

**OPIOID AND NON-OPIOID ACTIONS OF  
DYNORPHIN A ON SPINAL WIDE DYNAMIC RANGE  
NEURONS**

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## **ABSTRACT**

Dynorphin A is an endogenous peptide that can activate opioid receptors and bradykinin receptors; the latter has been proposed to be a mechanism of dynorphin A's pronociceptive actions. Sensitization of wide dynamic range (WDR) neurons in the spinal dorsal horn causes innocuous stimuli to be perceived as pain. Under conditions of chronic inflammation, elevated levels of dynorphin A are found in the spinal cord and could contribute to central sensitization by modulating WDR neuron function. To test the effect of dynorphin A on WDR neurons, we performed *in vivo* extracellular recordings on the spinal cord of anesthetized rats in the presence of a dose range of an opioid, dynorphin A (1-13), or non-opioid dynorphin A (2-13). WDR neurons' response was attenuated in naïve rats by dynorphin A (1-13). Dynorphin A (2-13) did not potentiate WDR response to nociceptive inputs in naïve rats. These findings suggest that the pronociceptive actions of intrathecal dynorphin A is unlikely to be mediated by activation of the WDR neurons in physiological conditions. A fragment of dynorphin A, LYS1044, retains high affinity for bradykinin receptors but has no agonist activity, and could provide a basis for therapeutics using dynorphin A as a new modality for pain.

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## **INTRODUCTION**

### **Neuropathic Pain**

Neuropathic pain is caused by direct injury to either peripheral or CNS neurons or disease affecting the part of the nervous system that signals pain. Symptoms include pain evoked by normally non-painful mechanical or thermal stimulus, or spontaneous pain (Baron, 2006). Pain signals are normally carried by C-fibers and A $\delta$ -fibers that respond primarily to noxious stimuli. A $\delta$ -fibers are myelinated, with larger diameter axons, and a conduction velocity on the order of 20 m/s; these neurons are responsible for acute pain sensations in response to weaker intensity of stimulus. C-fibers are unmyelinated fibers characterized by slow conduction velocity on the order of 2 m/s. C-fibers are also polymodal, responding to mechanical, thermal, and chemical stimuli, active not only in pain sensation, but also for itch, warmth, touch, and cramp. Specifically, C-fiber nociceptors respond to strong intensity stimuli and produce slow, dull, long-lasting pain (Purves, 2004).

Changes may occur after nerve injury, which promote spontaneous neuron firing, or firing in response to innocuous stimuli. These changes may include upregulation of sodium channels, presynaptic N-type calcium channels, or vanilloid receptors like TRPV1, causing heat hyperalgesia (Baron, 2006). Release of proinflammatory mediators also creates ectopic pain in noninjured nociceptors in response to cytokines like TNF- $\alpha$ .

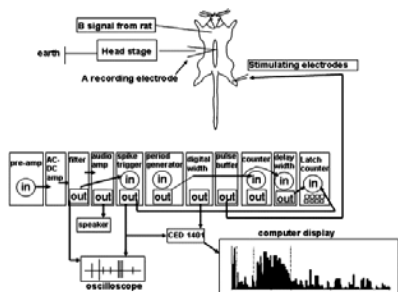
C-fibers synapse to second-order neurons in the spinal cord dorsal horn. These second-order neurons include wide dynamic range, high threshold, and low threshold neurons. They exhibit windup, or temporal summation of signals upon repeated stimulation. These neurons ascend to the brain stem and thalamus, forming the spinothalamic tract (Chung, 1979).

Some of the current treatments for neuropathic pain involve opioids, non-steroidal anti-inflammatory drugs (NSAIDs), and anticonvulsants, namely gabapentin and pregabalin, that act upon the  $\alpha_2 \delta_1$  and 2 subunits of voltage gated calcium channels (Baron, 2006). Many patients respond differently to medication, and not one drug can work for all conditions in the vast number of afflictions and categories of neuropathic pain. Thus, there is still a pressing need to investigate the mechanisms underlying neuropathic pain with the goal of developing better drugs to treat more targets.

## Wide Dynamic Range Neurons

Wide dynamic range neurons (WDR) are second order neurons in the deeper laminae that receive mechanical and thermal sensory input from peripheral non-nociceptive as well as nociceptive input from C-fibers, A $\beta$ -fibers, and A $\delta$ -fibers. WDR neurons are differentiated from high and low threshold second order neurons by their response to both weak and strong mechanical stimuli (Chung, 1979). They also receive inhibitory input from GABA releasing interneurons and descending pathways from the brain. In abnormal pain states, spontaneous C-fiber firing results in WDR sensitization so that input from non-nociceptive fibers becomes painful (Baron, 2006).

Alterations in WDR firing can be directly measured by performing extracellular electrical recording of these neurons in anesthetized rats. A schematic of the recording setup and equipment is displayed in Figure 1 below.



**Figure 1:** Single neuron signals from the recording electrode within the spinal cord are characterized by depth, A- and C- fiber thresholds, and receptive field size. Action potentials above certain amplitude are amplified and filtered into audio signals and oscilloscope display. They are displayed by the computer on a rate histogram and as numerical action potentials per set time frame. Electrical stimulation is administered via stimulating electrodes in the neuronal receptive field (Urch, 2003).

By directly recording from the WDR neurons, it is possible to characterize their neurophysiological properties, what may modulate these properties, and how these properties may change under pathological conditions like peripheral nerve injury.

## Central sensitization

Central sensitization has been presented as a mechanism underlying neuropathic pain, alongside peripheral changes. It is characterized by alterations in the spinal cord dorsal horn leading to WDR hyperexcitability, transforming innocuous sensations transmitted by A $\beta$  and A $\delta$  fibers into painful ones; these effects are initiated and maintained by the hyperexcitability of C-fibers. Repeated stimulation of C-fibers causes glutamate release, acting on post-synaptic NMDA receptors.

Peripheral nerve lesion and sensitization of A and C afferents may lead to central sensitization in several ways. Upregulation of N-type calcium channels on C-fibers results in increased glutamate release, contributing to dorsal horn sensitization. Substance P, a nociceptive neuropeptide, is also released and maintains dorsal-horn sensitivity (Luo et al., 2001). Dramatic upregulation of the voltage gated sodium channel subtype, Nav1.3, in injured primary afferents is thought to promote spontaneous ectopic discharge characteristic of injured fibres, while another sodium channel subtype, Nav1.8, may prolong the excitability of primary afferents through its distinctive slow-inactivating, fast-repriming kinetics. Post-synaptically Nav1.3 channels also become upregulated (Hains et al., 2004). Altered expression of potassium-chloride exporters shifts the transmembrane anion gradient, which may cause GABA release to provide an excitatory instead of inhibitory effect in lamina I neurons, contributing to central sensitization (Coull et al., 2003). Non-neural glial cells may contribute by releasing proinflammatory cytokines (Wieseler et al., 2005). Thus, functional perturbation of one or more of these pre-synaptic and post-synaptic signaling molecules in the ascending nociceptive pathway upon an injurious insult to the peripheral nervous system could contribute to central sensitization.

The current working model proposes that central sensitization is essential to establishing a persistent pain state by inducing a long-term adaptation of the sensory nervous system to inputs. This systems adaptation may involve a loss of descending inhibition of pain transmission from the brain, as evidenced by the clinical efficacy in some cases of serotonin and noradrenalin reuptake blocking antidepressants. Preclinical findings also suggest that sensory hypersensitivity may be due to enhanced descending pain facilitatory pathways in structures such as the mesencephalic reticular formation

(Ossipov et al., 2006). Such adaptations at the systems level are likely to produce long-term, fundamental changes in an individual's sensory function, sometimes in spite of an apparent recovery from the initial injury.

## **Dynorphin A and Bradykinin**

### **Structure of Dynorphin A**

The Lai lab pioneered in uncovering dynorphin A's agonist action at bradykinin receptors and its potential relevance for peripheral nerve injury induced pain. Dynorphin A is an endogenous opioid peptide that is widespread throughout the CNS, but exists in higher concentrations in the hypothalamus, medulla, pons, midbrain, and spinal cord (Goldstein and Ghazarossian, 1980). It has a broad range of actions including appetite (Lambert et al., 1993), as well as stress and depression regulation through an opioid mechanism (Land et al., 2008). It is produced from prodynorphin, and on occasion, is released as big dynorphin with dynorphin B. Dynorphin A is selective for  $\mu$ -opioid receptors, and seems to exhibit affinity for NMDA and bradykinin receptors (Lai et al., 2006). Like other opioids, dynorphin A is stored in large dense-core vesicles that require more intense stimuli for release into the synaptic cleft (Drake, 2007).

The sequence of dynorphin A (1-17) is Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln. The N-terminal Tyr is essential for opioid binding; in-vivo aminopeptidases rapidly reduce dynorphin A to its des-Tyrosyl form, thereby removing its opioid affinity, but retaining its non-opioid effects (Young et al., 1987). Rapid -COOH shortening has also been observed. However, the peptide retains its opioid affinity as C terminal amino acids are removed. Dyn (1-5) is also known as Leu-enkephalin, but Dynorphin A and Leu-enkephalin do not exhibit the same cross-reactivity in tissues, so the smaller opioid may be alternatively derived (Goldstein and Ghazarossian, 1980).

### **Physiological Effects of Dynorphin A**

Dynorphin A is upregulated in the dorsal horn of the spinal cord under a number of pathologies that result in chronic pain states (Campillo et al., 2010). Evidence supporting this includes increased dynorphin A immunoreactivity in both the superficial and deeper laminae 10 days after SNL surgery (Malan et al., 2000). The physiological effects of dynorphin A are dose dependent and at higher doses, contrary to its potent in-vitro opioid effect. Some studies have found that intrathecal injection of 10  $\mu$ g dynorphin A into the spinal cord produces a long-lasting analgesic effect, more potent than even morphine on a per molar basis in the spinal cord (Han and Xie, 1982), whereas intracerebroventricular injection of dynorphin A antagonizes morphine's opioid effects. Others have reported that at non-paralytic doses, intrathecal injection of dynorphin A has no anti-nociceptive effect despite potent in-vitro kappa opioid agonism; this led people to believe that perhaps the tail-flick or other motor tests were not the best assessment of motor impairment in rats (Stevens and Yaksh, 1986). However, it remains clear that at and above doses of 20 nmol, dynorphin A impedes motor function and leads to paralysis (Ren et al., 1985). Dynorphin A's upregulation correlates with spinal cord injury and is directly involved in neuronal cell death in both the dorsal and lumbar horns; NMDA receptor activation and Asp release are directly responsible for this effect (Skilling et al., 1992). The excitotoxic effects of dynorphin A have also been investigated via its activation of caspase-3 activity, promoting neuronal apoptosis at concentrations of 10  $\mu$ M (Singh et al., 2003).

The mechanism by which Dynorphin A plays a role in spinal sensitization remains unclear. However, Dynorphin's ultimate effects on NMDA receptor function may be central to its excitotoxicity at high dose. Prodynorphin knockout mice that underwent SNL surgery exhibited a return to baseline by day 10, whereas neuropathic pain was sustained in wild type mice. Intrathecal dynorphin antiserum reversed this pain in wild type mice at post-SNL day 10. The decreased pain thresholds in both wild type and knock out and time dependent antihyperalgesic effect of dynorphin antiserum indicated dynorphin was not necessary to initiate nerve injury-induced pain, but maintained it. MK-801, an NMDA antagonist, caused early and later reversal of pain thresholds, revealing that the pain itself was NMDA receptor dependent, but could be activated by two separate systems, including dynorphin's upregulation after post-

SNL day 10 (Wang et al., 2001). Dynorphin A's actions on NMDA have also been observed; NMDA-evoked prostaglandin release stimulated by Dynorphin A (2-17) has been linked to activation of p38 mitogen activated protein kinase (Svensson et al., 2005); p38 MAP kinases are involved in the body's response to cytokines and stress, maintaining nerve injury and inflammation-induced pain (Ji et al., 2002a).

Dynorphin's non-opioid effects are not limited to NMDA and excitatory amino acid release. The phenomenon of opioid-induced hyperalgesia also implies increased sensitivity may be opioid pathway related. Prolonged morphine exposure may lead to enhanced expression of Dynorphin A's pronociceptive actions, and may also play a role in the brain's descending pain facilitation. Morphine enhanced capsaicin induced calcitonin gene related peptide (CGRP) release in opioid induced hyperalgesia is blocked by dynorphin A antiserum; dynorphin A expression also increases in response to prolonged morphine exposure. CGRP is a potent vasodilator and pro-nociceptive peptide, and this implies dynorphin A may contribute to an abnormal state of central sensitivity caused by chronic morphine use (Gardell et al., 2002).

Additional evidence for dynorphin A's pronociceptive effects include studies where inhibition of dynorphin A activity has consistently normalized painful responses in rat behavioral tests. This, coupled with the observations that mice with a null mutation in the prodynorphin gene do not exhibit persistent pain after peripheral nerve injury indicate dynorphin A activity is critical for the maintenance of chronic pain (Lai et al., 2008).

### **Dynorphin A's actions on Bradykinin receptors**

Dynorphin A elicits an intracellular calcium response that is NMDA independent, indicating a non-opioid, non-NMDA component to neural excitation (Tang et al., 2000). The bradykinin receptor is another non-opioid target of dynorphin A.

The bradykinin peptide is a pro-inflammatory, pronociceptive peptide released from blood vessels after injury. Two types of bradykinin receptors exist centrally and peripherally, B1 and B2. B2 is constitutively expressed while B1 expression is induced by injury. Bradykinin receptors are Gq coupled GPCRs that result in mobilization of intracellular calcium through IP3 activation. Dynorphin A activates bradykinin receptors resulting in calcium influx through voltage gated calcium channels in sensory neurons, providing functional evidence for dynorphin A's interaction with bradykinin receptors. It has been observed, however, that the Ca<sup>2+</sup> mobilization resulting from dynorphin A acting on bradykinin receptors is not PKC coupled, but instead may be cAMP pathway dependent, with downstream activation of L-type and P/Q type calcium channels.

Interestingly, there is no evidence of de novo action of the kinins in the spinal cord. In addition, kinins would be degraded before reaching the spinal cord. Injection of bradykinin into the cord produces conflicting responses involving hyperalgesia at low dose and antinociceptive effects at high dose. As prodynorphin and bradykinin receptors are both localized to the superficial laminae of the spinal cord, and bradykinin receptors are expressed on DRG neurons expressing substance P or CGRP, a possible pathway for dynorphin A's potentiation of excitatory neuropeptide release emerges.

In-vivo blockade of B1 and B2 receptors, using the antagonists DALBK and HOE-140 respectively, reverse neuropathic pain in the presence of elevated levels of dynorphin in rats. Dynorphin A injection in wild type rats produced hypersensitivity indicated by lowered pain thresholds, which was reversed in a dose-dependent manner by bradykinin antagonists. The same dose-dependent reversal of pain thresholds was observed for SNL animals treated with DALBK and HOE-140 without additional dynorphin A injection. Reversal of hyperalgesia by HOE-140 followed the same time dependency for dynorphin A upregulation, matching dynorphin A's profile for maintaining but not initiating neuropathic pain. Thus, dynorphin A's maintenance of neuropathic pain is likely to occur through interaction with bradykinin receptors. (Lai et al., 2006).

This project investigates dynorphin A's actions on the bradykinin receptor, using dynorphin A peptide fragments as a model for design of neuropathic pain-specific bradykinin receptor antagonists. Additionally, as sensitization of wide dynamic range neurons is central to neuropathic pain transmission,

this project also investigates dynorphin A's potential role in wide dynamic range neuron sensitization after nerve injury under elevated conditions in the deeper laminae.

#### **RATIONALE/HYPOTHESIS/SIGNIFICANCE**

The specific actions of dynorphin A in the spinal cord have yet to be defined. Although in vitro intracellular calcium mobilization in response to bradykinin receptor activation by dynorphin A has been observed to explain dynorphin's in vivo maintenance of neuropathic pain, the location and precise mechanism is still unclear. The contrast between dynorphin A's potent in vitro opioid effects and in vivo excitotoxic effects indicate opioid and non-opioid actions of dynorphin A. A possible non-opioid mechanism could link dynorphin A to sensitization of wide dynamic range neurons and other neurons in neuropathic pain.

Our working hypothesis is that at physiological levels, spinal dynorphin A acts predominantly at opioid receptors of which it has high affinity (with dissociation constants ranging from 1 nM at the kappa receptor to 150 nM at the delta receptor) and selectivity. However, when its expression levels are significantly enhanced under pathological conditions, the higher synaptic concentrations of dynorphin A and its proteolytic fragments exhibit cross reactivity at other molecular targets including bradykinin receptors. These interactions of non-opioid dynorphin A (2-13) with bradykinin receptors expressed on wide dynamic range neurons, may contribute directly to the non-opioid, excitatory, and pronociceptive effects of dynorphin A witnessed in vivo.

Testing the des-Tyrosyl fragment of dynorphin A reveals lower affinity, non-opioid targets and their resultant effects. Dynorphin A (2-13) is a minimal fragment that exhibits physiologically relevant pharmacological effects. Tests could also be accomplished with the original opioid fragments by antagonizing opioid receptors with naloxone for  $\mu$ , nor-BNI for  $\kappa$ , and naltr indole for  $\delta$ , but mixing multiple compounds complicates their combined effects. Opioid antagonists could also influence the body's natural tone, altering pain thresholds prior to dynorphin A testing. Thus, both opioid and non-opioid fragments were analyzed separately.

The project's design directly measures changes in wide dynamic range neuron activity in response to proteolytic fragments of dynorphin A in naïve animals. The response at various concentrations is recorded using the frequency of action potentials at several time points. If dynorphin A is found to have a predominantly inhibitory effect on neuronal activity, and  $\delta$ -opioid receptor antagonists will be administered to determine the specific opioid receptor responsible for inhibition. If dynorphin A activates wide dynamic range neurons, B1 and B2 receptor antagonists DALBK and HOE-140 will be administered; normalization of activity will indicate an interaction between dynorphin A and bradykinin receptors contributing to wide dynamic range neuron sensitization.

In most disorders, pain is a symptom that disappears with resolution of the underlying disease. However, in conditions where the underlying disease cannot be cured, chronic pain management becomes a priority. Diabetes mellitus, osteoarthritis, and bone cancer are examples of these conditions. Neuropathic pain resulting directly from damage to the nervous system can present similarly in many disease states. Thus the need for effective chronic pain treatment touches a widespread population. In addition, response to pain treatment varies greatly from person to person (Baron, 2006). Existing opioid pain medications including oxycodone, fentanyl, and morphine, also cause drowsiness, nausea, and digestive problems. Long-term consequences of opioid treatment include tolerance, drug dependency, as well as hyperalgesia. Clinically, pain treatment disparities are related to consequences regarding opioid use for pain relief, as some physicians wary of drug seeking behavior will refuse to prescribe painkillers. Physicians have been documented to have given patients over the counter medication instead of stronger medication due to racial biases, resulting in potential under treatment of pain (Green et al., 2003). In addition, pain relief is central to workers with high occupational hazards who are commonly minorities. In these ways, providing new routes for pain treatment without the negative consequences associated with powerful analgesics may also reduce disparities in pain treatment for minority populations.

Additionally, although NMDA receptor antagonists are developing, these antagonists could have blanket effects throughout the nervous system. Exploring dynorphin A as a new, more specific modality with less harmful side effects is crucial to combating chronic pain (Wang et al., 2000).

With the hopes of bolstering drug efficacy by personalizing treatment for a genetically diverse population, as well as reducing adverse and addictive side effects in existing treatment, there is a necessity for additional pain drug development. Investigating the key players behind central sensitization and hyperalgesia will introduce new routes for pain treatment.

## **MATERIALS AND METHODS**

### ***Chemicals***

With the exception of dynorphin A (1-13) (purchased from American Peptides), all dynorphin A analogs were provided by Dr. Yeon Sun Lee at the Department of Chemistry and Biochemistry, University of Arizona. Opioid receptor antagonists were purchased from Sigma.

### ***Transfection of HEK-293 cells***

HEK-293 cells were transfected with cDNA for the human B1 receptor (GenBank ACC# AY275464) or that for the human B2 receptor (GenBank ACC# AY275465) incorporated in vector pcDNA3.1. A midi plasmid prep was performed by transforming DH5 $\alpha$  competent E. coli cells to express B2 plasmid. 2  $\mu$ l of 50  $\mu$ l stock, 200 ng plasmid DNA was added to 50  $\mu$ l DH5 $\alpha$  cells and mixed gently. The mixture was incubated on ice for 30 min and heat shocked for 30 sec at 42°C. Tubes were placed on ice for 2 min, and 950  $\mu$ l SOC+LB medium was added to each tube, incubated for 1 hour at 37°C at 225 rpm. Plates containing 15 ml LB agar and ampicillin were inoculated at concentrations from 10  $\mu$ l, 100  $\mu$ l, and 250  $\mu$ l over night at 37°C. Single colonies were picked from plates and inoculated in a 2.5 ml culture of LB media with ampicillin, and incubated for 8 hours at 37°C at 300 rpm. 200  $\mu$ l of starter culture was added to 100 ml of LB media and ampicillin, incubated for 16 hours at 37°C at 300 rpm. Bacterial cells were harvested by centrifugation at 6000xg (6250 rpm JA-14 rotor) for 15 min at 4°C. A Qiagen Midi Plasmid Prep kit was used to extract and purify the plasmid. After DNA was precipitated and centrifuged, the pellet was air-dried and dissolved in 10 mM Tris Cl, pH 8.5 to a final concentration of 0.5  $\mu$ g/ $\mu$ l. Plasmid was sequenced prior to transfection and evaluated for purity with a 280/260 ratio of 1.8.

HEK-293 cells were prepared 1 day before transfection in log growth phase, incubated at 37°C, 5% CO<sub>2</sub>. 6 wells of 100,000-300,000 cells were plated in 2 ml per well of HEK media without antibiotics (50 ml/L fetal bovine serum (FBS), Minimum Essential Medium (MEM)). DNA was mixed with FuGene 6 Transfection Reagent (Roche) and incubated 15-30 min, then 100  $\mu$ l of mixture was added to each well and shaken. Cells were incubated under normal growth conditions (37°C, 5% CO<sub>2</sub>). 2 ml HEK media was added to each well after 6 hours incubated. After 48 hours, HEK media was prepared with 1 mg/ml G-418 (neomycin) for selection. 2 cell culture petri dishes per transfection ratio were created in a 1:15 dilution from 6-well plates to dishes. When colonies grew containing 100-200 cells, a 100  $\mu$ l pipet tip was used to scrape colonies under a microscope and transferred into 24 well plates with 1.5 ml of 600  $\mu$ g/ml geneticin. Once wells were confluent, cells were suspended in 500  $\mu$ l of 600  $\mu$ g/ml G-418 in 25 cm<sup>2</sup> flasks with 5 ml HEK media. Transfected cells with a stable expression of the receptors were screened using radioligand binding and cloned based on neomycin (1 mg/mL) resistance. Saturation analysis was performed on stably transfected cell lines.

### ***PCR Analysis of Transfected Cell Lines***

Transfection plasmids were analyzed using BamHI, XbaI, XhoI, and EcoRI restriction enzyme digests. 0.5  $\mu$ l enzyme was incubated for 1 hr at 37°C with 2  $\mu$ l of 0.5  $\mu$ g DNA in 1  $\mu$ l enzyme buffer and 6.5  $\mu$ l ddH<sub>2</sub>O and run on a 1.2% agarose gel with 5  $\mu$ l/50ml GelStar Stain and imaged at 300 nm.

hB1 and hB2 HEK-293 transfected cell lines were tested for expression hB1 and hB2. Forward and reverse primers for specific sequences were selected using PrimerBLAST, and are as follows: hB1

forward primer: 5'-TGCACAGAGTGCTGCCGACA-3', hB2 reverse primer: 5'-GGCCTCATGGGGGAGGAGCA-3,' hB2 forward primer: 5'-TGCTGCTATTCATCATCTGC-3,' hB2 reverse primer: 5'-CTGAATGGGTTCTGACCTG-3.' RNA was extracted from lysed cells using a Biorad Total RNA Miniprep Kit Aurum #732-6820. RT-PCR was then used to create cDNA from RNA extract. 1ug RNA was mixed with 10 ug DEPC water. 2 ul oligonucleotides were added with primers to separate tubes and heated to 80°C for 3 min. MMRTase, RNase inhibitor, dNTPs, and RT buffer from Ambion Retroscript #AM1710. RNA was cycled at 42°C for 1 hour then at 92°C for 10 min.

The resulting cDNA was then amplified using PCR using Biorad iTaq polymerase, MgCl<sub>2</sub>, dNTP mix, and iTaq buffer. Cycles were run at 95°C for 5 min, followed by an annealing cycle at 55°C and 72°C cycle. Resulting DNA was run on a 1.2% agarose gel with 5 ul/50ml GelStar Stain and imaged at 300 nm.

### ***Membrane preparation from rat brain and transfected HEK 293 cells***

Rat brains were harvested from naïve Sprague-Dawley rats and placed in 50 ml ultracentrifuge tubes, 4 brains per tube. Brains were homogenized in 20 ml of 50 mM Tris buffer pH 7.4 with 2 uM/ml PMSF for 30 sec and spun at 2000xg (4000 rpm, JA-18 rotor) for 15 min at 4°C. The supernatant was recovered and homogenized. The spin and homogenization was repeated two times, and finally spun at 17,500 rpm for 30 min at 4°C. The pellet was resuspended in Tris buffer, 2 brains/12 ml.

Cells were cultured in 150 cm<sup>2</sup> flasks until 95% confluence. The growth media was aspirated, and cells were rinsed with 10 ml PD buffer. 10 ml of 50 mM Tris buffer pH 7.4 with 2 uM/ml PMSF was added. HEK-293 cells were scraped off and combined into centrifuge tubes and incubated on ice for 15 min for complete lysis. The cells were centrifuged at 2000xg (4000 rpm, JA-18 rotor) for 15 min at 4°C. The supernatant was poured off, and the pellet was resuspended in 10 ml Tris buffer with PMSF to each tube and homogenized for 30 sec on ice. The homogenate was centrifuged at 40,000xg (17,500 rpm, JA-18 rotor) for 30 min at 4°C. The pellet was resuspended in Tris buffer with PMSF, 20 ml per flask.

### ***Saturation Analysis***

Transfected HEK293 cells expressing hB1 or hB2 receptors were harvested and resuspended in 50 mM Tris, pH 7.4. Cell were incubated with [<sup>3</sup>H]Bradykinin (hB2) or [<sup>3</sup>H]Kallidin (hB1) (concentration range: 0 – 4 nM) for 3 hours at 25°C. Non-specific binding was defined by that in the presence of 10 μM bradykinin or kallidin, respectively. Data were analyzed by non-linear least squares analysis using GraphPad Prism.

### ***Competitive radioligand Binding***

[<sup>3</sup>H]Kallidin/ligand competition analyses were carried out using crude membrane preparations from rat brains which contain bradykinin B2 receptors. Membranes were resuspended in 50 mM Tris pH 7.4 buffer. Binding reactions were done using 10 concentrations of a test article (0.1 pM – 100 μM), 1 nM [<sup>3</sup>H]Kallidin, 50 μg brain membranes, protease inhibitor cock-tail, at 25°C for 3 hours in a shaking water bath. Non-specific binding was defined by that in the presence of 10 μM kallidin. Membranes were rapidly filtered through Whatman GF/B filter paper and washed with 4 mL of cold 0.9% saline. Radioactivity was determined by liquid scintillation counting in a Beckman LS6000. Data from three independent experiments were analyzed by non-linear least squares analysis using GraphPad Prism.

### ***Phosphatidylinositol hydrolysis Assay***

24 well plates were seeded with B2 transfected HEK-293 cells, 150,000 cells per well, and incubated overnight. Growth media was exchanged with IMDM containing 4% FBS and 0.2 uM [<sup>3</sup>H] Inositol and incubated for 20 hours. Tracer growth media was exchanged for IMDM for 1 hour, then replaced by IMDM with 500 mM LiCl for 1 hour. Media was aspirated and replaced with test compounds for 1 hour.

Cells were washed with MeOH and scraped into tubes containing [ratio?] ddH<sub>2</sub>O and chloroform and spun at 4000 rpm, 5 min. Supernatant was transferred into tubes containing ddH<sub>2</sub>O and poured into

anion exchange columns. After 3 H<sub>2</sub>O washes, a 5 mM Sodium Tetraborate, 60 mM Sodium Formate, 0.2 Ammonium Formate, 0.1 Formic acid solution was washed twice through columns. Columns were eluted with 2 ml 0.2 mM Ammonium Formate/0.1M Formic Acid solution into 10 ml scintillation vials, to which 9 ml scintillation fluid was added to each vial, and the radioactivity was determined by scintillation counting in a Beckman XX. Data were analyzed by non-linear least squares analysis using GraphPad Prism.

### ***Electrophysiology***

Male Sprague-Dawley rats (Central Biological Services, University College London, UK) weighing 250-270g at time of electrophysiological experiments were employed for this study. All experimental procedures were approved by the UK Home Office and followed the guidelines under the International Association for the Study of Pain (Zimmermann, 1983)

Animals were anesthetized with isoflurane (1-1.2%) delivered in a gaseous mix of N<sub>2</sub>O (66%) and O<sub>2</sub> (33%). A laminectomy was performed to expose the L4-5 segments of the spinal cord. Extracellular recordings were made from ipsilateral deep dorsal horn neurons (lamina V-VI) using parylene coated tungsten electrodes (A-M Systems, USA). One neuron was recorded from each animal which all had defined receptive fields in the toe regions of the hind paw.

Wide dynamic range neurons were located in lamina V by lowering the electrode into the cord at 500-1000 $\mu$ m depth. Oscilloscope signals from firing neurons were observed while tapping the hindpaw ipsilateral to the site of electrode insertion. The characteristic, large amplitude firing pattern of WDR neurons continued after removal of stimulus. Once a potential WDR was identified it was isolated by shifting the electrode within the cord so that the amplitude of a single neuron was 4 times greater than background. Cell death resulted at times by overexciting neurons or puncturing with the electrode; this property was also used to isolate single cells by killing an interfering cell. To prevent WDR death, stimuli were spaced so that spontaneous firing in an excited cell was allowed to taper off before testing began. WDR neurons exhibit windup, tested by inserting two electrodes into the receptive field on the hindpaw and applying a train of 16 transcutaneous electrical stimuli (2ms wide pulses, 0.5 Hz). Input values were recorded; windup occurred when number of action potentials from the final pulse was significantly larger than 16 times initial input. Threshold of a selected WDR was also tested by raising the administered current in single pulses until two signals are heard, the second being delayed C-fiber firing.

Three sets of controls were then run on the identified WDR cell. A train of 16 transcutaneous electrical stimuli (2ms wide pulses, 0.5 Hz) were applied at 3 times the threshold current for C-fibers, following which a post-stimulus histogram was constructed. Responses evoked by A- $\beta$  (0-20ms), A- $\delta$  (20-90ms) and C-fibers (90-350ms) were separated and quantified on the basis of latency. Responses occurring after the C-fiber latency band were taken to be the post-discharge of the cell (350-800ms).

The peripheral receptive field was also stimulated using a range of natural stimuli (dynamic brush, von Frey filaments, 2g, 8g, 16g, 26g, and heat, 40, 45, 48°C) over a period of 10 seconds. Heat was applied with a constant water jet onto the centre of the receptive field. To account for water jet mechanical pressure, a control is recorded at 35°C. Data was captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions).

The testing procedure was carried out every 20 minutes and consisted of a train of 16 electrical stimuli followed by natural stimuli as described above. Following three consecutive stable control trials (<10% variation) neuronal responses are averaged to give the pre-drug control values.

### ***Drug administration***

Three doses of each drug (dynorphin A (1-13) and dynorphin A (2-13)) were tested on each neuron in successively higher concentrations so that a series of dose-response curves could be generated and the results were then pooled for the population of cells for 100 nM, 250 nM. A few tests were carried out at 500 nM. The effect of each dose was followed over an hour, with tests carried out at 10, 30 and 50 minutes before subsequent doses were applied and effects again followed at the same time points.

## Data Analysis

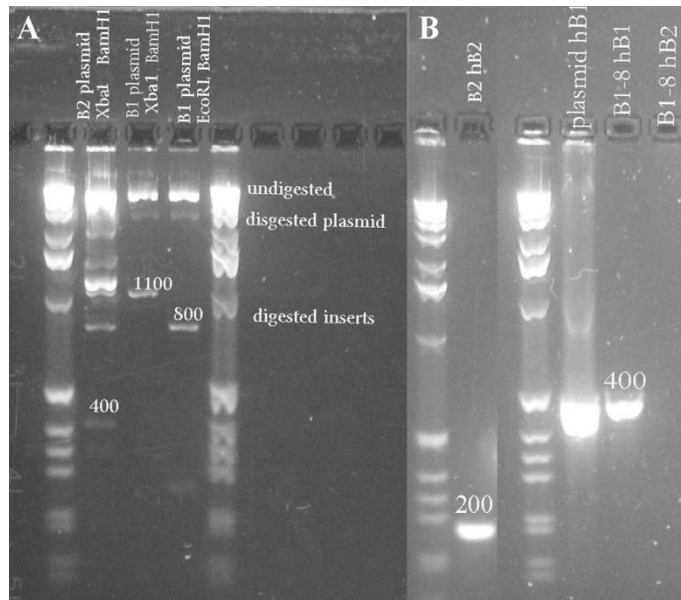
All data are presented as mean  $\pm$  standard error of mean (S.E.M.). Behavioral data were analyzed using the non-parametric Mann-Whitney *U*-test. Pre-drug control neuronal responses between groups were compared one-way analysis of variance (ANOVA). Drug effects were expressed as the mean maximal evoked neuronal response for each dose. Statistical analysis was performed on raw data with drug effects assessed using analysis of variance with repeated measures (RM-ANOVA). Where a significant effect was seen with increasing dose, Dunnett's post hoc tests were then used to assess individual dose effects. All statistical testing was performed by using Prism 4.0 software (GraphPad/Prism, San Diego, Calif). Level of significance was set at \*  $P < 0.05$ .

## RESULTS

### HEK-293 transfected cells express high levels of hB2 receptor

The hB2 and hB1 transfected HEK-293 lines were produced as in-vitro models to test for B2 and B1 receptor function using the phosphatidylinositol hydrolysis assay. No endogenous expression of bradykinin receptors was found in untransfected HEK-293 cells, consistent with the fact that HEK-293 is receptor and channel poor kidney cell line, and bradykinin receptors may be found in neuronal, lung, and smooth muscle tissues. HEK-293 cells provided a resilient and simple system for in-vitro analysis, but required screening and verification of stable bradykinin receptor expression. The identities of the transfection plasmids were first analyzed using restriction enzyme digests. Restriction enzyme analysis of the of hB2 and hB1 cDNA incorporated into the 5.4kb pcDNA3.1+ expression vector produced expected fragments of 1000 and 400 for hB2 with XbaI and BamHI, 1082 for hB1 with XbaI, Bam HI, and 860 for hB1 with EcoRI and BamHI.

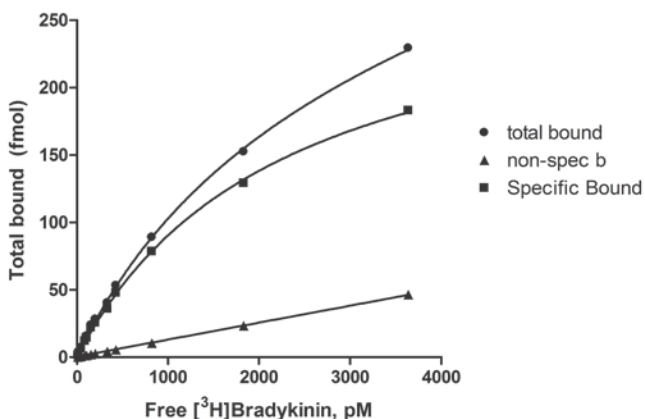
PCR analysis of mRNA extracted from the transfected hB2-HEK-293 cells produced an expected band at 230 bp. Similar analysis of the hB1 transfected cells produced an expected band at 400 bp. Although expression levels were not quantified during PCR, the brightness of the bands suggested significant expression of the receptors as a result of stable transfection. The hB1 transfected line has been prepared for future functional assays and is not used in the present study. The hB2 transfected cell line is routinely maintained and used for the purpose of this study.



**Figure 1:** PCR analysis of hB1 and hB2 plasmid for transfection (A) and of hB1 and hB2 expressing HEK-293 transfected cells (B). Plasmid digests by restriction enzymes correspond to predicted fragments expected from cleavage of XbaI, BamHI, EcoRI, in plasmid vector pcDNA3.1. Transcript fragments of expected size based on primer forward and reverse positioning confirmed existence of hB2 transcript in hB2 transfected HEK-293 cells, and hB1 transcript in hB1 transfected HEK-293 cells. No hB2 expression was detected in hB1 transfected cells, indicating no endogenous hB2

expression in HEK-293 cells.

Receptor density was quantified using saturation binding analysis of [<sup>3</sup>H]Bradykinin to the B2 receptor. The K<sub>D</sub> of bradykinin at cells overexpressing human B2 receptor was found to be 2.25 ± 0.0 nM, with a B<sub>max</sub> of 540 fmol/10<sup>6</sup> cells, or 1.5 pmol/mg protein. These data are consistent with published K<sub>D</sub> values for bradykinin binding to bradykinin receptors, and further confirms stable transfection of a high level of B2 receptors in the cells.



**Figure 2:** Saturation analysis of [<sup>3</sup>H]Bradykinin at the transfected hB2 receptor in stably transfected HEK-293 cells. The dissociation constant (K<sub>D</sub>) of [<sup>3</sup>H]Bradykinin is 2.25 ± 0.0 nM. The binding is saturable, showing a B<sub>max</sub> value of 540 fmol/10<sup>6</sup> cells.

### Dynorphin A fragments exhibit specific binding to bradykinin receptors

The first step to validating dynorphin A's direct interaction at the bradykinin receptor involves determining dynorphin A's binding affinity to B2 receptors in neuronal tissues. Non-opioid fragments of dynorphin A were designed to determine the nature of dynorphin A's specific, structure activity relationship (SAR) at the B2. Competitive radioligand binding analysis of Dynorphin A (2-13) and a number of fragments derived from it against [<sup>3</sup>H]Kallidin binding in rat brain membrane and transfected B2 cell membrane preparations is shown in Table 1.

Compound	Sequence	Rat Brain Membranes			hB2 Transfected Cells		
		Log IC50 ± s.e.m.	Ki (nM)	n	Log IC50 ± s.e.m.	Ki (nM)	n
Bradykinin	RPPGFSPFR	-7.23 ± 0.04	38	1	-7.15 ± 0.07	46	1
Dyn (1-17)	YGGFLRRIRPKLKWQ				-6.90 ± 0.11	89	3
Dyn (2-13)	GGFLRRIRPKLK	-6.85 ± 0.07	100	13	-6.52 ± 0.22	172*	4
LYS 1002	GGFLDDIRPKLK		>10,000	1			
LYS 1026	FLRRIRPKL	-6.86 ± 0.06	86	3			
LYS 1004	GFLRRI	-5.63 ± 0.27	1410	7	-5.63 ± 0.27		
LYS 1044	FLRIRPK	-6.79 ± 0.15	97	5	-6.85 ± 0.04	94*	1

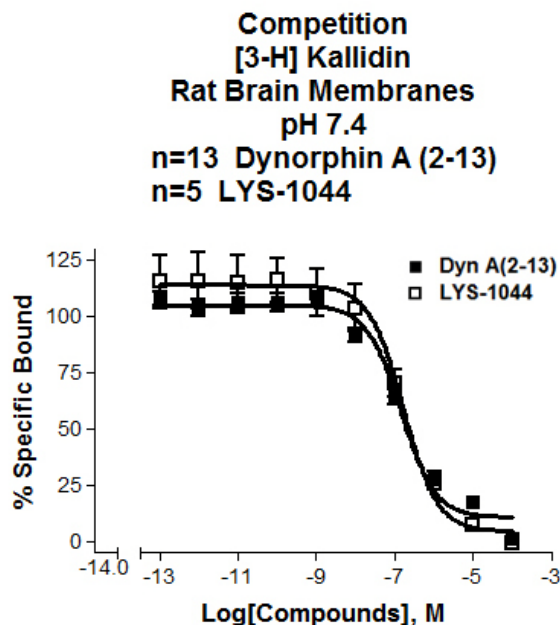
**Table 1:** [<sup>3</sup>H]-Kallidin competition analysis of dynorphin A fragment binding to bradykinin receptors in rat brain membrane and hB2 transfected cells. These data suggest that a C terminal positive charge and internal positively charged groups at positions 6 and 7 are essential for high affinity binding of dynorphin A to the B2 receptor. A structurally dependent, pH dependent binding indicates a specific interaction between dynorphin A and B2. \* indicates competition against [<sup>3</sup>H]-Bradykinin.

These dynorphin A fragments suggest the minimal peptide requirements for the peptide's specific binding to the B2 receptor. Binding data from LYS 1002 suggests that when internally positive charged groups at positions 6 and 7 are switched with negatively charged groups, the peptide becomes noncompetitive. Peptides with C-terminal positive charge compete with higher affinity, such as LYS 1026 and LYS 1044. Loss of N-terminal Tyr and C-terminal peptides does not impact Ki if C-terminal and

internal positive charges remain intact. Overall dependency upon sequence and charge location indicate that dynorphin A fragment binding to B2 receptors exhibits structure activity dependence, supporting a model for dynorphin A binding specifically to B2 receptors to effect physiological function. The  $K_i$ 's found for rB2 in rat brain membranes does not differ significantly from ones found for human receptors expressed in the hB2 transfected cell line (David Rankin, personal communications). Additionally, the binding affinities at pH 8.5 are decreased 10-fold from those at pH 7.4, and are optimal at pH 6.8. This particular pH dependence bears a similarity to the optimal binding of bradykinin to the B2 receptor at pH 6.8, implying some similarity in binding despite their differing amino acid sequences. Decreased pH in pathological conditions may enhance this interaction (Sara Hall, personal communications).

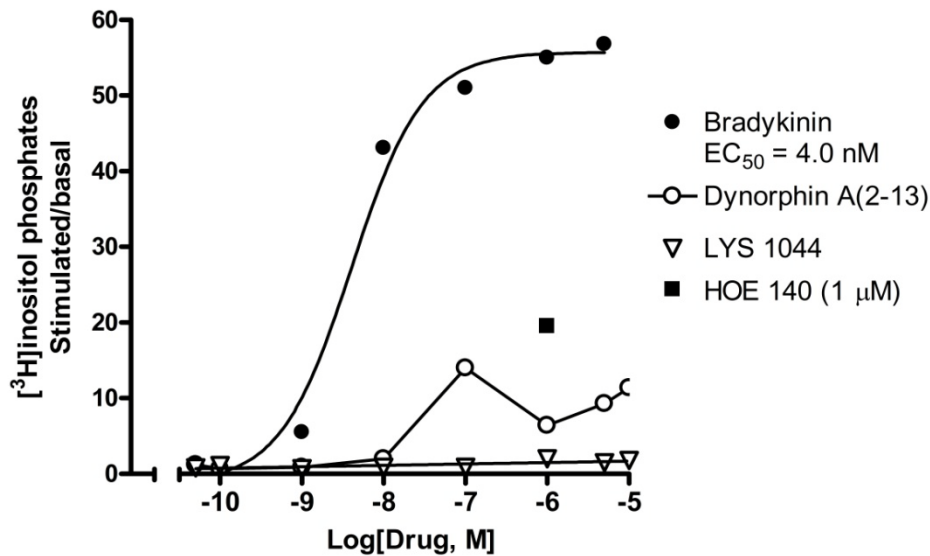
### LYS 1044 retains binding affinity for B2 receptor but does not stimulate PI

Figure 3 directly compares the cumulative competitive binding data for the parent compound, dynorphin A (2-13) and the short fragment LYS 1044 against [ $^3$ H]kallidin binding in rat brain membranes. The data suggest a single binding site for both dynorphin A (2-13) and LYS 1044, and that the affinity for the two ligands at the bradykinin receptors are very similar. Thus, LYS 1044 represents a key structure that confers the affinity of dynorphin A binding to bradykinin receptors. Because adult rat brain membranes express predominantly B2 receptors, we can assume that the structure of LYS 1044 is critical for the interaction of dynorphin A at the B2. This is further supported by similar affinity of dynorphin A and 1044 at the transfected hB2 receptors (Sara Hall, personal communications).



**Figure 3:** Competition binding analysis of LYS 1044 and Dyn A (2-13) against [ $^3$ H]Kallidin at pH 7.4 in rat brain membranes. The inhibition constant ( $K_i$ ) of LYS 1044 at the B2 receptor is 97nM, and Dyn A (2-13) is 85nM at pH 7.4.

The canonical pathway of bradykinin receptors is a coupling to phospholipase C via Gq resulting in the production of inositol phosphates. Figure 4 shows a robust dose response curve of bradykinin stimulated PI hydrolysis in the hB2 expressing HEK293 cells. The  $EC_{50}$  value is 4.0 nM based on 3 independent experiments.



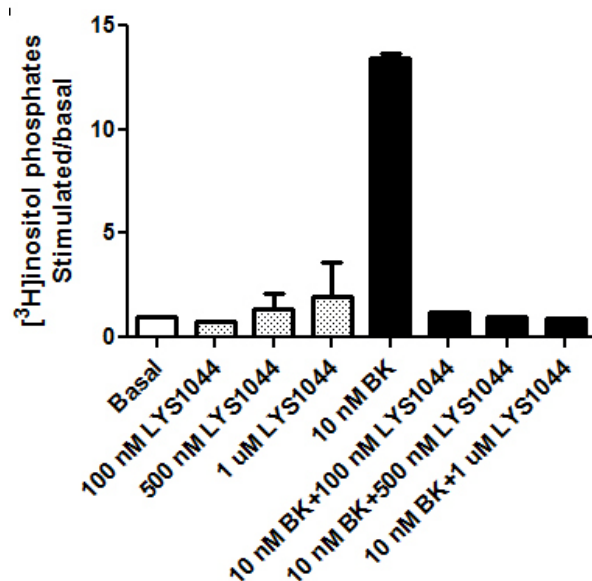
**Figure 4:** Phosphoinositol release is stimulated by bradykinin activation of bradykinin receptors in B2 transfected HEK-293 cells. LYS 1044 does not stimulate PI, while Dynorphin A (2-13) and HOE-140 act as partial agonists.

We have noted previously that dynorphin A (2-13) is very poor in activating this pathway, and instead activates calcium influx through the B2 (Lai et al., 2006). In these cells, perhaps because of the high level of B2 expression as evidenced by saturation analysis and the  $B_{max}$  value seen in the PI hydrolysis assays, we could detect a small stimulatory response by dynorphin A (2-13) at the higher concentration range of 100 nM and above when compared with that of bradykinin; the response seen here is consistent with the characteristic of a weak, partial agonist as suggested previously (Figure 4). The dose response is also in line with the affinity of dynorphin A (2-13) at the B2 receptor (Table 1 and Figure 3). It is interesting to note that HOE 140, which has been defined as a B2 selective antagonist, behaves as a partial agonist at the hB2.

These data suggest that we can use the PI assay to routinely measure the potential intrinsic activity of dynorphin A fragments and other novel ligands at the transfected hB2 receptor, in comparison to the reference compound bradykinin. Furthermore, we can also use this cell line and the PI assay as a functional assay to determine the effect that any compound has on the activity of bradykinin and dynorphin A (2-13) at the hB2 receptor, i.e., to screen for antagonist at the hB2 against bradykinin and/or dynorphin A.

LYS 1044 was examined for its functional activity. As seen in Figure 4, LYS 1044 did not stimulate PI hydrolysis up to 10  $\mu$ M, suggesting a lack of agonist activity at the hB2 receptor. At concentrations above 10  $\mu$ M, preliminary data suggest a small response (data not shown); however, at these concentrations, whether the response is specific to B2 activation is not clear, as peptides at these high concentrations commonly produce non-specific effects. Thus, unlike dynorphin A (2-13), LYS 1044 shows little or no stimulatory effect at phospholipase C via the hB2 receptor.

To determine if LYS 1044 blocks bradykinin response at the hB2, transfected cells were treated with several concentrations of LYS 1044 alone, with 10 nM bradykinin alone, or with 10 nM bradykinin plus LYS 1044. Figure 5 shows that LYS 1044 at 100 nM, 500 nM or 1  $\mu$ M does not significantly alter the level of inositol phosphates in the transfected cells, while 10 nM bradykinin, the  $EC_{50}$  concentration for half of maximal PI stimulation, stimulates PI hydrolysis. All three concentrations of LYS 1044 completely blocked the stimulatory effect of 10 nM bradykinin. This data suggest that LYS 1044 is likely an antagonist at the B2 receptor.

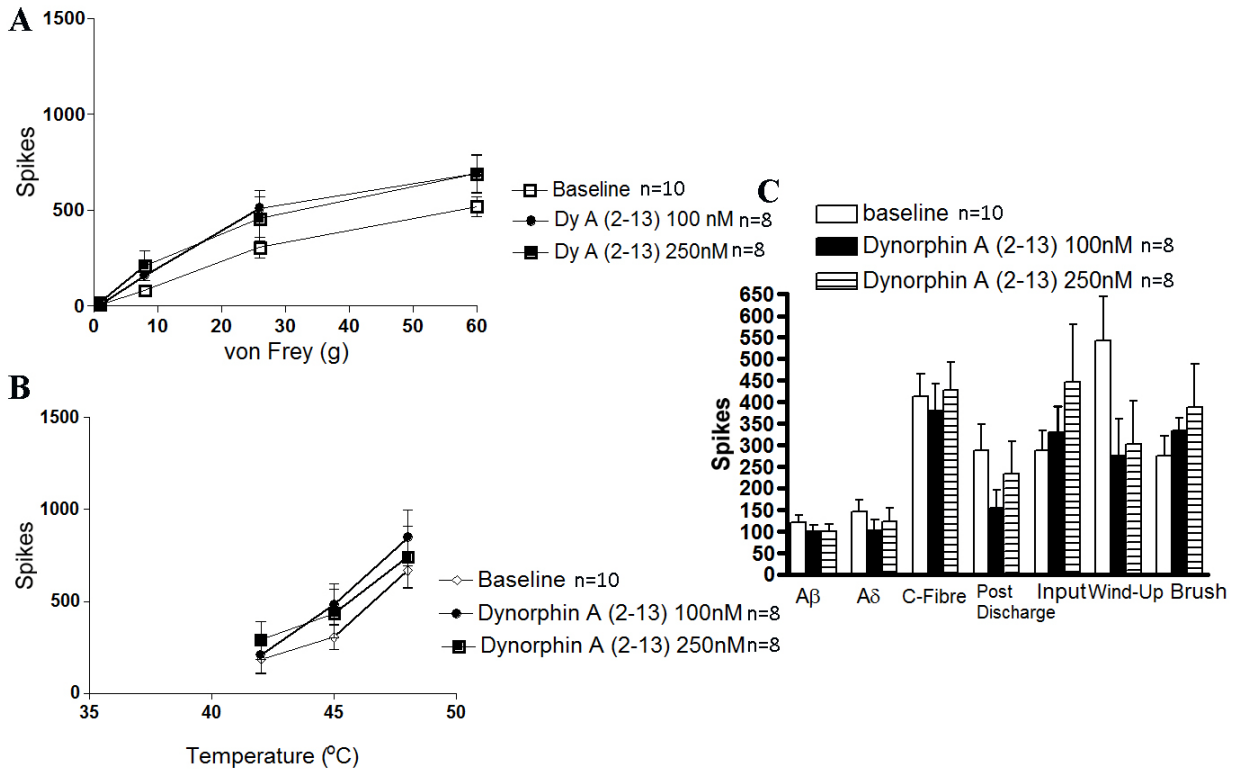


**Figure 5:** LYS 1044 antagonizes 10 nM bradykinin PI stimulation at concentrations >100 nM.

**Dynorphin A (2-13) does not significantly activate spinal wide dynamic range neurons.**

Although Dynorphin A (2-13)'s role as a partial agonist for the bradykinin receptor was established in-vitro, the specific location and mechanism of action remains unknown. To test the hypothesis that dynorphin A could activate bradykinin receptors to excite wide dynamic range neurons, dynorphin A (2-13) was administered directly onto the spinal cord of naïve anesthetized rats. The results reflect wide dynamic range neuron responses influenced by surrounding interneurons and glial cells. Although a slight increase in activity was observed in response to dynorphin A administration, this response was not dose-dependent or significant. A-β, A-δ, and C-fiber activity were virtually equivalent to baseline, and wind-up exhibited no consistent pattern. No link between dynorphin A (2-13) administration could be drawn to wide dynamic range neuron sensitization in the naïve rat model, so no B1 or B2 receptor antagonists were tested. The existence of B2 receptors on wide dynamic range neurons also remains uncertain, although they may exert indirect effects on wide dynamic range neurons through nearby glial cells. It remains to be seen if dynorphin A (2-13) has an impact in SNL animals due to the physiological changes in a chronic pain state from naïve conditions.

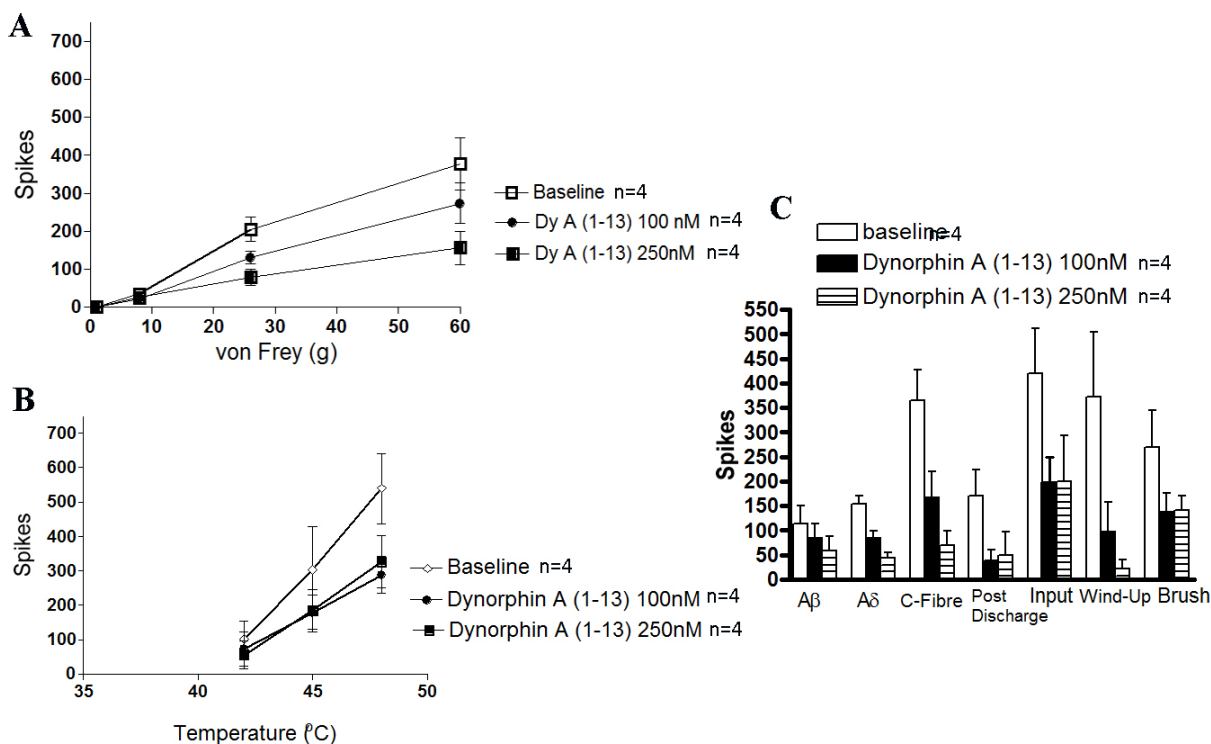
Additionally, doses of 500 nM were initially attempted, but were discontinued as cell viability decreased with high doses of dynorphin A (2-13). This may be directly related to dynorphin's neurotoxic effects at high doses, as seen in the PI hydrolysis assay with decreased HEK-293 viability and a drop in PI stimulation. This neurotoxic effect may not be B2 receptor mediated.



**Figure 6:** Mean  $\pm$  SEM altered behavioral activity and response profile of dorsal horn wide dynamic range spinal neurons after 50  $\mu$ l dynorphin A (2-13) exposure in rats at concentrations of 100 nM and 250 nM. After 10 min, 30 min, and 50 min, evoked neuronal response to mechanical (A), thermal (B), and electrical (C) stimuli were recorded in naive animals ( $n=8$ ). Based on latency measurements, neuronal responses were subdivided into A- $\beta$ , A- $\delta$ , and C-fibers, or postdischarge. Dynorphin A (2-13) exposure did not elicit a significant dose-dependent activation of wide dynamic range neuron firing in naïve rats.

### Dynorphin A (1-13) reduces wide dynamic range activity at doses of 100-250 nM.

The opioid dynorphin A (1-13) acted as predicted to lower WDR activity in a dose dependent manner in mechanical, thermal, and electrical tests. C-fiber spikes and wind-up in particular were significantly reduced. This inhibition was so great that 500 nM doses were not tested, as higher doses of opioid tended to depress respiration and increase the chance of animal death under anesthesia. This correlates to the potent opioid effects observed natural to dynorphin A's  $\kappa$ -opioid and  $\mu$ -opioid binding. Preliminary experiments indicate that attenuation of activity by the opioid fragment dyn A (1-13) was of even greater magnitude in SNL rats, including low intensity thermal and mechanical stimulation. The next step is to examine the effect of dynorphin A (2-13) in these animals, as well as other non-opioid dynorphin A fragments. Preliminary electrophysiological recording data with LYS 1044 also indicates it exerts an inhibitory effect on wide dynamic range neurons in SNL rats.



**Figure 7:** Mean  $\pm$  SEM altered behavioral activity and response profile of dorsal horn wide dynamic range spinal neurons after 50  $\mu$ l dynorphin A (1-13) exposure in rats at concentrations of 100 nM and 250 nM. After 10 min, 30 min, and 50 min, evoked neuronal response to mechanical (A), thermal (B), and electrical (C) stimuli were recorded in naïve animals (n=4). Based on latency measurements, neuronal responses were subdivided into A- $\beta$ , A- $\delta$ , and C-fibers, or postdischarge. Dynorphin A (1-13) exposure elicited a dose-dependent inhibition of activity in naïve rats, especially in C-fiber electrical firing and wind-up.

## DISCUSSIONS

Our results indicate that dynorphin A (2-13) direct activation of B2 receptors on WDR neurons is unlikely to be an underlying mechanism behind WDR sensitization. The simplest explanation is that dynorphin A promotes pain transmission through a different pathway, for example, pre-synaptically instead of post-synaptically, or through neurons traversing the superficial lamina. Dynorphin A's post-SNL expression is predominately increased in lamina I and only to a lesser degree in lamina V, so it would be worthy to pursue recording in a different area.

Dynorphin A may not independently evoke responses in vivo. It has already been shown that dynorphin A potentiates morphine induced capsaicin-evoked CGRP release; therefore it may not be surprising that administering only dynorphin A (2-13) on the spinal cord produces no excitatory effect in naïve animals. A similar situation exists for substance P and glutamate potentiation for pain transmission (citation). Within a chronic pain model, e.g. OIH or SNL, an animal's physiology differs significantly enough that dynorphin A could promote WDR activity or enhance a newly upregulated chemical's actions to promote sensitivity. Therefore, recording from SNL animals as a model for chronic neuropathic pain is one of the most necessary follow ups.

The results of this study confirmed dynorphin A (1-13)'s inhibitory capability in vivo through its opioid actions. However, initial results indicate naloxone does not produce strong reversal of dynorphin A (1-13)'s effects. This hints at non-opioid mediated anti-excitatory effects. Other non-opioid targets of dynorphin A outside of bradykinin and NMDA receptors also include ASIC channels and melanocortin

type 1, 3, and 4 receptors. Dynorphin A and big dynorphin have been found to limit steady-state desensitization of ASIC 1a channels in cortical neurons, promoting neuronal damage during pathological acidosis (Sherwood and Askwith, 2009). The GPCR system of MC receptors, normally involved in pigmentation, has appeared also in inflammation and promotion of nociception. Dynorphin A (2-13) exhibits moderate binding affinity with dissociation constants ( $K_D$ 's) of 40 nM-150 nM as an antagonist of MC receptors (Quillan and Sadee, 1997). Testing MC antagonists alongside opioid antagonists might reveal the receptors responsible for the inhibition. Nevertheless, it is simply more likely that further testing will reveal dynorphin A (1-13)'s effects are opioid mediated due to its high opioid affinity. Furthermore, the des-Tyrosyl fragment dynorphin A (2-13) did not produce a dampening of WDR activity; if MC receptors were responsible, both dynorphin A (1-13) and (2-13) would have produced results, although dynorphin A (1-13) had slightly more potent antagonism against  $\alpha$ -MSH.

The apparent disconnect between dynorphin A (2-13)'s promotion of allodynia and hyperalgesia during behavioral analysis and none during electrophysiological recording can also be attributed to limitations in the process of recording. The state of the anesthetized animal under isoflurane is obviously different from that of a conscious animal. Although the anesthesia should not have affected peripheral signaling, it could possibly increase the descending tone from the brain to the spinal cord, creating a higher threshold to conquer for any activating effects.

The size and scope of the system was ideal for examining the cumulative effects on WDR from the system even if the drug did not interact directly with receptors expressed on WDR but on interneurons or glial cells. However, that too had its drawbacks. The process of isolating a suitable cell and exacting hours of controls limits the test to a very specific population of WDR neurons; the testing process eliminates cells that are hyperexcitable or high threshold. At the same time, only effects on WDR were recorded, not effects on other second order neurons. As mentioned previously, this may be the wrong location to examine dynorphin A's effects.

Certain fragments of dynorphin A from the structure activity relationship table are under further investigation as potential analgesics. In particular, LYS 1044 has been found to reduce pain thresholds in behavioral studies. Preliminary data also indicates that LYS 1044 lowers wide dynamic range neuron excitation in SNL animals. Whether or not this effect is through activation of an opioid pathway or through blocking a pronociceptive pathway, namely bradykinin receptor activation, remains to be seen. We will continue investigating the physiological relevance of dynorphin A upregulation in the spinal cord and determining which fragments are agonists or antagonists at B2 receptors. Other dynorphin A non-opioid fragments will also be tested for their activity at the WDR.

## SUMMARY

Dynorphin A's specific opioid effects are well characterized, but cannot explain its neurotoxicity, nor its role in maintaining persistent hyperalgesia and allodynia *in vivo*. A variety of non-opioid targets, including bradykinin, and melanocortin GPCRs, as well as ASIC channels and ionotropic NMDA receptors, have appeared as dynorphin's companions as possible mediators of pain transmission and potentiation. Our focus on dynorphin A's interactions with bradykinin led to development of dynorphin A fragment based bradykinin receptor antagonists, and also an investigation of dynorphin A's actions on wide dynamic range neurons. Wide dynamic range neurons are integrators of peripheral signals and key players in central sensitization; we hypothesized that dynorphin A could activate wide dynamic range neurons to promote neuropathic pain. Results indicated that the non-opioid fragment dynorphin A (2-13) had little excitatory effect, but the opioid fragment dynorphin A (1-13) exhibited a potent inhibitory effect on normal WDR activity in naïve animals. From these data it is unlikely that dynorphin A (2-13) is acting to promote sensitization of WDR neurons. Other sites of action, including neurons in the superficial laminae or presynaptic sites may be responsible for *in vivo* hyperalgesia. Much testing remains to be accomplished in the SNL model with the bradykinin antagonist LYS 1044 and other dynorphin A fragments with potential for reversing behavioral hyperalgesia, which will elucidate their mechanisms of action. Fragments of interest as bradykinin receptor antagonists that reverse SNL-pain *in vivo* will be advanced as potential therapeutics for chronic pain.

## REFERENCES

- Purves, Dale. (2004). *Neuroscience*. Massachusetts: Sinauer Associates, Inc.
- Baron, R. (2006). Mechanisms of Disease: neuropathic pain—a clinical perspective. **2**(2) 95-106
- Chung, J.M., Kenshalo, J.R., Gerhart K.D., Willis, W.D. (1979). Excitation of Primate Spinothalamic Cutaneous C-Fiber Volleys. *Journal of Neurophysiology*. **42**(5), 1354-1369
- Urch, C., Dickenson, A. (2003). In vivo single unit extracellular recordings from spinal cord neurons of rats. *Brain Research Protocols*. **12**, 26-34
- Luo D.Z., Chaplan, S.R., Higuera, E.S., Sorkin, L.S., Stauderman K.A., Williams, M.E., Yaksh, T.L. (2001). Upregulation of Dorsal Root Ganglion  $\alpha 2\delta$  Calcium Channel Subunit and Its Correlation with Allodynia in Spinal Nerve-Injured Rats. *Journal of Neuroscience*. **21**(6), 1868-1875
- Hains, B.C., Saab, C.Y., Klein, J.P., Craner, M.J., Waxman, S.G., (2004). Altered Sodium Channel Expression in Second-Order Spinal Sensory Neurons Contributes to Pain after Peripheral Nerve Injury. *Journal of Neuroscience*. **24**(20), 4832-4839
- Coull J.A., Bordreau, D., Bachand K., Prescott, S.A., Nault, F., Sik, A., Koninck, P., Koninck, Y. (2003). Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*. **424**, 938-942
- Wieseler-Frank J., Maier, S.F., Watkins, L.R. (2005). Central Proinflammatory Cytokines and Pain Enhancement. *Neurosignals* **14**, 166-174
- Ossipov, M.H., Lai, J., Malan P., Porreca, F. (2006). Spinal and Supraspinal Mechanisms of Neuropathic Pain. *Annals of the New York Academy of Sciences*. **909**(1), 12-23
- Lambert, P.D., Wilding, J., al-Dokhayel, A.A., Bohuon, C., Comov, E., Gilbey, S.G., Bloom, S.R. (1993). A role for neuropeptide-Y, dynorphin, and noradrenaline in the central control of food intake after food deprivation. *Endocrinology* **133**(1), 29-32
- Land, B.B., Bruchas, M.R., Lemos, J.C, Xu, M., Melief E.J., Chavkin, C., (2008). The Dysphoric Component of Stress is Encoded by Activation of the Dynorphin-Opioid System. *Journal of Neuroscience*. **28**(2), 407-414
- Drake, C.T., Chavkin, C., Milner, T.A. (2007). Opioid systems in the dentate gyrus. *Prog. Brain Res.* Progress in Brain Research **163**: 245–63
- Young, E.A, Walker, J.M., Houghten, R., Akil, H. (1987). The degradation of dynorphin A in brain tissue in vivo and in vitro. *Peptides*. **8**, 701–707
- Goldstein A. and Ghazarossian, V.E. (1980). Immunoreactive dynorphin in pituitary and brain. *Proceedings of the National Academy of Sciences USA*, **77**, 6207–6210
- Campillo, A., Cabanero, D., Garcia-Nogales, P., Romero, A., Milanes V., Laorden L., Puig M. (2010). Increased Spinal Dynorphin Levels and Phospho-Extracellular Signal-Regulated Kinases 1 and 2 and c-Fos Immunoreactivity after Surgery under Remifentanyl Anesthesia in Mice. *Molecular Pharmacology*. **77**, 185-194
- Malan, T.P., Ossipov, M.H., Gardell, L.R., Ibrahim, M., Bian, D., Lai, J., and Porreca, F. (2000). Extraterritorial neuropathic pain correlates with multisegmental elevation of spinal dynorphin in nerve-injured rats. *Pain*. **86**, 185–194
- Han J.S. and Xie C.W. (1982). Dynorphin: Potent Analgesic Effect in Spinal Cord of the Rat. *Life Sciences*. **31**, 1781-1784
- Stevens, C.W. and Yaksh, T.L. (1986). Dynorphin A and related peptides administered intrathecally in the rat: A search for putative kappa opiate receptor activity *Journal of Pharmacology and Experimental Therapeutics*, **238**, 833-838
- Ren, M.F., Lu, C.H., Han, J.S. (1985). Dynorphin A (1-13) Antagonizes Morphine Analgesia in the Brain and Potentiates Morphine Analgesia in the Spinal Cord. *Peptides*. **6**, 1015-1020
- Skilling, S.R., Sun, X., Kurtz, H. J., Larson, A.A. (1992). Selective potentiation of NMDA-induced activity and release of excitatory amino acids by dynorphin: possible roles in paralysis and neurotoxicity. *Brain Research*. **575**, 272-278
- Singh, I.N., Goody, R., Goebel, S.M., Martin K.M., Knapp, P.E., Marinova, Z., Hirschberg, D., Yakovleva, T., Bergman, T., Bakalkin, G., Hauser, K.F. (2003). Dynorphin A (1-17) induces

- apoptosis in striatal neurons in vitro through alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor-mediated cytochrome c release and caspase-3 activation. *Neuroscience*. **122**, 1013-1023
- Wang, Z., Gardell, L.R., Ossipov, M.H., Vanderah T., Brennan, M., Hochgeschwender, U., Hruby, V.J., Malan P., Lai, J., and Porreca, F. (2001). Pronociceptive Actions of Dynorphin Maintain Chronic Neuropathic Pain. *Journal of Neuroscience*. **21**(5), 1779-1786
- Svensson, C.I, Hua, X.Y., Powell, H.C., Lai, J., Porreca, F., Yaksh, T.L. (2005). Prostaglandin E2 release evoked by intrathecal dynorphin is dependent on spinal p38 mitogen activated protein kinase. *Neuropeptides* **39**, 485-494
- Ji, R.R, Befort, K., Brenner, G.J., Woolf, C.J. (2002). ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. *Journal of Neuroscience*. **22**, 478-485
- Gardell, L.R., Wang, R., Burgess, S.E., Ossipov, M.H., Vanderah, T.W., Malan P.T., Lai, J., Porreca, F. (2002). Sustained Morphine Exposure Induces a Spinal Dynorphin-Dependent Enhancement of Excitatory Transmitter Release from Primary Afferent Fibers. *Journal of Neuroscience*. **22**(15), 6747-6755
- Lai, J., Luo M, Chen Q, Porreca F. (2008). Pronociceptive action of dynorphin via bradykinin receptors. *Neuroscience Letters*, **437** (3),175-179
- Tang, Q., Lynch, R.M., Porreca, F., Lai, J. (2000). Dynorphin A Elicits an Increase in Intracellular Calcium in Cultured Neurons Via a Non-Opioid Non-NMDA Mechanism. *Journal of Neurophysiology* **83**, 2610-2615
- Lai, J., Luo M.C., Chen, Q., Ma, S.W., Gardell, L.R., Ossipov, M.H. Porreca, F. (2006). Dynorphin A activates bradykinin receptors to maintain neuropathic pain. *Nature Neuroscience*. **9**, 1534-1540
- Carmen R. Green, Anderson, K.O., Baker, T.A., Campbell, L.C., Decker, S., Fillingim, R.B., Kaloupek, D.A., Lasch, K.E., Myers, C., Tait, R.C., Todd, K.H., Vallerand, A.H. (2003). The Unequal Burden of Pain: Confronting Racial and Ethnic Disparities in Pain. *Pain Medicine*. **4**(3), 277-294
- Zimmermann M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. (Guest Editorial). *Pain*. **16**, 109-110
- Kim S.H, Chung J.M. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain*. **50**, 355-363
- Sherwood, T., Askwith, C.C. (2009). Dynorphin Opioid Peptides Enhance Acid-Sensing Ion Channel 1a Activity and Acidosis-Induced Neuronal Death. *Journal of Neuroscience*. **29**(45), 14371-14380
- Quillan, J.M., Sadee W. (1997). Dynorphin peptides: antagonists of melanocortin receptors. *Pharm Res* **14**(6): 713-719.