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G protein-coupled receptors in arthropod vectors: Omics and pharmacological approaches to elucidate ligand-receptor interactions and novel organismal functions

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Abstract

Regulation of many physiological processes in animals, certainly those controlled by neuropeptide hormones, involves G protein-coupled receptors (GPCRs). Our work focusing on endocrine regulation of diuresis and water balance in mosquitoes and ticks started in 1997 with the kinin receptor, at the dawn of the omics era. After the genomic revolution, we began work on the endocrinology of reproduction in the red imported fire ant. We will use the template of this comparative work to summarize key points about GPCRs and signaling, and emphasize the most recent developments in the pharmacology of arthropod neuropeptide GPCRs. We will discuss omics' contributions to the advancement of this field, and its influence on peptidomimetic design while emphasizing work on blood feeding arthropods.

Keywords

GPCRs; neuropeptides; tick; mosquito; blood feeding arthropods; High throughput screening; receptor modeling

Introduction

GPCRs, also known as seven transmembrane (7TM) receptors, are cell surface receptors and integral membrane proteins that transduce signals across cell membranes. The most comprehensive structural and functional database for GPCRs is GPCRdb [1]. GPCRs are classified in 5 major classes A-F [2], or according to the human classification GRAFS: Glutamate (Class C), Rhodopsin (Class A), Adhesion (Class B2), Frizzled (Class F) and Secretin (Class B1) [3], and additionally in vertebrates, Taste 2 and orphan GPCRs [4]. Because of the diversity in signals they transduce, these receptors regulate most physiological processes in metazoans. These signals are diverse: light, biogenic amines,

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

neuropeptides and peptide hormones, glutamate, lipoglycoproteins, and protons. Some GPCRs have endogenous activity without the requirement for ligand binding and others have self-proteolytic and activating properties. Upon activation, GPCRs undergo a conformational change that transduces the signal across the membrane.

The GPCR designation derives from their coupling to, and activation of intracellular trimeric G proteins (Gα, Gβ and Gγ), and the G designation for the latter refers to the **G**TP-binding property of the GTPase subunit α (guanine nucleotide binding and GTP hydrolase). The receptor interacts with the Gα subunit through TM3, TM5, TM6 and intracellular loop 2 (ICL2) and ICL3 [5]. The Gα subunit that exchanges cGDP for cGTP, separates upon receptor activation from the two other subunits, βγ (which remain bound to each other). Downstream, both (α and βγ) modulate activity of different proteins [5]. Once activated, the GPCR signal ends when the αGTPase hydrolyzes cGTP to cGDP, and αGTPase reassociates with Gβγ [5]. Four major Gα-protein families are recognized: G_s (stimulatory, increase cAMP), $G_{i/o}$ (inhibitory, decrease cAMP), $G_{q/11}$ (trigger IP_3-mediated release of Ca^{2+} from calcium stores [5]) and $G_{12/13}$ (activate RhoGTPase nucleotide exchange factors, RhoGEFs) [6]. In sum, a limited number of G protein classes link to a diverse and high number of GPCRs. All these types of Gα proteins are predicted in the *Drosophila* genome [7]. The *Drosophila* eye-specific subunit Gβe is conserved in all arthropods analyzed and most invertebrates encode two Gγ genes [8].

While the above description summarizes a generalized model for GPCR function, other signaling modalities exist, i.e. upon activation GPCRs may be phosphorylated by GPCR kinases (GRKs) and then couple to arrestins [5]; GPCRs may oligomerize [9] or heterodimerize [10]. In *Drosophila* GPCR desensitization by arrestin encoded by the *Kurtz* gene functions either at the plasma membrane (low arrestin affinity) or by internalization (high arrestin affinity) [11]. Several silkworm (*Bombyx mori*) GPCRs for neuropeptides are internalized by arrestin [12].

This review summarizes our opinion on the current state of GPCR research in entomology, with emphasis on arthropod vectors of diseases. Our opinion is based on our academic experience in arthropod endocrinology and neuropeptide receptors. Since the basics of GPCR signaling were summarized above, what follows is the contribution of “omics” to the advancement of GPCR deorphanization and characterization. In addition, the recent incorporation of computational structural biology to modelling of arthropod GPCRs is discussed. Until crystal structures from arthropod GPCRs are available, these models should accelerate efforts of insect physiologists and chemists to design more potent and stable receptor ligands. To end, work on insect kinins will exemplify the evolution of work in this area.

Omics aids GPCR discovery, functional characterization and deorphanization

We have summarized in Fig. 1 the general process of GPCR identification and functional characterization and we will use this framework to highlight work on specific arthropod GPCRs.

Identification of receptors and ligands

Kinin peptides and cognate receptors are not present in all insect species and in those where they exist, there are variations in the number of ligands and receptors. Omics and fluorescent analogues revealed in the coleopteran *Tribolium castaneum* kinin signaling is absent [13]. The tick *Ixodes scapularis* exhibits an amplification of the kinin signaling system, with 19 peptides predicted in the propeptide and four genes for cognate kinin receptors predicted, one of which we cloned (HM807526) [14]. Transcriptomics of the CNS of the triatomine *Rhodnius prolixus* supported the genome annotation of GPCRs for neuropeptides and neurotransmitters, with 62 GPCRs annotated [15]. Kinin has been detected in the brain of the kissing bug *Triatoma infestans* by nano-LC and ESI-OrbiTrap MS/MS in a comparative study supported by transcriptomics [16]. Transcriptome analyses of the southern cattle tick, *Ripicephalus microplus*, synganglion identified GPCRs, although the low depth of 454 sequencing did not allow to detect the kinin receptor in samples of ticks from Texas [17] which should be present [18], underscoring the often low expression of GPCRs as a challenge for detection. Without genomes available, GPCRs from the foreleg of another cattle tick, *Ripicephalus australis*, have been predicted from transcriptomic analyses and although no cDNAs were cloned or validated, a model was produced for a class C $GABA_B$ receptor [19]. Transcriptome analyses of the Haller's organ in the dog tick using Illumina Hi-seq only yielded two GPCR transcripts, and expression of one was verified by qRT-PCR [20]. Both of these reports acknowledge limitations in the prediction of tick GPCRs.

Insect genome databases [21,22] facilitate the reverse genetics approach for the discovery of novel neuropeptides [23] and GPCRs. Efforts by the expert community on arthropod neuropeptides are now summarized in the Database for Insect Neuropeptide Research (DINeR) [24]. The predictions of gene products from insect genome sequences complements *de novo* neuropeptide identification from tissues, followed by transcriptomic studies of specific organs or tissues (Fig. 1). This approach was applied to the discovery of locust tryptopyrokinin genes [25]. The resources mentioned above are identified as input knowledge in Fig. 1 (panels on right).

Functional characterization of GPCRs

Functional characterization of arthropod GPCRs is most often achieved through recombinant expression in mammalian cells, such as Chinese Hamster Ovary cells (CHO-K1) and human embryonic kidney cells (HEK293), or embryonic insect cell lines (*Drosophila* Schneider S2) [26] (Fig. 1, center, Processes). Functional analyses of arthropod receptors utilizes calcium mobilization assays (using bioluminescence [27] or fluorescence), or by measurements of cAMP [28]. These are G-protein dependent assays often used in academic settings for forward pharmacology and can be low-throughput or high-throughput (Fig. 1). Other G-protein-dependent or G-protein-independent assays (β-arrestin recruitment assay, receptor trafficking and label-free assays) are commercially available [29].

Omics has provided abundant sequences for similarity searches and sequence alignments that provide information to infer the critical residues of both receptors and peptides necessary for activity. From the receptor side, this is best exemplified by the discovery that a single residue distinguishes insect periviscerokinin receptors (PVKr) from PBAN receptors

[30]. Analyses of PBAN/pyrokinin (PK)/PVK receptors identified a residue in TM3 (Y125) specific for PVKr, and residue Y/T/W/L 265 in TM5, also as specific for PVKr; these residues are predicted to be involved in specific ligand recognition. We found that Y122 is present in the tick PVKr, and this was relevant to our experiments to differentiate tick pyrokinin from periviscerokinin receptors [31,32]. GPCRs for biogenic amines and most for short neuropeptides belong to the Rhodopsin-like, class A GPCRs. These GPCRs have a conserved three-amino acid residue motif, DRY or ERY, present at the cytosolic extension of TM3 region. This motif is present in tick neuropeptide GPCRs we functionally characterized for kinin, pyrokinin and periviscerokinin [18,31–33], and for serotonin receptors from tick and mosquito [34–36]. Allatostatin C in the mosquito *Aedes aegypti* inhibits juvenile hormone synthesis by inhibiting the citrate mitochondrial carrier [37]. In allatoregulatory peptides, a clear differentiation of function is associated with variations in this motif that are conserved across different orders: allatostatin C receptors feature the DRY motif; MIP/allatostatin B receptors, QRY; allatotropin receptors, DRW, and FGLa/allatostatin A receptors, DRF [38]. The above and other evidence [39] points to a potential role of the DRY motif in G protein interaction, but receptors also interact throughTM5, TM6 and intracellular loop 2 (ICL2) and ICL3 [5]. A limited number of G proteins display the specific patterns of amino acid residues (selective bar code) for receptor recognition, but different receptors recognize the G protein residues through distinct regions on the receptors [5,40].

If the specific ligand or ligand-type is known, then this constitutes a forward pharmacological approach (Fig. 1, left, Processes). Our work on characterizing the sNPF receptor from fire ants was complicated because all the canonical sNPF peptide ligands that end in the amino acid sequence “LRLRFa” (a= amide) failed to activate the receptor. We tested several *Drosophila* and mosquito sNPF peptides in both calcium mobilization and cAMP assays without success. It was not until the release of the fire ant genome and confirmation by cloning a cDNA encoding an unusual sNPYa that we succeeded: Two potential ligands predicted from the translated sequence activated the recombinant receptor by decreasing cAMP in CHO-K1 cells. Mass spectrometry of queen brain confirmed the presence of one peptide fragment [28].

Receptor deorphanization involves identifying ligands for those GPCRs that have no known activating molecule(s). This is at the core of reverse molecular pharmacology utilizing screens of candidate- or random ligands. Once a receptor is deorphanized, the physiological function of both ligand and receptor can be elucidated. A phylogenetic analysis of the *Drosophila* GPCRs and a list of *Drosophila* G proteins was recently published, where the existence of orphan receptors is acknowledged [7]. *Drosophila* continues to be the best studied insect and FlyAtlas 2 (www.flyatlas2.org) has recently been released [41]. This database provides tissue expression information with links to a number of fruit fly and malaria mosquito resources for each gene of interest, including GPCRs, and their associated literature. Despite the fact that some orthologs of insect GPCRs have not been identified in the *Drosophila* genome, the comprehensive genomic sequencing data served as the basis for novel receptor discovery in the model lepidopteran, *B. mori*. In silkworm, genome mining of neuropeptide GPCRs based on homology to those in *Drosophila* allowed the identification of contigs with partial GPCR genomic sequences. Cloning of GPCR cDNAs was followed by qRT-PCR expression analyses of tissues of interest (corpora allata and corpora cardiaca), and

receptors with highly abundant transcripts were selected for recombinant expression in mammalian cells. This yielded the characterization of the first insect allatotropin receptor [42]. This is an example of reverse pharmacology (Fig. 1, left, Processes). Homologous receptors were later characterized in other insect orders [38].

GPCRs as successful targets in the druggable genome

Arthropod GPCRs are of great interest as potential new targets for pesticide development [43–45]. Human GPCRs are the targets of about 34% of marketed therapeutic drugs [46]. Further, 50% of the marketed drugs targeting peptidergic GPCRs are small molecules [47]. Chemical validation (Fig. 2) of a potential target by a small synthetic molecule is critical to generate initial commercial interest. Current drug discovery approaches for the identification of new compounds that activate or inhibit GPCRs integrate molecular informatics, structural biology, combinatorial library design, and high-throughput screening (HTS) (Fig. 1, left). HTS involves assay development, miniaturization, and automation. For assays relying on G protein coupling, it is a common practice to manipulate the signaling pathway via use of chimeric, mutated, and/or a promiscuous G protein to allow non-Gq-coupled receptors to couple to PLC-IP_3-Ca^{2+} pathway [48]. Most work on functional characterization of arthropod GPCRs or HTS report results after testing one signaling modality (i.e. calcium or cAMP) or using $G_{\alpha 16}$ for universal signaling through the calcium cascade. This is a limitation in advancing knowledge of arthropod GPCRs because the same GPCR may signal through more than one pathway. Other GPCR technologies for HTS are described elsewhere [29,49].

Perhaps the major obstacle in the exploitation of the GPCR superfamily for insect management is that only one group of GPCRs, the tick octopamine and tyramine/octopamine receptors (biogenic amines GPCRs) are believed to be successful targets of the commercial formamidine acaricide, amitraz [50]. Gross *et al.* characterized the previously named octopamine receptor as a tyramine/octopamine receptor (TAR1) which is also activated by the amitraz metabolite BTS27271, but only in the presence of tyramine [51]. The presence of mutations associated with resistance to amitraz in another tick octopamine receptor (β-adrenergic-like) gene [52], points to the biogenic amine receptors, β-AOR and perhapsTAR1, as the likely targets of formamidines in ticks. In the lepidopteran *Bombyx mori*, α- and β-like adrenergic octopamine receptors responded differentially to the amitraz metabolite N2-(2,4-Dimethylphenyl)-N1-methyformamidine (DPMF), being DPMF more potent (at pMolar concentrations) on recombinant β-AOR in a cAMP synthesis assay [53]. Altogether, these results point to insect and tick β-AORs as targets for amitraz metabolites. In an academic setting, dopamine receptors have been targeted for these HTS approaches in both tick *Ix. scapularis* [54] and mosquitoes [43]. D1-like receptor antagonists were toxic to mosquito larvae [55]. Several vertebrate biogenic amine receptor crystal structures are available to facilitate homologous arthropod receptor modelling [1].

Omics informs computational biology in absence of crystal structures: structural modeling and design of receptor-ligand interactions

The elucidation of GPCR crystal structures has advanced drug design for specificity and potency for medical pharmacology [56,57]. The structural characterization of GPCRs has been possible by baculovirus-mediated expression in insects cells [58]. While there are no published crystal structures for arthropod GPCRs [1], the first homology model for red pigment-concentrating hormone receptor from the water flea crustacean is available [59,60]. While advances have been done in the crystallization of GPCRs, this is still a highly technically challenging field [61]. A strategy to produce abundant allatostatin C receptor protein from silkworm involved expressing both receptor and combinations of eight endogenous G proteins in the baculovirus system. Receptor co-expression in cells with a Gα4β3γ trimer produced higher protein yields, perhaps by stabilizing the receptor [62]. Selecting the optimal G proteins for co-expression may help in producing abundant GPCR protein for other receptors [62].

The lack of crystal structures for insect or tick GPCRs is a major obstacle to conducting *in silico* screens on them and for the rational design of active ligands targeting them (Fig. 1, below, center). However, an alternative approach is to predict the structure by computational modeling. Sahbaz et al. made *in silico* structure models of an allatostatin receptor from a stick insect *Carausius morosus*, and computationally docked a putative peptide ligand into the receptor [63]. They concluded the N-terminus and extracellular loops (ECL2 and ECL3) form the binding pocket. From these modeled receptor-ligand interactions they proposed binding motifs on the receptor extracellular loops and performed receptor site-directed alanine-mutagenesis accordingly. By measuring binding forces between mutant receptors and the ligand through atomic force microscopy, they experimentally validated the importance of a 5-residue motif in ECL3 in the receptor-ligand interaction [63]. Similar computational protocols including sequence and phylogeny analyses, protein structure prediction, protein-ligand docking, and sometimes ligand redesign were used in other studies for arthropod GPCRs [19,64–66]. In the absence of crystal structures these studies point to the power of computational biology, especially when combined with structure-activity relationship (SAR) experiments (including receptor mutagenesis), in studying GPCR-ligand binding and for designing ligands. Meanwhile, obstacles remain in modeling the often-flexible receptor ECLs for structure prediction or ligand docking.

Aided by the accurate peptide structure prediction and the ability of massive peptide production, the trend of small proteins as drugs has started in pharmacological drug design [67]. The European Union funded project nEUROSTRESSPEP focuses on discovery of novel biocontrol agents for insect pests from neuroendocrinology that would be harmless to pollinators. The project aims at testing in field trials rationally designed and selective peptides against arthropod pests by 2020 (http://www.neurostresspep.eu/home).

The arthropod kinin signaling system as a model of neuropeptide GPCR validation towards pesticide discovery

Invertebrate kinins, also known as “leucokinins” are multifunctional neuropeptides [68]. They are part of the neuropeptide “variable set” in insect genomes, not present in all insects [69]. We have studied the kinin signaling system utilizing chemical and reverse genetics tools for validation, as summarized in Fig. 2. Several kinin functions have emerged since their discovery as myotropins: are diuretic in dipterans [70], control pre-ecdysis movements during development, centrally regulate the size of a meal, modulate desiccation and cold tolerance [71] and olfaction. In *Ae. aegypti* three kinin peptides are encoded in a single mRNA and all three activate the receptor [72]. We recently discovered that the kinin receptor is expressed in labella and tarsi and modulates sugar taste perception in *Ae. aegypti* females, results that were unexpected considering the known involvement of the kinin receptor in mosquito diuresis [73]. Kinin signaling is crucial for tracheal clearance and air filling prior to ecdysis in *Drosophila* [74].

The selection of kinin peptides to initiate a validation pipeline of neuropeptide GPCRs as targets for arthropod control fulfilled the *a-priori* requirement of being a small peptide, i.e. the kinin core analog is 5 amino-acid residues long. This is an important criterion because small peptides may have lesser interactions with the receptor and thus, it should be easier to design or randomly screen libraries to find a small synthetic molecule [75] as ligand that may have commercial potential as lead compounds (pronounced [lēd]) (Fig. 1). The “Lipinski rule of five” which defines small molecules for therapeutic purposes (oral drugs <500 Da) [75], is often violated for many agrochemicals and antibiotics.

The cloning of the *R. microplus* kinin receptor represented the first neuropeptide GPCR cloned from the Acari and the first kinin arthropod receptor known [18]. For this, we used degenerate primers similar to the lymnokinin receptor from a snail (Fig. 1). Sequence similarity searches allowed us to predict the gene CG10626 encoded the *Drosophila* kinin receptor [18,76]. The development of CHO-K1 cell lines stably expressing the cattle tick kinin receptor and the *Ae. aegypti* kinin receptor allowed us to confirm receptor function in calcium bioluminescence assays [33,72] (Fig. 1), and perform comparative structure-activity relationship studies of core kinin analogs and alanine scans to determine critical residues for activity [77]. These studies verified that F at position 1 and W at position 4 of the core kinin pentapeptide of sequence FFSWGa are critical for activity. With this information other analogues were designed by R. Nachman. We validated a potent biostable kinin peptidomimetic “K-Aib-1” (also named 1728; contains α-**a**mino**i**so**b**utyric acid) in functional assays with recombinant kinin receptors from the tick and *Ae. aegypti* and showed it had equal or greater potency than endogenous kinins [78]. Further, this analogue showed antifeedant activity and caused mortality in aphids [79]. Subsequently, Chinese scientists have evaluated a shorter lead Aib analog [80] related to K-Aib-1 (and derivatives thereof) and found that it exhibited two-fold greater potency, underscoring the aphicidal potential for these lead compounds [81]. The K-Aib-1 analog reduced the blood meal size in *R. prolixus*, which by failing to fully engorge also exhibited a reduced rate of normal ecdysis [82]. Parallel to chemical validation of diuretic peptides in *Ae. aegypti* females, we began

validation of the kinin receptor [70] by RNAi, a reverse genetics approach (Fig. 2). Silencing of the kinin receptor reduced fluid excretion by 50% after a blood meal [70]. We discovered a novel function of the kinin receptor by chemical and genetic validation (Fig. 2). When we provided the K-Aib-1 analog in a sugar solution to starved *Ae. aegypti* females, contact of tarsi and labella with the mixture elicited their aversive behavior (ET_{50} ~ 6s). Females walked-, jumped- or flew-away after contacting the mixture [73] and they did not ingest it. The elicited aversive behaviors point to the possibility of exploiting GPCR-modulated sensory perception in mosquitoes by developing environmental or human protection compounds that are deterrent *before* the female feeds.

Conclusions

The practical exploitation of the insect and Acari (ticks and mites) “GPCRome” for pest control is still in its infancy. Genomics, proteomics and peptidomics should provide needed information on tick GPCRs and ligands for advancing endocrinology. The possible role of TAR1 as a target site for amitraz advances efforts in molecular acaricide resistance monitoring in tick populations. Loss-of-function studies of GPCRs by generating stable mutant strains with CRISPR-Cas9 technology for both receptor and ligand should accelerate discovery in insects, as shown for *Ae. aegypti* [83]. Further functional analyses of diverse sequences will provide a clearer picture of constraints, commonalities and behaviors of these receptors between vertebrates and invertebrates.

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Highlights

- Arthropod GPCRs are under-exploited targets for pest management.
- Omics contributes to functional characterization of GPCR-ligand pairs.
- Insect GPCR structural models will improve small molecule design.
- Kinin peptidomimetics reduced feeding in insect pests.

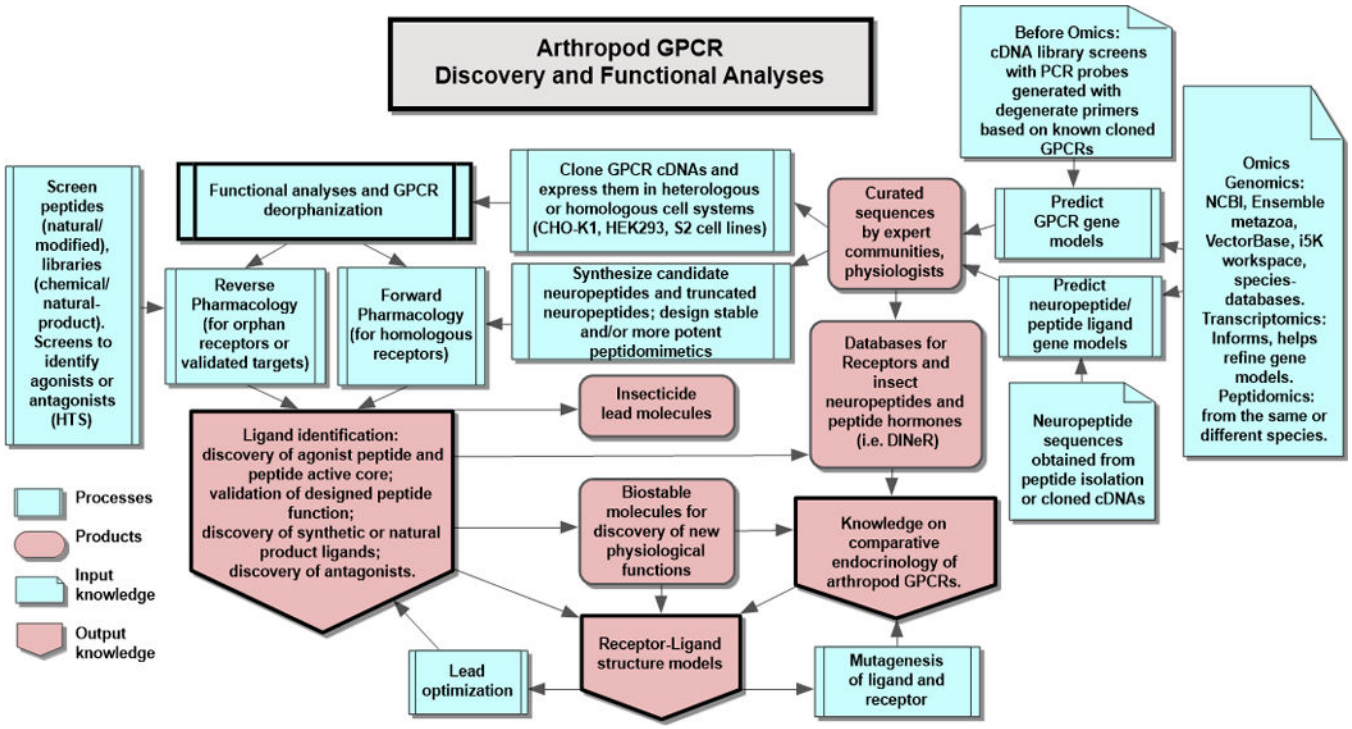

Figure 1. Omics contribution to arthropod GPCR discovery and functional analyses

Genomes, transcriptomes, peptidomes and cDNA clones (input knowledge) aid in the curation of protein sequences for both GPCRs and peptide ligands. Curated sequences are organized in databases such as the Database for Insect Neuropeptide Research (DINeR [24]). GPCRs are expressed in cell systems for verification of function. If they are highly similar to other deorphanized receptors this constitutes a forward pharmacology approach. Alternatively, they are deorphanized through a reverse-pharmacological approach to discover either endogenous or synthetic ligands or natural small molecule active ligands by high-throughput screening (HTS). Once a ligand is a “hit” on the receptor, as agonist or antagonist, bioassays are performed, or the ligand is used as a tool to probe suspected functions in tissues *(in vitro*). These processes or activities result in different products, and converge as output knowledge of new ligands, receptor function, comparative endocrinology and receptor-ligand structure models. “Ligand-docking” informs ligand (= lead) optimization, and models are improved after site-directed mutagenesis experiments.

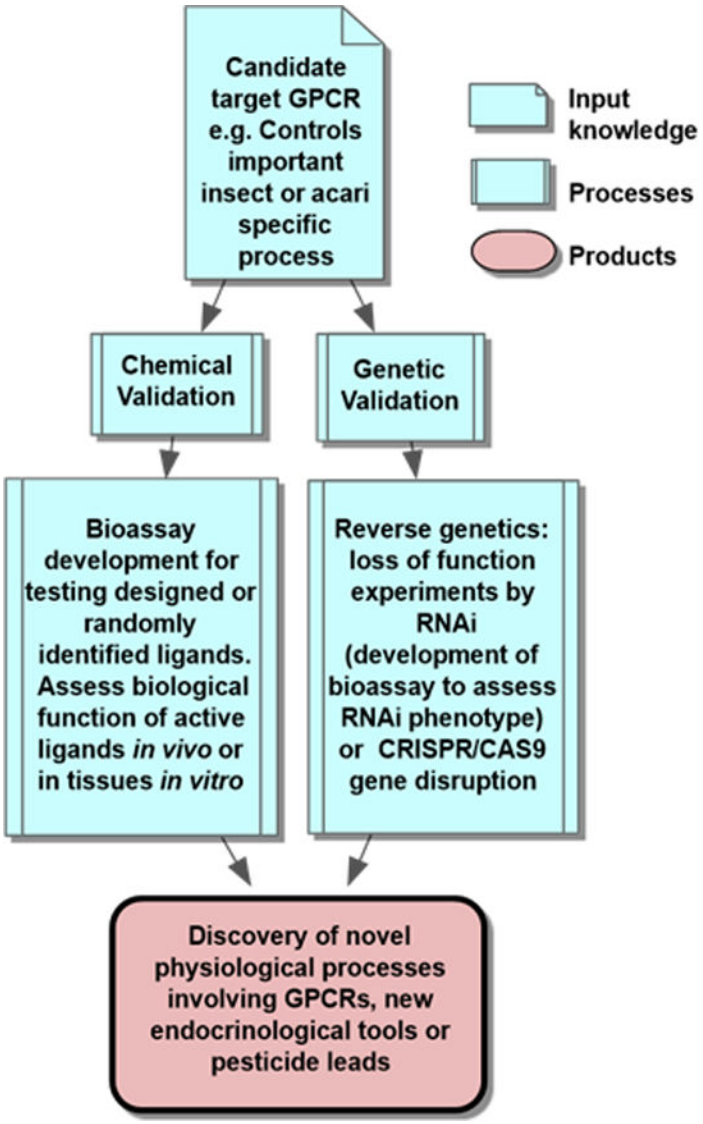

Figure 2. Key elements of GPCR target validation

For chemical validation of a GPCR as a candidate target, the designed or identified ligand that is active on the recombinant receptor is applied *in vivo* and *in vitro* in tissues. Bioactivity must be determined, either as mortality or by another adverse biological effect derived from its action as antagonist or superagonist (i.e. paralysis). For genetic validation, “loss of function” experiments most typically, or “gain of function” experiments, must confirm the disruption of receptor function has a measurable effect. Through these processes, we validated stable potent peptidomimetics for, and discovered novel functions of the kinin receptor [73].