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δ-2 Opioid receptor plasticity and GM-1

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The native cardiac enkephalin, methionine-enkephalin-arginine-phenylalanine (MEAP) altered vagally induced bradycardia when introduced into the sinoatrial (SA) node by microdialysis. The responses to MEAP were bimodal in character with lower doses enhancing bradycardia while higher doses suppressed bradycardia. The opposing vagotonic and vagolytic effects were mediated respectively by δ-1 and δ-2-subtypes of the same δ-opioid receptor. The opposing responses were blocked by sub-type specific antagonists. When the mixed agonist, MEAP was evaluated after treatment with the monosialosyl ganglioside, GM-1, the δ-1-mediated vagotonic response was enhanced and the δ-2-mediated vagolytic response was reduced. Subsequent studies were conducted to test the hypothesis that increased GM-1 content in the SA node reduced the δ-2-mediated vagolytic response independent of a coincident increase in competing δ-1-mediated vagotonic activity. The selective δ-2-agonist, deltorphin was introduced into the SA node by microdialysis to evaluate initial δ-2-vagolytic responses. The right vagus nerve was stimulated and the expected decline in heart rate was significantly attenuated by deltorphin. GM-1 was then perfused into the nodal interstitium for one hour without a significant change in vagal transmission. Following GM-1, deltorphin was reintroduced and a clear attenuation of the deltorphin's vagolytic response was observed. Similar results were obtained in time controls when GM-1 was omitted. In both cases the δ-1 selective antagonist 7-benzylidenaltrexone (BNTX) failed to restore

the vagolytic response when added afterward. However when added to the time controls early in the protocol, BNTX completely prevented the loss in the vagolytic response. When both the initial deltorphin and GM-1 were omitted the vagolytic response was significantly improved. In summary, the initial study with the mixed agonist, MEAP suggested that GM-1 reduced the δ-2-vagolytic response. This was confirmed when the relatively selective δ-2-agonist, deltorphin, was substituted for MEAP. Subsequent protocols suggested that deltorphin and GM-1 produced qualitatively similar losses in the vagolytic response that were not restored by subsequent δ-1-receptor blockade. Thus, the attenuation of the δ-2 response was not due to the addition of competing δ-1-mediated vagotonic activity. The elimination of the deltorphin mediated attrition of the δ-2 response by BNTX indicated that the declining response was mediated by δ-1-receptors. Thus GM-1, deltorphin, and time all interact to modify subsequent δ-2-mediated vagolytic responses. The specific contribution of deltorphin in this process was mediated by the activation of δ-1-receptors. Whether deltorphin has intrinsic δ-1 activity or causes the release of an endogenous δ-1 agonist is unclear. The specific mechanism by which the δ-1 and δ-2 opioid receptors interact likewise remains to be determined.

δ-2 OPIOID RECEPTOR PLASTICITY AND GM-1

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PUBLICATIONS

1. **Stanfill A, Jackson KE, Farias M, Barlow M, Deo S, Johnson S, Caffrey JL.** Leucine-Enkephalin interrupts sympathetically mediated tachycardia prejunctionally in the canine sinoatrial node. Exp Biol Med: 228(8): 898-906, 2003

UNDER REVIEW

1. **Vinogradoca TM, Lyashkov AE, Zhu W, Yang D, Deo S, Barlow MA, Johnson S, Caffrey JL, Zhou Y, Maltsev VA, Lakatta EG.** Pacemaker cells decode and translate protein kinase A signals into intrinsic rhythmic Ca^{2+} oscillations that regulate the heart's beating rate. Nature, 2005 (**submitted**)

2. **Olivencia-Yurvarti AH, Mallet RT, Ortolano GA, Paul G, Barlow MA, Deo S, Daniel N, Johnson S, Caffrey JL.** Leukocyte filtration for off-pump coronary artery bypass. Filtration 2005 (**in press**)

ABSTRACTS

1. **Barlow MA, Daniel N, Deo S, Johnson S, Yoshishige D, Caffrey JL**. Vagotonic effects of enkephalin are not mediated by sympatholytic mechanisms. (12th annual Research Appreciation Day) University of North Texas Health Science Center, 2004.

2. **Caffrey JL, Deo S, Barlow MA, Johnson S, Farias M**. Opioid-Ganglioside interactions during vagal bradycardia. FASEB J. 18: A1074, 2004.

3. **Stanfill A, Jackson K, Farias M, Barlow M, Deo S, Johnson S, Caffrey JL**. Kappa-opioid receptors in the cardiac pacemaker decrease sympathetic tachycardia. (11th annual Research Appreciation Day) University of North Texas Health Science Center, 2003.

4. **Barlow MA, Deo S, Johnson S, Caffrey JL.** Vagotonic effects of enkephalin are not mediated by sympatholytic mechanisms. FASEB J. 19: A1303, 2005.

5. **Johnson-Davis S, Deo S, Barlow MA, Yoshishige D, Caffrey JL.** GM-1, Deltorphin, and d-2 receptor plasticity in the SA Node. FASEB J. 19: A1322, 2005.

6. **Deo S, Barlow MA, Johnson S, Daniel N, Caffrey JL**. Repeated arterial occlusions improve vagal transmission in the sinoatrial node without eliminating the vagolytic response to opioids. FASEB J. 19:A708, 2005.

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CHAPTER I

Introduction

Opium is a mixture of pharmacologically active alkaloids derived from the opium poppy, *Papaver Somniferum*. Opium and its principal biologically active constituent, morphine, have been used therapeutically as analgesics and antidiarrheals for several thousand years. Unfortunately, the addictive properties of opiates and their potential for abuse have often limited the use of opiates as medicinal tools. As a result, the pharmaceutical industry has invested heavily in a concerted effort to separate the benefits of morphine from its liabilities. Despite the synthesis of thousands of opiate derivatives and 200 years of research, that goal remains largely unattained.

Prior to 1970, the collective pharmacological experience with synthetic opiates led to the suggestion that there were receptors for morphine in mammalian systems. In the early 1970's evidence began to accumulate for the existence of specific opiate binding sites in suspensions of brain cell membranes. This logically led to the proposal that apart from morphine, there must be an endogenous ligand that normally interacts with these specific binding sites. Soon thereafter, Hughes et al. reported on the structures of two endogenous opioids which they named enkephalins (10). The endogenous opioids now include a large number of related hormones and neurotransmitters. The endogenous opioids are primarily small peptides produced by the body that bind to opiate receptors and possess biological activities in common with the opiate alkaloids. Opioids most often function as neuromodulators by moderating neurotransmitter release.

There are four major classes of endogenous opioids: endomorphins, enkephalins, dynorphins, and endorphins. The latter three are derived from well studied precursor molecules, respectively proenkephalin, prodynorphin and proopiomelanocortin. Since their discovery, much of opioid research has concentrated on endogenous opioids in the brain and spinal cord regarding their role in pain and addiction. However, opioids are widely distributed in the periphery and in particular enkephalins, dynorphins and endorphins have been extracted from cardiac homogenates (1, 3, 14, 17, 18, 21). Most of the data dealing with the effects of opioids on cardiovascular function involve either enkephalin or dynorphin. Initial reports indicated that the amount of preproenkepahlin mRNA in heart was quite abundant, which suggested that the cardiac enkephalins were derived primarily from the resident cellular constituents within the myocardium and not the extrinsic innervation (9). The mRNA and its products have since been identified respectively in isolated cardiomyocytes and the culture medium surrounding them (12). Not surprisingly, the enkephalins are capable of altering cardiovascular function.

Like most opioids the cardiac enkephalins often function as neuromodulators and appear to exert acute effects on myocardial function through interactions with the autonomic innervation of the heart. In this regard the effects of administered enkephalin have been difficult to demonstrate under resting conditions in the absence of active nerve traffic. Thus, the primary opioid receptors in heart are most likely localized prejunctionally on sympathetic and parasympathetic nerve terminals where they modulate the release of norepinephrine and acetylcholine. At the nerve ending, neuromodulation is generally accomplished by opening potassium channels, by

opening/closing calcium channels and by increasing/decreasing the activity of adenylylcyclase. Thus the opioids may increase or decrease neurotransmitter release depending on which of the cellular mechanisms predominates.

The heart contains enkephalins in the form of cryptic sequences within the inactive precursor, proenkephalin. Proenkephalin may be differentially processed through a variety of intermediates but when fully processed four opioids should be released in a ratio of 4:1:1:1. These four active opioids are methionine enkephalin (ME), met-enkephalin-arg-phe (MEAP), met-enkephalin-arg-phe-gly-leu (MEAGL), and leucine enkephalin (LE), respectively. Despite the stoichiometric disadvantage, MEAP is generally the most abundant enkephalin found in heart. The enkephalins are particularly active versus vagal input to the heart. In early studies enkephalins reduced the effects of vagal stimulation on heart rate, atrial contractility, and coronary blood flow (2, 3). ME, LE and MEAP have all been demonstrated to reduce vagally induced bradycardia when introduced directly into the sinoatrial (SA) node by microdialysis (3, 8, 11, 16). Though details of this mechanism are unclear the response is mediated by specific opioid receptors.

MEAP is particularly abundant in heart and recent studies have increasingly focused on it biological properties. There are three classes of endogenous opioid receptors originally named: mu (μ), kappa (κ), and delta (δ). The functional specificity of different endogenous opioids for these receptors can display a significant degree of overlap. However, the enkephalins are generally viewed as potent δ-agonists and thus are often described as the native agonist for the δ-receptor (15). MEAP suppressed

vagal transmission through a δ-opioid receptor mediated response (11). The vagolytic response was blocked by a δ-receptor antagonist and duplicated by the addition of a δ-agonist (11). The effect of MEAP was subsequently demonstrated as bimodal in character (8). MEAP also improves vagal transmission (vagotonic) at very low doses (10^{-15} mol/min) and reduces vagal transmission (vagolytic) at higher doses (10^{-12} mol/min) (8). The two opposing effects appeared to have been mediated by subtypes of the δ-opioid receptor since each effect was blocked by sub-type specific antagonists. These studies led us to examine the mechanisms by which these opposing responses might interact.

Excitatory responses in opioid systems, though not uncommon, are often overlooked in favor of classical opioid-mediated inhibitory responses. Crain and Shen have even suggested that opiate receptor polarity can be shifted from inhibitory to excitatory modes by altering the local membrane environment (6). In this regard, neuronal plasma membranes are rich in a specific group of charged glycolipids collectively called gangliosides. Gangliosides are amphipathic molecules with paired fatty-acid side chains which asymmetrically anchor the molecules in the outer membrane leaflet. They also have hydrophilic oligosaccharide head groups that project outward into the aqueous interstitium surrounding the cell as part of the glycocalyx that determines many of the surface properties of cells. The head contains variable numbers of sialic acid residues that provide the external portion of the molecule with a negative charge. The ganglioside GM-1 has one sialic acid residue. GM-1 is particularly interesting because of it close association with $G_{s\alpha}$ -mediated signal transduction mechanisms. Evidence for this association derives from observations that

GM-1 specifically binds cholera toxin and provides the toxin with functional access to the nearby G-protein, $G_{s\alpha}$. The toxin then exerts its toxic effect by altering the intrinsic GTPase activity associated with $G_{s\alpha}$. This association between GM-1 and $G_{s\alpha}$ provides a potential point of interaction between the quality of the membrane environment and its constituent signal transduction mechanisms. Thus, neuronal membrane GM-1 represents a logical candidate to influence opioid receptors that use $G_{s\alpha}$-based signal transduction systems.

Crain and Shen argued that membranes rich in the monosialosyl-ganglioside, GM-1, favored excitatory opioid responses that comprise one limb of a positive feedback loop that stimulates the synthesis of more GM-1. In adapting their hypothesis to our observations we suggest that ultra-low opioid concentrations stimulate δ-1 opioid receptors and activate adenylyl cyclase. The resulting increase in the cyclic-AMP dependent protein kinase, phosphorylates glycosyltransferase, and increases the synthesis of GM-1. This increase in GM-1 theoretically improves the efficiency of excitatory opioid receptor coupling and counteracts the inhibitory opioid receptor effects. In the absence of GM-1 the opioids preferentially couple through G_i/G_o-coupling and suppress adenylylcyclase (4, 5). Opioids traditionally shorten action potentials in sensory neurons and reduce neurotransmitter release. However the opposite response was observed when low-dose opioids were applied to dorsal root ganglion cells. Ultra-low doses of morphine extended action potential duration through a GM-1-$G_{s\alpha}$-adenylylcyclase coupling mechanism (7). Longer action potentials presumably increase the effective time for neurotransmitter release (7). Wu et al., also hypothesized that the increases in GM-1 may increase the number of excitatory opiate

receptors by recruiting additional receptors from among those previously coupled through G_i-G_o (19, 20). This rationale led us to propose to study the interaction of GM-1 and sub-types of δ-opioid receptors within the SA node.

Our initial study in this regard demonstrated that GM-1 enhanced the vagotonic effect of low-dose MEAP and reduced the vagolytic effect of higher-dose MEAP. MEAP was instilled into the SA node at a sub-threshold dose just below that needed to improve vagal transmission. The right vagus nerve was stimulated and the expected results were observed. The low dose had no discernable effect and the high dose attenuated the vagally-mediated decline in heart rate by more than 70%. GM-1 was administered and the MEAP/vagal interactions were reassessed. GM-1 did not alter the control response to vagal stimulation. However, the sub-threshold dose of MEAP now produced a measurable vagotonic effect and the vagolytic effect of the higher dose was reduced by a similar proportion. These observations led us to propose that GM-1 reduced the δ-2-mediated vagolytic response by an independent mechanism, distinct from the arithmetic effect of a coincident increase in the opposing δ-1-mediated vagotonic response. Thus, the following studies were designed to test the hypothesis that GM-1 reduces δ-2-vagolytic responses independent of a coincident increase in the opposing δ-1-vagotonic response.

Specific Aims

1. To test whether administering GM-1 reduces the vagolytic response to deltorphin II, a selective δ-2-agonist.
2. To test whether the lost vagolytic response is restored by blockade of opposing δ-1-mediated vagotonic activity with the δ-1-antagonist, BNTX.
3. To test the stability of the vagolytic response in the absence of added GM-1.

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CHAPTER II

δ-2 Opioid Receptor Plasticity and GM-1

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Abstract

The native cardiac enkephalin, methionine-enkephalin-arginine-phenylalanine (MEAP) altered vagally induced bradycardia when introduced into the sinoatrial (SA) node by microdialysis. The responses to MEAP were bimodal in character with lower doses enhancing bradycardia while higher doses suppressed bradycardia. The opposing vagotonic and vagolytic effects were mediated respectively by δ-1 and δ-2-subtypes of the same δ-opioid receptor. The opposing responses were blocked by sub-type specific antagonists. When the mixed agonist, MEAP was evaluated after treatment with the monosialosyl ganglioside, GM-1, the δ-1-mediated vagotonic response was enhanced and the δ-2-mediated vagolytic response was reduced. Subsequent studies were conducted to test the hypothesis that increased GM-1 content in the SA node reduced the δ-2-mediated vagolytic response independent of a coincident increase in competing δ-1-mediated vagotonic activity. The selective δ-2-agonist, deltorphin was introduced into the SA node by microdialysis to evaluate initial δ-2-vagolytic responses. The right vagus nerve was stimulated and the expected decline in heart rate was significantly attenuated by deltorphin. GM-1 was then perfused into the nodal interstitium for one hour without a significant change in vagal transmission. Following GM-1, deltorphin was reintroduced and a clear attenuation of the deltorphin's vagolytic response was observed. Similar results were obtained in time controls when GM-1 was omitted. In both cases the δ-1 selective antagonist 7-benzylidenaltrexone (BNTX) failed to restore the vagolytic response when added afterward. However when added to the time

controls early in the protocol, BNTX completely prevented the loss in the vagolytic response. When both the initial deltorphin and GM-1 were omitted the vagolytic response was significantly improved. In summary, the initial study with the mixed agonist, MEAP suggested that GM-1 reduced the δ-2-vagolytic response. This was confirmed when the relatively selective δ-2-agonist, deltorphin, was substituted for MEAP. Subsequent protocols suggested that deltorphin and GM-1 produced qualitatively similar losses in the vagolytic response that were not restored by subsequent δ-1-receptor blockade. Thus, the attenuation of the δ-2 response was not due to the addition of competing δ-1-mediated vagotonic activity. The elimination of the deltorphin mediated attrition of the δ-2 response by BNTX indicated that the declining response was mediated by δ-1-receptors. Thus GM-1, deltorphin, and time all interact to modify subsequent δ-2-mediated vagolytic responses. The specific contribution of deltorphin in this process was mediated by the activation of δ-1-receptors. Whether deltorphin has intrinsic δ-1 activity or causes the release of an endogenous δ-1 agonist is unclear. The specific mechanism by which the δ-1 and δ-2 opioid receptors interact likewise remains to be determined.

Introduction

The sinoatrial (SA) node is the pacemaker of the heart. The SA node is located in the superior lateral wall of the right atrium near the entry of the superior vena cava. The SA node is composed of specialized cardiac muscle cells, the pacemaker cells. The nodal cells are spontaneously active and electrically coupled (11). This coupling promotes coordinated dispersion of the electrical signal and subsequent organized contraction. The cardiac pacemaker is densely innervated by sympathetic and parasympathetic nerve fibers that moderate automaticity through the local nodal release of norepinephrine and acetylcholine. In addition to direct innervation, multiple factors within the node modify both spontaneous activity and neurotransmitter release. Among these moderators, cardiac opioids appear to be potentially important neuromodulators.

There are four major classes of endogenous opioids: enkephalins, endorphins, endomorphins, and dynorphins. Proenkephalin, the presumed source of endogenous enkephalin, has seven constituents of enkephalins embedded in its primary sequence. When completely processed proenkephalin produces four copies of methionine enkephalin (ME) and one each of met-enkephalin-arg-phe (MEAP), met-enkephalin-arg-phe-gly-leu (MEAGL), and leucine enkephalin (LE). Surprisingly, proenkephalin mRNA is more abundant in the heart than most other tissues including the brain (9, 21). Despite the stoichiometric advantage afforded ME, MEAP concentrations are higher in the heart than any of the other three enkephalins (13, 21). ME, LE and MEAP have all been demonstrated to alter vagally induced bradycardia when introduced into the

sinoatrial (SA) node by microdialysis. (8,10,21). The enkephalins identified in heart alter heart rate by binding to specific opiate receptors which are probably located prejunctionally on sympathetic and parasympathetic nerve terminals.

Opioid receptors moderate a wide variety of physiological systems primarily through the regulation of neurotransmitter release (14). There are three classes of endogenous opioid receptors originally designated as mu (μ), kappa (κ), and delta (δ) receptors. The enkephalins are potent δ-agonists and are generally viewed as the primary ligands for the δ-receptor. Though behavioral and pharmacologic studies have provided support for distinct receptor subtypes of the δ-receptor (1,10,15,18,22), biochemical studies have identified a single protein transcript (1,5,12). In heart, δ-receptor stimulation produced a bimodal response during vagal stimulation (16). Lower doses (10^{-15} mol/min) of enkephalin enhanced vagal transmission (vagotonic) while higher doses (10^{-12} mol/min) suppressed vagal transmission (vagolytic) (8). The two opposing effects appeared to have been mediated by subtypes of the δ-opioid receptor since each effect was blocked by sub-type specific antagonists.

The observations made in heart are consistent with those made in sensory neurons that indicated that opioids were excitatory in some circumstances and inhibitory in others (2). Crain and Shen proposed that the quality and sensitivity of the response was governed by the ganglioside content of the cell membrane surrounding the opiate receptor. Membranes rich in the monosialosyl-ganglioside, GM-1 favored excitatory opioid responses at very low doses. The excitatory response was further proposed to activate a positive feedback loop that increased its own excitatory activity by stimulating the synthesis of more GM-1. Ultra-low opioid concentrations stimulate

δ-1-opioid receptors and activate adenylyl cyclase. The hypothesis suggested that the resulting increase in the cyclic-AMP dependent protein kinase, phosphorylated glycosyltransferase, and increased the synthesis of GM-1. This increase in GM-1 theoretically improved the efficiency of excitatory opioid receptor coupling and counteracted the inhibitory opioid receptor effects. In the absence of GM-1 these same opioids reduced cyclase activity through G_i/G_o-coupling (2,3). Thus, the environment around the receptor modified the response to opioids in isolated systems.

Since only one transcript has been isolated for the δ-receptor, we have suggested that the δ-receptor coupling in the SA node is fluid and the receptor subtype dependent responses might be inter-convertible. In support of this thesis, a decrement in the intensity of δ-2-mediated vagolytic responses was noted following a lengthy exposure of the SA node to δ-1-receptor stimulation. These preliminary observations prompted the suggestion that a similar ganglioside mediated plasticity might be operative in parasympathetic nerves regulating heart rate. The following studies were designed to test that hypothesis that introducing additional GM-1 into the interstitium of the SA node will reduce the intensity of δ-2-mediated vagolytic responses secondary to an increase in competing δ-1-mediated vagotonic responses. The following report will provide evidence that introducing additional GM-1 into the interstitium of the SA node increased the intensity of δ-1-receptor mediated vagotonic responses at very low doses of enkephalin while reducing the potential opposition by δ-2-receptor mediated vagolytic responses at higher doses. The data will also support the thesis that the decline in the δ-2-response following exposure to GM-1 was dependent on prior δ-1-stimulation but was independent of coincident opposition by δ-1-mediated vagotonic

activity. In summary, experimental protocols were conducted to test the hypothesis that the neural membrane ganglioside GM-1 reduces the δ-2 response independent of the increase in the opposing δ-1 response.

Materials and Methods

Surgical Preparation. Thirty-two mongrel dogs of either gender weighing 15-25 kg were assigned at random to various experimental protocols. All protocols were approved by the Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals. The animals were anesthetized with sodium pentobarbital (32.5 mg/kg), intubated, and mechanically ventilated initially at 225 mls/min/kg with room air. Fluid filled catheters were then inserted into the right femoral artery and vein and advanced into the descending aorta and inferior vena cava, respectively. The arterial line was attached to a Statham PD23XL pressure transducer to monitor heart rate and arterial pressure during the remainder of the surgical preparation. The venous line was used to administer supplemental anesthetic, as required. The acid-base balance and the blood gases were determined periodically with an Instrumental Laboratories Blood Gas Analyzer. The pO2 (90-120 mmHg), the pH (7.35-7.45) and the pCO2 (30-40 mmHg) were adjusted to normal by administering supplemental oxygen, bicarbonate or modifying the minute volume.

The right and left cervical vagus nerves were isolated through a ventral midline surgical incision. The nerves were double ligated with umbilical tape to prevent afferent nerve traffic during electrical stimulation. The isolated nerves were then returned to the prevertebral compartment for later retrieval. Surgical anesthesia was carefully monitored, and a single dose of succinylcholine (50 μg/kg) was administered intravenously to temporarily reduce involuntary muscle movements during the 10-15

minutes required for electrosurgical incision of the chest. The costosternal cartilage for ribs 2-5 were severed to permit access to the thoracic cavity and the heart was exposed from the right aspect. The pericardium was opened and the dorsal pericardial margins were sutured to the body wall to support the heart. The left femoral artery was isolated and a high fidelity, catheter-tip, pressure transducer (Millar) was inserted and then advanced into the abdominal aorta to measure heart rate and blood pressure continuously on-line thereafter (PowerLab).

Nodal Microdialysis. A 25g stainless steel spinal needle containing the microdialysis line was inserted into the center of the sinoatrial node along its long axis (6). The needle was removed and the probe was then positioned so that the dialysis window was completely within the substance of the sinoatrial node. The microdialysis probes were constructed of a single 1cm length of dialysis fiber from a Clirans TAF08 (Asahi Medical) artificial kidney (200 μm ID, 220 μm OD) and hollow silica (SGE; Austin, Texas) inflow and outflow tubes (120 μm ID, 170 μm OD). The dialysis tubing permits molecules with a molecular mass of 35,000 kDa or less to cross from the lumen into the nodal interstitium. This technique allows the precise introduction of agents directly into the nodal interstitium for extended periods without provoking complicating systemic reflexes. After placement of the probe in the SA node, the preparation was allowed to equilibrate for one hour while perfused with saline at 5μl/min.

Materials. MEAP and Deltorphin II were synthesized by American Peptide Co., Sunnyvale, CA GM-1 was obtained from Sigma Chemical Company, St. Louis, MO and 7-benzylidenaltrexone (BNTX) was obtained from Tocris Cookson, Ellisville, MO.

Statistical Methods. All data were expressed as means and standard errors. Differences were evaluated with an ANOVA; a repeated measures approach was employed where appropriate. Individual treatment differences were determined by post hoc analysis with Dunnets or Tukey respectively, when multiple comparisons to control or multiple comparisons among treatments were necessary. Differences determined to occur by chance with a probability of $p < 0.05$ were accepted as statistically significant.

Experimental protocols:

Graphic representations for all protocols are illustrated in the accompanying Figure 1.

Protocol 1. Interactions between GM-1 and the native agonist, MEAP. After equilibration for one hour, the right cervical vagus nerve was stimulated at a supra-maximal voltage (15 v) for 15 seconds at low (1-2 Hz) and high (3-4 Hz) frequencies selected to produce respectively 10-20 bpm and 30-40 bpm decreases in heart rate. Sub-threshold vagotonic (5 fmol/min, Lo-MEAP) and sub-maximal vagolytic (1.5 nmol/min, Hi-MEAP) doses of MEAP were administered by dialysis for 5 minutes each. After 5 minutes exposure to the first dose, the two step heart rate/vagal frequency evaluation was repeated. The dose was then increased and the vagal transmission was re-evaluated 5 minutes later. The MEAP infusion was then discontinued and the line was washed with vehicle until control vagal function was restored. GM-1 was then added to the dialysis inflow (5 nmol/min) and continued for 30 minutes. After 30 minutes, the GM-1 was discontinued and the vagal responses to the two doses of MEAP used earlier were tested again.

Protocol 2: Interactions between GM-1 and the δ-2-agonist, deltorphin II.

(DGD) After the initial equilibration, control vagal responses were obtained by sequentially stimulating the right vagus nerve in two steps as described above. The δ-2 agonist, deltorphin, was then introduced into the SA node by microdialysis at a sub-maximal dose of 0.7 nmol/min for five minutes (10). The two-step vagal evaluation was repeated to quantify the initial δ-2 (vagolytic) response prior to exposure to GM-1. This test of efficacy was designated δ2-5 to indicate the time in the protocol. After determining the δ-2-response, deltorphin was discontinued and the system was washed out with saline (45-60 min) until the control vagal responses were restored. GM-1 (5nmol/min) was perfused for one hour and the vagus was stimulated every 15 minutes to evaluate the effects of GM-1 alone. Excess GM-1 was washed out for 30 minutes and post-GM-1 control responses were retested. Deltorphin was reintroduced and after 5 minutes, the vagolytic, δ_2-responses were also retested and designated δ2-155. Deltorphin was discontinued but was introduced again 25 minutes later for another vagal test designated δ2-180 to evaluate the progression of changes in the δ-2-response. The deltorphin was discontinued and washed out (45-60 min). The δ-1 selective, antagonist (BNTX) was then introduced (5nmol/min) for five minutes and the right vagus nerve was stimulated to evaluate further the effects of δ-1-receptor blockade with BNTX alone. BNTX and deltorphin were then introduced together (1:1) for five minutes and a two-step vagal stimulation designated δ2-250 was conducted to determine (by subtraction) the contribution of δ-1 mediated (vagotonic) response to any change in the δ-2 response. The treatments were then discontinued, the area was washed and the vagal responses were tested periodically until the reappearance of the original control response.

Protocol 3: Controls: vehicle, duration, and repeated deltorphin II. (DSD) The purpose of this study was to determine the potential influence of the duration of the protocol, the repeated vagal stimulation, and/or the repeated exposure to deltorphin on the subsequent deltorphin mediated δ-2-vagolytic responses. This protocol was identical to the first 150 minutes in Protocol 2 except vehicle (saline) was substituted for GM-1 during the treatment period.

Protocol 4: Controls: vehicle, duration, and naïve deltorphin II. (_SD) The purpose of this study was to remove the influence of prior deltorphin exposure on the δ-2 response. This protocol is similar to Protocol 2 and 3 except both the initial exposure to deltorphin (δ2-5) and the treatment with GM-1 were omitted. Vehicle was perfused for two and half hours with vagal stimulations every 15 minutes during the second hour as in Protocol 2. The subsequent deltorphin challenges without and with BNTX were then applied in a sequence equivalent to δ2-155, δ2-180 and δ2-250 in Protocol 2.

Protocol 5: GM-1 and naïve deltorphin (no wash). (_GD) The purpose of this study was to test whether GM-1 was effective alone. The protocol was designed to test by omission of δ2-5, whether the decline in the δ-2-vagolytic response depended on an interaction between the initial exposure to deltorphin (δ2-5) and the subsequent addition of GM-1. The initial two-step vagal stimulation was conducted followed by one hour of perfusion with vehicle to simulate the δ2-5 exposure to deltorphin and its washout. GM-1 was infused, at a dose of 5nmol/min for the second hour and the right vagus nerve was tested at 15-minute intervals as described in the other protocols, to evaluate progressive effects of GM-1. GM-1 was discontinued and immediately afterward, the deltorphin challenges without and with BNTX were then applied in a

sequence equivalent to δ2-155, δ2-180 and δ2-250 as described in Protocol 2.

Protocol 6: GM-1 and naïve deltorphin (with wash). (_GWD) The purpose of this study was to test whether the influence of GM-1 was sustained after discontinuing its perfusion or did the δ2-response recover. The sequence of the protocol was identical to that in Protocol 5 above through to δ2-180 except that a 30 minute wash was inserted between GM-1 and the first two δ2-evaluations now designated δ2-180 and δ2-205. BNTX was not tested in this protocol.

Protocol 7: Influence of δ-1 blockade on δ-2 response. (DBD) The purpose of this study was to test whether apparent contributions of deltorphin to the erosion of the δ2-response observed in Protocols 2 and 3 depended upon δ-1-receptor activity. This protocol was identical to the control in Protocol 3 except that the δ-1-antagonist, BNTX was added to the dialysis inflow at after equilibration but before δ2-5 deltorphin. BNTX was then continued throughout the following two hours and during the subsequent δ-2-challenges at δ2-155 and δ2-180.

Results

Basal cardiovascular parameters for all subjects across all treatments are presented in Tables 1, 2a, and 2b. Animal subjects were assigned randomly to various protocols and there were no significant differences in blood pressure or heart rate among groups prior to treatment. Resting heart rate and blood pressure were unaltered by any of the treatments applied during the protocols, except for subjects in protocol 7 in which the initial heart rates were lower.

Protocol 1: Interactions between GM-1 and the native agonist, MEAP (Fig. 2). The filled squares in Figure 1 illustrate the two step decline in heart rate following right vagal stimulation at 2 and 4 Hz (Control). Pretreatment with a sub-threshold dose of MEAP (Lo MEAP) had no effect on vagal transmission. The higher dose (Hi MEAP) reduced the vagally mediated decline in heart rate by approximately two thirds. GM-1 had no demonstrable effect on the control response (GM-1). However after pretreatment with GM-1, the vagolytic effect of Hi MEAP was reduced and a clear vagotonic effect of Lo MEAP emerged. These data led to the hypothesis that GM-1 improved the δ-1-mediated vagotonic effect of MEAP at the expense of a decline in its δ-2-mediated vagolytic effect. The subsequent protocols in this report were designed to evaluate the δ-2-portion of this thesis with the aid of the selective δ-2-agonist, deltorphin.

Protocol 2: Interactions between GM-1 and the δ-2-agonist, deltorphin II (Fig 3). (DGD) Like MEAP, deltorphin produced a significant vagolytic response when first introduced in to the nodal interstitium (δ2-5). The GM-1 treatment had no

measurable effect on the vagal responses during the 60-minute treatment period, but the subsequent vagolytic responses at δ2-155 and δ2-180 were progressively reduced in magnitude. The δ-1 selective antagonist, BNTX had no effect on vagal transmission when added alone and did not restore the vagolytic response when combined with deltorphin (represented in Figure 10 below). In fact, the vagolytic effect of deltorphin was reduced further. These data suggests that GM-1 suppresses the δ-2-receptor response without a coincident contribution from enhanced δ-1-mediated vagotonic activity.

Protocol 3: Controls: vehicle, duration, and repeated deltorphin II (Fig 4). (DSD) The purpose of this study was to test whether the reduction in the δ-2-response observed in earlier protocols occurs in the absence of added GM-1. Thus vehicle was substituted for GM-1 during the treatment period. The initial vagolytic effect of deltorphin was similar to the initial response in the first protocol at 1 and 3 Hz. Surprisingly, after a vehicle-only infusion for a time interval matching the treatment period in Protocol 1, there was similar progressive loss in the δ-2-mediated vagolytic effect of deltorphin during the δ2-155 and δ2-180 evaluations. Once again, the vagolytic response was almost gone after 30 minutes. These data suggests that there is attrition of the δ-2 response during the protocol, perhaps due to the length of the protocol or the repeated deltorphin administration. Once again, the lost vagolytic response was not restored by blocking the δ-1-receptors with BNTX (represented in Figure 10 below).

Protocol 4: Controls: vehicle, duration, and naïve deltorphin II (Fig 5). (_SD) The purpose of this study was to test whether repeated exposure to deltorphin

contributed to the loss of the δ-2-response observed in Protocol 3. In this protocol the initial deltorphin exposure was omitted but the remainder of the three hour protocol through δ2-180 was replicated. The two δ2-receptor evaluations at δ2-155 and δ2-180 were both significantly different from control. A clear vagolytic response was observed at δ2-155 that was more intense than any of the prior deltorphin responses at δ2-5 or δ2-155 observed in Protocols 2 and 3. When deltorphin was retested 25 minutes later, the subsequent attrition though apparent was mild compared to that observed in Protocols 2 and 3. These data suggest that in the absence of treatment the δ_2 –mediated vagolytic response grows stronger. Furthermore, the initial exposure to deltorphin at δ2-5 in Protocols 2 and 3 may have contributed to the greater rate of attrition of the δ-2 response in those earlier protocols.

Protocol 5: GM-1 and naïve deltorphin (no wash) (Fig 6). (_GD) The purpose of this protocol was to evaluate the contribution of GM-1 to the loss of the δ-2 response in the absence of a prior exposure to deltorphin. Sixty minutes perfusion with vehicle was substituted for the initial exposure to deltorphin and subsequent wash. GM-1 was then introduced into the dialysis inflow and vagal function was tested at 15-minute intervals for one hour. Vagal responses during this treatment were not significantly different from the original control. GM-1 was continued in combination with the first introduction of deltorphin (δ2-155). When the vagus was tested after 5 minutes, a clear but comparatively weaker vagolytic response was observed. This response was similar to the initial deltorphin responses observed in Protocols 2, and 3 at δ2-5 and less intense than the δ2-155 evaluation observed without GM-1 in Protocol 4. (Figure 5) Deltorphin was discontinued and then reintroduced twenty-five minutes

later. When the vagus was retested again at δ2-180, there was significant attrition of the vagolytic response. Thus, GM-1 alone did not appear to have diminished the δ2-response however GM-1 did appear to have interacted with deltorphin to accelerate the rate of loss in the δ-2-mediated response. Once again, blockade of the δ-1-receptors afterward did not restore the vagolytic response (represented in Figure 10 below). The vagolytic effect of deltorphin was in fact reduced further.

Protocol 6: GM-1 and naïve deltorphin (with wash) (Fig 7). (_GWD) The purpose of this study was to test whether the influence of GM-1 was sustained after discontinuing its perfusion. In this protocol the initial (δ2-5) deltorphin exposure was omitted. After an hour period equivalent to the interval typically needed to test deltorphin and wash it out, a control vagal response was evaluated. GM-1 was then introduced into the SA node by microdialysis for a second hour. Vagal responses during and after GM-1 treatment were similar to initial control stimulations. GM-1 was discontinued and 25 minutes later, near to the point when the maximal attrition of the deltorphin response was typically observed (Protocols 2-5), deltorphin was introduced for the first time (δ2-180). Five minutes later when the δ-2-response was evaluated, a significant but comparatively weak vagolytic effect was observed. This response was identical to that observed without the wash (Protocol 5) and also less intense than that observed without GM-1 (Protocol 4). The washout of GM-1 appeared to have little effect on the vagolytic response at δ2-155. Deltorphin was discontinued and then reintroduced twenty-five minutes later (δ2-205). When vagal transmission was tested again 30 minutes later, the vagolytic response was reduced further. Thus, time and GM-1 appear to modify the δ-2 mediated vagolytic response in opposite directions.

Time increases the intensity of the vagolytic response and GM-1 prevents that increase.

Protocol 7: Influence of δ-1-antagonist pretreatment on the δ-2 response (Fig 8). (DBD) The purpose of this study was to test whether δ-1-blockade prevents the loss of the δ-2 response. BNTX was first introduced into the SA node alone. After five minutes exposure to BNTX, the vagal stimulations were repeated and there was no significant difference between this response and the control response. BNTX was then combined with deltorphin for five minutes and the vagus was retested. A typical deltorphin mediated vagolytic response was observed. Deltorphin was discontinued and Protocol 3 was then repeated with BNTX added throughout. In this case the δ2-5, δ2-155, and δ2-180 evaluations were virtually identical to one another with no erosion in the subsequent vagolytic responses. These data led to the suggestion that the loss of the δ-2 response was indeed mediated by activation of δ-1 receptors. Thus, either deltorphin has intrinsic δ-1 activity or facilitates the activity of an endogenous δ-1-agonist.

Discussion

GM-1 facilitates the attrition of δ-2-mediated vagolytic responses? The initial data reported above for MEAP supported the primary hypothesis that GM-1 enhanced the δ-1-mediated vagotonic effect of MEAP and reduced the opposing δ-2-mediated vagolytic effect. When the more selective δ-2-agonist, deltorphin was employed to verify the δ-2-components of the observations with MEAP, a similar reduction in the vagolytic response was observed when the vagolytic effect of deltorphin was compared before and after the administration of GM-1. Surprisingly, a very similar rate of attrition in the vagolytic effect was observed in time controls in which the GM-1 was omitted; suggesting a more complex interaction between the agonist, the ganglioside, and the protocol.

The initial studies with deltorphin thus suggested several potential conclusions. The rate of attrition produced by the deltorphin protocol without added GM-1 may have already been maximal. GM-1 may not be involved in the process or the endogenous content may have rendered the added GM-1 superfluous. The attrition in the response may have been due to the protocol itself or the prior exposure to the δ-2-agonist, deltorphin. Opioid mediated down regulation of opiate receptors is a widely recognized phenomenon however prior studies with MEAP in the SA node had provided little evidence for down regulation of vagolytic response during two hours of continuous exposure (7). Deltorphin and MEAP do differ in that MEAP is a mixed δ1/δ2-agonist and deltorphin is reportedly more δ2-selective. The vagolytic effect of deltorphin was also very slow to wash out (45-60 min) when compared to MEAP (10-20 min). The

slower off-responses might reflect longer residence times within the δ-receptor site and thus a greater likelihood of down regulation.

An additional protocol was conducted to test whether the erosion of the δ-2-response depended on the duration of the protocol or the prior exposure to deltorphin. When the early exposure to deltorphin at 5 minutes was omitted and the first exposure was delivered late in the protocol at 155 minutes, the vagolytic response was stronger than that observed in any of the previous protocols at 5 minutes or at 155 minutes. This unexpected result suggested that the vagolytic response in untreated controls had improved during the protocol. This observation also suggested that the attrition observed in the two earlier protocols was actually more substantial than apparent, since the vagolytic response should have gotten stronger. The stronger vagolytic response in the absence of prior deltorphin also suggested that prior exposure to deltorphin contributed to the subsequent erosion of the vagolytic response two hours later.

Pretreatment with GM-1 in naive animals without prior exposure to deltorphin prevented the improved δ-2-vagolytic response (Figure 9). This observation suggested that the improved δ-2-mediated vagolytic response may have resulted from the gradual metabolism of endogenous GM-1 during the protocol. This must be tempered by the observation that imposition of a 30 minute wash between the GM-1 treatment and the δ-2-evaluation also produced an intermediate δ-2-response that was identical to that observed immediately after GM-1 (Figure 9). The lost vagolytic response was once again not restored by the subsequent blockade of the nodal δ-1-receptors indicating that the reduced vagolytic response was not a consequence of competition from an increased

vagotonic response (Figure 10). Thus, fluid changes in GM-1 content may contribute to the expression of the δ-2-response since maintaining GM-1 content favors the down regulation or uncoupling of the δ-2-response.

Reduced δ-2-mediated vagolytic responses are not the net arithmetic effect of increased δ-1-mediated responses. The clear loss in the vagolytic response in Protocols 2 and 3 was not the result of opposition from δ-1-mediated vagotonic activity since the lost vagolytic effect was not immediately restored by δ-1-blockade with BNTX. In each of these two deltorphin-protocols, the vagolytic effect of deltorphin was clearly eroded further when evaluated afterward in combination with BNTX. Since BNTX alone had no effect, the continued erosion of the deltorphin response may represent a later stage in the progressive loss of the δ-2-vagolytic response started prior to introducing BNTX (Figure 10).

δ-1-receptor stimulation contributes to the loss of the δ-2-mediated vagolytic response. The third part of the study was designed to test whether stimulation of δ-1-receptors was responsible for the observed decline in the δ-2-mediated vagolytic response. In this regard, nodal δ-1-receptor blockade applied early in the protocol completely prevented the attrition of the δ-2-mediated vagolytic response. In each of the five earlier protocols, a quickly repeated exposure to deltorphin after 30 minutes resulted in a consistent reduction in its vagolytic effect (Fig 3-7). However, when the paired evaluations were preceded by BNTX throughout the treatment period, the two sequential deltorphin evaluations were nearly identical (Fig 8). As indicated above adding BNTX afterward did not prevent the acute progressive loss in the vagolytic

response. Thus, δ-1-receptor blockade prevents the loss of the δ-2-mediated responses when applied in advance but is unable to reverse the responsible process once it is permitted time to proceed.

These observations in the SA node are consistent with the hypothesis that the coupling of inhibitory (vagolytic) opioid responses is both dynamic and responsive to δ-1-receptor stimulation. GM-1 facilitates excitatory responses to opioid receptor stimulation in isolated systems. CHO cells have little endogenous GM-1 and when these cells were transfected with cloned δ-opioid receptors they displayed the typical inhibitory coupling between the agonist and the adenylyl cyclase second messenger system. Adding GM-1 to these same cells shifted the coupling so that a clear excitatory increase in cyclase activity was then demonstrated (19). The role of adenylyl cyclase in this process was further verified in electrophysiological studies on dorsal root ganglion cells. In these cells the excitatory effects of opioids were selectively blocked by cholera toxin and the inhibitory effects were selectively blocked by pertussis toxin indicating the two opposing effects were mediated respectively by G_s and G_i-G_o coupling (4, 17). The opioid influence observed at any given concentration may be determined by the net result of G_s coupled excitatory and G_i-G_o coupled inhibitory effects mediated by opioid receptors in the same cells. Thus, changes in receptor coupling may be required to explain the observed plasticity in δ-mediated responses since only one δ-receptor protein has be identified thus far (1,3).

In conclusion, the initial study with MEAP suggested that GM-1 reduced the vagolytic δ-2 opioid response when combined in the SA node with MEAP. When the

relatively selective δ-2 agonist deltorphin was used to compare δ-2 mediated vagolytic responses before and after GM-1 exposure, the response afterward was reduced. When the GM-1 treatment was omitted, the loss in the vagolytic effect, though still observed, was quantitatively less dramatic. These observations led to the suggestion that duration of the experiment or prior exposure to deltorphin interacted with GM-1. The elimination of the pre-post attrition of the δ-2 response by BNTX suggested that the declining response was mediated by δ-1 receptors. In this regard BNTX failed to restore the δ-2 response when added afterward. This suggests that the attenuation of the δ-2 response is not due to the addition of competing δ-1 mediated vagotonic activity. Thus, GM-1, deltorphin, and time all interact to modify subsequent δ-2-mediated vagolytic responses. The specific effect of deltorphin in this process is mediated by the activation of δ-1-receptors. Whether deltorphin has δ-1 activity or causes the release of an endogenous δ-1 agonist remains to be verified. The specific mechanism by which the δ-1 and δ-2 opioid receptors interact is not fully understood. Furthermore, the physiological consequences of observed shifts in vagal transmission also remain to be determined.

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Legends

Figure 1: This figure is a graphic representation of temporal events in Protocols 1-7.

Figure 2: Changes in heart rate mediated by low (1-2 Hz) and high (3-4 Hz) frequency stimulation of the right vagus nerve are illustrated during exposure to sub-threshold vagotonic (5 fmol/min, Lo-MEAP) and sub-maximal vagolytic (1.5 nmol/min, Hi-MEAP) doses of MEAP, introduced into the interstitium of the SA node by microdialysis. Values are means and standard error of the mean from seven subjects.

Figure 3: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during treatment with the neuronal membrane ganglioside, GM-1. The evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin are illustrated before and after treatment in the lower panel. The designations δ2-5, δ2-155, and δ2-180 indicate the δ_2-evaluations at 5, 155, and 180 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean for four subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P<0.05$ and $P<0.01$ respectively. The symbol (##) indicates the change in the heart rate was significantly different from δ2-5 ($P<0.01$).

Figure 4: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during treatment with vehicle (saline). The evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin are

illustrated before and after treatment in the lower panel. The designations δ2-5, δ2-155 and δ2-180 indicate the δ_2-evaluations at 5, 155 and 180 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean from four subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P<0.05$ and $P<0.01$ respectively. The symbol (##) indicates the change in the heart rate was significantly different from δ2-5 ($P<0.01$).

Figure 5: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during two hours treatment with vehicle (saline) without prior exposure to deltorphin. The evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin are illustrated after vehicle treatment in the lower panel. The designations δ2-155 and δ2-180 indicate the δ_2-evaluations at 155 and 180 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean from four subjects. The symbols (* and **) indicate the change in the heart rate was significantly different from control at $P<0.05$ and $P<0.01$ respectively. The symbol (##) indicates the change in the heart rate was significantly different from δ2-155 ($P<0.01$).

Figure 6: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during two hours treatment with the neuronal membrane ganglioside, GM-1 without prior exposure to deltorphin. The

evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin after GM-1 treatment are illustrated in the lower panel. The designations δ2-155 and δ2-180 indicate the δ_2-evaluations at 155 and 180 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean from four subjects. The symbol (*) indicates the change in the heart rate was significantly different from control ($P<0.05$). The symbol (##) indicates the change in the heart rate was significantly different from δ2-155 ($P<0.01$).

Figure 7: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during two hours treatment with the neuronal membrane ganglioside, GM-1 without prior exposure to deltorphin. A 30 min wash was conducted prior to the first δ-2-evaluation to test whether the influence of added GM-1 was sustained after its infusion was discontinued. The evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin after GM-1 + wash treatment are illustrated in the lower panel. The designations δ2-180 and δ2-205 indicate the δ_2-evaluations at 180 and 205 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean from four subjects. The symbol (**) indicates the change in the heart rate was significantly different from control ($P<0.01$). The symbol (##) indicates the change in the heart rate was significantly different from δ2-180 ($P<0.01$).

Figure 8: Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated in the upper panel during treatment with δ_1-antagonist, BNTX. The evaluation of vagolytic δ_2-responses with the δ_2-agonist, deltorphin are illustrated before and after treatment in the lower panel. The designations δ2-5, δ2-155 and δ2-180 indicate the δ_2-evaluations at 5, 155 and 180 minutes respectively after the initial control stimulations. The numerical values on those bars indicate the percent inhibition from the original control. Values are means and standard error of the mean from four subjects. The symbol (**) indicates the change in the heart rate was significantly different from control ($P<0.01$).

Figure 9: The δ2-mediated percent inhibition of vagal bradycardia (3 Hz) at δ2-155 is compared across treatments. The designation δ2-155 indicates the δ_2–evaluation at 155 minutes after the initial control stimulation. The label DGD for study 2 represents prior exposure to deltorphin and treatment with GM-1 before the δ2-155 evaluation. The label DSD for study 3 represents prior exposure to deltorphin and saline treatment before the δ2-155 evaluation. The label _SD for study 4 represents saline treatment only before the δ2-155 evaluation. The label _GD for study 5 represents GM-1 treatment immediately followed by the δ2-155 evaluation. The label _GWD for study 6 represents GM-1 treatment followed by 30 min wash before δ2-180 evaluation. The label DBD for study 7 represents prior exposure to deltorphin and treatment with BNTX before the δ2-155 evaluation. Values are means and standard error of the means for subjects in each group.

Figure 10: The mock data in the upper panel illustrates the expected response to acute δ1-blockade with BNTX if the δ2-response was lost as a result of competition from increased δ1-activity. The middle panel illustrates the expected response if δ2-response was lost as a result of down regulation. The lower panel illustrates the actual data from Protocol 2. Changes in heart rate mediated by low and high frequency stimulations of the right vagus nerve are illustrated during sequential δ_2-evaluations before and after treatment with the neuronal membrane ganglioside, GM-1. The designations δ2-5 and δ2-180, indicate the δ_2-evaluations at 5 and 180 minutes after the initial control stimulations. The designation δ2-250 + BNTX indicates the δ_2-evaluation at 250 min when the δ-2 agonist and BNTX were combined. The numerical value on those bars indicates the percent inhibition from the original control. Values are means and standard error of the mean for four subjects. The symbols (*) and (**) indicate the change in heart rate was significantly different from control at $P< 0.05$and $P<0.01$, respectively. The symbol (##) indicates the change in heart rate was significantly different from δ2-5 ($P<0.01$).

Study 1: Interactions between GM-1 and the native agonist, MEAP							
	Control	Lo MEAP	High MEAP	GM-1	GM-1+Lo MEAP	GM-1+ High MEAP	
MAP (mmHg)	102 ±6	97 ±7	99 ±8	94 ±9	96 ±10	90 ±8	
Heart Rate (bpm)	132 ±7	128 ±5	128 ±6	131 ±6	130 ±5	126 ±5	

Resting Cardiovascular Indices

Table 1

Study 2: Interactions between GM-1 and the δ-2 agonist, deltorphin II (DGD)							
	Control	δ2-5	GM-1	δ2-155	δ2-180	BNTX	BNTX + δ2-250
Heart Rate (bpm)	123 ±3	126 ±3	124 ±6	121 ±9	122 ±9	123 ±10	119 ±11
MAP (mmHg)	102 ±4	100 ±6	97 ±6	100 ±6	98 ±6	87 ±9	85 ±9
Study 3: Controls: vehicle, duration, and repeated deltorphin II (DSD)							
	Control	δ2-5	Saline	δ2-155	δ2-180		
Heart Rate (bpm)	126 ±6	123 ±6	124 ±8	120 ±7	120 ±7		
MAP (mmHg)	99 ±4	96 ±7	97 ±8	92 ±6	95 ±5		
Study 4: Controls: vehicle, duration, and naïve deltorphin II (_SD)							
	Control	Saline	Saline	δ2-155	δ2-180	BNTX	BNTX + δ2-250
Heart Rate (bpm)	128 ±3	127 ±5	122 ±4	121 ±6	121 ±7	120 ±8	119 ±8
MAP (mmHg)	106 ±7	104 ±11	103 ±8	101 ±7	100 ±9	92 ±8	91 ±8

Resting Cardiovascular Indices

Table 2a

Resting Cardiovascular Indices

Study 5: GM-1 and naïve deltorphin (no wash) (_GD)							
	Control	GM-1	GM-1 + δ2-155	GM-1 + δ2-180	BNTX	BNTX + δ2-250	
Heart Rate (bpm)	126 ±6	120 ±2	119 ±4	123 ±5	121 ±5	121 ±5	
MAP (mmHg)	99 ±10	96 ±8	90 ±7	93 ±8	84 ±7	86 ±5	
Study 6: GM-1 and naïve deltorphin (with wash) (_GWD)							
	Control	GM-1	δ2-180	δ2-205			
Heart Rate (bpm)	126 ±6	123 ±6	124 ±8	120 ±7			
MAP (mmHg)	99 ±4	96 ±7	97 ±8	92 ±6			
Study 7: Influence of δ-1 blockade on δ-2 response (DBD)							
	Control	BNTX	BNTX + δ2-5	BNTX + δ2-155	BNTX + δ2-180		
Heart Rate (bpm)	108 ±4	109 ±5	106 ±6	99 ±5	94 ±3		
MAP (mmHg)	94 ±5	94 ±3	95 ±3	94 ±5	92 ±4		

Table 2b

Protocol 1

Control	Lo-MEAP	High-MEAP	GM-1	Lo-MEAP	High-MEAP
60 min	5 min	5 min	30 min	5 min	5 min

Protocol 2 (DGD)

Control	δ2-5	GM-1	δ2-155	δ2-180	BNTX	BNTX+ δ2-250
60 min	5 min	60 min	5 min	5 min	5 min	5 min.

Protocol 3 (DSD)

Control	δ2-5	Saline	δ2-155	δ2-180	BNTX	BNTX+ δ2-250
60 min	5 min	60 min	5 min	5 min	5 min	5 min.

Protocol 4 (_SD)

Control	Saline	δ2-155	δ2-180	BNTX	BNTX+ δ2-250
60 min	120 min	5 min	5 min	5 min	5 min.

Protocol 5 (_GD)

Control	GM-1	δ2-155	δ2-180	BNTX	BNTX+ δ2-250
60 min	120 min	5 min	5 min	5 min	5 min.

Protocol 6 (_GWD)

Control	GM-1	Saline (wash)	δ2-180	δ2-205	BNTX	BNTX+ δ2-250
60 min	60 min	30 min	5 min	5 min	5 min	5 min.

Protocol 7 (DBD)

Control	δ2-5	Saline	δ2-155	δ2-180	BNTX	BNTX+ δ2-250
60 min	5 min	60 min	5 min	5 min	5 min	5 min.

Figure 1

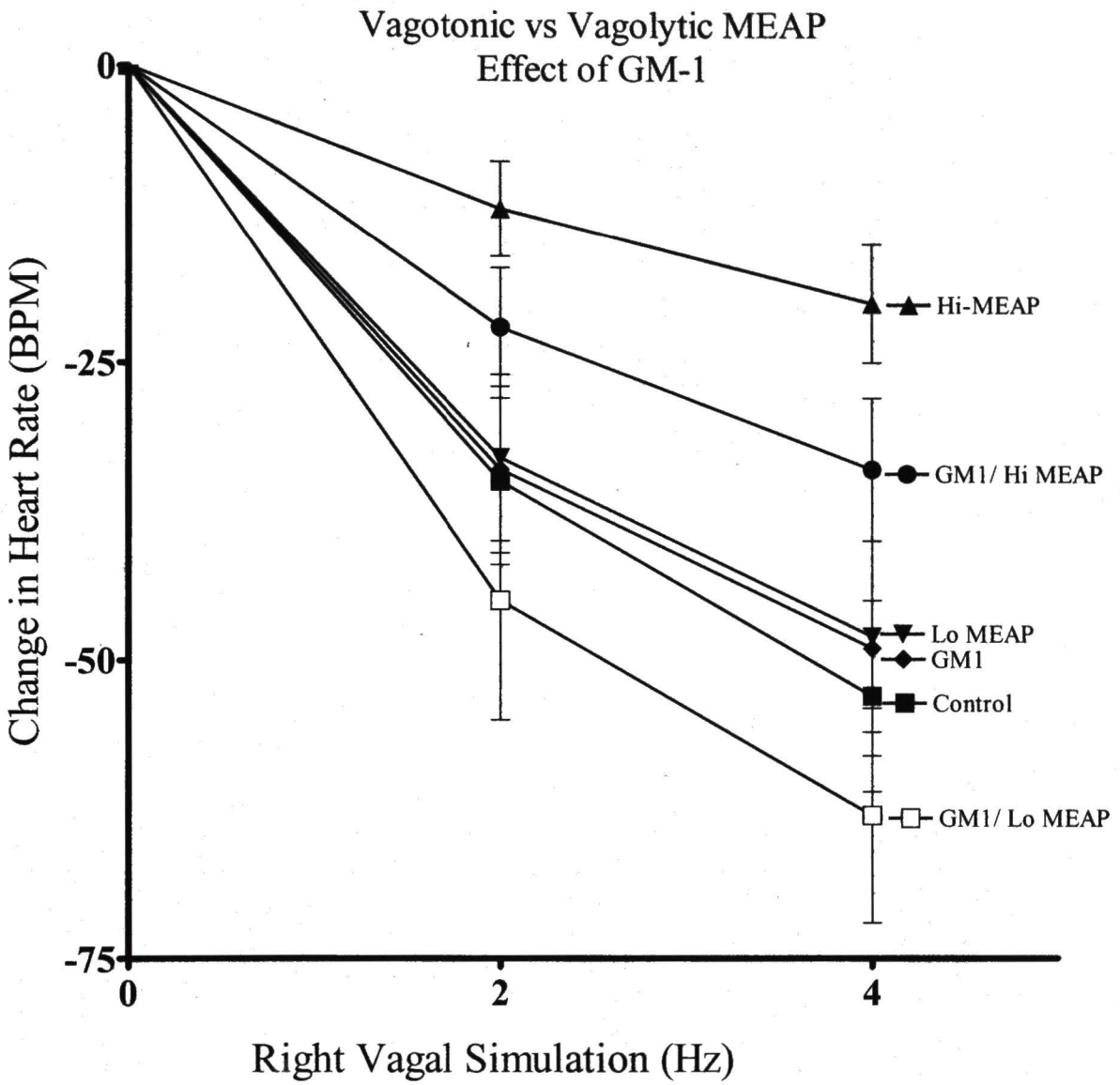
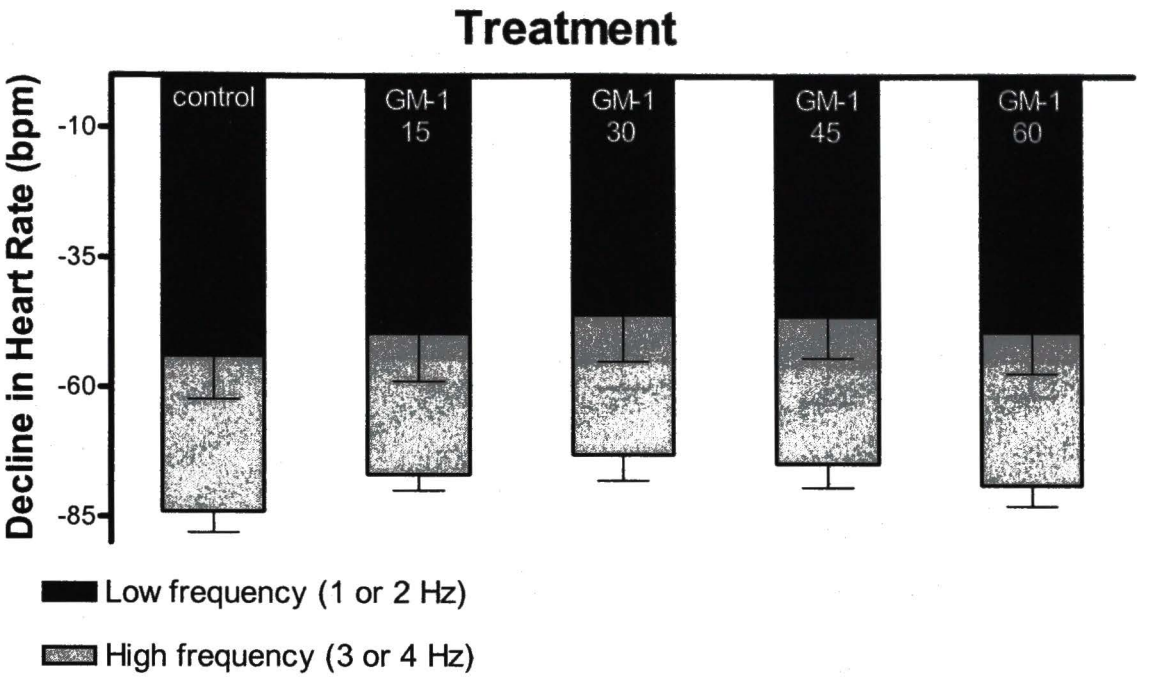

Vagotonic vs Vagolytic MEAP
Effect of GM-1
0
-25
-50
-75
Change in Heart Rate (BPM)
0
2
4
Right Vagal Simulation (Hz)
Hi-MEAP
GM1/ Hi MEAP
Lo MEAP
GM1
Control
GM1/ Lo MEAP

Figure 2


Treatment
control
GM-1 15
GM-1 30
GM-1 45
GM-1 60
Decline in Heart Rate (bpm)
-10
-35
-60
-85
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

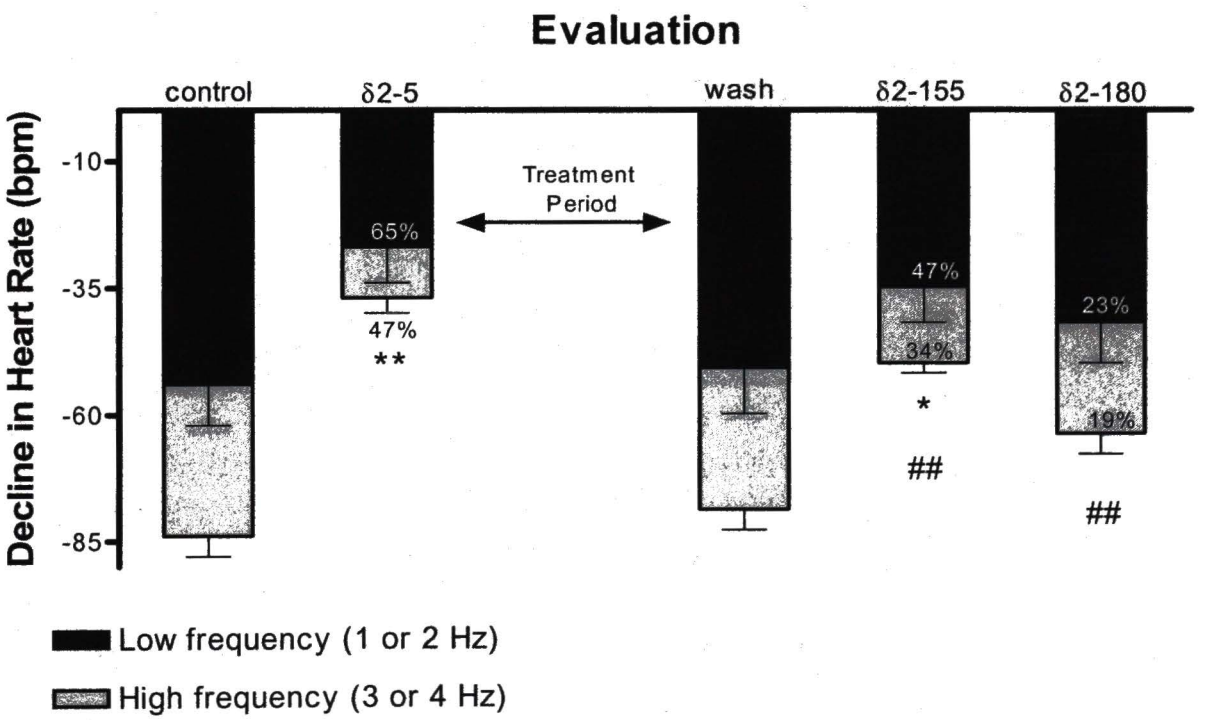
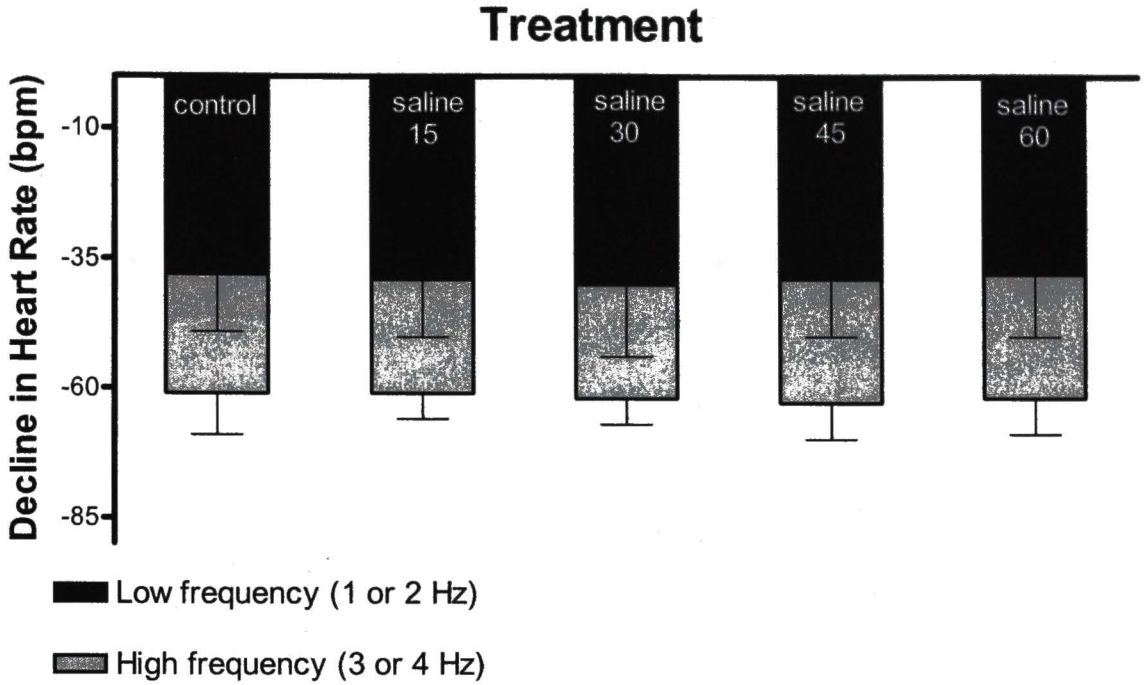

Evaluation
control
δ2-5
wash
δ2-155
δ2-180
Treatment Period
Decline in Heart Rate (bpm)
-10
-35
-60
-85
65%
47%
**
47%
34%
*
##
23%
19%
##
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 3


Treatment
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
saline 15
saline 30
saline 45
saline 60
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

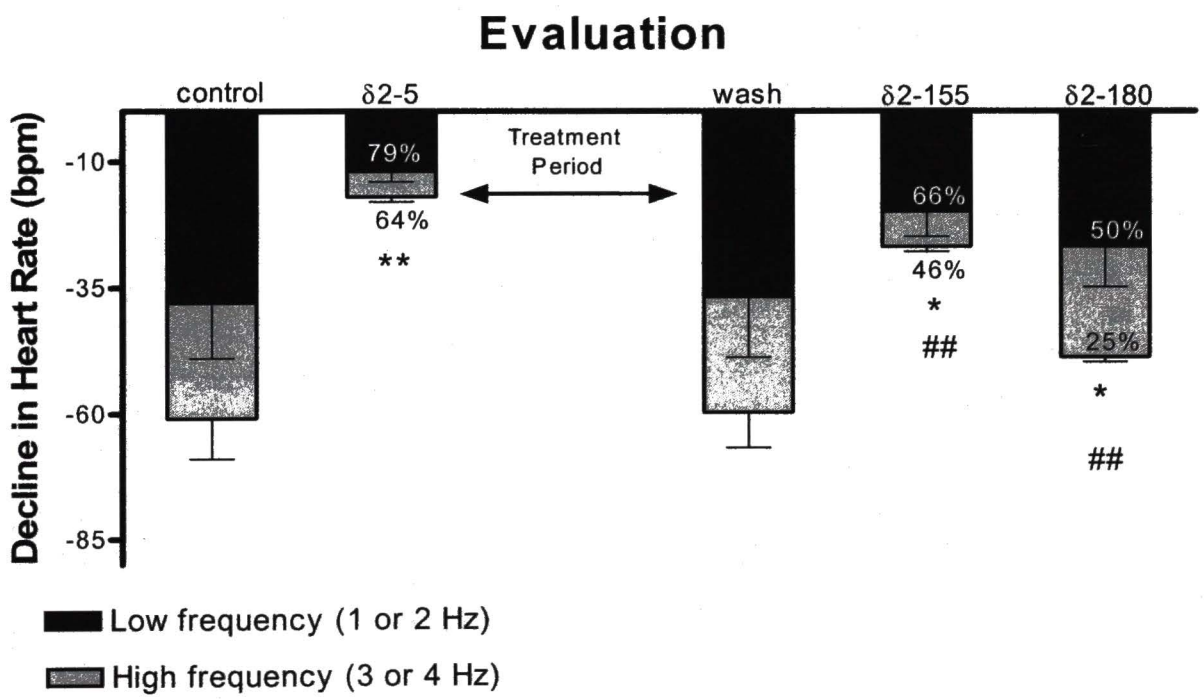

Evaluation
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
δ2-5
wash
δ2-155
δ2-180
79%
64%
**
Treatment Period
66%
46%
*
##
50%
25%
*
##
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 4

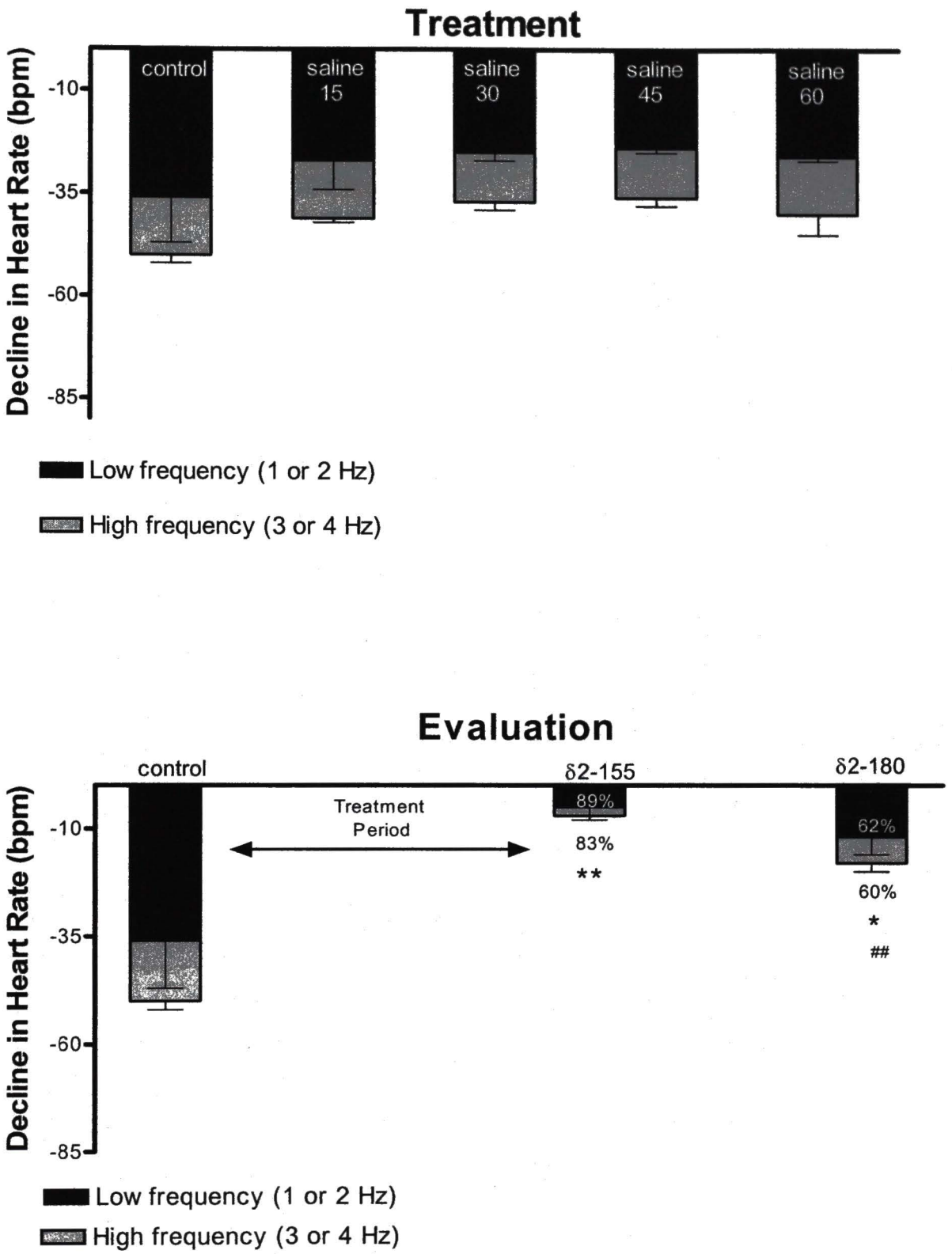
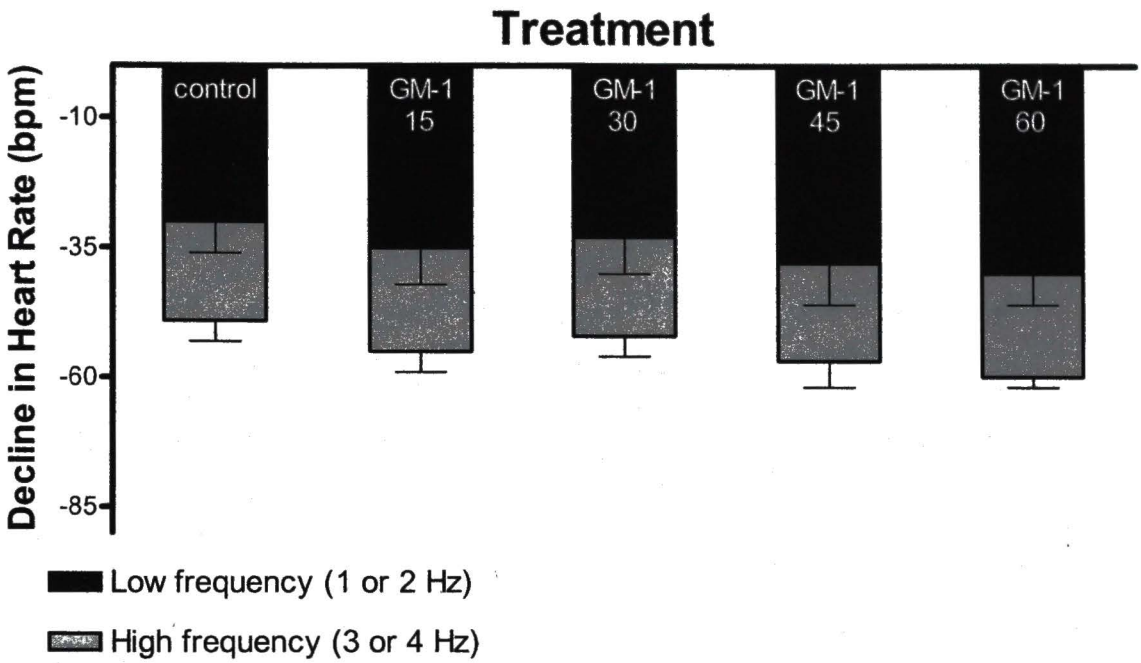

Treatment
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
saline 15
saline 30
saline 45
saline 60
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)
Evaluation
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
Treatment Period
δ2-155
89%
83%
**
δ2-180
62%
60%
*
##
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 5


Treatment
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
GM-1 15
GM-1 30
GM-1 45
GM-1 60
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

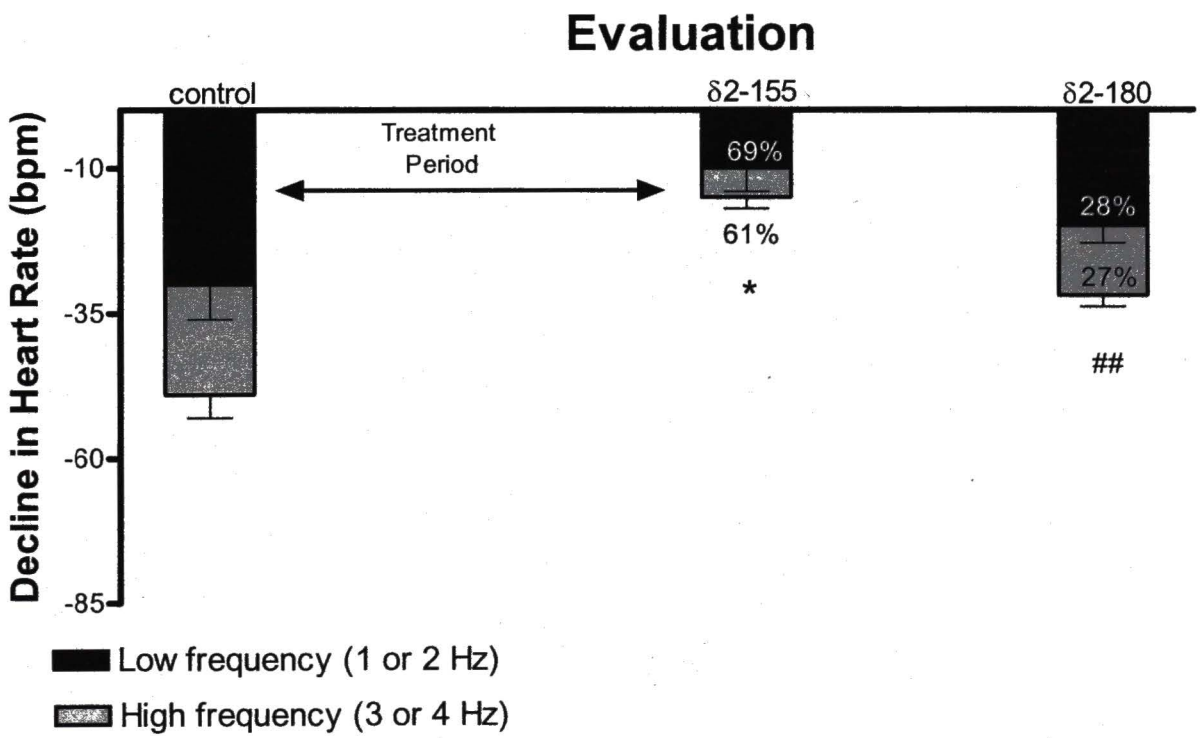
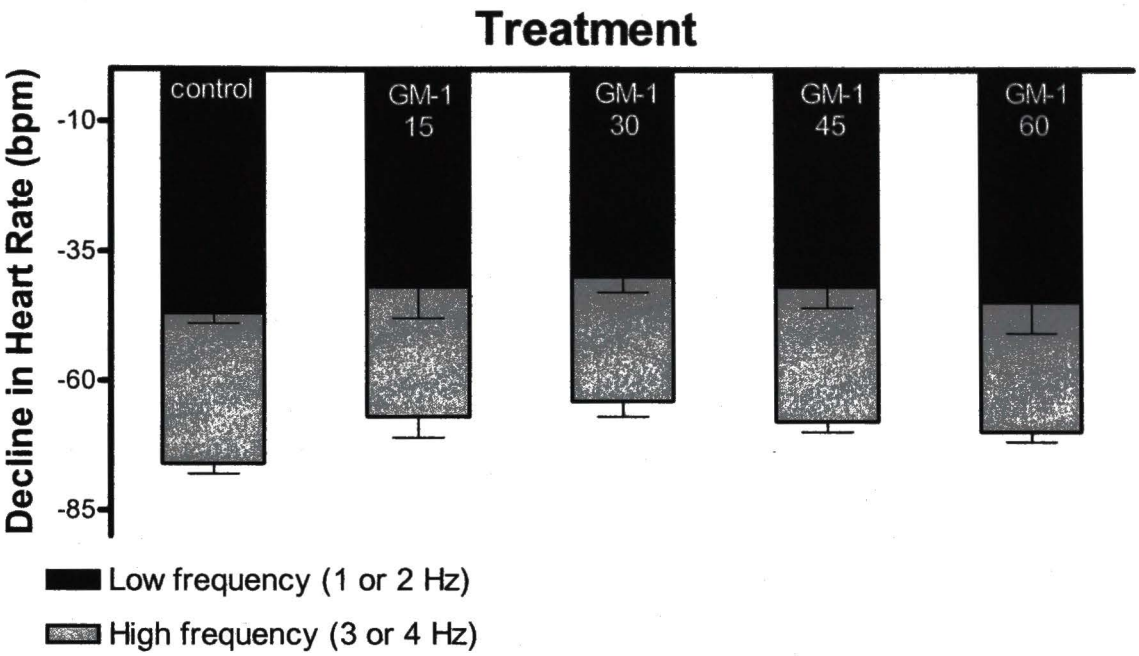

Evaluation
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
Treatment Period
δ2-155
69%
61%
*
δ2-180
28%
27%
##
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 6


Treatment
control
GM-1 15
GM-1 30
GM-1 45
GM-1 60
Decline in Heart Rate (bpm)
-10
-35
-60
-85
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

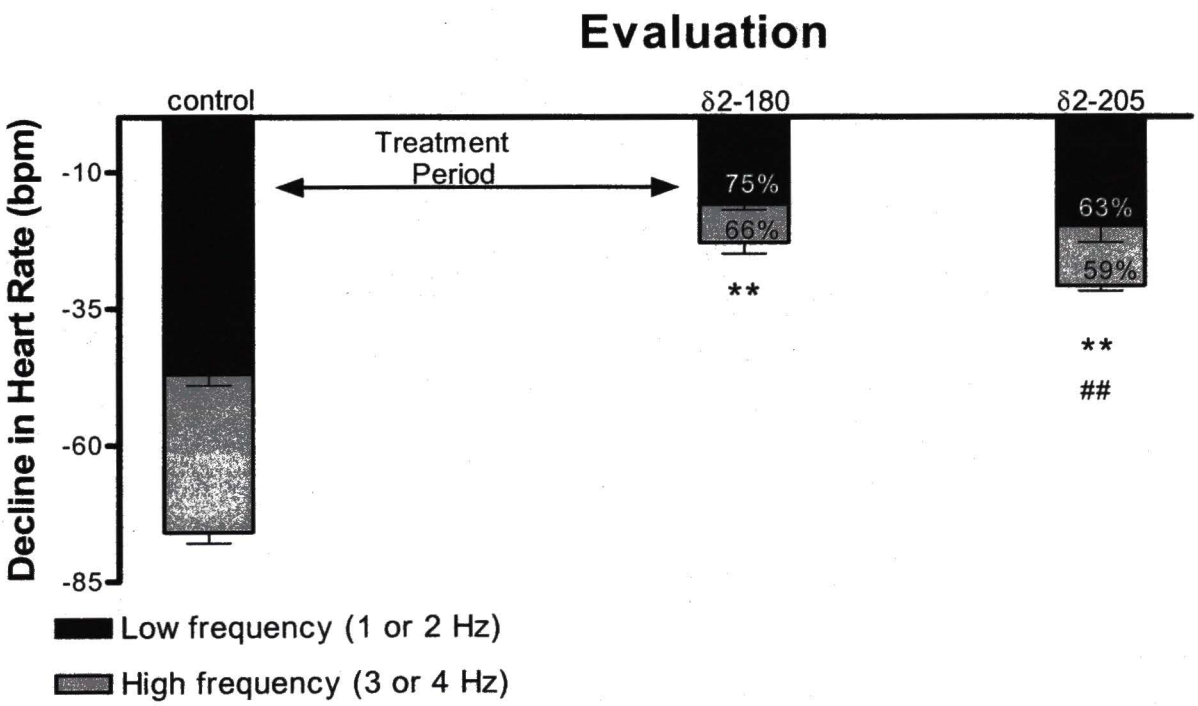

Evaluation
control
Treatment Period
δ2-180
δ2-205
75%
66%
63%
59%
**
**
##
Decline in Heart Rate (bpm)
-10
-35
-60
-85
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 7

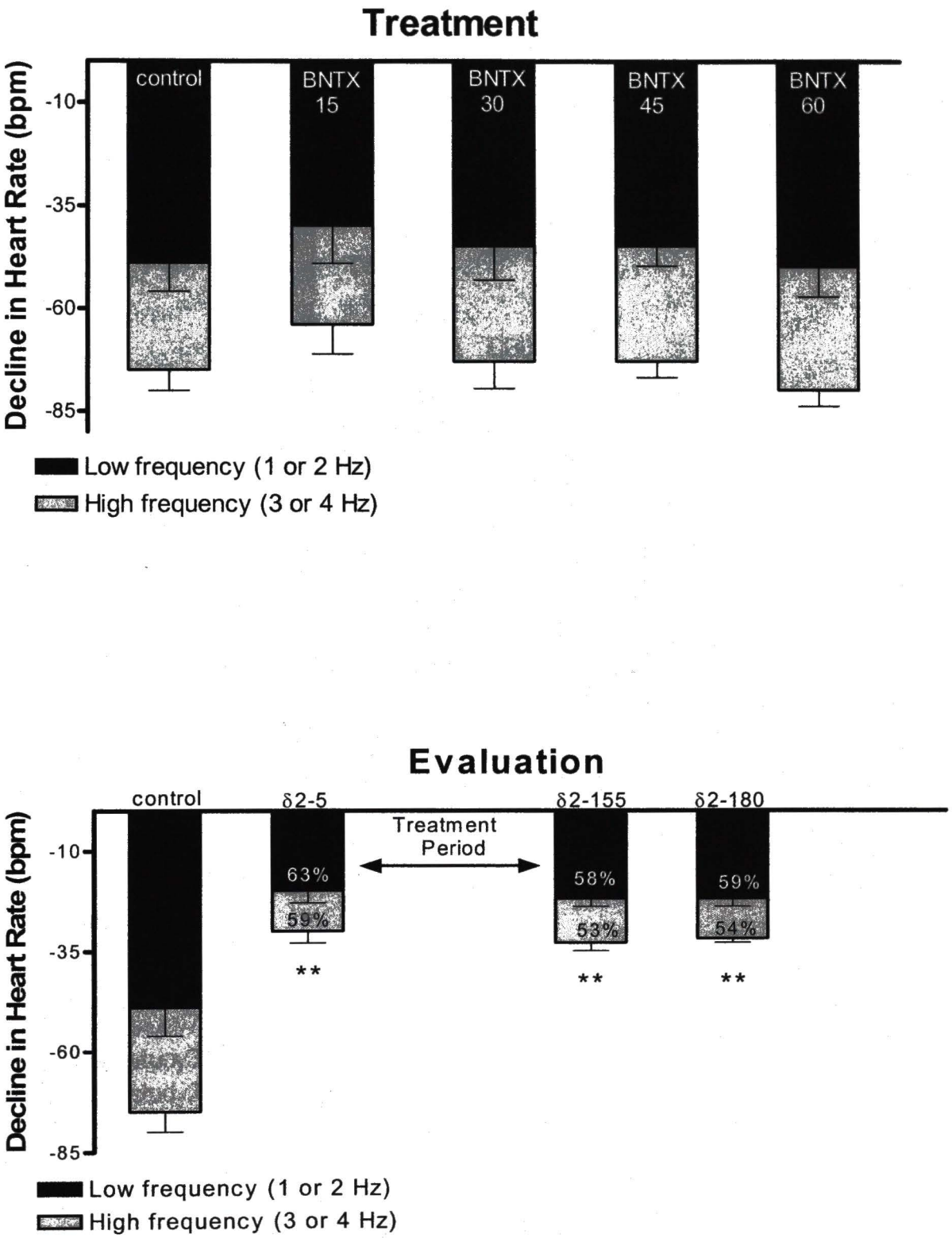

Treatment
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
BNTX 15
BNTX 30
BNTX 45
BNTX 60
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)
Evaluation
Decline in Heart Rate (bpm)
-10
-35
-60
-85
control
δ2-5
Treatment Period
δ2-155
δ2-180
63%
59%
58%
53%
59%
54%
**
**
**
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 8

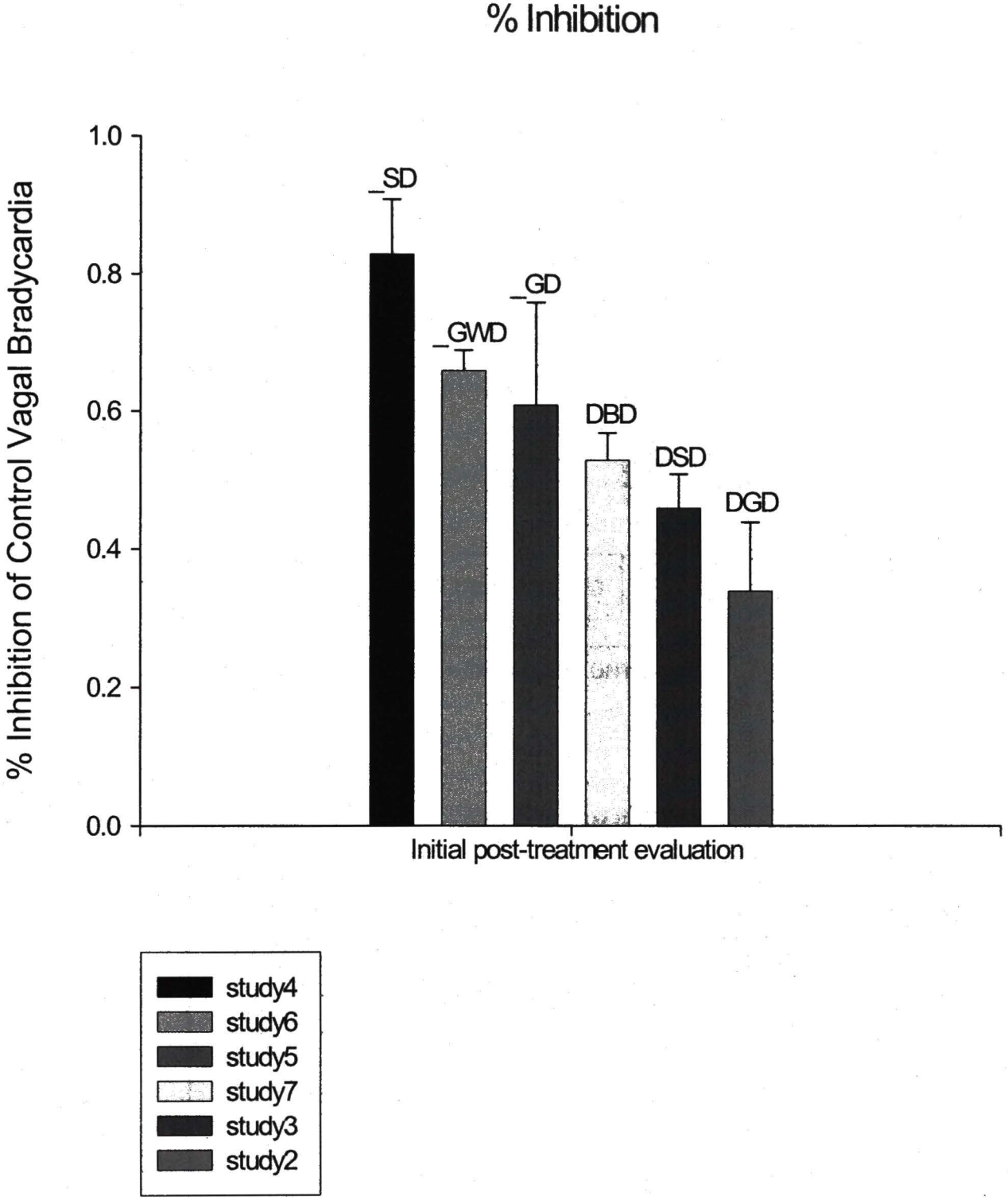
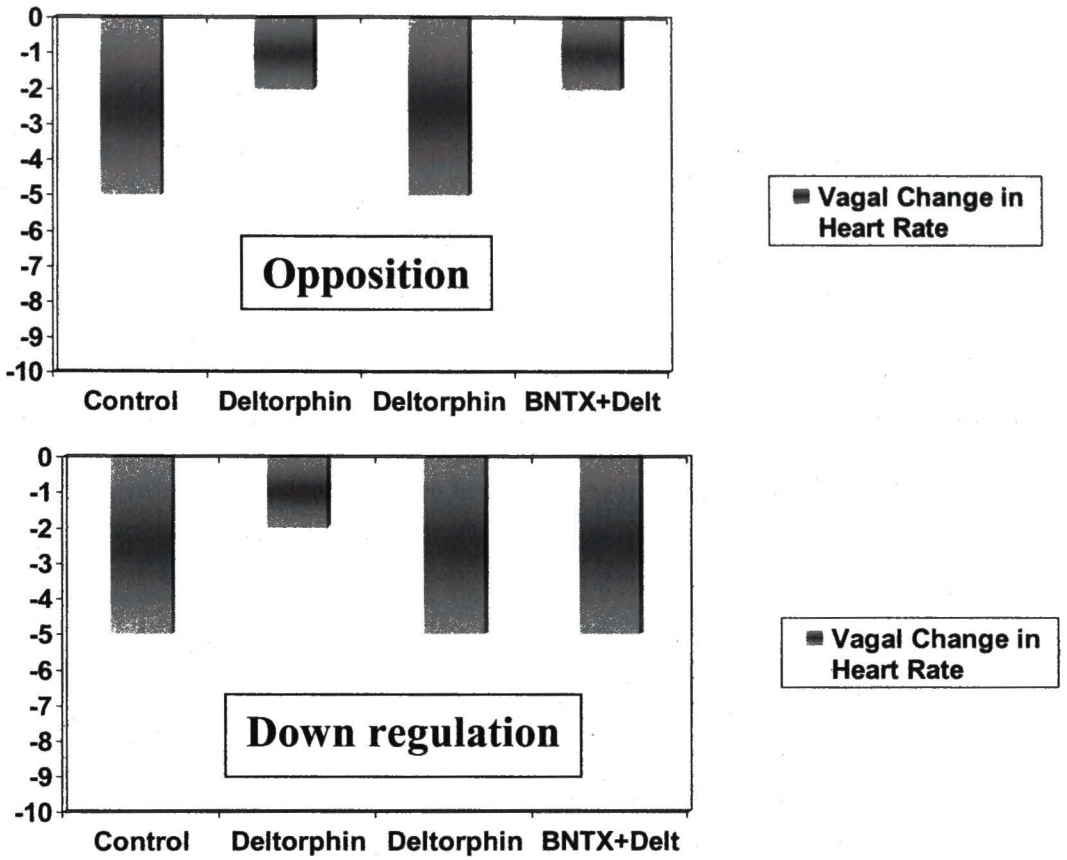

% Inhibition
% Inhibition of Control Vagal Bradycardia
1.0
0.8
0.6
0.4
0.2
0.0
_SD
_GWD
_GD
DBD
DSD
DGD
Initial post-treatment evaluation
study4
study6
study5
study7
study3
study2

Figure 9


0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
Opposition
Control
Deltorphin
Deltorphin
BNTX+Delt
Vagal Change in Heart Rate
Down regulation
Control
Deltorphin
Deltorphin
BNTX+Delt
Vagal Change in Heart Rate

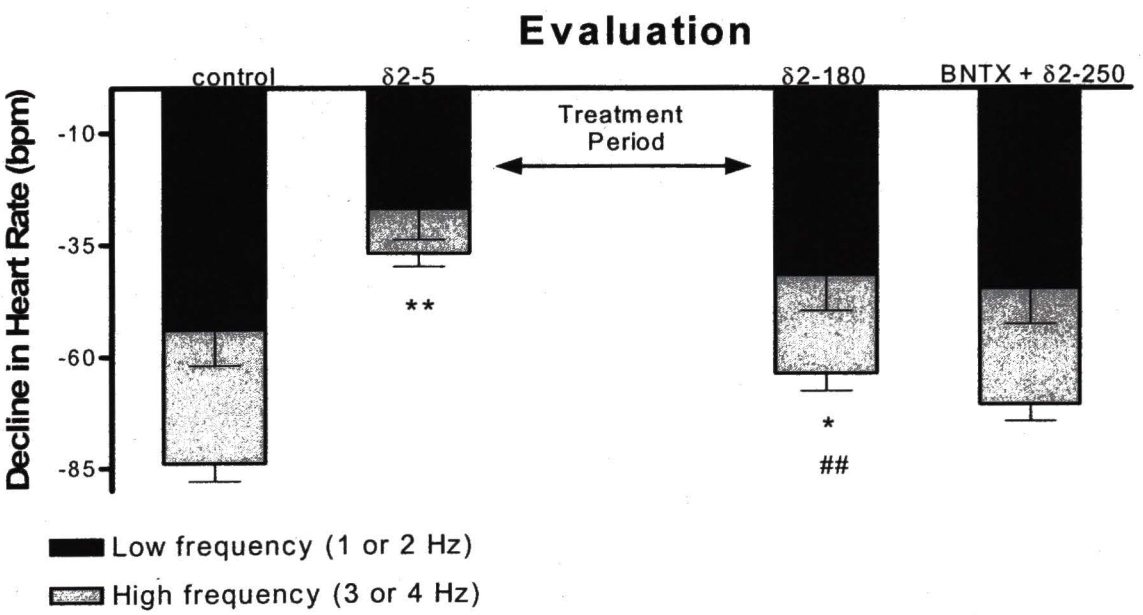

Evaluation
control
δ2-5
δ2-180
BNTX + δ2-250
Treatment Period
Decline in Heart Rate (bpm)
-10
-35
-60
-85
**
*
##
Low frequency (1 or 2 Hz)
High frequency (3 or 4 Hz)

Figure 10

CHAPTER III

SUMMARY AND CONCLUSIONS

1. Time and/or external GM-1 appear to modify the δ-2 mediated vagolytic response in opposite directions. The δ-2-mediated vagolytic response becomes more intense over time and GM-1 prevents that improvement..

2. The δ-2 response becomes less intense after prior exposure to deltorphin, and the loss of the δ-2 response is blocked by BNTX. Therefore the attrition of the response is a δ-1 receptor dependent response.

3. The attenuation of the δ-2 response is not due to the addition of competing δ-1 mediated vagotonic activity since the lost response is not restored by the subsequent blockade of the δ-1-receptors with BNTX.

FUTURE STUDIES

The following studies are proposed to further clarify the influence of the neural ganglioside on loss of the δ-2 response.

1. The above findings indicate that GM-1 and deltorphin administered alone may be less effective than when administered in combination. Since the loss of the δ-2-response was prevented by BNTX, additional studies of the δ-1-contribution to their interaction will be needed.
2. Develop an immunohistochemistry approach to test whether sustained δ-1 stimulation increases the membrane ganglioside, GM-1 in nerve terminals within the SA node.
3. Develop a micro-analytical method to quantify tissue GM-1 content directly.
4. Develop biochemical and pharmacological approaches to determine whether deltorphin diminishes it own vagolytic effect through intrinsic δ-1 activity or through the release of an endogenous δ-1 agonist.