UNIVERSITY OF CALIFORNIA, SAN DIEGO

Investigating the neurohormonal basis of courtship behavior in the medicinal leech, *Hirudo verbana*

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of James Frederic Bratka is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2012
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The Results contain work that was done in conjunction with Dr. Krista Todd, and presented here with her permission. These data may at a later date be prepared for submission for publication.
ABSTRACT OF THE THESIS

Investigating the neurohormonal basis of courtship behavior in the medicinal leech, *Hirudo verbana*

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Master of Science in Biology

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Professor Kathleen French, Chair

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Neurohormones in the vasopressin/oxytocin family of peptides are responsible for eliciting social behaviors ranging from kin recognition (Ferguson et al., 2002) to pair-bonding (Winslow et al., 1993) and other aspects of reproductive behavior in various organisms (Donaldson and Young, 2008; Northrop and Erskine, 2008). In the medicinal leech, exogenous homologs of vasopressin, such as conopressin and annetocin, and the endogenous *Hirudo* homolog, hirudotocin (Salzet, 2007), initiate
stereotyped courtship behavior in the medicinal leech *Hirudo verbana*.

*Hirudo sp.* is a valuable model organism for investigating pattern generator networks due to its readily accessible central nervous system, relatively small number of neurons, many of which are well characterized, and similarities between mid-body ganglia (Macagno, 1980; Muller et al., 1981; Pearce and Friesen, 1984). Vasopressin analogs interact with a central pattern generator in the medicinal leech that initiates and maintains a variety of behaviors that play a role in reproduction (Wagenaar et al., 2010). Using immunohistochemistry, we determined that a vasopressin-like molecule was reliably present in specific identified neurons within the leech nervous system. Most of those cells are as yet uncharacterized, but one pair of large, well-known neurosecretory cells, the Leydig cells, showed a positive reaction in every ganglion.

In addition, to look for potential targets of this vasopressin-like molecule, we probed ganglia with antibodies that recognize receptors for mammalian vasopressin. We discovered a set of neurons that labeled positively for vasopressin receptor 1B. In future work, we will use the results to further study the neurohormonal basis of reproductive behavior.
**Introduction**

For almost 50 years, the medicinal leech (*Hirudo sp.*) has been a highly useful model organism for studying the nervous system. Leeches are annelid worms consisting of a fixed number (32) of body segments. An easily accessible nerve cord that consists of a head brain, 21 mid-body ganglia that innervate the 21 mid-body segments, and a tail brain runs through the body (Fig. 1 A, B) (Muller et al., 1981). Almost all mid-body ganglia are nearly identical to one another and consist of approximately 400 neurons that can readily be seen with light microscopy (Macagno, 1980). The fifth (G5) and sixth (G6) midbody ganglia, however, are specialized. They contain approximately 500–600 neurons and innervate the segments of the animal that control reproductive function. G5 is the male ganglion and innervates the penis and associated ducts, while G6, the female ganglion, innervates the ovaries and their associated ducts and includes neurons that extend into the male segment (Zipser, 1979; Jellies and Kristan, 1988).

Even though each adult leech contains sexually mature male and female reproductive organs, they are not self-fertilizing. Instead, fertilization is internal, requiring two leeches to cooperate if they are to line up their gonopores, which are located on the ventral side of the animal (Fig. 1A). However, leeches spend most of their time with their ventral body surface against a solid substrate, which would prevent apposition of ventral surfaces. Leeches overcome this potential impediment by executing moderately stereotyped courtship behavior, part of which is a dorsal-ventral twisting motion (“telephone-cording”) that can persist for hours until the pair has one
**Figure 1:** *Hirudo verbana* anatomy and mating. A) A full length *Hirudo verbana* showing the ventral (V) and dorsal (D) surfaces of the organism. The area where the male (♂) and female (♀) gonopores are located is enlarged and marked. B) A cartoon of the leech’s entire nervous system, showing the head brain, tail brain, and mid-body ganglia (Carus, 1891). C) Spontaneous leech mating behavior, demonstrating mate searching along ventral faces and the “telephone-cording” behavior (Wagenaar et al., 2010).
Leeches do not have to match up both sets of gonopores simultaneously for successful mating, and in fact, examples of mutual copulation seem to be rare (Mann, 1962).

We are interested in determining the neural basis of the twisting motion in particular, but mating leeches are extremely sensitive to external disturbances, such as the electrophysiological procedures that would normally be required for such studies (Wagenaar et al., 2010). Fortunately, when conopressin or hirudotocin —homologs of the mammalian 9-amino acid hormones, vasopressin and oxytocin —is injected into an adult leech at dosages producing nanomolar to micromolar concentrations in the body, it vigorously twists for hours, ignoring all perturbation. A series of studies revealed that the origin of the twisting motion was in ganglia G4 through G6, leading to the hypothesis that in this region there is at least one physiologically accessible central pattern generator (CPG) that initiates and maintains this behavior (Wagenaar et al., 2010).

With this knowledge, we wanted to find a way to use electrophysiology to study the neurons involved in this putative CPG. A method that has in the past proved highly effective for bridging the gap between coordinated neuronal firing (fictive behavior) and real behavior involves using a semi-intact preparation (Gray et al., 1938; Kristan et al., 2005), in which part of the nerve cord is exposed while the rest of the animal remains intact. This allows for direct observation of changes in a ganglion’s motor activity at the neuronal level and the effect those changes have on other segments of the leech. It works best for movements that are small, slow, and involve the entire animal, such as bending (Kristan Jr., 1982), shortening (Shaw and
Kristan, 1995), swimming (Stent et al., 1978), and crawling (Baader and Kristan, 1992). Following injection of conopressin, the longitudinal twisting behavior of telephone-cording becomes a strong, large movement in only the mid-body of the leech (Wagenaar et al., 2010). In addition, in our lab, we have demonstrated that injection of other homologues of mammalian vasopressin, such as hirudotocin and annetocin, produced the same behavior. Arginine-vasotocin at high concentrations (200x higher than other hormones) produced the behavior, but it was weaker. Injecting mammalian vasopressin or oxytocin did not produce any behaviors, even at high concentrations. These findings made courtship behavior accessible to study at the neuronal level. However, first we needed to discover which specific neurons to target. Several years ago, Rounak Nassirpour, working with Dr. Brian Norris, discovered that antibodies to mammalian vasopressin reliably bind to specific neurons in adult leech ganglia, demonstrating significant species cross-reactivity (Nassirpour and Norris, 2001). This observation is highly useful, but in retrospect, not entirely surprising. Vasopressin and oxytocin are closely related 9-amino acid neuropeptides that are known to control reproductive functions in mammals (Winslow et al., 1993; Wang et al., 1994), and the amino acid sequences among peptides in the vasopressin/oxytocin family of molecules are highly conserved among both vertebrates and invertebrates, explaining the cross-reactivity of the antibody (Fig. 2).

Based on the results reported by Nassirpour and Norris, we developed antibody staining protocols to reveal neurons that could serve as a source for the native leech hormone. We used a polyclonal antibody that recognizes mammalian vasopressin (AVP) to probe the leech ganglion for neurons that contained a reproductive hormone
**Hirudotocin** (*Hirudo medicinalis*) – Cys-Phe-Ile-Arg-Asn-Cys-Pro-Leu-Gly

**Annetocin** (*Eisenia foetida*) – Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly

**Arg-conopressin** (*Conus sp.*) – Cys-Ile-Ile-Arg-Asn-Cys-Pro-Arg-Gly

**Vasopressin** (most mammals) – Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly

**Oxytocin** (most mammals) – Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly

**Figure 2:** Amino acid sequences of 9-amino acid neurohormones in the vasopressin family. Amino acids that are conserved across all peptides are shown in black; other colors show divergence. The eighth amino acid is underlined, as the charge (at pH 7) at that position determines whether the molecule is a –tocin (neutral) or –pressin (positive)
similar to vasopressin. Additionally, we used a polyclonal antibody to mammalian 
vazopressin receptor 1B (AVPR1B) to determine which cells had receptors to the 
leech’s native vasopressin-like molecule.

Our lab is also highly interested in the developmental of the nervous system in 
*Hirudo sp.* (French and Kristan, 1992; Kristan et al., 1993; Marin-Burgin et al., 2006; 
Todd et al., 2010). We wondered if our AVP antibody could stain neurons in leeches 
that had not reached full maturity. If so, we might be able to track the formation of the 
putative reproductive CPG as the leech matured. Using embryonic leeches, and 
characterizing their age based on external features (percent of embryonic 
development, % ED) (Reynolds et al., 1998), we probed leeches at 50% and 100% 
ED. Leeches at 100% ED that have just hatched from their cocoon are called 
juveniles. We probed both stages for the presence of the neuropeptide, and only 
juveniles for the presence of hormone receptors.

We considered positively stained neurons to be candidate cells contributing to 
the CPG that generates the “telephone cording” behavior and perhaps other leech 
reproductive behaviors as well.
**Methods**

**Organisms**

Adult *Hirudo verbana* were obtained from a commercial supplier [Niagra Medicinal Leeches (Cheyenne, WY) or Carolina Biological Supplies (Burlington, NC)] and maintained in colonies in our laboratory at 15°C. Adult leeches were anaesthetized in ice-cold Hirudo saline (115 mM NaCl, 4 mM KCl, 1.8 mM CaCl$_2$, 1.5 mM MgCl$_2$, 10 mM glucose, 10 mm HEPES; pH 7.4), the ventral nerve cord was exposed, and individual ganglia or sections of the nerve cord were removed from the animal. Nervous tissue was pinned in a Sylgard-bottomed dish for further manipulation. In adults, individual ganglia were removed from the leech in normal leech saline (Muller et al., 1981) and pinned out in a Sylgard-bottomed Petri dish. In addition, we desheathed some ganglia — that is, we removed the connective tissue capsule from around the ganglion — to increase the access of antibodies.

Embryos were released from cocoons when they were at ~50% or 100% ED and held at 20-24°C in “embryo water,” sterile-filtered Arrowhead spring water (Arrowhead Water, Brea, CA) with 32 mol of MgCl$_2$ and 40 mol of CaCl$_2$ added per liter. The developmental stage of each embryo was determined using external morphological features. Stages run from 0% (egg deposition) to 100% ED (juvenile) (Reynolds et al., 1998). For immunohistochemistry in embryos, we anesthetized them in 8% ethanol in a Sylgard bottomed dish, exposed mid-body ganglia 3-12 (still in 8% ethanol), and stained the intact nerve cord.
Immunohistochemistry (IHC)

After dissection, all preparations (whether adult or embryonic) were immediately fixed either with 2% paraformaldehyde overnight (15-20 hours) or with 4% paraformaldehyde for 2 hours with gentle agitation at 4° C. Ganglia were then washed with phosphate buffered saline (PBS - per liter: add 2.56 g NaH₂PO₄, 11.94 g Na₂HPO₄ to 800 mL DI H₂O, adjust to pH 7.4; add 87.66 g NaCl and bring to final volume of 1 liter with DI H₂O) three times for one hour each or six times for 30 minutes each. Ganglia were permeabilized with 10% goat serum and 0.3% Triton X-100 in phosphate buffered saline (PBX) for 30 minutes or one hour. Next, while still in a 10% goat serum and 0.3% PBX solution, one of two primary polyclonal antibodies was applied. The antibodies were either mammalian anti-vasopressin (AVP –AbCam, ab68669) at a 1:50 or 1:100 concentration, or mammalian anti-vasopressin receptor 1B (AVPR1B –AbCam, ab66052) at a 1:50 concentration. Tissues were incubated with antibody overnight with gentle agitation at 4° C. Washes were performed using PBS as described above. The secondary antibody, Goat pAb to Rabbit IgG conjugated to a fluorophore (Cy3) (AbCam: ab6939), was applied at a 1:500 concentration in 10% Goat Serum with 0.3% Triton X-100 in PBX overnight with gentle agitation at 4° C. Finally, the preparation was washed with PBS six times for 10 minutes each, dehydrated through an ethanol series (30%, 50%, 70% for five minutes each, then 100% three times for five minutes each), cleared in methyl salicylate, and mounted on a glass slide using DePex mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).
Imaging

Preparations were imaged with a Leica laser scanning confocal microscope and Leica software. All final images shown in the Results are compressed z-stacks of individual ganglia. The following parameters were constant for all ganglia: format: 1024x1024; speed: 400Hz; line accu: 3; line avg: 1; frame accu: 1; frame avg: 2; z-step size: 3.01 μm; 543 nm laser power: 20%; scanning range: 550nm to 650nm. Total magnification, gain, and offset values are as noted in individual images.

In order to determine whether staining was significant, the images were processed according to a protocol developed by Dr. Krista Todd. ImageJ (http://rsbweb.nih.gov/ij/) was used to determine the average pixel intensity around the ganglion and the standard deviation of the intensity. A threshold was set at the average intensity + 2 standard deviations above the average. A custom Look-Up-Table (LUT) was then created that assigned pixels with intensities above the threshold to be black while all other pixels were displayed in white. Black cells were scored as positively stained with the antibody (“significantly stained” neurons).
Results

We probed individual leech ganglia with multiple antibodies from various companies and determined that anti-mammalian vasopressin (AVP) and anti-mammalian vasopressin receptor 1B (AVPR1B) antibodies from AbCam gave reliable, positive staining on ganglia from all developmental stages studied. To tentatively identify neurons stained by the antibody we noted the soma size and position and the visible morphology of processes and compared these properties with a published map showing the somas of the neurons in an adult *Hirudo sp.* ganglion (Muller et al., 1981).

Vasopressin Immunohistochemistry

We used immunohistochemistry to identify neurons that contained peptides that antibodies to arginine-vasopressin could bind to. In this part of the study, we used adult mid-body ganglia 4 through 9 (G4 through G9). In these ganglia, AVP antibodies produced one reliable staining pattern in G5, 6, and 7 (e.g., Fig. 3). At least some cells toward the anterior and lateral edges were labeled in every preparation, and based on the map (Muller et al., 1981), we identified these neurons as cells 50, 120 or 121 and 167/168/169/170. Of these cells, one is well known. Cell 50, also called the Leydig cell, is known to have a neuroendocrine function. Previous work has shown that this pair of neurons contains the neuromodulator myomodulin (Keating and Sahley, 1996). None of the black cells in the middle of the ganglion were labeled sufficiently reliably to identify them.
We saw a different staining pattern in adult G4 ganglia (Fig. 4). The Leydig cells (cell 50) were labeled reliably, but although the staining of some neurons in the middle of the ganglion was above the threshold, the pattern was not sufficiently reliable to allow us to identify these cells.

We saw yet another staining pattern in adult G8 and G9 ganglia with AVP (Fig. 5). In these ganglia, both the Leydig cells (cell 50) and a pair of small cells (cells 120/121) were labeled reliably.

In juvenile leeches, we saw a consistent staining pattern in all ganglia from G5 through G12 (Fig. 6). The Leydig cells (cell 50) were reliably labeled in all of these ganglia, as were two small cells (cells 120 or 121). In embryonic leeches (50% ED) only the Leydig cells (cell 50) were labeled in the ganglia we examined, G4 to G9 (Fig. 7). The maps of the adult ganglia were used to determine the potential identities of the stained neurons based on location in the ganglion and morphology of the processes. This approach is justified by repeated results in the Kristan lab showing that the somata of leech ganglionic neurons occupy their definitive position from 50% ED and do not move during subsequent development (Stent et al., 1992).

**Vasopressin-receptor Immunohistochemistry**

To label cells within the ganglia using an antibody that recognizes the receptor for arginine vasopressin (AVPR1B), the connective tissue capsule surrounding the ganglion had to be dissected away ("desheathed"). This procedure increased access to the neurons and improved labeling. Using this protocol, several cells with small somata were labeled significantly in adult G5 through G8 (see Methods); most of them
have not yet been identified. However, cell 251 was stained reliably in all ganglia, and cell 255 was stained frequently (Fig. 8).

Although cell 255 remains unidentified, cell 251, (also called the Nut cell) has been studied previously. Its morphology resembles many leech motor neurons, but its projection pattern is unique (Stewart et al., 1991). Because our antibody showed only the position of the soma, we could not positively identify the cell with our immunohistochemical results alone. Dr. Krista Todd confirmed the identification by finding and filling a cell 251 in an adult G6 ganglion using Alexafluor 488 (a green fluorescent dye). She then treated the ganglion with antibodies as previously described, using a Cy-3-tagged antibody (red). When she imaged the ganglion with confocal microscopy and merged the images from the green and red channels, the two fluorophores were co-localized (Figure 9).

In juvenile ganglia (G5 through G10), we only detected cell 255 reliably (Fig. 10).

To guard against false positives, we ran a secondary antibody control (Fig. 11). We followed all procedures for a normal stain, with the omission of a primary antibody. We saw no significant fluorescence or any artifacts, demonstrating that the staining we observed was not due to selective binding by the secondary antibody.

Acknowledgments

The work in this section may be prepared for future publication. The data in figure 9 were collected in collaboration with Dr. Krista Todd and were used here with her permission.
Figure 3: Anti-vasopressin (AVP) staining in an adult G7 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in eight imaged ganglia, which were a combination of G5, 6, and 7 ganglia. A) Raw image of G7 ganglion labeled with 1:50 AVP. 200x magnification, gain 881 volts, offset -0.4%. B) Image processed as described in Methods. C) Ventral face of an adult Hirudo medicinalis ganglion, showing the identities of many neurons. Neurons marked in black correspond to cells that were reliably labeled by anti-vasopressin. Gray neurons correspond to other reliably stained, but less readily identified neurons. D) Dorsal face of the ganglion, using the same convention as in C to indicate labeled cells. Maps in panels C and D were adapted from (Muller et al., 1981).
Figure 4: Anti-vasopressin (AVP) staining in an adult G4 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in three imaged G4 ganglia.  

A) Raw image of G4 ganglion labeled with 1:100 AVP. 200x magnification, gain 958 volts, offset -1.0%.  

B) Image processed as described in Methods.  

C) Ventral face of an adult *Hirudo medicinalis* ganglion. Neurons marked in black correspond to reliably stained and readily identifiable cells, in this case Leydig cells (cell 50).  

D) Dorsal face of the ganglion using the same convention as in C to indicate labeled cells. Maps in panels C and D were adapted from (Muller et al., 1981).
Figure 5: Anti-vasopressin (AVP) staining in an adult G8 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in four imaged ganglia, which were a combination of G8 and G9 ganglia. A) Raw image of G8 labeled with 1:100 AVP. 200x magnification, gain 881 volts, offset -0.4%. B) Image processed as described in Methods. C) Ventral face of an adult Hirudo medicinalis ganglion. Neurons marked in black correspond to reliably stained and readily identifiable neurons. D) Dorsal face of the ganglion using the same convention as in C to indicate labeled cells. In addition, neurons marked in gray correspond to other reliably stained cells and their possible identities. Maps in panels C and D were adapted from (Muller et al., 1981).
Figure 6: Anti-vasopressin (AVP) staining in a juvenile leech (100% E.D.) G7 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in 14 imaged ganglia, which were a combination of G4 through G12. A) Raw image of G7 labeled with 1:100 AVP. 400x magnification, gain 1012 volts, offset -0.6%. B) Image processed as described in Methods. C) Ventral face of an adult Hirudo medicinalis ganglion. Neurons marked in black were reliably stained and readily identifiable. D) Dorsal face of the ganglion using the same convention as in C to indicate labeled cells. In addition, neurons marked in gray correspond to other reliably stained cells and their possible identities. Maps in panels C and D were adapted from (Muller et al., 1981).
**Figure 7:** Anti-vasopressin (AVP) staining in an embryonic (50% E.D.) G5 ganglion. In all images, anterior is towards the top of the page. Binding results were observed in 12 imaged ganglia, which were a combination of G3 through G9. 

A) Raw image of G5 labeled with 1:100 AVP. 200x magnification, gain 738 volts, offset -1.9%. 

B) Image processed as described in Methods. 

C) Ventral face of an adult *Hirudo medicinalis* ganglion. Neurons marked in black correspond to reliably stained and readily identifiable cells, in this case Leydig cells (cell 50). 

D) Dorsal face of the ganglion using the same convention as in C to indicate labeled cells. Maps in panels C and D were adapted from (Muller et al., 1981).
Figure 8: Anti-vasopressin receptor 1B (AVPR1B) staining in a desheathed adult G5 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in 6 imaged ganglia, which were a combination of G4 through G8. A) Raw image of G5 labeled with 1:50 AVPR1B. 200x magnification, gain 613 volts, offset -1.0%. B) Image processed as described in Methods. C) Ventral face of an adult *Hirudo medicinalis* ganglion. Neurons marked in black correspond to reliably stained and readily identifiable cells; neurons marked in gray correspond to another reliably stained cell pair and its possible identity. No cells with somata on the dorsal surface were labeled. Map in panel C was adapted from (Muller et al., 1981).
Figure 9: Nut cell staining and morphology. An adult G6 ganglion was dissected and desheathed. The nut cell (cell 251) is marked by a white arrow. A) Confocal image showing AVPR1B staining. B) Nut cell (cell 251) filled with Alexafluor 488 (a green fluorescent dye, which was allowed to diffuse through the cell’s processes. C) Overlay of A and B demonstrating the colocalization of both the antibody and dye in the soma of the nut cell in yellow.
**Figure 10:** Anti-vasopressin receptor 1B (AVPR1B) staining in a juvenile leech (100% E.D.) G5 ganglion. In all images, anterior is toward the top of the page. Binding results were observed in nine imaged ganglia, which were a combination of G4 through G10. **A)** Raw image of G5 labeled with 1:50 AVPR1B. 400x magnification, gain 1012 volts, offset -0.6%. **B)** Image processed as described in Methods. **C)** Ventral face of an adult *Hirudo medicinalis* ganglion. Neurons marked in grey correspond to reliably stained cells and their possible identity in the adult. No cells with somata on the dorsal surface were labeled. Map in panel C was adapted from (Muller et al., 1981).
Figure 11: Negative control of an isolated adult G4 ganglion. Procedure was identical to that described in the Methods, but we omitted the primary antibody. In both images, anterior is toward the top of the page. A) Raw image of an adult G4. 200x magnification, gain 881 volts, offset -0.4%. B) Image processed as described in Methods. We detected no significant staining; several somata are dimly visible in the raw image due to typical auto-fluorescence.
Discussion

Our immunohistochemical studies showed an increase in the number of neurons stained by both the AVP and AVPR1B antibodies as the leech grows and matures. With the AVP antibody, we reliably stained two cells at 50% E.D. (cell 50), four at 100% E.D. (cells 50 and 120/121), and up to six in the fully mature adult (cells 50, 120/121, and 167/68/69/70). Using the AVPR1B antibody, we observed two major cells reliably labeled at 100% E.D. (cell 255) and four at full maturity (cells 251 and 255). The two antibodies labeled different neurons, indicating that the neurons containing the vasopressin-like peptide and those containing the receptor formed two different subsets of ganglionic neurons.

Because leech neurons were labeled by these antibodies produced to recognize mammalian molecules, we reasoned that leeches must produce a molecule that belongs to this family. Indeed, a member of the family, hirudotocin, has been previously described (Salzet, 2007).

When we used antibodies that recognize mammalian AVP, we saw consistent and strongly positive labeling of cell 50, commonly known as the Leydig cell. This identification was confirmed based on morphology, position of the soma, and projection of major processes (Keyser et al., 1982).

Researchers have shown that Leydig cells cross-react with antibodies that recognize myomodulin (Keating and Sahley, 1996). Leydig cells are strongly electrically coupled to each other across each ganglion and to Leydig cells in neighboring ganglia to form a ladder-like network that runs through the leech's body.

Our study has revealed several neurons that may contribute to the production
and regulation of leech reproductive behavior, so it represents an important step
towards elucidating the neuronal basis of that behavior. Behavioral studies in our lab
have revealed that injecting vasopressin homologs into an adult leech produces
courtship behavior, so it is reasonable to hypothesize that neurons containing the
peptide and neurons that express its receptor are likely to be part of the neuronal
circuit that controls leech mating and copulation behaviors. Our work suggests that a
leech develops this circuit slowly with more neurons contributing to the regulation of
the behavior as those neurons begin producing vasopressin-like molecules and
receptors. The strong interconnectivity among Leydig cells could coordinate and
amplify the release of this important hormone during mating behavior.

Physiological work progressing toward identification of neurons in the
reproductive central pattern generator (CPG) strongly suggested that the circuitry is
located within G4, 5, and 6 (Wagenaar et al., 2010). Directly bathing G5 or 6 in
conopressin produced characteristic rhythmic bursts in motor neurons that project to
the body wall in those two segments. However, we observed labeling of Leydig cells
and other neurons up and down the nerve cord. We suggest that these neurons provide
the hormonal signal that initiates and maintains the courtship behavior, but they are
not part of the central pattern generator. The neurons that were labeled by the antibody
to the AVP receptor are more likely to be part of the central pattern generator, but
confirmation remains for the future.

Future experiments will further confirm the specificity of the antibody, the
identities of the cells that were labeled in this project, and characterize the
electrophysiology of the circuit underlying reproductive behavior. The work described
in this paper provides an important first step into the larger project of understanding how endocrine and neuronal signals work together to produce and maintain reproductive behavior in the medicinal leech.
References


