E-06 ‡	6.005E-05	1.1E-05 ‡
A5	DVPGALRF-amide	1.921E-05	6.51E-04 ‡	2.523E-04	3.85E-05 ‡
A6	DVPGVARF-amide	4.052E-05	3.6E-05 ‡	-	-
A7	DVPGVLAF-amide	-	2.99E-05 ‡	-	7.53E-05 ‡
A8	DVPGVLRA-amide	-	-	-	-

* Cohen et al., 2009

‡ Ruiz-Lancheros and Geary, unpublished result

5.3. Expression systems affect receptor activity

Although we were unable to functionally express *Bma*-NPR-5 in *S. cerevisiae*, experiments with *Bma*-NPR-4 heterologously expressed in yeast confirmed the importance of the (PG)VLRF-amide motif for receptor activation in this system. It suggests that differences in expression systems can affect the characterisation of ligand-receptor interactions. Discrepancies of efficacies and potencies are commonly observed among different expression systems, because these systems are equipped with different

accessory factors and employ different reporters for readouts (Miret et al., 2002). For example, the concentration-response relationship of *C. elegans* FLP-18 peptides for NPR-5 has been studied in *Xenopus* oocytes, CHO cells and *S. cerevisiae*, and each study showed different EC_{50} values (Cohen et al., 2009; Kubiak et al., 2008; Ruiz-Lancheros and Geary, unpublished observation).

The aequorin-based Ca^{2+} bioluminescence assay has several advantages over the Alamar Blue-based yeast proliferation assay. First, the bioluminescence assay measures Ca^{2+} concentration, which results in higher sensitivity and a larger dynamic range compared to the Alamar Blue assay. In addition, the Ca^{2+} bioluminescence assay has an instant readout following compound addition; without any incubation time, there is less chance for contamination. Higher risk of contamination was observed in the yeast proliferation assay, due to its long incubation time of 44 – 48 hr. Moreover, several variable factors were accounted for and minimized in the aequorin-based Ca^{2+} bioluminescence assay, ensuring that the adjusted data reflect solely the effect of receptor activation by the ligands. For instance, the cell count was normalised against total Ca^{2+} for each well, but this was not achievable in the yeast proliferation assay, in which the initial cell number per well cannot be normalised, because cell count is the varying factor and the final read-out. Greater variation may especially exist between biological replicates, as the cell dilution processes were carried out separately for each biological replicate. The growth rate of yeast is also affected by variations in growth media sources (Saghbini et al., 2001).

However, the maintenance of mammalian cell lines is more labour intensive and has higher technical requirements in the lab setup. Culturing mammalian cells needs very

specific conditions, including a constant temperature, CO_2 level, humidity and a sterile working environment to avoid contamination. Transfection reagents and the bioluminescent substrates are expensive. In addition, this Ca^{2+} bioluminescence assay utilises a microplate reader equipped with automated liquid handling and simultaneous luminescence measurements. The yeast assay, on the other hand, is relatively easy to carry out. The Alamar Blue can provide fluorescence as well as colorimetric indication. One driving force for using the yeast assay is for local drug discovery in Africa. African scientists have access to rich biodiversity of natural products, while at the same time somewhat limited resources for research (Geary et al., 2012). Local drug discovery programs can adapt the yeast-based assay as an initial screen to search for compounds with activity against nematode GPCRs. Even without a plate reader to quantify yeast cell growth, it is possible to make qualitative observations based on the colorimetric property of Alamar Blue. Those initial hits can be verified and further studied in collaboration with other laboratories, using other expression systems. This provides a more sustainable strategy for combating helminthiases in endemic regions and a new approach for local anthelmintic discovery. The strategy of using the yeast-based system to study receptor activity and to search for compounds with potential therapeutic effects, complemented by using mammalian cell expression system for confirmation, is not limited to anthelmintic discovery; it has also been proposed in various studies for investigating human GPCRs and the treatment of human disorders (Ficociello et al., 2018; Ladds et al., 2003).

VI. Conclusion

In this study, we gained insights into the activation of *Bma*-NPR-4 and *Bma*-NPR-5 in response to neuropeptide ligands *in vitro* using two different heterologous expression systems. Expressed in CHO cells, *Bma*-NPR-4 and *Bma*-NPR-5 can both be activated by the same peptides encoded on the *flp-18* precursor gene from *C. elegans*. The two receptors exhibit differential preferences for FLP-18 peptides, suggesting that they may have different physiological functions in the parasite *B. malayi*. Further structure-activity relationship analysis indicates that the (PG)VLRF-amide motif of FLP-18 peptides is essential for agonism, as its truncation or modification diminishes receptor activation. The importance of C-terminal amino acids and amidation of FLP-18 peptides confirms previous studies on *C. elegans* NPR-4 and NPR-5. We postulate that the FLP family of neuropeptides and their receptors are conserved across the phylum Nematoda; thus, studies carried out using *C. elegans* can likely be extrapolated to other parasitic species.

Only *Bma*-NPR-4 was functionally expressed in *S. cerevisiae*, and we observed more variability and decreased sensitivity in response to FLP-18 peptides. However, the SAR studies showed consistent results, confirming the essential role of the (PG)VLRF-amide motif in receptor activation. The yeast expression system provides a low-cost alternative for receptor characterisation and discovery of non-peptide ligands in regions with limited resources. The knowledge gained from characterising *Bma*-NPR-4 and *Bma*-NPR-5 can contribute to drug development processes in combating parasitic infections.

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