Short neuropeptide “F” and the nutritional and reproductive status of the red imported fire ant colony

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Outline

• Invasive species with high reproductive output
• Neuropeptide control of nutrition and reproduction: the sNPF system as candidate
• Molecular, biochemical characterization of the fire ant sNPF receptor:
  - Queens: a link between nutrition and reproduction
  - Workers: The sNPF receptor in worker division of labor and sensing nutritional status
• My work: Deorphanization of the sNPF receptor
Red imported fire ant was introduced to the USA from South America in 1930 and rapidly spread throughout the southern United States.

Recently to California and other regions of the world Australia, New Zealand, Taiwan, Hong Kong, Macao and Mainland China.

Mature mounds reach ~35 cm (1 foot) in height and have no openings except just before and after a mating flight.

*Photo: Texas imported fire ant research and management project.*

- serious agricultural, medical, and urban pest
- damage to property in the United States alone is greater than $6 billion annually (Lard et al. 2006)

The most important organizing feature of an ant colony is the number of queens

- Monogynous colonies: headed by a single queen
- Polygynous colonies: headed by multiple queens

Completely associated with variation at the marker gene Gp-9, which has two alleles, ‘B’ and ‘b’

Fire ant colony organization

Reproductive
- Queen
- Male

Sterile
- Workers

According to task performance workers can be divided into nurses, foragers and reserves.

Food flow in the fire ant colony

• All the members of the colony feed by sharing the liquid food via trophallaxis

• Workers forage for liquid and solid foods

• Only 4th instar larvae can feed upon solid foods and digest protein for the colony

Liquids: sugars oils

Solid proteins

Larval stage

Fire ant colony in the laboratory

Protein and sugar source

Oil and protein source

Sugar source

Brood
Neuropeptides signaling system controls physiology

• Neuropeptides control physiological functions of insects such as feeding, locomotion, development, and reproduction

• Social organization in insects highlights the challenge of understanding the physiological functions of a “superorganism”: what genes control social behavior?

• Neuropeptide Y: the first feeding-stimulatory neuropeptide discovered in mammals (Stanley and Leibowitz 1985)

• In insects, long and short NPF have been identified as orthologues of mammalian NPY
• Insect NPF peptides are ~36 amino acid residues in length (“long”)
• Insect sNPF peptides are 6-11 amino acid residues (“short”)
Candidate gene approach for the analysis of genes involved in regulation of nutritional status

sNPF peptides have been identified in several insect species, the Colorado potato beetle, fruitfly, locust etc (Spittaels et al. 1996; Broeck 2001; Schoofs et al. 2001)

In fruit fly sNPF peptide controls food intake and regulates body size (Lee et al. 2004)

sNPF receptor has been first identified from *Drosophila* (Mertens et al. 2002)

What is the significance of sNPF in social insects and in colony nutritional status?
In invertebrates, neuropeptide F (NPF) peptides share structural similarity with vertebrate neuropeptide Y, which regulates food consumption, circadian rhythms, anxiety, and other physiological processes. The insect neuropeptide F receptors belong to the G protein-coupled receptor (GPCR) rhodopsin family. We have cloned the fire ant putative short NPF receptor using PCR and RACE methods. The complete 2,185-bp cDNA encodes a 387-residue protein with a predicted GPCR seven transmembrane region structure. We propose that the sequence of the honey bee short NPF receptor, which has not yet been annotated, encodes a protein of 393 residues. In fire ant mated queens, receptor transcripts were detected in the brain, midgut, hindgut, Malpighian tubules, fat body, and ovaries. The highest transcriptional expression was found in the brain. The downregulation of the fire ant short NPF receptor transcriptional expression in the brain with starvation suggests that the short NPF signal transduction cascade may play a role in feeding regulation in fire ant mated queens. Arch. Insect Biochem. Physiol. 61:195–208, 2006.
Short neuropeptide F receptor transcript was identified in various organs of the fire ant queen.

sNPF receptor transcript relative abundance at the central level is sensitive to changes in nutritional status

sNPF receptor transcript decreased in the starved fire ant mated queen’s brain (semi-RT PCR)

Subsequent research supports sNPF signaling system controls feeding behavior in insects: negative and positive correlations found with fed status

- In *Bombyx mori* larvae sNPF1, sNPF2 and sNPFR transcripts decrease upon starvation (Nagata et al. 2012)

- Colorado potato beetles are devoid of sNPF peptides during diapause, a period of starvation (Huybrechts et al. 2004)

- *Schistocerca gregaria* sNPF signaling plays inhibitory role in feeding (Dillen et al. 2013; 2014)

- In contrast, sNPFR is overexpressed in starved fruit flies, and cockroaches (Root et al. 2011; Mikani et al. 2012)
sNPF signaling system: linking reproductive control and nutritional status

- Copulation and competition with the potential mates need enormous energy
- Reproduction requires substantial metabolic energy for synthesis of vitellogenin for oocyte maturation
- Potential gonadotropin in locust

Is sNPF signaling related to fire ant ovary development and reproduction?

*(De Loof et al., 2001)*
Starvation causes the reduction of egg laying in the mated queens

First demonstration of sNPF receptor protein in the ovary and brain of fire ant: sNPF peptide as potential brain-ovary neurohormone

Immunolocalization of the short neuropeptide F receptor in queen brains and ovaries of the red imported fire ant (Solenopsis invicta Buren)

Hsiao-Ling Lu and Patricia V Pietrantonio

Conclusions: The analysis of sNPF receptor immunolocalization shows that the sNPF signaling cascade may be involved in diverse functions, and the sNPF peptide(s) may act in the brain as neurotransmitter(s) or neuromodulator(s), and in the ovaries as neurohormone(s). To our knowledge, this is the first report of the cellular localization of a sNPF receptor on the brain and ovaries of adult insects.
Detection of the sNPFR protein in fire ant queen brain and ovary

<table>
<thead>
<tr>
<th>KDa</th>
<th>M</th>
<th>Brain VQ</th>
<th>Brain MQ</th>
<th>Ovary VQ</th>
<th>Ovary MQ</th>
</tr>
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<tbody>
<tr>
<td>250</td>
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<td>25</td>
<td></td>
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</tbody>
</table>

VQ: vergin queen
MQ: mated queen

55.3 kDa
51.1 kDa
46.2 kDa

Lu and Pietrantonio. 2011. BMC Neuroscience 12:57
Immunolocalization of sNPFR in the fire ant queen brain

An anterior view and a posterior view of the fire ant queen brain showing the localization of sNPFR. The brain is labeled with various regions marked as C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, and a scale bar of 100 µm is present in both views.

Lu and Pietrantonio BMC Neuroscience 2011 12:57
Localization of sNPFR protein in the mated queen ovary

- Receptor signals were detected in the posterior end of the oocytes at mid-oogenesis stage (arrows).
- First GPCR found might be involved in oocyte polarity.

Oo: oocyte; Fc: Follicle cell; TC: Trophocytes; F: follicle
Our working model for endocrine control of reproduction in fire ant

Mated queen

Schematic by Dr. Pietrantonio
Nutritional status of fire ant is maintained by worker subcastes

Role of sNPF signaling in division of labor?
First demonstration of differential expression of sNPF receptor protein in the brain of workers of social insect

Differences in sNPF Receptor-Expressing Neurons in Brains of Fire Ant (Solenopsis invicta Buren) Worker Subcastes: Indicators for Division of Labor and Nutritional Status?

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Number of sNPF cells found in minor than in major workers. Those sNPF cells detected in all worker subcastes appear to be involved in olfaction or SEG functions. The differential expression of clusters in subcastes suggests that sNPFR signaling is involved in regulating behaviors associated with specific subcastes and thus, division of labor. Some sNPFR cells appear to be involved in nutrient sensing and/or brood care, feeding behavior and locomotion. In colonies without brood, workers showed a lower cluster number, and an overall reduced sNPFR signal. Our results suggest the sNPF signaling system is a candidate for the neurobiological control of worker division of labor and sensing brood presence, perhaps correlating with protein requirements and availability.
sNPFR in the brain of worker subcastes

Increase in the total number of cells expressing sNPFR

Nutritional need of the colony changes sNPFR expressing cells

Nutritional need of the colony changes sNPFR expressing cells

Nutritional need of the colony changes sNPFR expressing cells

## The type and number of immunoreactive sNPFR cell changes in worker brain

<table>
<thead>
<tr>
<th>Workers from colonies with brood</th>
<th>Workers from colonies without brood</th>
<th>Queens from colonies (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Majors</td>
<td>Majors</td>
<td>Majors</td>
</tr>
<tr>
<td>Cell N° per brain/SEG hemisphere (1)</td>
<td>Total N° of cells per brain</td>
<td>Cell N° per brain/SEG hemisphere (1)</td>
</tr>
<tr>
<td>C1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C2*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>-</td>
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</tr>
<tr>
<td>C4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C5</td>
<td>2-3</td>
<td>4-6</td>
</tr>
<tr>
<td>C6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C7*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C9*</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C12*</td>
<td>N/A</td>
<td>2-3</td>
</tr>
<tr>
<td>C13</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C15*</td>
<td>3-5</td>
<td>6-10</td>
</tr>
<tr>
<td>C16</td>
<td>N/A</td>
<td>5</td>
</tr>
<tr>
<td>Total cell number (range)</td>
<td>19-26</td>
<td>29-39</td>
</tr>
<tr>
<td>Percent change in cell N° (3)</td>
<td></td>
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</tr>
</tbody>
</table>
• Short neuropeptide F signal may play important role in the fire ant subcaste division of labor.

• The change in the expression of sNPF receptor in the fire ant workers’ brain in presence/absence of brood may be related to the nutritional need of the colony.

What activates the receptor?
Grand challenge in deorphanizing the short neuropeptide F receptor

• Pre-genome era

• sNPF ligand not known from the fire ant
Invertebrate sNPF sequences are conserved

- short neuropeptide F (sNPF) peptides are universally characterized by a LRLRFamide C-terminus

Alignment of invertebrate sNPF sequences

Mosquitoes
- Aedes aegypti_sNPF1
- Aedes aegypti_sNPF2
- Aedes aegypti_sNPF3
- Aedes aegypti_head_peptide1
- Aedes aegypti_head_peptide2
- Anopheles gambiae_sNPF1
- Anopheles gambiae_sNPF2
- Anopheles gambiae_sNPF3
- Anopheles gambiae_sNPF4
- Anopheles gambiae_sNPF5
- Drosophila melanogaster_sNPF1
- Drosophila melanogaster_sNPF1(4-11)
- Drosophila melanogaster_sNPF2
- Drosophila melanogaster_sNPF2(12-19)
- Drosophila melanogaster_sNPF3
- Drosophila melanogaster_sNPF4
- Bombyx mori_sNPF1
- Bombyx mori_sNPF2
- Bombyx mori_sNPF3
- Bombyx mori_sNPF1(4-11)
- Bombyx mori_sNPF2(3-10)
- Bombyx mori_sNPF3(4-11)

Fruit fly
- Drosophila melanogaster_sNPF1
- Drosophila melanogaster_sNPF1(4-11)
- Drosophila melanogaster_sNPF2
- Drosophila melanogaster_sNPF2(12-19)
- Drosophila melanogaster_sNPF3
- Drosophila melanogaster_sNPF4

Silkworm
- Bombyx mori_sNPF1
- Bombyx mori_sNPF2
- Bombyx mori_sNPF3
- Bombyx mori_sNPF1(4-11)
- Bombyx mori_sNPF2(3-10)
- Bombyx mori_sNPF3(4-11)

Hypothesis: sNPF receptor is Gi-protein coupled receptor

- G\(\alpha_i\) inhibits adenylyl cyclase decreasing intracellular cyclic adenosine monophosphate (cAMP)

Garczynski et al. 2007. Peptides. 28,: 109 - 118

Dillen et al. 2013. PLoS ONE 8: e53604
Expression of fire ant sNPFR in mammalian cell line (CHOK-1 cells)

Bajracharya et al. (2014) PLoS ONE
Ligands tested on the *SisNPFR-C6E8* cell line
CHO-K1 stably expressing the sNPF receptor
(Hsiao-Ling Lu’s Ph.D. work)

<table>
<thead>
<tr>
<th>Peptide ligands</th>
<th>Amino acid sequence</th>
<th>Activity ($EC_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DromesNPF1</em></td>
<td>AQ&lt;sub&gt;RS&lt;/sub&gt;P&lt;sub&gt;SP&lt;/sub&gt;S&lt;sub&gt;LR&lt;/sub&gt;RF&lt;sub&gt;a&lt;/sub&gt;</td>
<td>not active 1µM</td>
</tr>
<tr>
<td><em>DromesNPF2</em></td>
<td>WFGD&lt;sub&gt;VDNQKPIR&lt;/sub&gt;SP&lt;sub&gt;SLR&lt;/sub&gt;RF&lt;sub&gt;a&lt;/sub&gt;</td>
<td>not active 1µM</td>
</tr>
<tr>
<td><em>Drome sNPF2</em></td>
<td>SP&lt;sub&gt;SL&lt;/sub&gt;RLRF&lt;sub&gt;a&lt;/sub&gt;</td>
<td>not active 1µM</td>
</tr>
<tr>
<td>12-19</td>
<td></td>
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</tr>
</tbody>
</table>

Pre-OMICs era offered no solution
Draft genomes of six species of ants were released. 

Using the honey bee short neuropeptide F sequence we identified the sNPF gene in the fire ant genome, but............
The endogenous “sNPF propeptide” reveals a sNPY ligand!
cDNA cloning and predicted amino acid sequence

Different from the universally conserved LRLRFamide

Bajracharya et al. (2014) PLoS ONE. 9: e109590
The fire ant predicted active “sNPF” ligand is different from that of other invertebrates and different from the other ant species.

Bajracharya et al. (2014) PLoS ONE. 9: e109590
The predicted sNPY peptide is expressed in fire ants

Identification of sNPY from the fire ant eggs and larvae in MALDI-TOF MS

Bajracharya et al. (2014) PLoS ONE. 9: e109590
The predicted peptide activates the fire ant “sNPF” receptor

Endogenous sNPY decreases intracellular cAMP in CHO-K1 cells

Bajracharya et al. (2014) PLoS ONE. 9: e109590
Tyrosine (Y) at the peptide C-terminus is critical for receptor-ligand binding: Phe (F) renders fire ant peptide inactive.

Bajracharya et al. (2014) PLoS ONE. 9: e109590
### Ligands tested on the fire ant sNPF receptor

<table>
<thead>
<tr>
<th>Peptide ligands</th>
<th>Amino acid sequence</th>
<th>Activity (EC₅₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse PYY</td>
<td>AKPEAPGEDASPEELSRYYA&lt;sub&gt;a&lt;/sub&gt;SLRHYLNLVTRQRY&lt;sub&gt;a&lt;/sub&gt;</td>
<td>not active 1µM</td>
</tr>
<tr>
<td>Apime NPY</td>
<td>EPEPMARPTRPEIFTSPEELRRYIDHVSDY&lt;sub&gt;a&lt;/sub&gt;LYLSGKARY&lt;sub&gt;a&lt;/sub&gt;</td>
<td>not active 1µM</td>
</tr>
</tbody>
</table>

- **Honey bees though, encode the long NPF peptide which ends in NPY**
- It is unknown if fire ants encode a canonical long NPF peptide.

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*Bajracharya et al. (2014) PLoS ONE. 9: e109590*
Conclusions

• Endogenous sNPY ligand was identified in the fire ant

• Fire ant sNPY ligand is different from the consensus sequence xPxLRLRFamide universal among invertebrates characterized so far

• sNPF receptor is a Gi-coupled protein receptor because it decreases cAMP
The Red Imported Fire Ant (*Solenopsis invicta* Buren) Kept Y not F: Predicted sNPY Endogenous Ligands Deorphanize the Short NPF (sNPF) Receptor

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**Abstract**

Neuropeptides and their receptors play vital roles in controlling the physiology and behavior of animals. Short neuropeptide F (sNPF) signaling regulates several physiological processes in insects such as feeding, locomotion, circadian rhythm and reproduction, among others. Previously, the red imported fire ant (*Solenopsis invicta*) sNPF receptor (*S. invicta* sNPFR), a G protein-coupled receptor, was immunolocalized in queen and worker brain and queen ovaries. Differential distribution patterns of *S. invicta* sNPFR protein in fire ant worker brain were associated both with worker subcastes and with presence or absence of brood in the colony. However, the cognate ligand for this sNPFR has not been characterized and attempts to deorphanize the receptor with sNPF peptides from other insect species which ended in the canonical sequence LRLRFAamide, failed. Receptor deorphanization is an important step to understand the neuropeptide receptor downstream signaling cascade. We cloned the full length cDNA of the putative *S. invicta* sNPF prepropeptide and identified the putative “sNPF” ligand within its sequence. The peptide ends with an amidated Tyr residue whereas in other insect species sNPFs have an amidated Phe or Trp residue at the C-terminus. We stably expressed the HA-tagged *S. invicta* sNPFR in CHO-K1 cells. Two *S. invicta* sNPFs differing at their N-terminus were synthesized that equally activated the sNPFR, SLRSAALAAGHLRYa (EC$_{50}$ = 3.2 nM) and SALAAGHLRYa (EC$_{50}$ = 8.6 nM). Both peptides decreased the intracellular cAMP concentration, indicating signaling through the $G_{q/11}$-subunit. The receptor was not activated by sNPF peptides from other insect species, honey bee long NPF (NPY) or mammalian PYY. Further, a synthesized peptide otherwise identical to the fire ant sequence but in which the C-terminal amidated amino acid residue ‘Y’ was switched to ‘F’, failed to activate the sNPFR. This discovery will now allow us to investigate the function of sNPY and its cognate receptor in fire ant biology.
Future directions

1. Identify the long NPY/F from the fire ant.

2. Localize the sNPY producing cells in the fire ant queen and workers brain by *in situ* hybridization.

3. Understand the physiological role of sNPY and sNPFR in the fire ant (RNAi).
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