The Spinal Neuropeptide Y System is a Potential Target for Chronic Pain Therapeutics

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Millions of people are affected by chronic pain—pain that persists for weeks, months, and even years. Chronic pain costs the economy billions of dollars in health care and disability and the effected individuals lose sleep, money and time from their lives due to their suffering. There are not any successful, long-term treatments for chronic pain, and development of new medications is hindered unfortunately because the precise organization and function of the neural mechanisms underlying nociception are still unclear. For these reasons, it is important to study the nociceptive pathway to learn the identity and function of neurons and transmitters involved, with the goal of identifying new targets for pain therapeutics.

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INTRODUCTION

Many degenerative diseases, nerve disorders, and nerve malfunctions can result in the impairment of physical sensations, such that an affected individual no longer has any sense of being touched, or perceive and ordinary stimulus as painful. These individuals also commonly suffer from chronic pain, which destroys their quality of life. While medicine has many effective ways to treat acute pain, numerous procedures for treating chronic pain have been developed, but have had limited success. Some of these procedures include local electric stimulation, deep brain stimulation, surgeries, alternative medicines like acupuncture, meditation and relaxation techniques and medications. Gaining a better understanding of the signaling molecules and neural networks involved in the pain pathway would be extremely beneficial to creating pain therapies and defining new targets for drug interventions. The goal of this research would be to take advantage of the natural pain transmission pathways and endogenous antinociceptive mechanisms to provide effective pain relief. The dorsal horn of the spinal cord is a prime location for this research. It is a key area of spinal pain transmission[1], but the precise organization and wiring of the neurons is unknown. 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[2]. While morphine 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 possible candidate is neuropeptide Y, because recent studies have shown that the spinal neuropeptide Y system is potentially involved in the modulation of nociceptive information[3].

Neuropeptide Y (NPY), a 36 amino acid peptide that is widely distributed throughout the central and peripheral nervous systems[4], has a variety of physiological functions including blood pressure control, feeding, anxiety, and memory[5]. There are at least five different receptor subtypes for NPY (Y1-Y5), with the Y1 and Y2 receptors being the most abundant[6,7]. Acting through its different receptors, neuropeptide Y has been shown to have an excitatory, inhibitory and biphasic effect on cells[8,9]. While more research is needed to confirm if the neuropeptide Y system could be a potential target for chronic pain therapies, the link between neuropeptide Y and nociception has been confirmed by anatomical, behavioral, and pharmacological studies. This review will examine the results from these studies and discuss the potential of using the spinal neuropeptide Y system as a target when developing therapeutics to treat chronic pain.

THE NEUROPEPTIDE Y MEDIATED SYSTEM IN THE DORSAL HORN

In order for NPY to exert a direct effect on nociception, its receptors would need to be located in key sites of nociception. The major spinal cord region involved in nociceptive modulation is the substantia gelatinosa, or the superficial layers (lamina I-II) of the dorsal horn[1].

Neuropeptide Y Y1 receptors in the dorsal horn

The neuropeptide Y Y1 receptor (Y1R) in the

dorsal horn is located primarily post-synaptically and is generally considered to exert an inhibitory effect[9,12]. Neuropeptide Y acts through a G-protein coupled receptor with $G_{i/o}$ subunits to inactivate adenylate cyclase[7,10]. This has an inhibitory effect as the signaling cascade normally activated by G-proteins is inactive. Additionally, the Y1 receptor can activate G-protein coupled inwardly rectifying potassium channels (GIRK). This hyperpolarizes the cell, resulting in its inhibition. Y1 receptors can also influence intracellular calcium levels by activating L-type Ca^{2+} channels[7,10].

There are at least seven different populations of Y1 receptor-expressing neurons in the dorsal horn and area X of the spinal cord. These neuron populations have been classified into types 1-7, with type 1 and type 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 and are found in lamina I. Some were identified to be projection neurons by retrograde labeling with Cholera Toxin-B subunit injected at the 9th thoracic segment[11].

It is likely that the Type 1 cells represent the same population of cells described by Zhang *et al.*, as small somatostatin-expressing interneurons[12]. This would indicate that Type 1 cells are excitatory interneurons through the indirect evidence that dorsal horn cells expressing somatostatin have been found to co-express the vesicular glutamate transporter 2 (VGLUT-2)[13], making the excitatory transmitter, glutamate, the primary neurotransmitter of those cells. Since NPY peptide co-localizes with γ-aminobutyric acid (GABA) in lamina II[14], NPY may be acting to reduce pain signals through inhibition 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. It is unknown under which circumstances these neurons are activated, but it is possible that 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[11].

Neuropeptide Y Y2 receptors in the dorsal horn

Spinal neuropeptide Y Type 2 receptors (Y2R) 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[15]. 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[15]. Since the Y2R regulates N-type calcium channels[16], it can allow more Ca^{2+} to enter the cell and trigger neurotransmitter release. Conversely, activation of the Y2 receptor in the dorsal horn has a net inhibitory effect, since it reduces Ca^{2+} currents and stops the release of excitatory amino acid neurotransmitters.[8] These processes are not yet completely understood and more research is still needed to clarify the data.

INTRATHECAL NEUROPEPTIDE Y REDUCES NOCIFENSIVE REFLEX BEHAVIORS

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 latency response latency in the $52^{o}C$ hotplate test[3]. (Typically the response measured in a hotplate test is paw-withdrawal and an "increased latency" indicates that the rat was slower to respond to the stimulus and is therefore interpreted as having decreased nociception.) This research was confirmed by Taiwo & Taylor who found increased paw-withdrawal latency in response to a radiant heat source, in addition to increased hotplate latency[17]. Additional evidence that NPY could be involved in regulating the spinal transmission of nociception came from intrathecal injections of NPY into anesthetized animals, resulting in a reduced nociceptive flexor reflex[18,19]. These behavioral tests show that i.t. NPY reduces protective reflex responses to acute noxious stimuli, but do not necessarily predict an effect in situations of persistent nocifensive stimulation or chronic pain.

Neuropeptide Y is antinociceptive after peripheral inflammation and nerve injury

A common way to model persistent nociception is to inject inflammogens into the plantar surface of the hindpaw. One such inflammogen is complete Freund's adjuvant (CFA), which causes thermal and mechanical hyper-sensitivity for several days[20]. CFA-induced hyperreflexia can be inhibited by i.t. injection of NPY, as shown by increased paw withdraw latencies in the hotplate test[16]. A model of acute peripheral inflammation is the formalin test, where a dilute formalin solution is injected into the plantar hindpaw surface. This damages the tissue, instantly causing intense behavioral and physiological responses that can be measured in terms of licking and flinching behaviors during the 90-minute test, which consists of two distinct phases separated by a relatively quiescent interphase period[21]. NPY dose-dependently inhibited licking behaviors in Phase I

Hyperreflexia
An increased reflexive response to a noxious stimulus.

Formalin Test
A model of acute peripheral inflammation where formalin is subcutaneously injected into the hind paw, where it damages the tissue, instantly causing intense behavioral and physiological responses that can be measured in terms of licking and flinching behaviors.

and[22] licking and flinching behaviors during Phases I and II of the formalin test[21,23].

Persistent nociception can also be induced through nerve injury. The spared nerve injury (SNI) model involves unilateral transection of two out of the three terminal branches of the sciatic nerve[23]. The peroneal and tibial nerves are cut, leaving the sural nerve intact. This results in robust mechanical and thermal nocifensive hyperreflexia (an increased response to a noxious stimulus). The behavioral effects of this injury are seen within 24 hours and last for at least six months[24]. Neuropeptide Y, when administered two weeks after SNI surgery, completely inhibited the enhanced nocifensive responses to mechanical, heat and cold stimuli produced by the nerve injury[22]. These studies indicate that intrathecal injection of NPY is effective in reducing nocifensive reflex responses after peripheral inflammation and nerve injury.

Spinal neuropeptide Y system changes after inflammation and nerve injury

The behavioral studies described above suggest that there might be a link between neuropeptide Y and inflammatory or neuropathic pain. It has been found that peripheral inflammation leads to increased levels of NPY and Y1R mRNA transcripts in the dorsal horn[25,26]. This indicates that following CFA injection there are more Y1 receptors, and thus more places for NPY to bind. Additionally, after nerve injury there is increased NPY binding in the dorsal horn[27]. These changes to the NPY system suggest increased NPY signaling and therefore increased inhibition of nociceptive signals. The results support a possible role for neuropeptide Y in the modulation of inflammatory or neuropathic pain.

NEUROPEPTIDE Y ANALGESIA IS BLOCKED BY 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[16]. This presumably was the result of blocking endogenous NPY from binding to the receptors. When BIBO3304 was given concurrently with NPY, the analgesic effect of NPY was completely inhibited. These effects were similar in the SNI experiments where BIBO3304, when administered along with NPY, completely reversed the anti-allodynic effects of NPY. The Y2 antagonist BIIE0246 also was effective in reducing the anti-allodynic effects of NPY when they were

administered together[23]. These experiments provide evidence that the antinociceptive effects of intrathecal NPY can positively be attributed to action of the peptide at its spinal receptors.

NEUROPEPTIDE Y ANTINOCICEPTION IS INHIBITED IN Y1 RECEPTOR KNOCK-OUT MICE

The antagonist studies showed that both the NPY Y1 and Y2 receptors play a role in modulating nociception. Naveilhan *et al.* further investigated the role of the Y1 receptor in nociception using Y1 receptor knockout (Y1R-KO) mice that were developed at the Karolinska Institute using homologous recombination. The Y1R-KO mice demonstrated a marked nocifensive hyperreflexia, 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[28]. The Y1R-KO mice also had a much reduced mechanical threshold, which was measured using the Von Frey test[27,29]. They also showed 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. Additionally, the response of the knock-out mice to nerve injury was tested using a partial sciatic nerve ligation model. The nerve injury caused mechanical hyperreflexia in wild-type mice, which was notably increased in the knock-out mice[27].

These Y1R knock-out mice experiments were confirmed and elaborated upon by Kuphal *et al.*, who used knockout 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 hyperalgesia in the Y1R-KO mice. CFA also produced mechanical hypersensitivity 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 hypersensitivity. The anti-hyperreflexia effects of i.t. NPY were reduced in the Y1R-KO mice compared to the wild-type[30].

The enhanced nocifensive reflex responses caused by knocking out the Y1 receptor can likely be attributed to the fact that the 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

Tail flick test
A test to measure thermal sensitivity where a beam of radiant light is focused on the tail until a response is emitted.

Von Frey Test
A test used to measure mechanical sensitivity, involving nylon monofilaments that, when pressed against tissue until they bend, exert a calibrated amount of force.

Neuropathic Pain
Pain arising as a direct consequence of a lesion or disease affecting the somatosensory system.

Intrathecal
The fluid-containing space around the spinal cord, also called the spinal canal.

Response latency
Measure of time elapsed from application of stimulus to response.

have increased transcript levels of Substance-P and CGRP, but lower levels of the peptides compared to wild-type. This could indicate that they have an increased release of the excitatory peptides, with a rapid transport of the peptides from the cell bodies, leading to increased nociception[28]. The inability of i.t. NPY to cause antinociceptive effects in the knock-out mice strongly suggests that the antinociceptive reflex effects of NPY are modulated primarily through the NPY-Y1 receptors. Of course, null mice lack Y1R everywhere in the nervous system raising the possibility that the behavioral effects observed were due to changes at supraspinal sites, in addition to any spinal changes.

CONCLUSIONS AND FUTURE DIRECTIONS

Neuropeptide Y receptors are located at the major spinal site of nociceptive regulation. While there is debate over the role of the Y2 receptor in nociception, it is clear that neuropeptide Y acting through its spinal Y1 receptor has an antinociceptive effects. Intrathecal NPY reduced reflexive responses to noxious thermal stimuli and was also very effective at reducing nocifensive reflex responses in situations of inflammation and nerve injury, which are widely used as models of chronic pain. That these effects are specifically linked to the injection of NPY is verified by the fact that they can be blocked by simultaneously injecting a NPY antagonist along with the peptide. Furthermore, the evidence given by the Y1R knockout mice, where no NPY analgesia could be produced, supports an important role for the Y1 receptor in nociception.

Neuropeptide Y receptors have potential as a target for chronic pain therapeutics

The data reviewed in this paper provides a strong foundation for the idea that the neuropeptide Y system could be a target for developing therapeutics for chronic pain, however, there is still more research needed to be done before such a statement can be made for sure. A glaring shortcoming of the research that has been done to date is that all of the behavioral tests used only measure protective reflexes. When looking for a treatment for clinical pain, it is important to use tests that measure what is clinically relevant. Tonic clinical pain is generally associated with prolonged input from c-fibers, which can be activated by low rates of heat transfer[31]. Reflexive tests may not be clinically relevant for testing chronic pain. Additionally, since reflexes involve only the spinal cord, and can be observed in decerebrate animals[17,18], they may not provide reliable information as to what the animal is experiencing. Operant behavioral tests may be better suited for chronic pain research because they force the animal to make decisions on how to deal with noxious stimuli.

Reflexive tests
Measure involuntary movements made in response to a stimulus.

Operant tests
Measure behaviors that require spinal‑cerebrospinal integration and specifically require a learned, motivated behavior that terminates exposure to the noxious stimulus.

They can use less intense stimuli and involve cerebral processes. The amount of time spent in contact with noxious stimuli can give researchers an idea of what the animal is experiencing[32]. Until NPY is tested in an operant setting, all we know for sure is that it is an effective reflex modulator.

Additionally, we need a more precise way to investigate what is happening at the cellular level in the dorsal horn—which receptors are involved and which cells express them? The answers to these questions are important since potential therapeutics would act on the spinal NPY receptors. The knock-out animals are a good start, but there are two major downfalls to using them. First, the animals develop without the Y1 receptor and second, the animals have no Y1 receptor throughout their entire neuraxis[28,29]. These issues are problematic since much pain modulation occurs at levels of the brainstem and above, not to mention the other functions of NPY that might be affected by the lack of the Y1 receptor. A better model would be a knockout that can be conditionally turned on after development, or to specifically kill the cells in the spinal cord that express the Y1 receptor using new targeted toxin technology.

The potential for neuropeptide Y to be used as a therapeutic agent in treating chronic pain certainly exists and the actions of NPY after inflammation and nerve injury suggest that it is effective as much more than a reflex modulator. Researchers in this area are on the right track and with the right additional experiments we could possibly have a new peptide system for drug companies to target.

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FURTHER INFORMATION

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