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Mass Spectrometry Methods and Applications for Functional Characterization of the Crustacean Neuropeptidome

By

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A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctorate of Philosophy

(Chemistry)

at the

UNIVERSITY OF WISCONSIN-MADISON

2020

Date of final oral examination: 06/11/2020

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Chapter 10

Using Crabs to Study Neuropeptides and Their Role in the Nervous System with Mass Spectrometry Methods

Written by Kellen DeLaney, Lingjun Li Research Group

Written for the Wisconsin Initiative for Scientific Literacy in order to describe this thesis for a broader audience

This chapter was written to explain the research I have conducted to a broad, non-scientific audience. Scientific research is generally performed with the goal of being useful to the greater population. As such, it is critical that our findings are communicated with others. Science communication is a fundamental component of our responsibilities as scientists. I acknowledge the Wisconsin Initiative for Science Literacy at the University of Wisconsin-Madison for providing this platform and for sponsoring and supporting the creation of this chapter.

Abstract

The human nervous system is comprised of billions of cells that work together to transport messages throughout the body. These messages are transported partly by molecules called neuropeptides. My thesis focuses on developing and applying tools to study these neuropeptides. I use crabs as a model organism because their nervous system is much simpler than that of humans but is still similar. By changing various ways the neuropeptides are analyzed, we were able to identify more neuropeptides and even new, never-before-detected neuropeptides. These neuropeptides were tested to see if they had a function in the nervous system related to feeding, a universal process, and heartbeat. Those neuropeptides that did have a function may provide useful insight into how the nervous system works in the body and information on their function may lead to improvements in how we treat neurological disorders.

Crabs as a model for the nervous system

The nervous system is a beautifully complex system within the human body that includes the brain, spinal cord, and nerves running through every part of the body. Overall, the nervous system is made up of 86 billion cells, called neurons, that transport messages throughout the body. While this vast complexity enables the body to perceive and respond to our surroundings in a variety of sophisticated ways, it makes studying this system difficult. If we can understand the way neurons communicate within the body, we can be better equipped to understand and treat neurological disorders, such as Parkinson's disease and Alzheimer's disease. However, in order to understand specifically how the neurons communicate with each other in this system, we would have to map connections between each of these 86 billion neurons, and each can have multiple connections to various other neurons.

To address this daunting task, we look to crabs, specifically species such as *Cancer borealis*, or Jonah crab. These ten-legged, shelled critters seem far removed from humans on the surface, but the basic workings of their nervous system is surprisingly similar to that of humans. The difference is that the crab nervous system is much simpler. While a human nervous system has billions of neurons, a crab has only one hundred thousand neurons, which is one ten-thousandth of a percent of the number in humans. The small number of neurons in the crab nervous system means that it is easier to map how neurons connect to each other and influence each other. For example, twenty-five neurons are involved in controlling a crab's chewing and are connected as shown in **Figure 1**, where circles represent neurons and lines represent connections.[1] The arrows represent the direction molecules move between the neurons. The connections between neurons typically act as gates; sometimes they are open and let things pass through and sometimes they are closed so nothing can pass through. These gates are selective, in

that they only let certain molecules pass through, similar to how different keys allow access to different doors in an apartment building.

Neuropeptides: The molecules that make things happen

Just like gates, the connections between neurons can open with the right "key." Some molecules are shaped in a way that they can fit into a part of the gate and tell it to open. One group of molecules that can do this is neuropeptides, which are made out of the same substance as proteins, but are smaller in size. These neuropeptides act as messengers, entering and leaving neurons throughout the body to change the way they behave. As a result, neuropeptides are incredibly important for helping to control virtually all processes within the body. Because of this, understanding how they work not only improves our understanding of the nervous system, but it can help us develop treatments for neurological diseases by designing drugs that mimic the shape and function of neuropeptides.

However, the characteristics that make them great signaling molecules are also the reason they are difficult to study. They are present in very small amounts and degrade rapidly over time. This is helpful for conveying messages. For example, if you touch a hot burner with your hand, your hand releases a burst of neuropeptides to communicate this to your brain, telling your hand to move away from the hot burner. If you then decide for whatever reason to immediately touch that burner again, another burst of neuropeptides will be released. If the previous burst is still circulating around in the body, the next message won't be as easily recognized and it will take longer to tell your hand to move away from the burner. In other words, by having a small number of something, differences are more noticeable and so the response is more striking. Additionally, neuropeptides come in a wide variety of forms, which is useful for conveying more specialized

messages, but also makes them challenging to study because there are more of them to keep track of.[2]

Because neuropeptides are present in small amounts, do not last long outside the body, and are present in many different varieties, finding a way to study what each one is and how it works is difficult.

Developing ways to detect neuropeptides

Most methods of studying neuropeptides can only look at one neuropeptide at a time. These methods give lots of helpful details, but can be tedious when there are hundreds of neuropeptides in the body, and impossible when we don't know which neuropeptides we're looking for. In order to study all of the neuropeptides at once, we use mass spectrometry, which is a technique that detects neuropeptides (and other molecules) based on their mass-to-charge ratio, or how big they are versus how much charge they have on them. This method is good because we see almost all neuropeptides at once and we don't need to know what we're looking for ahead of time. However, we still encounter challenges with neuropeptides being present in low amounts, making it hard to detect them with a mass spectrometer.

In order to better detect neuropeptides, we need to separate them from each other, like spreading out a crowd of people to see the individuals more clearly. We also need to make sure they have a charge because if a molecule has no charge, the mass spectrometer cannot detect it. An overview of the general steps involved in neuropeptide analysis, including separation, charging, and mass spectrometry analysis, is shown in **Figure 2**. Most of the time, mass spectrometry is used with a separation method called liquid chromatography. Molecules are pushed through a tube filled with a substance. The speed at which they travel through the

substance depends on how much they stick to the substance in the tube. This is shown in **Figure 2B**. They are then given a charge by applying a voltage as they are being emitted from the tip of the tube. This method works to detect some neuropeptides, but not all neuropeptides can be separated from each other this way, and not all neuropeptides can become charged in liquid form this way. As neuropeptides come in many varieties, there isn't one perfect way to separate them. Just like how you can separate people in a crowd in multiple ways (e.g. how tall they are or what color shirt they're wearing), molecules can be separated in multiple ways that makes it easier to see different ones, and we can give them a charge in different ways as well.

To increase the number of neuropeptides detected, we developed a method that used a different separation mechanism, called capillary electrophoresis, shown in **Figure 2B**, and combined it with a method that gave molecules a charge in a different way. With this method, molecules were separated as they moved through a tube based on how quickly they could move in the presence of an electric field, similar to electricity in a wire. When they leave the tube, they are deposited on a plate attached to a continuously moving platform and allowed to dry. Instead of giving charge to the neuropeptides in liquid form with a voltage, the charge is given to the neuropeptides in solid form with a laser. The overall setup is shown in **Figure 3**.[3] By changing both the separation method and the method of giving charge, different neuropeptides are able to be detected within the same sample. In this way, complementary methods are combined to increase the overall number of neuropeptides able to be detected.

Developing ways to characterize neuropeptides

In addition to simply detecting the presence of neuropeptides in a sample, we need to know more about the details of the neuropeptides, such as what components they are made of.

This can be done by performing a second round of mass spectrometry after the first. The initial mass spectrometry measurement identifies the mass-to-charge ratio of the neuropeptides. We can then select individual neuropeptides, break them into pieces, and see the mass-to-charge ratio of the individual pieces. This tells us more about what pieces make up the neuropeptide. However, selecting every individual neuropeptide to be broken apart would take too long, so it isn't feasible. Typically, only the most prominent mass-to-charge ratios are selected and broken into pieces. This method is called data dependent acquisition (DDA) because what is selected to be broken depends on the relative amount of molecules in the data. As a result, many molecules that are present in low amounts, such as neuropeptides, get overlooked and we end up not knowing what pieces they are made of.

To address this problem, we used a method that selected a wide range of mass-to-charge ratios to be broken into pieces simultaneously. This method is called data independent acquisition (DIA) because it doesn't depend on the amounts of each molecule in the data. The difference between DDA and DIA can be thought of like picking apples, where each apple is a mass spectrometry scan, and ripe apples are neuropeptides. The DDA method is like picking the 5 apples on each tree that are closest to you and putting them each in its own basket. The DIA method is like picking all of the apples that you can reach and putting them each in one of the 5 baskets. At the end of the day, you look through the baskets and determine which ones are actually ripe. With the DDA method that only has 1 apple per basket, it's faster and easier to determine apple ripeness. With the DIA method, there are maybe 10 to 20 apples in each basket that have to be filtered for ripeness. It takes longer to find the ripe apples in the basket, but you ultimately end up with more ripe apples. **Figure 4** shows a depiction of the two different methods.

While this new method made the secondary mass spectrometry measurements more complicated, it ensured that all molecules were getting broken into pieces. We then used software developed in another lab that could match the individual pieces back to the original mass-to-charge ratio they came from.[4] In this way, we were able to get detailed information about more of the neuropeptides that were in lower amounts in the sample, resulting in information about twice as many neuropeptides compared to the original method with good reproducibility, as shown in **Figure 5**.[5] In this way, we can increase the number of neuropeptides we're able to characterize to get a deeper understanding of what neuropeptides are present within the nervous system.

How neuropeptides influence feeding behavior

Seeing what neuropeptides are present in the nervous system is important, but we also need to know what these neuropeptides do (if they even do anything). There are numerous ways to get an idea of what a neuropeptide does. The simplest way is to compare the neuropeptides between two conditions, such as before or after a crab eats food. Looking at neuropeptides involved in feeding behavior is particularly interesting in crabs because their nervous system is located in their stomach. This system controls the chewing and filtering of food and receives instruction from neuropeptides. We compared the neuropeptides in crabs that were fed tilapia for 30 minutes to those that were not fed anything, and we found that, in general, the amount of neuropeptide increased in many parts of the nervous system, such as the brain. However, in other parts of the crab that release neuropeptides as hormones, like the sinus glands, the amount of many neuropeptides decreased. Some of these results are shown in **Figure 6**. The fact that certain neuropeptides change when the crab is fed indicates that they may have an influence on

the feeding process in some respect. This finding is helpful because instead of trying to get functional information on hundreds of neuropeptides, we can focus on the subset that do change, reducing the workload and making studies more meaningful.

The discovery of never-before-seen neuropeptides and their effect on heart rate

With the improvements in detection and characterization of neuropeptides, we made an exciting discovery. Over the course of studying these neuropeptides, we found numerous neuropeptides that have never been detected before. These neuropeptides looked similar to those previously identified in that some of the pieces were the same. However, each had unique pieces that made it different from anything we've seen before. Furthermore, the amount of two of these neuropeptides increased in the crab after feeding, indicating that they may have a biological role. This is exciting because we're uncovering more of the unknown parts of the crab nervous system and gaining a deeper understanding of all the molecules working together.

These two neuropeptides were identified in the tissue on either side of the heart that typically releases neuropeptides into the heart, called the pericardial organs. In order to get a better idea of how these two neuropeptides are involved in the crab's bodily functioning, we tested their effect on crab hearts. A notable benefit to using crabs as a model organism is that they have robust central pattern generators. This means that you can remove the heart from the crab and it will continue to beat. We removed the heart, placed it in a dish filled with saline, and infused saline through the major artery on the tail-side. The arteries on the other side of the heart were tied to a force displacement transducer, a small instrument that measures force, or how hard something pulls on it. Every time the heart contracted, it pulled on the force displacement transducer, which measured the strength of the heartbeat.[6] Using this setup, shown in **Figure 7A**,

we could measure the normal heartbeat of the crab, then infuse a neuropeptide into the heart and see how the heartbeat changed. When we infused the first neuropeptide, the heartbeat became stronger and faster, as shown in **Figure 7B** and **C**. When we infused the second neuropeptide, the strength of the contractions didn't change much, but the heartbeat became slower. These results indicate that these two neuropeptides are biologically active and may have a role in modulating heartbeat that is feeding-related.

What this means for neuroscience

Throughout the course of my Ph.D. research, I developed improved tools for studying neuropeptides and have applied these methods to identify which neuropeptides are biologically active. While my research focused on a global understanding of neuropeptides, more detailed functional analyses can be performed on the neuropeptides that we identified to be biologically active. Once a better understanding of these neuropeptides is achieved, the findings can be extended to more complex nervous systems, such as that of insects, mice, and even humans. Ultimately, understanding how neurons use these neuropeptide messengers to communicate throughout the body can improve our ability to generate treatments for neurological diseases and disorders.

Acknowledgements

The research described here was conducted with funding from a predoctoral fellowship supported by the National Institutes of Health, under Ruth L. Kirschstein National Research Service Award T32 HL 007936 from the National Heart Lung and Blood Institute to the University of Wisconsin-Madison Cardiovascular Research Center and the National Institutes of

Health-General Medical Sciences F31 National Research Service Award (1F31GM126870-01A1). KD also acknowledges guidance and editorial support provided by the science communications program of the Wisconsin Institute for Scientific Literacy.

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Figures

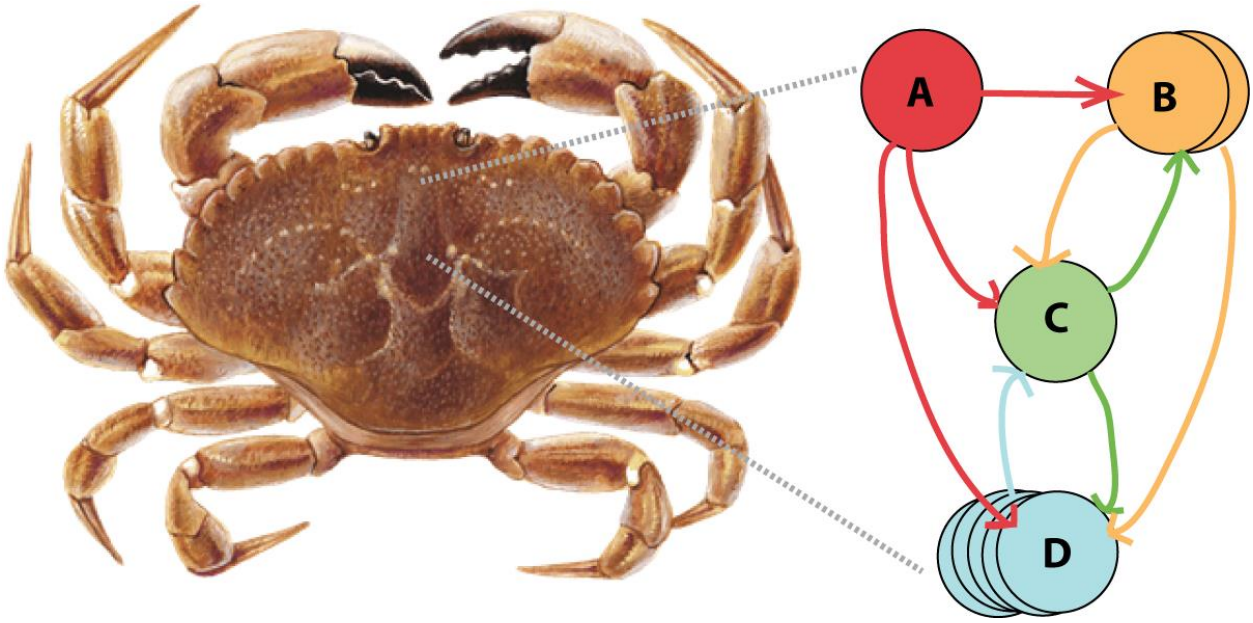

Figure 1. Depiction of the neurons in the crab nervous system that are responsible for controlling chewing. Circles indicate neurons, with different neurons labeled by different letters and colors, and lines indicate connections between neurons.[1]

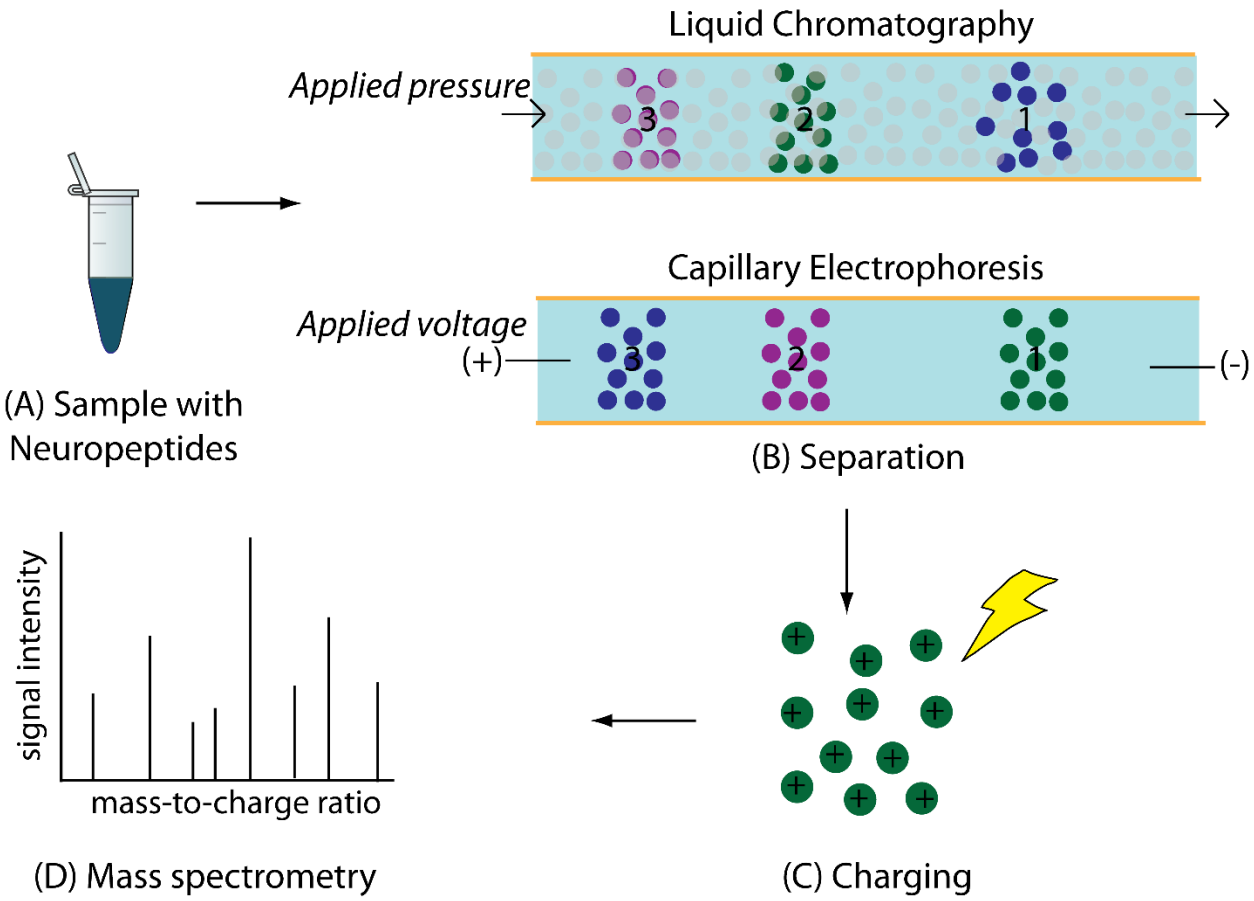

Figure 2. Overview of a typical mass spectrometry workflow, (A) starting with a sample containing a mixture of neuropeptides, (B) separation using two common methods, (C) providing a charge to the molecules so that they can be detected, and (C) mass-to-charge ratio measurements of the molecules using mass spectrometry.

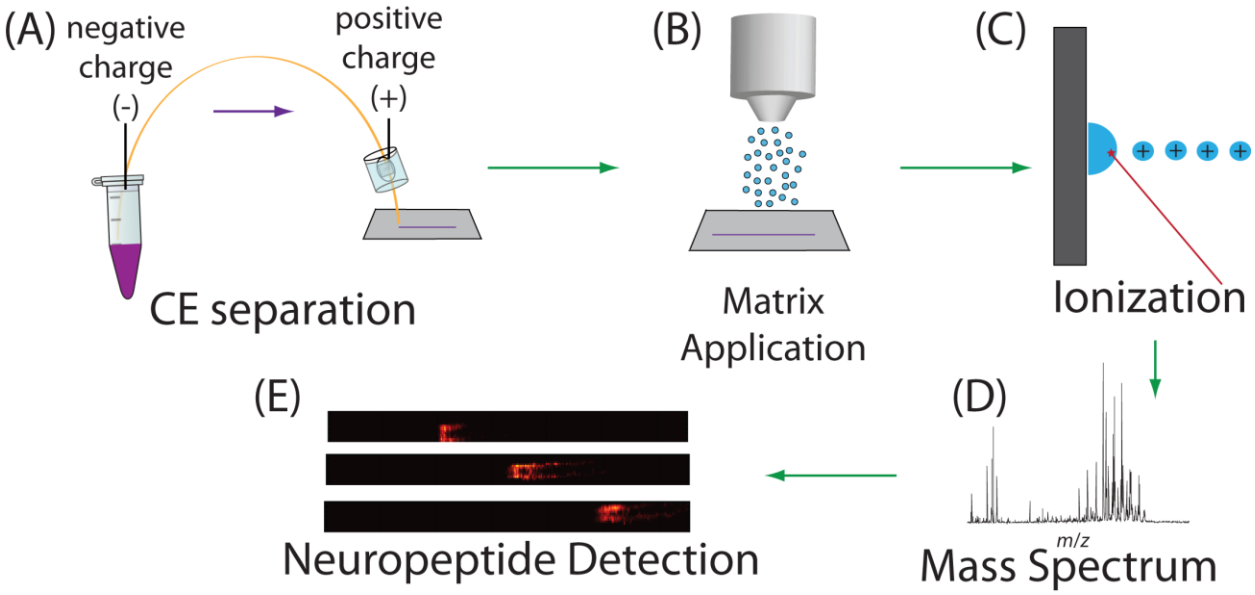

Figure 3. Depiction of the alternative separation and charging method for detection of neuropeptides. (A) A voltage is applied to a tube with neuropeptides, allowing them to move through the tube at different speeds and land on a metal plate in a process called capillary electrophoresis (CE). The metal plate is attached to a continuously moving platform so that neuropeptides are deposited in a continuous line. (B) A special molecule (matrix) is sprayed on the plate to help absorb the laser energy. (C) A laser is shot at the dry neuropeptide and matrix, causing the molecules to ionize, or obtain a charge. (D) The molecules are analyzed with mass spectrometry and detected based on their mass-to-charge ratio (*m/z*) in the mass spectrum. (E) Neuropeptides are detected at different points along the line so that their signals are separated from each other.[3]

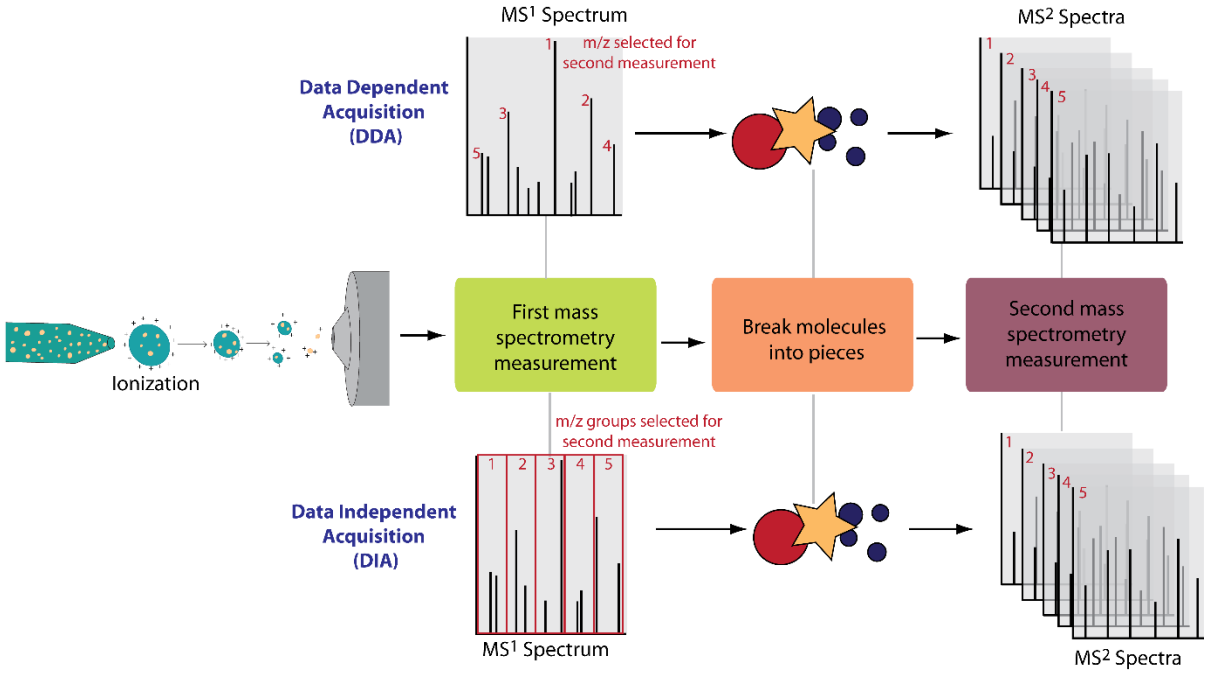

Figure 4. Depiction of different methods of identifying neuropeptides in a mass spectrometry experiment, including (top) data dependent acquisition (DDA) and (bottom) data independent acquisition (DIA). First, molecules are ionized, or given a charge. Next, an initial mass spectrometry measurement is made to determine the mass-to-charge ratio (*m/z*) of each molecule. Then, certain *m/z* values or groups of *m/z* values are selected and broken into pieces. The resulting pieces are detected by their *m/z* values in a second mass spectrometry measurement.

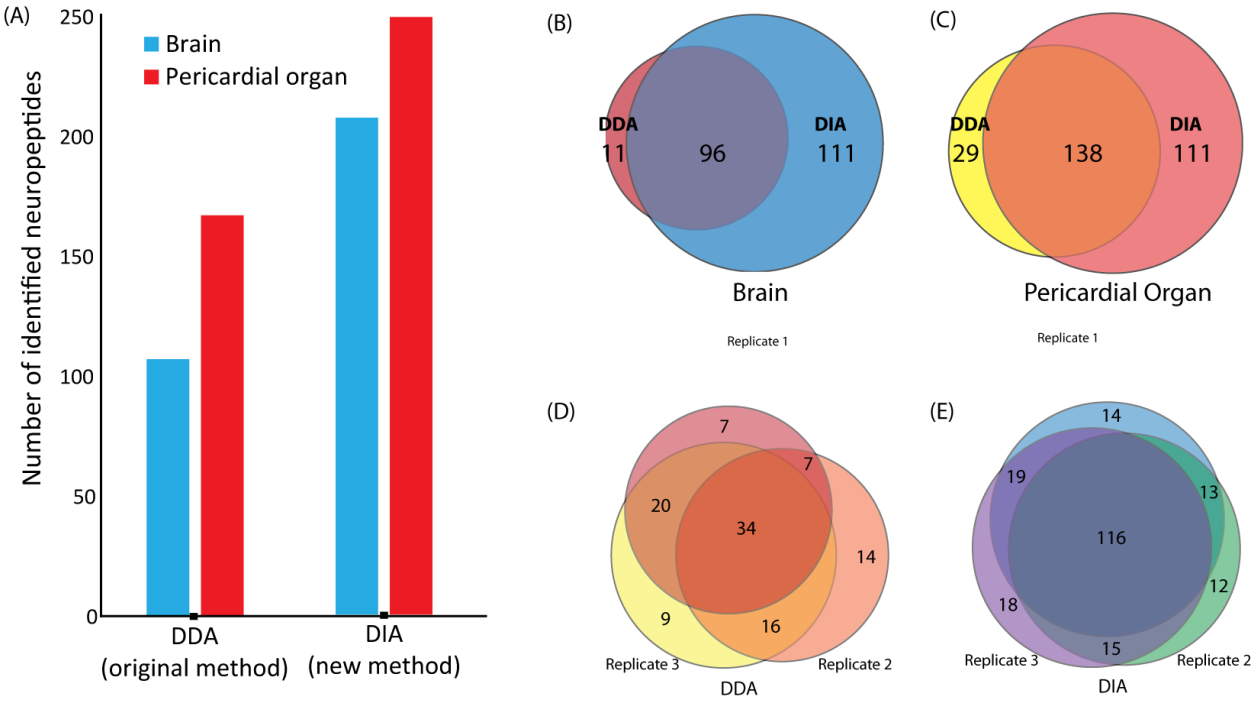

Figure 5. Resulting data comparing the original data dependent acquisition (DDA) identification method with the new data independent acquisition (DIA) identification method for mass spectrometry. (A) The bar graph shows the number of neuropeptides identified with each method for two different tissues, the brain and pericardial organs. Pericardial organs are located on either side of the crab heart and release neuropeptides into the heart. The overlap of neuropeptides identified in the (B) brain and (C) pericardial organs show that most neuropeptides identified with DDA were also identified with DIA, indicating that the new method wasn't missing neuropeptides the original method detected. The overlap of neuropeptides identified in replicate samples in (D) the DDA method was lower than that of the (E) DIA method, showing that the results of the new method were reproducible.[5]

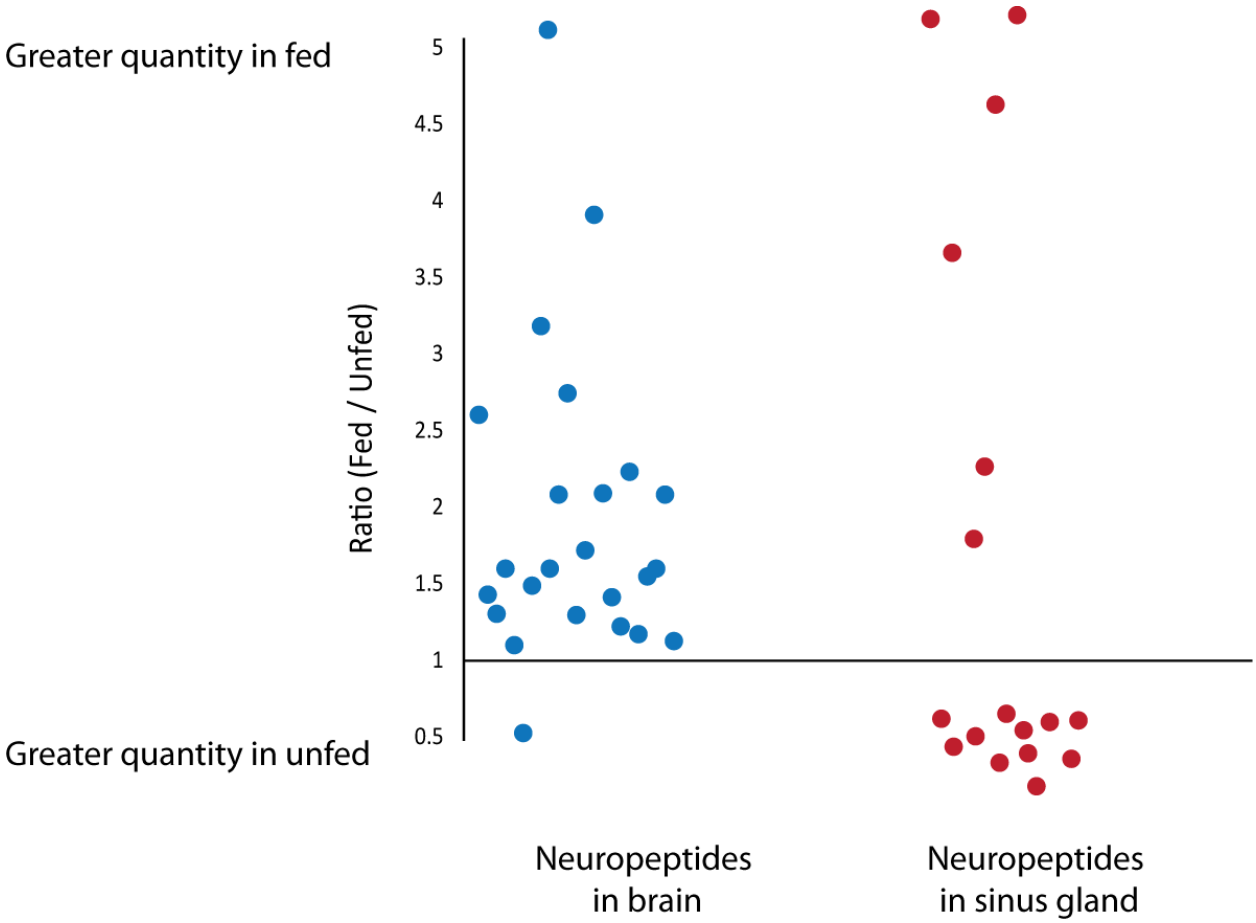

Figure 6. Differences in the amount of neuropeptide in tissue in crab between fed and unfed state in brain and sinus gland tissue. Each circle represents a different neuropeptide, and the value shown for each is the ratio of amount in fed crab vs amount in unfed crab. Ratios greater than 1 indicate an increase in neuropeptide in fed tissue, and ratios less than one indicate a decrease in neuropeptide in fed tissue.

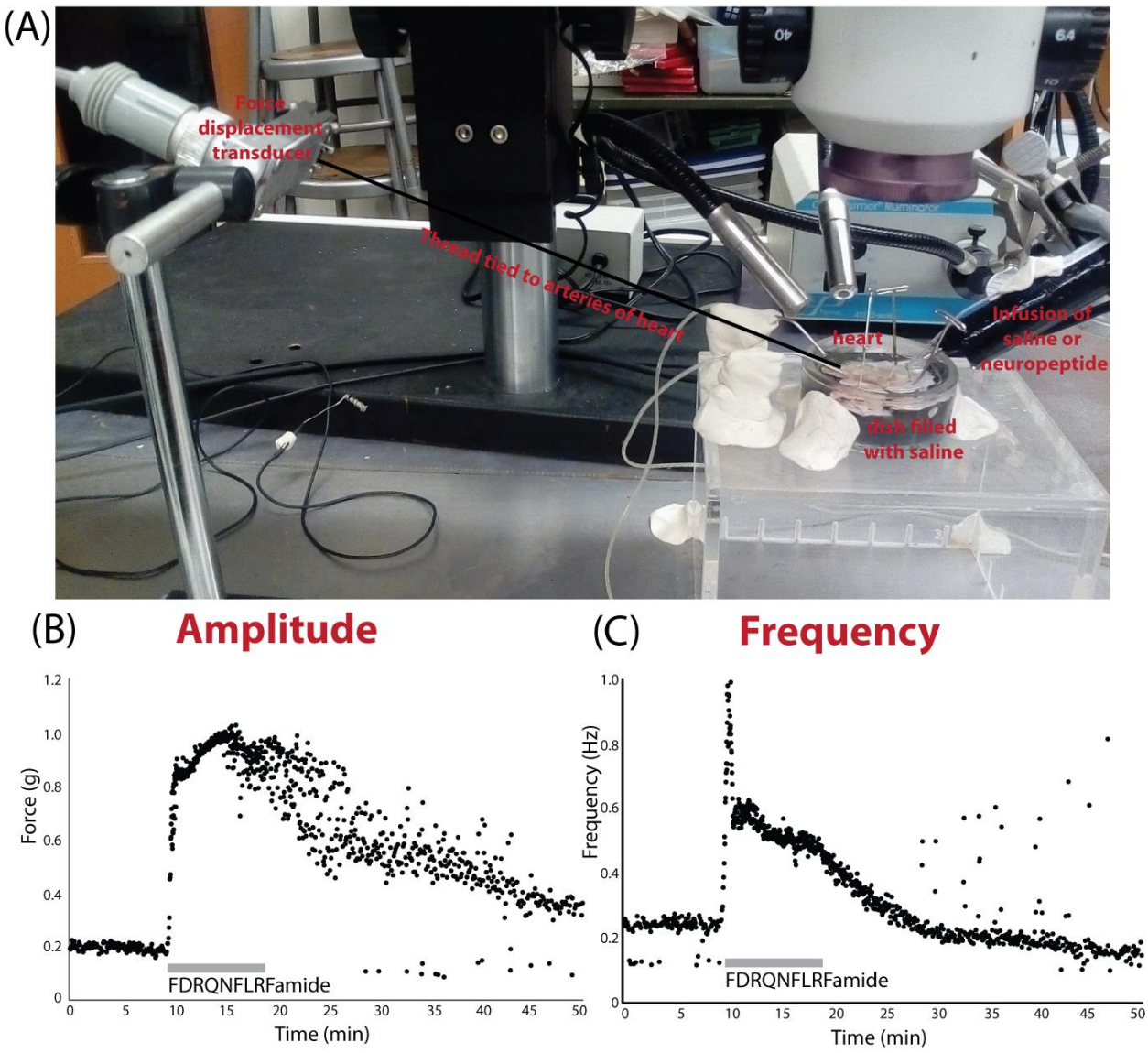

Figure 7. Measurements were made on the effects of neuropeptides on crab heartbeat. (A) The heart is removed from the crab and placed in a dish filled with chilled saline. Saline and neuropeptides are infused into the heart through the tail-side artery. The head-side arteries are tied with thread to a force displacement transducer that measures the strength at which the heart pulls on the thread when it contracts. Recordings of the (B) force and (C) frequency are collected over the course of time as a neuropeptide is infused into the heart. In this way, we can see how the presence of a neuropeptide changes the heartbeat.