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Comparative study of Allatostatin and ChAT expression between insect and crustacean forebrains for visual processing, learning, and memory functions

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Abstract
Understanding the evolutionary relationship between insect and crustacean brains hinges on the similarity of their structure and function. Is there conservation among the forebrains in both types of organisms? The expression of two different proteins, allatostatin and acetylcholine transferase (ChAT) in ghost shrimp (crustacean) brains was determined and compared with their expression in fruit flies and honeybees (insects). Using immunohistochemistry, shrimp brains were treated with protein-binding fluorescent antibodies and cell-body stains and then imaged with confocal microscopy. For both shrimp and flies, allatostatin was found in the optic lobe medulla, indicating a similar role played in visual activity. Allatostatin was also expressed in the understudied medulla terminalis of the shrimp. Prior research showed that allatostatin was found in a region of the honeybee brain that sends signals to the mushroom body (a learning and memory structure). This region could be homologous to the medulla terminalis and these results might help determine the latter's function. ChAT was found in the medulla and lobula regions of shrimp and flies, again indicating conservation of visual processing. ChAT was also found in the mushroom body of shrimp and flies, implying that conservation of learning and memory structures is plausible but requires another antibody and more research to reach a definite conclusion. These findings are significant in providing evidence for common ancestor evolution of organisms with mushroom bodies. They can further help understand the anatomical region precursor(s) for the evolution of optic lobes and mushroom bodies. Additionally, this study sets a baseline to identify homologous structures in humans for further discoveries in human neurobiology.

Keywords
Neuroscience, Evolutionary biology, Arthropods, Evolutionary neuroscience, learning and memory, allatostatin, acetylcholine transferase

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Introduction and Summary

The overall topic of study for this experiment is the evolution of learning and memory brain structures in crustaceans. The hypothesis being tested was that visual, learning, and memory structures are evolutionarily conserved between crustaceans and insects. It was expected that crustaceans express allatostatin and choline acetyltransferase (ChAT) in the same forebrain regions as insects when exposed to the same antibodies.

Drosophila, also known as the fruit fly, is an organism that shares genetic, cellular, electrophysiological, and chemical properties with the human nervous system. In an experiment testing the effects of ChAT on *Drosophila* brains, it was found that peripheral sensory neurons and neurons associated with the visual systems were labeled by the ChAT antibody (11). Another experiment tested the expression of allatostatin in honeybee brains. This experiment showed that visual processing and surrounding mushroom body regions were labeled by the antibody (4). The objective of this experiment was to replicate these experiments on crustacean brains to see if similar anatomical brain regions were labeled. The experiment was performed using immunohistochemistry (IHC). IHC involves the process of binding each antibody to a target molecule as well as to a fluorescent detector. Then, brain tissue slices treated with the antibodies are viewed under a confocal microscope (6).

Background

Crustacean and Insect Evolution

Crustaceans and insects are both members of the phylum Arthropoda, which means that they share a common ancestry that dates back hundreds of millions of years. Arthropods are characterized by their segmented bodies, jointed limbs, and exoskeletons made of chitin (1). The exact evolutionary relationship between crustacean and insect brain structures is still debated. Despite their similarities, crustaceans and insects have evolved distinct adaptations to suit their different environments. Crustaceans are primarily aquatic, with many living in marine or freshwater environments, while insects are predominantly terrestrial (5). In spite of these differences, studying the evolution of the brain specifically will give insight into the origin of the mushroom body, which will then help to better understand how the environment shapes brain structure/function.

In this experiment, *Palaemonetes paludosus*, or Ghost shrimp, were used as the crustacean species. This was because they are relatively easy to obtain and maintain in the laboratory. Ghost shrimp are widely available and can be purchased from many local pet stores. They are also relatively inexpensive, which made them an excellent choice to buy in bulk (3). In addition to their accessibility and low cost, ghost shrimp have a number of other qualities that made them useful for this experiment. They have a small, simple nervous system that is similar to that of other crustaceans, but they are much easier to handle and study than larger species such as lobsters or crabs. The mushroom bodies of these organisms are located in their eye stalks. Ghost shrimp are

also transparent, which makes it easier to dissect and extract their brains.

Key Antibodies

The two antibodies used in this experiment were ChAT and Allatostatin. These antibodies were chosen because they showed conclusive results in similar experiments using insects.

Acetylcholine Transferase (ChAT)

The ChAT antibody is an immunoglobulin that specifically recognizes acetylcholine transferase (ChAT), an enzyme that is involved in the synthesis of acetylcholine. Acetylcholine is a neurotransmitter that plays a key role in the communication between neurons and other cells in the nervous system (2). The ChAT antibody is often used in research to study the distribution and activity of acetylcholine in the brain and other tissues. The ChAT antibody has also been used in the development of therapies for diseases that involve abnormalities in acetylcholine signaling, such as Alzheimer's disease and myasthenia gravis.

Allatostatin

Allatostatin is a type of neuropeptide hormone that is found in insects and other invertebrates. It belongs to a larger family of molecules called allatostatins, which are involved in the regulation of various physiological processes, including feeding, growth, and reproduction. Allatostatin is produced and released by certain neurons in the brain and other parts of the nervous system, and it acts on target cells by binding to specific receptors on their surface (7). The allatostatin receptor is a protein that is expressed on the surface of cells and is specific to allatostatins. A monoclonal antibody that targets the N-terminal sequence of allatostatin was used as the label.

Hypothesis

The investigational hypothesis was that insects and crustaceans evolved divergently from a common ancestor. Therefore, crustaceans were expected to express allatostatin and ChAT in the same forebrain regions as insects, when exposed to the same antibodies.

Materials

The materials used were: Phosphate Buffer Saline (PBS, Sigma-Aldrich #P4417), 16% Paraformaldehyde (Electron Microscopy Sciences #15710), Triton X-100 (Sigma-Aldrich #G7893), Agarose (Boston Bioproducts #P73050G), Choline acetyltransferase monoclonal antibody (Developmental studies hybridoma bank #ChAT4B1), Allatostatin monoclonal antibody (Developmental studies hybridoma bank #5F10), SYTO™ 13 Green Fluorescent Nucleic Acid Stain (ThermoFisher #S7575), Normal Donkey Serum (NDS, Jackson ImmunoResearch #017-000-121), Cy3 Donkey anti-Rabbit (Jackson ImmunoResearch #711-166-152), Cy5 DonkeyGoat anti-Mouse (Jackson ImmunoResearch #715-175-150) and Mowiol 4-88 (Sigma-Aldrich #81381).

Methods

An immunohistochemical protocol used to test the effect of ChAT and allatostatin antibodies. The first step was to dissect the ghost shrimp and place the extracted brains in a cold fixative. The fixative was prepared using 0.75mL PBS and 0.25 mL of 16% paraformaldehyde. After extracting the brain and placing them in the

fixative, they were kept on ice for 24 hours in microcentrifuge tubes.

After the brains and mushroom bodies had been kept in the fixative for 24 hours, they were washed twice with PBS solution. The washing process included draining the fixative solution from the microcentrifuge tubes and then pipetting in the PBS solution. Once the brain and mushroom bodies were washed, they were embedded in agarose and sliced into thin sheets. The embedding process consisted of placing each individual brain well spaced apart on a petri dish and pouring agarose solution over it. The agarose solution was made using 3.8 g of agarose and 50 mL of double-distilled water. After the agarose had set, the jelly-like substance was removed from the petri dish and each brain was cut into a block using a sharp blade. Then, each block was ready to be sliced.

To slice, a machine called a vibratome was used. Each block would be glued to the surface of the vibratome plate and submerged in PBS to make the slicing smoother. The blocks were cut into 60 μm sections. All the slices from one brain were placed in one well of a 24-well plate and submerged in PBS solution. This process was repeated for all the brains.

After the blocks were sliced and immersed in the PBS solution, they were washed with PBS-TX, a soapy mixture of 50 mL PBS and 250 μL TritonX-100. These tissues were washed and then placed on a shaker so the material could be continuously and evenly distributed. This process was repeated twice. After the second wash, 50 μL of normal donkey serum (NDS) was added to block the antibodies from binding proteins that were not being studied. An hour after the NDS was added, 10 μL of each primary antibody (ChAT and Allatostatin) were distributed to each well. The well plates were covered in foil and left on a shaker overnight.

The next day, the slices were washed using PBS-TX solution 6 times for twenty minutes per wash. The NDS was then added. After an hour had elapsed, 2.5 μL of the secondary antibody, Alexa 647 Goat anti-Mouse was added to each well. The well plates were then covered and left on the shaker again overnight.

The next day, the slices were washed again, but with a normal PBS solution. This process was repeated six times for twenty minutes each. After the second wash, 0.5 μL of Syto13 was added to each well to dye the cell bodies. After the sixth wash, the slices were ready to be mounted on slides. To do this, the slices were carefully removed from their well plates, dried, and aligned linearly on a glass slide. They were then coated in elvanol. The elvanol was made using 5 g Mowiol, 20 ml PBS, and 10 ml glycerol. A micro coverslip was then placed on top of the slide. The elvanol was used as an adhesive substance to bind together the slide and the coverslip. The slides were then examined under the confocal microscope.

Results

Allatostatin

Allatostatin expression was observed in two distinct areas of the ghost shrimp's forebrain. First, in the visual structures of the eye stalks. Specifically, allatostatin was found to be expressed in the medulla region of the optic lobes (Figure 1A, 1B). In crustaceans, the

medulla receives visual information from the retina and then processes and conveys it to structures that integrate it with information from other sensory organs. Expression in the medulla region indicated that allatostatin was involved in visual processing. In pevious research, Kreissl S et. al., found that honeybee brains that were stained with allatostatin antibodies also showed expression in their medulla regions (Figure 1C) (4). This illustrated that allatostatin was used for visual processing in both insects and crustaceans.

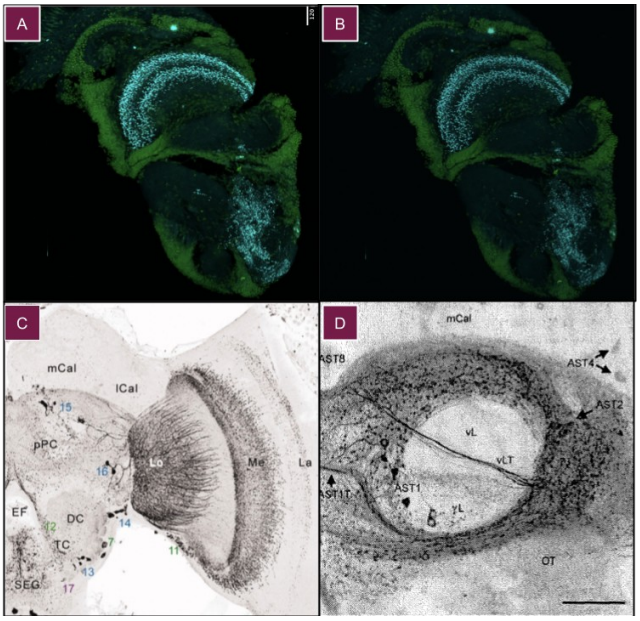

Figure 1. Comparative allatostatin expression in ghost shrimp and honeybees
Blue highlighted regions depict the areas in which allatostatin is expressed in the ghost shrimp. Green highlighted regions indicate the Syto-13 nucleic acid stain. (A, B) Allatostatin expression is clearly seen in the medulla region. Expression is also seen in the lower medulla terminalis. (C) Darkened areas depict allatostatin expression in honeybees. The honeybee model also shows expression in the medulla region of the visual processing area (4). (D) The region of the honeybee brain that surrounds the mushroom body illustrates allatostatin expression.

Second, allatostatin was found in the significantly understudied medulla terminalis (Figure 1A, 1B). Kreissl S et. al., found that allatostatin was also found in a region that surrounds the mushroom body and sends signals to it (Figure 1D) (4). The medulla terminalis in crustaceans is adjacent to the mushroom body. Since allatostatin was found in both of these regions, it is plausable that both of these could be homologous structures. This could indicate that the medulla terminalis plays a role in sending signals to the mushroom body.

Acetylcholine Transferase (ChAT)

ChAT expression was observed in three distinct areas of the shrimp's forebrain. First, in two visual processing structures. Like allatostatin, ChAT was expressed in the medulla of the optic lobes in the eye stalk (Figure 2A, 2B). ChAT was also expressed in another visual processing structure called the lobula (Figure 2A, 2B). Kouji Yasuyama K, et. al. Found that fruit flies expressed ChAT throughout their optic lobes (Figure 2C) (11). Therefore, ChAT is likely to be involved in visual processing for both species of insects and crustaceans.

ChAT expression was also found at low levels in the mushroom body itself of the ghost shrimp (Figure 2A, 2B). However, we cannot be confident in the location of this expression without a second stain that shows the mushroom bodies. Yasuyama's research also demonstrates this phenomenon since their images show light spotting of ChAT in the mushroom body of the fruit fly (Figure 2D) (11). Hence, both shrimp and fruit flies expressed ChAT in the mushroom body region. This also indicates the possible conservation of the mushroom body structure, even though the data needs to be further verified. Regardless, it is tempting to speculate that acetylcholine plays some role in the learning and memory functions of the mushroom body in both insects and crustaceans. However, more research needs to be done to confirm this theory.

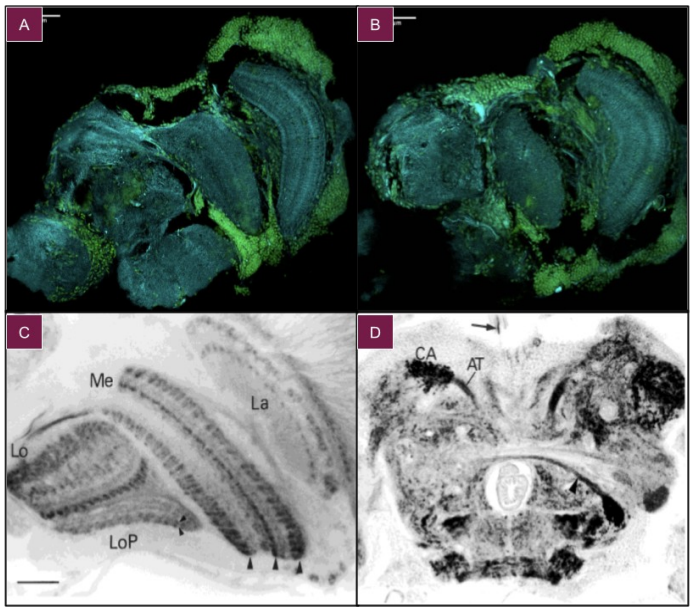

Figure 2. Comparative acetylcholine transferase (ChAT) expression in ghost shrimp and fruit flies.
Blue highlighted regions depict the areas in which ChAT is expressed. Green highlighted regions are a result of the general syto-13 stain. (A, B) ChAT expression is clearly seen in the ghost shrimp medulla and lobula regions. Expression is also seen to a lesser extent in the mushroom body. (C) Darkened areas depict allatostatin expression insects. The honeybee model also shows expression in the medulla and lobula regions of the visual processing area (11). (D) ChAT expression is localized in the mushroom body of the fruit fly brain. One highlighted region, labeled as CA, is the calyx of the mushroom body (11).

Discussion

Allatostatin

The results supported the hypothesis that visual processing areas are conserved in crustaceans and insects. Allatostatin was observed in the medulla region for both the ghost shrimp and in honeybees. Thus, the medulla regions of both insects and crustaceans are likely conserved. In regards to the conservation of learning and memory structures, allatostatin was labeled in the medulla terminalis area of the ghost shrimp and a region of the honeybee that sends signals to the mushroom body. In crustaceans, some neural networks are relatively straightforward to understand. Researchers are able to pinpoint connections between those structures to then identify their functions. However, the medulla terminalis consists of an overwhelming number of seemingly disorganized neural connections. This has made it challenging for researchers to determine the exact function of the medulla terminalis. However, this research indicates a possible theory for its function. Since allatostatin was found in this region in both the crustaceans and insects, it may be speculated that these structures are homologous. This means that allatostatin might play a role in the signaling and communication for learning and memory processes and that this communication is conserved in both crustaceans and insects.

ChAT

The results supported the hypothesis for the visual processing areas. ChAT is conserved in the medulla and lobula regions of ghost shrimp and honeybees. The lobula is also involved in the visual processing of specific features, such as color, contrast, and movement, and in controlling visual attention and the selection of relevant visual information. Therefore, the expression of ChAT provides further evidence for the conservation of visual processing structures in insects and crustaceans. For the learning and memory aspect of the hypothesis, ChAT was found to be expressed in the mushroom body as well. This expression is also demonstrated in the fruit fly model. This lends credence to the hypothesis that there is a conservation of the mushroom body in insects and crustaceans. However, it cannot be definitively concluded that ChAT is expressed in the learning and memory structures. Further research with another antibody may confirm this hypothesis.

Context

A key question in the overall study of crustacean and insect brain evolution is whether the mushroom body developed independently in insects and crustaceans or if it diverged evolutionarily from a common ancestor. Previous research indicated the latter conclusion and this research corroborates it. In a study by Wolff and Strausfeld, this conservation is demonstrated through a comparison of expression in the brains of animals from different phyla using a different set of antibodies. Their research provides evidence of “mushroom body-like centers sharing a neuroanatomical ground pattern and proteins required for memory formation” (10). These researchers have further lent credence to this theory by studying mushroom-body-like structures in crustaceans (9).

Conclusion

The results presented in this paper support the hypothesis that insects and crustaceans evolved

divergently from a common ancestor and therefore express allatostatin and Choline acetyltransferase (ChAT) in the same forebrain regions. There are some nuances that can be addressed using further experimentation. In allatostatin and acetylcholine transferase, the results clearly show evidence for the conservation of visual processing structures among insects and crustaceans. For allatostatin, potential conservation is shown for the medulla terminalis. This conservation can also help determine the medulla terminalis' function since more is known about the function of insect structures. Some expression of ChAT could be seen in the mushroom body itself for both shrimp and fruit flies. Further experimentation need to be performed to definitively demonstrate a conservation of learning and memory processes in crustaceans and insects. Part of this further experimentation could include pairing ChAT with another antibody against DC0; a protein that is known to be expressed in the mushroom body region. It is important to understand brain evolution in order to draw conclusions about the origins of the brain. While the conservation of these two proteins cannot conclusively prove the divergent evolution of brain structures, they do add to the larger body of evidence. By identifying the common ancestry of structures in arthropods, features can be identified that may be present in homologous structures in mammals. This provides insight into how the functioning of the mammalian brain and how it can go awry, potentially leading to new treatments and therapies for neurological disorders. Additionally, understanding the principles for building a brain structure for vision or learning can help in developing better models for artificial intelligence and machine learning. This could lead to more advanced and effective AI systems.

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