Nematode neuropeptide receptors and their development as anthelmintic screens

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SUMMARY

This review addresses the potential use of neuropeptide receptors for the discovery of anthelmintic agents, and particularly for the identification of non-peptide ligands. It outlines which nematode neuropeptides are known and have been characterized, the published information on drug discovery around these targets, information about existing high- and low-throughput screening systems and finally the likely safety of neuropeptide mimetics.

Key words: Nematodes, anthelmintic, discovery, FMRFamide, G protein-coupled receptors.

INTRODUCTION

The control of parasitic organisms in plants and mammals is limited to very few available drugs. Routine use of these drugs has lead to widespread resistance within the invertebrate population (Sangster, 2001). Therefore, the need for discovery of novel drugs with new modes of action, invertebrate selectivity and environmental safety has increased in urgency. An ideal drug target should have several key attributes. Firstly, it must be essential for the function of the parasite. It must be a non-redundant target, i.e. if it is knocked out, there must be no 'back-up' system that can perform the same function. It should be unique to the parasite (not present in the host), or at least sufficiently different from the host homologue to provide for pharmacological distinction.

The emerging paradigm for antiparasitic drug discovery in the pharmaceutical industry relies on the identification of compounds that specifically target critical parasite proteins (Geary, Thompson & Klein, 1999*a*). Neuropeptidergic systems of parasites have been proposed as useful targets for this purpose (Geary *et al*. 1995; Maule *et al*. 2002; Mousley, Marks & Maule, 2004*a*). Once the function of a neuropeptide receptor is ascertained, and that function found to be essential for the maintenance of the parasite in the host, it can be considered a target for drug discovery. Progress toward the development of screens based on nematode neuropeptide receptors is described in this review.

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MOLECULAR CHARACTERIZATION OF NEMATODE NEUROPEPTIDE G PROTEIN-COUPLED RECEPTORS (GPCRS)

Many laboratories have contributed to the discovery of neuropeptides in free-living and parasitic nematodes by direct purification and identification (Davis & Stretton, 1996; Day & Maule, 1999) and by cloning neuropeptide precursor genes (Nathoo *et al*. 2001; Kim & Li, 2004). Searches of the *C. elegans* genome with neuropeptide precursor detection algorithms has led to the identification of at least 23 *flp* genes (these encode precursors of *F*MRFamide-*l*ike *p*eptides; Kim & Li, 2004), but there are more (P. McVeigh & A. G. Maule, Queen's University Belfast, 2004, personal communication). In addition to the *flp* gene family, genome searches revealed the existence of a large family of *nlp* genes in *C. elegans*; these *n*europeptide-*l*ike *p*rotein genes encode structurally distinct peptides that putatively function in the neuromuscular system. Thirty-two *nlp* genes have been tentatively assigned in the *C. elegans* genome (Nathoo *et al*. 2001).

Assuming that neuropeptides encoded on a given precursor gene act at common G protein-coupled receptors (GPCRs) (Bowman *et al*. 2002), one could reasonably expect to find ~60 neuropeptide GPCR–encoding genes in the *C. elegans* genome. An initial survey of this database led to the annotation of 54 neuropeptide GPCRs, 18 biogenic amine GPCRs, 4 glutamate GPCRs and 3 GABA GPCRs (Bargmann, 1998). It has become apparent that many of the *C. elegans* neuropeptide GPCRs occur in differentially spliced forms (Lowery *et al*. 2003; Komuniecki *et al*. 2004), but the functional consequences of this variation have not been defined.

The first report of functional expression of a nematode GPCR appeared in 1997; a *C. elegans*

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5-HT receptor was expressed in murine Ltk$^-$ cells, as detected by [^{125}I]LSD binding and 5-HT-mediated suppression of forskolin stimulation of cAMP levels in transfected cells (Olde & McCombie, 1997). Subsequently, a variety of nematode GPCRs activated by non-neuropeptide neurotransmitters have been functionally expressed in insect cells, mammalian cells or *Xenopus laevis* oocytes (Komuniecki *et al*. 2004 for review).

In contrast, functional expression of *C. elegans* neuropeptide GPCRs in typical receptor expression systems was more problematic (unpublished observations). To our knowledge, functional expression of the NPR-1 GPCR (C39E6.6) was first obtained in transiently transfected CHO cells after including a temperature-shift step (37 to 28 °C) in the protocol (Kubiak *et al*. 2003*a*; Geary & Kubiak, 2005). Receptor activation, detected by measuring neuropeptide-stimulated increases in binding of [^{35}S]GTPγS, revealed that a peptide encoded on *flp-21* (GLGPRPLRF-NH_2, also known from *Ascaris suum* as AF9) was a potent agonist of NPR-1. That pertussin toxin ablated the [^{35}S]GTPγS response suggests that NPR-1 coupled to G_o/G_i in these cells. Additional evidence for receptor expression was obtained in experiments that measured binding of [^{125}I]YGLGPRPLRF-NH_2 to membranes from transfected CHO cells. The only known behavior associated with NPR-1 in *C. elegans* is regulation of feeding behaviour. Worms expressing NPR-1 with valine at position V215, feed in isolation, whereas individuals expressing the F215 variant clump during feeding even in the presence of abundant *E. coli* (de Bono & Bargmann, 1998). The activity of GLGPRPLRF-NH_2 was much more profound in the V215 (solitary) allele than in the F215 variant, with EC_{50} values for activation of 2·5 vs. 60 nm, respectively.

Generally, similar results were obtained for NPR-1 expressed in *Xenopus laevis* oocytes (Rogers *et al*. 2003). Oocytes are maintained at 19 °C, which may permit the same receptor processing observed when temperatures were lowered for CHO cells as described above. Expression was detected by coupling G_o/G_i activation to opening of inwardly rectifying K^+ channels (GIRK1 and GIRK2). GLGPRP-LRF-NH_2 was a more effective agonist against the V215 receptor than the F215 variant. In addition, the V215 receptor of the NPR-1 receptor was activated by peptides on the *flp-18* precursor (-PPGVLRF-NH_2), though they were considerably less efficacious than the *flp-21* peptide.

In the same series of experiments, the NPR-1 receptor was ectopically expressed in the *C. elegans* pharynx, permitting electrophysiological analysis in an endogenous tissue (Rogers *et al*. 2003). In this case, both *flp-21* and *flp-18* peptides activated both alleles of the receptor with similar potency and efficacy; with signalling appearing to be mediated through $G\alpha_q$. This result underscores the context-dependent pharmacology of GPCRs expressed in heterologous systems, which must be taken into consideration when interpreting data from high-throughput screens.

A temperature shift was also necessary to achieve functional expression of the *C. elegans* C10C6.2 GPCR in transiently transfected CHO cells (Kubiak *et al*. 2003*b*). As detected by measuring increases in [^{35}S]GTPγS binding to CHO cell membranes, peptides encoded on the *flp-15* precursor (e.g. GGPQGPLRF-NH_2 and related peptides) were matched to this receptor. Based on experiments with pertussis toxin, signalling appeared to be through $G\alpha_{o/i}$. Interestingly, an insect FaRP, GNSFLRF-NH_2, also activated the receptor, though it was about 10-fold less potent than the nematode peptides. The biology of neither the C10C6.2 GPCR nor *flp-15* has been determined in *C. elegans*.

An additional report of a match of the *C. elegans* C26F1.6 peptide GPCR with a peptide ligand was achieved in transiently transfected HEK293 cells (Mertens *et al*. 2004) co-transfected with the promiscuous G protein, $G\alpha_{16}$. A fluorescence assay that measures Ca^{2+} flux was used to demonstrate that peptides encoded on the *flp-7* (TPMQRSSMVRF-NH_2) and *flp-11* (AMRNALVRF-NH_2) precursors activated the receptor. Structurally related peptides encoded on the same precursors were ineffective, presenting an unusual structure-activity relationship. Both active peptides had EC_{50} values $>1\ \mu$M, which is not very potent. It is possible that this reflects the heterologous expression system or the presence of a non-nematode G protein. As for the C10C6.2 – *flp-15* system, little is known about the biology of the C26F1.6 receptor or the *flp-7*/*flp-11* precursors.

A patent describing expression of nematode GPCRs and additional matched FaRP ligands has been granted (Lowery *et al*. 2003). A summary of the matched receptors is shown in Table 1.

An initial large-scale RNA interference (RNAi) survey of the *C. elegans* genome revealed few phenotypes associated with peptide GPCR suppression (Kamath *et al*. 2003). Notably, interference with one of the 'tachykinin-like' neuropeptide GPCRs, AC7.1, produced a 'sick' phenotype. A subsequent targeted analysis of 60 putative neurotransmitter and neuropeptide GPCRs (Keating *et al*. 2003) identified 13 with phenotypic consequences (uncoordinated movement or changes in egg-laying) after RNAi. It should be noted that most of these were not detected in the previous screen. The most profound *unc* responses were found with RNAi for the NPY-like receptor C10C6.2 and the F59D12.1 receptor, which is of less certain phylogeny. Less marked effects were found in worms subjected to RNAi for the peptide GPCRs T05A.1 and T02E9.1, two dopamine GPCRs and a muscarinic cholinergic GPCR. Interestingly,

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Table 1. Summary of matched *C. elegans* neuropeptide GPCRs

Designation	Close Invertebrate GPCRs[1]	Ligands[2]	Reference
C39E6.6	CeT05A1·1a/b/−84 DmCG7395 (NPFR)/−46	*flp-21*	Kubiak *et al*. 2003*a*
C10C6.2	CeC39E6.6/−43 DmCG7395/−39	*flp-15*	Kubiak *et al*. 2003*b*
C26F1.6	CeT19F4.1/−41 DmCG2114/−34	*flp-7*/−*11*	Mertens *et al*. 2004
C16D6.2	CeZC412.1/−43 CeC39E6.6/−43 DmCG7395/−41	*flp-18*	Lowery *et al*. 2003
C25G6.5	CeT22D1.12/−52 DmCG1147/−49	*flp-21*	Lowery *et al*. 2003
C53C7.1	CeC16D6.2/−74	*flp-3*	Lowery *et al*. 2003
F41E7.3	CeC39E6.6/−46 DmCG7395/−62	*flp-21* SchistoFLRFamide[3]	Lowery *et al*. 2003
Y58G8A.4	CeZC412.1/−59 DmCG7395/−48	*flp-18*	Lowery *et al*. 2003

[1] Based on BLAST analysis in WormBase (*www.wormbase.org*). Numerical value is exponential value of the similarity comparison.
[2] *C. elegans* precursor gene on which the best-known ligands are encoded.
[3] A FaRP purified from the locust, *Schistocerca gregaria*, PDVDHVFLRF-NH_2.

RNAi for the AC7.1 receptor led to statistically insignificant effects on motility, in contrast to the earlier survey, though the worms appeared mildly sluggish.

Egg-laying (and consequently brood size) was increased in the presence of double-stranded RNA (dsRNA) for an NPY-like GPCR (F35G8.1), a 5-HT GPCR and a GPCR related to a *Drosophila* FMRFamide receptor (C26F1.6), whereas these parameters were decreased following RNAi for three NPY-like GPCRs (C16D6.2, C25G6.5 and F41E7.3).

Although RNAi experiments fail to identify phenotypes for the majority of putative *C. elegans* peptide GPCRs, physiological experiments have shown most of the FaRPs to have potent and profound effects on nematode neuromuscular systems (Maule *et al*. 2002). This discrepancy suggests that agonists rather than antagonists at peptide GPCRs will generally be needed as anthelmintics. Historically, neuroactive anthelmintics are almost all agonists, so this condition is not without precedent. In other peptide GPCR discovery exercises, non-peptide antagonists are discovered much more often than agonists (Beeley, 2000). This consideration must be factored into discovery programmes based on screening with nematode GPCRs.

SCREENING FOR AGENTS THAT ACT AT NEUROPEPTIDE G PROTEIN-COUPLED RECEPTORS (GPCRS): STATUS REPORT

The drive to discover non-peptide ligands for neuropeptide receptors was markedly stimulated by the realisation that opiates (such as morphine and codeine) are agonists at receptors for mammalian neuropeptides in the enkephalin/endorphin family (Lord *et al*. 1977). The first directed screening exercise to target a neuropeptide receptor was developed to discover non-peptide ligands for the mammalian cholecystokinin (CCK) receptor (Evans *et al*. 1988). Following its success, identification of non-peptide mimetics of neuropeptides has become well known in human drug discovery, with such compounds identified by random file screening and directed design. Non-peptide ligands have been discovered for a wide range of neuropeptide receptors, including those for angiotensin II, endothelin, bradykinin, neurokinin, vasopressin, cholecystokinin and substance P (Giannis & Kolter, 1993; Pettibone & Freidinger, 1997). These include both agonists and antagonists, though, as noted, the latter are over-represented among leads found in screening exercises. This background encouraged the development of a programme designed to discover non-peptide ligands for parasite neuropeptide GPCRs.

Although non-peptide ligands have not yet been developed for invertebrate neuropeptide receptors, efforts have been made in insecticide discovery. The non-peptide benzethonium chloride was noted to have some structural similarity to the locust FaRP SchistoFLRFamide (Lange *et al*. 1995; Nachman *et al*. 1996). This compound bound to the SchistoFLRFamide receptor and acted as a SchistoFLRFamide agonist in insect tissue preparations with reasonable potency, signalling through an arachidonic acid pathway like the native peptide. Benzethonium chloride analogues have not been further developed, but these data demonstrate that it is possible to identify non-peptide agonists for invertebrate neuropeptide receptors. In an alternative approach, the

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USDA Agricultural Research Service have developed stable versions of sulfakinin neuropeptides that include 'effective and stable mimics of a critical but unstable portion' the Tyr(SO_3H) moiety, which was replaced by the aliphatic diamino acid α-aminosuberic acid. The stable peptidomimetics inhibit the feeding response in a number of insects (Nachman *et al*. 2005). This approach could also be applied to designing biologically active peptide analogues for nematode neuropeptide receptors.

The only reported discovery exercise based on identifying non-peptide ligands for nematode peptide GPCRs is a screen for compounds that displace [^{125}I]AF2 (KHEYLRF-NH_2) from binding sites in *A. suum* muscle membranes (Lee *et al*. 1999). Results of biochemical studies suggest that the AF2 receptor is a GPCR (Kubiak *et al*. 2003*c*). None of the hits from this screen has yet been developed to commercial use.

HIGH- AND LOW-THROUGHPUT SCREENING SYSTEMS

Many test systems have been developed to identify the function of neuropeptides in nematode species. Such systems can also be used to test potential non-peptide agonists or antagonists of peptide receptors. These studies can be carried out using whole organisms or selected tissues. Application of the isolated peptide or non-peptide ligand to whole organisms allows observation of phenotypic or behavioural responses on many parts of a life cycle, including egg laying, larval development, adult motility or responses of isolated tissues.

Injection of neuropeptides into the pseudocoelom has demonstrated behavioural effects on posture and locomotion in *A. suum* (Davis & Stretton, 2001), and body waveforms and cAMP production in *A. suum* and *C. elegans* (Reinitz *et al*. 2000). Studies have also shown that neuropeptides have effects on feeding and reproduction in nematodes by modulation of pharyngeal pumping (Brownlee *et al*. 1995) and ovijector activity (Fellowes *et al*. 2000; Marks *et al*. 1999*a*). Neuropeptide effects on the social feeding behaviour have also been demonstrated in *C. elegans* (Kubiak *et al*. 2003*a*; Rogers *et al*. 2003). *A. suum* appears to be the organism of choice for partial tissue studies on nematodes. Using isolated dorsal and ventral somatic body wall tissue, muscle tension studies can be used to demonstrate the excitatory, inhibitory or more complex effects of a peptide (Maule *et al*. 1995; Trim *et al*. 1997, 1998; Marks *et al*. 1999*b*).

One method for investigating the role of neuropeptides and mimetics thereof is observation of their effects on whole organisms and tissue preparations. If appropriate pharmacological tools are available, the mode of action of a molecule can be determined. However, this may be hampered by breakdown of the test molecule (peptide/compound) by peptidases, therefore masking activity. These assays are time consuming, can only be used to test small numbers of molecules and require large quantities of peptide or compound. As a screening tool, these systems are more appropriate for functional investigations for the effects of the test molecule.

Ligand binding assays, such as that mentioned previously, using *A. suum* membranes to screen for displacers of AF2 provide a useful format for investigating pharmacology, or to screen using competitive inhibition at the receptor. However, the need to use membrane preparations from parasite species renders the screens relatively time-consuming and difficult to run. The signal may be partially masked by non-specific binding to the large quantities of proteins and other factors present in the crude preparations and there are limits to the number of compounds that can be screened based on a limited membrane supply. The discovery exercise based on identifying small molecule ligands for nematode peptide GPCRs mentioned above did not involve recombinant material. Instead, the programme screened for non-peptide compounds that displaced [^{125}I]AF2 (KHEYLRF-NH_2) from binding sites in *A. suum* muscle membranes (Lee *et al*. 1999), and potent small molecules were identified (though, as noted, these have not progressed to the market).

Advances in chemical, biochemical and genetic modification techniques have provided the tools to investigate the binding of neuropeptides to their receptors in a format that can be optimised into high throughput. This is essential for the screening of large libraries of compounds against a single target. Isolation and cloning of GPCRs and ion channels and subsequent transformation into cell systems, yeast or bacteria allows the development of *in vitro* screening assays in 96, 384 or even 1536 well plates. These cell systems can link the activation of the GPCR to pathways resulting in a measurable endpoint, e.g. proliferation, growth, bioluminescence (Stables *et al*. 1997) or enzyme production (Kuroda *et al*. 1999). This type of system is used routinely in drug discovery for GPCR targets, screening thousands or even millions of compounds per target. Adapting nematode GPCRs to such systems is a necessary prelude to performing state-of-the-art high throughput screening (HTS).

Since the sequencing of the *C. elegans* genome, cDNAs encoding many neuropeptide GPCRs have been cloned, transfected and expressed in a variety of systems (Lowery *et al*. 2003), as described earlier in the paper. However, the function of most of these GPCRs has not been reported and their de-orphanisation using peptide libraries has only begun. Difficulty in achieving functional expression of nematode GPCRs in mammalian systems has made this task far from straightforward (Geary & Kubiak, 2005).

WILL NEMATODE NEUROPEPTIDES OFFER BROAD-SPECTRUM ACTIVITY?

While it may be possible to develop agents that act against single species of parasite, these would remain essentially niche products within the anthelmintic market. Given the inescapable costs of drug discovery this is not a viable situation and it is important that an agent kill all parasitic helminths. Indeed, agents that act to prevent infestation by both endo- and ectoparasites are desirable, as with the macrocylic lactones (e.g. ivermectin, doramectin, moxidectin and milbemycin oxime). Fortunately, while some of these neuropeptides are unique to particular species, some show cross phyla activity. Marks *et al*. (1997) showed excitatory effects of nematode neuropeptides in the flatworm *Fasciola hepatica*. Arthropod neuropeptides have also been shown to have effects in *A. suum* somatic muscle (Maule *et al*. 1996; Mousley *et al*. 2004*b*) and ovijector (Mousley *et al*. 2004*b*). Peptides like these, once matched to their receptors, may lead to the discovery of a drug with broad-spectrum activity.

SAFETY AND SELECTIVITY OF INVERTEBRATE NEUROPEPTIDES AS ANTIPARASITIC AGENTS

When FaRPs and FaRP receptors were first advanced as antiparasitic drug targets, it was proposed that the absence of close homologues of these neuropeptides from mammals would limit selectivity concerns over their use in animals. This assertion was based on comparisons between mammalian peptides and the invertebrate FaRP motif (aromatic)-(aliphatic)-Arg-Phe-NH_2 (Geary *et al*. 1999*b*). However, peptides closely resembling FaRPs have been found in vertebrates (Dockray, Sault & Holmes, 1986; Hinuma *et al*. 2000), and several mammalian peptides such as neuropeptide Y and PYY have structurally similar motifs. Subsequent work has confirmed that invertebrate FaRPs are active in some mammalian tissues. Studies using anti-FMRFamide antibodies demonstrated immunoreactivity in a wide range of mammalian tissues, including the brain (Yang *et al*. 1985), spinal cord (Majane, Casanova & Yang, 1988) and intestine (Feher & Burnstock, 1989; Kubben, van Assche & Bosman, 1986), and also in chicken brain (Dockray, Sault & Holmes, 1986). Yang used FMRF-NH_2 immunoreactivity to isolate two bovine neuropeptides that fall into the FaRP family, NPFF (FLFQPQRF-NH_2) and NPAF (AGEGLSSPFWSLAAPQRF-NH_2). Analogues were subsequently identified in humans (Perry *et al*. 1997), mice and quail (Ukena & Tsutsui, 2001), chickens (LPLRF-NH_2 and related peptides described by Dockray *et al*. 1986), and ox gamma-melanocyte stimulating hormone (Panula, Aarnisalo & Wasowicz 1996). The human NPFF receptor, HLWAR77, has been cloned and expressed (Elshourbagy *et al*. 2000).

The mammalian neuropeptides NPFF and NPAF and their analogues have multiple functions in mammals (Panula *et al*. 1996; Elshourbagy *et al*. 2000). They have been implicated in pain modulation, with anti-opioid activity (Gouarderes *et al*. 1993). Other effects include induction of morphine abstinence in dependent rats (Malin *et al*. 1990) and control of opioid-mediated defeat analgesia following biological stress (Kavaliers & Yang, 1991). They are also involved in cardiovascular regulation (Roth *et al*. 1987) and inhibited aldosterone release from adrenal slices (Labrouche *et al*. 1998). Up-regulation of NPFF and its receptor during inflammatory hyperalgesia in rats has been described (Yang & Iadarola, 2003).

FMRF-NH_2 binds to the cloned human NPFF receptor with nanomolar affinity (Kotani *et al*. 2001). It also acts through the MERF (Met-enkephalin-Arg-Phe) non-opioid receptor (Benyhe *et al*. 1997) and opioid receptors (Zadina & Kastin, 1986). It has weak activity in some isolated rat spinal cord preparations targeting opiate dependency and spinal responses to pain, in which NPFF is potent. These assays are for systems involved in modulation of spinal responses to afferent stimulation including pain, and for opiate dependency models (Huang *et al*. 1998).

Given this, it is unsurprising that FMRFamide has been shown to have a number of physiological effects on mammals, including modulation of food intake (Robert *et al*. 1989; Dockray, 2004), antinociception in the paw pressure test in rats (Pittaway *et al*. 1987), attenuation of antinociceptive effects of MERF and morphine (Tang, Yang & Costa, 1984), increasing mean arterial blood pressure and heart rate in rats (Thiemermann *et al*. 1991), modulation of opioid tolerance or dependence (Raffa, 1991), suppression of cholecystokinin (CCK)-stimulated amylase secretion (Garry & Sorenson, 1988) and inhibition of glucose stimulated insulin release and somatostatin release from isolated rat pancreas (Sorenson, Sasek & Elde, 1984).

The only other invertebrate FaRP that has been directly studied in mammals is pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2, originally isolated from the ganglia of the mollusc *Helix aspersa*. It caused rapid dose-dependent increases in the blood pressure and heart rate of anaesthetised rats, with the blood pressure effects acting via the sympathetic nervous system, as demonstrated by α-adrenoreceptor blockade (Deigin *et al*. 1988).

The implications of these studies affect considerations of the use of neuropeptides as antiparasitic agents and the development of small molecule mimetics of the peptides. In the case of the former, the invertebrate peptides may activate mammalian receptors for related neuropeptides.

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Production of small molecule mimetics may result in less specificity for particular invertebrate receptors and broaden the spectrum, so cross-reactivity will have to be tested against known mammalian neuropeptide receptors such as those for NPFF and NPAF. This does not reduce the likelihood of identifying a neuropeptide-based antiparasitic agent, but brings the approach in line with other antiparasitic approaches, in which selectivity must be built into lead compounds to ensure safety, as opposed to the idyllic situation that compounds targeted to invertebrate neuropeptide receptors would miraculously be free of such hazards.

CONCLUSION

Identification of non-peptide mimetics of neuropeptides is well known in human drug discovery, with such compounds identified by random file screening and directed design. Non-peptide neuropeptide ligands are known for a wide range of receptors, including those for angiotensin II, endothelin, cyclophilin, integrin, bradykinin, neurokinin, vasopressin, cholecystokinin and substance P. These include both agonists and antagonists. This history suggests that identification of non-peptide agonists and antagonists of nematode neuropeptide receptors is only a matter of time and effort.

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