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Localization of CCK(+) Neurons in the Sensory Pathways of Mus Musculis

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Localization of CCK(+) Neurons in the Sensory Pathways of Mus Musculis

Abstract
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. Our research identifies neurons that are implicated in the hyperanalgesic response to the cessation of opiate-medication after dependence has been established. These neurons are identified by the Cholecystokinin protein (CCK). The first chapter of this research focuses on localizing these neurons in regions of the central nervous system that are responsible for transducing painful signals from the periphery to the brain. Our research supports the hypothesis that neurons with high levels of CCK expression (CCK(+) neurons) are involved in the transmission of pain from the periphery to the brain. We did not find that the CCK(+) neurons communicate through GABA neurotransmission.

The second chapter of this research focuses on an experimental observation that CCK expression was localized in the vasculature of the spinal cord, but not cortical tissue. Lipophilic vasculature staining through the use of DiI provided contrary results, and indications that CCK-expression may be capillary-dependent.

Keywords
Cholecystokinin, Vasculature, Transgenic, Opiate, Opioid, Analgesia

Subject Categories
Medicine and Health Sciences

This senior honors thesis is available at University of New Hampshire Scholars' Repository: https://scholars.unh.edu/honors/346
LOCALIZATION OF CCK(+) NEURONS IN THE
SENSORY PATHWAYS OF MUS MUSCULIS

Zachary Tepper

A Thesis submitted to the faculty of
University of New Hampshire
In partial fulfillment of the requirements for the degree of

Bachelors of Science

Department of Biological Sciences

2017, May
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Finally, I owe a great debt to my friends and family. To all those too numerous to mention individually, you are the structure and support of everything I value. Without you, I would be far less fortunate, and this project may never have taken form. Thank you all.
ABSTRACT

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. Our research identifies neurons that are implicated in the hyperalgesic response to the cessation of opiate-medication after dependence has been established. These neurons are identified by the Cholecystokinin protein (CCK). The first chapter of this research focuses on localizing these neurons in regions of the central nervous system that are responsible for transducing painful signals from the periphery to the brain. Our research supports the hypothesis that neurons with high levels of CCK expression (CCK(+) neurons) are involved in the transmission of pain from the periphery to the brain. We did not find that the CCK(+) neurons communicate through GABA neurotransmission.

The second chapter of this research focuses on an experimental observation that CCK expression was localized in the vasculature of the spinal cord, but not cortical tissue. Lipophilic vasculature staining through the use of DiI provided contrary results, and indications that CCK-expression may be capillary-dependent.
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INTRODUCTION

Rationale

With incidences of opioid-dependence steadily increasing amongst the American public, the withdrawal symptoms associated with disuse are receiving much more attention. From regular appearances in pop culture songs to the underlying theme in many independent films, opioid addiction and withdrawal have become common conflicts that Americans are exposed to in everyday life. 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.

Unfortunately, scientific understanding of the complex actions of opioid painkillers and the implications of chronic use are lacking. This research project aims to identify particular neurons that are involved with the withdrawal symptoms of opioid-dependence. More specifically, this research aims to localize neurons in the sensory pathways that express cholecystokinin (CCK), a protein that has been implicated in the regulation of opiate-mediated pain transmission. By locating the distribution of these neurons throughout the central nervous system, and then beginning to characterize their function, we aim to provide insight into the biological mechanism(s) responsible for the regulation of opiate-mediated analgesia. These findings may serve to undermine high levels of opioid prescriptions, or suggest alternative drug therapies for combating pain pathologies.

Previous Research into the Role of CCK

Cholecystokinin (CCK) is an enzyme that was first identified in the duodenum (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. Recent evidence indicates that CCK also plays a role in the
experience of painful sensations. Such research implicates neurons that express CCK (hereon termed CCK(+) neurons) in the sensation of hyperalgesia in response to the acute discontinuation of opiate medication\[2\]. Inversely, while using opiate medication, CCK may be involved in depressing the effects of opiate-induced analgesia, leading patients to require larger and larger doses to alleviate the same level of pain.

**Anatomy/Physiology**

The central nervous system (CNS), 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 CNS is organized in a very particular manner. The ventral, or motor, portion of the spinal cord is home to the neurons that are responsible for controlling our muscles. The dorsal, or sensory, portion of the spinal cord receives information from the periphery and transmits it to the brain in order to perceive it \[4\]. However, the spinal cord is limited in that it only receives information from below the neck.

In order to perceive information from the neck and above, we rely on the sensory nucleus of cranial nerve 5, also known as the sensory region of the trigeminal nucleus. The trigeminal nucleus is a cluster of neurons located in the brainstem, which is organized from medial to lateral. The sensory areas of the brainstem are found most lateral \[5\]. Thus, sensory information from the neck, face, and head (including pain) must be transmitted through the outermost cell bodies of the trigeminal nucleus.

**Neuronal Communication**

Neurons in the body communicate through the use of neurotransmitters. Neurotransmitters are chemical compounds that are transmitted from one neuron to another; in this way, information is passed throughout the nervous system \[6\]. CCK(+) neurons in the CNS
belong to one of two main subcategories: glutamatergic or GABAergic, depending on which neurotransmitter they use to relay information \(^7\)[8]. Although neurotransmitters may have different actions depending on their location, this experiment targets glutamate and GABA because they are highly abundant and highly specific. A neurotransmitter will either be excitatory, like glutamate, or inhibitory, like GABA. Excitatory neurotransmitters increase the likelihood that a neuron will generate an action potential and propagate a signal. Inhibitory neurotransmitters decrease the likelihood of signal propagation. Glutamate is the most common excitatory neurotransmitter in the CNS, while GABA is the most common inhibitory neurotransmitter in the CNS \(^9\).

**Research Objectives**

The goal of this research experiment is to localize CCK(+) cell bodies within the sensory pathways and categorize them as either glutamatergic or GABAergic. In order to localize these neurons, genetically-modified mice (CCK-TdTomato) were bred. Neuronal tissue from these mice was stained with various immunohistochemical reagents and analyzed through confocal microscopy.

After the CCK(+) neurons had been localized, antibody staining techniques indicated the presence or absence of the GABA neurotransmitter. Successful acquisition of this information will provide data for future studies into the biological and electrical properties of these neurons, and thus inspire insight into the behavioral implications involved in the sensation of pain.
RESEARCH QUESTION

Are CCK(+) neurons in the spinal cord and trigeminal nucleus involved in the transmission of pain? What biological mechanisms do CCK(+) neurons use to communicate and modulate the pain pathway?

MATERIALS & METHODS

Reagents

- Isoflurane (B72J15B, Piramal)
- Euthasol (200-071, Virbac)
- PBS (P3813, Sigma-Aldrich)
- Paraformaldehyde (HT501128-4L, Sigma-Aldrich)
- SeaPlaque Brain Slice Agar (100262, MP Biomedicals)
- Low Melting Point Agarose (50101, Lonza)
- TEA Buffer (1610773, BioRad)
- Aluminum Foil (obtained at retail store)
- AAA Battery (obtained at retail store)
- Superglue (obtained at retail store)
- Triton X-100 (T8787, Sigma-Aldrich)
- SYTO 13 Immunohistochemical Stain (S7575, ThermoFisher)
- Bovine Syrum Albumin (BSA) (A9647-50G, Sigma-Aldrich)
- Anti-GAD65/67 Primary Antibody (PA1-84572, ThermoFisher)
- Anti-GABA Primary Antibody (AB175, Sigma-Aldrich)
- Goat Anti-Mouse Secondary Antibody (A32723, ThermoFisher)
- Donkey Anti-Rabbit Secondary Antibody (A-21206, ThermoFisher)
• Vectashield Mounting Medium for Fluorescence (H-1400, Vector Laboratories)
• Clear nail polish (obtained at retail store)

**Equipment**

• 26 ½ Gauge Needles
• Mini-Pump Variable Flow Perfusion Device (Fisher Scientific)
• Dissection Equipment
• Plexi-glass Chamber
• Three-way stopcocks
• 50mL conical tubes
• 12-well plate
• Soft-tip Paintbrush
• Microscope slides
• Standard glass coverslips (no. 1.5)
• Vibratome (VT1000s, Leica)
• Confocal Microscope (LSM 510 Meta, Zeiss)

**REAGENT SETUP**

**2% Brain Slice Agarose**

Add .4g Brain slice agar to 20mL of 1x TAE Buffer. Pour into petri dish. Store at 4 °C.

**2% Low Melting Agarose**

Use microwave to dissolve 0.2g in 10ml 1x TAE Buffer. Cool at RT and use immediately.
0.2% PBT

Mix 1mL Triton X-100 detergent with 500mL of PBS solution. Store at Room Temperature.

SYTO 13 Work Solution

Dilute 5mM stock 1:10 in PBT.

5% BSA Work Solution (for 12 wells)

Dissolve 0.3g of BSA stock in 6mL of 0.2% PBT.

PROCEDURES

Cardiac Perfusion

1. In ice bucket, cool PBS and PFA in separate 50mL conical tubes
2. Fill mini-pump tubes with respective liquid; ensure no air is in lines
3. Saturate plexi-glass chamber with Isoflurane
   a. Wet tissue paper in 50mL conical tube with Isoflurane and place in chamber
4. Prepare syringe with Euthasol
   a. .05mL for female mice; .07mL for male mice
5. Place mouse in Isoflurane chamber until breathing is slow
6. Remove mouse by grasping the neck fur; place on lab mat and check paw reflex by pinching any of the paws
   a. If the animal reacts, anesthetize further; if not, proceed.
7. Inject the mouse intraperitoneal with Euthasol and place on dissecting tray; hold the mouse in place with its tail until euthanasia has occurred
8. Pin the mouse through its four paws, leaving the thoracic cavity exposed

9. Open the thoracic cavity to expose the heart by first cutting through the skin, then through the viscera

10. Pull ribcage back and ensure the heart is fully accessible

11. Insert 26 ½ gauge needle into the left ventricle while supporting with hemostat; once punctured, clamp with hemostat

12. Cut right atrium with scissors and begin perfusion (at slowest possible rate)

13. Perfuse with cold PBS until perfusate runs clear (at least 10 minutes)
    a. Intestines and liver will turn a clear color, indicating a successful perfusion

14. Switch to cold 4% PFA and perfuse for 10 minutes, then switch back to PBS for 10 minutes

15. Collect brain and spinal cord tissue and place in 4% PFA overnight at 4 °C to complete fixation

16. Dispose of mouse accordingly

**Spinal Cord Prep for Slicing**

1. Tear off thin strip of aluminum foil approx. 4 inches long

2. Roll aluminum foil around AAA battery, leaving positive terminal exposed
    a. Fold foil around the bottom of the battery and press against lab bench to ensure a closed bottom

3. Remove battery from foil; fill foil cylinder with 2% low melting agarose, leaving approx. 1cm for displacement

4. Using forceps, embed spinal cord lengthwise in liquid agarose and allow to cool, ensuring the tissue remains upright and parallel to cylinder walls
**Tissue Slicing**

1. Prepare Vibratome with PFA well
2. Soak razorblade in ethanol for 2 minutes
3. Using forceps, evenly attach blade to PFA-specific mount and screw into place
4. Cut brain slice agarose into two rectangles, mounting a base and backbone to secure animal tissue in Vibratome well
   a. Mount with the minimum amount of superglue to ensure agarose is in place, but not overly saturated
5. Prepare tissue for sectioning
   a. Trim brain with razor to ensure a flat surface for mounting
   b. Remove agarose-embedded spinal cord and ensure a flat surface for mounting
6. Place a conservative amount of superglue on top of the base piece of agarose
7. Using a spatula, add brain or spinal cord flush with the back piece of agarose, directly centered on base agarose
8. Fill well to the brim with PBS, making sure the blade and tissue are submerged
9. Set the Vibratome to 100µm per slice
10. Move blade close to the top of the tissue, set start position
11. Slice through the top of the tissue, setting the endpoint within the back piece of agarose
12. Hit the start button to automatically slice 100 microns below last cut
13. Save desired slices in 12-well plate with 1mL of PBS in each well
   a. Add slices to wells using soft-tip paintbrush
      i. Approx. 3-4 brain slices per well
      ii. Approx. 4-5 spinal cord slices per well
**SYTO 13 Staining**

All washes are 10 minutes long with 1mL of wash per well; washes and incubation take place on rotor at low speed (constant agitation)

1. Wash slices 2 times with 1mL 0.2% PBT
   a. Washes are done by discarding liquid into waste and adding 1mL of wash
   b. Always cover the 12-well plate with aluminum foil to prevent light exposure
2. After second wash, remove remaining PBT
3. Add 500µL 0.2% PBT
4. Add SYTO 13 work solution, allowing for 500nM final concentration
5. Incubate for 1 hour at room temperature
6. Wash 3 times with 0.2% PBT
7. Mount slices on microscope slides

**Immunohistochemical Staining Protocol**

All washes are 10 minutes long with 1mL of wash per well; washes, incubations, and washes take place on rotor at low speed (constant agitation)

1. Add 1mL of PBS to each well of a 12-well plate
2. With soft-tip paintbrush, gently add tissue slices to wells and sink to the bottom
3. Wash slices 1 time in PBS and 2 times in 0.2% PBT
4. Block for 2 hours at room temperature in 1mL 5% BSA/PBT per well
   a. 5% BSA/PBT made by dissolving .6g BSA in 12mL of 0.2% PBT
5. Leave slices in blocking buffer overnight at 4 °C with constant agitation
   a. Using a moist Kimwipe and Parafilm, create a humidity chamber by taping the Kimwipe to the roof of the 12-well plate and ensuring a good seal
6. After 36 hours, remove 12-well plate from cold room

7. Wash slices 3 times in PBT

8. Add 500 µL 5% BSA/PBT per well

9. Add primary antibody at room temperature and block for 1 hour
   a. 1µL per well for Anti-GABA (~1:500 dilution)
   b. 2.5µL per well for Anti-GAD65/67 (~1:200 dilution)

10. Leave primary antibody on shaker at 4 °C for 36 hours
    a. Create humidity chamber

11. Wash 3 times in 0.2% PBT

12. Add 500µL 0.2% PBT per well

13. Add 2.5µL of secondary antibody per well (~1:200 dilution)
    a. Goat Anti-Mouse for Anti-GABA
    b. Donkey Anti-Rabbit for Anti-GAD65/67

14. Leave at room temperature for 3 hours with constant agitation

15. Wash 3 times 0.2% PBT; 1 time PBS

16. Mount slices on microscope slides

**Microscope Mounting**

1. After mounting slices with soft-tip paintbrush, remove excess PBS from slide with Kimwipe

2. Add Vectashield drop-wise to ensure all tissue slices are protected from fading

3. Mount coverslip and label slide

4. Wrap in aluminum foil to protect from light exposure

5. Store at 4 °C for 30 minutes
6. Cover edges of coverslip with a thin layer of clear nail polish to increase adhesion and protect tissue from dehydration

7. Store at 4 °C in black box to prevent light exposure

**Confocal Microscopy**

1. Setup LSM 510 Meta microscope for multi-channel imaging
   a. For SYTO 13 collection, use 488nm excitation and collect using BP 505-530 filter
   b. For TdTomato collection, use 543nm excitation and collect using LP 560

2. Use on-screen command to select the desired objective

3. Set pinholes for each channel to 1

4. Isolate area of interest in the optical objective

5. Select ‘Find’, to collect an image
   a. adjust offset/gain as needed
RESULTS

Through the use of confocal microscopy, the presence of CCK(+) neurons in the sensory areas of the spinal cord and trigeminal nucleus were identified (Figure 1). Three regions of the spinal cord were examined: the lumbar, thoracic, and cervical (Figure 2). Individual slices from the cervical, thoracic, and lumbar spinal sections demonstrated strong red fluorescence (TdTomato) in the dorsal region of the spinal cord. Slices stained with SYTO 13 also demonstrated bright green fluorescence throughout all of the gray matter. 630x magnification of these slices 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 II/III (Figure 1).

The SYTO 13 stain of the trigeminal nucleus resulted in a large yellow/orange fluorescence in the lateral region of the brainstem. Slices from the rostral region of the trigeminal nucleus showed an increased amount of yellow/orange fluorescence compared to the caudal slices.

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) (Figure 4). Primary and secondary antibody staining of both the spinal cord and trigeminal nucleus slices showed many red fluorescent neurons, fewer yellow/orange fluorescent neurons, and very little green fluorescent neurons.
Figure 1. **(Top)** TdTomato co-localizes with SYTO 13 in the spinal cord. (Left) A 5x image of the cervical spinal cord. (Right) A 20x magnified image of the dorsal region of the cervical spinal cord showing (L) SYTO 13 fluorescence indicating the presence of neuronal cell bodies; (M) TdTomato fluorescence indicating the presence of CCK; (R) A composite image indicating the co-localization of TdTomato and SYTO 13. CCK(+) cell bodies are identified by a yellow fluorescence.

**(Bottom)** TdTomato co-localizes with SYTO 13 in the trigeminal nucleus. (Left) A 5x image of the rostral trigeminal nucleus. (Right) A 20x magnified image of the lateral region of the trigeminal nucleus showing (L) SYTO 13 fluorescence indicating the presence of neuronal cell bodies; (M) TdTomato fluorescence indicating the presence of CCK; (R) A composite image indicating the co-localization of TdTomato and SYTO 13.
**Figure 2.** The spinal cord of a mouse separated into distinct regions:
(A) Cervical
(B) Thoracic
(C) Lumbar
(Top) 5x cervical spinal cord.
(Middle) 5x thoracic spinal cord.
(Bottom) 5x lumbar spinal cord.
All sections have been stained with SYTO 13 to identify neuronal cell bodies.
Figure 3. Presence of the GABA neurotransmitter in the spinal cord.
(Top) Anti-GABA IHC stain showing (L) Anti-GABA; (M) TdTomato; (R) Composite
(Bottom) Anti-GAD 65/67 IHC stain showing (L) Anti-GAD 65/67; (M) TdTomato; (R) Composite
Figure 4. Presence of the GABA neurotransmitter in the Trigeminal Nucleus. (Top) Anti-GABA IHC stain showing (L) Anti-GABA; (M) TdTomato; (R) Composite (Bottom) Anti-GAD 65/67 IHC stain showing (L) Anti-GAD 65/67; (M) TdTomato; (R) Composite
DISCUSSION

**Localization of CCK in the Sensory Pathways**

As hypothesized, confocal analysis of the spinal cord shows extensive red/green (TdTomato/SYTO 13) co-localization in the dorsal horn of the gray matter, indicating the presence of CCK(+) cell bodies. This region of the spinal cord, particularly Lamina I/II, houses the sensory neurons responsible for transducing signals of painful information from the periphery to the brain. Interestingly, CCK expression was very well conserved throughout the different regions of the spinal cord, suggesting its importance in the role of peripheral pain transduction.

Similar analysis of the spinal nucleus of the trigeminal nucleus shows high levels of TdTomato/SYTO 13 co-localization in the lateral region of the brainstem. This region is responsible for transducing painful information from the neck/head to the brain. There was also a conservation of CCK expression in the spinal nucleus of the trigeminal nucleus from rostral to caudal. These findings likely indicate a connection between CCK(+) neurons and pain transduction. The nature of this relationship will be the topic of future research.

**GABA Antibody Staining**

Unexpectedly, immunohistochemical stains for the presence of the GABA neurotransmitter did not yield strongly positive results in the spinal cord. Confocal analysis of two different immunohistochemical stains (Anti-GABA and Anti-GAD65/67) indicate a very low co-localization of CCK(+) neurons and GABA(+) cell bodies throughout the spinal cord. These findings indicate that CCK(+) cell bodies in the sensory regions of the spinal cord do not use the GABA neurotransmitter to communicate.

The same immunohistochemical analysis of the trigeminal nucleus did not yield such definitive results. Rather, the Anti-GABA stain appears to indicate significant overlap of red/green signal (TdTomato/Anti-GABA). These results are not supported by the Anti-
GAD65/67 stain, which does not indicate an overlap between CCK(+) neurons and GABA(+) neurons.

Further research into nature of CCK(+) neurons of both the spinal cord and trigeminal nucleus is required to definitively identify the neurotransmitters these neurons use to communicate. Preliminary results potentially indicate an interesting difference between the methods of communication between CCK(+) neurons of the spinal cord and CCK(+) neurons of the trigeminal nucleus. If these two distinct regions transduce painful information by different means of communication, further research may indicate specific therapies that can be better targeted toward the intended area of effect.
FUTURE DIRECTIONS

The course of our research has opened up a number of avenues for future research. The inconsistent results achieved when staining the spinal cord and trigeminal nucleus with Anti-GABA and Anti-Gad65/67 immuno-fluorescent markers will be further investigated. Our research team is currently working with a new mouse model, DLX5/6-CCK-TdTomato that will allow us to definitively isolate CCK(+) neurons that use GABA as a neurotransmitter.

Further studies can be done to identify what neurotransmitter other CCK(+) neurons use to communicate, such as the sensory neurons of the spinal cord and trigeminal nucleus. These findings will generate a broader understanding of how these neurons contribute to the pain pathways, and may just as well provide new targets for the administration of analgesic medication.
REFERENCES


DIFFERENTIAL STAINING OF BLOOD VESSELS USING CCK-CRE IN MUS MUSCULIS BRAIN AND SPINAL CORD

Zachary Tepper

A Thesis submitted to the faculty of

University of New Hampshire

In partial fulfillment of the requirements for the degree of

Bachelors of Science

Department of Biological Sciences

2017, May
INTRODUCTION

Rationale

The mammalian vasculature system is a highly researched subject. Considering its influence on the progression of disease, distribution of toxins/nutrients, deliverance of drugs, etcetera, it’s no wonder so much time and money is dedicated to understanding and manipulating the vasculature system. The variety of functions and dysfunctions of the vasculature system calls for a wide breadth of research in order to understand it entirely. This research project aims to further characterize the mammalian vasculature system.

Over the course of the research defined in the previous chapter, our lab team identified a striking difference in the expression of CCK within the vasculature of the spinal cord and the vasculature of the brain. Analysis through confocal microscopy indicated a high level of CCK/TdTomato fluorescence in what appeared to be large vessels of the spinal cord. However, when similar vessels were identified in the brain, no such fluorescence was found. As covered previously, CCK is a paracrine peptide hormone, found in the gut, that signals the release of bile and enzymatic proteins responsible for the breakdown of fatty foods \[^{[1]}\]. Furthermore, CCK has been implicated in the hyperanalgesic response to the disuse of opiate medications \[^{[2]}\]. Thus, CCK is involved in the modulation of painful signals from the spinal cord and trigeminal nucleus to the sensory regions of the brain responsible for perceiving painful stimuli. Our observations imply that CCK may also be an endocrine hormone; that is, a compound that travels through the blood stream to effect distant organ systems. If so, this may be one mechanism by which CCK modulates painful signals from the periphery.
**Previous Research into CCK and the Vasculature System**

Research into the role of CCK as an endocrine protein has mostly focused on its role in the breakdown of fatty foods. As such, CCK acts as a gallbladder constriction factor\(^3\). Recent research evaluated the release of CCK as a neurotransmitter in the brain, and hypothesized that overstimulation of CCK\(^+\) neurons may result in elevated levels of CCK in the blood plasma\(^4\). However, this relationship has yet to be positively correlated, and there appears to be species-specific differences in the level of stimulation required to result in CCK in the plasma\(^4\).

Interestingly, CCK has also been found as a relevant tumor growth factor in metastatic tumors, particularly in the thyroid and adrenal glands\(^5\). As such, CCK has proven to be an effective tumor indicator when present in plasma at higher than normal levels\(^5\).

**Research Objectives**

The goal of this research experiment is to evaluate the noted expression of CCK in vasculature of the spinal cord, but not the cortical tissue. Furthermore, our research aims to identify which cells within the vascular tissue are expressing CCK. In order to identify these cells, genetically-modified mice (CCK-Chr2) were perfused with a lipophilic endothelial cell label (DiI) and analyzed through confocal microscopy in order to identify the cells responsible for the fluorescence seen in the vasculature.
RESEARCH QUESTION

Do blood vessels in the spinal cord and trigeminal nucleus differ in their expression of CCK?

MATERIALS & METHODS

Reagents

• Isoflurane (B72J15B, Piramal)
• Euthasol (200-071, Virbac)
• PBS (P3813, Sigma-Aldrich)
• Paraformaldehyde (HT501128-4L, Sigma-Aldrich)
• SeaPlaque Brain Slice Agar (100262, MP Biomedicals)
• Low Melting Point Agarose (50101, Lonza)
• TEA Buffer (1610773, BioRad)
• Aluminum Foil (obtained at retail store)
• AAA Battery (obtained at retail store)
• Superglue (obtained at retail store)
• Triton X-100 (T8787, Sigma-Aldrich)
• DiI C(18) Lipophilic Vasculature Stain (D3911, Thermofisher)
• Vectashield Mounting Medium for Fluorescence (H-1400, Vector Laboratories)
• Clear nail polish (obtained at retail store)

Equipment

• 26 ½ Gauge Needles
• Three 10mL Syringes
• Dissection Equipment
• Plexi-glass Chamber
• Three-way stopcocks
• 50mL conical tubes
• 12-well plate
• Soft-tip Paintbrush
• Microscope slides
• Standard glass coverslips (no. 1.5)
• Vibratome (VT1000s, Leica)
• Confocal Microscope (LSM 510 Meta, Zeiss)

**REAGENT SETUP**

*2% Brain Slice Agarose*

Add .4g Brain slice agar to 20mL of 1x TAE Buffer. Pour into petri dish. Store at 4 °C.

*2% Low Melting Agarose*

Use microwave to dissolve 0.2g in 10ml 1x TAE Buffer. Cool at RT and use immediately.

*0.2% PBT*

Mix 1mL Triton X-100 detergent with 500mL of PBS solution. Store at Room Temperature.

*5% (wt/vol) Glucose*

Dissolve 50g of glucose in 1000mL of MilliQ water. Filter through a 0.22-µm filter.
**Diluent Stock**

Mix PBS and 5% glucose at a ratio of 1:4.

**DiI Stock Solution**

Dissolve 100mg of DiI crystal in 16.7mL of 100% ethanol overnight with constant agitation. Store at room temperature in the dark. Keep covered with aluminum foil at all times.

**DiI Work Solution**

Immediately before use, add 200µL of DiI stock solution to 10mL of diluent. Quickly shake to mix.

**PROCEDURES**

**Cardiac Perfusion**

1. Fill the three syringes with perfusion fluids.
   
   a. One with 10mL PBS
   
   b. One with 10mL PFA
   
   c. One with 10mL DiI work solution

2. Backflow the perfusion line first with PFA, then DiI, then PBS to ensure no air is in the line

3. Saturate plexi-glass chamber with Isoflurane
   
   a. Wet tissue paper in 50mL conical tube with Isoflurane and place in chamber

4. Prepare syringe with Euthasol
   
   a. .05mL for female mice; .07mL for male mice

5. Place mouse in Isoflurane chamber until breathing is slow
6. Remove mouse by grasping the neck fur; place on lab mat and check paw reflex by pinching any of the paws
   a. If the animal reacts, anesthetize further; if not, proceed.
7. Inject the mouse intraperitoneal with Euthasol and place on dissecting tray; hold the mouse in place with its tail until euthanasia has occurred
8. Pin the mouse through its four paws, leaving the thoracic cavity exposed
9. Open the thoracic cavity to expose the heart by first cutting through the skin, then through the viscera
10. Pull ribcage back and ensure the heart is fully accessible
11. Insert 26 ½ gauge needle into the left ventricle while supporting with hemostat; once punctured, clamp with hemostat
12. Cut right atrium with scissors and begin perfusion (at slowest possible rate)
13. Perfuse with PBS until perfusate runs clear (at least 10 minutes)
   a. Intestines and liver will turn a clear color, indicating a successful perfusion
   b. All perfusion fluids should be administered at ~1mL/minute
14. Switch to Dil work solution and perfuse for 10 minutes
15. Switch to 4% PFA and perfuse for 10 minutes
16. Collect brain and spinal cord tissue and place in 4% PFA overnight at 4 °C to complete fixation
17. Dispose of mouse accordingly
**Spinal Cord Prep for Slicing**

5. Tear off thin strip of aluminum foil approx. 4 inches long

6. Roll aluminum foil around AAA battery, leaving positive terminal exposed
   
   a. Fold foil around the bottom of the battery and press against lab bench to ensure a closed bottom

7. Remove battery from foil; fill foil cylinder with 2% low melting agarose, leaving approx. 1cm for displacement

8. Using forceps, embed spinal cord lengthwise in liquid agarose and allow to cool, ensuring the tissue remains upright and parallel to cylinder walls

**Tissue Slicing**

14. Prepare Vibratome with PFA well

15. Soak razorblade in ethanol for 2 minutes

16. Using forceps, evenly attach blade to PFA-specific mount and screw into place

17. Cut brain slice agarose into two rectangles, mounting a base and backbone to secure animal tissue in Vibratome well
   
   a. Mount with the minimum amount of superglue to ensure agarose is in place, but not overly saturated

18. Prepare tissue for sectioning
   
   a. Trim brain with razor to ensure a flat surface for mounting
   
   b. Remove agarose-embedded spinal cord and ensure a flat surface for mounting

19. Place a conservative amount of superglue on top of the base piece of agarose

20. Using a spatula, add brain or spinal cord flush with the back piece of agarose, directly centered on base agarose
21. Fill well to the brim with PBS, making sure the blade and tissue are submerged

22. Set the Vibratome to 100µm per slice

23. Move blade close to the top of the tissue, set start position

24. Slice through the top of the tissue, setting the endpoint within the back piece of agarose

25. Hit the start button to automatically slice 100 microns below last cut

26. Save desired slices in 12-well plate with 1mL of PBS in each well
   a. Add slices to wells using soft-tip paintbrush
      i. Approx. 3-4 brain slices per well
      ii. Approx. 4-5 spinal cord slices per well

**SYTO 13 Staining**

All washes are 10 minutes long with 1mL of wash per well; washes and incubation take place on rotor at low speed (constant agitation)

8. Wash slices 2 times with 1mL 0.2% PBT
   a. Washes are done by discarding liquid into waste and adding 1mL of wash
   b. Always cover the 12-well plate with aluminum foil to prevent light exposure

9. After second wash, remove remaining PBT

10. Add 500µL 0.2% PBT

11. Add SYTO 13 work solution, allowing for 500nM final concentration

12. Incubate for 1 hour at room temperature

13. Wash 3 times with 0.2% PBT

14. Mount slices on microscope slides
**Immunohistochemical Staining Protocol**

All washes are 10 minutes long with 1mL of wash per well; washes, incubations, and washes take place on rotor at low speed (constant agitation)

17. Add 1mL of PBS to each well of a 12-well plate
18. With soft-tip paintbrush, gently add tissue slices to wells and sink to the bottom
19. Wash slices 1 time in PBS and 2 times in 0.2% PBT
20. Block for 2 hours at room temperature in 1mL 5% BSA/PBT per well
   a. 5% BSA/PBT made by dissolving .6g BSA in 12mL of 0.2% PBT
21. Leave slices in blocking buffer overnight at 4 °C with constant agitation
   a. Using a moist Kimwipe and Parafilm, create a humidity chamber by taping the Kimwipe to the roof of the 12-well plate and ensuring a good seal
22. After 36 hours, remove 12-well plate from cold room
23. Wash slices 3 times in PBT
24. Add 500 µL 5% BSA/PBT per well
25. Add primary antibody at room temperature and block for 1 hour
   a. 1µL per well for Anti-GABA (~1:500 dilution)
   b. 2.5µL per well for Anti-GAD65/67 (~1:200 dilution)
26. Leave primary antibody on shaker at 4 ºC for 36 hours
   a. Create humidity chamber
27. Wash 3 times in 0.2% PBT
28. Add 500µL 0.2% PBT per well
29. Add 2.5µL of secondary antibody per well (~1:200 dilution)
   a. Goat Anti-Mouse for Anti-GABA
b. Donkey Anti-Rabbit for Anti-GAD65/67

30. Leave at room temperature for 3 hours with constant agitation

31. Wash 3 times 0.2% PBT; 1 time PBS

32. Mount slices on microscope slides

_Microscope Mounting_

8. After mounting slices with soft-tip paintbrush, remove excess PBS from slide with Kimwipe

9. Add Vectashield drop-wise to ensure all tissue slices are protected from fading

10. Mount coverslip and label slide

11. Wrap in aluminum foil to protect from light exposure

12. Store at 4 °C for 30 minutes

13. Cover edges of coverslip with a thin layer of clear nail polish to increase adhesion and protect tissue from dehydration

14. Store at 4 °C in black box to prevent light exposure

_Confocal Microscopy_

6. Setup LSM 510 Meta microscope for multi-channel imaging
   a. For DiI collection, use 488nm excitation and collect using a custom filter for wavelengths between 527-540nm
   b. For TdTomato collection, use 543nm excitation and collect using LP 560

7. Use on-screen command to select the desired objective

8. Set pinholes for each channel to 1

9. Isolate area of interest in the optical objective
10. Select ‘Find’, to collect an image

   a. adjust offset/gain as needed
RESULTS

Through the use of confocal microscopy, extensive segments of mouse vasculature were identified in the spinal cord and cortical tissue. Spinal cord slices of CCK-Chr2 mice indicate very strong green fluorescence (Chr2) throughout the apparent vasculature of the spinal cord, but not cortical tissue (Figure 1).

Analysis of DiI-stained tissue in both the spinal cord and cortical areas indicate successful vasculature staining. Vasculature of the brain indicates a strong co-localization of Chr2 expression and DiI labelling (Figure 2). Images of the spinal cord show moderate, but incomplete Chr2 co-localization with DiI-stained vascular tissue (Figure 3).

Figure 1. Vasculature of cortical tissue and spinal cord tissue
(Left) Cortical vasculature (amygdala).Chr2 expression (green) indicates the presence of CCK. The boxed region indicates what appears to be a large cortical vessel.
(Right) Spinal cord vasculature. Chr2 expression (green) indicates the presence of CCK. Large vascular tissue appears to express CCK, as well as surrounding neuronal tissue.
Figure 2. Chr2/Dil Co-localization in cortical tissue (striatum)
(Left) Chr2 label (green) indicates CCK expression. (Middle) Dil stain (red)
indicates vasculature. (Right) Co-localization of Chr2 and Dil (orange)
Figure 3. Chr2/DiI expression in spinal cord
(Left) Chr2 label (green) indicates CCK expression. (Middle) DiI stain (red) indicates vasculature. (Right) Co-localization of Chr2 and DiI images
DISCUSSION

CCK Expression in Vasculature

The lipophilic DiI stain worked very well for labelling the vasculature of both the spinal cord and brain. Co-localization analysis of Chr2 labelling (indicating CCK expression) and DiI staining (indicating vasculature) demonstrated very unexpected results from what was seen in the initial observations. Initially, CCK expression appeared to be co-localized with the vasculature of the spinal cord, but not of the cortical tissue. The results of this study indicate a very high level of CCK/DiI co-localization in cortical tissue, but only low to moderate levels of co-localization in the spinal cord. A number of factors may contribute to this unexpected result. Firstly, the emission spectra for both DiI and Chr2 are difficult to separate. It is likely that interference between the signals may produce images in which there appears to be signal where it does not actually exist. Secondly, the region of analysis may also be a confound the results. In the analysis of cortical tissue, there are small circular cell bodies that protrude from the vascular tissue. These are believed to be pericytes, which help maintain the structure of capillary vasculature. In images of the spinal cord, the vasculature appears to lack these pericytes, indicating that the image is taken of a vein or artery. Thus, it is possible that CCK-expression in vascular tissue is present only in the capillary beds. Further research is required before any conclusions about CCK-expression in vascular tissue can be made.
FUTURE DIRECTIONS

Although analysis of CCK-expression in the vasculature of the spinal cord and cortical tissue presented confounding data, future research into capillary-dependent CCK expression and the communication of CCK (+) neurons and blood vessels are still viable pursuits. The unmistakable connections made between CCK (+) terminal buttons and vasculature walls may be an important aspect in the control of blood flow throughout the central nervous system. More research into these connections is required to better understand how CCK (+) neurons function in the mammalian nervous system.
REFERENCES


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 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 florescence in the sensory region when stained with SYTO 13. The spinal cord (right) also shows a yellow/orange florescence in the sensory region indicating CCK(+) neurons.](image)
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.

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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