THE ROLE OF Y1R-EXPRESSING DORSAL HORN INTERNEURONS IN PAIN

By

Laurie Lee Lemons

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Approved:

Dr. Ronald Wiley

Dr. Craig Kennedy

Dr. Li Min Chen

Dr. Sukhbir Mokha

Dedicated to Brayden,

Benjamin,

Mom and Dad.

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INTRODUCTION

Pain

The International Association for the Study of Pain (IASP) defines pain as

"an unpleasant sensory and emotional experience associated with actual or

potential tissue damage, or described in terms of such damage"(IASP, 2011).

As the definition indicates, pain is a complex entity made up of sensory, affective,

motivational, and cognitive dimensions. The sensory dimension, nociception, is

the physiological process by which primary sensory neurons detect pain-

producing stimuli. The brain perceives these inputs as pain and suffering.

Pain perception is subjective and response to the same stimulus can differ

between individuals. This is due in part to the affective component of pain

processing, which attributes emotional coloring to the experience. Some of the

emotions that accompany pain include fear, anxiety, depression, anger, and

disgust. Commonly, one's pain perception is also influenced by previous

experiences with pain, feelings about the treatments, and overall emotional state.

The motivational and cognitive dimensions refer more to the self- protection

aspects of pain perception, which is why we evolved pain sensory systems in the

first place. Pain can motivate learning processes in order to avoid current and

future tissue damage and can also interfere with learning if it becomes too

intense.

Short term protective pain is referred to as acute pain. Acute pain is characterized by being limited in time and disappearing with resolution of the pathological cause or aversive stimuli. Pain becomes chronic when it persists for an extended period of time, is associated with chronic pathological processes, and affects multiple physiologic, psychological, and social functions. Chronic pain affects millions of people worldwide and is frequently accompanied by hyperalgesia, hypersensitivity to painful stimuli, and allodynia, perceiving a normally innocuous stimulus as painful(IASP, 2011). This persistent pain interferes with a person's productivity and ability to carryout normal everyday activities. Each year, 7100 billion dollars are spent on health care, lost time and wages, unemployment, and disability due to poorly controlled chronic pain. For sufferers of chronic pain, there is great need for improved long-term treatments. Current treatments are invasive, ineffective, or plagued with serious side-effects. Our current limited understanding of the exact mechanisms and wiring of the body's pain transmission system is a significant obstacle to the development of more specific and effective chronic pain treatments; a better understanding of the neural systems underlying and modulating pain perception could be instrumental in developing better treatments for chronic pain.

Neurobiology of Pain and Nociception

Primary Afferent Nociceptors

All sensory systems, including the pain system, have mechanisms to convert environmental stimuli into electrochemical signals, including specific

receptors and neural pathways. Pain sensation results from the activity of dedicated nociceptive primary afferents, not from the over-stimulation of other kinds of receptors, such as touch receptors. Nociceptors are a primary sensory neurons activated by stimuli capable of causing tissue damage(Sherrington, 1906). The cell bodies of these primary afferent nociceptors originate in the trigeminal (head) or dorsal (body) root ganglia and send out one branch of their T-shaped axons into the dermal and epidermal layers of the skin or viscera where they terminate as free nerve endings, while the other branch terminates in the dorsal horn of the spinal cord(Julius and Basbaum, 2001).

Primary afferent nociceptors are classified into two types, Aδ and c fibers, based upon their structure, diameter, and conduction velocity. Aδ fibers are lightly myelinated with diameters ranging from 2-6 μm and conduction velocities of 12-30 m/s. They are responsible for rapidly transmitting sensations evoked by higher intensity stimuli with short latencies and for rapidly triggering fast withdrawal responses. There are two types of Aδ fibers. Type 1 responds to noxious thermal, mechanical, and chemical stimuli. It gradually increases its response to heat and will sensitize to burn and chemical injury. Type 2 responds to thermal and chemical stimuli, responding with an early peak and slowly adapting response. Aδ fibers are responsible for what is commonly referred to as 1st pain, or the rapid onset, sharp, brief pain felt immediately after a noxious stimulus(Julius and Basbaum, 2001).

C fibers are unmyelinated with diameters ranging from 0.4-1.2 μm and conduction velocities of 0.5-2 m/s. They propagate information more slowly than

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Aδ fibers, with prolonged, slowly adapting potentials, and induce dull, burning, or aching pain, also called 2nd pain. C fibers can be specifically thermo-, mechano-, or chemically sensitive, or polymodal, responding to two or all three stimulus modalities. There are also several special types of c fibers, such as those that respond to high intensity thermal stimuli and mediate the flare response after tissue damage; the slow-conducting, mechano-insensitive fibers activated by histamine that are likely involved in burning and/or itching sensations; and the "silent receptors" that don't normally respond to noxious stimuli, but can be activated in presence of inflammation. Typically, Aδ fibers are associated with producing acute pain and c fibers with chronic pain (Julius and Basbaum, 2001;Serpell, 2006).

The axons of nociceptors terminate as free nerve endings in the skin, where they possess membrane receptors sensitive to various stimuli in the environment. Temperatures are detected by several members of the TRP (transient receptor potential) family of channels that act as molecular thermometers. TRPV1 is a nonselective plasma membrane cation channel that is activated by temperatures greater than 43°C and capsaicin, the compound that gives peppers their heat and causes a burning effect when it comes in contact with the skin. It is expressed by type 2 Aδ fibers and many c fibers. TRPV2, expressed by type 1 Aδ fibers, is activated by high intensity thermal stimuli with a threshold of 52°C, but is capsaicin insensitive(Caterina et al., 1997;Szallasi and Blumberg, 1999;Clapham et al., 2001).

The transmission of cold pain is much less understood than heat pain. Both menthol, a naturally occurring component of mint oils that produces a cooling sensation when applied to the skin, and icilin, a more potent, synthetic compound, have been valuable tools in finding transducers of noxious cold. TRPM8 is a cool specific channel that is located in the peripheral projections of c fibers and is activated by menthol, icilin, and temperatures around 25°C. It does not colocalize with any known markers of nociceptive fibers, such as CGRP, Substance P, and TRPV1(McKemy et al., 2002;Peier et al., 2002;Foulkes and Wood, 2007;McKemy, 2007). The TRPA1 channel (formerly called Anktm1), is specifically expressed in a subset of sensory neurons that express CGRP, Substance P, and TRPV1. It is activated at temperatures at or below 17°C (which are reportedly painful in humans) and icilin, but is menthol insensitive. TRPA1 is also responsible for generating the nociceptive currents in response to mustard oil and is upregulated after nerve injury and inflammation, indicating that it likely contributes to the cold hypersensitivity seen in those conditions as well(Story et al., 2003;Bandell et al., 2004;Jordt et al., 2004;Story and Gereau, 2006). Additionally, several other TRP channels have been identified with a range of temperature thresholds that might be responsible for detecting warm and cool sensations (Patapoutian et al., 2003;Foulkes and Wood, 2007). To date, no mechanical transducers have been definitively identified.

The Withdrawal Reflex

Primary sensory neurons do not carry their message directly to the brain, but synapse in the spinal cord or brainstem. The simplest pathways of sensory neurons are flexor reflex arcs. A reflex is an automatic, usually rapid, response to an adverse stimulus. Many reflex actions are protective and the action happens involuntarily, meaning the brain is not required to generate the response. Bypassing the brain allows the actions to happen quickly and without delay, although sensory information about the stimulus and response are usually sent to the brain after the reflex action has occurred.

There are several types of reflex arc, but, when discussing painful stimuli, it's the withdrawal, or flexor, reflex that's involved (Figure 1). The withdrawal reflex occurs through a polysynaptic reflex arc. When the receptor detects painful stimuli, it responds and sends the information to the spinal cord via myelinated sensory neurons. The primary afferents synapse onto interneurons in the spinal cord, which transfer the signal to motor neurons that excite relevant muscles to contract (and antagonistic muscles to relax), producing a withdrawal movement. Usually, there are also projections sent contralaterally to stimulate, or inhibit, muscles on the opposite side of the body. These automatic actions are referred to as crossed reciprocal inhibition and extensor reflexes, respectively, and are necessary for maintaining balance during the flexor reflex.

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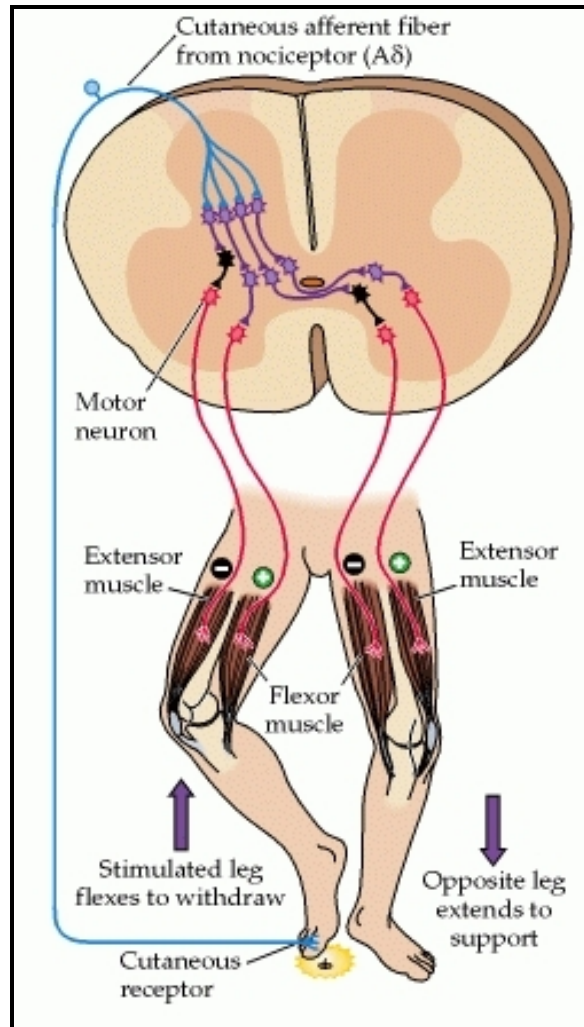
Figure 1: Spinal cord circuitry of the withdrawal reflex. Stimulation of nociceptors in the foot activates local circuits in the spinal cord that cause withdraw of the stimulated extremity and extension of the other extremity to provide compensatory support (Purves, 2004).

Spinal Modulation and Ascending Pathways

Since many nociceptors are polymodal, coding of the type of stimuli detected must occur at spinal, or higher, levels. The neural pathways the signal follows to reach the brain are more complicated and less understood than the reflex arcs.

Again, most nociceptive primary afferents terminate in the spinal cord before being processed and sent to higher centers. Most nociceptive sensory neurons terminate in the superficial dorsal horn of the spinal cord (laminae I and II) with a small percentage terminating in lamina V, where they synapse onto local interneurons or projection neurons(Melzack and Wall, 1965). The primary neurotransmitter used for these connections is glutamate, which elicits a fast excitatory response in the post-synaptic neurons, however, many of the primary afferents also release other neurotransmitters (such as Substance P)(Lawson et al., 1997;Julius and Basbaum, 2001).   These other neurotransmitters, along with the robust network of excitatory and inhibitory interneurons in the dorsal horn and input from axons descending to the dorsal horn from various parts of the brain and brainstem, all act to modulate transfer of the pain signal from the primary afferent nociceptor to nociceptive projection neurons that transfer the signal to the brain.

The organization of the neurons involved in this network is decidedly complex and little is known about the specific neuronal circuits involved in the dorsal horn. Discerning the connections and neurotransmitters involved in this process is a key area of pain research as there are many potential targets for pain therapeutics in the dorsal horn.

After modulation, the nociceptive message is transmitted to second order projection neurons. The most common ascending path for pain and temperature information to the brain is called the spinothalamic tract, but there are also other

ascending tracts that carry nociceptive information, such as the spinoreticular, and spinomesencelephalic tracts.

The spinothalamic tract (Figure 2) has lateral and medial components. Axons cross the midline of the spinal cord through the anterior white commissure and ascend in the contralateral ventrolateral quadrant to the thalamus. The lateral spinothalamic track projects to the lateral thalamus, specifically the ventral posterior lateral, the ventral posterior inferior, and the posterior lateral nuclei, which then project to the primary and secondary somatosensory cortexes. The medial spinothalamic track projects to the intralaminar and some diffuse projection nuclei of the medial thalamus, which then project to the insula and anterior cingulated cortices. Also, as the fibers pass through the brainstem, projections are sent into various regions, including the reticular formation of the medulla and the mescencephalic periaquaductal gray (PAG), which likely activates a descending analgesia system(Almeida et al., 2004).

The spinoreticular tract travels along with the spinothalamic tract in the ventrolateral spinal cord and brainstem. It projects to the parabrachial nucleus and the locus ceruleus. Reticular neurons respond preferentially to noxious stimuli and likely activate endogenous analgesia systems and relay information to trigger motivational and affective responses. The spinomesencephalic tract ascends to the midbrain in the lateral funiculus and terminates in several midbrain nuclei including the PAG, cuneiform nucleus, and superior colliculus. These nuclei play a role in activating the descending modulatory systems and orientating to the stimulus(Almeida et al., 2004).
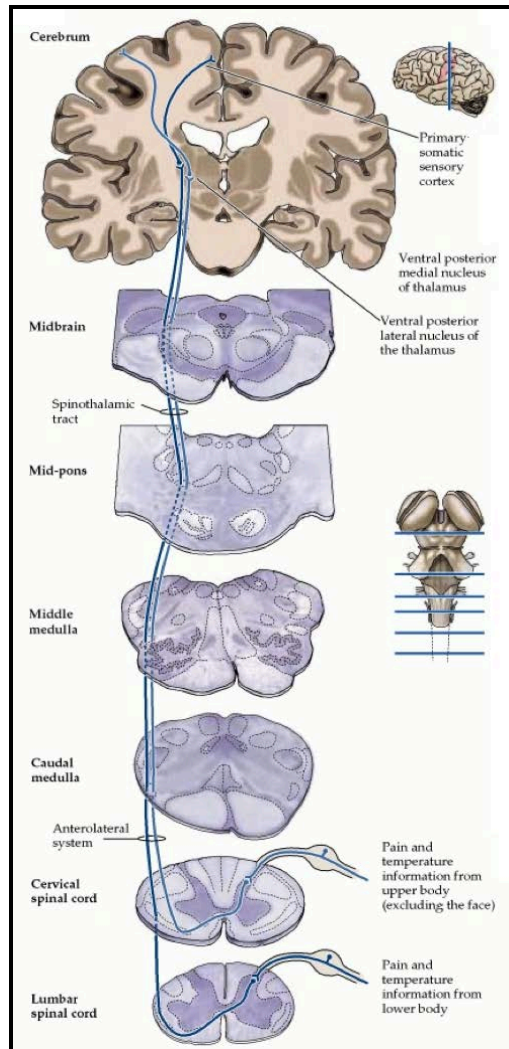
Figure 2: The Spinothalamic Tract. Pain and temperature information is carried from nociceptors into the spinal cord, where the information then follows the contralateral spinothalmaic pathway to the thalamus and higher brain structures (Purves, 2004).

Measuring and Studying Pain

As described above, pain is a subjective experience that varies among individuals, and as thus, measuring it can be challenging. Pain, including suffering, motivational, emotional, and cognitive responses, is experienced in the brain. In a clinical setting, when a patient complains of pain, the physician often

will ask the patient to rate the severity of the pain. Typically this is done on a number scale, for example 0-10, where a response of "0" indicates no pain and "10" is the worst pain they've ever felt. Note that the pain rating scale depends on the patient's prior experience with pain compared to their perception of their current pain. The same stimulus would very likely be rated differently by different people.

Measuring pain in humans depends on the patient being able to describe the sensations they're feeling, and their locations, and rate their pain. Clinicians and investigators can then gauge the efficacy of therapeutic interventions based upon the changes described by the patients. Since animals don't have language skills, investigators have had to devise ways to measure their behavioral responses in order to study nociception and pain in non-human models.

Some early attempts to assess nociception in non-human animals involved observing spontaneous behaviors or physiological reactions that were believed to represent pain or distress. Some of these spontaneous events include vocalizations, autotomy/ overgrooming, and sleep disruption; however, each of these events can be caused by non-painful stimuli as well. These spontaneous behaviors may indicate, in some cases, baseline levels of ongoing discomfort and can be used to compliment, but not replace, direct measures of nociception or hyper-sensitivity(Vierck et al., 2005b;Vierck et al., 2008).

In order to directly measure nociception, investigators began to look at stimulus → response experiments, where a potentially noxious stimulus is applied and the reflexive responses measured. Typically the response measured

is the latency to first response, where an "increased latency" indicates that the animal was slower to respond to the stimulus, which is interpreted as decreased nociception. These types of tests have been used to measure responses to heat, cold, and mechanical stimuli. A radiant heat source is applied to the tail in the "tail flick" task and the time from stimulus application to the rat moving its tail away from the heat is measured. This radiant heat source can also be applied to the hind paw in the paw-withdrawal task or heat can be applied to hind paws using a hotplate set to a specific temperature. To assess response to cold, acetone is applied to the hindpaw as the stimulus and mechanical sensitivity can be measured using a pin prick test or Von Frey filaments. In each case, the latency to lift/ guard or lick the paw is measured.

This latency to first response is not always an accurate measure of sensitivity, as the tests are very susceptible to experimenter bias and a normal footstep can easily be mistaken for paw withdrawal response. An alternative is to use a modified hotplate test where a less intense stimulus is applied over an extended period of time. This method is beneficial for several reasons: 1- experimenters can collect lick/guard behaviors over time, 2- the total number of responses and duration of responding can be measured, yielding a less biased result, and 3- the heat can be set to a lower temperature that will preferentially activate C nociceptors over $A\delta$ nociceptors, which are more relevant in chronic pain studies. These reflex tests are widely used by researchers and drug development companies since they can yield large amounts of data in little time and require little to no training of the animal subjects or investigators.

The third type of behavioral testing investigators use to assess pain in animals are the operant tasks. These tests involve cerebral processing and decision making, because in each test the animal is asked to make a choice about which environment it prefers: in the place preference test, the animal chooses between two temperatures, typically one noxious and the other aversive, but not overtly painful; in the escape task, the animal can choose to escape from the thermal stimulus to a brightly lit, angled shelf; and in the feeding interference task the animal can remain in a dark, neutral temperature start box, or step onto the thermal floor plate in order to consume sweetened condensed milk. By measuring the amount of time the animal spends in each environment, we can determine the extent that the unpleasant thermal stimuli are affecting the choices made by the animal. These choices reveal the aversiveness or motivational qualities of the stimuli, therefore these responses can be considered to be more reliably representative of pain than simple reflex responses(Mauderli et al., 2000;Vierck et al., 2005b). Actually, when direct comparisons have been made between reflex responses and cortically dependent operant tasks, different, and sometimes opposite, effects have been demonstrated. In each case, the results from operant tests are more consistent than reflexive tests with what would be expected from human studies(Vierck et al., 1990;Vierck and Light, 1999;Vierck et al., 2004;Vierck et al., 2005b;Wiley et al., 2007). This makes logical sense since chronic pain sufferers don't complain about repeated limb flexion or tail flicks, they complain about discomfort, which is more accurately modeled and measured using the operant tasks.

Models of Chronic Pain

Each of the methods described above for testing and measuring nociception and pain are effective only for determining baseline responses to acute stimuli. We have adequate treatments for acute pain, but the clinically relevant problem is chronic pain. Sufferers of chronic pain often have heightened sensitivities to noxious (hyperalgesia) and non-noxious stimuli (allodynia). These symptoms are a result of a nerve injury or persistent inflammation that have been reported to cause physiological changes leading to changes in gene expression and plasticity in sensory neurons, the spinal cord, and supraspinal structures. Inflammation and nerve injury have been modeled several ways by researchers.

A common way to model persistent nociception is to inject inflammogens into the plantar surface of the hindpaw. Two such inflammogens are the common food additive carrageenan and complete Freund's adjuvant (CFA), which cause thermal and mechanical hyper-sensitivity that can last several hours (carrageenan) or two weeks or more (CFA)(Zukowska and Feuerstein, 2005). An intermediate model of peripherally induced pain is the formalin test, where a dilute formalin solution is injected into the hindpaw. This damages the tissue, instantly causing intense behavioral and physiological responses that can be measured in terms of licking and flinching behaviors for approximately 90 minutes following injection. These behaviors usually consist of two distinct phases of responding separated by an interphase period with suppressed responding(Tjolsen et al., 1992). The formalin test is often described as a model

of persistent pain and has been extensively studied; however, the clinical relevance of the formalin test is not clear.

Persistent enhanced nociception can also be induced through nerve injury. Some examples of injuries used by researchers to mimic chronic pain states are axotomy, the spared nerve injury (SNI), and the chronic constriction injury (CCI).   These can either be unilateral or bilateral. An axotomy is the cutting or severing of a neuron's axon and usually results in death of the neuron. In the chronic constriction injury model, several sutures are tied loosely around the sciatic nerve, resulting in robust mechanical and cold sensitivities (Vierck et al., 2005a). Finally, the spared nerve injury model involves transection of two out of the three terminal branches of the sciatic nerve, which also results in robust mechanical and thermal hypersensitivities(Decosterd and Woolf, 2000). Both the CCI and SNI produce enhanced nocifensive responses within 24 hours that can last for several months.

Summary

Chronic pain affects millions of people and there is a growing need for successful, long-term treatments that aren't plagued with side effects. Unfortunately, development of new medications is hindered because the precise organization and function of the neural mechanisms underlying nociception are still unclear. Therefore it's important to study the nociceptive pathway to learn the identity and function of neurons and transmitters involved, with the dorsal horn of the spinal cord being a prime location for this research. Several pain-

related peptidergic targets have been identified to date in the spinal cord, such as Substance-P and the opioids, and researchers have already taken advantage of these systems to create pain therapeutics. For example, the commonly used analgesic, morphine is an agonist of the endogenous mu-opiate receptor. While morphine often works well to treat acute pain, the hope is that other neuropeptide systems could be targeted in a similar way to relieve chronic persistent pain. One excellent candidate is neuropeptide Y, as recent studies have shown that the spinal neuropeptide Y system is involved in the modulation of nociceptive information.

The Neuropeptide Y System in the Spinal Cord

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed throughout the central and peripheral nervous systems (Tatemoto et al., 1982) and has a variety of physiological effects on blood pressure control, feeding, anxiety, and memory(Hokfelt et al., 1998). There are at least five different receptor subtypes for NPY (Y1-Y5), with the Y1 and Y2 receptors being the most abundant in the spinal cord(Larhammar et al., 1998;Silva et al., 2002). A role has also been identified for Neuropeptide Y in pain based on both behavioral studies using intrathecal injections of NPY and anatomical evidence showing that both the Y1 and Y2 receptors are present in the superficial dorsal horn, a key area of nociceptive gating.

Neuropeptide Y - Y1 Receptors

Neuropeptide Y1 receptors are generally considered to be inhibitory(Zhang et al., 1999;Brumovsky et al., 2007). They are G-protein coupled receptors with $G_{i/o}$ subunits that inactivate adenylate cyclase when neuropeptide Y is bound, which has an inhibitory effect on the cell. Additionally, the Y1 receptor can activate G-protein coupled inwardly rectifying potassium channels (GIRK), which hyperpolarizes the cell, resulting in its inhibition. Y1 receptors can also influence intracellular calcium levels by activating L-type Ca^{2+} channels(Sun et al., 2001;Silva et al., 2002).

Neuropeptide Y1 receptors are located primarily postsynaptically in the dorsal horn and at least seven different populations of Y1 receptor-expressing neurons have been identified in the dorsal horn and area X of the spinal cord, characterized by location and morphology, but not necessarily function. These neuron populations have been classified into types 1-7, with type 1 and 2 neurons localized in the superficial dorsal horn. Type 1 neurons are found in lamina I-II and are tightly packed, fusiform shaped cells, with rapidly dividing bipolar processes. Type 2 neurons are larger than type 1, are found in lamina I, and some were identified to be projection neurons by retrograde labeling using Cholera Toxin-B injected at the 9th thoracic segment(Brumovsky et al., 2006).

It is likely that the Type 1 cells represent the same population of cells described by Zhang et al., as small somatostatin-expressing interneurons(Zhang et al., 1999). One could then infer that these Type 1 interneurons are excitatory, since dorsal horn cells expressing somatostatin have been found to co-express

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the vesicular glutamate transporter 2 (VGLUT-2), making glutamate the primary neurotransmitter of those cells(Todd et al., 1992). Since NPY co-localizes with γ-aminobutyric acid (GABA) in lamina II interneurons(Rowan et al., 1993), NPY may be acting to reduce pain signals through co-inhibition (along with GABA) of the type 1 excitatory interneurons or by acting directly to inhibit the type 2 projection neurons.

Neuron types 3-7 are found throughout lamina III – X and include: type 3, small neurons in lamina III; type 4, large, multipolar neurons in the area between lamina III and IV; type 5, large, multipolar, projection neurons in lamina V and VI; type 6, large, multipolar, projection neurons around the central canal in lamina X; and type 7, large neurons in lamina VIII(Brumovsky et al., 2006). It is unknown under which circumstances these neurons are activated, but it is possible that some of these populations could be activated in situations of inflammation or nerve injury and involved in mechanisms of descending inhibition or transmission of nociceptive information to higher brain centers.

Neuropeptide Y - Y2 Receptors

Spinal Y2 receptors are located on cell bodies in the dorsal root ganglion (DRG) and are found presynaptically on nerve terminals in the dorsal horn; however the anatomy of the Y2 receptor has only been studied in the mouse to date(Hokfelt et al., 2007), and no reliable antibodies are available for immunohistochemistry. Activation of the Y2 receptor in the DRG is generally considered to exert an excitatory effect on the cell, which is increased after nerve

injury(Hokfelt et al., 2007), however these processes are not yet completely understood and more research is still needed to clarify the data.

Intrathecal Injection of Neuropeptide Y

Intrathecal (i.t.) administration of NPY has been shown to have an antinociceptive effect in the rat. This was first published by Hua et al., who found that NPY dose-dependently increased the hindpaw lick/guard latency response on the 52°C hotplate test(Hua et al., 1991). This research was confirmed by Taiwo & Taylor who found increased paw-withdraw latency in response to a strong radiant heat source, in addition to increased hotplate latency at 52°C(Taiwo and Taylor, 2002) in rats injected with intrathecal NPY. Additional evidence that NPY could be involved in regulating the spinal transmission of nociception is that intrathecal injections of NPY into anesthetized animals results in a reduced nociceptive flexor reflex(Xu et al., 1994;Xu et al., 1999). These behavioral tests show that i.t. NPY reduces protective reflex responses to acute noxious stimuli, however, different models involving persistent nocifensive stimulation must be used to make inferences about effects on chronic pain.

Complete Freund's adjuvant (CFA) causes heat and mechanical hyper-sensitivity that can last up to two weeks. The CFA-induced hyper-reflexia can be inhibited by i.t. injection of NPY, as shown by increased paw withdraw latencies in the 52°C hotplate test(Taiwo and Taylor, 2002). Intraplantar injection of formalin instantly causes intense responses that can be measured in terms of licking and flinching behaviors. NPY dose-dependently inhibited licking

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behaviors in Phase I and licking and flinching behaviors during Phases I and II of the formalin test(Mahinda and Taylor, 2004;Intondi et al., 2008).

Chronically enhanced nociception can also be induced through nerve injury. Neuropeptide Y, when administered two weeks after SNI surgery, completely inhibited the mechanical and cold hyper-nociception, produced by the nerve injury(Intondi et al., 2008). These studies indicate that intrathecal injection of NPY is effective in reducing nociception after peripheral inflammation and nerve injury, suggesting a possible role for neuropeptide Y in modulating inflammatory or neuropathic pain.

It has been found that peripheral inflammation leads to increased levels of NPY and Y1 mRNA transcripts in the dorsal horn(Ji et al., 1994;Zhang et al., 1994). This could indicate that following CFA injection there are more Y1 receptors, and thus more places for NPY to bind, but changes in static levels do not necessarily correlate to dynamic changes in function. However, increased NPY binding in the dorsal horn has been shown after nerve injury(Brumovsky et al., 2004), further supporting the idea that there may also be increased NPY involvement in modulating nociception after inflammation. Increased NPY binding could result in increased inhibition of nociceptive signals, which relates to the observation that the antinociceptive action of intrathecal NPY is enhanced in situations of persistent nociceptive stimulation compared to acute stimulation. Thus, endogenous NPY in the superficial dorsal horn may represent a counter-regulatory mechanism for suppressing chronic pain.

Intrathecal Injection of Neuropeptide Y Receptor Antagonists

The antinociception produced by i.t. NPY can be blocked by simultaneously injecting a NPY antagonist. Two days after unilateral hindpaw CFA injection, Taiwo and Taylor intrathecally administered the NPY Y1 receptor antagonist BIBO3304 with or without NPY. BIBO3304 given alone slightly enhanced the CFA-induced thermal hypersensitivity, indicated by a slight decrease in paw-withdraw latency. This presumably reflected blocking the antinociceptive effect of endogenous NPY binding to Y1 receptors. When BIBO3304 was given concurrently with NPY, the antinociceptive effect of NPY was completely inhibited. These effects were similar in experiments with SNI animals, where BIBO3304 co-administered with NPY, completely reversed the antinociceptive effects of NPY. The Y2 antagonist BIIE0246 also was effective in reducing the antinociceptive effects of NPY when they were administered together(Taiwo and Taylor, 2002); however, BIIE0246 is overtly neuro-toxic at doses similar to those in this study, raising concerns about the interpretation of these experiments. Taken together, these experiments provide evidence that the antinociceptive effects of intrathecal NPY can positively be attributed to action of the peptide at Y1 dorsal horn spinal receptors, but the role, if any, for Y2 receptors remains to be determined.

When these same antagonists were used in the formalin test, however, the results were more complicated. As previously stated, i.t. NPY injection alone reduces licking behaviors in phases 1 and 2, and flinching behaviors in phase 2 of the formalin test. The Y1 antagonist BIBO3304 did not change the formalin-

induced licking and flinching behaviors when administered alone. When NPY and BIBO3304 were administered together, the inhibitory effect of NPY was reduced when measuring flinching behaviors, but licking responses were not altered, raising the possibility that NPY is likely working at other sites to produce antinociception in the formalin test. When the Y2 antagonist BIIE0246 was administered alone, there was no effect on the formalin-induced licking and flinching and there was also no change in the antinociceptive effect of i.t. NPY on licking and flinching behaviors when NPY was co-administered along with BIIE0246, somewhat arguing against a contribution of spinal Y2 receptors in NPY antinociception in the formalin test(Intondi et al., 2008).

There are several caveats to the results obtained in the formalin tests. First, the Y2 antagonist used in the studies does not have a very high affinity for Y2 receptors. It's also highly toxic and had to be administered at low doses, further compounding the problem of having a low affinity. These problems call into question the validity of the Y2 antagonist experiments. Second, the relationship of the formalin-test to any particular clinical pain problem is unclear.

Even with the complications introduced with the formalin test results, the NPY antagonist experiments do support the hypothesis that intrathecal NPY is antinociceptive by action of the peptide at its spinal Y1 receptors. The role of Y2 receptors in nociception remains to be determined.

Neuropeptide Y1 Receptor Knock Out Mice

The antagonist studies strongly suggest that the NPY Y1, and possibly Y2, receptors play a role in modulating nociception. Naveilhan et al. further investigated the role of the Y1 receptor in nociception using an Y1 receptor knock out (Y1R-KO) mouse that was developed at the Karolinska Institute using homologous recombination. The Y1R-KO mice demonstrated a marked nociceptive hyper-reflexia compared with wild-type mice. They showed reduced latencies on hotplate temperatures of 50°, 52°, 55°, and 58^oC and also in the tail flick test at temperatures tested between 46° and 54^oC. Intrathecal NPY, which has an antinociceptive effect in wild-type mice, had no effect in the Y1R-KO mice on the hotplate tests. The Y1R-KO mice also had a much reduced mechanical threshold, which was measured using the Von Frey test(Naveilhan et al., 2001).

The Y1R-KO mice also had increased behaviors in response to inflammation and nerve injury. They exhibited increased licking and flinching events during Phase 1 of the formalin test and demonstrated increased pain-related behaviors in response to inflammation caused by capsaicin applied to the hindpaw. The response of the knock-out mice to nerve injury was tested using a partial sciatic nerve ligation model. The nerve injury causes mechanical hyper-nociception in wild-type mice, measured using Von Frey filaments, which was notably increased in the knock-out mice(Naveilhan et al., 2001;Shi et al., 2006).

These Y1R knock-out mice experiments were confirmed and elaborated upon by Kuphal et al., who used knock out mice developed at the University of Lausanne by Thierry Pedrazzini. Using the CFA model of peripheral

inflammation, they found that the dose of CFA required to evoke thermal hypersensitivity for one day in wild-type mice, produced a much longer lasting hypersensitivity in the Y1R-KO mice. CFA also produced mechanical hyper-nociception in both wild-type and KO mice, which was reduced by i.t. injection of NPY in the wild-type, but not the KO mice. Next they tested the mice using the SNI model, which causes thermal hyper-nociception. The antinociceptive effects of i.t. NPY were reduced in the Y1R-KO mice compared to the wild-type(Kuphal et al., 2008).

The hyper-nociception and sensitivity caused by knocking out the Y1 receptor can likely be attributed to the fact that endogenous NPY had no available receptors to bind, similar to the NPY antagonist studies. Another theory for the hyper-sensitivity observed in knock-out mice is that they have increased RNA transcript levels of Substance-P and CGRP, but lower levels of the peptides compared to wild-type(Brumovsky et al., 2004). This could indicate that they have an increased release of the excitatory peptides, with a more rapid transport of the peptides from the cell bodies, leading to increased nociception. The inability of i.t. NPY to cause any antinociceptive effects in the knock-out mice strongly suggests that the antinociceptive effects of NPY are modulated primarily through the NPY-Y1 receptors. However, it must also be taken into consideration that Y1R-KO mice lack Y1 receptors throughout the CNS and may have developmental consequences due to life long absence of effective receptors.

Intrathecal Injection of Neuropeptide Y-Saporin

All of the above anatomical and behavioral data strongly suggest a role for the spinal Neuropeptide Y system in nociception, specifically that Y1R-expressing neurons in the superficial dorsal horn can modulate reflex responses to noxious or aversive stimuli. Since Neuropeptide Y exerts an inhibitory effect when acting through its Y1 receptor, one would predict that destroying Y1R-expressing neurons would also inhibit nocifensive reflex responses. Destroying the neurons that express the Y1 receptor and removing them completely from the neural network allows researchers to ask the question "What is the role of the cells that express the Y1 receptor in nociception?" rather than focusing on the receptor itself the function of the receptor itself.

Conjugating saporin, a ribosomal inactivating toxin, to Neuropeptide Y, creates a targeted toxin called NPY-sap that, when injected into the lumbar intrathecal space, creates a lesion of Y1R-expressing neurons in the dorsal horn of the spinal cord. 750ng of NPY-sap led to about 45% loss of Y1R-expressing dorsal horn neurons, but did not affect cells in the dorsal root ganglion. The behavioral effects of this lesion were as expected from previous reflex studies. Loss of Y1R-expressing dorsal horn neurons was associated with increased first response latencies on the 44°C hotplate, decreased lick/ guard durations on the 44° and 47°C hotplate, and reduced formalin-induced nocifensive behaviors during both the interphase and Phase 2 of the formalin test(Wiley et al., 2009).

The reflex data from the NPY-sap experiments confirmed the hypothesis that killing Y1R-expressing superficial dorsal horn neurons mimics the inhibitory

effects of NPY. These results, both behavioral and anatomical, also confirm that NPY-sap is effective in targeting Y1R-expressing neurons.

Summary

The superficial dorsal horn (lamina I-II) of the spinal cord is made up of a dense network of neurons and is a key area of spinal pain transmission and gating. These neurons communicate using a number of classical transmitters and neuropeptides that work together to modulate and propagate incoming sensory information to higher centers. Neuropeptide Y is one of these transmitters. It and two of its receptors, Y1 and Y2, have been found in the dorsal horn of the spinal cord and studies have shown that intrathecal injection of NPY leads to decreased noficensive reflex behaviors, indicating a role for NPY in nociceptive modulation. Further studies using antagonists, knock-out mice, and targeted toxins, have implicated the Y1 receptor as the primary receptor through which NPY inhibits nociception. Since all of the behavioral data reported to date measured reflexive responses to stimuli, it is unclear whether the Y1R-expressing neurons also modulate pain.

As previously stated, pain differs from nociception in that it is a complex experience made up of sensory, affective, motivational, and cognitive dimensions. In order to reliably evaluate pain sensitivity, measures in laboratory animals must assess cortically mediated behavioral responses to noxious stimuli, such as occurs with the operant behavioral tasks.

Specific Aims

There is sufficient evidence that the spinal Neuropeptide Y system is involved in negatively modulating nociception and that this is primarily mediated through the Y1 receptor. Studies using NPY-sap show that the targeted destruction of Y1-expressing cells also exerts an inhibitory effect on nociception. In order to determine if the spinal NPY system also modulates pain, and not just nocifensive reflexes, operant behavioral tasks must be used, as these require cerebral processing of stimuli and comparison with similar past experiences in order for the animals to respond. It is hypothesized that spinal Y1 receptor-expressing neurons also play an inhibitory role in the transmission of pain to the brain.

In order to test this hypothesis, neurons in the dorsal horn will be selectively destroyed by intrathecal injection the targeted toxin NPY-sap, which is composed of the ribosomal-inactivating toxin, saporin, coupled to a NPY. After the targeted neurons are destroyed, three operant behavioral assays will be used to measure the effects that removal of these neurons from the neural network has on responses to noxious stimuli. Finally, lumbar spinal cord sections from each rat will be processed using immunohistochemical staining for the Y1 receptor, and for other cellular markers, to assess the extent and selectivity of the effects of NPY-sap and to correlate the degree of target neuron depletion to the magnitude of behavioral responses for each rat, i.e. the relationship between target neuron loss and behavioral changes.

Aims 1 and 2: Determine the effect(s) of intrathecal NPY-sap on operant responses to noxious thermal stimuli under (1) normal conditions and (2) pathological conditions.

Baseline operant nocifensive responses of NPY-sap treated rats will be compared to controls, injected with a nonsense peptide-saporin conjugate, using three different operant paradigms: thermal place preference, escape, and feeding interference tasks.   In order to assess the effects of NPY-sap on the common clinical pain-related problems of hyperalgesia and allodynia, NPY-sap and control conjugate-injected rats will be tested on the operant tasks before and after producing hindpaw inflammation (complete Freud's adjuvant).   Since there are reported similarities in electrophysiologic actions between NPY and opiate agonists, morphine analgesia will also be assessed in the operant tasks with toxin and control rats before and after inducing hyperalgesia/ allodynia, thus providing information about any NPY-sap effect on opiate analgesia.

Aim 3: Anatomically identify and define the targeted interneuron populations in the dorsal horn.

This set of experiments will identify which cells were killed by the NPY-sap and where they were located in the dorsal horn. After completion of behavioral testing, lumbar spinal cord sections from each rat will be stained for both the Y1 and the mu opiate receptors (MOR) to define the extent and location of the lesion produced by the toxin.   Additionally, lumbar spinal cord sections from rats treated with two other toxin conjugates to inhibitory neuropeptides, dermorphin-

sap, which kills cells expressing the mu-opiate receptor, and Galanin-sap, which

kills cells expressing the Gal-R1 receptor, will be stained for the Y1 receptor to

determine if there is overlap in the respective neuronal populations.

CHAPTER 2

MATERIALS AND METHODS

Subjects

All experiments in this study were conducted using adult female Long

Evans hooded rats obtained from Harlan Industries, Inc (Indianapolis, IN, USA).

Rats were housed two or three to a shoebox cage, depending on weight, in a

temperature controlled environment with 12 hour light/dark cycles and free

access to food and water. All procedures were approved by the Vanderbilt

University institutional animal care and use committee and conformed to the

National Institutes of Health (NIH) guide for care and use of laboratory animals.

All procedures were closely monitored to minimize animal discomfort.

Lesioning Y1R-expressing Dorsal Horn Neurons

Neuropeptide Y-saporin, Galanin-saporin, Dermorphin-saporin, and a

control nonsense peptide-saporin conjugate, blank-saporin, were supplied by

Advanced Targeting Systems (San Diego, CA, USA). Toxins were dissolved in

sterile, preservative-free normal saline (Sigma Chemical, St. Louis, MO, USA).

Concentrated stock solutions were stored at -4°C and working dilutions were

made fresh and stored on ice until used the same day and then discarded.

Rats were anesthetized by i.p. injections of a ketamine-xylazine-

acepromazine mixture supplemented with additional ketamine as needed to

maintain insensibility. As previously described(Kline and Wiley, 2008;Wiley et al., 2009), lumbar intrathecal catheters (Recathco LLC, Allison Park, PA, USA) were inserted into the spinal subarachnoid space through a small incision in the atlantooccipital membrane, as per the technique of Yaksh and Rudy, to a depth of 8.5 cm from the dural incision. The toxin was injected in a volume of 10 μl followed by a 10 μl flush of saline and the catheters were removed after 10 minutes. The wounds were closed with Michel clips and rats were warmed until awake then returned to home cages.

Behavioral Testing

All behavioral testing was completed in a dimly lit, dedicated room with low level white noise in the background. Each day rats were allowed to acclimate to the testing room for 30-60 minutes prior to behavioral testing. The rats were coded to blind the experimenter to their identities during behavioral testing.

Reflex Testing Procedures

A modified thermal test, as previously described(Kline and Wiley, 2008;Wiley et al., 2009), was used to assess effects of depletion of Y1 receptor-expressing dorsal horn neurons on innate nocifensive reflex responses to aversively cold stimuli. The cold-plate test chamber consisted of a clear Plexiglas box (25cm x 15cm x 16cm) with a ventilated lid sitting on a custom-built aluminum plate containing internal circulation channels to assure uniform surface temperature. The temperature of the plate was controlled by a thermally

regulated circulator (Polyscience circulating bath model 9105, Washington, PA, USA) as previously described. Prior to each test trial, rats were placed in an identical enclosure with floor temperature at 20°C for 10 minutes and then transferred to the test chamber. Trials were run at 0.3°C, 5°C, and 10°C with each trial lasting 600 seconds. Hind paw licking and guarding (sustained lifting) behaviors were captured in real time by continuous observation using a custom computer program, which records latency and duration of each response, as well as total numbers of responses and total amount of time spent responding. Rats do not require training prior to testing on the cold plate. Typically, they are placed in the test enclosure with the plate off a couple of times before testing begins so that they are acquainted with the environment and the newness doesn't distract them from the thermal floor.

Operant Behavioral Test Procedures

Thermal Preference Task

The thermal preference task involves two temperature-regulated aluminum plates (same as described above) that are placed end to end and separated by a piece of plexiglass with a hole cut in it for the rat to walk though. The entire area is enclosed in a plexiglass box (30cm x 13cm x 16cm), creating two compartments whose temperatures can be independently adjusted. Thermal preference trials were 300s and the rat was allowed to freely move back and forth between the two compartments for the entire trial. Stimulus temperatures for thermal preference testing were set at 15°C and 45°C. Training for the

thermal preference task occurred over a four day period with the floor plates set to 38° for the first two trials and 20° and 40° for the second two trials. This allowed the rats to acclimate to the test chamber, while exploring freely from compartment to compartment and learning that the floor on each side was a different temperature. The amount of time spent on the 45ºC side and the number of crossovers between compartments were recorded. A rat was considered to have crossed over when all four feet were in the new compartment.

Escape Task

As described by Mauderli et al(Mauderli et al., 2000), the escape task consists of a plexiglass enclosure (35cm x 11cm x 24cm) atop a temperature regulated aluminum plate (same as described above), which serves as the floor. At one end of the enclosure is a brightly lit, angled, room temperature shelf that rats can climb on to escape the stimulus.

Rats require extensive training on the escape task before data can be collected. The first two times a rat is placed into the test box, the floor plate and light are off and the shelf is flat; for the next two trials, the floor plate and light remain off, but the shelf is angled; and for the following two trials, the light is turned on low above the angled shelf, but the floor plate remains off. Once each rat is adjusted to the test box and is climbing onto and off of the shelf, the floor plate is turned on at 38°C and moved either up or down 1-2° at a time until the desired test temperatures are reached. This is critical to ensure the rats don't develop an avoidance response.

Escape trials were 360 seconds and responses were measured at 10°, 15°, 20°, 38°, 45°, and 47°C. At test temperatures 38° or above, rats were pre-warmed on a 38° plate and at temperatures below 38° rats were pre-cooled on a 20° plate for 360 seconds before escape testing. The number of times rats escaped to the shelf and the amount of time spent on the shelf were recorded.

For morphine trials, preservative-free morphine sulfate sterile solution (Baxter, Deerfield, IL) was diluted in preservative-free sterile physiological saline for subcutaneous injection 30 minutes before escape testing. Subcutaneous morphine doses were 0.0, 0.5, and 1.5 mg/kg.

Feeding Interference Task

The feeding interference task is another two-chambered task, in which a plexiglass box (40cm x 13cm x 43cm) with a room temperature start area is adjacent to the thermal plate (same as described above). At the opposite end of the thermal plate from the start box is a feeding tube with sweetened condensed milk (diluted 1:32 for these experiments). The rat must walk across and remain standing on the thermal plate in order to drink the milk, but can return to the neutral temperature start area at any time during the trial. Trials were run for 480 seconds and the amount of time spent on the thermal plate was measured.

The first time the rats are introduced to the feeding interference task, the floor plate is set to a neutral temperature, or off, and there is full strength sweetened condensed milk in the sipper tube. These conditions are repeated several trials in a row until all rats discover the milk and drink from the sipper. For these experiments, any rat that didn't drink from the sipper by, or during, the

third trial, or who didn't spend the majority of the trial on the plate consuming milk, was removed from the experiment. Once all of the rats were consuming the milk, it was slowly diluted, one trial at a time (1:2, 1:4, 1:8, 1:16, and 1:32), until the rats no longer spent the entire trial drinking. Then the temperature of the floor plate was gradually raised or lowered 1-2° at a time until the test temperatures were reached.

Complete Freund's Adjuvant

Rats were anesthetized by i.p. injections of a ketamine-xylazine-acepromazine mixture.and then injected bilaterally with 0.1ml Complete Freund's Adjuvant (CFA) in the plantar surface of their hindpaws. CFA injection was 45 days after intrathecal toxin injection. Rats were tested 2-7 days post-CFA injection.

Anatomical Procedures

Spinal cord sections were prepared as previously described(Kline and Wiley, 2008;Wiley et al., 2009). Two weeks after toxin/vehicle injections, or at the conclusion of behavioral testing, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 500ml of cold normal saline containing 5mM sodium phosphate, pH 7.5, 1g/l sodium nitrite (vasodilator) and 1000 units/liter sodium heparin (anticoagulant), followed by 1L of cold 4% formaldehyde prepared from paraformaldehyde in 100 mM sodium phosphate, pH 7.5.

Spinal cords were postfixed for 4 hours and then equilibrated overnight in 30% sucrose in 5mM sodium phosphate, pH 7.5, the night before sectioning. Transverse sections of the lumbosacral spinal cord were cut at 40μm thickness on a freezing sliding microtome (American Optical). Spinal cord sections were collected six to a well in 24-cell tissue culture plates. Spinal cord sections were equilibrated with antifreeze solution consisting of glycerol-ethylene glycol-phosphate buffer and stored at -20°C until processed for peroxidase or fluorescent immunohistochemistry.

Peroxidase Immunohistochemistry

As previously described (Kline and Wiley, 2008;Wiley et al., 2009), control and toxin treated sections were processed in parallel. One in six series of transverse spinal cord sections were removed from antifreeze at room temperature and washed in Tris-buffered saline followed by incubation for 15 minutes in 5% normal serum at room temperature. Then the free-floating sections were transferred to primary antibody diluted in 1% serum and incubated for 4 hours at room temperature. Then sections were washed and processed for peroxidase immunohistochemistry using the standard biotin–avidin technique (ABC elite kit, Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine/nickel as chromogen. Antibody #96106 raised against Y1R and antibody #96202 raised against Gal-R1 were provided by the CURE Digestive Diseases Research Center (Antibody/RIA Core, NIH grant #DK41301). The MOR1 antibody was from Chemicon, now Millipore, (Temecula, CA).

Reacted sections were washed and mounted on gelatin-coated slides,

dehydrated, cleared and examined using a Leica (Bannockburn, IL, USA)

DM6000B automated photomicroscope. Blank-sap and toxin sections were

processed simultaneously in parallel using the same solutions and conditions.

Y1R, MOR, and Gal-R1 measurements

As previously reported(Kline and Wiley, 2008;Wiley et al., 2009), we used

computer-assisted quantitative densitometry to evaluate Y1R, MOR, and Gal-R1

staining in the superficial dorsal horn. Using randomized coded sections to blind

the operator to experimental condition, user-defined areas of interest

encompassing the entire medio-lateral extent of lamina I and II were digitally

captured. Both right and left dorsal horns from 8 to 10 stained sections of lumbar

segments L4 and L5 from each spinal cord were photographed. The darkest

pixels (intensity 0–100 out of a range of 0–250 gray levels) were chosen as

consistently representing specific staining as determined by comparing the

distribution of computer selected stained pixels to visual inspection of the

peroxidase-stained sections. Mean pixel counts for each rat were computed

from the 8 to 10 L4/5 sections measured (16-20 dorsal horns) from each rat. All

measurements were performed on raw digital images, no transformations or

other photo processing manipulations were applied to the original images from

which measurements were taken counted from the coded slides to obtain the

staining intensity for each dorsal horn region of interest.

Fluorescence Immunohistochemistry

Control and toxin treated sections were processed in parallel. One in six series of transverse spinal cord sections were removed from antifreeze at room temperature and washed in 0.3M phosphate-buffered saline. Then the free-floating sections were incubated in a mixture of rabbit anti-NPY and guinea pig anti-MOR, diluted in phosphate buffered saline, and incubated 48 hours at 4°C. The sections were washed and incubated for 3 hours in species-specific fluorescent secondary antibodies: anti-guinea pig Alexa 488 and anti-rabbit Alexa 594 (Invitrogen, Carlsbad, CA, USA). Reacted sections were washed and mounted on gelatin-coated slides using antifade mounting media (Invitrogen, Carlsbad, CA, USA). Slides were stored at 4°C until ready to be analyzed.

MOR/Y1R Cell Counting Procedures

Fluorescent stained sections were viewed using a Zeiss LSM510 (Carl Zeiss, Inc, North America) Inverted confocal microscope. Sections were scanned sequentially with a 40x oil-immersion lens to produce a z-series of 24 optical sections separated by 1μm z-steps. The most medial part of the superficial dorsal horn was imaged to ensure the same area of each spinal cord section was compared.

The images were viewed and analyzed using Confocal Assistant and ImageJ (Free Programs). For each toxin group, six spinal cord sections from each of three rats were analyzed. The sixth section from each z-series was viewed and counted. Cells that stained positive for MOR and Y1R individually

were marked and counted by viewing the red (Y1R) and green (MOR) channels independently. Then the individual images were merged and the cells that were marked twice were counted as expressing both Y1R and MOR. An average number of MOR-expressing, Y1R-expressing, and MOR/Y1R co-expressing cells was calculated for each rat.

Statistical Procedures

All data were tested for equal variances, and satisfied, the Shapiro-Wilk criteria for normality prior to use of parametric statistical analyses. Raw anatomical data, or stained pixel counts (spinal cord sections), as described above, was compared using a standard t-test. For raw behavioral data (duration of nocifensive or escape responding, etc.), t-test and two- or three-way analysis of variance (ANOVA) techniques were used. In each case a minimum significance level of $P<0.05$ was required to reject the null hypothesis. As appropriate, Tukey's test or the Holm-Sidak Method was used for pairwise, post hoc comparisons within the ANOVA. All graphing and statistics were done with SigmaPlot v11 software (SPSS, Inc., Chicago, IL, USA).

CHAPTER 3

BEHAVIORAL EFFECTS OF NPY-SAP

Abstract

The spinal Neuropeptide Y system is a potential target for development of new pain therapeutics. Lumbar intrathecal injection of NPY is antinociceptive, reducing hyper-reflexia to thermal and mechanical stimulation, particularly after nerve injury and inflammation. We have previously shown that intrathecal injection of Neuropeptide Y-sap (NPY-sap) is also antinociceptive, reducing nocifensive reflex responses to noxious heat and formalin. In the present study, we sought to determine the role of dorsal horn Y1R-expressing neurons in pain by destroying them with NPY-sap and testing the rats on three operant tasks. Lumbar intrathecal NPY-sap 1- reduced CFA-induced hyper-reflexia on the 10°C cold plate, 2- reduced cold aversion on the thermal preference and escape tasks, 3- was analgesic to noxious heat on the escape task, 4- reduced the CFA-induced allodynia to cold temperatures experienced on the thermal preference, feeding interference, and escape tasks, and 5- did not inhibit or interfere with morphine analgesia.

Introduction

The superficial dorsal horn of the spinal cord is the first key area of nociceptive gating and modulation within the CNS (Melzack and Wall, 1965), and

therefore, a potential target for pain therapeutics (Cougnon et al., 1997;Silva et al., 2002). Several neuropeptides in the superficial dorsal horn are thought to play an important role in modulating nociception, including Neuropeptide Y (NPY). Intrathecal injection of NPY is antinociceptive, reducing hypersensitivity to thermal and mechanical stimulation.(Hua et al., 1991;Xu et al., 1994;Xu et al., 1998). This NPY-mediated antinociception is particularily evident after nerve injury and inflammation (Taiwo and Taylor, 2002;Mahinda and Taylor, 2004;Intondi et al., 2008), when levels of NPY and Y1R mRNA transcripts are increased in the dorsal horn (Ji et al., 1994).

The Y1 receptor is a key player in the NPY-mediated nociception. When a Y1R antagonist was intrathecally administered to animals with nerve injury or inflammation, the animals showed increased nocifensive hyper-reflexia and when it was adminstered along with NPY, the antinociceptive effect of NPY was effectively blocked (Taiwo and Taylor, 2002). Additionally, studies involving Y1R knock-out mice (Naveilhan et al., 2001;Shi et al., 2006;Kuphal et al., 2008) and conditional knock-down mice (Solway et al., 2011) have shown that mice lacking the Y1R have increased inflammatory and injury related nocifensive hyper-reflexia, which is not affected by intrathecal NPY.

Neurons that express the Y1 receptor can be permanently and selectively destroyed using NPY-sap, a conjugate of NPY and saporin, a ribosomal inactivating toxin. We have previously shown that intrathecal injection of NPY-sap produces a selective lesion of Y1R-expressing dorsal horn neurons, without affecting primary afferent neurons in the dorsal root ganglion. Rats treated with

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NPY-sap showed decreased nocifensive reflex responses to aversive heat and formalin (Wiley et al., 2009). These results suggest a significant role for Y1R-expressing neurons in modulating nocifensive reflexes, but do not necessarily reflect changes in the degree of discomfort experienced by the animal subject. In order to make inferences about analgesia, one must use responses that require cerebral processing of nociceptive information, such as those obtained from operant behavioral tasks (Vierck et al., 2005b).

In this study we looked at the effects of intrathecal NPY-sap on analgesia and the common clinical pain-related problems of hyperalgesia and allodynia by testing rats on three different operant paradigms before and after inducing CFA inflammation. In order to bridge the operant data obtained in this study to the existing literature on the effects of spinally administered NPY and NPY-sap, rats were first tested on a cold plate reflex task before and after inducing CFA inflammation. Then baseline operant nocifensive responses were assessed using three different operant paradigms. Each operant task asks the subject to make a context-specific choice about which stimulus condition it prefers, thus making possible inferences about the degree of discomfort/aversiveness experienced by the subject. Three operant paradigms are used here in order to rule out potential sources of confounding that may occur when relying on only one task. Given the anatomic and physiologic similarities between NPY and opioid peptides in the superficial dorsal horn, we examined morphine analgesia before and after inducing hyperalgesia/ allodynia, to assess any effect of NPY-sap on opiate analgesia.

Results

Coldplate Hindpaw Lick/Guard Responses

Prior to CFA injection, Blank-sap and NPY-sap rats were tested on the cold plate at 0.3°, 5°, and 10°C. At 0.3° and 5°C, rats from both groups responded equally with minimal hindpaw licking or guarding and none of the rats from either group responded by licking or guarding at 10°C (Figure 3). After CFA injection, all of the rats engaged in hindpaw licking or guarding at 10°, showing that CFA induced cold hypersensitivity in both experimental groups (Blank-sap: P=0.002, $F_{2,15}$=36.433, 2-way ANOVA; NPY-sap: P=0.023, $F_{2,12}$=49.079, 2- way ANOVA; Figures 3 and 4), however, the CFA-injected NPY-sap rats responded by licking or guarding significantly less than the CFA-injected Blank-sap rats (P=0.023, $F_{1,33}$=4.22, 2-way ANOVA, Figures 3 and 4).
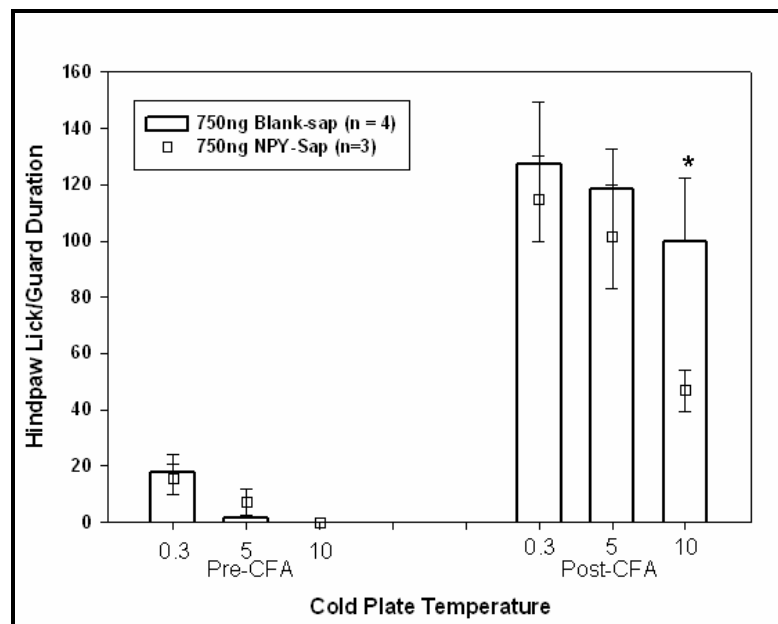
Figure 3: Effects NPY-sap on cold plate reflex responses. Graph shows effects of i.t. NPY-sap on lick/guard nocifensive responses on 0.3°, 5°, and 10°C cold plate before and after CFA-induced inflammation. Bilateral CFA increased reflexive responses of both Blank-sap and NPY-sap treated rats at all temperatures, with the NPY-sap rats responding significantly less than the Blank-sap rats at 10°C. Data was collected 5-7 weeks after NPY-sap injection and 4 days post CFA injection.

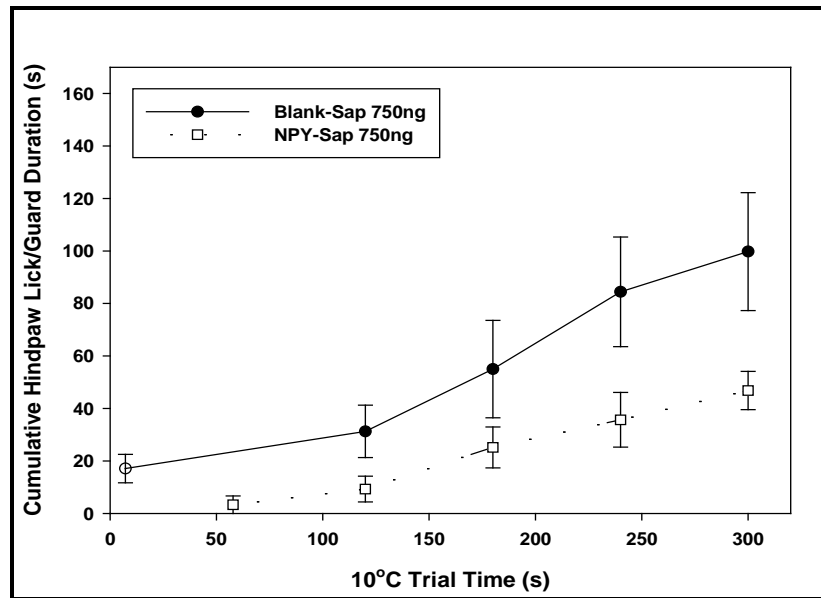**Figure 4: Effect of NPY-sap on 10°C cold plate after CFA. NPY-sap reduces CFA-induced hyper-nociception on the 10°C cold plate reflex test. Data is presented as a cumulative time course of lick/guard durations throughout the 300 second trial.**

Operant Behavioral Responses

Thermal Preference Task: Blank-sap control rats spent significantly more time on the hot (45°C) side of the thermal preference task than NPY-sap rats both before (P=0.03, $F_{1,4}$= 16.614, 2-way ANOVA, Figure 5A and B) and after CFA inflammation (P=0.01, $F_{1,4}$= 9.790, 2-way ANOVA, Figure 5C and D). After CFA injection, Blank-sap rats increased their time spent on the hot side, almost completely avoiding the 15°C side after the first minute of the trial (P=0.015, $F_{1,4}$=9.017, 2-way ANOVA, Figure 5C). NPY-sap rats responded the same before (Figure 5B) and after CFA inflammation (Figure 5C), spending about half the trial on each side of the apparatus.
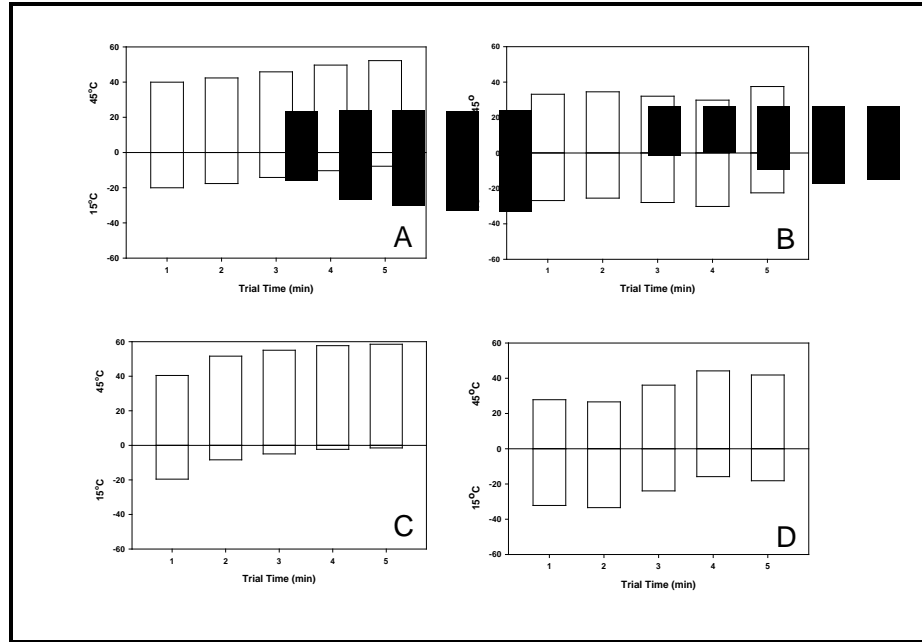
Figure 5: Effects of NPY-sap on thermal preference task. Time course data of thermal preference task shows that NPY-sap reduces cold aversion before and after CFA-induced inflammation. Each bar represents one minute of the trial and the rats' preference during that minute (black: 45°, white 15°). Blank-sap rats (A and C) spent more time on the hot side than NPY-sap rats (B and D) both before and after CFA inflammation. After CFA, Blank-sap rats almost completely avoided the 15° side (C). NPY-sap rats responded the same before (B) and after CFA inflammation (D). Data was collected 5-7 weeks post NPY-sap injection and 5 days post CFA injection.

Feeding Interference Task: Before CFA injection, both NPY-sap and Blank-sap rats spent the majority of the trial standing on the 10°C plate consuming the sweetened condensed milk (Figure 6A). After CFA injection, Blank-sap rats spent significantly less time on the cold plate feeding than they had previously ($P<0.001$, $F_{1,128}=14.465$, 3-way ANOVA, Figure 6B). NPY-sap rats showed no change in behavior after CFA, spending the same amount of time on the cold plate before and after CFA; with the result that NPY-sap rats spent significantly more time feeding than Blank-sap rats post-CFA injection ($P<0.001$, $F_{1,128}=11.555$; 3-way ANOVA, Figure 6B).
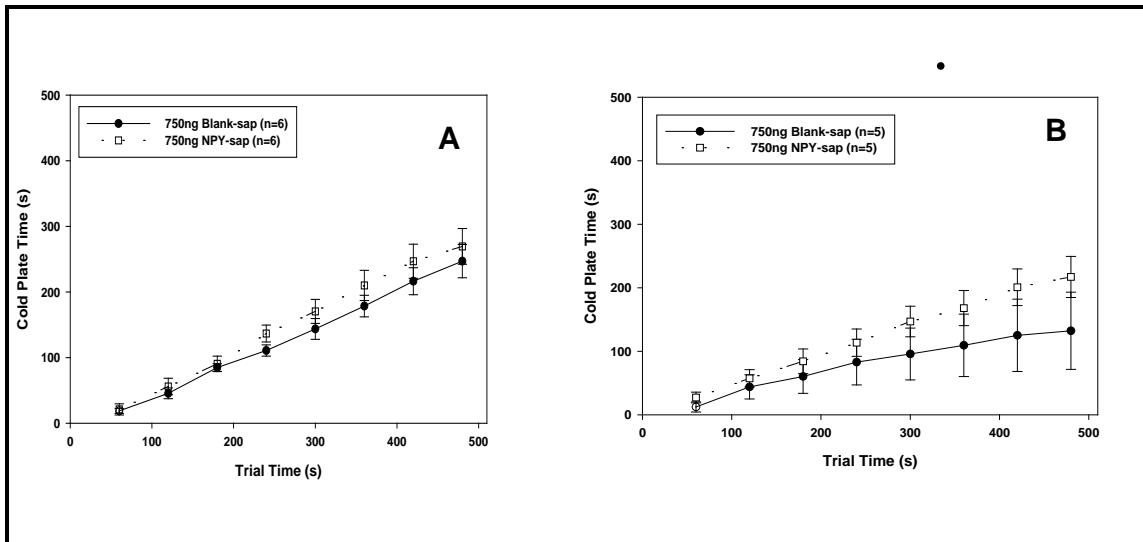
45

Figure 6: Effects of NPY-sap on feeding interference task. NPY-sap and Blank-sap rats responded similarly before CFA inflammation (A). After CFA (B), Blank-sap, but not NPY-sap, rats spent less time on the cold plate than they had previously, showing NPY-sap reduces CFA-induced thermal allodynia. Data were collected from 2-5 weeks post NPY-sap injection and 2-4 days post CFA injection.

Escape Task: NPY-sap treated rats chose to climb onto the escape shelf significantly less than Blank-sap rats at 10°C (p<0.001, $F_{1,120}$=62.659, 2-way ANOVA, Figure 7A), 15°C (p<0.001, $F_{1,120}$=12.572, 2-way ANOVA, Figure 7B), 44°C (P<0.001, $F_{1,120}$= 12.999; 2-ANOVA; Fig 7E), and 47°C (P<0.001, $F_{1,120}$=30.769; 2-way ANOVA; Fig 7F), indicating that all of these temperatures were perceived as less aversive to the toxin-treated rats than controls. There was no difference between control and NPY-sap rats in escape responding at the non-aversive temperatures of 20° and 38°C. Administration of both 0.5mg/kg and 1.5mg/kg systemic morphine significantly reduced escape duration of Blank-sap (0.5mg/kg: P=0.012; 1.5mg/kg: P<0.001; 2-way RM ANOVA, Figure 8A) and NPY-sap rats (0.5mg/kg: P=0.02; 1.5mg/kg: P<0.001; 2-way RM ANOVA; Figure 8B) from the 47°C hotplate. There was no significant interaction between NPY-sap and morphine.
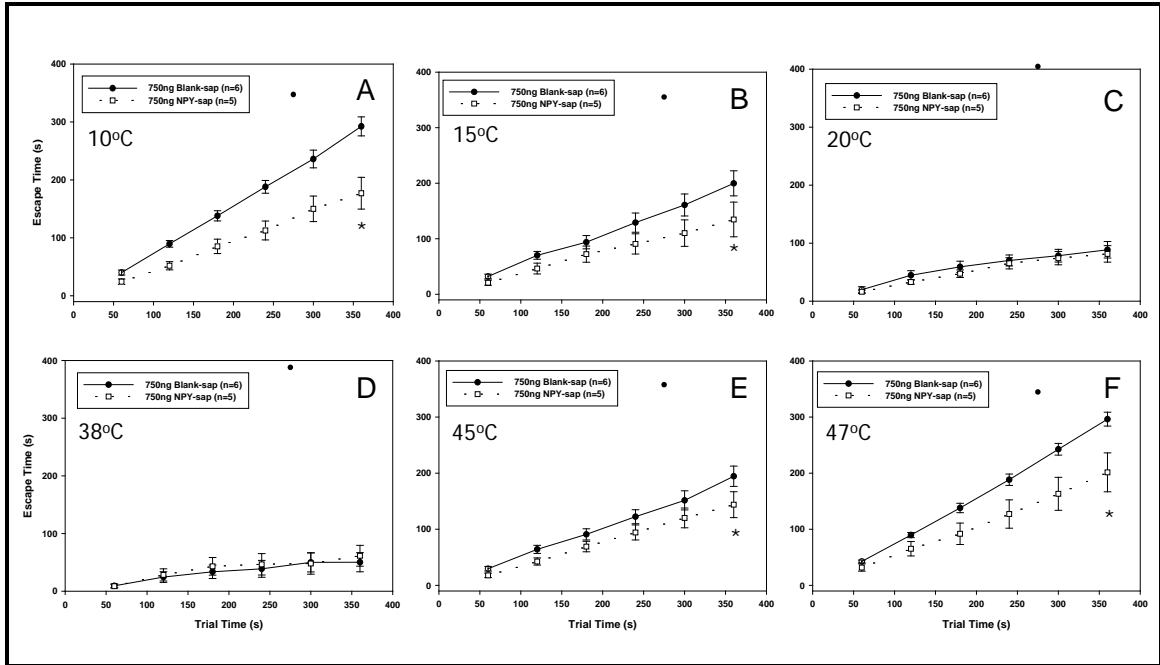
Figure 7: Effects of NPY-sap on the escape task. Time course data showing decreased escape responding (shelf time) by NPY-spa rats compared to Blank-sap rats at the aversive temperatures of 10°, 15°, 44°, and 47°C . There was no difference between NPY-sap and Blank-sap rats in escape responding at the neutral temperatures of 20°(C) and 38°C (D). Data were collected 2-5 weeks post NPY-sap injection.
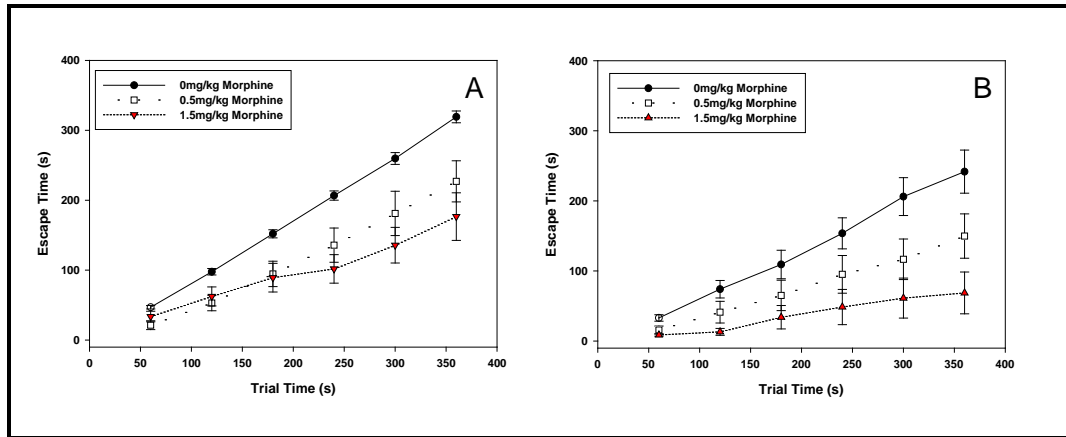
Figure 8: Effects of NPY-sap on morphine analgesia. Escape task time course data shows that administration of both 0.5mg/kg and 1.5mg/kg systemic morphine 30 minutes prior to testing significantly reduced escape duration of Blank-sap (A) and NPY-sap rats (B) from the 47° hotplate. There was no significant interaction between NPY-sap and morphine. Data was collected 5 weeks post NPY-sap injection.

After administration of CFA, Blank-sap rats escaped significantly more

from 15°C than they did prior to CFA injection (P<0.001, 3-way ANOVA, $F_{1,102}$=

22.075, Figure #A and C). Again, NPY-sap rats escaped less than Blank-sap

rats (P<0.001, $F_{1,102}$= 31.652, 3-way ANOVA, Figure 6A and C).
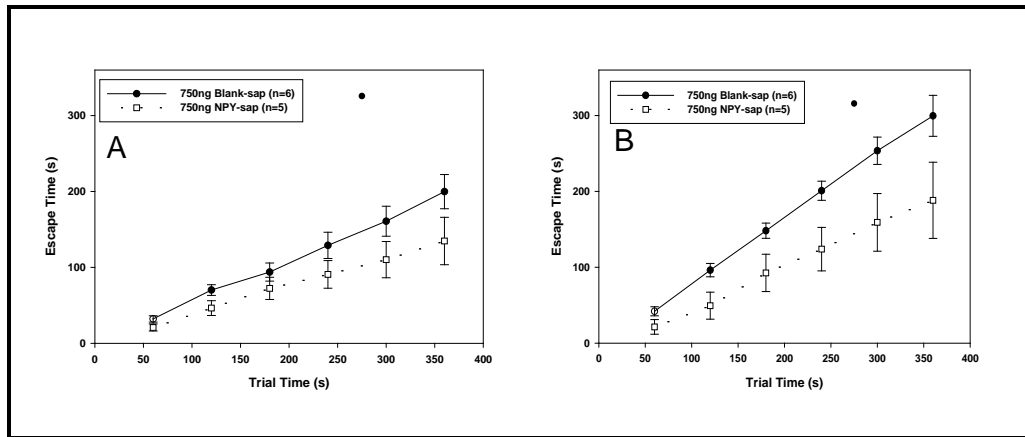
Figure 9: Effects of NPY-sap on escape task after CFA. NPY-sap reduces thermal allodynia on the escape task compared with controls. After administration of CFA (B), Blank-sap rats escaped significantly more from 15° than they did without inflammation (A). Data was collected 5 weeks post NPY-sap injection and 2-4 days post CFA injection.

Finally, both groups of rats were given systemic morphine injections and

tested on the escape task at 45°C. CFA inflammation did not affect baseline

responding (saline injections) of either group, compared with their pre-CFA

responding, while 1.5mg/kg morphine significantly decreased escape time of

both Blank-sap (P=0.012, $F_{1,9}$= 21.735, 2-way RM ANOVA) and NPY-sap

(P=0.007, $F_{1,9}$= 21.735, $F_{1,9}$= 18.436, 2-way RM ANOVA) rats (data not shown).

Again, there was no significant interaction between NPY-sap and morphine

analgesia.

Discussion

The principal observations of this study are: 1- bilateral plantar injection of

CFA led to marked hyper-reflexia in Blank-sap control rats, that was significantly

less in NPY-sap rats; 2- intrathecal NPY-sap reduced baseline cold aversion in normal rats with CFA on both the thermal preference and escape tasks; 3- intrathecal NPY-sap was analgesic to noxious heat in the escape task; 4- bilateral CFA caused cold hyperalgesia and allodynia that was less in NPY-sap rats, as measured using three operant tasks; and 5) intrathecal injection of NPY-sap did not interfere with morphine analgesia before or after CFA inflammation.

Prior to operant behavioral testing, rats were tested on the 10°C cold plate and reflexive lick/guard responses were measured. This was done to bridge the data from the operant tasks used in this study to the current literature on NPY and nociception to establish that the long evans female rats in these experiments were showing the same reflex changes previously reported. Before CFA injection, the 10°C cold plate was aversive enough to make the rats uncomfortable, but not enough to elicit reflex nocifensive responses from rats in either treatment group. After CFA injection, both groups of rats responded significantly more, showing that the CFA application was effective in inducing cold hyper-reflexia, however, CFA-induced hyper-reflexia was reduced by targeted elimination of the Y1R-expressing neurons, which concurs with previous reports that NPY is antinociceptive after inflammation.

Next rats were tested on each of three operant tasks. In the thermal preference task, rats were asked to choose between two compartments with different temperature floors, one noxious heat (45°C) and the other aversively cool (15°C). In baseline testing, the control rats showed some preference for the hot side, while the NPY-sap rats split the trial evenly between both

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compartments. These results could be interpreted two ways, that NPY-sap either increases heat sensitivity or decreases cold sensitivity. Taking into consideration data from previous reflex experiments (Wiley et al., 2009) and the other operant tasks in the present study, it is evident that NPY-sap induced loss of Y1R-expressing neurons leads to primarily decreased cold sensitivity. After administration of bilateral CFA, the control rats avoided the 15° side after the first minute of testing, exhibiting the cold allodynia associated with inflammation. The behavior of the NPY-sap rats did not change after CFA, indicating that loss of Y1R dorsal horn neurons blocked inflammation-induced cold allodynia.

While the control rats chose to avoid the 15° plate in the thermal preference task, they could be motivated to stand on a 10° plate by the availability of sweetened condensed milk in the feeding interference task. Before CFA inflammation, control and NPY-sap rats spent the same amount of time standing on the cold plate consuming milk at 10°C, however, at 8°C, the control rats spent less time on the thermal plate than the NPY-sap rats (data not shown). The temperatures used in the feeding interference task are slightly lower than in other tasks, presumably due to the rewarding properties of the milk, which motivates the rats to occupy an uncomfortable area that they would normally avoid. After CFA, however, the control rats spent significantly less time on the 10°C cold plate than the NPY-sap rats, again illustrating the cold allodynia produced by inflammation in control rats and the anti-allodynic effect of intrathecal NPY-sap.

Rats were next tested in the escape task, where they must choose to either stand on the thermal floorplate or escape to an angled, brightly lit, neutral temperature shelf. Rodents find bright lights aversive and they have to work to stay on the shelf due to its angle, so rats would not typically escape without motivation. NPY-sap rats escaped from the thermal surface to the neutral shelf less overall than control rats at 10°, 15°, 45°, and 47°. NPY-sap treatment was analgesic at both aversively cold and hot temperatures. The escape times of both groups were the same at neutral temperatures, indicating that the control rats had not developed an avoidance response and were sampling the floor temperature before escaping to the shelf. After CFA-induced inflammation, control rats escaped more from 15°C than they had previously, again showing that the allodynia produced by CFA inflammation in control rats was reduced in NPY-sap rats.

The present study is the first to demonstrate a role for NPY receptor-expressing neurons in pain, as opposed to nociception. The data from each of the three operant tasks agrees with what would be expected from the current knowledge of the spinal NPY system. The Y1 receptors are likely located on excitatory interneurons, which presumably provide excitatory drive to projection neurons, which are inhibited when NPY binds. NPY-sap kills the cells expressing Y1 receptors, permanently inhibiting them (see Chapter 4). Rats injected with 750ng NPY-sap were less sensitive to cold temperatures and not susceptible to CFA-induced cold allodynia, indicating a clear role for Y1R-expressing neurons in pain modulation, particularly after peripheral inflammation. Inflammatory pain is

typically associated with increased sensitivities to tactile and cold stimuli. We are currently lacking adequate treatments for chronic pain and the go-to drug for many clinicians is morphine, however, morphine is not very effective in minimizing cold sensitivities. In our experiments, bilateral CFA did not affect sensitivity to noxious heat, thus posing Y1R-expressing neurons as excellent candidates to be targeted for the development of analgesic drugs. It is important that any new treatment not interfere with morphine analgesia, so we injected the rats with systemic morphine and tested them on the escape task before and after CFA injection. We found that intrathecal NPY-sap did not interfere with or interact with the ability of morphine to produce analgesia.

It was not the intent of this study to examine the relationship between the spinal NPY and opiate systems. While Y1 receptors and μ-opiate receptors have been shown to interact and overlap within the spinal cord and several supra-spinal regions (Moran et al., 2004;Wang, 2004;Upadhya et al., 2009) and have similar electrophysiological properties (Moran et al., 2004), further anatomical studies and behavioral testing using intrathecal morphine injections would be valuable in determining if the lesion produced by NPY-sap effects spinalmu opiate mechanisms of analgesia.

Conclusions

The present results show that lumbar intrathecal injection of 750ng NPY-sap reduces sensitivity to cold stimuli and eliminates the allodynia associated with CFA-induced inflammation, without interfering with morphine analgesia,

indicating that the spinal NPY system would be an appropriate target for

development of analgesic drugs.

CHAPTER 4

ANATOMICAL EFFECTS OF NPY-SAP

Abstract

The spinal Neuropeptide Y system is involved in the modulation of pain and nociception, primarily through action on the Y1 receptors (Y1R), which are inhibitory. Selective destruction of Y1R-expressing neurons in the dorsal horn using NPY-sap significantly reduces dorsal horn Y1R staining and has also been shown to be antinociceptive, using both reflexive and operant measures. In this study, we attempt to further characterize the lesion produced by NPY-sap to assess the extent and selectivity of targeted neuron depletion. The effects of NPY-sap, Derm-sap, and Gal-sap on Y1R staining intensity are compared, along with the effect of NPY-sap on Gal-R1 and MOR expression. We found that only NPY-sap affects Y1R-expressing dorsal horn neurons. Additionally, we show that there are cells in the dorsal horn that likely co-express both Y1R and MOR. These observations of the anatomical effects of intrathecal NPY-sap form the basis for attributing the behavioral changes described previously in NPY-sap rats to a specific anatomic lesion.

Introduction

The superficial dorsal horn of the spinal cord is the first key area of nociceptive gating and modulation within the CNS (Melzack and Wall, 1965), and

therefore, a potential target for pain therapeutics. However, the precise organization of the dorsal horn remains incompletely understood. Several neuropeptides in the superficial dorsal horn are thought to inhibit, or negatively modulate, nociception, including neuropeptide Y (NPY), galanin, and μ-opioid peptides. While much is known about these peptide systems, their precise role in modulating nociception is unclear.

As previously described (Chapter 1), two NPY receptors, Y1R and Y2R, are localized in the dorsal horn. Y1 receptors are primarily post-synaptic and exert an inhibitory effect (Brumovsky et al., 2002;Brumovsky et al., 2006), while the Y2 receptors are pre-synaptic and excitatory (Hua et al., 1991;Brumovsky et al., 2005). The presence of these two receptors likely accounts for the biphasic effects sometimes seen after intrathecal injection of NPY. Similarly, intrathecal injection of galanin has also been shown to produce both pro and antinociceptive effects. Again, this is likely due to the presence of two receptors types, GalR1 and GalR2, in the dorsal horn. GalR1 receptors are primarily expressed post-synaptically on dorsal horn interneurons, while both GalR1 and GalR2 receptors are pre-synaptic (Xu et al., 1996;O'Donnell et al., 1999). Typically, GalR1 receptors are inhibitory (Zhang et al., 1995) and GalR2 receptors are excitatory (Liu et al., 2001;Liu and Hokfelt, 2002). Finally, the μ-opioid peptides exert their antinociceptive effects through the inhibitory μ-opiate receptor (MOR), which is located both pre- and post-synaptically.

While it is likely that each of these neuropeptide systems play a unique role in nociception, they have much in common. Galanin, NPY, and μ-opiate

peptides have each been shown to co-exist with GABA, and galanin and NPY

each co-localize with enkephalins, however, galanin and NPY are found in

different populations of neurons (Todd et al., 1992;Rowan et al., 1993;Simmons

et al., 1995;Zhang et al., 1995;Landry et al., 2000;Tiong et al., 2011). As

previously mentioned, each of these neuropeptides can have an antinociceptive

effect when administered intrathecally, likely exerted through action on the Gal-

R1 receptor, the NPY-Y1 receptor (Y1R), and the μ-opiate receptor (MOR),

respectively. Each of these receptors couples with $G_{i/o}$ subunits, consistent with

the observation that ligand binding exerts an inhibitory effect on target neurons

(Wang et al., 1998;Sun et al., 2001). Additionally, the Y1 receptor has similar

electrophysiological characteristics to the mu-opiate receptor (MOR) (Moran et

al., 2004) and intrathecal galanin has been shown to potentiate the

antinociceptive action of morphine in reflex testing (Wiesenfeld-Hallin et al.,

1990). With these similarities, it is unclear whether the peptidergic neurons,

and/or the corresponding neurons expressing the neuropeptide receptors,

represent three separate populations, or if there is overlap among them.

In order to attribute the behavioral changes seen after intrathecal NPY-sap

to the destruction of Y1R-expressing cells, it is critical to know whether the NPY-

sap targets only Y1R-expressing neurons or if it affects other populations, as

well. Our previous work with NPY-sap has shown that intrathecal NPY-sap

(750ng) significantly reduces dorsal horn Y1R staining in lamina I and medial

lamina II, without affecting DRG neurons(Wiley et al., 2009). In the current study,

we elaborated upon these findings to assess the extent and selectivity of the

targeted neuron depletion. We compared the Y1R lesion produced using NPY-sap with that produced using Derm-sap, which kills cells expressing the μ-opiate receptor, and Gal-sap, which kills cells expressing the Gal-R1 receptor. Also, we looked at the populations of Y1R-, Gal-R1-, and MOR-expressing neurons to determine if there's any overlap.

Results

Immunohistochemistry

Sixteen rats were injected with either 750ng Blank-sap, 750ng NPY-sap, 625ng Derm-sap, or 500ng Gal-sap. The dosages selected are the same as those used in behavioral experiments, since they yield significant behavioral changes without causing any adverse toxin effects. Blank-sap, composed of the saporin toxin conjugated to a nonsense peptide, was used as a control, the same as in the behavioral studies. Intrathecal injection of 750ng NPY-sap has previously been shown to produce a 40% reduction in dorsal horn Y1R staining. In the present experiments the reduction in stain was slightly less, with about 30% loss of Y1R staining, (p=0.019, t-test, Figure 10) but still resulted in significant behavioral effects as described previously (Chapter 3). The receptor loss in both cases was mainly in lamina I and medial lamina II of the dorsal horn. There was no reduction in Y1 receptor stain in tissue from Blank-sap, Derm-sap, or Gal-sap rats.
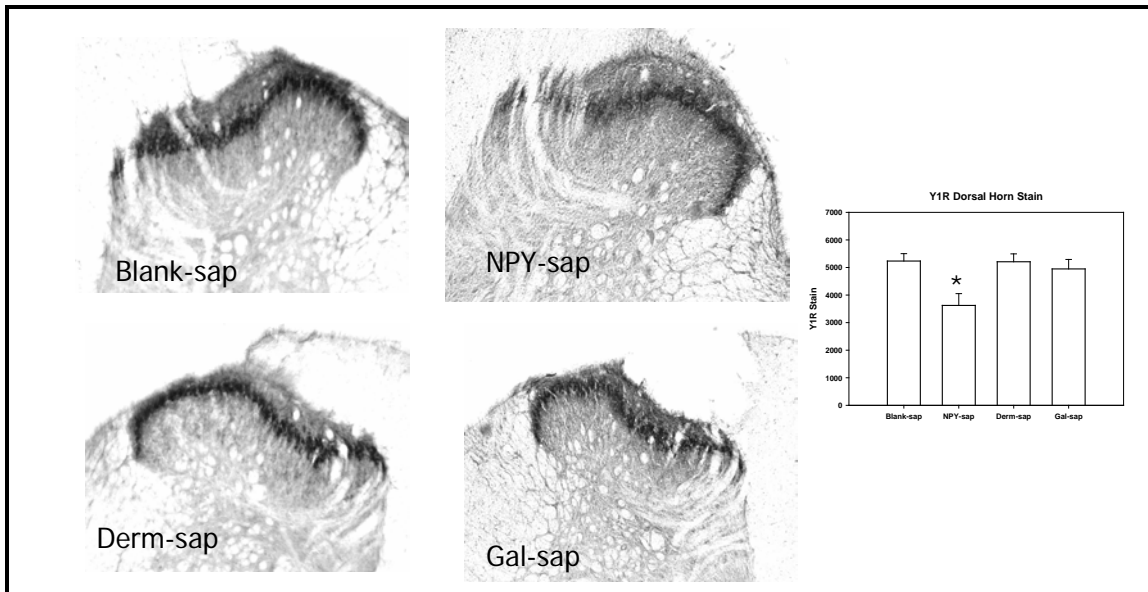
Figure 10: Intrathecal NPY-sap leads to a reduction in dorsal horn Y1R stain. Tissue from rats injected with Blank-sap, NPY-sap, Derm-sap, or Gal-sap was stained for Y1R. Only NPY-sap caused a lesion in Y1R-expressing cells.

Next the tissue was stained for MOR and Gal-R1. In the Derm-sap and Gal-sap tissues, staining intensities for their intended targets, MOR and Gal-R1, respectively, were each reduced by approximately 40% (MOR p=0.01; Gal-R1 p=0.03, t test). Neither toxin affected staining intensities of the other receptor. Staining for both receptors was decreased in the NPY-sap tissue (Figure 11), but the MOR stain counts just missed the significance level (Gal-R1 p=0.04; MOR p=0.05, t test).

Fluorescence

Tissue from the same rats injected with Blank-sap, NPY-sap, and Derm-sap, was stained for both Y1R and MOR and labeled using fluorescent secondary antibodies. The cells that express Y1R, MOR, and Y1R/MOR were counted and it was found that approximately 9% of the total cells counted

express both Y1R and MOR in control rats. This percentage was the same in NPY-sap treated rats, but the percentage of cells that co-express both receptors was increased in the Derm-sap rats at about 13%.
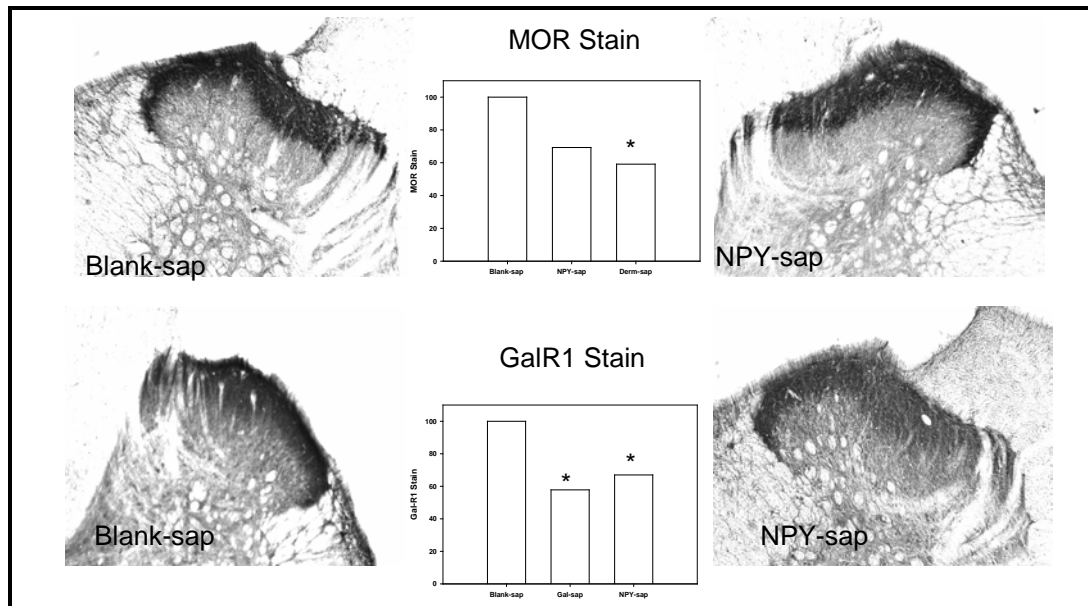
Figure 11: Effects of NPY-sap on MOR and Gal-R1 stain. Tissue from rats injected with Blank-sap, NPY-sap, Derm-sap, or Gal-sap was stained for MOR and Gal-R1. As expected, derm-sap rats had reduced MOR stain and gal-sap rats had reduced Gal-R1 stain, compared with Blank-sap rats. NPY-sap rats also had significantly less Gal-R1 stain and a decrease in MOR stain.

Discussion

We have previously published that intrathecal injection of 750ng NPY-sap leads to a 40% lesion of Y1R-expressing superficial dorsal horn neurons (Wiley et al., 2009). In the present experiments, the lesion caused by the same dosage of toxin was less, with about 30% loss. There could be several reasons for the difference, but it is most likely that, since the same stock of toxin was used for the injections in both experiments, which were several years apart, the toxin had begun to break-down over time and the dosage injected in the latter experiments

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was actually less than 750ng. Further justification for this is given in the same paper where we showed that tissue from rats injected with 750ng NPY-sap had less Y1R staining intensity than rats injected with 500ng NPY-sap, showing a relationship between dosage of toxin injected and extent of lesion produced.

The other two toxins used in these experiments, derm-sap and gal-sap, also create significant dorsal horn lesions. Derm-sap targets cells that express the μ−opiate receptor and Gal-sap targets cells expressing the Gal-R1 receptor. Tissue from rats injected with these toxins did not have decreased Y1R stain, indicating that the toxins do not affect Y1R-expressing neurons and are selective in targeting specific neuronal populations.

To further investigate the possibility of overlap in these neuronal populations, the NPY-sap tissue was stained for MOR and Gal-R1. Intrathecal injection of NPY-sap led to a decrease in staining intensity of Gal-R1 and, to a lesser degree, MOR. This data implies that the NPY-sap toxin also targets cells expressing Gal-R1 and MOR, however, if there were overlaps in the neuronal populations, you would have expected to see a decrease in Y1R staining intensity from injection of Gal-sap or Derm-sap, and we did not.

It is unknown how reliable the results using the Gal-R1 antibody are, since there is controversy in the literature over the specificity of the available antibodies and we weren't able to obtain consistent staining results using our antibody.

In the case of the MOR-expressing cells, these experiments were repeated several times and the MOR-expressing cell loss in NPY-sap rats was right around the significance point each time. It is possible that the immuno-

staining techniques being used were not sensitive enough to detect the loss of small populations of cells. This warranted a closer look at the cells themselves.

Tissue from Blank-sap, NPY-sap, and Derm-sap rats was double stained for both Y1R and MOR and analyzed under confocal microscopy. The main conculsion that can be drawn from these experiments is that some dorsal horn neurons express both Y1R and MOR. This is only a small percentage of the total Y1R and MOR-expressing cells. A probable explanation for the decrease in MOR staining seen with NPY-sap, when no decrease in Y1R was seen with Derm-sap is that these cells aren't targeted and destroyed by Derm-sap, but are by NPY-sap. One possible explanation is that the toxins might be more or less effective in destroying mono- vs. dual-receptor-expressing neurons. One such mechanism for this might be if one population of neurons is under more intense endogenous control than the other, there might be more endogenous ligand present to compete with the toxin for binding to receptors and the neurons might be less likely to be killed by the toxin.

Due to several complications that arose while these experiments were being conducted, this question of receptor co-localization still needs to be further investigated. Strategies for improving these experiments include: 1 – using a higher dose of toxin to produce more consistent lesions, reduce variability, and enlarge statistical sensitivity; 2 – sacrificing and processing tissue immediately prior to analyzing to ensure the brightest stain; and 3 – using a third label, such as NeuN, or other neuronal marker, to identify neurons, which would aid in the neuron-counting process.

Conclusion

Intrathecal injection of NPY-sap creates a significant lesion of Y1R-expressing neurons in the dorsal horn, which is clearly different from the lesions produced by other toxins, such as Derm-sap and Gal-sap. There is potential overlap between the populations of Y1R- and Gal-R1-expressing neurons, since NPY-sap also reduced Gal-R1 stain, which could be further investigated pending the availability of an improved Gal-R1 antibody. Finally, there also is evidence that some of the Y1R-expressing dorsal horn neurons co-express MOR.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

General Discussion

Millions of people worldwide suffer from poorly controlled chronic pain that

interferes with their productivity and ability to carryout normal everyday activities.

Unfortunately for those affected by chronic pain, we are currently lacking

effective long-term treatments without side effects. The development of new

treatments has been hindered, in part, by our limited knowledge of the

connectivity, wiring, and specific functions of the neurons involved in the pain

pathways, specifically those in the dorsal horn of the spinal cord, the first key

area of nociceptive gating and processing.

It has long been established that there are several neurotransmitters and

neuropeptides located in the dorsal horn that are involved in nociceptive

transmission. These consist of both excitatory and inhibitory signaling molecules

that act to either propagate or dampen the nociceptive signal, respectively.

In these studies, we undertook to investigate the spinal Neuropeptide Y

system by determining the role Y1R-expressing interneurons play in pain

modulation. The combination of the anatomical and behavioral results presented

here support the conclusion that Y1R-expressing neurons in the dorsal horn are

involved in modulation of thermal nociception, particularly cold pain and the cold

hyperalgesia seen after CFA inflammation, and loss of these neurons produces

thermal analgesia, consistent with the hypothesis that Y1R-expressing cells are excitatory interneurons.

We also compared our findings with NPY-sap rats to those treated with toxins that target two other groups of putative excitatory interneurons: GalR1-expressing neurons and MOR-expressing neurons. The present results with the lesion produced by intrathecal NPY-sap differ from: 1 - selective destruction of dorsal horn GalR1-expressing dorsal horn neurons by Gal-sap, which strongly decreases operant and reflex responses to aversive heat, bur may increase sensitivity to cold(Lemons and Wiley, 2011); 2 - selective destruction of dorsal horn MOR-expressing neurons with derm-sap, which has no effect on baseline reflex responses to heat(Kline and Wiley, 2008); and, 3 - selective destruction of dorsal horn MOR-expressing neurons with derm-sap and selective destruction of dorsal horn GalR1-expressing dorsal horn neurons by Gal-sap, in response to morphine injection, where both derm-sap and gal-sap treated rats show profoundly decreased antinociceptive effect of morphine(Kline and Wiley, 2008;Lemons and Wiley, 2011). The anatomical and behavioral results described in this work indicate that the population of neurons targeted by NPY-sap is unique and different from those targeted by either Gal-sap or Derm-sap. Once completed, the combined results from studies using these toxins and several others will greatly aid in decoding the pain pathways in the dorsal horn.

Our limited knowledge of the detailed wiring and connectivity of the pain pathway is not the only obstacle in the development of new, improved, pain treatments. The methods commonly used for testing pain also need to be

revised in order to increase our knowledge of the innate pain processing systems. The present studies also contributed to the field of pain research through their use of operant behavioral tasks, which are relatively new to the field of pain research and have been met with some resistance from pain investigators.

As described in Chapter 1, pain is a complicated, subjective, and individualized experience, generated from a combination of sensory, affective, motivational, and cognitive dimensions. Pain is measured in humans based upon their verbal descriptions or ratings of the sensations they're experiencing. Since animals are unable to describe their experiences, pain researchers have to decipher the animal's feelings from its behavior. The behaviors that are most commonly measured are reflexive responses to an applied stimulus. These reflex tests have been traditionally used by researchers and drug companies due to their ease of use and speed of generating large amounts of data. The problem with these tests is that they aren't clinically relevant. There is no cortical processing necessary for a reflexive response and these tests use such intense stimuli that the animal subject may actually develop a fear response that may skew the results.

The operant tasks involve cerebral processing and decision making, because in each test the animal is asked to make a choice about which environment it prefers, as previously described in Chapter 1. By observing the animals' choices, we can determine the extent that they are affected by the unpleasant thermal stimuli. These choices reveal the aversiveness, or

motivational qualities, of the stimuli and are therefore more reliably representative of pain than simple reflex responses. These tasks can also be set up for automatic data collection, which removes user bias and reduces error. Investigators have been reluctant to change from reflexive to operant behavioral testing due to the extensive training (sometimes up to a month) required of the animals before data can be collected. There are also very few publications using operant tasks for pain testing, so the literature isn't available for comparison of results.

The experiments in this work contributed more data and experience to the field of pain testing with using operant testing. We've illustrated the usability and validity of using operant tasks and shown that significant, meaningful data can be obtained in a short time and using few animals.

In regards to the current study, the operant tasks provided much more information than the reflex tasks on the role Y1R-expressing dorsal horn neurons play in pain and nociception. The reflex results suggest that Y1R-expressing neurons are more involved in modulating withdrawal reflexes driven by activation of C, rather than Aδ nociceptors, since NPY-sap treated rats had increased response latencies to 44°, but not 47°, or 52°C. Loss of Y1R-expressing neurons also led to decreased response events and total durations of responding at 47°C in Sprague Dawley male rats, but not Long Evans female rats (which were used in the current study)(Wiley et al., 2009). A simple cold-plate test was also unable to detect any effect of NPY-sap injection on 0.3°, 5°, or 10°C in normal rats, where both the thermal preference and escape tasks show that rats choose to

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avoid temperatures at or below 15°C when possible, unless they're rewarded for

remaining on the cold plate, as in the feeding interference task. Since the

operant tasks make use of comparisons and force the animals to choose an

environment, we can discern the motivations behind their behavior and gain

insight into their perception of the stimulus. Also, since they're more sensitive,

less noxious temperatures can be used, better allowing testing of animals with

enhanced pain states like inflammation or nerve injury. Thus, the results gained

from using operant tasks are more representative of pain and more clinically

relevant than results from simple reflex tests.

Future Directions

While the studies described in this work provide insight into the role of

Y1R-expressing dorsal horn interneurons in pain, a more complete picture of the

anatomical and functional connectivity of these neurons and their role in

nociceptive modulation can be gained with the completion of a couple additional

experiments.

First, to better define the site of toxin action, it would be worthwhile to

simultaneously inject NPY-sap and an Y1R or Y2R antagonist. We have shown

that lumbar intrathecal NPY-sap reduces dorsal horn Y1R staining, but has no

effect on Y1R-expressing DRG neurons and there is no evidence of cytotoxicity

in DRG neurons after injection of 1000ng NPY-sap(Wiley et al., 2009). Since

there is no mRNA for Y2R in the dorsal horn (Gustafson et al., 1997) and in

mouse dorsal horn Y2R protein appears to be almost entirely

presynaptic(Brumovsky et al., 2007), we conclude it's highly likely that only Y1R-expressing dorsal horn neurons are targeted by intrathecal NPY-sap. Since there aren't any antibodies available to stain for Y2R, co-administration of NPY-sap along with either Y1R or Y2R antagonists could be used to confirm the site of toxin action. The lack of toxin effect on presynaptic neurons is not specific to intrathecal NPY-sap injection. Intrathecal derm-sap has also been shown to selectively destroy MOR-expressing neurons in the superficial dorsal horn, without affecting MOR-expressing DRG neurons. A possible explaination for this is that the toxins are not internalized into the axons or terminals of DRG neurons, or that once internalized, the NPY-sap is not effectively transported to the cell bodies in the DRG.

Additional markers that can be stained for to help identify and define the neurons killed by the toxin and to help place the targeted neurons within the dorsal horn circuitry include: VGlut2 (excitatory interneurons), GAD/GABA (inhibitory interneurons), and retrograde tracers WGA/CTB (projection neurons). The behavioral data strongly indicates that the Y1R-expressing dorsal horn neurons targeted by the toxin are excitatory interneurons, staining for these markers can confirm this and also show if other types of cells express Y1R.

The behavioral data presented here shows that Y1R-expressing cells are involved in modulating CFA-induced cold hyperalgesia and allodynia. This finding agrees with the reflex literature, where the antinociceptive effect of intrathecal NPY in increased after CFA inflammation and nerve injury. Also, as described in Chapter 1, peripheral inflammation and nerve injury cause several

changes in the expression of NPY and its receptors in the dorsal horn and DRG.
For these reasons, it would be informative, and give a sense of completion to the
study, if these operant behavioral tasks were repeated using a model of
neuropathic pain, such as bCCI or SNI.

Inflammation and nerve injury aren't the only effective means of inducing
thermal hypersensitivity. As described in Chapter 1, capsaicin and mustard oil
activate the TRPV1 and TRPA1 receptors, respectively, when applied to the
surface of the skin, resulting in transient hyperalgesia(Patapoutian et al.,
2003;Foulkes and Wood, 2007). Since Y1R-expressing neurons also appear to
be involved in the modulation of pain from noxious heat, which is transduced by
the TRPV1 receptor, it would also be worthwhile to look at the effects of NPY-sap
after applying bilateral capsaicin to the rats' hindpaws. Moreover, since the
TRPA1 receptor is activated by noxious cold temperatures and Y1R-expressing
dorsal horn neurons are involved in modulating cold pain, it would be especially
valuable to investigate the effect of intrathecal NPY-sap on mustard oil-induced
hyperalgesia. Finally, it would be interesting to observe the effects of both
capsaicin and mustard oil in the NPY-sap injected rats before and after inducing
inflammation or nerve injury.

During the course of these experiments, there were two instances where
the toxin-injected and operant-task-trained animals were unable to be tested for
2-3 months. Before the testing hiatus, the rats' behavior clearly corresponded
with the two experimental groups, NPY-sap and Blank-sap, with the toxin rats
showing the same NPY-sap effects documented above. However, when testing

resumed, there was no longer a distinguishable difference between the two groups of animals. Without further investigation, it is unclear whether this effect was due to behavioral: from the rats not being handled for a length of time, or anatomical: neuronal plasticity, or re-wiring, of the dorsal horn, reasons. In order for NPY-sap, or any of the toxin-saporin conjugates, to be considered as effective pain therapeutics, they would need to exert a lasting effect. It would be worthwhile to inject rats with NPY-sap and monitor their behavioral responses over a long period of time. Since changes were noticed after just 2-3 months of inactivity, that time-range would be a good starting point for these experiments.

Finally, for a thorough comparison between the lesions, further behavioral testing using Gal-sap and Derm-sap injected animals should be conducted. The results from our initial experiments testing Gal-sap rats on the operant tasks suggest that Gal-sap might increase cold sensitivity.   This could be further explored by repeating the tests using cool and cold temperatures and also enhancing cold sensitivity through CFA inflammation or nerve injury. Our current data with Derm-sap rats is limited to reflexive tests, so testing those animals on the operant tasks would provide insight into the role of MOR-expressing dorsal horn neurons in pain. Furthermore, since both inflammation and nerve injury lead to a reduction of opioid receptors in the dorsal horn and DRG(Zhang et al., 1998), resulting in decreased efficacy of morphine after injury, it would be interesting to examine the effect of Derm-sap on pain responses in injury models and compare with the other toxins.

Conclusions

The data presented and discussed in this dissertation provides insight into the role of Y1R-expressing dorsal horn interneurons in pain. Rats injected with 750ng NPY-sap were less sensitive to warm and cold temperatures and did not experience CFA-induced cold allodynia, indicating that Y1R-expressing dorsal horn neurons play an important role in pain modulation, particularly after peripheral inflammation. Inflammatory pain is typically associated with increased sensitivities to tactile and cold stimuli. We are currently lacking adequate treatments for chronic pain and the go-to drug for many clinicians is morphine, however, morphine is not very effective in minimizing cold sensitivities. The involvement of Y1R-expressing neurons in modulating cold pain and the fact that intrathecal injection of NPY-sap does not interfere with morphine analgesia, along with the observation that that intrathecal NPY-sap doesn't interfere with protective reflexes, all pose Y1R-expressing dorsal horn neurons as excellent candidates to be targeted for the development of analgesic drugs.

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