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Research Article

Pain Perception: Investigating Links Between Pain Transmission and CCK(+) Neurons, with Regard to the Opioid Crisis

—Sumeet Panesar and Zachary Tepper

With dependence upon opioids, such as codeine, morphine, and heroin, steadily increasing amongst the American public, the withdrawal symptoms associated with disuse are receiving much more attention. From its regular appearance in pop culture songs to serving as the underlying theme in many independent films, opioid addiction and withdrawal has become part of many Americans' everyday life. Often, patients experiencing the withdrawal symptoms of opioid dependence complain of the devastating impact of hyperalgesia (Angst, Koppert, Pahl, Clark, & Schmelz, 2003). Hyperalgesia is a heightened sensitivity to pain, and it is very common in those who quit using opioid medications. Opioid dependence has increased in tandem with opioid prescription rates, and it's only right that research into the complicated processes of addiction and withdrawal keep up with the high usage levels.

Unfortunately, scientific understanding of the complicated actions of opioid painkillers and the implications of their chronic use is lacking. Our research was directed by the interests of our neuroscience lab mentor, Dr. Arturo Andrade, who is an avid researcher in the field of pain perception. Our project, funded through the Summer Undergraduate Research Fellowship (SURF) program at the University of New Hampshire, aimed to identify particular neurons, or cells of the nervous system, that are involved with the withdrawal symptoms of opioid dependence. More specifically, our research aimed to find, or localize, neurons in the sensory pathways that express a protein called cholecystokinin (CCK), which has been associated with the hypersensitivity to pain that is symptomatic of opiate withdrawal (Angst, Koppert, Pahl, Clark, & Schmelz, 2003). CCK is a protein; as such, the blueprint for producing it resides in the



The authors analyzing brain slices using the confocal microscope. *Sumeet (left) Zach (right).*

genetic code. Most cells don't utilize this part of their genetic code, and thus most cells don't make, or express, CCK. By targeting the CCK protein, we hoped to isolate those particular cells that are responsible for the hyperalgesia that so commonly occurs during opiate withdrawal. We aimed to provide insight into the biological mechanism(s) responsible for opioid-induced hyperalgesia by locating the distribution of these neurons throughout the central nervous system, and then beginning to characterize their function. These findings may serve to undermine high levels of opioid prescriptions or suggest alternative drug therapies for combating hyperalgesia.

CCK and the Pain Perception Mechanism of the Brain and Spinal Cord

The protein cholecystokinin (CCK) is an enzyme that was first identified in the small intestine of mammals. In the gut, CCK plays a role in signaling the release of bile from the gallbladder and digestive enzymes from the pancreas. In this way, CCK acts as a precursor to the breakdown of fat and protein. Current evidence, which has directly influenced the direction of our research, indicates that CCK also plays a role in the experience of painful sensations (Wiesenfeld-Hallin, Xu, & Hokfelt, 2002).

The central nervous system, consisting of the brain and spinal cord, plays a necessary role in making sure that exposure to pain is perceived correctly. In order to do so, the nervous system is organized in a very particular manner. The spinal cord can be divided into two sections, ventral (front/anterior) and dorsal (back/posterior). The ventral portion is commonly referred to as the "motor" area because it is home to the neurons that are responsible for controlling our muscles. The ventral area allows us to move our bodies how we want to. The dorsal portion of the nervous system is known as the "sensory" area because it receives information from the periphery (anywhere outside the brain and spinal cord) and transmits it to the brain so that we can perceive it. If an individual were to step on a pin, for example, it is the responsibility of the dorsal area of the spinal cord to relay that information to the brain to be perceived as pain. However, the spinal cord is limited in that it receives information only from below the neck.

In order to perceive information from the neck and above, we rely on an area of the brainstem called the sensory nuclei of the trigeminal nucleus. The trigeminal nucleus consists of three clusters of neurons in the brainstem. Two of these clusters are dedicated solely to sensory perception, while the third is split between sensory perception and motor control. The brainstem is different from the spinal cord; it can be considered a transitional point between the spinal cord and the brain itself. Instead of being organized by ventral and dorsal areas, the brain stem is organized from medial (central) to lateral (outer). The sensory areas of the brainstem are found in the outermost area. A cross-section of the brain stem would look like the letter "O." The outer ring of the "O" would be the sensory region and the inner portion would be the motor region. Visualizing the spinal cord and brainstem as a stack of Os, the sensory pathways travel vertically through the outer portion of the Os. Thus, sensory information from the neck, face, and head (including pain) must be transmitted through the outermost cell bodies of the trigeminal nucleus.

Research into the sensation and perception of pain has highlighted that neurons that express CCK (hereon termed CCK(+) neurons) play an important role in the acute tolerance that is observed in

response to analgesic opiate medicine (Kissin, Bright, & Bradley, 2000). In other words, CCK(+) neurons in the sensory areas of the central nervous system are responsible for making a patient need larger and larger doses of opiates in order to relieve the same amount of pain. Before delving into how to tackle such an issue, a thorough understanding of how neurons work and how they communicate is needed.

Neurons in the body communicate through neurotransmitters, which are chemical compounds transmitted from one neuron to another. In this way, information passes throughout the nervous system. CCK(+) neurons in the central nervous system belong to one of two main subcategories, depending on which neurotransmitter they use to relay information: glutamatergic or GABAergic (GABA is an acronym for gamma aminobutyric acid). For our experiment, we focused solely on the GABA neurotransmitter.

A neurotransmitter is either excitatory or inhibitory. Excitatory neurotransmitters increase the likelihood that a neuron will continue to pass along information. Inhibitory neurotransmitters decrease this likelihood. Glutamate is the most common excitatory neurotransmitter in the central nervous system, while GABA is the most common inhibitory neurotransmitter in the nervous system. The goal of our research was to localize CCK(+) cell bodies within the sensory pathways and categorize them as either excitatory or inhibitory; we also aimed to identify the specific neurotransmitters that these neurons used to communicate.

Transgenic Mice Background and Staining Techniques

In order to localize, or find, these neurons, we acquired two separate breeds of genetically modified mice. When bred together, the heterozygous offspring of these two breeds contained a label in its genetic code. This label, known as TdTomato, emits a bright red light under proper conditions. The genetic modifications of these mice ensured that the TdTomato label was paired with the expression of CCK. That way, any cell that expressed CCK would also express TdTomato, and would be identifiable by its red color when viewed under a confocal microscope. Unfortunately, CCK is found throughout the entire anatomy of the neurons that express it, and not just the cell bodies. Thus, although the cell bodies themselves will have a red label, so will the long axons and dendrites that project from the cell body. This creates some confusion, because neurons are extraordinarily close together, which may cause one cell's dendrites, axons, and cell body to overlap with another cell's dendrites, axons, and cell body. As a result, it becomes very difficult to differentiate which cells truly express CCK and which do not. While TdTomato helped identify the general area within the spinal cord and trigeminal nucleus where CCK(+) could be found, we needed to be more specific.

In order to identify which neurons were truly expressing CCK, we used the fluorescer SYTO 13. A fluorescer is a molecule that emits light under the proper conditions. We applied SYTO 13 through a specific staining process. SYTO 13 is useful because it enters the cell body and binds to the nucleic acids of DNA and RNA. SYTO 13 produces a bright green light, indicating the presence of a neuron's cell body. Thus, any CCK(+) cell body would be labelled by the red TdTomato as well as the green SYTO 13, producing an identifiable yellow/orange color. Within the sensory region of the spinal cord

and trigeminal nucleus, we were able to distinguish clearly the yellow/orange color under the confocal microscope.

After we localized the CCK(+) neurons with the yellow/orange color, we used antibody staining techniques to indicate the presence or absence of the GABA neurotransmitter in the CCK(+) cell bodies. Antibody staining uses immune-system proteins to identify a specified target. Our target was GABA, and we used an antibody known as Anti-GAD 65/67 to indicate its presence. Under the confocal microscope, both SYTO 13 and the Anti-GAD stains emit a green fluorescence. Therefore, separate samples were used to stain with SYTO 13 and Anti-Gad. Staining for GABA, if both the spinal cord and trigeminal nucleus demonstrated a positive result, there would be evidence that CCK(+) neurons in the sensory regions of the pain pathway communicate through the use of GABA-mediated inhibition. This information could guide future studies into the biological and electrical properties of the CCK(+) neurons, and thus inspire insight into the behavioral implications involved in the sensation of pain.

Mysteries Behind the Fluorescing Light Revealed

Through the use of confocal microscopy, we identified the presence of CCK(+) neurons in the sensory areas of the spinal cord and trigeminal nucleus (Figure 1). The red fluorescence from TdTomato indicated the presence of CCK. Green SYTO 13 fluorescence indicated the presence of neuronal cell bodies. Therefore, the co-presence of the red TdTomato and green SYTO 13 emissions, as shown by a yellow/orange color, indicated a CCK(+) neuronal cell body.

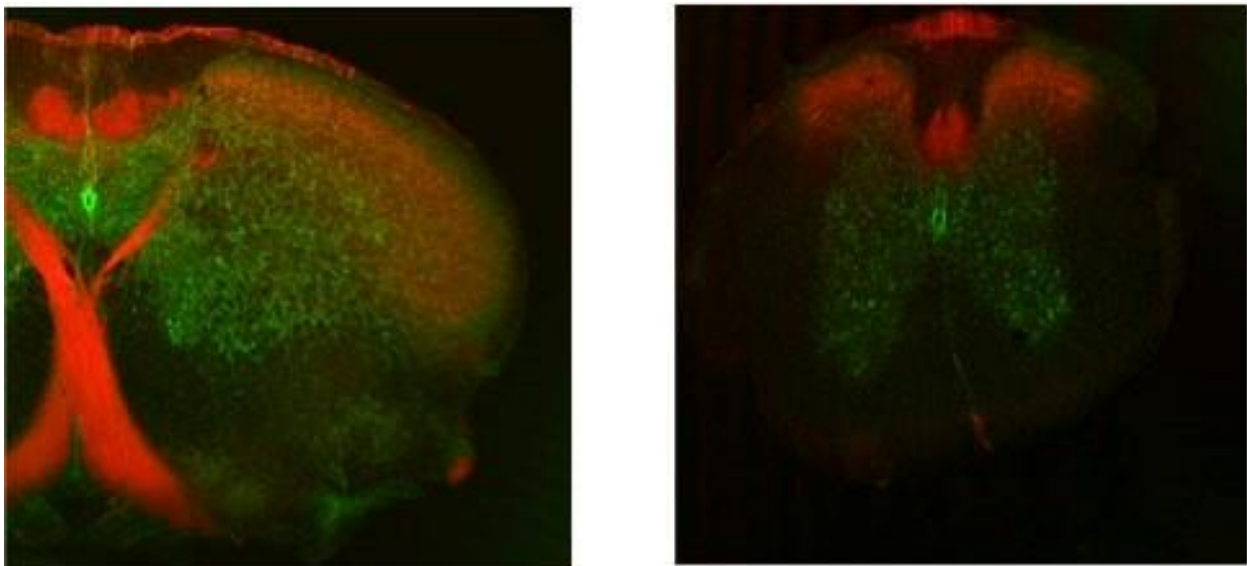


Figure 1: The trigeminal nucleus (left) shows yellow/orange fluorescence in the sensory region when stained with SYTO 13. The spinal cord (right) also shows a yellow/orange fluorescence in the sensory region indicating CCK(+) neurons.

We were curious to see if there were any differences in the amount of CCK(+) neurons in varying regions of the spinal cord, so we examined the lumbar, thoracic, and cervical regions (Figure 2). Individual slices from each of the three regions demonstrated an equal amount of red fluorescence throughout the sensory areas of the spinal cord. When stained with SYTO 13, there was also a bright green fluorescence throughout all of the gray matter (neural tissue where neuronal cell bodies are located). The images indicate an overlap of the red/green fluorescence (as indicated by a yellow/orange color) in the dorsal areas of the sensory regions, specifically lamina (layers) II/III (Figure 1). The lamina II/III of the spinal cord, also known as the substantia gelatinosa, is involved with the transmission of painful signals from the body, where they occur, to the brain, where they are perceived (Cervero & Iggo, 1980). This finding supports our hypothesis that CCK(+) neurons reside in the most dorsal areas of the sensory region of the spinal cord. This finding also supports the hypothesis that CCK(+) neurons are involved with the transmission of painful signals from the body. After analyzing the spinal cord, our attention shifted toward the trigeminal nucleus in an attempt to localize CCK(+) neurons there.

Figure 2: Image of a dissected mouse spinal cord. From top to bottom the sections are cervical, thoracic, and lumbar.



The neuronal cell bodies in the trigeminal nucleus are responsible for the perception of pain from the neck and head (Wilcox et al., 2015). The caudal (lower) portion of the trigeminal nucleus contains a cluster of cells known as the spinal nucleus of the trigeminal nerve. This cluster of cells is responsible for the transmission of painful signals, as opposed to other sensory information (such as touch and pressure) that is also carried through the trigeminal nucleus (Wilcox et al., 2015).

By staining the trigeminal nucleus with SYTO 13 we were able to see a bright yellow/orange fluorescence in the lateral, or outer, region of the brain stem. The trigeminal nucleus sample contained both rostral and caudal (higher and lower, respectively) slices. We observed an increased amount of yellow/orange fluorescence in the rostral slices compared to the caudal slices, meaning there are more CCK(+) cell bodies in the higher sections of the trigeminal nucleus.

These findings support our hypothesis that CCK(+) neurons are involved in the perception of pain from the neck and head. We then turned to additional immunostains to determine whether co-localization actually existed within the CCK(+) cells.

Anti-GAD65/67 and Anti-GABA immunostains, which fluoresce bright green, demonstrated fluorescence throughout the gray and white matter of both the spinal cord and trigeminal nucleus (Figure 3). Primary and secondary antibody staining of the trigeminal nucleus slices showed many red fluorescent neurons, fewer green fluorescent neurons, and very few yellow/orange fluorescent neurons.

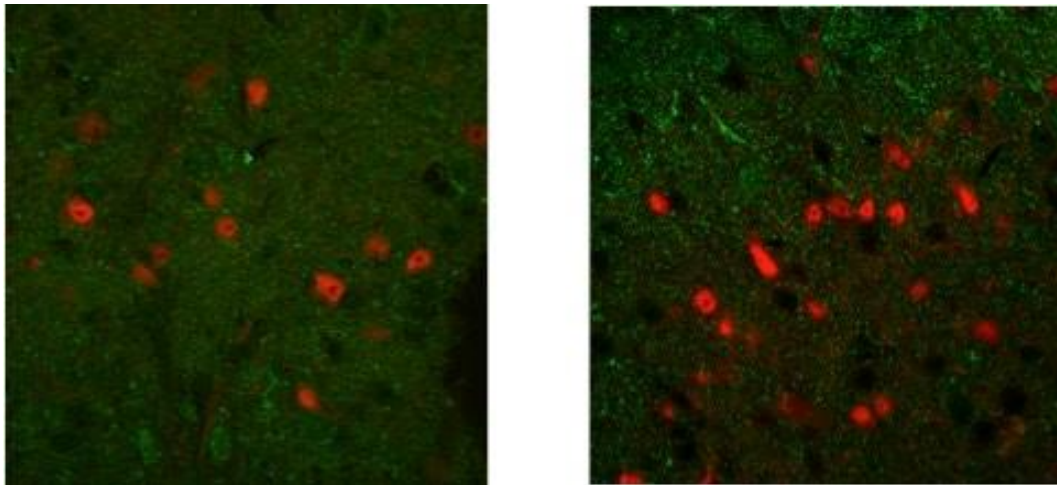


Figure 3: The trigeminal nucleus (left) with the Anti-GABA stain shows a mix of red, green, and yellow/orange neurons. The spinal cord (right) with the Anti-GABA stain shows a similar mix of neuronal fluorescence.

Thus, in both the spinal cord and trigeminal nucleus, there was very little co-localization of CCK and GABA. Rather, CCK was generally confined to the sensory regions, while GABA was spread throughout. We identified individual GABAergic neurons in the sensory regions, but there was minimal overlap between GABA and CCK. This does not support our hypothesis that CCK(+) neurons act through the transmission of GABA, but does not eliminate the possibility that these neurons are inhibitory. Although GABA is the most common inhibitory neurotransmitter in the central nervous system, there are numerous other inhibitory chemicals that may linked CCK(+) neurons or to pain inhibition (Beato & Nistri, 1998; Surmeier, Ding, Day, Wang, & Shen, 2007). Future experiments need to identify the exact neurotransmitter(s) that is/are released from CCK(+) neurons, as well as the effects that these neurotransmitters have on the rest of the central nervous system.

Final Thoughts and Future Endeavors

Our research has progressed quite far given the time frame of our experiments. Although we answered several questions throughout the early experimentation phase, many more uncertainties arose that will need further clarification. Localizing CCK(+) neurons is merely a scratch on the surface. Our research will not stop here and will continue for many semesters to come.

At the time we published this article, we had spent only a couple of months compiling data on this research project. In that time, we took away much more than expected. Throughout the project, we were able to apply our in-class knowledge and have many intellectually stimulating discussions; this experience will benefit us for years to come. For Sumeet, who plans to pursue a career in dental medicine, this experience has been invaluable, as he will be relied upon for his acute awareness and understanding of neck/head pain. This experience has also been advantageous to Zachary, who intends to practice medicine for the United States military, where an intimate understanding of the central nervous system will serve him well.

With regard to the overall aim of the project, we hope that by building upon our current research we eventually will be able to understand the underlying mechanisms that contribute to pain perception and modulation within the CCK(+) neurons. With this new information, it would be easier to specifically target the mechanisms that contribute to the hyperalgesic effects of opioid withdrawal and to alleviate these symptoms altogether.

There are many individuals who deserve our highest regards; without them our experiment and research could never have begun. We would like to thank first and foremost our lab mentor, Dr. Arturo Andrade, for helping originate the idea and aid in the experimental setup. We would also like to thank the Hamel Center for Undergraduate Research for giving us the opportunity to receive a Summer Undergraduate Research Fellowship to work in our lab over the summer. Finally, we would like to thank our families for their continuous support and encouragement throughout the duration of the grant.

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Author and Mentor Bios

Neuroscience major **Sumeet Panesar**, from Hooksett, New Hampshire, realized at the beginning of his research project that there was a lot to learn about pain transmission: “The process is quite complex and there are many mysteries behind the pain-sensing neurons, mysteries we are still trying to uncover.” But with the support of his research partner, Zach, their mentor, Dr. Arturo Andrade, and a Summer Undergraduate Research Fellowship (SURF), Sumeet tackled many concepts that aren’t covered in a normal classroom setting. The hands-on experiments in the lab, he says, “helped cement critical ideas within our minds.” His new understandings about the functions in the trigeminal nucleus, which controls pain perception from the neck and head, will be valuable in his future work as a dentist or dental researcher. He hopes that sharing his research through *Inquiry* will show others “exactly what undergraduates are capable of accomplishing.” Sumeet will graduate from the University of New Hampshire in May 2018.

Zachary Tepper, a neuroscience and behavior major from Danbury, Connecticut, says that “working in the lab and performing real experiments has taught me far more about my field of study than I've learned in classroom lectures.” Zach’s research project with Sumeet was suggested by their mentor, Dr. Arturo Andrade, and began with a 2016 Summer Undergraduate Research Fellowship (SURF). Their research has extended beyond their summer work and has become the basis for Zach’s senior thesis for the University Honors Program. In fact, Zach considers his research as still unfinished, but he points out that this is one of the things he learned about the research process: “There will always be more questions to tackle, and they often arise unexpectedly.” After graduating from the University of New Hampshire in May 2017, Zach plans to teach English overseas and then look into attending medical school through the United States armed forces. The “hands-on skills and mental exercises” he learned by studying pain transmission with Sumeet and Dr. Andrade will be valuable in any future endeavor.

Arturo Andrade is an assistant professor of neurobiology in the Department of Biological Sciences at the University of New Hampshire (UNH). He teaches introductory and advanced courses in neuroscience and his research focuses on the neurobiology of pain and anxiety. Relatively new to UNH, Dr. Andrade first met Zach when his lab was still full of unpacked boxes. He mentored many students at his previous institution, Brown University, but Zach and Sumeet were his first mentees at UNH. Dr. Andrade admires the persistence that Zach and Sumeet demonstrated despite setbacks during their research. He points out that “their resilience to frustration will absolutely make them successful professionals.” Dr. Andrade feels that writing for *Inquiry*’s audience is very beneficial for students in scientific fields, as there is always a need to be able to explain their complicated work to those without the same technical knowledge.