

NEUROPEPTIDE Y MODULATION OF SYMPATHETIC  
ACTIVITY IN  
MYOCARDIAL INFARCTION

BY

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## ABSTRACT

**Objectives.** This study examines the effect of neuropeptide Y (NPY) in modulating the central sympathetic activity after myocardial infarction (MI) in rats.

**Background.** Previous studies have shown the co-existence of NPY with norepinephrine (NE) in the brain and possible functional interaction between the two. NPY inhibits the release of NE at the presynaptic level and can be considered to act as a neuromodulator.

**Methods.** Two groups of rats were examined in this study. An "experimental group" defined as those rats undergoing left coronary artery ligation and a "sham group" without coronary ligation serving as controls. The animals of both groups underwent microdialysis in paraventricular nucleus (PVN) of the brain at 2, 4 and 8 weeks later. Microdialysis samples were collected with and without injecting NPY at a concentration of  $10^{-8}$  M in the brain. Concentration of released NE was determined by injecting purified microdialysate samples in the high performance liquid chromatography (HPLC). To explore the possibility of the role played by the receptors, autoradiographical localization of NPY receptors in PVN was also carried out between the experimental and sham groups at 8 weeks after coronary ligation.

**Results.** Concentration of NE measured in the samples was decreased by 50% with NPY in 2- and 4- but only 20%, ( $p < 0.05$ ) in 8-week old post infarcted rats. The diminished inhibitory effect of NPY on NE release was associated with increased

sympathetic activity which was reflected by NE level in blood plasma. 8-week old post infarcted rats had almost 2 fold , ( $p < 0.05$ ) increase in the level of NE in blood plasma as compared to the shams. Autoradiography revealed a significant decrease in density of NPY receptors in the PVN at 8 weeks post infarcted rats ( $p < 0.05$ ).

**Conclusions.** Our study suggests that abnormality in the control of sympathetic system by NPY may result in an elevated level of NE during congestive heart failure (CHF).

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## LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CHF	congestive heart failure
DA	dopamine
E	epinephrine
NE	norepinephrine
NPY	neuropeptide Y
CNS	central nervous system
PNS	peripheral nervous system
SNS	sympathetic nervous system
HPLC	high performance liquid chromatography
MI	myocardial infarction
CSF	cerebro spinal fluid
SD	sprague-dawley
ANF	atrial natriuretic factors
PVN	paraventricular nucleus
NTS	nucleus tractus solitarius
LRN	lateral reticular nucleus
VLM	ventro lateral medulla
IR	immuno reactivity
AP	area postrema

SHR	spontaneously hypertensive rats
PHN	posterior hypothalamic nucleus
MPN	median preoptic nucleus
AHA	anterior hypothalamic area
PPH	posterior pituitary hormone

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## 1. INTRODUCTION

There are many studies to indicate that a large North American population is affected by congestive heart failure (CHF). Both clinically as well as experimentally this pathophysiological disorder is accompanied by a hyperactivity of neurohormones that includes an increase in the circulatory level of norepinephrine (NE), (Cohn et al., 1986), plasma renin activity (Dzau et al., 1981), vasopressin (Creager et al., 1986) and atrial natriuretic factors (ANF) (Cody et al., 1986). Studies also show that neuroendocrine excitation precedes the development of clinically recognizable heart failure (Francis et al., 1990) which strengthens the pathogenetic role of neurohormonal activation in CHF.

Plasma NE has been used as the indicator and marker of the sympathetic nervous system (SNS) in the pathophysiological as well as in the normal situation. Several views have been proposed regarding high levels of NE in CHF. This increase in level of NE may be due to excessive release of the related catecholamines from the adrenal medulla, higher sympathetic outflow from the central nervous system (CNS), or NE release from the adrenergic nerve terminals and reuptake of NE peripherally. It has also been shown that NE clearance does not function normally in CHF (Davis et al., 1988) which results in the accumulation of NE in the systemic circulation. In fact, plasma analysis of NE which provided an indirect estimation of the SNS is accompanied by the recent use of direct recordings of efferent sympathetic nerve activity in patients with heart failure. Patients with

elevated levels of NE in systemic circulation reflects a higher level of sympathetic outflow.

It has been known for years that brain sympathetic system controls peripheral sympathetic action therefore, the possibility exists that the CNS may be involved in CHF. This is also strengthened by the fact that NE contained within central neurons is involved in cardiovascular sympathetic regulation. The role of CNS might be the causal agent of CHF and in turn is mediated by SNS. However, in spite of an active research in this field very little data is available to understand the involvement of brain in CHF.

In this regard, neuropeptide Y, a 36 amino acid peptide, has gained much interest in the recent past. It is present in the brain in high amounts and is widely distributed within neurons of the CNS and PNS. It occurs in mammalian brain in higher concentration than all other peptides studied to date. It also co-exists and is co-released with epinephrine (E) and NE in the catecholaminergic systems in brain. Studies have shown that NPY is increased in the circulation in MI, hypertension and other diseases where the sympathetic system is activated. Thus there is a good possibility that a functional interaction between NE and NPY may exist within brain (Woo et al., 1993). NPY may play a modulatory role in situation with heightened sympathetic activity. Unfortunately no work has been done till today to show the involvement of NPY in CHF.

In the brain, NPY is present in the highest concentration in PVN where it is co-localised with NE. Since it has been found that mechanisms normally involving

NPY as a neuromodulator in PVN are altered in hypertension and other cases of heightened sympathetic activity, it is postulated that CHF may precipitate changes in mechanisms involving brain NPY and increased sympathetic activity. In an effort to clarify this aspect, the present study examines whether or not the central catecholamine system in PVN are modulated by NPY in CHF.

## **2. REVIEW OF THE LITERATURE**

### **2.1 Sympathetic System in Congestive Heart Failure**

The circulating levels of catecholamines are increased in order to support the depressed heart function in CHF. The mechanisms for this abnormal activation of the SNS however are poorly understood. The evidence favours a view that the role of CNS may be a causal factor (Lown et al., 1976) which is mediated by the SNS. Studies by Schomig et al., 1988 have shown that elevation of catecholamines in the systemic circulation causes an activation of peripheral sympathetic nerve activity by central mechanisms (increased sympathetic drive). Psychoemotional factors and stress are thought to be involved in this kind of activation of sympathetic activity (Lown et al., 1973). Unfortunately, there are only few experimental data available on this aspect. In addition, inspite of active research in this field, the exact reason for heightened sympathetic activity following myocardial infarction is unknown at present.

Increased sympathetic nervous activity acts as a hallmark of symptomatic CHF (Francis et al., 1991). Sympathetic nervous activity is increased in patients with CHF as a means of maintaining homeostasis in the face of a decrease in affecting circulating blood volume (Francis et al., 1984). Enhanced circulating levels of catecholamines have been reported in patients with CHF, reflecting sympathetic activation (Strange et al., 1978, Bertel et al., 1982). Changes in sympathetic nerve activity are reflected in changes in plasma E and NE concentration. Sympathetic

concentration of catecholamine reflect the extent of MI and hemodynamic alteration evoked by acute MI. Reflex activation of the sympathetic system, induced by both cardiovascular reflexes and by activation of afferent nerve fibres originating in the infarcted zone, as well as pain and anxiety are regarded as causes of an enhanced adrenergic activity in patients with CHF. Plasma levels of sympathetic neurotransmitter have been used as a prognostic indicator and NE in particular is raised significantly in patients with CHF. NE is liberated as a result of the reflex induced sympathoneural and sympathoadrenal simulation occurring in the presence of MI and coronary occlusion was reported as early as 1971 by Stazewska et al. Patients with class IV heart failure have increased plasma concentration of NE and poor prognosis for survival (Thomas et al., 1978, Cohn et al., 1984). Leimbach et al., 1986 have shown that microneurographically measured central nervous system traffic to peroneal muscle is increased in patients with CHF and also correlates with plasma NE levels. Thomas et al., 1978, have reported that the level of plasma catecholamine in patients with heart failure correlates with that of the New York Heart Association Functional Classification.

CHF has always been associated with increased sympathetic activity, but the exact mechanism of the association is unknown at present. It has been known for a long time that neurons residing within the brain play a crucial role in regulating the sympathetic activity. However, their exact location and distribution until recently has been uncertain. In the last decade, attention has been focused upon neurons in restricted regions of the hypothalamic structures which give rise to anatomical

pathways that can eventually modulate the activity of sympathetic preganglionic neurons. With the recent findings of a regulatory peptide NPY, which is co-released and co-localised with NE there has been an increasing possibility that the CNS may play a crucial role in the overall activation of the SNS in association with NPY.

## **2.2 NPY: Chemistry and Localization**

NPY is a 36 amino acid peptide neuromessenger originally isolated from the porcine brain (Tatemoto et al., 1982). Named after its carboxy terminal and amino terminal tyrosine residues, it is a putative sympathetic neurotransmitter that possesses some remarkable characteristics of its own. It is associated with negative inotropic effect (Minson et al., 1989, Rigel et al., 1989, Zukowska et al., 1987), direct vasoconstrictory action (Zakowska et al., 1986, Hanko et al., 1986), pre and post synaptory modulatory effects of NE release (Lundberg et al., 1985) and NE induced vasoconstriction (Edvinsson et al., 1984).

NPY has also been recently shown to be co-localized with catecholamines specially NE in CNS. NPY like IR has been found within discrete areas throughout the CNS except the cerebellum (Allen et al., 1986, Maccorrone et al., 1986). Using immunohistochemical techniques Everitt et al., 1984, and Blessing et al., 1986 showed NPY like IR localized in the noradrenergic/adrenergic cell bodies of A1/C1 cell groups of ventrolateral medulla oblongata of rats. Co-localization of NPY with catecholamines has also been reported in the dorsal motor nucleus of vagus, nucleus of the tractus solitarius and the lateral reticular nucleus (Hokfelt et al., 1983a,

1983b). Peripheral chromaffin storage vesicles also contain catecholamines with NPY (Winkler et al. , 1988).

NPY is present in high amounts in both PNS (Lundberg et al., 1982) and CNS (Chronwall et al., 1985) of mammals. In the brain it is unevenly distributed with highest concentration in hypothalamus, an area which is well known to be a rich catecholaminergic site (Chronwall et al., 1985). In the last decade, enormous progress has been made in the identification of forebrain structures which plays a crucial role in regulating the sympathetic activity. In this regard, attention has been focused upon neurons in restricted regions of the hypothalamic structures which can eventually modulate the activity of sympathetic preganglionic neurons.

While several forebrain structures including posterior hypothalamic nucleus (PHN), median preoptic nucleus (MPN), anterior hypothalamic area (AHA) have been implicated for sympathetic regulation, the paraventricular nucleus (PVN), because of its well defined nuclear boundaries, has received the most attention and has now been extensively investigated using a variety of electrophysiological (Ciriello et al., 1980), neuroanatomical and pharmacological methods (Sawchenko et al., 1985).

### **2.3 Role of NPY in PVN**

Recent electrophysiological evidence suggests that PVN might play an important role in the regulation of cardiovascular responses which might regulate the sympathetic tone. These observations have been based on the findings that PVN projects directly into the sites involved in cardiovascular regulation.

NPY fulfils the criteria for a neurotransmitter or neuromodulator. At low doses NPY injection into the ventricles reduces NE release in the hypothalamus notable in the PVN (Vallego et al., 1986). Furthermore, a microdialysis study also demonstrated that NPY injection in the medial hypothalamus reduced extra cellular NE levels (Shimizu et al., 1989). Binding sites for NPY have been identified throughout the brain by autoradiography.

The highest concentrations of NPY IR is found in the PVN region. Extensive NPY system has been observed where NPY appears to be co-localized within catecholamine terminals (Gray et al., 1986). Since the NPY IR perikarya have also been reported in the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM), the NPY in the PVN most likely arises from catecholamine cell groups within the brainstem particularly the A1 / C1 noradrenergic and adrenergic cell groups in the VLM and the C2 adrenergic cell group in the NTS. Since both VLM and NTS are involved in regulating blood pressure, it is quite possible that NPY plays a role in cardiovascular regulation at the level of the hypothalamus.

In the brain the highest densities of NPY containing fibers and perikarya are found in the hypothalamic PVN (Chronwall et al., 1985). Stimulation of the PVN increases sympathetic outflow (Kannan et al., 1989). The PVN of hypothalamus is an integration site for autonomic function (Swanson et al., 1983). PVN is also one of the areas intensely studied for its prominent role in cardiovascular control as well as having neurons that project to sites in ventro lateral medulla (VLM) involved in pressure control (Saper et al., 1983).



In an interesting research report Iseabail M. Macrae and John L. Reid, 1988, and Harfstrand et al., 1987 has stated that intracisternal administration of NPY (microinjection at a dose of 1.25 nmol) into the hypothalamus (PVN and periventricular nuclei) and thoracic spinal cord gave rise to a big fall in blood pressure and heart rate 1 hour postinjection. The fall in blood pressure and heart rate was dose dependent. This modulatory function of NPY had been a question until recently. In the last decade it was found that receptors of NPY may play role and might be responsible for this behaviour of this regulatory peptide.

## **2.4 NPY Receptors**

NPY receptors are involved in 3 different actions at sympathetic neuroeffector junctions. The 3 actions are the direct post junctional effect, the indirect post junctional effect (amplifying effect) and the pre junctional inhibition of NE release (Edvinsson et al., 1989).

Receptors for NPY are subdivided into post synaptic Y-1 receptors and pre synaptic Y-2 receptors on the basis of the affinity of the 13-36 fragment of NPY, using a preparation of the SNS. The receptors of Y-1 type can recognize the full parent peptide porcine NPY 1-36. The Y-1 receptor has been shown to mediate several effects of NPY in the periphery like vasoconstriction and anxiolytic effect in animal models of anxiety.

NPY receptors of the Y2 type, which can also recognize C-terminal fragments of porcine NPY, especially porcine NPY 13-16 (Wahlestedt et al., 1988).

Experiments with receptor autoradiograms have shown that  $I^{125}$  NPY 1-36 predominantly labels the Y-1 receptors since the C-terminal porcine NPY fragment 13-36 only can displace  $I^{125}$ -NPY 1-36 from its binding sites in very high concentration (over  $1\mu\text{M}$ ). A third type of receptor for NPY has been recognized as Y-3 receptor but its functional importance still remains to be established

A considerable body of evidence now suggests that the vasodepressor actions of centrally administered NPY are at least in part mediated via activation of high affinity of Y-1 NPY receptors located at the cardiovascular centres in the brain (Fuxe et al., 1983). In the CNS, it has been shown that Y-2 receptors of NPY reduces NE release in the medulla and hypothalamus of SD rats (Woo et al., 1991, Woo et al., 1992) and that NPY partially stimulates the presynaptic  $\alpha_2$ -adrenergic receptors in these regions. Large number of high affinity  $I^{125}$ -NPY binding sites have been mapped out within the central nervous system of the rat brain (Harfstrand et al., 1986). However, as with several other peptides, there are unexplained discrepancies between the distribution of binding sites and the density of NPY-IR innervation, for example, binding sites are relatively sparse in the PVN (Martel et al., 1986).

Available data also indicate the existence of interactions between NPY receptors of the Y1 and Y2 type. One possibility is that the activation of the NPY receptor of Y2 type may lead to an increase in the affinity of the NPY receptors of the Y1 type. The first evidence of specific  $I^{125}$ -NPY binding sites in the rat brain was provided by Unden et al., 1985a, 1985b. They demonstrated that these binding sites were sensitive to proteolytic enzyme, thiol agents and pH alterations.

Autoradiographic studies have revealed that NPY binding sites are widely distributed in the rat brain. High densities are found in e.g. the olfactory bulb, superficial layer of cerebral cortex, ventral hippocampus, lateral septum, various thalamic nuclei, area postrema and NTS (Nakajima et al., 1985, Harfstrand et al., 1986b, Martel et al., 1986). As in the case with many neuronal messengers, the distribution of NPY and that of its binding sites are mis-matched, and they do not always overlap. For example IR studies on NPY shows the greatest amount of distribution of NPY IR fibres in PVN region of hypothalamus where as binding studies have revealed poor receptor site in the PVN. Cases in CHF, coronary heart disease and hypertension have revealed decrease in the presence of NPY receptors. Therefore the level of NPY in a heightened sympathetic activity situation as in CHF is very important in understanding the development of MI and subsequent CHF.

## **2.5 NPY Level During Increased Sympathetic Activity**

It is possible that a massive release of catecholamines in CHF which represents an increased sympathetic activity may be due to the redistribution of NPY in the brain. Since peripheral chromaffin storage vesicles also contain catecholamines with NPY (Winkler et al., 1988), following heightened sympathetic activity NPY release increases which in turn elevates its level in parallel with NE release (Haass et al., 1989). Release of NPY is revealed by overflow from the human heart as well as increases in the systemic plasma levels in man upon sympathetic activation (Lundberg et al., 1986). The ratio between the overflow of

NPY and that of NE increases with the frequency of stimulation of sympathetic activity. There is also report of increased plasma concentrations of neuropeptide Y in healthy people during physical exercise (Lundberg et al., 1986) and patients with acute and chronic heart failure (Hulting et al., 1990, Maissek et al., 1989). NPY has been reported to exert both negative cardiac inotropic and chronotropic actions (Zukowska et al., 1987, Franco et al., 1985, Rious et al., 1986, Allen et al., 1986). Thus prolonged release of NPY during sympathetic activation in the development of CHF might contribute to some extent in the deterioration of ventricular function.

Increased plasma level of NPY could be detrimental in situation of CHF and appropriate NPY antagonist could be developed to combat the ill effect of increased level of plasma NPY.

## **2.6 Central Sympathetic System: Interaction with NPY**

NPY inhibits release of NE at the presynaptic level (Waeber., 1990). This neuropeptide has been postulated to act as a neuromodulator in the central and peripheral sympathetic nervous systems (Tsuda et al., 1990, Woo et al., 1991). In addition, injections of NPY into the third ventricle and PVN significantly depresses the sympathetic activity. Numerous CNS effects of NPY have been suggested (Wahlestedt et al., 1989). At the brain stem level, NPY is involved in the control of basic vegetative function, such as respiratory and cardiac regulation (Fuxe et al., 1983). NPY effects hypothalamic mechanisms including regulation of hormone secretion (Wahlestedt et al., 1987), circadian rhythm (Alber et al., 1984) and food

intake (Levene and Morley., 1984). Involvement of NPY in the regulation of behaviour and in certain psychopathological states has also been suggested (Heilig et al., 1990).

Interactions between NPY and monoaminergic system in the brain have attracted a vast amount of interest amongst researchers. In the PNS, NPY is mainly co-localised with NE in post ganglionic sympathetic neurons and seems to potentiate post synaptic responses to the amine transmitter while presynaptically inhibiting its release (Lundberg et al., 1982). The actions of NPY at sympathetic neuroeffector junctions have attracted maximum interest. Edvinsson et al., 1987 studied the physiological significance of NPY/NE co-existence and reported that NPY may affect the sympathetic neuroeffector junction in at least 3 different ways: 1) direct post junctional effect for example vasoconstriction; unrelated to adrenoreceptors, 2) potentiation of NE evoked response and 3) pre-junctional suppression of stimulated NE release. The possible end result of these diverse actions may be an improvement in the economy at the sympathetic neuroeffector junctions reflected in a suppression or shortening of NE release process after nerve stimulation. It appears that NPY and NE can be released differentially from sympathetic nerves and they may act in concert to maintain adequate neurotransmission (Lundberg and Hakfelt., 1986, Wahlestedt et al., 1987a).

There are several well known views regarding the interaction between NPY and NE. Since NPY and NE are found to co-exist and co-localise in the neurons of the brain, NPY may affect the release of NE directly by modulation autoreceptors

that control its release. NPY may also affect the release of NE through interneurons which in turn modulates presynaptic autoreceptors. NPY may enhance NE's postsynaptic effects by inhibiting its reuptake. NPY may facilitate the action of NE by acting on the postsynaptic membrane via receptor-receptor interactions (Kyrkouli et al., 1992). NPY fails to enhance the turnover of NE and may in fact depress its release (Fuxe et al., 1986, Pernow., 1988, Stjarne et al., 1986, Westfall et al., 1988).

Major co-existence occurs in the noradrenergic A1 and A4 groups. Partial co-existence is reported in noradrenergic A2 and A6 (the locus coeruleus) groups, whereas noradrenergic cells in the area postrema and in the A5 and A7 group seem to be devoid of NPY (Everitt et al., 1984). Functionally NPY produces NE like effects on hormone release (Kalra and Crowley., 1984). The co-localisation of NPY and NE is an interesting feature of post ganglionic sympathetic nerves to the cardiovascular system (Edvinsson et al., 1985, Matsuyama et al., 1985, Allen et al., 1985, Mattiasson et al., 1985, Uddman et al., 1984, Gu et al., 1984, Lundberg et al., 1983, Furness et al., 1983). Presence of NPY may well be a reliable marker of those sympathetic nerves which serve the cardiovascular system (Mc Lachlan et al., 1986). There is a general tendency for NPY to decrease NE concentration at lower doses of NPY. However with higher doses the release of NE remains unaffected or increased. This biphasic effect of NPY on NE system is also reported by Harfstrand et al., 1987. In another work done by Alan et al., 1978 it was reported that NE levels in excess of 1800 pg/ml were required to produce hemodynamic effects. Under usual condition the biologic action of NE can be attributed only to its

sympathetic neurotransmitter function.

The interaction of NPY with the central sympathetic system and the action of NPY as a neurotransmitter and neuromodulator has raised an interesting topic in the field of medical research. However no study has been conducted so far to look at the brain NPY and its interaction with sympathetic system in CHF.

### 3. HYPOTHESIS AND OBJECTIVES

The work in this thesis is based on the hypothesis that neuropeptide Y is involved in ischemic heart disease by modulating sympathetic activity in catecholamine-rich cardiovascular centres within the brain. The objectives are as follows:

Objective #1: To examine the effect of NPY on catecholamine levels in the PVN. These experiments were conducted to test the hypothesis that the effect of NPY in the PVN area of coronary ligated rats is different from that of sham operated controls. Microdialysis studies were carried out at 2, 4, and 8 weeks following coronary occlusion in rats. Catecholamine sampling from the PVN area of the brain with and without NPY injection was performed by stereotaxically implanting a microdialysis probe in the PVN. Analyses of the released catecholamine levels in the brain samples were performed by injecting purified microdialysate sample in the HPLC with an electrochemical detector.

Objective #2: To examine the relationship between NPY effects in the brain and changes in the sympathetic activity as measured by plasma levels of NE following coronary occlusion in rats. Plasma levels of NE were analyzed by injecting purified plasma samples in the HPLC with an electrochemical detector.



Objective #3: To examine changes in NPY receptor distribution by autoradiographic techniques in coronary ligated animals. Tissue sections from experimental and sham-operated animals were incubated with radiolabelled I<sup>125</sup>-NPY and exposed to radioactivity sensitive film to determine the extent of binding of NPY receptors.

## **4. MATERIALS AND METHODS**

### **4.1 Materials**

Male SD rats were used for all the experiments and were obtained from the University of Manitoba Central Animal Care Services. The microdialysis probes were purchased from Bioanalytical Systems (West-Lafayette, USA). The HPLC eluent reagent was purchased from the Waters division of Millipore, Milford, Massachusetts, USA. The porcine-NPY was acquired from Sigma (St. Louis, USA). The [<sup>3</sup>H] Hyperfilm was obtained from Amersham (Oakville, Canada). The <sup>125</sup>I-Bolton-Hunter labelled porcine NPY was purchased from NEN Dupont (Boston, USA). All other reagents used in the following experiments were analytical grade.

### **4.2 Preparation of Experimental Animals**

Male SD rats were housed for one week after their arrival, before any experiments were conducted. The rats were housed on a 12/12 hours dark-light cycle in a temperature controlled environment ( $22 \pm 1^{\circ}\text{C}$ ) with free access to food and water.

#### **4.2.1 Coronary Ligation**

MI was produced in male SD rats (200-250 g) by occlusion of the left coronary artery as described by Johns and Olson., 1954 and modified by Selye et al., 1960. The animals were anaesthetized with ether, the skin incised along the left sternal

border, the fourth rib was cut proximal to the sternum and retractors were inserted. The pericardial sac was perforated and the heart was exteriorized through the intercostal space. The left coronary artery was ligated about 2 mm from its origin with a suture of 6-0 silk, and the heart was repositioned in the chest. Left coronary artery occlusion was ascertained by the paling to the suture. Throughout the course of the operation, rats were maintained on a positive pressure ventilation delivering a mixture of 95% Oxygen and 5% Carbondioxide mixed with ether. Closure of the wound was accomplished by a purse-string suture. The mortality of the surviving animals operated upon in this fashion was about 35% within 48 hours. Sham-operated animals were treated similarly except the suture around the coronary artery was not tied. Animals were allowed to recover and received food and water ad libitum and maintained for a period of 2, 4, and 8 weeks prior to the microdialysis technique.

#### 4.3 Microdialysis *In Vitro*

In vitro probe recovery was done prior to each experiments to determine the relative recovery of the probe and is defined as concentration of catecholamines in perfusate as a percentage of the concentration of the outer medium. The microdialysis probe (CMA/12, Bioanalytical Analytical Systems, BAS, West Lafayette, USA) was placed in an eppendorf vial containing 100 ng/ml of NE dissolved in the perfusate (containing in mM: 147 NaCl, 4 KCl, 1.2 CaCl<sub>2</sub>, 1.1 MgCl<sub>2</sub>, at pH 6). After a 30 minute stabilizing period, 3 samples were collected at a flow rate of 2.2 µl/min. The mean of the three samples were used to correct the basal

microdialysis experiments.

#### 4.4 Microdialysis *In Vivo*

Male SD rats were anesthetized by an intraperitoneal injection of Ketamine (4.0 mg/100g) and Xylazine (0.4 mg/100g). The anesthetic was given every hour at the same dose for the remainder of the experiment. Body temperature was maintained at 37°C using a homeothermic blanket control unit (Harvard Apparatus, USA). Rats were placed in a Kopf stereotaxic frame with the incisor bar set at 5 mm above the interaural line. A microdialysis probe (CMA/12, BAS) with an exposed dialysis membrane of 1 x 0.5 mm and 20,000 Dalton molecular weight cut-off was lowered in the PVN of the brain using the following co-ordinates : AP +1.0/ ML +0.5/ DV -8.0 mm for the 2- and 4-week group of rats, with bregma and the dura as the reference zero point. 8 week old group had co-ordinates of AP +1.1/ ML +0.5/ DV -8.3 from the zero bar in mm respectively. The inlet port on the microdialysis probe was connected to a CMA/110 liquid switch (BAS) which in turn was connected to microsyringes placed in a CMA/100 microinjection pump (BAS). The outlet port of the probe was connected to a piece of tubing placed in a CMA/140 microfraction collector (BAS). Figure I A represents a schematic diagram of microdialysis instrument connected to the HPLC. Figure I B shows a photograph of a microdialysis experiment *in vivo*.

A stabilization period of 2 hour was given before the collection of samples because studies have shown that released concentration of substances of interest takes approximately 1.5-2 hours to return to the basal levels after damage caused by

Figure I A. Schematic diagram showing the instrumentation for the microdialysis experiment. An anesthetized rat is fixed in the David Kopf stereotaxic frame and the probe inserted into the region of interest in the brain. The inlet opening of the probe is connected to a syringe selector which in turn is connected to the syringes in the microdialysis pump. The outlet opening of the probe is connected to the tubing which ends into the collecting microvials inside the fraction collector. The body temperature of the anaesthetized rat is kept at 37°C using a homiothermic pad.

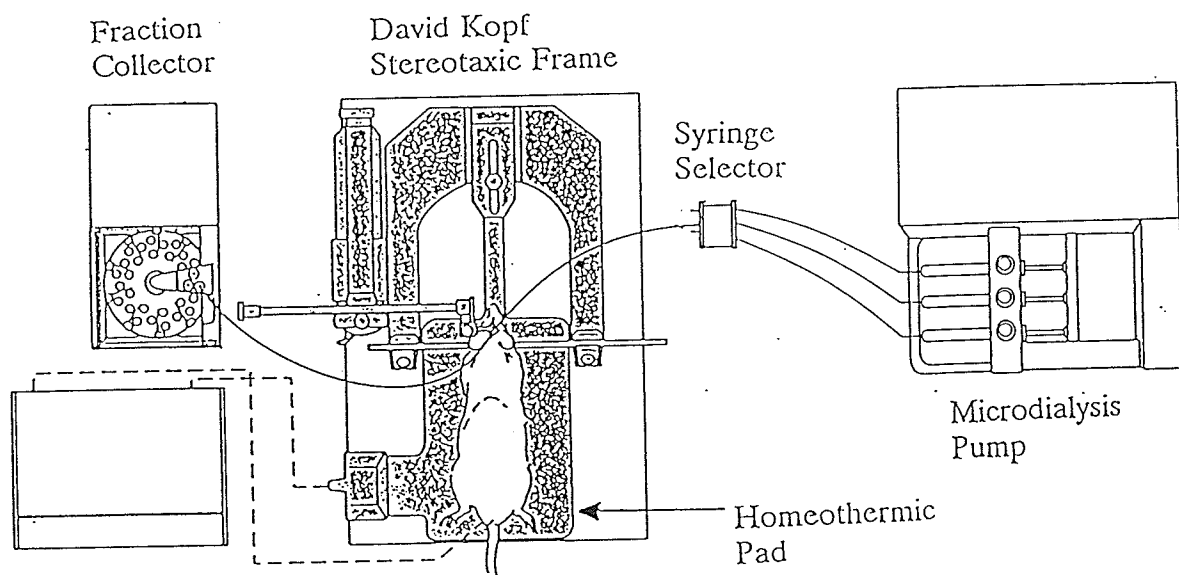
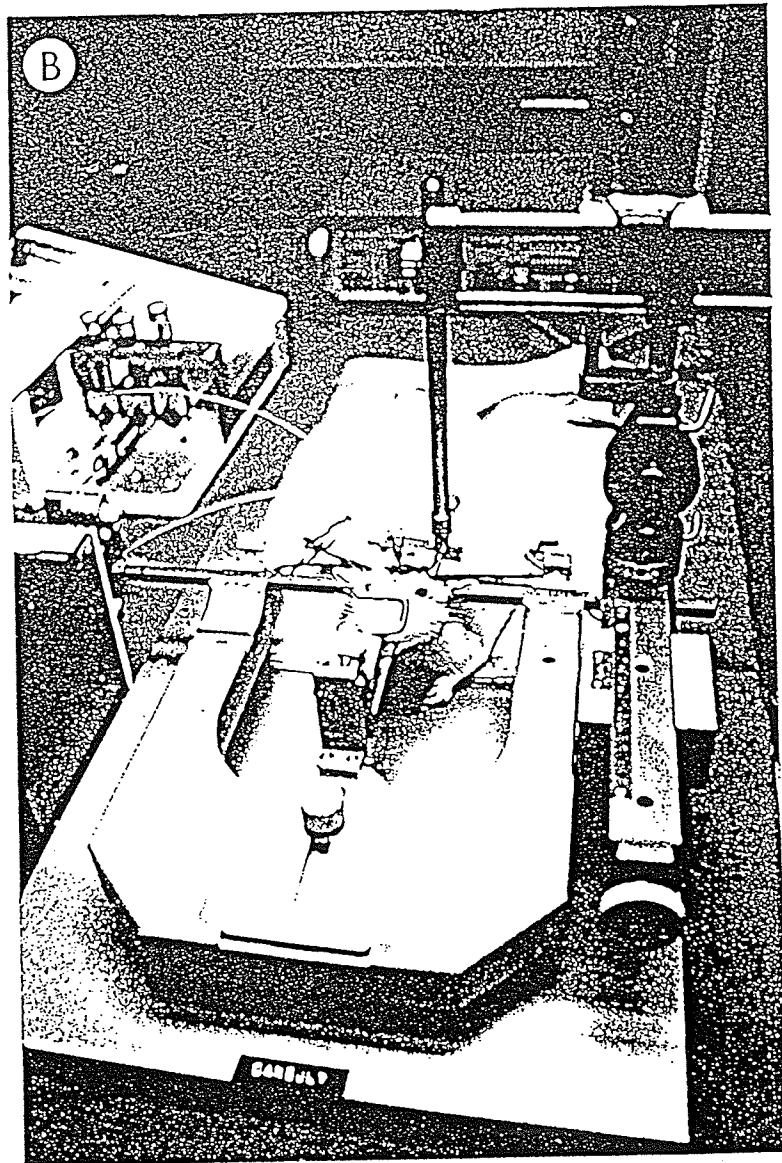


Figure I B. Photograph of an anesthetized rat undergoing microdialysis experiment. The microdialysis probe is inserted into the PVN region of the brain. PVN; paraventricular nucleus.





implantation of a microdialysis probe (Kendrick., 1989). The perfusate was microdialysed in the brain at a flow rate of 2.2  $\mu$ l/minute. The microdialysate samples were collected after a period of 2 hours of stabilization and was immediately taken for purification procedure before the catecholamine analysis in the HPLC. The animals were then intracerebrally exposed to a freshly prepared solution of neuropeptide Y at a concentration of  $10^{-8}$  mol/L dissolved in the perfusate. After another stabilisation period of 1 hour, samples were collected for the analysis of catecholamine to examine the effect of neuropeptide Y. 100  $\mu$ l of samples were collected before and after exposure of neuropeptide Y.

#### **4.4.1 Purification of Microdialysate Samples**

Microdialysate samples underwent the purification procedure through an alumina extraction protocol (Waters Millipore, Mississauga, Ontario, Canada). The purification protocol involved adsorption, washing and elution steps which is shown in detail in flow chart form in figure II. The purified samples were then injected into the HPLC for detection and analysis of the concentration of released catecholamines.

#### **4.4.2 High Pressure Liquid Chromatography**

20  $\mu$ l of the purified sample were directly injected in a reverse-phase HPLC system coupled to an electrochemical detector (Waters, Mississauga, Canada). The HPLC system consisted of Waters Resolve C18 5 $\mu$ m dimethyloctadecylsilyl bonded silica particle size in a 3.9 mm x 150 mm column, 510 pump, U6K liquid injector, and

Figure II. Flow chart representing steps for purification of NE from the brain microdialysate sample. 100  $\mu$ l of microdialysate was collected from the PVN region of the brain which underwent purification procedure before being injected into the HPLC. NE; norepinephrine, PVN; paraventricular nucleus, HPLC; high performance liquid chromatography, EDTA; ethylene dinitrilo tetraacetic acid.

100  $\mu$ l of brain microdialysate sample



Add 200  $\mu$ l of 2 M Tris; pH 8.7



Add 1 ruby spatula full alumina oxide



Vortex for 10 minutes



Centrifuge for 1 minute at 2000 r.p.m.



Remove the supernatant and add 200 $\mu$ l of 0.2% Tris; pH 8.1



Vortex for 1 minute



Centrifuge for 1 minute at 2000 r.p.m.



Remove the supernatant and add 40  $\mu$ l of acetic acid (1 : 100) + 10% of 10  $\mu$ l of sodium bisulphite + 8% of 10  $\mu$ l of EDTA to the residue



Vortex gently



Take 20  $\mu$ l of supernatant and inject into the HPLC

460 electrochemical detector. The glassy carbon working electrode was set at +0.60 Volts vs a Ag/AgCl reference electrode. The mobile phase was filtered and degassed regularly a day prior to the experiments. NE was eluted within 5 minutes of the injection when the flow rate on the HPLC pump was set at 0.9 ml/min. The chromatographic data was collected on a microcomputer using Baseline 810 chromatographic workstation software. Standard samples containing 100 ng/ml of NE were used to quantify the samples.

#### **4.5 Plasma Catecholamine Analysis**

For determination of the level of sympathetic activity the blood plasma of the sham and experimental rats underwent purification procedure and were subsequently, injected into the HPLC to detect and analyze the level of NE. Concentration of NE was obtained after chromatographic comparison to a standard of known concentration of catecholamines. 4 ml of blood was collected intracardially after the microdialysis and were centrifuged to extract a ml of plasma. The purification was according to an alumina extraction protocol (Waters-Millipore, Mississauga, Ontario, Canada), following different steps of adsorption, washing and elution shown in details in flow chart figure III.

#### **4.6 Perfusion and Histological Procedures**

After microdialysis sampling the rats were perfused transcardially with 0.9% saline followed by 4.0% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4).

Figure III. Flow chart representing steps for purification of NE from the blood plasma sample. 1 ml of blood plasma was collected and purified before being injected into the HPLC. NE; norepinephrine, HPLC; high performance liquid chromatography, EDTA; ethylene dinitrilo tetraacetic acid.

1 ml of blood plasma sample

↓

Add 400  $\mu$ l of 2M Tris EDTA; pH 8.0

↓

Add 1 ruby spatula full alumina oxide

↓

Vortex for 10 minutes

↓

Centrifuge for 10 minutes at 11000 r.p.m.

↓

Remove the supernatant and add 1 ml of 0.2% Tris EDTA; pH 8.1

↓

Vortex for 2 minutes

↓

Centrifuge for 10 minutes at 11000 r.p.m.

↓

Remove the supernatant and add 1 ml of 0.2% Tris EDTA; pH 8.1

↓

Centrifuge for 10 minutes at 15000 r.p.m.

↓

Remove the supernatant and add 100  $\mu$ l of 1 : 100 acetic acid + 10  $\mu$ l of sodium bisulphite + 10  $\mu$ l of EDTA

↓

Vortex gently

↓

Take 20  $\mu$ l of supernatant and inject into the HPLC

At the end of each experiment the heads were separated below the second cervical vertebra, and after craniotomy the brains were blocked on the stereotaxic apparatus with the incisor bar 5 mm above the interaural line. The brain segments were cryoprotected in 25% sucrose : 10% glycerine in 0.1 mol/L phosphate buffer for a period of 3 days. The sections were taken using a cryostat at a constant temperature of -17°C, at a thickness of 40 µm. Sections were mounted and stained with Thionine Nissl stain and probe insertion site was histologically confirmed (Figure IV A, IV B). Flow chart figure V shows the steps of staining procedure in details.

For the confirmation of the extent of infarction, histological sections of the heart of 2, 4, and 8 weeks old infarcted animals were taken and stained with Martius Scarlet Blue (MSB) trichome stain.

#### **4.7 NPY Receptor Autoradiography**

NPY receptor autoradiography was performed according to a previously standardized protocol (Martel, 1990). The experimental as well as sham animals were quickly decapitated and stereotaxically blocked in David Kopf apparatus with the incisor bar set at 5 mm above the interaural line. The blocked brains were immediately frozen in dry ice and were cut at 20 µm thickness on a cryostat at a constant temperature of -13°C. The sections were thaw mounted on treated gelatin coated slides and stored at -80°C for further use. Before the autoradiography experiments the sections were thawed at room temperature and then pre-incubated in 50 mM Tris-HCL, pH 7.4 at room temperature for 45 minutes. The tissue sections

Figure IV A. Photomicrograph represented in low magnification of the cross section of brain demonstrating the placement of a microdialysis probe at the site of PVN of brain in experimental group of rats. Corresponding sections from sham group also showed similar results. Arrowhead indicates tip of microdialysis probe in the PVN.



A

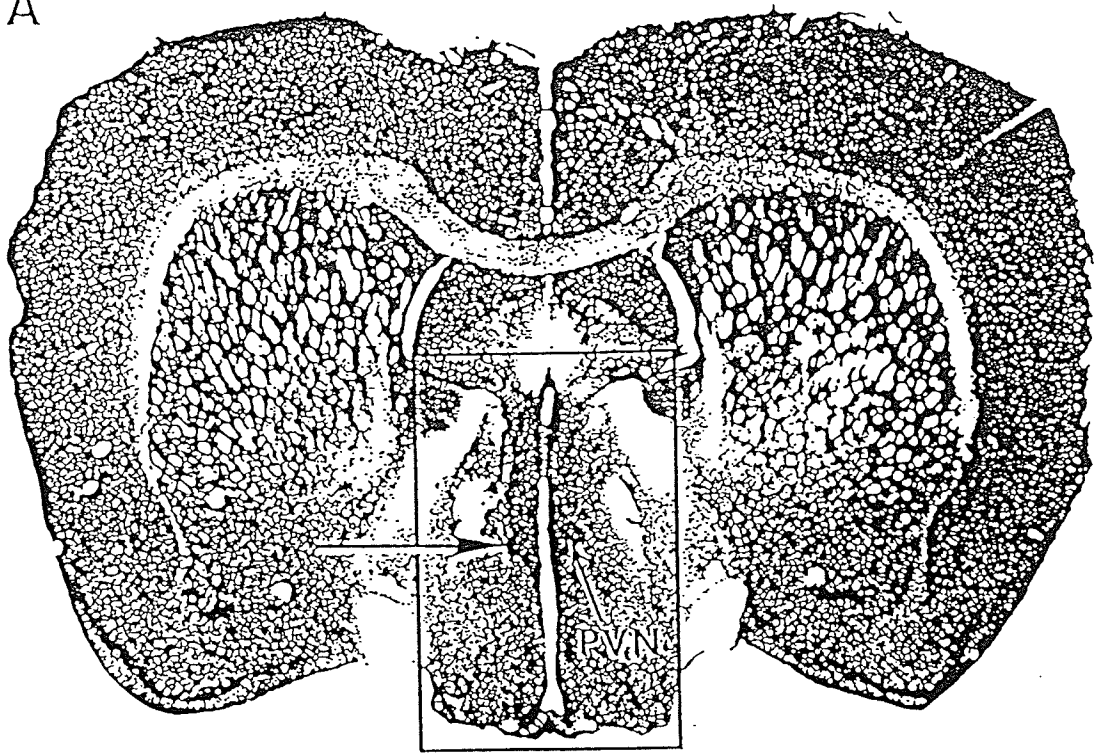


Figure IV B. Photomicrograph representing high magnification of the enclosed box of Fig. IV A. It shows an example of a typical lateral placement of a microdialysis probe in the PVN of the brain. Note the small amount of tissue damage caused by the insertion of probe.

PVN; paraventricular nucleus.

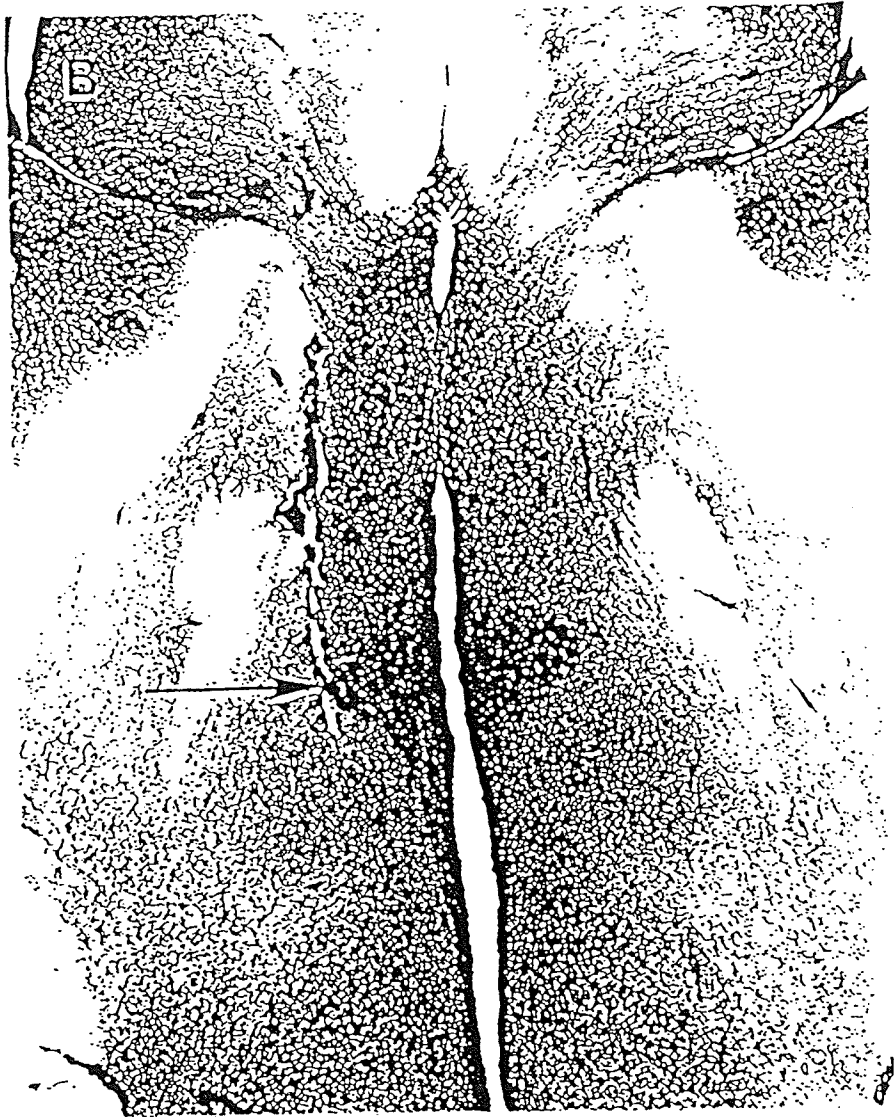


Figure V. Schematic representation of the steps for Nissl staining to stain the brain sections at a thickness of 40  $\mu\text{m}$ . Nissl staining provided a clear view of the probe insertion site in the PVN area of the brain. PVN; paraventricular nucleus.

Tissue section in 100% ethyl alcohol (5 minutes)



100% ethyl alcohol (5 minutes)



95% ethyl alcohol (2 minutes)



70% ethyl alcohol (2 minutes)



distilled water (2 minutes)



thionin stain (3 dips)



distil water (5 dips)



distil water (5 dips)



70% ethyl alcohol (2 minutes)



95% ethyl alcohol (2 minutes)



100% ethyl alcohol (2 minutes)



100% ethyl alcohol (2 minutes)



xylol (till coverslip)

were incubated for 120 minutes at room temperature in the 50 mM Tris buffer (pH 7.5) containing 0.05% bacitracin, 0.1% bovine serum albumin and 25 pM <sup>125</sup>I-Bolton-Hunter-porcine NPY (2000 Ci/mmol, NEN Dupont, Boston, USA). Specificity of the NPY receptors were determined by the difference in binding observed on adjacent sections in the absence and presence of 1  $\mu$ M porcine NPY. The brain sections were then washed in chilled Tris-HCL buffer four times for 4 minutes each followed by a dip in chilled double distil water. The sections were rapidly air dried and then tightly juxtaposed against Hyperfilm-<sup>3</sup>H (Amersham) sealed within Picker International cassettes. The boxes were stored at 4°C for 4 days before developing.

The optical density of the autoradiograms were scanned electronically to measure the proportion of constant light that passes through the tissue image. To accomplish this, the image is transmitted from a scanner to a monitor and digitized by a computer. The computer can then be used to create a gray scale for each pixel. Tissue density readings can be taken from anatomically defined areas.

The instrumentation for computer-assisted densitometry consisted of a light-box with a scanner and a high resolution colour monitor. This software stores a single image and converts this image to a set of digital values. Optical density measurements were taken using the Bioanalytical Molecular Image Analysis software by the Biorad.

## 5. Statistical Analysis

All data are presented as the mean  $\pm$  SEM. Group differences were analyzed using ANOVA and specific means were compared using Tukey's multiple comparison test. Statistical significance for all the tests was set at  $p < 0.05$ .

## 6. RESULTS

### 6.1 General Characteristics of Sham and Ligated Animals 2, 4 and 8 weeks Following MI

Table 1 summarizes the general characteristics of the experimental groups of animals at 2, 4 and 8 weeks post infarction. A study of scar weight, left ventricle, and right ventricle weight revealed significant differences between experimental (4 and 8 weeks following coronary occlusion) and sham operated group of animals ( $p < 0.05$ ). Specifically, evidence of cardiac hypertrophy in experimental group of 8-week old infarcted rats was observed by an increased mass of the remaining viable left ventricle. Signs of clinical CHF were also evident in experimental rats 8 weeks following MI as was evidenced by cardiac hypertrophy and pulmonary edema. When left ventricular weight was normalised with body weight a significant difference was evident in 4- and 8-week old ligated animals ( $p < 0.05$ ). Significant congestion of lungs in the experimental animals was noted by increased wet and wet/dry lung weight ratio in the 8-week old experimental group of rats ( $p < 0.05$ ). A progressive increase in the right ventricle weight was evident in 2-, 4- and 8-week old ligated groups which was significant specially in the 8-week old stage ( $p < 0.05$ ).

The heart of the experimental group of animals was hypertrophied at 8-week post infarction to a maximum extent (VI C) when compared with the heart of the animals of the other experimental groups of 2- and 4-week old ligated animals (Figure VI A, VI B).



Table 1. General Characteristics of Experimental Rats 2, 4, and 8 Weeks After Induction of Myocardial Infarction

Parameters	Week-2		Week-4		Week-8	
	Sham	Ligated	Sham	Ligated	Sham	Ligated
Lt. Ven. Wt.(g)	0.71 ± 0.06	0.85 ± 0.08*	0.84 ± 0.05	0.96 ± 0.04*	0.99 ± 0.09	1.26 ± 0.07*
Rt. Ven. Wt.(g)	0.20 ± 0.02	0.21 ± 0.04	0.23 ± 0.03	0.33 ± 0.04	0.24 ± 0.01	0.38 ± 0.05*
Lt. Ven. Wt. (10 <sup>-3</sup> )	2.17 ± 0.21	2.66 ± 0.25	2.02 ± 0.18	2.46 ± 0.19*	2.67 ± 0.26	2.41 ± 0.29*
Body Wt.						
Scar Wt. (g)	ND	0.14 ± 0.02	ND	0.14 ± 0.01	ND	0.26 ± 0.03
Lung Wet Wt.(g)	1.28 ± 0.18	1.21 ± 0.16	1.48 ± 0.12	1.66 ± 0.21	1.58 ± 0.17	1.88 ± 0.14*
Lung Dry Wt.(g)	0.29 ± 0.03	0.28 ± 0.07	0.37 ± 0.07	0.38 ± 0.08	0.39 ± 0.04	0.42 ± 0.03
Lung Wet Wt. Lung Dry Wt.	4.36 ± 0.33	4.36 ± 0.43	4.10 ± 0.31	4.27 ± 0.29	3.93 ± 0.23	4.38 ± 0.21*

Data are expressed as mean ± SEM of six experiments in each group. Left ventricular weight indicated for experimental animals does not include scar tissue. ND, not detectable.

\* p < 0.05.

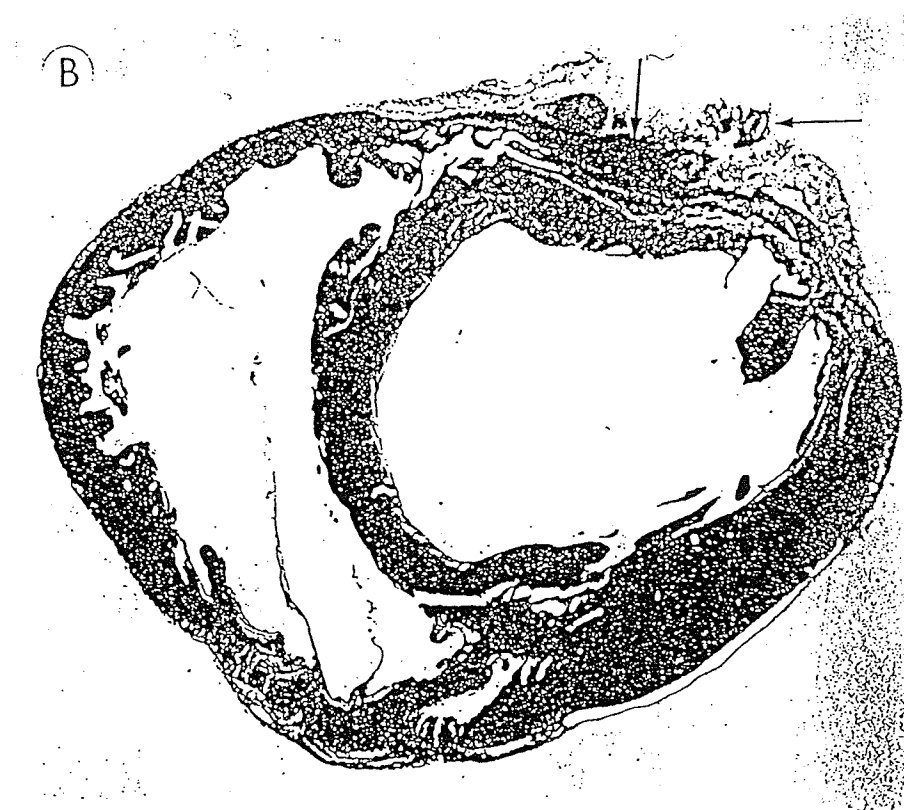
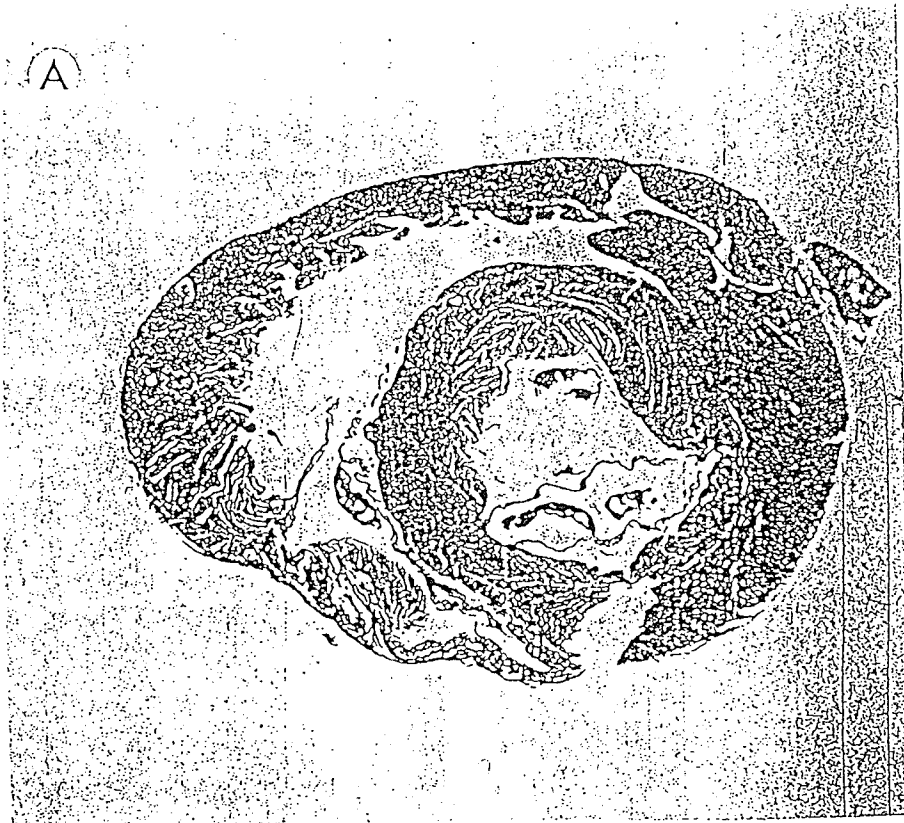
Figure VI A. Photomicrograph showing the cross section of the heart of a 2-week old infarcted rat, stained with MSB stain. MSB stain has the characteristic property to stain the collagen fibre with a blue colour. Note the presence of very less amount of collagen tissue stained in blue colour.

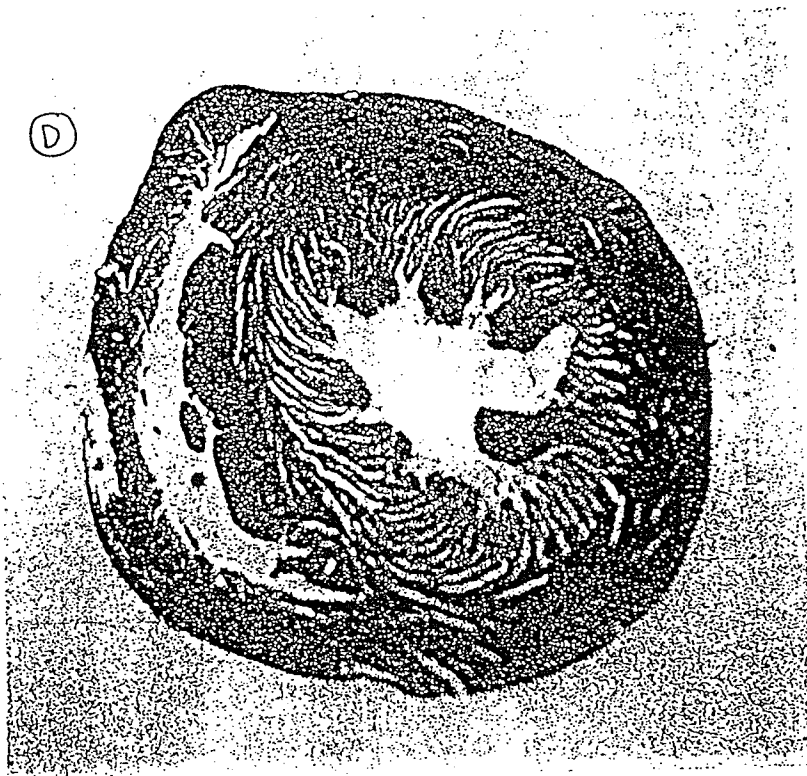
Figure VI B. Photomicrograph showing the cross section of the heart of a 4-week old infarcted rat, stained with MSB stain. Note the presence of moderate amount of collagen tissue in blue colour.

Figure VI C. Photomicrograph showing the cross section of the heart of a 8-week old infarcted rat, stained with MSB stain. Note the presence of extensive amount of collagen tissue stained in blue colour in the left ventricle of the heart. The cross section also gives a clear view of a well hypertrophied heart as compared to other two sections of the 2 and 4 week-old post infarcted animal.

Figure VI D. Photomicrograph of the cross section of the heart of a sham animal stained in MSB stain. Note the complete absence of collagen tissue and a very small outline of the heart with a well defined right and left ventricles.

MSB; martius scarlet blue.





Histological cross sections of the heart of 8-week old experimental group of animals showed extensive ventricular dilation, hypertrophy and collagen scar tissue deposition . Hearts of all the three experimental group of rats were hypertrophied and bigger in size as compared to the sham group as is evidenced in the cross section of heart of a sham animal (Figure VI D). At a period of 2 and 4 weeks after coronary ligation, the experimental animals were not significantly different from the sham operated animals in any other parameters except the left ventricular weight. A general trend of increase in scar weight was also evident with the progression of congestive heart failure in the coronary ligated rats.

## 6.2 Microdialysis *In Vitro*

Relative recovery rates of the microdialysis probes were performed every day prior to the experiments to determine the capacity of the probe to recover catecholamine within the brain. The concentration of baseline NE was adjusted according to the relative recovery rate of the probe. The recovery rate of the probes ranged between 10%-15%. Probes were perfused with NPY to assess the possible effects of NPY on the capability of the probe to recover NE. However, no differences was found in the recovery of NE in the dialysate, before and after the perfusion time of 1 hour.

### **6.3 Microdialysis *In Vivo***

#### **6.3.1 Baseline NE at 2, 4 and 8 weeks after coronary ligation**

Baseline NE levels released from the PVN reflected significant differences between the ligated and the sham animals in all the three groups. 2-week old experimental group of animals showed a significant increase of about 6 fold ( $p < 0.05$ ) in the baseline NE level of the PVN (figure VII), when compared to its sham controls. 4- week old experimental group of animals showed significant increase of 3.5 fold (figure VIII) with its sham group ( $p < 0.05$ ) while an increase of 7 fold was reflected in the baseline NE release in the PVN in the 8 week old ligated rats (figure XI) when compared with its respective shams ( $p < 0.001$ ).

#### **6.3.2 NPY - Induced NE Release**

Inhibition of NE was observed in general in both the groups of experimental and sham animals at different stage of 2, 4 and 8 weeks post infarction following administration of NPY at a concentration of  $10^{-8}$  mol/L in the PVN area of brain when compared with the original concentration of released NE at the basal level. This trend was visible in both the sham as well as in ligated animals of all the groups. NE levels of the PVN in all the sham operated control groups were decreased between 50%-60% when compared with the pre-drug baseline levels obtained before NPY infusion. Percentage level of reduction of NE from the pre-drug level in the 2-week old group of ligated animals was about 70% (figure X). 4-week old ligated animals showed the

Figure VII. Histogram showing basal level of NE in the PVN of sham and experimental groups of animal 2-week post infarction. Released concentration of NE values (pg/ $\mu$ L) sample are presented as mean  $\pm$  SEM of six animals in each group. Microdialysate samples for the baseline were collected 2 hours after the implantation of the microdialysis probe. \*  $p < 0.05$  compared to the sham group of animal. NE; norepinephrine, PVN; paraventricular nucleus.

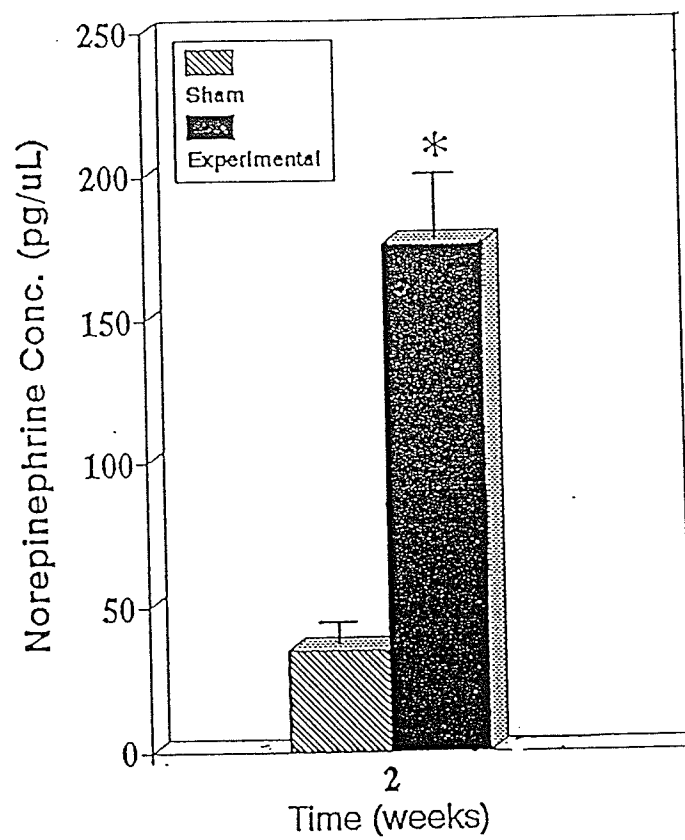




Figure VIII. Histogram showing basal level of NE in the PVN of sham and experimental groups of animal 4-week post infarction. Released concentration of NE values (pg/ $\mu$ L) sample are presented as mean  $\pm$  SEM of six animals in each group. Microdialysate samples for the baseline were collected 2 hours after the implantation of the microdialysis probe. \*  $p < 0.05$  compared to the sham group of animal. NE; norepinephrine, PVN; paraventricular nucleus.

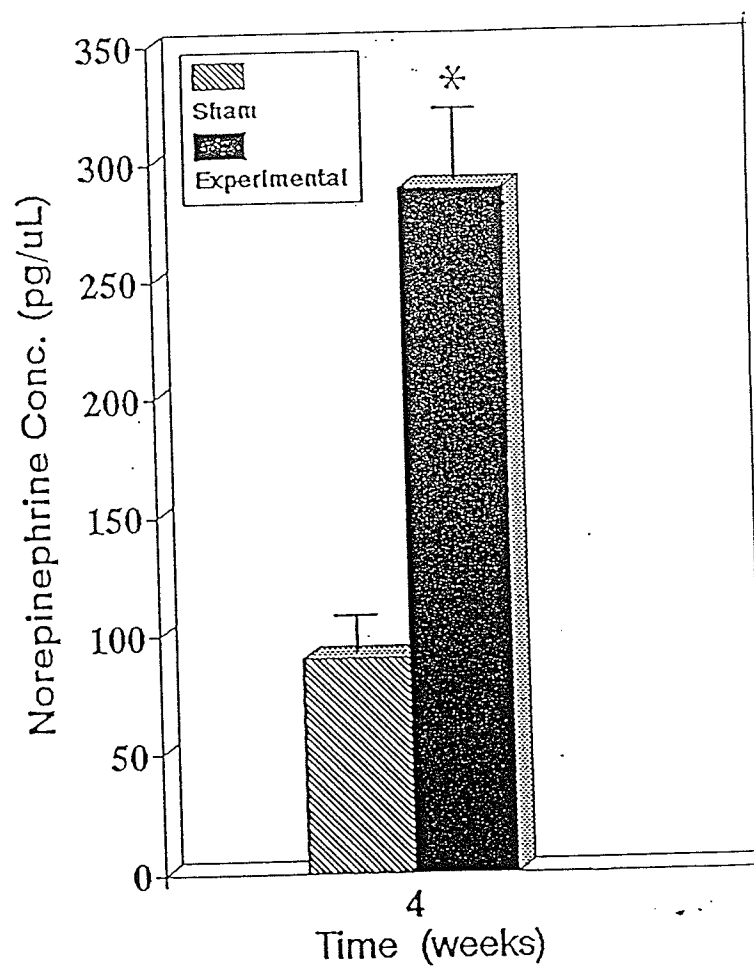


Figure IX. Histogram showing basal level of NE in the PVN of sham and experimental groups of animal 8-week post infarction. Released concentration of NE values (pg/ $\mu$ L) sample are presented as mean  $\pm$  SEM of six animals in each group. Microdialysate samples for the baseline were collected 2 hours after the implantation of the microdialysis probe. \*\*  $p < 0.001$  compared to the sham group of animal. NE; norepinephrine, PVN; paraventricular nucleus.

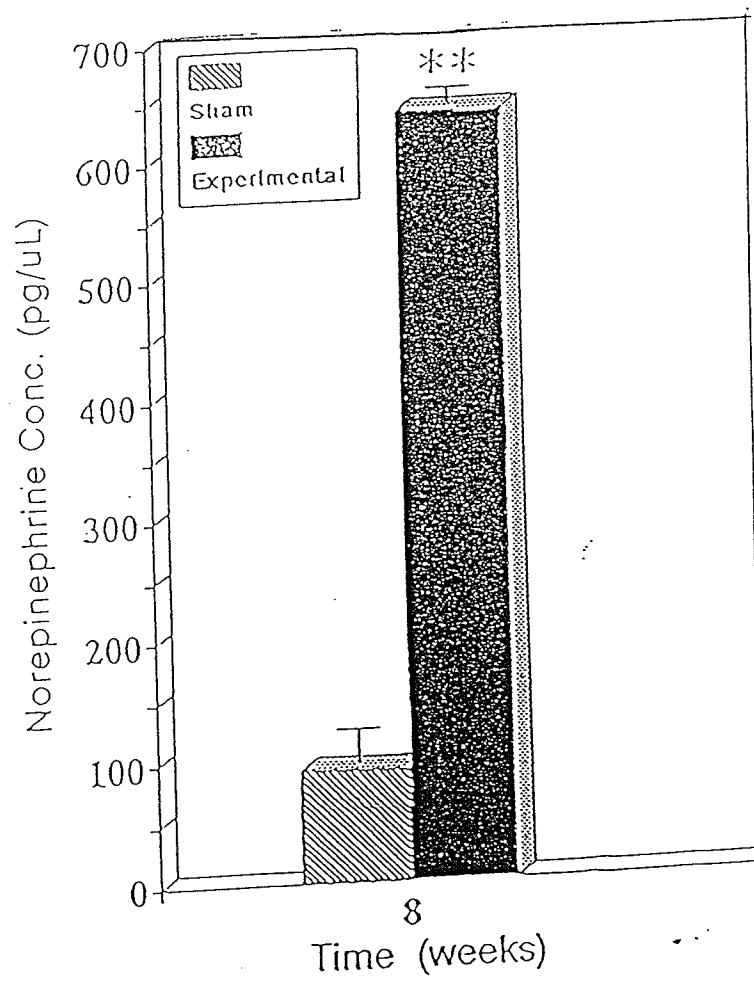
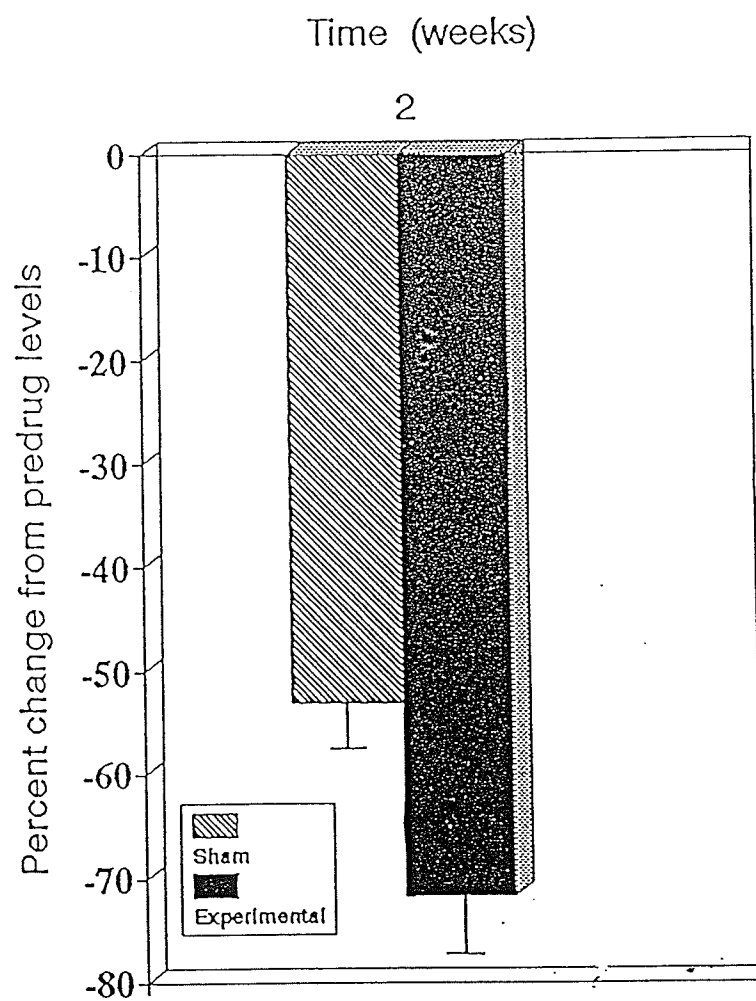


Figure X. Histogram showing effect of NPY at a concentration of  $10^{-8}$  M in the PVN of sham and experimental group of animals 2-week post infarction. NE release is expressed as a percentage change from the pre-drug baseline levels.

NPY; neuropeptide Y, PVN; paraventricular nucleus, NE; norepinephrine.



reduction about 50% before and after infusing NPY (figure XI). However, a significant observation was noticed in the 8 week old ligated animals in which the level of NE in the PVN changed by only 20% from the pre NPY level (figure XII) compared with that of the sham operated controls.

#### **6.4 Plasma NE Analysis**

NE levels of plasma in the sham and ligated animals of all the groups were analyzed to determine the level of sympathetic activity. Plasma NE level showed no differences between the sham and ligated animals in the groups of 2 and 4 weeks after MI. However, 100% increase in the level of NE was apparent and significant between the sham and ligated animals of the 8-week old group;  $p < 0.05$  (Figure XIII).

#### **6.5 Perfusion and Histology**

After the microdialysis sampling, for the confirmation of the probe site sections of brain were stained in the Nissle stain. Histological analysis of brain sites sampled by microdialysis not only confirms the probe site at the correct position but also shows the extent of damage in the tissue immediately surrounding the probe membrane. This is particularly important because extensive damage may lead to an incorrect basal level of NE outflow. In this study data from such animals were not taken into consideration in which the probe site was found to be in some other area of the brain other the PVN.

Figure XI. Histogram showing effect of NPY at a concentration of  $10^{-8}$  M in the PVN of sham and experimental group of animals 4-week post infarction. NE release is expressed as a percentage change from the pre-drug baseline levels.

NPY; neuropeptide Y, PVN; paraventricular nucleus, NE; norepinephrine.



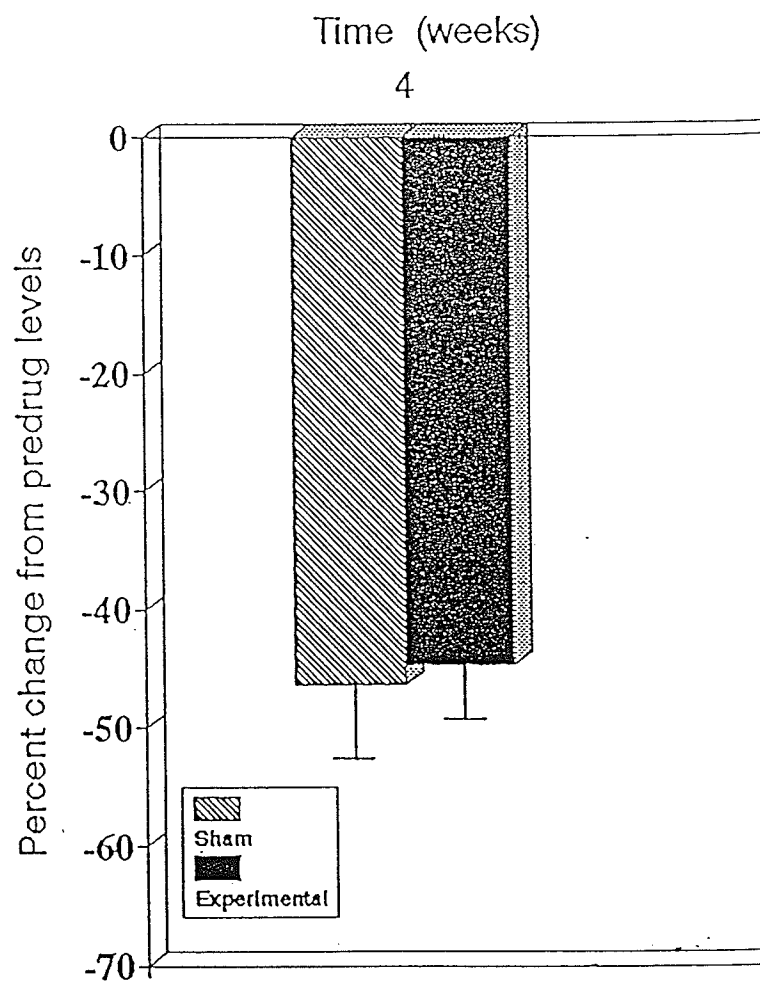


Figure XII. Histogram showing effect of NPY at a concentration of  $10^{-8}$  M in the PVN of sham and experimental group of animals 8-week post infarction. NE release is expressed as a percentage change from the pre-drug baseline levels.

NPY; neuropeptide Y, PVN; paraventricular nucleus, NE; norepinephrine.

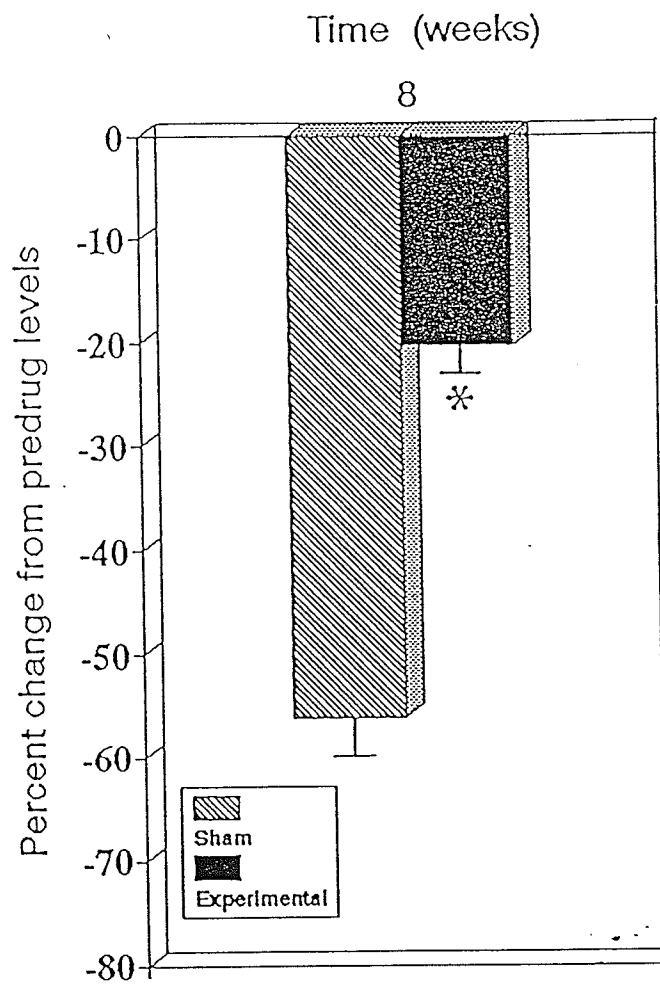
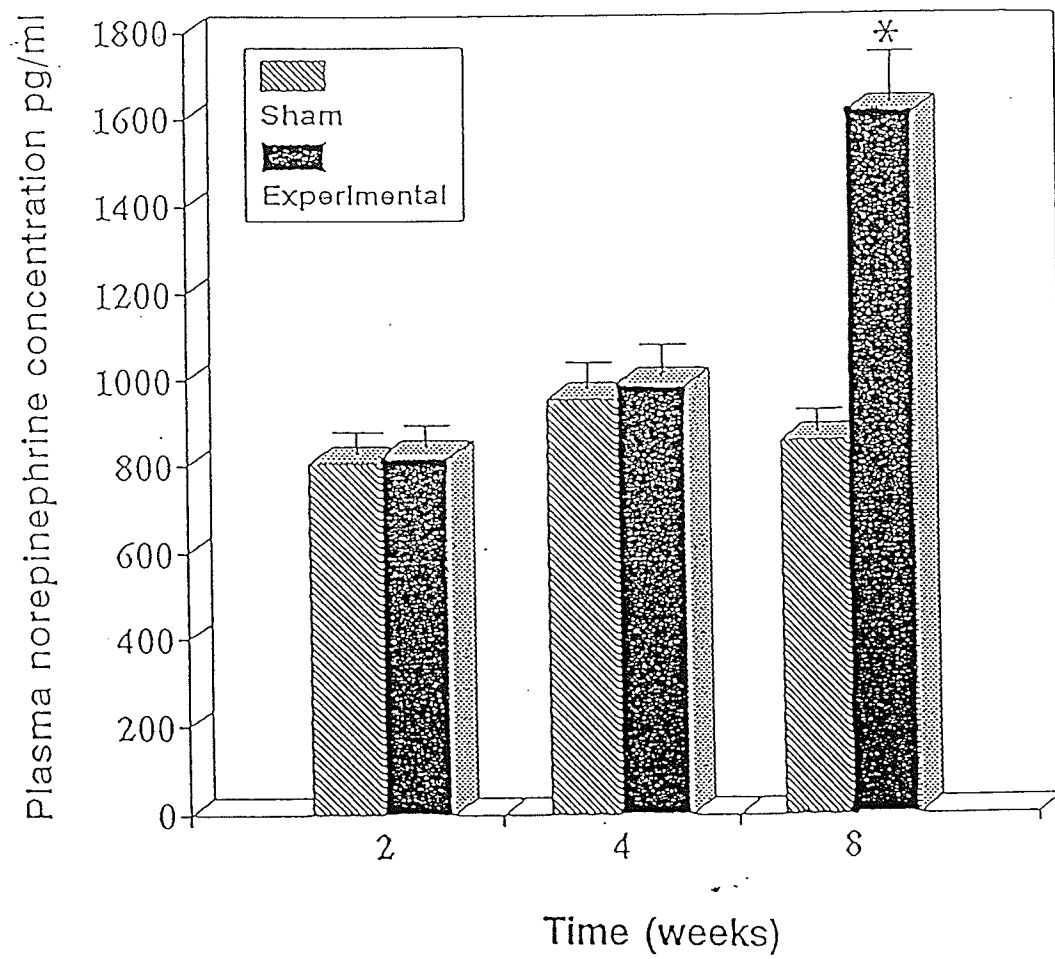


Figure XIII. Histogram showing plasma NE concentration in sham and experimental group of animals 2-, 4- and 8- week post infarction. In this graph the concentration of plasma as pg/ml of sample is represented as a mean  $\pm$  SEM of six animals in each group. \*  $p < 0.05$  compared to the sham group of animals. NE; norepinephrine.



## 6.6 NPY Receptors Study

Autoradiograms of the radio-autography experiments done at 8 weeks after induction of MI showed an extensive NPY receptor binding in the PVN region of the sham operated control animals (Figure XIV A). The ligated animals showed a significant decrease in the number of NPY receptor binding (Figure XIV B). Non-specific binding was determined as the binding observed in the presence of 1.0  $\mu$ M concentration of porcine-NPY (Figure XIV C). The densitometric measurements of these autoradiograms demonstrated a significant low level in the number of NPY receptors in the PVN of ligated rats compared to those of their sham operated controls as measured by their optical density units;  $p < 0.05$  (Figure XV).

Figure XIV A.      Autoradiograph of a brain section showing non-specific binding in the presence of 1.0  $\mu$ M porcine NPY. Arrow head points the PVN region of the brain.

Figure XIV B.      Autoradiograph of brain section showing  $^{125}$ I-NPY binding in the sham group of rats. Note the presence of extensive binding as represented by a dark shade of grey colour in the PVN region. Arrowhead points the PVN region of the brain.

Figure XIV. C.      Autoradiograph of brain section showing  $^{125}$ I-NPY binding in the experimental group of rats 8 weeks post infarction. Note the absence of binding in the PVN compared to that of the sham animals. Arrowhead points the PVN region of the brain.

Abbreviations: NPY; neuropeptide Y, PVN; paraventricular nucleus.

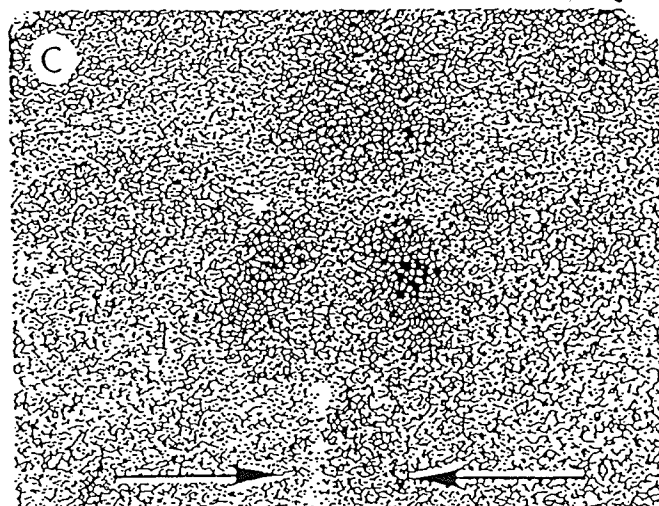
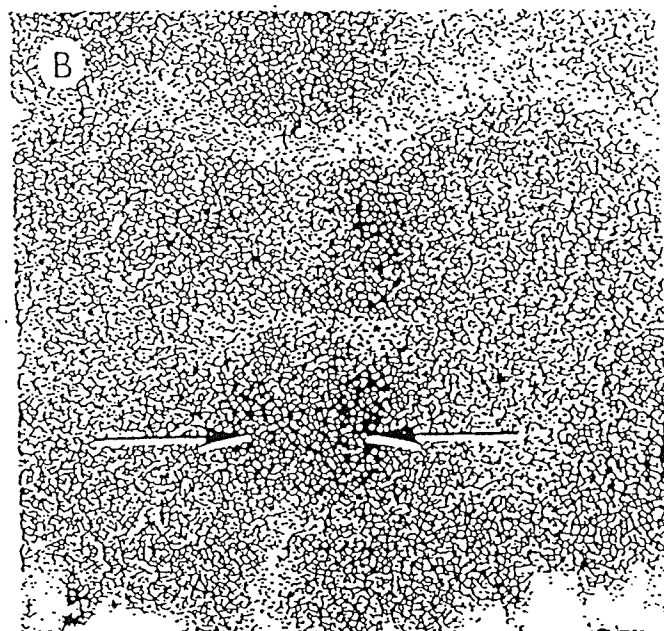
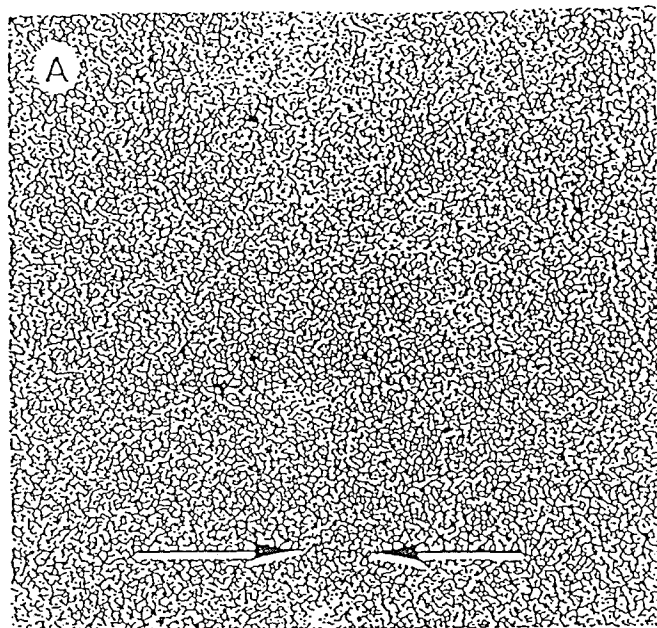
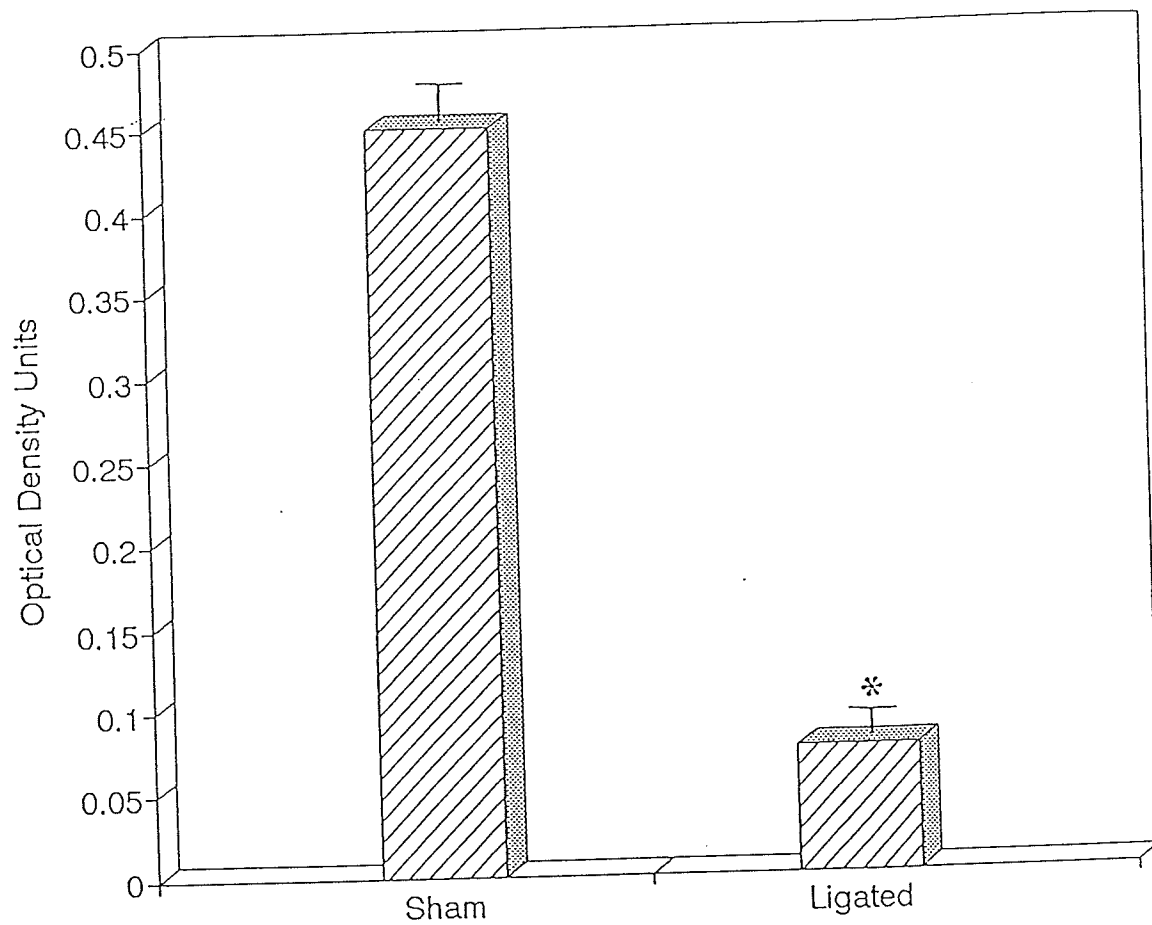




Figure XV. Histogram showing number of receptor binding in optical density units. Specific binding was determined as the difference in binding observed on an adjacent section in the presence and absence of 1.0  $\mu$ M porcine-NPY. Data is represented as mean  $\pm$  SEM of six animals in each group compared to the sham animals \*  $p < 0.05$ . Abbreviation: NPY; neuropeptide Y.



## **7. DISCUSSION**

### **7.1 General Characteristics of Sham and Ligated Animals**

Ligation of left descending coronary artery in rats has been widely used as an experimental approach to the induction of CHF (Dreuler et al., 1986) and extensively reviewed in the study by Curtes et al., 1987. CHF secondary to MI of the left ventricle, has been reported to occur in rats after surgical ligation of the left coronary artery (Dixon et al., 1990). The CHF model has also been widely accepted to study the pathophysiology of this disease and in fact, in our animals with large healed infarcts showed signs of pulmonary edema and cardiac hypertrophy. This was particularly evident at 8 weeks post-infarcted rats. Although additional hemodynamic data are required to establish congestive heart failure in our model, the animals had, indeed, a large myocardial infarction with an early sign of congestive heart failure.

### **7.2 Microdialysis in Vivo**

#### **7.2.1 Baseline Extracellular NE in PVN at 2, 4 and 8 Weeks After Coronary Ligation**

Our results showed that the baseline NE in PVN was elevated by several folds in all the experimental groups at 2, 4 and 8 weeks post infarction, whereas the basal level varies very little in the sham operated groups. These results were similar to our earlier findings where increased release of endogenous NE from the PVN was found

in aortic-banded and SHR (Woo et al., 1991, Woo et al., 1993). Studies reported by Quail et al., 1988 also showed similar observation in spontaneously hypertensive rats where they measured the basal release of NE by using a push-pull cannula technique.

Higher concentration of NE, released from the brain suggests an enhanced central noradrenergic activity, associated with high sympathetic activity that may be responsible for the development of CHF. Because sympathetic activity is increased in congestive heart failure models, higher levels of NE in the paraventricular nucleus might be associated with the increase in sympathetic drive. This augmented sympathetic drive was demonstrated by our plasma analysis of catecholamine level as will be discussed later in this section.

#### **7.2.2 NPY- Induced NE Release**

Exposing the PVN of all the three age groups in sham animals to NPY at a concentration of  $10^{-8}$  M resulted in a decreased amount of NE released in the microdialysate by 50% - 60%. The reduction was 70% in 2-week old and around 50% in 4-week old post infarcted rats. Because NPY did not affect NE recovery rates of the probes, these changes were most likely the result of NPY acting at the synaptic interface. This is further substantiated by the fact that NPY is known as a presynaptic inhibitor of NE release (Waeber., 1990). He also reported that a functional interaction between NPY and NE appears to exist centrally with NPY potentiating the effect of the catecholamine.

However, when NPY at a concentration of  $10^{-8}$  M was infused into the PVN

of the 8-week old group of experimental animals, a reduction of only 20% in NE value was observed. The mechanisms responsible for this lack of effect of NPY on NE release might induce increased sympathetic nerve activity in the hypothalamus. Similar observation has also been reported by Tsuda et al., 1990.

### **7.3 Analysis of Plasma NE**

In this study level of NE in the blood plasma of 8-week old post infarcted animals showed a 2 fold increase compared to the shams. A stage of 8 weeks post infarction represents an early sign of CHF as is evidenced by the cardiac hypertrophy and pulmonary edema. There are several reports of increased sympathetic activity associated the development of CHF.

Since PVN is an important integrative area for both autonomic and cardiovascular control (Swanson et al., 1983), an elevation of extracellular NE levels in PVN may then be correlated with an elevated central sympathetic activity (Woo et al., 1993). Enhanced central noradrenergic nerve activity has been suggested to be involved in the development of CHF. The sympathetic activity exerts an important direct effect on cardiac function which is mediated by the release of the neurotransmitter NE at the terminal sympathetic nerve endings. Since the SNS is the chief mediator of the left ventricular response to stress, the sympathetic activation results in an increase of arterial pressure by increasing the heart rate, myocardial contractility. As a result NE is released from the sympathetic nerves which in turn increases the distensibility and force of contraction of the ventricle which gradually

leads to the heart failure. The evidence of alteration of many of the function like a low inhibition of the NE level in PVN associated with CHF may be correlated with a change in sympathetic drive which is clearly depicted in this study.

#### **7.4 NPY Receptor Study**

It may be pointed out that decreased ability of NPY to act on the presynaptic site in PVN at an early stage of CHF portrays a series of causal factor for this disease. Keeping in view the role of receptors, our autoradiography experiment clearly shows a decrease in NPY receptor density, that may at least partially be responsible for the ineffectiveness of NPY to inhibit NE presynaptically. Compared with shams the number of receptors for NPY found in the PVN was significantly less in the experimental group of rats when analyzed statistically. This study was further supported by our previous experiments which show a similar trend of decrease in NPY receptors in the PVN area of the aortic banded rats (Woo et al., 1994).

Since NPY is known to co-exist with NE both centrally and peripherally and there is an increase in sympathetic activity during CHF, a downregulation of NPY receptors kinetics could be expected in response to an increased catecholamine level and release. This downregulation of NPY receptor following MI may aggravate the release of NE and myocardial dysfunction. Studies have also shown lower level of neuropeptide Y-immunoreactivity in the hypertensive brain with respect to normotensive rats where sympathetic activity is greater. Taken together, decreasing the sympathoinhibitory influence of neuropeptide Y in paraventricular nucleus,

through which a decrease in neuropeptide Y receptors is a possible mechanism, may reciprocally increase sympathetic activity in congestive heart failure.

## 7.5 Methodological Considerations

Before using microdialysis probes to measure release of substances in brain, it is essential first to assess their performance in recovering substances of interest *in vitro*. While such *in vitro* recoveries may not precisely reflect *in vivo* conditions, they do provide a basis for estimating extracellular concentration of substances of interest in the brain in conjunction with tissue homogenate data. It also allows some estimate of the relationship between extracellular and intracellular concentration (Kendrick, 1989).

The results of *in vitro* experiments performed in this study demonstrate the importance of probe site, flow rate of the perfusate and NE concentration in the medium outside the probe for relative recovery. The results from sampling at a flow rate of 2.2  $\mu\text{L}/\text{min}$  imply that the NE recovery is proportional to the length (i.e., area, since the probe diameter is constant) of the semipermeable part of the probe.

Relative recovery is inversely proportional to flow rate (Lindfors et al., 1987). Therefore at a flow rate of 2.2  $\mu\text{L}/\text{min}$  which is used in this study indicates that the total number of molecules recovered to the perfusate/time unit is largely constant around the above mentioned flow rate and that increasing the flow implies decreasing the concentration of recovered substance. The relative recovery of NE was found to be independent of the concentration, similar to that found *in vivo* experiments.

Different sets of co-ordinates were selected for the 2-, 4- and 8-week old rats due to the difference in age groups and body weight. This was necessary to ensure that the PVN was being sampled as the sizes of the animals were different. A stabilization period of 2 hour proved adequate before collecting the samples because studies have revealed that released concentration of substance of interest takes approximately 1.5 - 2 hours to return to the basal levels after damage caused to the brain tissue by the implantation of a microdialysis probe (Kendrick., 1989).

The size of the outer diameter of the probe was 500  $\mu\text{m}$  and keeping in view the proper placement of the probe in relation to the PVN, the probe was placed lateral to the PVN for a maximum uptake of substance of interest because of the greatest amount of membrane surface area of the probe was in contact with the PVN in this position. Placing the probe in the middle of the PVN would lead to a significant destruction of nuclei in the PVN whereas medial placement of the probe to the PVN would have made a risk of contamination by the third ventricle.

## **7.6 Conclusions**

The findings in this study suggest that there is a decrease in capability of NPY to affect the presynaptic inhibition of NE release in the PVN. This is evidenced by the level of NE at 8-weeks post infarction where the inhibition was just 20% as compared with the predrug NE level in the paraventricular hypothalamus. This phenomenon is also associated with higher sympathetic activity since the NE level in the plasma of experimental group was 2 fold higher than the control group. **It thus**



It thus strengthens the hypothesis that the chain of events that initiates congestive heart failure may involve NPY at the level of PVN. Increased sympathetic activity may be a resultant factor due to the changes in action of NPY at the level of PVN. Further studies to define the association between NPY and CHF are thus clearly needed.

## 8. SUMMARY

NE and NPY have been found to be co-localized in the brain. Based on this fact we examined the possible effect of NPY in modulating the central sympathetic activity in failing hearts after MI in rats. The left coronary artery was ligated and the rats underwent microdialysis in the PVN 2, 4 and 8 weeks later. Microdialysate samples were collected with and without injecting NPY. The concentration in purified microdialysate samples was assayed using HPLC. Concentration of NE released was decreased by 50% with NPY in 2- and 4- week but only 20% in 8- week old infarcted rats. The diminished inhibitory effect of NPY on NE release was associated with increased sympathetic activity as reflected by plasma NE. The 8-week old coronary ligated animals had almost two fold increase of plasma NE level as compared to the sham animals. Autoradiographical localization of NPY receptors in the PVN was also carried out in the sham and the coronary ligated animals which revealed a significant decrease in the density of NPY receptors at 8 weeks in the PVN of ligated rats as compared to the sham operated controls. Our results suggest that an abnormality in the control of sympathetic system by NPY may result in an elevated level of NE in CHF.

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