

**ROLE OF THE CENTRAL AMYGDALA AND THE  
MEDULLARY RAPHE IN TACHYCARDIC RESPONSES TO  
PSYCHOLOGICAL STRESSES**

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Thesis  
Entitled

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RAPHE IN TACHYCARDIC RESPONSES TO PSYCHOLOGICAL  
STRESSES**

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**ROLE OF CENTRAL AMYGDALA AND THE MEDULLARY RAPHE IN TACHYCARDIC RESPONSES TO PSYCHOLOGICAL STRESSES.**

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**ABSTRACT**

At present, little is known about the central mechanism of the stress-induced cardiac sympathetic drive responsible for stress-induced tachycardia. The aims of this study were to determine the effects of two brain regions involved in the increase of heart rate during psychological stress. The first part involved the examination of the effects of bilateral microinjections of GABA<sub>A</sub> receptor agonist, muscimol, into the amygdaloid complex on both heart and cardiac autonomic activity during restraint stress. The heart rate increased sharply after the onset of the restraint and reached a peak 1-2 min later. Subsequently, the heart rate began to fall, and during the next 10-15 min it approached the steady-state level. After injection of the vehicle, the mean heart rate during each of the three 10-min restraint periods was significantly higher compared to the pre-restraint level. After muscimol, the mean heart rate was significantly elevated only during the first 10 min of restraint. There was no difference in the early peak tachycardia between both conditions. Muscimol substantially accelerated the fall of the HR from the peak to the steady-state level, and thus the area under the curve value for muscimol was significantly smaller than that for the vehicle. The second part examined the effects of systemic and intra-medullary (raphe region) administration of the 5-HT<sub>1A</sub>-receptor agonist 8-OH-DPAT on cardiac changes elicited by restraint in rats. 8-OH-DPAT reduced the basal heart rate predominantly via a non-adrenergic, non-cholinergic mechanism. Restraint stress caused tachycardia (an initial transient increase with a sustained component).  $\beta$ -adrenoreceptor blockade with atenolol suppressed the sustained component, whereas muscarinic blockade with methyl-scopolamine abolished the initial transient increase, indicating that sympathetic activation and vagal withdrawal were responsible for the tachycardia. Systemic administration of 8-OH-DPAT at 3 doses (10, 30 and 100  $\mu$ g/kg) attenuated stress induced tachycardia in a dose-dependent manner and this effect was suppressed by the 5-HT<sub>1A</sub> antagonist WAY-100,635. Given alone, the antagonist had no effect. Systemically injected 8-OH-DPAT (100 $\mu$ g/kg) attenuated both the sympathetically-mediated sustained component and the vagally-mediated transient. Activation of 5-HT<sub>1A</sub> receptors in the medullary raphe by microinjection of 8-OH-DPAT mimicked the anti-tachycardic effect of the systemically administered drug but did not affect the basal heart rate. This suggests that tachycardia induced by restraint stress occurs due to a sustained increase in cardiac sympathetic activity associated with a transient vagal withdrawal.

**KEY WORDS: PSYCHOLOGICAL STRESS/ HEART RATE/ SYMPATHETIC TELEMETRY/ MEDULLARY RAPHY/ AMYGDALA**

119 pp.

บทบาทของเซินทรัลอะมิกดาลาและเมดัลลารีราเฟในการตอบสนองต่อการเต้นเร็วขึ้นของหัวใจ  
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### บทคัดย่อ

ในปัจจุบันมีความรู้ค่อนข้างมากเกี่ยวกับกลไกในระบบประสาทกลางที่ควบคุมการทำงานของหัวใจผ่านระบบซิมพาเทติกซึ่งสามารถถูกเหนี่ยวนำให้หัวใจเต้นเร็วขึ้นในระหว่างที่มีความเครียด การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาบริเวณในสมองสองส่วนที่ควบคุมการเต้นของหัวใจระหว่างที่มีความเครียดทางจิตใจ โดยการศึกษาส่วนแรกเกี่ยวข้องกับสารเพื่อกระตุ้นตัวรับกาบ้าชนิดเอที่ชื่อ muscimol เข้าไปโดยที่เซินทรัลอะมิกดาลาทั้งสองข้างระหว่างที่ถูกกระตุ้นให้เกิดความเครียด พบว่าอัตราการเต้นของหัวใจของหนูจะเพิ่มขึ้นอย่างรวดเร็วจนถึงจุดสูงสุดประมาณ ๑-๒ นาทีขณะที่หนูอยู่ในกล่องเครื่องมือที่ทำให้เกิดความเครียดเนื่องจากถูกจำกัดการเคลื่อนไหว หลังจากนั้นจะค่อยๆลดลงจนสู่ภาวะปกติภายใน ๑๐-๑๕ นาที หลังจากฉีดน้ำเกลือธรรมดาพบว่าเมื่อกระตุ้นให้เกิดความเครียดหัวใจจะเต้นเร็วขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับก่อนถูกกระตุ้น แต่หลังจากฉีด muscimol พบว่าหัวใจเต้นเร็วขึ้นอย่างมีนัยสำคัญเพียงสิบนาทีแรกและหลังจากนั้นลดลงจนกระทั่งเท่ากับก่อนการถูกกระตุ้น การศึกษาส่วนที่สอง ฉีดสารตอบรับตัวกระตุ้นของซีโรโทนิน 8- OH- DPAT เข้าทางใต้ผิวหนังและฉีดที่สมองส่วนราเฟ พบว่าสารนี้ลดอัตราการเต้นของหัวใจลงโดยผ่านระบบนอร์เอปิเนอรัลและระบบที่ไม่ใช่โคลิเนอรัล เมื่อฉีดสารยับยั้งตัวตอบรับ ของเบต้าอะดีนาลิก คือ Atenolol พบว่าสารดังกล่าวสามารถยับยั้งการเพิ่มขึ้นของอัตราการเต้นของหัวใจได้แต่ถ้าฉีดสารยับยั้งตัวตอบกลับ มัสคารินิก Methyl-scopolamine มีผลในทางตรงข้าม จากผลในการศึกษาครั้งนี้ชี้ให้เห็นว่า การกระตุ้นของเส้นประสาทซิมพาเทติก ร่วมกับการลดการทำงานของเส้นประสาทวากัส มีผลต่ออัตราการเต้นของหัวใจ นอกจากนี้ผลของ 8- OH- DPAT ขึ้นอยู่กับปริมาณการฉีดในแต่ละครั้งด้วย การฉีดสารต้านตัวตอบรับซีโรโทนินชนิดเอ WAY- 100, 635 เพียงอย่างเดียวไม่มีผลในการยับยั้งการทำงานของตัวตอบรับซีโรโทนินชนิดเออย่างมีนัยสำคัญ ข้อมูลการทดลองแสดงว่าการเต้นของหัวใจที่เร็วขึ้นเนื่องจากถูกจำกัดการเคลื่อนไหวเกิดขึ้นเพราะการเพิ่มการทำงานของระบบซิมพาเทติกที่ไปเลี้ยงหัวใจพร้อมกับการลดลงชั่วคราวของระบบวากัส

## CONTENTS

	page
ABSTRACT (ENGLISH)	IV
ABSTRACT (THAI)	V
LIST OF TABLES	VIII
LIST OF FIGURES	IX
LIST OF ABBREVIATIONS	XII
PUBLICATIONS AND PRESENTATIONS	XIV
CHAPTERS	
I    INTRODUCTION	1
II   OBJECTIVES	3
III  LISTERATURE REVIEW	4
Psychological stress and cardiac control	5
Central Brain control the heart during stress.	13
Role of Amygdala and cardiac response	16
Brainstem controls canters integrate stress-induced cardiac sympathetic neural outflow	20
GABA in cardiac control	23
Serotonin in cardiac control	26
Restraint stress	32
IV  MATERIALS AND METHODS	34
PART I : Effects of inhibition of the amygdala on stress-induced tachycardia in rats.	39
PART II: Effects of activation of 5-HT <sub>1A</sub> receptor on stress-induced tachycardia in rats.	45

**CONTENTS (cont.)**

V	RESULTS	54
	PART I : Effects of inhibition of the amygdala on stress-induced tachycardia in rats.	54
	PART II: Effects of activation of 5-HT <sub>1A</sub> receptor on stress-induced tachycardia in rats.	65
VI	DISCUSSION	94
VII	CONCLUSIONS	106
	REFERENCES	108
	BIOGRAPHY	119

## LIST OF TABLES

<b>TABLES</b>		<b>PAGES</b>
1	Changes in HRV indices elicited by restraint stress and effects of intracerebral microinjections of muscimol.	62
2	Restraint-induced tachycardia and effects of 5-HT <sub>1A</sub> receptor ligands.	74
3	Effects of autonomic blockade on restraint-induced tachycardia.	78

## LIST OF FIGURES

FIGURES		PAGES
1	The neural control of the heart.	7
2	The cardiac function is controlled by autonomic nervous system, the sympathetic and parasympathetic nerves, which act via adrenergic and cholinergic receptors.	10
3	The model of key central pathways that lead to the cardiovascular response to an acute stress.	15
4	The direct connections between the central nucleus of amygdala.	17
5	Medullary raphe controls cardiac function	22
6	The projections of the serotonin system arise from the nuclei of the dorsal raphe and the raphe magnus, which are located so as to monitor, and process ascending sensory input, and serve to facilitate information processing.	27
7	The example of restrainer	33
8	The telemetric ECG radio transmitter	36
9	The equipment needed for a surgical microinjection session	37
10	The restrainer consisted of transparent PVC tubing	38
11	The location of telemetry ECG transmitter described by Sgoifo in 1996	40
12	The procedure for the experiment of muscimol injection into bilateral amygdala prior to restraint stress.	42
13	Diagram illustrates of the ECG recording set-up with	44

## LIST OF FIGURES (cont.)

FIGURES		PAGES
	Power-Lab	
14	Effect of bilateral pharmacological inhibit of amygdale tachycardia elicited by the restraint stress.	57
15	The coronal sections of rat brain documenting the injection sites into the amygdala (A) and into striatum (B).	60
16	Control experiment: microinjection of muscimol into the ventral striatum did not affect stress-induced tachycardia.	64
17A	Systemic injection of 8-OH-DPAT, 100µg/kg s/c, reduces basal HR when given alone in 6 rats(n=6).	66
17B	Systemic injection of 8-OH-DPAT (100µg/kg s/c) after β-adrenergic blockade with atenolol.	68
17C	Systemic injection of 8-OH-DPAT (100µg/kg sc) after combined β-adrenergic and muscarinic-cholinergic blockade with atenolol and methyl-scopolamine.	70
18 A	Systemic pre-treatment with 8-OH-DPAT attenuated tachycardia elicited by the restraint stress.	72
18B	Results of regression analysis of data from A	73
19	Selective blockade of 5-HT1A receptors with WAY-100,635 (100µg/kg s/c.) prevents anti-tachycardic effects of 8-OH-DPAT (100µg/kg s/c) during restraint stress; n=6.	77
20	Selective blockade of 5-HT1A receptors with WAY-100,635 (100µg/kg s/c) did not affect tachycardia elicited by the restraint stress.	80
21	Autonomic blockade reveals components of restraint-elicited tachycardic responses.	82

**LIST OF FIGURES (cont.)**

<b>FIGURES</b>		<b>PAGES</b>
22	Systemically administered 8-OH-DPAT attenuates transient stress-induced tachycardia persisting after $\beta$ -adrenergic blockade with atenolol (2mg/kg).	84
23	Systemically administered 8-OH-DPAT attenuates stress-induced tachycardia persisting after muscarinic cholinergic blockade with methyl-scopolamine (50 $\mu$ g/kg).	86
24	Intra-raphé microinjection of 8-OH-DPAT does not affect basal heart rate.	88
25	Intra-raphé microinjection of 8-OH-DPAT attenuated tachycardia elicited by restraint stress.	90
26	Systemically administered WAY-100,635 (100 $\mu$ g/kg) prevents anti-tachycardic effects of intra-medullary microinjected 8-OH-DPAT.	93
27	The summary of this study	107

## LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-HT <sub>1A</sub>	5-hydroxytryptamine type A
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin
ACh	Acetylcholine.
ACTH	adrenocorticotropic hormone
Amb	nucleus ambiguous
ANS	autonomic nervous system
ATP	adenosine 5'-triphosphate
AUC	area under the curve
BPM	beat per minute
Ca	Calcium
CGRP	calcitonin gene-related peptide
CVLM	caudal ventrolateral medulla
DMH	dorsomedial hypothalamic nucleus
ECG	Electrocardiogram
ECu	external cuneate nucleus
et al.	et alii, and others
f	fonix
GABA	$\gamma$ -aminobutyric acid
Gi	gigantocellular reticular nucleus
HF	high frequency
HR	heart rate
HRP	Horseradish peroxidase
HRV	heart rate variability
Hz	Hertz
III	third ventricle

**LIST OF ABBREVIATIONS (cont.)**

LF	low frequency
LV	lateral ventricle
me	medial eminence
mt	mamillo-thalamic tract
NA	Sodium
NANC	Non-adrenergic Non-cholinergic
NE	norepinephrine
nl	nanoliter
nmol	nanomol
NO	nitric oxide
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
OT	optic tract
Py	pyramid
RVLM	rostral ventrolateral medulla
s.c.	subcutaneous
SEM	standard error of mean
SP	substance P
SpV	spinal nucleus of the trigeminal nerve
spV	spinal tract of the trigeminal nerve
T1/2	half life
VIP	vasoactive intestinal polypeptide
µg	microgram

## **PUBLICATIONS AND PRESENTATIONS**

### **PUBLICATIONS**

Ngampramuan, S., Baumert M., Beig, M.I., Kotchabhakdi, N., and Nalivaiko, E. (2008). Activation of 5-HT(1A) receptors attenuates tachycardia induced by restraint stress in rats. *Am J Physiol Regul Integr Comp Physiol* 294, R132-141.

Salome, N., Ngampramuan, S., and Nalivaiko, E. (2007). Intra-amygdala injection of GABAA agonist, muscimol, reduces tachycardia and modifies cardiac sympatho-vagal balance during restraint stress in rats. *Neuroscience* 148, 335-341.

### **PRESENTATIONS**

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# CHAPTER I

## INTRODUCTION

Stress is a major risk factor in the development of cardiovascular disease. Epidemiological and pathophysiological evidence shows that psychological or physical stress may be a specific trigger, precipitating myocardial infarction and sudden death (Leor, Poole et al. 1996). In animals, stress also influences cardiovascular, neuroendocrine and immunological function (Morris, Callahan et al. 1995; Glaser 2005). The relationship between stress and autonomic neural activity has been studied in basic and clinical studies. Tachycardia is a hallmark of emotional arousal induced by psychological stressors, and is predominantly mediated by cardiac sympathetic nerves (Barron and Van Loon 1989) and (Shapiro, Sloan et al. 1993).

At present; understanding of the brain pathways mediating stress-induced cardiac sympathetic is limited. Clearly, cardiac effects induced by psychological stress are initiated in the forebrain. Stimulation of the insular cortex (Oppenheimer, Wilson et al. 1991), the central amygdala (Markgraf and Kapp 1988), or the dorsomedial hypothalamus (Poisson, Christen et al. 2000), elicits arrhythmias in experimental animals, and cases of cardiac systole from temporal lobe (presumably amygdala) epileptic foci have been documented in humans (Liedholm, Duchek et al. 1992). Some previous studies (Galeno and Brody 1983; Gelsema, McKittrick et al. 1987; Soltis and DiMicco 1991) identified a number of brain sites whose stimulation results in tachycardia.

This study has identified two important brain centers, amygdala and medullary raphe. Earlier studies have established that the amygdala integrates both behavioral and vascular responses to acute psychological stress paradigm (Kubo, Okatani et al. 2004), but inhibitory effect of the amygdala on stress-induced cardiac

responses have not been studied so far. Interestingly, neurons in the medial amygdala are massively activated by the restraint, and their inhibition attenuates restraint-induced pressor responses. Whether the medial amygdala also contributes to the stress-induced tachycardia remains an open question, and requires a separate study.

Thus one of the aims of my thesis was to study the effect of pharmacological inhibition of the amygdaloid complex on both heart rate and cardiac autonomic activity that evaluated by the heart rate variability, HRV during psychological stress.

As far as intramedullary raphe region is concerned, recent evidence indicates that the medullary relay for the descending pathways which activate the heart during stress is located in this area and that relevant cardiomotor neurons are sensitive to, and could be inhibited by serotonin 1A (5-HT<sub>1A</sub>) receptor agonist (Zaretsky, Zaretskaia et al. 2003; Nalivaiko, Ootsuka et al. 2005). The ability of 5-HT<sub>1A</sub> receptors in cardiovascular control via central nervous system is well established, and their activation results in central sympathetic activity (McCall and Clement 1994; Ramage 2001). Most of the previous studies have been performed in anesthetized animals, where consistently found to have substantial and dose-dependent bradycardic and depressor effects (Nalivaiko 2006).

Recent studies conducted on conscious animals demonstrated the activation of 5-HT<sub>1A</sub> receptors which reduced cardiovascular changes elicited by psychological stresses (Nalivaiko, Ootsuka et al. 2005; van den Buuse and Wegener 2005). They suggested that antitachycardic effects of 5-HT<sub>1A</sub> receptors mediated by the suppression of stress-elicited activation of cardiac sympathetic nerves. The second aim of this study was to determine the activation 5-HT<sub>1A</sub> receptors in medullary raphe contributed to tachycardia induced by restraint stress.

This study used restraint which is a common and well described model to provoked psychological stress in rats. It consistently elicited tachycardia and raised arterial pressure (Barron and Van Loon 1989; Chen and Herbert 1995; McDougall, Widdop et al. 2005).

## CHAPTER II

### OBJECTIVES

In this chapter, I outline the main hypotheses, which are investigated in this thesis. The main data obtained from this study showed that pharmacological effect on two brain regions resulted in increased heart rate during psychological stress. The first part involved the examination of the effects of bilateral microinjections of GABA<sub>A</sub> receptor agonist, muscimol, into the amygdaloid complex on both heart and cardiac autonomic activity during restraint stress. The second part examined the effects of systemic and intra-medullary (raphe region) administration of the 5-HT<sub>1A</sub>-receptor agonist 8-OH-DPAT on cardiac changes elicited by restraint in rats.

The specific objectives of the work reported here in this thesis are outlined below:

**Hypothesis 1:** Does inhibition of the amygdaloid complex affect the tachycardic responses to restraint stress?

**Hypothesis 2:** How cardiac sympathetic and vagal activity contributes to tachycardia induced by restraint stress?

**Hypothesis 3:** Does activation of 5-HT<sub>1A</sub> receptors prevent this tachycardia, and what are the underlying mechanisms?

**Hypothesis 4:** What is the potential location of relevant 5-HT<sub>1A</sub> receptors?

**Hypothesis 5:** Are these receptors activated during restraint by intrinsically released 5-HT?

## **CHAPTER III**

### **LITERATURE REVIEW**

#### **1. Psychological stress and cardiac control**

Psychological stress is established risk factors for cardiac morbidity and mortality, but there is no satisfactory of the mechanistic link between mental and cardiac disorders (Nalivaiko 2006). The effects of psychological stress on such electrical activity of heart are largely mediated by autonomic nervous system (Sgoifo, de Boer et al. 1997; Inagaki, Kuwahara et al. 2004). It is generally recognized that the two divisions of the autonomic nervous system interaction to the heart are sympathetic and parasympathetic pathway (Levy 1971). The cardiac acceleration produced by strong sympathetic stimulation was usually overpowered, even by relatively weak parasympathetic activity.

#### **Psychological stress may induce life-threatening cardiac arrhythmias and sudden death in humans**

Psychological stress may induce life-threatening cardiac arrhythmias. This claim, often anecdotally supported, is well-documented by careful clinical studies. The arrhythmias were frequently precipitated by acute emotional disturbance, both in patients with coronary artery disease or other structural heart disease and in patients with no demonstrable lesions. In a recent well-documented study in patients predisposed to arrhythmias, psychological stress clearly precipitated ventricular tachycardia. Other studies reinforce the importance of the link between psychological stress and cardiac arrhythmias.

As examples, the Los Angeles earthquake in 1994 resulted in a six-fold increase in the sudden cardiac death rate on the day of the disaster (Leor *et al.*, 1996), and the incidence of arrhythmias in patients with implanted cardioverting defibrillators tripled following the World Trade Center terrorist attack (Kowalski & Steinberg, 2002). In patients with long QT syndrome, sudden alerting stimuli may trigger polymorphic ventricular tachycardia, with a latency of just a few seconds, indicating the neurogenic nature of these arrhythmias. Neurogenic mechanisms are likely to be involved in the genesis of lethal ventricular arrhythmia precipitated by abnormal brain activation during stroke or epileptic seizures.

### **Psychological stress induces life-threatening cardiac arrhythmias in animals?**

There have been few studies of this most important question, possibly reflecting the requirement for studies in conscious animals. Available studies document that cardiac arrhythmias occur in dogs and pigs with post-infarct myocardial damage when they are exposed to an unfamiliar environment, and in conditioned-fear paradigms (Corbalan *et al.*, 1974; Skinner *et al.*, 1975).

In study in conscious rabbits demonstrated that normally benign alerting stimuli can precipitate ventricular arrhythmias in animals with the myocardium rendered electrically unstable by pharmacological pre-treatment (i.v. dofetilide). In these animals, ectopic ventricular activity consistently occurred shortly after stressful stimuli, similar to the short-latency arrhythmias seen in humans with long QT syndrome.

### **Sympathetic-Parasympathetic interactions in the heart**

#### **A. Sympathetic Nervous System Innervate to Adrenergic neurons of heart:**

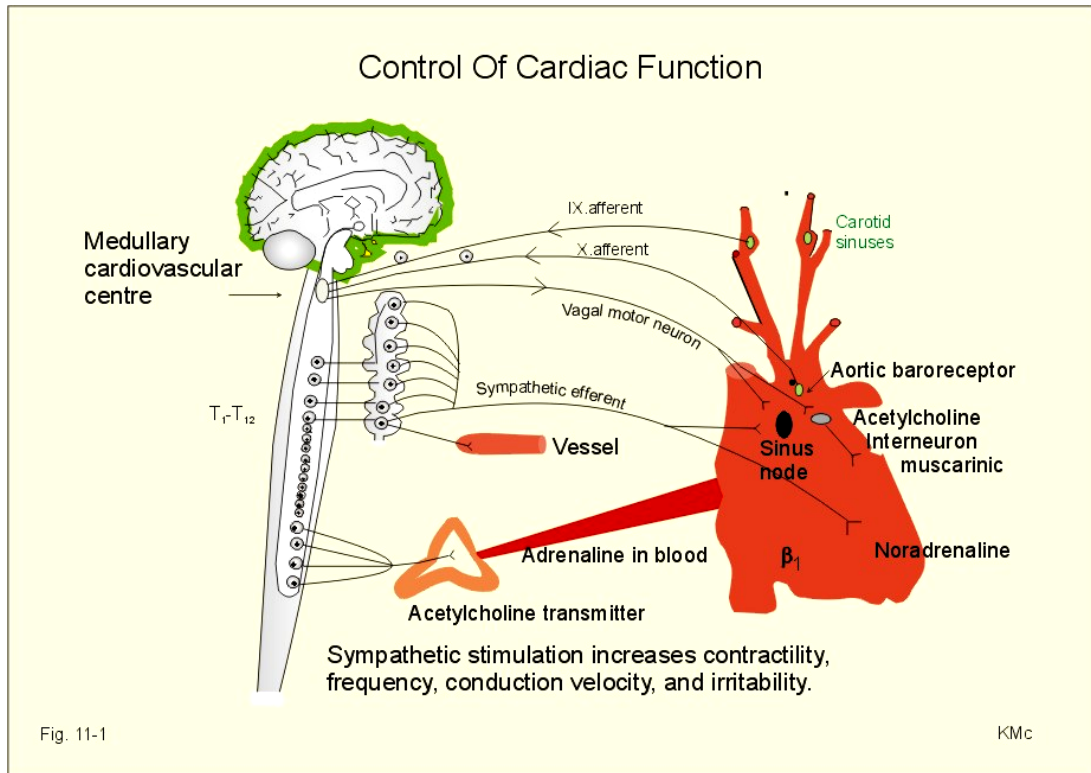
The sympathetic nervous system innervates all segments of the heart, pacemaker, conduction, and contractile cells. The effect of sympathetic stimulation on the heart is to increase the frequency of contraction, increase the velocity of spread of depolarization through the heart, and increase the contractility of the heart. On a

cellular level, sympathetic stimulation increases the rate of rise of phase zero, shortens phase two and increases the rate of repolarization of phase three of the pacemaker.

- 1. Anatomy of sympathetic innervation of the heart

From cell bodies in the intermediolateral columns of the spinal cord, axons of preganglionic sympathetic neurons exit the spinal cord at the lower two cervical and the upper six thoracic segments of the spinal cord and synapse in the bilateral stellate ganglia (combination of superior and middle cervical ganglia) or inferior cervical ganglia.

The postganglionic sympathetic fibers exit the ganglia and follow along the outer surface of the great blood vessels. Upon reaching the base of the heart, these fibers are distributed over the heart through an extensive epicardial plexus. Often these fibers penetrate the heart with the coronary vessels (Fig.1).



**Fig.1.** The neural control of the heart.

Sympathetic stimulation speeds up the sinus node (*sinus tachycardia*) and vagal activity slows the node (*sinus bradycardia*).

- 2. Mechanism of sympathetic action

Action potentials in the sympathetic fibers cause release of norepinephrine (NE) that activates  $\beta$ -adrenergic receptors located at the pacemaker tissue or in the myocardium. Cardiac  $\beta$  receptors (Beta-one receptors) are stimulated by the pharmacological agonist isoproterenol and are blocked by the antagonist propranolol.

Activation of  $\beta_1$  adrenergic receptors of pacemaker tissue increases the inward transmembrane  $\text{Na}^+$  current (if) and the  $\text{Ca}^{2+}$  current of pacemaker cells, increasing the rate of phase 4 depolarization of the pacemaker potential and enhancing automaticity.

Activation of  $\beta_1$  receptors on myocardial fibers increases  $\text{Ca}^{2+}$  uptake, sarcoplasmic release of  $\text{Ca}^{2+}$ , and the turnover rate of receptor stimulation serve to actin-myosin cross bridges. These actions of  $\beta_1$  increase cardiac contractility. Some  $\beta_1$  adrenergic receptors are coupled to  $\text{Ca}^{2+}$  channels through G proteins, and others are coupled through an adenylyl cyclase second messenger.

## B. Parasympathetic Nervous System Innervate to Cholinergic neurons of Heart

Parasympathetic fibers effectively innervate only the SA node, the AV node and the aerial conduction pathways. There is no effective parasympathetic innervation of the contractile fibers of the ventricles. Strong parasympathetic stimulation of the heart will produce slowing of the heart rate to the point that the heart may stop. It also produces slowing of conduction through the heart, especially in the AV node.

### 1. Anatomy of parasympathetic innervations of the heart

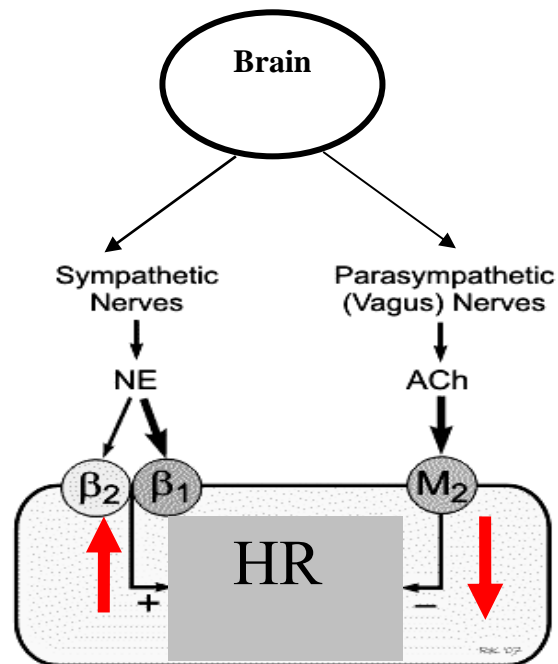
The cell bodies of the preganglionic neurons are located in vagal nuclei (dorsal motor n. of the vagus and nucleus ambiguus) of the medulla. Axons from these neurons form part of the bilateral Vagus nerves which pass caudally alongside the common carotid arteries and synapse on postganglionic neurons located on the surface of the heart, very close to the SA and AV nodes (Fig 1.).

Although considerable overlap occurs, the right vagus has greater innervation and impact on the SA node while the left vagus has greater effect on the AV node. Thus electrical stimulation of the right vagus decreases the automaticity of the SA node (and thus may slow or even stop the heart) while stimulation of the left vagus is more likely to produce various degrees of AV conduction block by slowing conduction through that tissue.

## 2. Mechanism of parasympathetic action

Action potentials in the postganglionic parasympathetic neurons cause release of the neurotransmitter, Acetylcholine. Acetylcholine (ACh) binds to muscarinic cholinergic receptors and directly (and rapidly) opens ligand-gated ion channels, causing K<sup>+</sup> conductance to increase in the pacemaker cells. The resulting outward current hyperpolarizes the membrane potential and decreases the rate of phase 4 depolarization and pacemaker automaticity. Slowing of heart rate results. Parasympathetic stimulation of the heart does not have a significant effect on ventricular contractility. Muscarinic receptors are stimulated by the parasympathetic agonist, muscarine and are blocked by the muscarinic antagonist, atropine.

Parasympathetic action on the cardiac muscle is rapidly terminated by the enzymatic degradation of acetylcholine by acetylcholinesterase. The high concentrations of cholinesterase present in the SA and AV nodes cause vagal inhibition of heart rate to be very brief (50 - 100 msec.). Because parasympathetic actions occur rapidly and dissipate just as rapidly, parasympathetic action is able to influence heart rate on a beat by beat basis. By contrast, the effects of  $\beta_1$  adrenergic receptor stimulation dissipate much more slowly (Fig.2).



**Fig.2** The cardiac function is controlled by autonomic nervous system, the sympathetic and parasympathetic nerves, which act via adrenergic and cholinergic receptors.  $\uparrow$  HR=Tachycardia,  $\downarrow$  HR=Bradycardia

**Non-adrenergic Non-cholinergic (NANC) mechanisms**

NANC transmitters in the heart can act directly in a number of ways, as well as exhibiting both cotransmitter and neuromodulatory functions. In addition to sympathetic, parasympathetic and sensory-motor nerves of extrinsic origin in the heart, combinations of various peptides, purines and nitric oxide have been demonstrated in the intracardiac neurons which project to specialized regions of the heart and to coronary vessels. These local neurons introduce another level of complexity in the system and provide the substrate for further interactions between the different types of nerve in the heart with consequent effects on the myocardium.

*Cotransmission and Neuromodulation*

In the recently, many studies of the neural control of peripheral functions and myocardial performance that have been restricted to the effects of noradrenaline (NA) and acetylcholine (ACh) released from the sympathetic and parasympathetic branches of the autonomic nervous system (ANS). The autonomic nerve fibers not only the supposed classical transmitters, NA and ACh, but also the other substances that mediated effects via non-adrenergic and non-cholinergic (NANC) mechanisms (Burnstock 1990).

NANC transmitters which are contained and transmitted by autonomic neurons can both function independently and work in cooperation among themselves and other classical transmitters such as NA or ACh. There are a lot of putative NANC neurotransmitters that act in the ANS together with purines such as adenosine 5'-triphosphate (ATP), monoamines such as 5-hydroxytryptamine (5-HT), neuropeptides such as neuropeptide Y (NPY), substance P (SP), vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide (CGRP) and most recently nitric oxide (NO) ((Lundberg, Hua et al. 1984; Furness, Morris et al. 1989; Burnstock 1990).

The major challenge to the one neuron can store and release more than one neurotransmitter (Cuello 1982; Burnstock 1990). Generally, the classical transmitters (NA or ACh), peptides and/or other NANC neurochemicals such as ATP act as a basic

for the processes of co-transmission and/or neuromodulation. In co-transmission, two or more substances must coexist within nerve fibers and each must have a direct action on specific postjunctional receptors. In contrast, a neuromodulator substance regulates the transmission process via pre- and/or postjunctional mechanisms. Thus, a neuromodulator can act on prejunctional receptors on nerve fibers to influence transmitter release, or it can act by altering the magnitude or time course of the action of the transmitter on the postjunctional cell. Co-transmission employing classic and NANC neurotransmitters can also act as neuromodulator in cardiac tissue (Morris, Gibbins et al. 1995).

## **2. Central Brain controls the heart during stress**

There are several reviews and studies providing comprehensive coverage of relevant neurophysiological mechanisms (Dampney 1994). Ter Horst GJ et al., 1996 confirmed the coherent complementary results of neuroanatomical retrograde tracing studies by injecting neurotropic virus into cardiac tissues. This knowledge revealed the brain areas that are involved in the regulation of cardiac function. The two basic principles of the neural control of the heart are its dual nature and the hierarchical organization. The nature is vagal and sympathetic pathway and the hierarchical organization is composed of intrinsic cardiac network, intrathoracic ganglia, spinal cord, lower brain stem, upper brain stem and forebrain.

### **Central regulatory mechanism controls sympathetic outflow to heart**

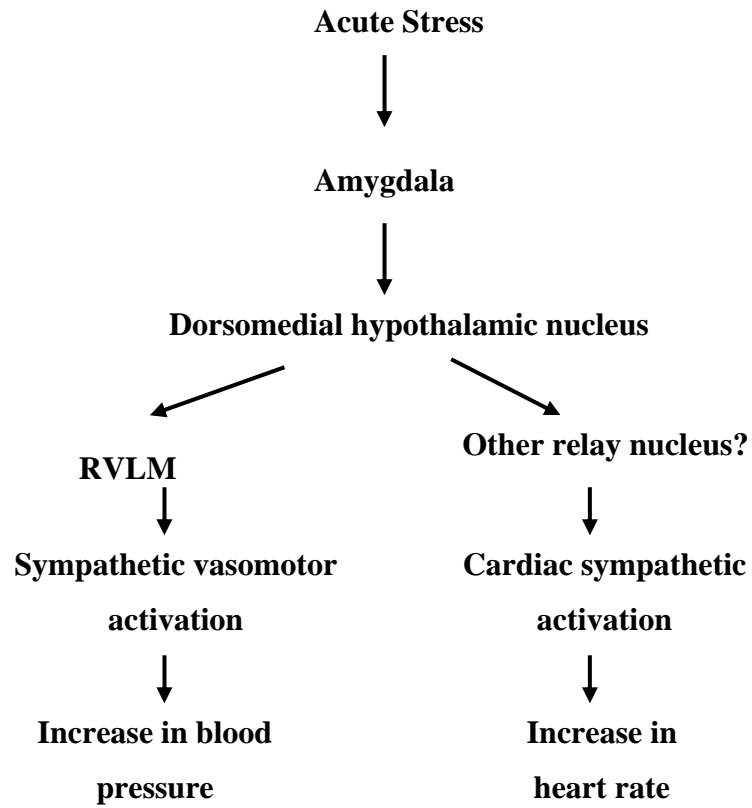
The neural mediated cardiovascular responses are evoked as part of more complex behavioural responses. It is well known that acute emotional or threatening stimuli can also elicit a marked cardiovascular response. Such a response has been observed in conscious animals or humans subjected to an acute alerting stimulus, such as air-jet stress or a loud noise (Davisson, Johnson et al. 1994; Schadt and Hasser 1998; Edwards, Marshall et al. 1999). This patterned response has the effect of increasing cardiac output and redistributing it preferentially towards the skeletal muscle beds and is thus appropriate for an animal that may need to fight or flee from a threatening situation.

It was first shown many years ago that electrical stimulation of a region in the hypothalamus, referred to as the 'defence area', elicits a cardiovascular response (Hilton 1975). It is not clear yet, however, whether this response is due to activation of neuronal cell bodies within this hypothalamic region or to fibres of passage that originate from higher centres, such as the amygdala.

More recently evidences suggest that the dorsomedial hypothalamic nucleus (DMH) plays a key role in integrating the cardiovascular response to acute stress. It is

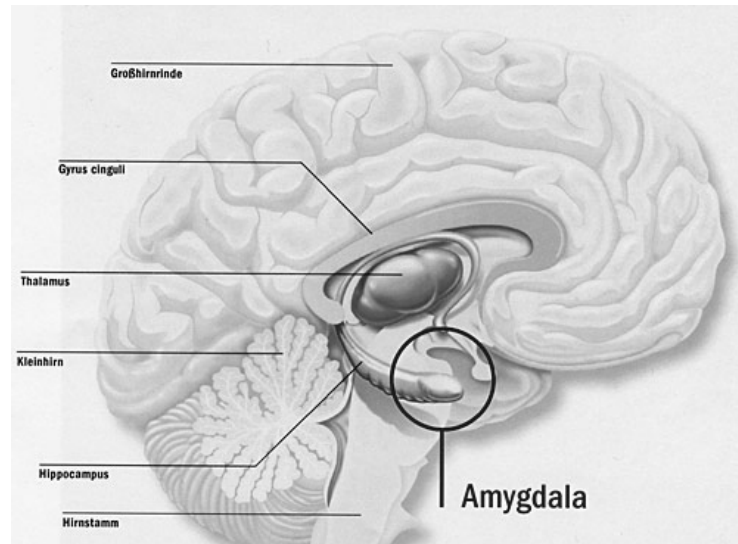
possible that this nucleus corresponds with the hypothalamic 'defence area', The observations of Di Micco JA et al. 1991 indicated that the DMH may be a critical region integrating the cardiovascular as well as other autonomic and nonautonomic components of the response to an acute emotional stress or alerting stimulus. Consistent with this, the DMH receives inputs from several forebrain nuclei that are believed to play a role in mediating the response to stress, including the amygdala (DiMicco, Stotz-Potter et al. 1996). In particular, activation of the basolateral nucleus of the amygdala generates a cardiovascular response very similar to that evoked by acute stress (Sanders and Shekhar 1991) and this evoked response is dependent on synaptic transmission in the DMH (Soltis and DiMicco 1991).

The previous study of Dampney et.al (Dampney, Coleman et al. 2002) demonstrated that vasomotor and cardiac responses evoked from the DMH are mediated by descending pathways of synaptic transmission within the RVLM (ventrolateral medulla). He designed a model of the key central connections mediating the cardiovascular response to an acute emotional stress (Fig.3)



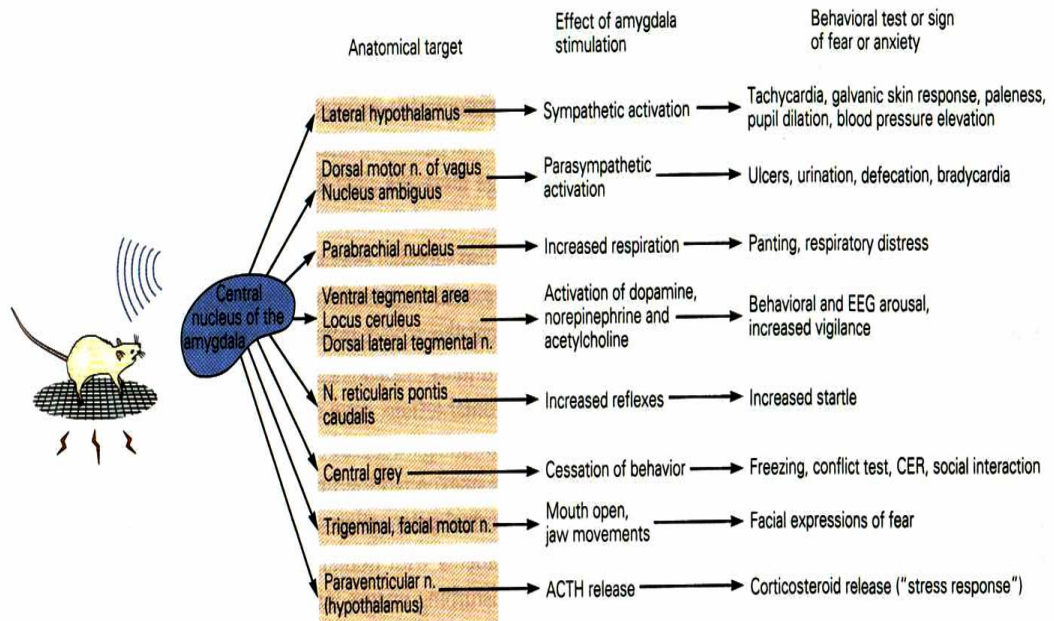
**Fig.3** This diagram shows the model of key central pathways that lead to the cardiovascular response to an acute stress. CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla.

## Role of Amygdala and cardiac response



The amygdala plays a key role in emotions and forming emotional memories. This almond-shaped structure integrates your senses and links them with your emotions. It also affects basic behaviors such as feeding, sexual arousal, and the “fight-or-flight” reaction to stress.

In the midbrain amygdala with adjacent areas (extended amygdala) integrates autonomic responses with emotional factors. Its functions are to interpret the emotional significance of incoming sensory information and to generate the appropriate autonomic, behavioral, motor, endocrine, and pain-suppressing responses to environmental stimuli (Amaral and Insausti 1992; LeDoux 1992) (Fig.4).



**Fig.4.** The direct connections between the central nucleus of the amygdala and a variety of hypothalamic and brain stem areas that may be involved in different animal tests of fear and anxiety.

The amygdala receives cardiopulmonary information and has direct projections to autonomic control sites, such as hypothalamus, parabrachial nucleus, NTS and the dorsal motor nucleus of the vagus which may be the anatomical substrate for descending control over the ANS (Cechetto and Gupta 2000). The amygdala is also an important cardiovascular control center within the limbic system with reciprocal connections with the insular cortex and direct projections to other autonomic control centers in the hypothalamus, pons and medulla (Cechetto and Gupta 2000). Stimulation of the central nucleus of the amygdala produces changes in BP, HR, respiration and gastric secretion and motility (al Maskati and Zbrozyna 1989; Davis 1992). In humans, electrical stimulation of the amygdala has been shown to produce fear sensations, and seizures involving amygdala and its connections may result in various autonomic manifestations, including serious cardiac arrhythmias (Benarroch 1997).

Tachycardia during restraint stress in rats is well-documented (Barron and Van Loon 1989; Chen and Herbert 1995; McDougall, Widdop et al. 2005). It occurs predominantly due to an increase in cardiac sympathetic nerve activity (Barron and Van Loon 1989). Substantial, but indirect evidence suggests that the amygdala could be the source of this elevated cardiac sympathetic drive.

Many animal studies report activation of amygdala neurones by stressful situations (Chen and Herbert 1995; Dayas, Buller et al. 1999; Crane, French et al. 2005; Trneckova, Armario et al. 2006). Electrical or chemical stimulation of the amygdala evoked increases in heart rate (Galeno and Brody 1983; Gelsema, McKittrick et al. 1987; Soltis and DiMicco 1991). Lesion of the amygdala consistently attenuated pressor responses to conditioned and unconditioned stressful stimuli, but produced controversial results with regard to the stress-induced cardiac changes (Galeno and Brody 1983; Iwata, LeDoux et al. 1986; Roozendaal, Koolhaas et al. 1991; Sanders and Shekhar 1991; Carter, Pinnock et al. 2004), most often being without effect. In the now classical study by LeDoux and colleagues (1986), they specifically emphasized that amygdala lesion did not affect tachycardic response to conditioned stimuli, in contrast to pressor and locomotor responses (LeDoux, Sakaguchi et al. 1986).

In humans, stress-induced amygdala activation correlates with the magnitude of associated tachycardia (Critchley, Rotshtein et al. 2005). All these findings indicate that the amygdala neurons responsible for tachycardia may be activated during stress; our findings provide first direct evidence that they are indeed activated. Using similar experimental strategy, Kubo et al. (2004) recently demonstrated that activation of the amygdala neurons is also essential for the restraint-induced pressor (vascular) responses (Kubo, Okatani et al. 2004).

## **Brainstem control centres integrate stress-induced cardiac sympathetic neural outflow**

The brain stem is the “stalk” of the brain below the cerebrum that connects to the spinal cord. It controls processes basic for survival, such as heart rate, breathing, digestion, and sleep. It is the main route of communication between the rest of the brain, the spinal cord, and the nerves that run throughout the body. It also has its own set of nerves that send and receive signals to the face, mouth, tongue, eye muscles, ears, and balance-sensing vestibular organs.

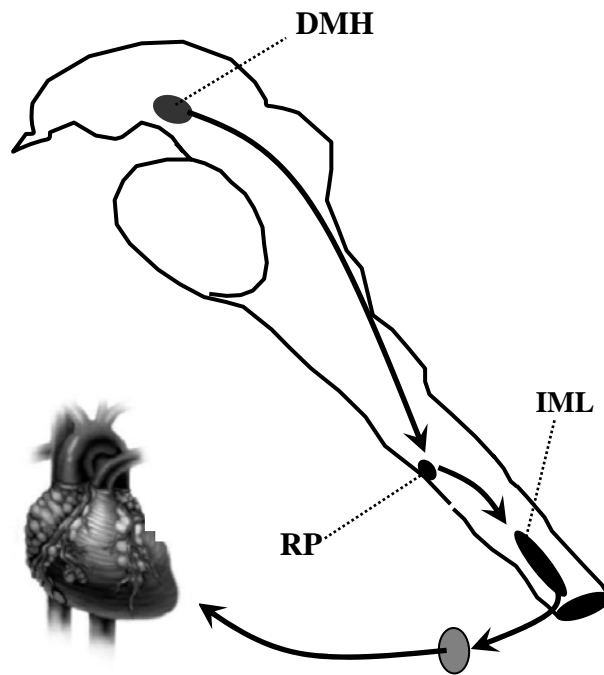
At present, understanding of the brain pathways mediating stress-related cardiac events is limited. Clearly, cardiac effects induced by psychological stress are initiated in the forebrain. Stimulation of the insular cortex (Oppenheimer, Wilson et al. 1991), the central amygdala (Markgraf and Kapp 1988) or the dorsomedial hypothalamus (Poisson, Christen et al. 2000) elicits arrhythmias in experimental animals, and cases of cardiac asystole from temporal lobe (presumably amygdala) epileptic foci have been documented in humans (Liedholm, Duchek et al. 1992). Vagal and sympathetic outflow to the heart constitute the two major efferent neural pathways. The lower brainstem contains integrative control centres that regulate these outflows.

The lower brain stem regulates integrative centres outflows. Until recently, the nucleus ambiguus and the dorsal vagal nucleus was considered as the origin of cardiac vagal activity. The sympathetic cardiac activity originates in the rostral ventrolateral medulla (RVLM) (Dampney, Coleman et al. 2002).

### **Medullary raphe controls cardiac function**

Recent novel and exciting studies suggest that medullary raphe neurons constitute the principal brainstem relay for stress-induced changes in cardiac function. In conscious rats, inhibition of neuronal function in the medullary raphe prevents stress-induced tachycardia (Zaretsky, Zaretskaia et al. 2003). In anaesthetized animals the medullary raphe is the principal brainstem relay for cardiac changes elicited by activation of the dorsomedial hypothalamus (DMH) (Samuels, Zaretsky et al. 2002). Medullary raphe neurons receive projection from neurons in region of DMH and project to spinal sympathetic center to evokes a pattern of cardiovascular changes (Morrison 2001; Samuels, Zaretsky et al. 2002). Thus, neurons in the raphe may provide the critical relay mediating hypothalamic influences on cardiac sympathetic nerve activity under condition of psychological stress (Fig.5).

Direct chemical activation of medullary raphe neurons causes marked increases in cardiac sympathetic nerve activity and heart rate (Cao and Morrison 2003). Neurons in the medullary raphe appear to integrate other autonomic processes activated during acute psychological or physical stress (Fields and Anderson 1978; Morris, Callahan et al. 1995).



**Fig.5** Medullary raphe controls cardiac function. DMH= Dorsomedial hypothalamus

IML= intermediolateral Cell Column, RP= Raphe Palidus

### **3. GABA in cardiac control**

In the last several years,  $\gamma$ -aminobutyric acid (GABA) has been shown to be a powerful, physiologically relevant inhibitory transmitter in the central nervous system. In particular, evidence has recently been accumulating that demonstrates that GABA may play an important role in the central regulation of cardiovascular homeostasis. GABA is well accepted as a major inhibitory neurotransmitter, occurring in 30-40% of all synapses (second only to glutamate as a major brain neurotransmitter). It is most highly concentrated in the substantia nigra & globus pallidus nuclei of the basal ganglia, followed by the hypothalamus, the periaqueductal grey matter ("central grey") and the hippocampus. The GABA concentration in the brain is 200-1000 times greater than that of the monoamines or acetylcholine. The biochemical components related to its synthesis, storage, release and degradation are well understood (Roberts 1976). Furthermore, the distribution of GABA and its receptors in various brain regions, including those important in cardiovascular regulation such as the hypothalamus, brainstem, and neocortex, has also received a great deal of attention (Meldrum 1982).

The best evidence for a physiological role of GABA in the central regulation of the circulation is in control of heart rate and reflex vagal responses. In early studies with the GABA antagonists bicuculline and picrotoxin, it was shown that the systemic administration of these agents resulted in a biphasic heart rate response: an initial bradycardia followed by a tachycardia. The bradycardia was vagal in origin, since it was prevented by vagotomy or atropine, whereas the tachycardia was sympathetically mediated, since it was prevented by spinal transection and bilateral extirpation of the stellate ganglia and adrenal (DiMicco, Gale et al. 1979).

### **Effects of GABA on Blood Pressure and Heart Rate**

The first report relating GABA to cardiovascular changes was by Takahashi et al. 1955, who found that GABA caused a fall of blood pressure in rabbits when injected intravenously and that its effect was the most powerful among  $\gamma$ -amino acids. In a later report (Takahashi, Tiba et al. 1958) these authors discussed the mechanism and site of action of GABA with remarkable prescience. In experiments performed in rabbits, dogs, and cats, they reached the following conclusions:

- (1) GABA caused centrally mediated reductions in blood pressure and heart rate, because GABA had no direct vascular or ganglionic blocking properties, but its effects were themselves prevented by sympathetic denervation or ganglion blockade.
- (2) The vagus, aortic depressor, and carotid sinus nerves were not necessary for the antihypertensive and bradycardiac actions of GABA.
- (3) The region of the CNS responsible for the effect of GABA was caudal to the cerebrum and probably in the medulla.

Since then, many other studies have confirmed the central hypotensive effects of GABA (Antonaccio, Kerwin et al. 1978). Because of the difficulties of working with large doses of GABA administered directly into the CNS, muscimol was used as a postsynaptic GABA agonist tool for further probing the potential role of GABA in the central regulation of blood pressure, and an attempt was made to systematically examine the central effects of GABA, glycine, and muscimol on blood pressure, heart rate, and renal sympathetic nervous discharge in anesthetized cats. It was demonstrated that both GABA and muscimol decreased blood pressure and heart rate, with parallel reductions in renal sympathetic nervous discharge, the latter effect being the first definitive proof for the central sympathetic inhibitor mechanism of GABA on blood pressure and heart rate. The hemodynamic, as well as the sympathetic, nerve effects were reversed by bicuculline, a reasonably specific GABA receptor antagonist (Antonaccio and Taylor 1977).

Furthermore, muscimol was found to be about 1,000 times as potent as GABA, thus eliminating the need for high doses of GABA previously required.

Although central glycine also reduced blood pressure and heart rate, its effects were small in comparison with those of GABA and required very large doses. In a subsequent study (Antonaccio, Kerwin et al. 1978) it was demonstrated that:

- (1) the effects of muscimol were totally through a central mechanism
- (2) its effects were due to specific postsynaptic GABA stimulation, because they were reversed by bicuculline but not strychnine
- (3) baroreceptors were not necessary for the effect.

Several other studies have demonstrated the central hypotensive action of muscimol and GABA in anesthetized cats (Antonaccio, Asaad et al. 1981). The pharmacological and anatomical specificity of the cardiovascular effects produced by central GABA receptor modulation has also been demonstrated. Clonidine is an antihypertensive agent whose central effects are mediated by a receptor stimulation. Muscimol administration after clonidine had no antagonistic effect but, rather, caused further reductions in blood pressure and heart rate. Similarly, bicuculline did not prevent the reductions in blood pressure and heart rate caused by clonidine. Conversely, the reductions in blood pressure and heart rate initially observed after GABA antagonists were reversed by muscimol, but not by piperhexane, an  $\alpha$ -receptor antagonist that reversed the cardiovascular effects of clonidine (Gillis and Quest 1979). Finally, clonidine decreases blood pressure and heart rate at a locus in the caudal medulla, whereas muscimol acts at the anterior medulla.

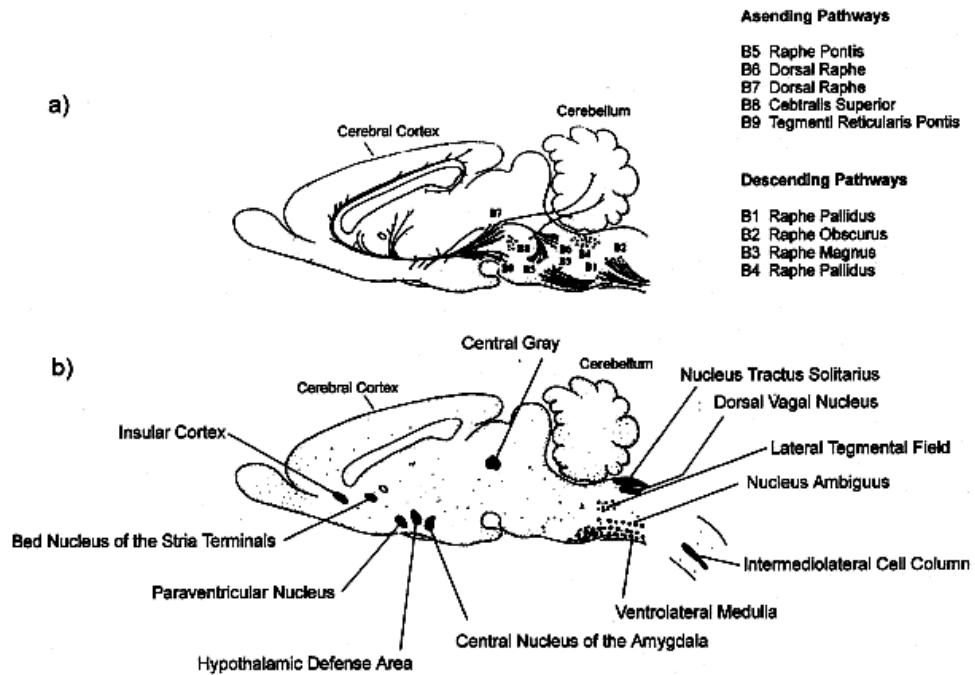
In conclusion, the stimulation of central GABA receptors with muscimol results in bradycardia that apparently is totally mediated by a reduction in sympathetic tone to the heart. The observation of Antonaccio and Taylor in 1977 has shown that the differences in heart rate reductions after muscimol in groups of cats with vagi intact, in comparison with those of vagi bilaterally sectioned (Antonaccio, Asaad et al. 1981).

## **4. Serotonin in cardiac control**

### **Historical overview**

The serotonin (5-HT) neurotransmitter has been implicated in the control of cardiovascular function by brain and spinal cord (McCall et al., 1994 and Ramage 2001). In the late 1980s, effects of serotonin in central cardiovascular generated the experimental evidence regarding site of action, receptors types and mechanism. The involvement of 5-HT<sub>1A</sub> receptors in cardiovascular control is well documented, and the consensus is that their activation results in central sympatholytic effects (McCall et al., 1994 and Ramage 2001). Most of the relevant studies were conducted either in anaesthetize animals or, if in conscious ones, drug effects were studied in the quite awake state.

5-HT neurons, located primarily in raphe obscurus, raphe pallidus, nucleus raphe magnus, and parapyramidal region project to autonomic areas of the lower brain stem and spinal cord, whereas forebrain areas involved in cardiovascular regulation receive their 5-HT input from the dorsal raphe (Mc Call RB and Clement ME 1994) (Fig.6).



**Fig.6** The projections of the serotonin system arise from the nuclei of the dorsal raphe and the raphe magnus, which are located so as to monitor, and process ascending sensory input, and serve to facilitate information processing. The key position of these serotonergic nuclei in the processing of sensory inputs underlies the concepts of sensory gating and directed attention. (A) the ascending and descending 5-HT contain pathways and (B) central areas involved in cardiovascular regulation.

Kuhn et al., 1980 have found that 5-HT and 5-HT precursors can elicit increases, decreases or biphasic changes in arterial blood pressure, heart rate, and sympathetic nerve activity, depending of the site on injection, the species, or the dose administered. 5-HT receptors can be divided into seven major subtypes (5-HT<sub>1</sub> to 5-HT<sub>7</sub>). The most widely used compounds to study the effects of central serotonergic regulation of cardiovascular system include the 5-HT<sub>1A</sub> receptor agonists 8-OH-DPAT.

### **Serotonin receptors**

There are three basic types of serotonin receptors; 5HT-1, 5HT-2, and 5HT-3. The 5HT-3 receptor is notably abundant in the area postrema, which stimulates emesis. There are at least four major subtypes of 5HT<sub>1</sub> receptors, the most important of which is 5HT-1a autoreceptors, which is expressed within the Raphe and Hippocampus. This implies that 5-HT<sub>1a</sub> modulates 5HT release from presynaptic neurons. 5HT-1a receptors are G-protein coupled, and play a role in thermoregulation, arteriolar vasomotor responses, hypotension, sexual behaviour, and sleep. 5HT-2 receptors are prevalent throughout the cortex and mediate platelet aggregation, vasomotor contraction and again possibly sleep.

### **The role of 5-HT<sub>1A</sub> receptor**

The administration of selective 5-HT<sub>1A</sub> receptors agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) reduced heart rate and arterial pressure in a number of species such as dog (Dabire H et al. , 1988), rat (Gradin K et al. , 1985, Fozard JR et al. ,1987) or rabbit (Hof RP et al. , 1989). Subsequently, it was demonstrated that both depressor and bradycardic effects of 5-HT<sub>1A</sub> receptors agonist are not due to peripheral action of the drug, because 8-OH-DPAT had no effect on cardiovascular parameters in pithed animals and did not alter effects of sympathetic nerve stimulation or of exogenous noradrenaline.

The large number of studies indicates that 5-HT<sub>1A</sub> receptors agonist act in the central nervous system to produce inhibition of sympathetic activity (McCall, Patel et

al. 1987). The study demonstrated the evidence that the effects of 5-HT<sub>1A</sub> receptors agonist were determined on spontaneous sympathetic nerve activity. 8-OH-DPAT produces a dose-related inhibition of sympathetic activity recorded from the inferior cardiac (McCall, Patel et al. 1987), renal (Ramage, Clement et al. 1992), or splanchnic nerve (McCall, Patel et al. 1987). The strong evidence provide that 5-HT<sub>1A</sub> receptors agonist act in central nervous system to inhibit sympathetic nerve activity, and therefore, decrease arterial blood pressure and heart rate (McCall and Clement 1994).

### **Medullary raphe as a potential site of action of 5-HT<sub>1A</sub> agonists**

The medullary raphe/parapyramidal area is a major centre that integrates the neural control of several autonomic functions during stress. This brain stem region also represents the major source of descending 5-HT projections to the spinal sympathetic neurons. Sympathetic preganglionic neurons located in the intermediolateral cell column of the spinal cord receive a very heavy serotonergic input (Wu, Elde et al. 1993). Interestingly, the 5-HT antagonists methysergide and metergoline decrease the spontaneous discharge rate of sympathetic preganglionic neurons in intact cats but not in spinally transected animals (McCall 1983). These data provide strong evidence to suggest that medullary 5-HT neurons provide an excitatory input to sympathetic preganglionic neurons to maintain the firing rate of these sympathetic neurons. This study concluded that 5-HT<sub>1A</sub> receptor agonists inhibit sympathetic nerve activity by decreasing the firing rate of medullary 5-HT neurons. There are several observations that support this hypothesis. Anatomically, medullary raphe nuclei contain a high density of 5-HT<sub>1A</sub> receptors (Thor, Blitz-Siebert et al. 1990).

### **5-HT<sub>1A</sub> receptors and Psychological stress in conscious animals**

Recently studies of the cardiovascular effects of 5-HT<sub>1A</sub> agonists on basal levels of heart rate and arterial pressure were limited. There are two studies demonstrating the potential action of 5-HT<sub>1A</sub> agonists on stress-elicited cardiovascular responses.

First study, van den Buuse and his colleagues (Van den Buuse et al., 2005) investigated drug effects in four rat strains (spontaneously hypertensive rats, fawn-hooded, Wistar-Kyoto and Sprague-Dawley) during the open-field test after systemic administration of one of three 5-HT<sub>1A</sub> agonists (buspirone, 8-OHDPAT or MDL73 005). They observed substantial antipressor and antitachycardic effects; however, they reported significant interspecies differences (e.g. both 8-OH-DPAT and MDL73 005 augmented, rather than reduced, pressor responses in Sprague-Dawley rats). Despite

these controversies, that study clearly demonstrates, for the first time, that 5-HT<sub>1A</sub> receptors are involved in cardiovascular stress responses.

Another study, Nalivaiko E. and his colleagues studied in conscious, unrestrained rabbits. They examined the effects of systemic 8-OH-DPAT on cardiovascular responses to several acute psychological stressors (loud sound, pinprick and air jet). They observed clear signs of hypermotility (a part of the serotonin syndrome) in their animals, starting from a dose of 20 mg/kg 8-OH-DPAT; this results that the drug had some action in the central nervous system, but did not affect cardiovascular centres. They then assessed the effects of intravenous 8-OH-DPAT on stress-induced changes.

Acoustic stimulation in rabbits usually cause vagally mediated bradycardia. The response was not affected by 8-OH-DPAT. In contrast, the drug substantially attenuated, in a dose-dependent manner, pin-prick- and air jet elicited tachycardiac responses. Because these effects were sensitive to WAY-100635 (a selective 5-HT<sub>1A</sub> receptor antagonist), they concluded that 8-OH-DPAT action was via activation of 5-HT<sub>1A</sub> receptors. In order to establish the anatomical location of drug action, they conducted another series of experiments where, during air jet stress, 8-OH-DPAT was microinjected into the medullary raphe area via a stereotactically pre-implanted guide cannula. They found that local administration of the drug reversed stress-induced tachycardia. Their major conclusion was that activation of 5-HT<sub>1A</sub> receptors in the medullary raphe–parapyramidal area causes suppression of neurally mediated cardiovascular changes during acute stress.

## 5. Restraint stress

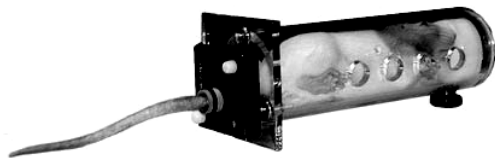
Restraint stress is known to evoke a discrete pattern of *c-fos* expression in the brain (Chen and Herbert 1995). The work reported that stress-induced tachycardia was maximal 10 min following the onset of restraint, and decreased thereafter. The peak value was not altered by repeated restraint, but levels fell towards baseline values more rapidly with increasing bouts of stress. Sixty minutes after the end of the first stress session, there was pronounced *c-fos* expression in the lateral septum, lateral preoptic area, lateral hypothalamic area, all divisions of the hypothalamic paraventricular nucleus, the medial (but not central) amygdala, the locus ceruleus and a brainstem structure (thought to be Barrington's nucleus), compared to rats transferred to the testing room but not restrained.

Restraint stress increased heart rate (Barron and Van Loon 1989), blood pressure (Barron and Van Loon 1989), blood flow (Lasbennes, Lestage et al. 1986), antinociceptive threshold, plasma catecholamine, adrenocorticotrophic hormone (ACTH), prolactin and corticosterone (Kvetnansky, Weise et al. 1979). It also caused tachycardia and pressor responses in both normotensive and spontaneously hypertensive rats (McDougall, Paull et al. 2000). The central control cardiovascular system primarily mediated at the level of medulla oblongata significant supramedullary modulation also occurs (Dampney 1994). The locus ceruleus (Miyawaki, Kawamura et al. 1993), dorsomedial hypothalamus (Zaretsky, Zaretskaia et al. 2003) and medullary raphe pallidus (Samuels, Zaretsky et al. 2002) play an important role in the initiation of stress response and can regulate sympathetic outflow.

The restraint stress is a plastic tube (internal diameter 5.5 cm) drilled with numerous pores to allow accessibility to fresh air. Rat placed in the restraint in the supine position. They were prevented from turning around by adjusting the pressure of a plastic bar applied to their abdomen, but this did not restrict respiration (Chen and Herbert 1995) (Fig.7).



(A)



(B)

**Fig. 7** The example of restrainer (A) and rat in restraint (B)

## **CHAPTER IV**

### **MATERIALS AND METHODS**

In this chapter, I briefly describe the surgical procedures used to prepare the rat with an implanted ECG electrode and guided cannula. The methods used to provide the ECG signal for heart rate recording and guide cannula for microinjection, are described. Also, the technique for producing high stress levels in the rat, by using a restrainer is described. The ECG recording in conscious rat is outlined and the computer data processing of heart rate is described here. Finally, the histological methods used to verify the microinjection in the amygdale and medullary raphe are outlined.

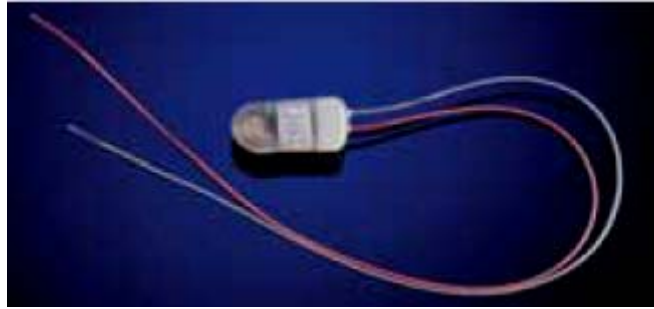
## **MATERIALS**

### **Chemicals**

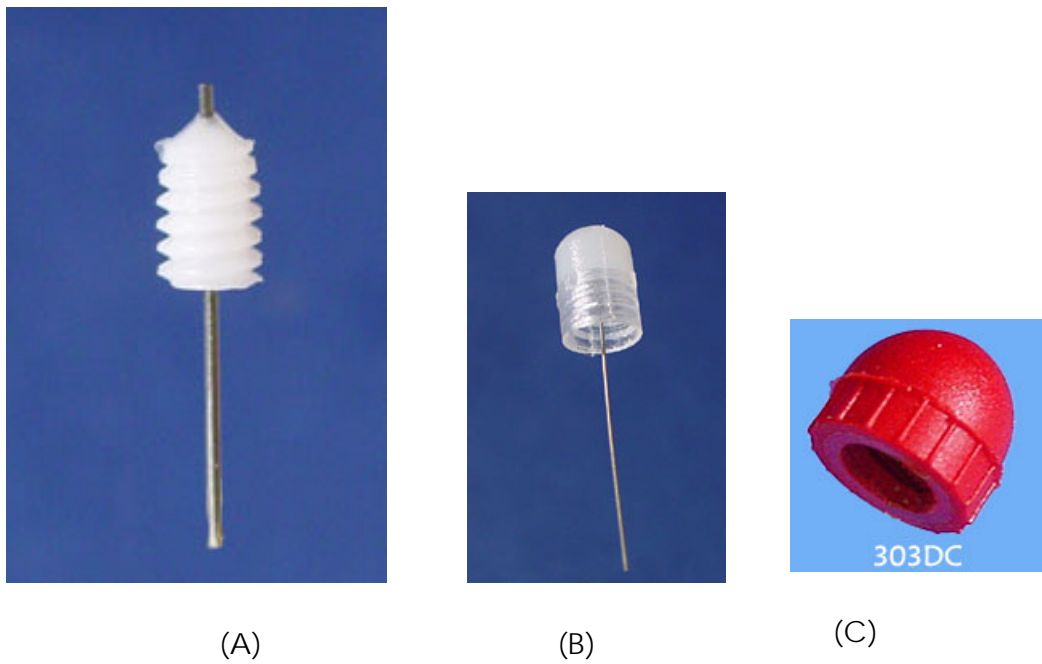
1. Isoflurane (1.5% in 100% oxygen) (2L per minute)
2. Carprofen 5 mg/kg from Sigma, USA
3. Muscimol 0.1 nmol in 500 nl sterile Ringer's solution from Sigma, USA
4. Ringer's solution
5. 8-OH-DPAT 100 µg/kg and 1 nmol in 100 nl from Sigma, USA
6. β-adrenergic blockage- Atenolol 2 mg/kg from Sigma, USA
7. Muscarinic blockage- Methylscopolamine bromine 50 µg/kg from Sigma, USA
8. WAY 100,635 100 µg/kg from Sigma, USA
9. Horradish peroxidase (HRP) 100nl of 0.1% solution

### **Equipments**

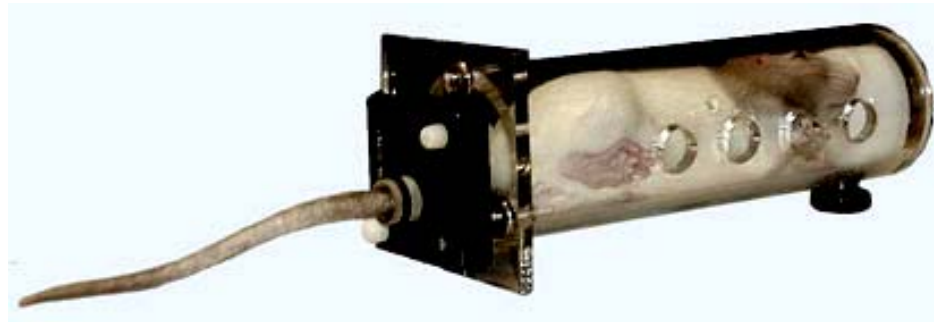
1. Telemetric ECG radiotransmitters (TA11CA-F40, Data Sciences International) (Fig.8)
2. Stainless steel guide cannula, dummy cannula and dust cap (Fig.9)
3. Cylindrical restrainer (Fig.10)
4. ECG power lab A/D and chart 5.4 software (AD Instruments, Australia) (Fig.13)



**Fig.8** The telemetric ECG radio transmitter (TA11CA-F40, Data Sciences International)



**Fig.9** The pictures show the equipment needed for a surgical microinjection session; (A)- a SPC Cannula Internal Acute to fit 5mm C31GA with 5mm projection, (B)- SPC Dummy cannula, 6mm and (C)- Dust Cap, red



**Fig.10** The restrainer consisted of transparent PVC tubing (I.D. 6 cm). Restraint is a well established experimental paradigm for provoking psychological stress in rats (Barron and Van Loon, 1989). It consistently elicits a robust rise in arterial pressure and heart rate (Barron and Van Loon, 1989; Chen et al., 2005; McDougall et al., 2000).

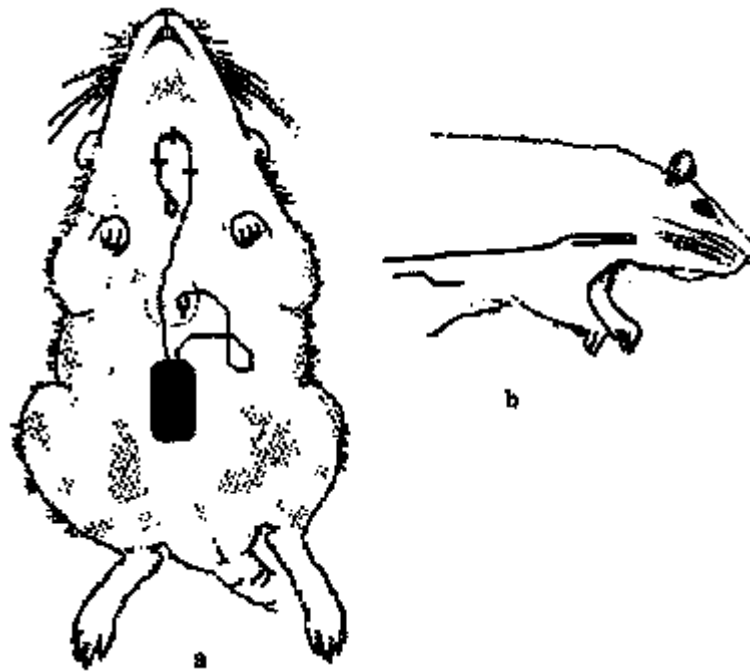
## **METHODS**

### **PART I: Effects of inhibition of the amygdala on stress-induced tachycardia in rats.**

#### **1.1 Animals and preliminary surgery**

The study was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and was approved by the Flinders University Animal Welfare Committee. Experiments were performed on 20 male Hooded rats (300-350g). Preliminary surgery was conducted under Isoflurane anaesthesia (1.5% in 100% oxygen).

Firstly, ECG electrodes were implanted according to the method described by Sgoifo in 1996 (Sgoifo et al., 1996) (Fig.10): one electrode was attached to the internal surface of the xyphoid process, and another was positioned in the mediastinum, along the trachea at the level of the left ventricle. Such electrode placement permits the recovery of 95-99% of heartbeats, even in moving animals. The leads of the ECG electrodes were tunnelled subcutaneously to the back of the neck, exteriorised and soldered to a headsocket. Animals were then placed in a stereotaxic apparatus, two burr holes were drilled, and two stainless steel guide cannula were placed bilaterally 2.8 mm caudal to the bregma and 4.0 mm lateral to the midline, with a tip located 5 mm below the surface of the skull. The headsocket and cannula were fixed to the skull with stainless steel screws and dental cement. The cannula was closed with obturators protruding 0.3 mm below the tip. Animals recovered from anaesthesia and were then returned to the animal house for at least one week before experimental studies.

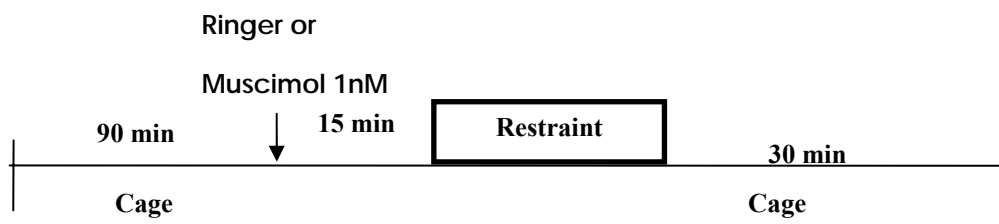


**Fig.11** The location of telemetry ECG transmitter described by Sgoifo in 1996. The body of the transmitter was positioned in the abdominal cavity (a) and the two leads (wire loops) on the dorsal surface of the xiphoid process and into the anterior mediastinum close to the right atrium (a and b) (Sgoifo et al., 1996).

## 1.2. Experimental protocol

On the day of experiments, the rats were brought up from the animal house, connected to the recording system via a flexible cable attached to a swivel, placed in a dark box (40 x 40 x 40 cm) and remained undisturbed for at least 60 min. They subsequently received bilateral injections of GABA<sub>A</sub> receptor agonist muscimol (0.1 nmol in 500 nl sterile Ringer's solution) or, on a different day, vehicle, in a counter-balanced manner. The tip of the injecting cannula was lowered either to the level of 8 mm or 6 mm below the skull surface, for the injections into the amygdala or for control injections into the striatum, respectively. Injections were made using a hand-held syringe, with visual control of the injected volume using a calibrated 10 µl glass capillary tube.

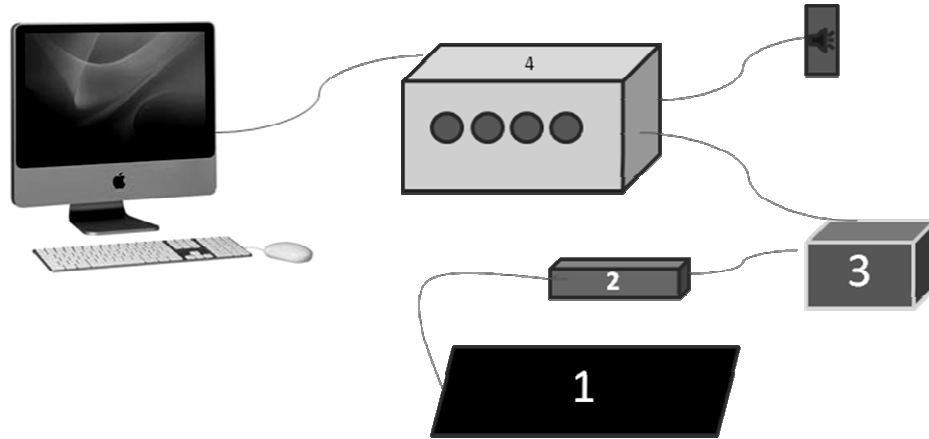
After injections, animals were then returned to the box for 30 min and then placed into a restrainer for the next 30 min (Fig.12). At the end of the experiment we labelled injection sites with horseradish peroxidase (HRP) dissolved in Ringers's solution (500 nl) using the same injection cannula. Animals were then euthanized, perfused transcardially with a fixative, and the brain was removed and sectioned. Sections were processed for HRP, stained with Neutral red, and the location of the cannula tip was photographed. (Muscimol was from Sigma- USA.)



**Fig.12** The procedure for the experiment of muscimol injection into bilateral amygdala prior to restraint stress.

### 1.3 Data acquisition and analysis

The analogue ECG signal was digitised at 1 KHz and acquired using PowerLab A/D converter and Chart 5.4 software (ADInstruments, Australia). The results measured peak stress-induced tachycardia, mean heart rate and heart rate changes ( $\Delta$ HR) during four 10-min epochs (one pre-stress and three during stress), and computed the area under the curve (AUC) values for three epochs during stress. Delta AUC was defined as ( $AUC_{\text{Vehicle}} - AUC_{\text{Muscimol}}$ ). Heart rate variability analysis in the frequency domain was performed using Chart 5.4 software (ADInstruments, Australia). Artefact-free data segments containing at least 1200-1400 heart beats were selected just before restraint (“pre-stress”), during the first 5 min of restraint (“onset-stress”) and during the last 10 min of restraint (“end-stress”). The detections of this study were total spectral power, the percentage of the low-frequency (LF, 0.4-0.67 Hz) and high-frequency (HF, 0.67-3 Hz) components, and the LF/HF ratio. All values are means $\pm$ SEM. A repeated measures two-way ANOVA (time x drugs) was followed by HSD post hoc test. Linear regression was used for assessing Delta AUC/AUC dependence.



**Fig.13** Diagram illustrates of the ECG recording set-up with Power-Lab

1. Receiver receive signal from the rat was implanted ECG activity probe
2. Dataquest box for ECG
3. Power supply
4. Mac Lab-Power lab connected to the computer

## **PART II: Effects of activation of 5-HT<sub>1A</sub> receptors on stress induced tachycardia in rats.**

### **2.1. Experimental Animals**

Male Hooded rats weighing 280-320g (n=77) were used in all experiments. All efforts were made to reduce animal pain or discomfort. Experiments were conducted in accordance with the European Community Council Directive of 24 November 2006 (86/609/EEC) and were approved by the Flinders University Animal Welfare Committee.

### **2.2 Preliminary surgery**

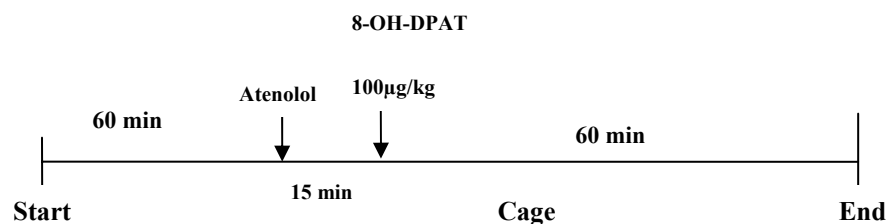
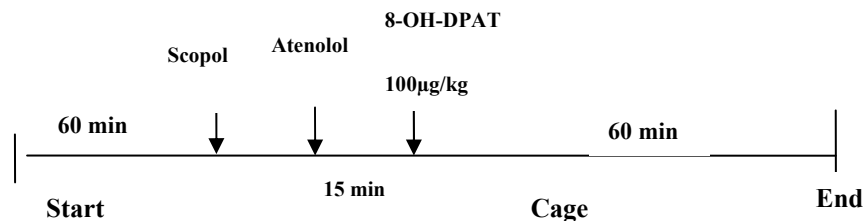
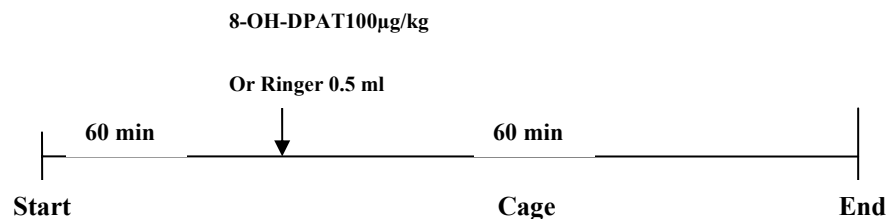
Preliminary surgery was conducted under Isoflurane (1.5% in 100% oxygen) anaesthesia. Carprofen (5 mg/kg) and Baytril (1 mg/10 kg) was used as an analgesic after the surgery. The telemetric ECG radio transmitters (TA11CA-F40, Data Sciences International) were implanted into the peritoneal cavity. Electrodes were placed according to the method described by Sgoifo (Sgoifo et al., 1996): on the internal surface of the xiphoid process and in the mediastinum, along the trachea at the level of the left ventricle. These placements permit recovery of 95-99% of heartbeats, even in vigorously moving animals. In some animals, during the same surgical session, a stainless steel guide cannula was stereotaxically positioned 2.8 mm caudal to the inter-aural line at the midline, inserted vertically to the IV ventricle, fixed to the skull using stainless steel screws and dental cement and closed with an obturator. The animals recovered from anaesthesia and were returned to the animal house for at least one week before experimental studies. They were kept on a reverse 12h/12h light-dark cycle.

### **2.3 Experimental protocol**

Experiments were carried out between 9 am and 3 pm. ECG probes were switched on and the animals remained in their home cage for at least 1h. Drugs were administered either subcutaneously, diluted in 0.5 ml Ringer's solution (Experiments 1-7 below), or microinjected into the medullary raphe (Experiments 8-10).

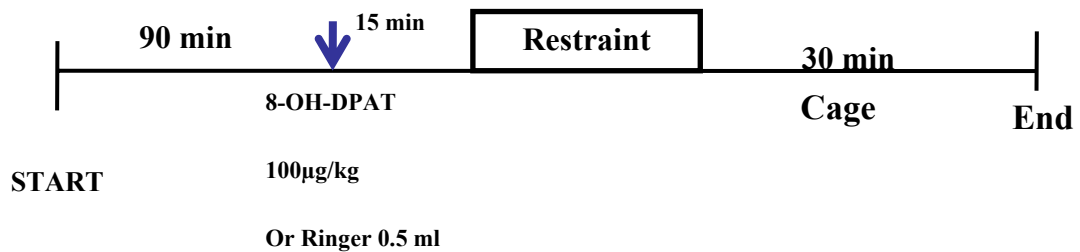
### 2.3.1 Experiment 1: Does systemic treatment with 8-OH-DPAT affect basal heart rate?

In the first group of rats (n=6), either 8-OH-DPAT (100 µg/kg) or, on different days, Ringer's solution, was administered and recordings were obtained for an hour. Similar injections were made in the second (n=6) and third (n=6) groups after β-adrenergic blockade (Atenolol 2 mg/kg) or after a combined muscarinic and β-adrenergic blockade (Methyl-scopolamine bromide 50 µg/kg plus Atenolol 2 mg/kg), respectively.



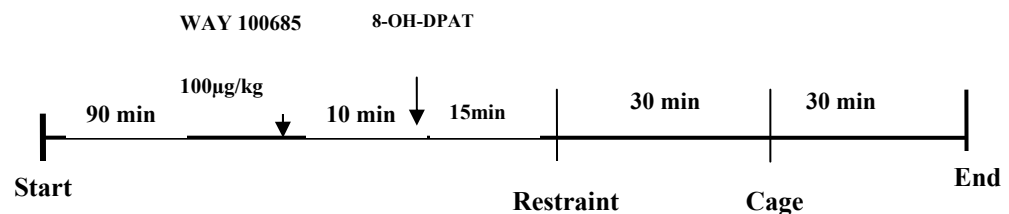
### 2.3.2 Experiment 2: Does systemic treatment with 8-OH-DPAT affect stress-induced tachycardia?

On different days, either 8-OH-DPAT (10, 30 or 100  $\mu\text{g}/\text{kg}$ ) or Ringer's solution was administered and 15 min later the rats were placed into a restrainer (transparent plastic tube, with 60 mm ID) for 30 min (n=7).



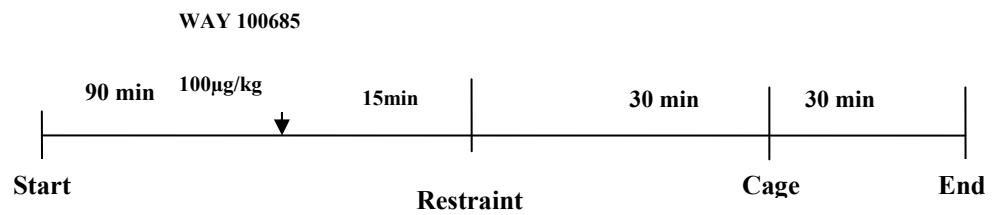
### 2.3.3. Experiment 3: Does systemic treatment with 8-OH-DPAT affect stress-induced tachycardia following 5-HT<sub>1A</sub> receptor blockade with WAY-100,635?

Prior to the restraint, animals received, on different days, the following combination of drugs: a) Ringer/Ringer; b) Ringer/8-OH-DPAT(100  $\mu\text{g}/\text{kg}$ ); c) WAY-100,635/8-OH-DPAT (both at 100  $\mu\text{g}/\text{kg}$ ) (n=6).



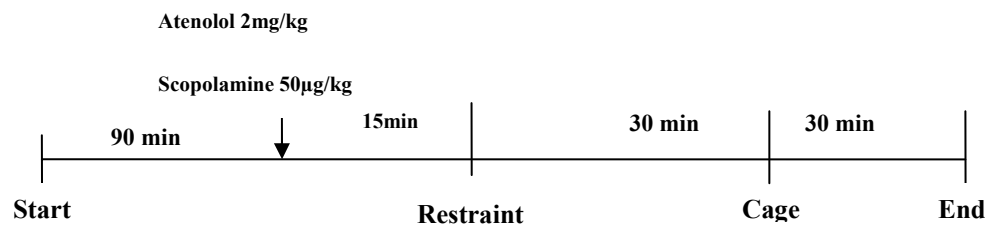
**2.3.4. Experiment 4: Does systemic treatment with WAY-100,635 affect stress-induced tachycardia?**

Prior to restraint, animals received, on different days, either WAY-100,635 (100 µg/kg) or Ringer’s solution (n=6).



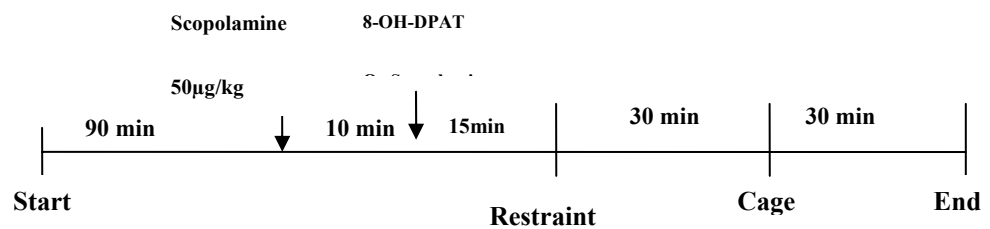
**2.3.5 Experiment 5: Does autonomic blockade affect restraint-induced cardiac responses?**

Prior to restraint, animals received, on different days: a) Ringer’s solution; b) Atenolol (2 mg/kg); c) Methyl-scopolamine bromide (50 µg/kg) (n=8).



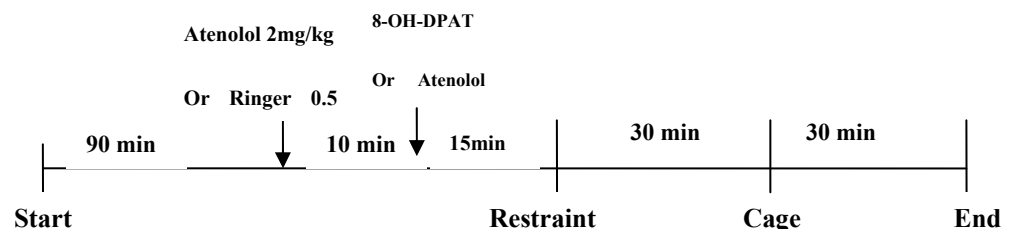
### 2.3.6 Experiment 6: Does systemic treatment with 8-OH-DPAT affect stress-induced tachycardia following vagal blockade?

Prior to restraint, animals received, on different days, the following combination of drugs at 10-min intervals: a) Methyl-scopolamine (50  $\mu\text{g}/\text{kg}$ )/Ringer; b) Methylscopolamine (50  $\mu\text{g}/\text{kg}$ )/8-OH-DPAT (100  $\mu\text{g}/\text{kg}$ ) (n=6).



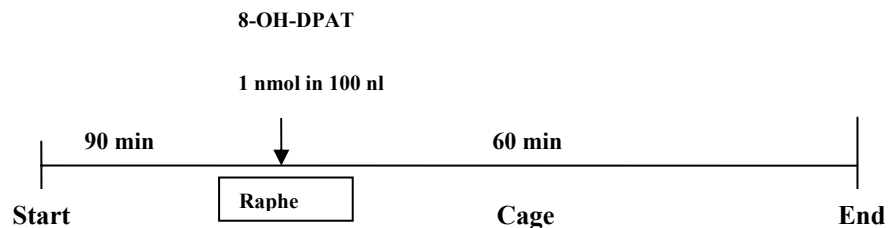
### 2.3.7. Experiment 7: Does systemic treatment with 8-OH-DPAT affect stress-induced tachycardia following $\beta$ -adrenergic blockade?

Prior to restraint, animals received, on different days, the following combination of drugs at 10-min intervals: a) Atenolol (2 mg/kg)/Ringer; b) Atenolol (2mg/kg)/8-OH-DPAT (100  $\mu\text{g}/\text{kg}$ ). N=6.



**2.3.8 Experiment 8: Does intracerebral microinjection of 8-OH-DPAT affect basal heart rate?**

Animals received an intra-medullary microinjection of either 8-OH-DPAT (1 nmol in 100 nl) or, on a different day, the equivalent volume of sterile Ringer’s solution, and recordings were continued for one hour. An injection cannula (OD 0.2 mm stainless steel wire, Small Parts,USA) was inserted 11 mm below the surface of the skull. Injections were made using a hand-held syringe, and the injection volume was assessed by observing the meniscus in a glass capillary attached to the injection cannula. Injections were made slowly (~20 s) and the cannula remained in place for 1 min after injection (n=6).



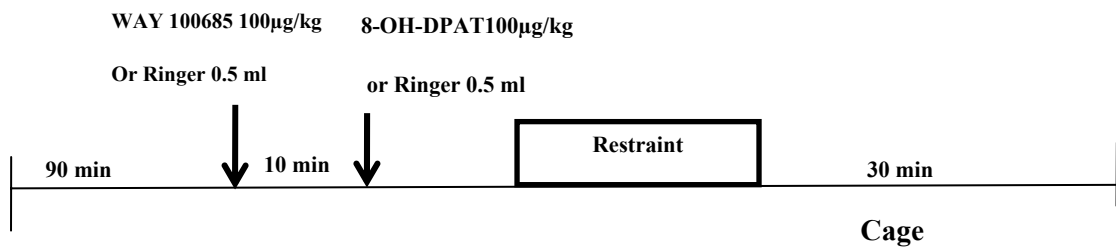
**2.3.9 Experiment 9: Does intracerebral microinjection of 8-OH-DPAT affect stress-induced tachycardia?**

Prior to restraint, animals received an intra-medullary microinjection of either 8-OH-DPAT (1 nmol in 100 nl) or, on a different day, the equivalent volume of sterile Ringer’s solution. In another 5 animals (control group), similar injections were performed 2.5 mm more dorsal (8.5 mm below the surface of the skull). Microinjections were performed similarly to the previous experiment (n=8).



### 2.3.10 Experiment 10: Does 5-HT<sub>1A</sub> receptor blockade with WAY-100,635 prevent anti-tachycardic effects of intra-medullary administered 8-OH-DPAT?

Prior to restraint, animals received, on different days, the following combination of drugs: a) Ringer (s.c) / Ringer (brain microinjection, 100 nl); b) Ringer (s.c) / 8-OH-DPAT (brain microinjection, 1 nmol in 100 nl); c) WAY-100,635 (s.c., 100 µg/kg / 8-OH-DPAT (brain microinjection, 1 nmol in 100 nl) (n=6).



Thus, this study was conducted in 77 rats (57 with systemic administration and 20 with intramedullary administration of 8-OH-DPAT). Each animal cohort was used for just one type of experiment. All experimental procedures were performed at least 48 h apart. To avoid serial effects, we used a counterbalanced or rotational design. All chemicals were from Sigma (USA).

## 2.4 Visualization of microinjection sites

Medullary microinjection sites were labelled with Horseradish Peroxidase (HRP) (100 nl of 0.1% solution), which was administered into the medulla immediately after the termination of the last experiment using the same injection cannula. Rats were euthanized with Lethobarb and perfused transcardially with formaldehyde fixative. Brains were removed and sectioned (50  $\mu$ m thickness).

HRP was visualised by incubating sections in 0.05% solution of di-aminobenzidine for 10 min, with the subsequent addition of a 0.01% solution of hydrogen peroxide. Sections were dried, dehydrated in alcohol, mounted on slides, stained with neutral red and photographed.

## 2.5 Data acquisition and analysis

Analog ECG signals were digitized at 400 Hz and acquired using a PowerLab A/D converter and Chart 5.4 software (ADInstruments). Heart rate was calculated from the ECG recording records using the same software. After removing artefacts, heart rate was automatically averaged for every minute. Spectral indices of heart rate variability were computed using the heart rate variability (HRV) module of the Chart 5.4 software. The low-frequency (LF) band was set at 0.15-1.0 Hz and the high-frequency (HF) band at 1.0-3.0 Hz. HF power is a measure of vagally-mediated respiratory sinus arrhythmia. We also computed the root-mean-square of the beat-to-beat interval differences (RMSSD), a standard HRV index reflecting fast vagal modulation of the inter-beat intervals. For characterising the recovery of the HR after handling-related tachycardia, we used T1/2 – time period during where HR fell to 50% of the peak increase. The dose-dependence of 8-OH-DPAT induced effects were assessed using linear regression. Group data was analysed by ANOVA, with Fishers's protected t-test and with the significance threshold set at the 0.05 level. All data is presented as mean $\pm$ SEM and, where possible, data values are embedded in our illustrations.

## **CHAPTER V**

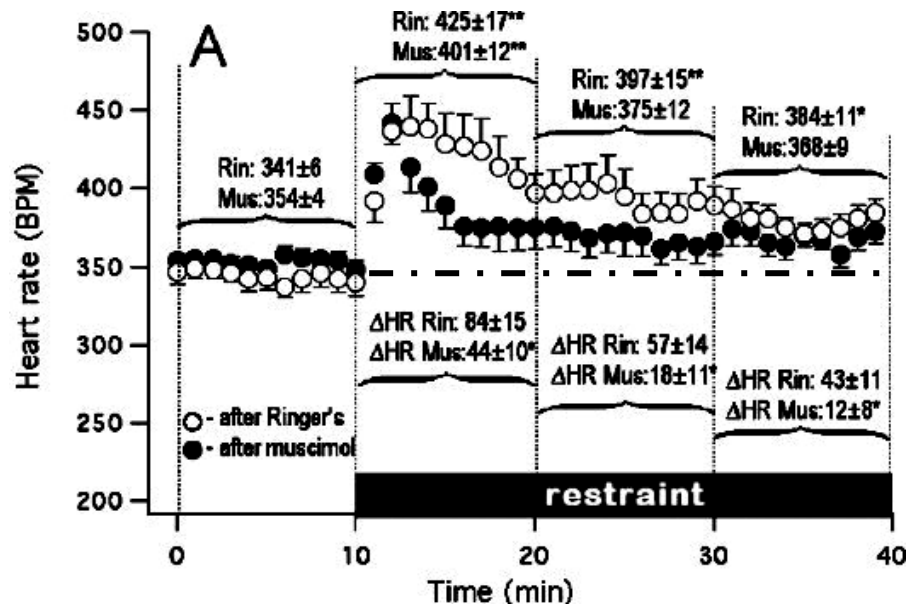
### **RESULTS**

#### **PART I: Effects of inhibition of the amygdala on stress-induced tachycardia in rats.**

##### **1.1 Effects of intra-amygdala muscimol injections on restraint-induced tachycardia.**

Tachycardia associated with handling during brain injections was short-lasting, so that within 10-15 min after injection the heart rate returned to the basal level in all animals after both vehicle and muscimol. Before stress, there was no difference in the HR values between vehicle and muscimol conditions. In rats that received intra-amygdala injection of vehicle, restraint stress caused tachycardia that peaked at about 425 BPM within 2-3 min and then declined, approaching a steady-state level of 40-45 BPM above the baseline within the next 10-15 min and remaining at this level until the end of restraint (Fig. 14 A for illustrations and data values).

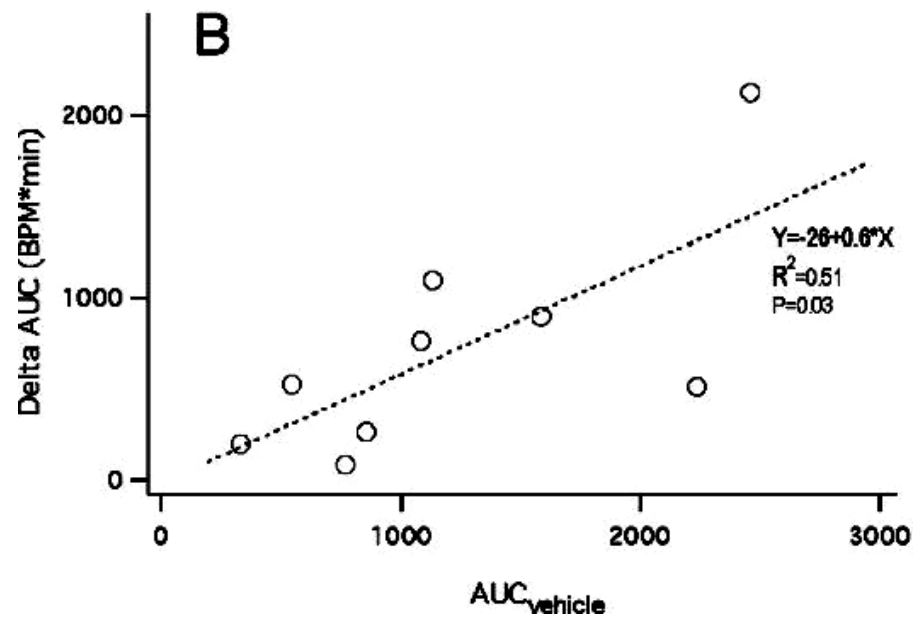
Although peak tachycardia did not differ between the two conditions (absolute values:  $443 \pm 15$  and  $441 \pm 13$  BPM and deltas:  $+94 \pm 13$  and  $+86 \pm 11$  BPM for vehicle and muscimol, respectively;  $p > 0.05$ ,  $n = 9$  for both data sets), increases in heart rate during each of the 10-min restraint epochs were significantly lower after muscimol. For the muscimol condition, mean heart rate was significantly elevated (ie different from pre-restraint) during only the first 10 min of the restraint. Mean  $\Delta$ HR values for each epoch were significantly lower after muscimol (Fig. 14A).



**Fig. 14** Effect of bilateral pharmacological inhibit of amygdale on the tachycardia elicited by the restraint stress.

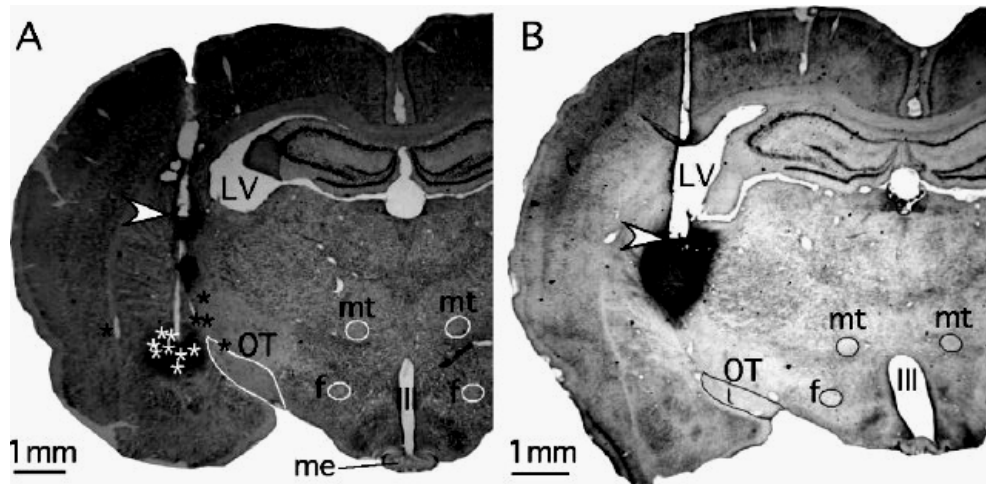
- A. The changes in heart rate elicited by a 30-min restraint stress in rats with bilateral intra-amygdala injection of vehicle (O) or with muscimol (●). Each data point is the mean of 1-min averages from the 9 animals. Data above the traces show absolute values of the heart rate for 10-min periods; \*\* -  $p < 0.01$ ; \* -  $p < 0.05$  for each condition during stress vs. pre-stress. Data below the traces show delta heart rate values (with regard to pre-stress); \* -  $p < 0.05$  for comparison of Vehicle vs. muscimol conditions.

Muscimol substantially accelerated the fall of heart rate from the peak to the steady-state level (Fig. 14A), and thus the area under the curve value for muscimol ( $503 \pm 162$  BPM\*min;  $P < 0.05$ ,  $n=9$ ) was significantly smaller than that for vehicle ( $1221 \pm 231$  BPM\*min). The inhibitory effect of muscimol on stress-induced tachycardia (assessed as an area under the curve) was directly proportional to the initial response (Fig. 14B).



**Fig. 14B** Effect of muscimol is directly proportional to the magnitude of the control tachycardic response to the restrain stress. The dashed line and data values represent the result of the liner regression analysis. Data expressed as an area under the curve (AUC).

Intra-amygdala locations of microinjection sites in 9 animals are illustrated in Fig. 15A. The experiment did not find any dependence of the magnitude of muscimol effect on the location of the actual injection site. Fig. 15A also shows five cases of unsuccessful injections, where missed the target. In two of these five cases that were closer to the target, the experiment observed some effects of muscimol (the area under the curve was reduced from 1361 to 1097 BPM\*min and from 962 to 887 BPM\*min, respectively), whereas at three other sites the drug was without effect. The results did not observe any gross behavioural changes after administration of either vehicle or muscimol to the amygdala.



**Fig.15** The coronal sections of rat brain documenting the injection sites into the amygdala (A) and into striatum (B). Arrowheads indicate the location of the tip of the guide cannula. White asterisks show the centre of successful injection sites; black asterisks are the center of injection sites considered unsuccessful.

Abbreviations: f- fonix; me- medial eminence; LV- lateral ventricle; mt- mamillo-thalamic tract; OT-optic tract; III- third ventricle

## **1.2 Effects of intra-amygdala muscimol injections on restraint-induced changes in heart rate variability.**

Restraint stress altered both low-frequency (LF) and high-frequency (HF) components of the heart rate power spectra. For the vehicle condition, the normalized high-frequency power decreased and the low-frequency power increased at the beginning, but not at the end of the restraint, resulting in a significant rise of the LF/HF ratio during this period. Following pre-treatment with muscimol, rats exhibited no significant changes in the HRV in response to stress. Data values for HRV changes are presented in Table 1.

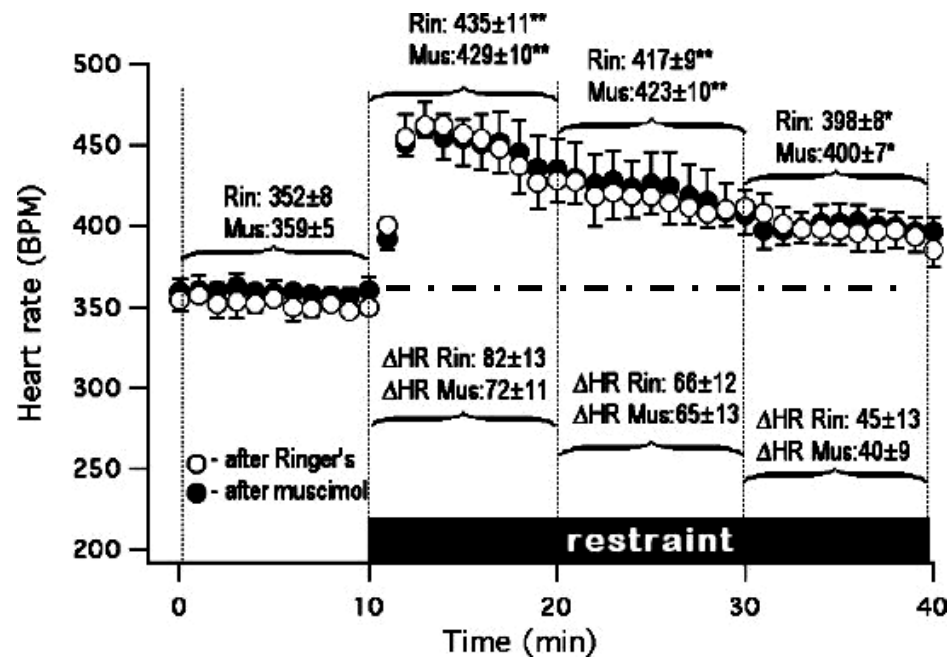
**Table 1. Changes in HRV indices elicited by restraint stress and effects of intracerebral microinjections of muscimol.**

	Pre-stress	Onset-stress	End-stress
A. Intra-amygdala injection - Vehicle			
LF (%)	51±3	67±3*	52±5
HF (%)	48±4	30±4**	46±4
LF/HF ratio	1.2±0.2	2.8±0.6**	1.3±0.2
B. Intra-amygdala injection - Muscimol			
LF (%)	61±3	52±3	47±5
HF (%)	37±4	44±4	50±4
LF/HF ratio	1.8±0.2	1.3±0.2 <sup>##</sup>	1.1±0.2
C. Intra-striatal injection - Vehicle			
LF (%)	58±5	71±4*	53±5
HF (%)	40±4	27±3*	45±4
LF/HF ratio	1.4±0.3	2.6±0.6**	1.2±0.2
D. Intra-striatal injection - Muscimol			
LF (%)	47±3	64±5*	52±4
HF (%)	52±4	32±4*	45±3
LF/HF ratio	0.9±0.1	2±0.5*	1.2±0.2

The data presented above as is a percentage of the total power. LF – low-frequency power, HF – high-frequency power. \* - significantly different from pre-stress,  $p < 0.05$ ; \*\* - significantly different from pre-stress,  $p < 0.01$ ; <sup>##</sup> - significantly different from the corresponding time point for the vehicle condition,  $p < 0.01$ .

### **1.3 Effects of intra-striatal muscimol injections on restraint-induced cardiac changes.**

In six animals (control group) either vehicle or muscimol were injected, on different days, into the ventral striatum, 2 mm dorsal to the amygdala site. Restraint-induced tachycardic responses and changes in heart rate variability in these rats did not differ between the two conditions (Fig. 16 and Table 1), and did not differ from responses observed in our major experimental group after vehicle injection. Intra-striatal locations of microinjection sites are illustrated on Fig. 15B.

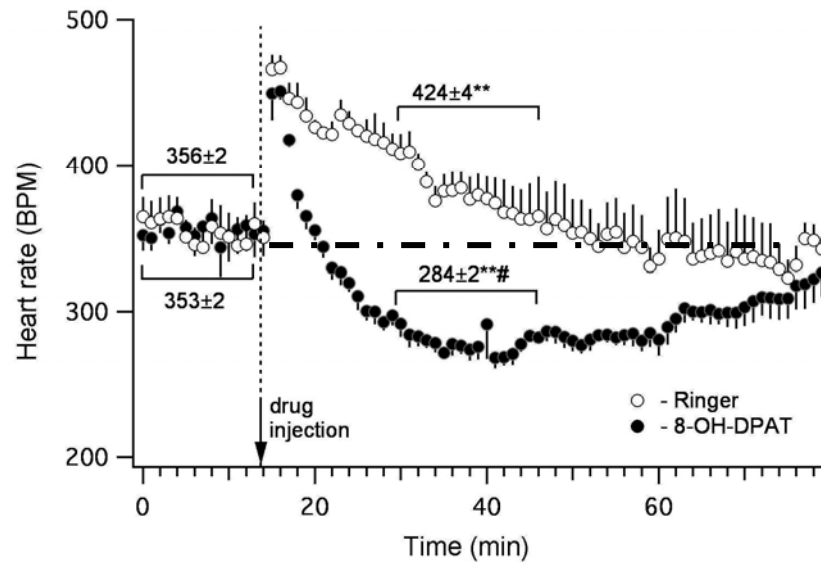


**Fig. 16.** Control experiment: microinjection of muscimol into the ventral striatum did not affect stress-induced tachycardia. Each data point is the mean of 1-min average from six animals, after injection of either vehicle (○) or muscimol (●). Data above the traces show absolute values of heart rate for 10-min periods; \*\*  $P < 0.01$ ; \*  $P < 0.05$  for each condition during stress vs. pre-stress. Data below the traces show delta heart rate values (compared with pre-stress).

## **PART II: Effects of activation of 5-HT<sub>1A</sub> receptors on stress induced tachycardia in rats.**

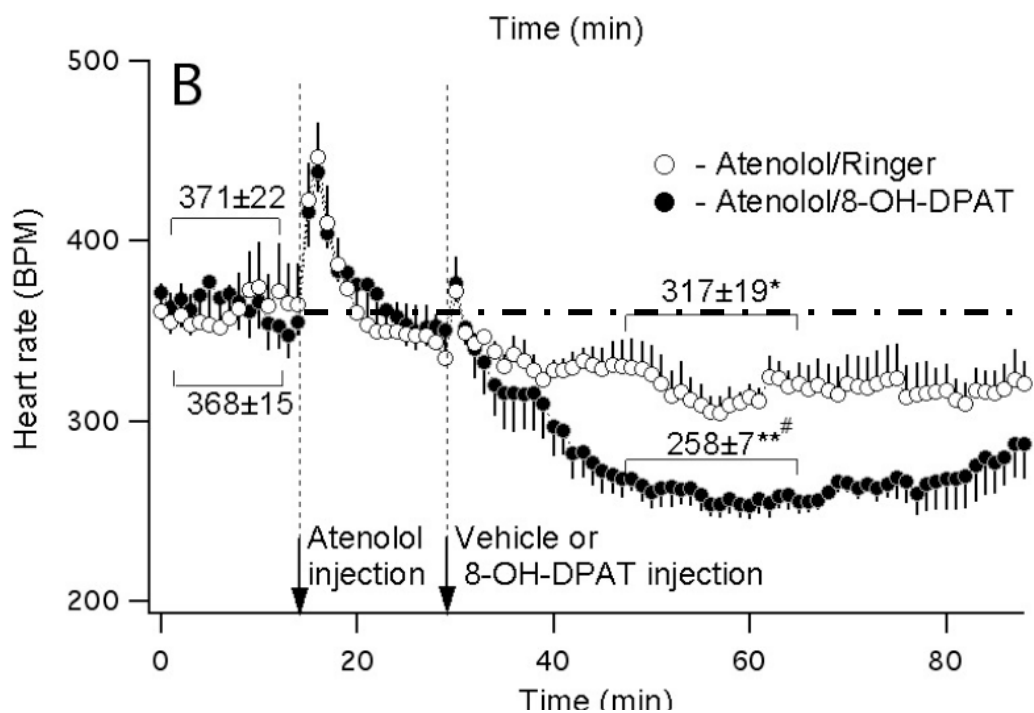
### **2.1 Effect of systemic 8-OH-DPAT on the basal heart rate and on HRV indices.**

Subcutaneous injection of either vehicle or 8-OH-DPAT (100 $\mu$ g/kg) caused transient tachycardia of similar magnitude. After the drug, HR fell within 10-15 min to a level significantly lower than the basal, and remained at this low level from about 15 to 40 min post-injection (Fig. 17A). In contrast, after vehicle, reversion of injection-induced tachycardia was quite slow, so that during the period from 15 to 40 min post-injection, HR was still different from basal level and from the corresponding values after 8-OH-DPAT. There was also a significant difference in the speed of HR decay from the peak:  $T_{1/2}$  was  $2.5\pm 0.7$  min and  $13\pm 1.2$  min after vehicle and drug, respectively ( $p<0.01$ ,  $n=6$ ). Treatment with 8-OH-DPAT substantially and significantly elevated HRV indices that reflect vagal modulation of the heart rate – RMSSD ( $1.6\pm 0.1$  and  $4.8\pm 0.7$  ms after vehicle and drug, respectively;  $p<0.01$ ,  $n=6$ ) and high-frequency power of the HRV ( $0.9\pm 0.2$  and  $4.9\pm 0.3$  ms<sup>2</sup> after vehicle and drug, respectively;  $p<0.05$ ,  $n=6$ ).



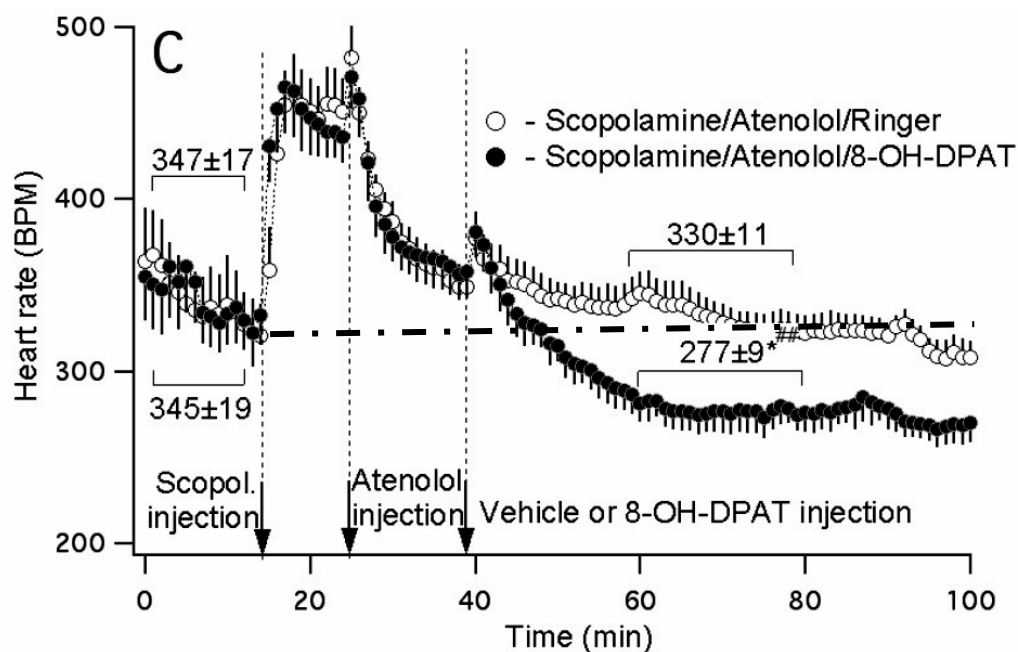
**Fig. 17 A.** Systemic injection of 8-OH-DPAT, 100 $\mu$ g/kg s/c, reduces basal HR when given alone in 6 rats(n=6). White circles (O) – vehicle; black symbols (●) – 8-OH-DPAT (100  $\mu$ g/kg s.c.). Mean values are presented near corresponding traces. Significantly different from pre-injection basal level: \*\* - significantly different from the pre-injection basal level:  $p < 0.01$ , respectively. ## - significantly different from vehicle for the same time point:  $p < 0.01$ , respectively.

Next experiment, then tried to determine whether 8-OH-DPAT-induced bradycardia could be prevented by beta-adrenergic blockade (Fig. 17B). Administration of Atenolol caused short-lasting tachycardia, so that 15 min later, just before the injection of vehicle or 8-OH-DPAT, HR did not differ from basal values for both conditions. This second injection provoked tachycardic responses that were minor compared to those after the first injection. While the HR after injection of vehicle still continued to fall, the bradycardia after the drug was more prominent, with a clear downward deflection (Fig.17B). There were compared HR during the period of maximal action of 8-OH-DPAT (detected in the previous experiment); the values were significantly different between the two conditions, and were lower compared to the corresponding basal (pre-atenolol) values.



**Fig. 17B** Systemic injection of 8-OH-DPAT (100µg/kg s/c) after β-adrenergic blockade with atenolol. White circles (O) – vehicle; black symbols (●) – 8-OH-DPAT (100µg/kg s.c.). Mean data values are presented near corresponding traces. \* and \*\* - significantly different from the pre-injection basal level,  $p < 0.05$  and  $p < 0.01$ , respectively. ## - significantly different from vehicle for the same time point,  $p < 0.01$ , respectively.

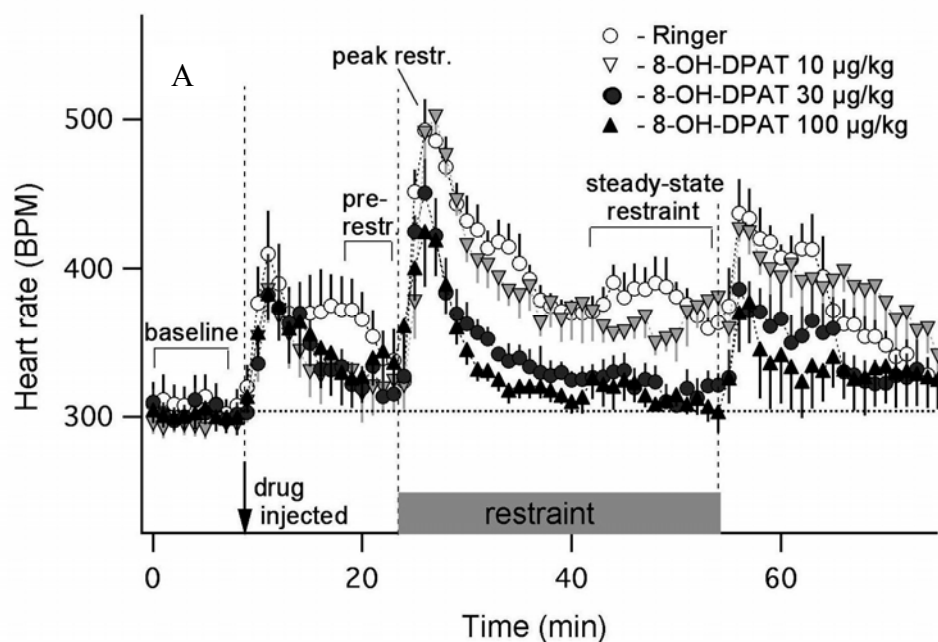
In the following experiment tested whether 8-OH-DPAT-induced bradycardia persists after a combined vagal and sympathetic blockade (Fig. 17C). Administration of methyl-scopolamine caused sustained tachycardia; injection of atenolol 10 min later caused a fall in HR after a small injection-related tachycardia. The time course of HR change did not differ for both conditions prior to the third injection. As shown in Fig. 16C, 8-OH-DPAT still produced a substantial bradycardic effect, so during the maximum of this effect, HR values were significantly different from both corresponding values after the vehicle and from the basal (pre-scopolamine) values.



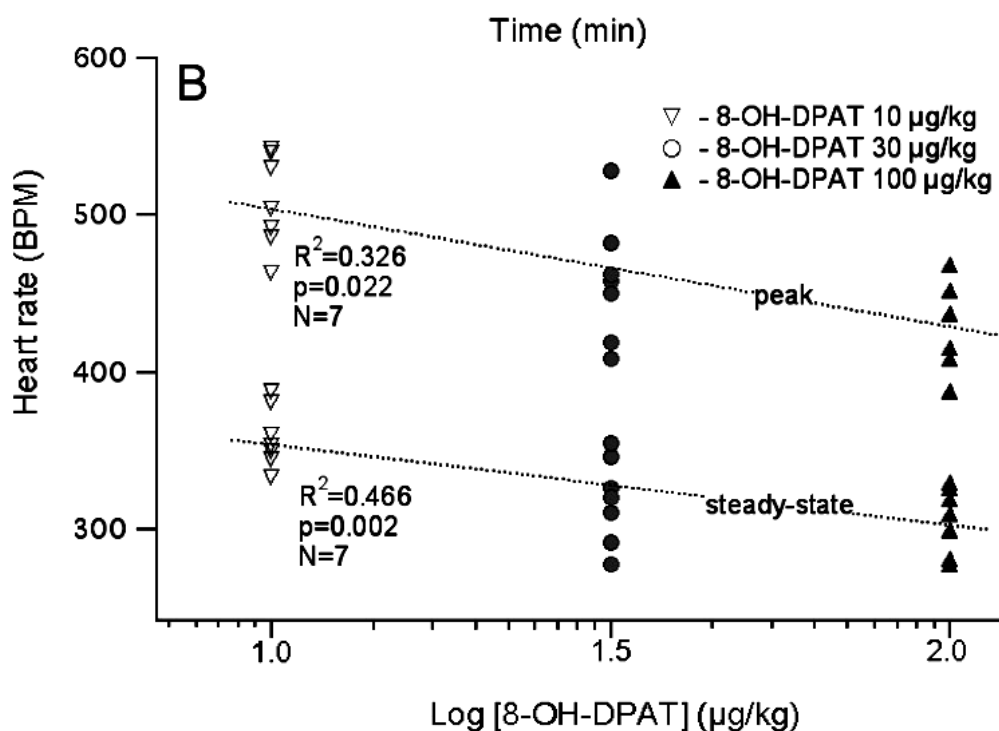
**Fig. 17C** Systemic injection of 8-OH-DPAT (100 $\mu$ g/kg sc) after combined  $\beta$ -adrenergic and muscarinic-cholinergic blockade with atenolol and methylscopolamine. These 3 experiments were conducted in 3 separate groups of rats (n=6 for each). White circles (O) – vehicle; black symbols (●) – 8-OH-DPAT (100 $\mu$ g/kg s/c.). Mean data values are presented near corresponding traces. \*- significantly different from the pre-injection basal level,  $p < 0.05$ , respectively. # - significantly different from vehicle for the same time point,  $p < 0.01$ , respectively.

## **2.2 Effect of systemic 8-OH-DPAT on cardiac responses elicited by restraint stress.**

In this experiment (n=7) tested whether activation of 5-HT<sub>1A</sub> receptors with 8-OH-DPAT (administered systemically at doses of 10, 30 and 100µg/kg) affects restraint-induced cardiac responses. Mean group data are shown in Fig. 18A, and data values are presented in Table 2A. Tachycardia associated with drug or vehicle administration reverted to the basal level within 15 min, so there was no difference for the pre-restraint values between the four conditions. After vehicle, restraint stress caused tachycardia which peaked at about 500 BPM within 1-1.5 min and then started to decline, approaching the steady-state level within 10-15 min and remaining at this level (or sometimes slowly declining) until the end of the restraint. In subsequent sections were thus refer to these data points as “peak restraint” and “steady-state restraint”. Effects of pre-treatment with 8-OH-DPAT depended on the dose used. After the dose of 10µg/kg, restraint induced tachycardia did not differ from the control condition. At the dose of 30µg/kg, 8-OHDPAT substantially reduced the steady-state increase in HR, and at the dose of 100µg/kg, the drug attenuated both initial peak tachycardia and the steady-state increase in the HR (Table. 2). Fig. 18B shows results of the linear regression analysis.



**Fig.18** Systemic pre-treatment with 8-OH-DPAT attenuated tachycardia elicited by the restraint stress. A - Traces showing changes in the heart rate in animals (n=7) pre-treated, on different days, with either vehicle or three doses of 8-OH-DPAT (10, 30 and 100µg/kg s/c.). Time of injection and the restraint period are indicated on the time axis. Also marked are the time periods (baseline, pre-restraint, peak restraint and steady-state restraint) during which the data value is statistically assessed. The data values from this experiment are presented in Table 2.



**Fig.18 B** Systemic pre-treatment with 8-OH-DPAT attenuated tachycardia elicited by the restraint stress. B – Results of regression analysis of data from (A) showing dose-dependence of 8-OH-DPAT action for both peak (upper graph) and steady-state (bottom graph) components of restraint-induced tachycardia.

**Table 2 Restraint-induced tachycardia and effects of 5-HT<sub>1A</sub> receptor ligands.**

	Baseline	Pre-restraint	Peak restraint	Steady-state restraint
A. Effects of 8-OH-DPAT				
Vehicle	318±3	353±15	492±21** (+137±19)	379±12** (+27±13)
8-OH-DPAT 10 µg/kg	311±3	321±4	501±10** (+181±16)	365±3* (+43±15)
8-OH-DPAT 30 µg/kg	307±2	320±4	450±23** (+132±17)	320±2##@ (+2±18)
8-OH-DPAT 100 µg/kg	310±2	337±5	419±17**##@ (+81±14##@)	314±2##@ (-23±14##@)
B. WAY-100,635 prevents effects of 8-OH-DPAT				
Vehicle + Vehicle	327±3	387±16	514±8** (+187±21)	386±14* (+58±17)
Vehicle + 8-OH-DPAT	318±3	334±12	400±23** (+84±16)	318±18 (+1±15)
WAY-100,635 + 8-OH-DPAT	331±2	414±13	508±6** (+175±19)	392±6* (+60±17)

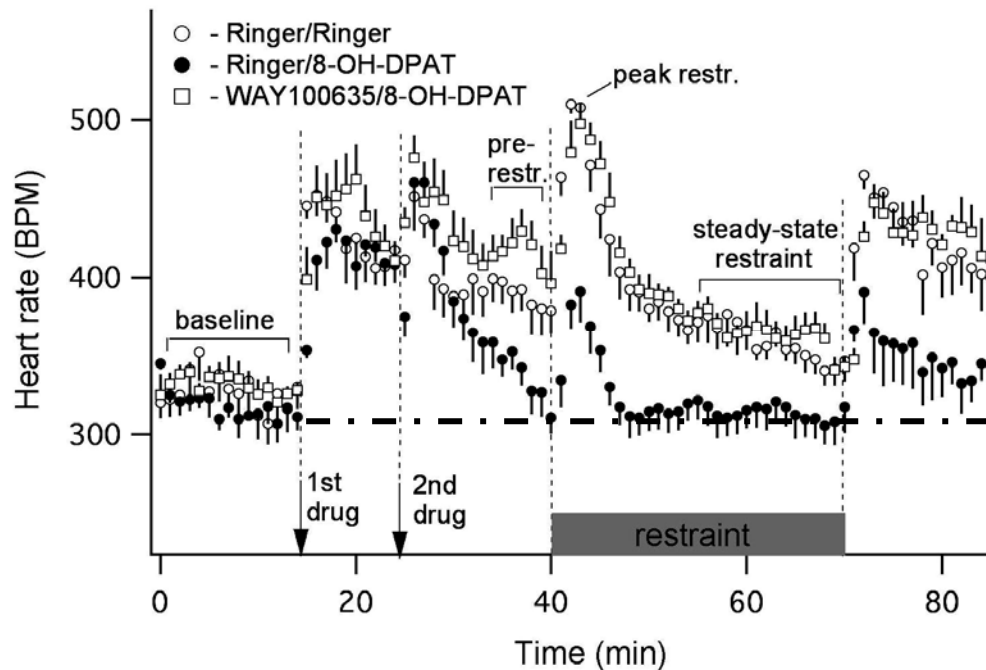
The above data is presented as mean±S.E.M. (BPM). For each set of data, the top line shows absolute values (Table 2A and B), and bottom line shows the difference between the pre-restraint value and peak or steady-state value during restraint (Table 2A). Because pre-restraint level was different between conditions in Table 2B, the bottom lines show here the difference between the basal level and peak or steady-state value during restraint. \*\* - significantly different from corresponding basal level value,  $p < 0.01$ ; # - significantly different from vehicle,  $p < 0.05$ ; ## - significantly different from vehicle,  $p < 0.01$ ; @ - significantly different from the lowest dose of 8-OH-DPAT,  $p < 0.05$ ; @@ - significantly different from the lowest dose of 8-OH-DPAT,  $p < 0.01$ ; \_ - significantly different from Vehicle/Vehicle condition,  $p < 0.05$ ; \_\_ - significantly different from Vehicle/Vehicle condition,  $p < 0.01$ ; - significantly different from WAY-100,635 + 8-OH-DPAT condition,  $p < 0.05$ ; \_ - significantly different from WAY-100,635 + 8-OH-DPAT condition,  $p < 0.01$ .

### **2.3 Effect of systemic 8-OH-DPAT on restraint-induced tachycardia after selective blockade of 5-HT<sub>1A</sub> receptors.**

In order to determine whether selective blockade of 5-HT<sub>1A</sub> receptors prevents anti-tachycardic effects of 8-OH-DPAT during stress. There were compared the restraint-induced changes in HR in three conditions using the following drug combinations:

- i) Ringer solution/Ringer solution
- ii) Ringer solution/8-OH-DPAT
- iii) WAY-100,635/8-OH-DPAT

Pre-restraint, peak and steady-state values did not differ between Ringer/Ringer and WAY-100,635/8-OH-DPAT conditions; both were however substantially and significantly different from the Ringer/8-OH-DPAT condition (Fig.19 and Table2B). Thus, selective blockade of 5-HT<sub>1A</sub> receptors completely abolished the effects of 8-OH-DPAT.



**Fig. 19** Selective blockade of 5-HT<sub>1A</sub> receptors with WAY-100,635 (100 $\mu$ g/kg s/c.) prevents anti-tachycardic effects of 8-OH-DPAT (100 $\mu$ g/kg s/c) during restraint stress; n=6. Traces show changes in heart rate in animals pre-treated, on different days, with the following drug or vehicle combination: vehicle/vehicle (Control), vehicle/8-OH-DPAT (5-HT<sub>1A</sub> receptor activation) and WAY-100,635/8-OH-DPAT (5-HT<sub>1A</sub> receptor agonist after receptor blockade). Time of injection and the restraint period are indicated on the time axis. Also marked are the time periods (baseline, pre-restraint, peak restraint and steady-state restraint) during which data values are statistically assessed. The data values from this experiment are presented in Table 3.

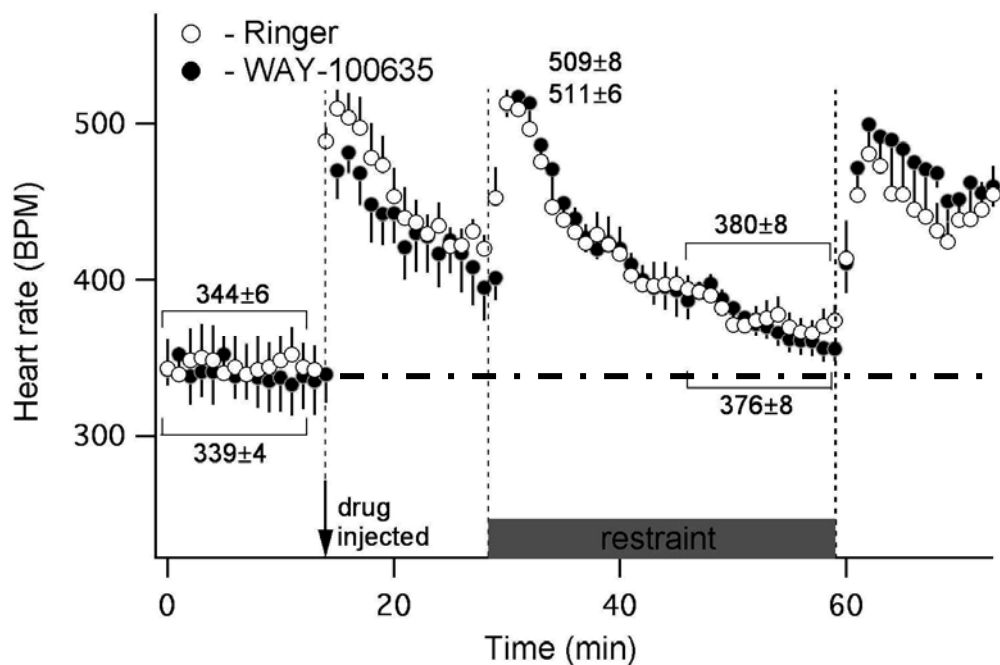
**Table 3. Effects of autonomic blockade on restraint-induced tachycardia.**

	$\Delta$ (Peak/Pre-restraint)	$\Delta$ (Steady-State/Pre-restraint)
Ringer solution	124±11	25±6
Atenolol	28±4*	1±4*
Methylscopolamine	97±11*	52±6*

The above data is presented as differences between peak during restraint and pre-restraint values (left column) and between “steady-state” restraint and pre-restraint values (right column). \*\* -significantly different ( $p < 0.01$ ) from both Ringer and Methyl-scopolamine conditions; ## -significantly different ( $p < 0.05$ ) from both Ringer and Atenolol conditions.

#### **2.4 Effects of systemically administered WAY-100,635 on restraint-induced tachycardia.**

In order to test whether there are any 5-HT<sub>1A</sub> receptor-dependent cardiac effects due to intrinsic 5-HT release during stress, rats were placed to the restraint after injection of either vehicle or WAY-100,635 (100µg/kg s/c), a selective 5-HT<sub>1A</sub> receptor antagonist. The experiment did not find any differences between vehicle and drug conditions (see Fig. 20 for graphs and data values, n=6).

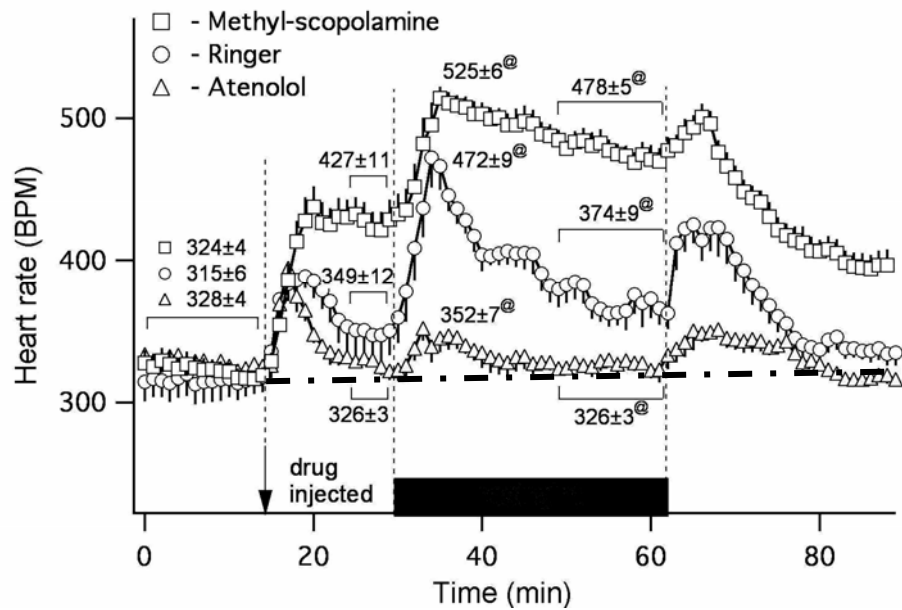


**Fig.20** Selective blockade of 5-HT<sub>1A</sub> receptors with WAY-100,635 (100µg/kg s/c) did not affect tachycardia elicited by the restraint stress. Traces show changes in heart rate in animals pre-treated, on different days, with either vehicle or WAY-100,635; n=6. Time of injection and the restraint periods are indicated on the time axis. Mean data values are presented near corresponding traces.

## **2.5 Effects of autonomic blockade on restraint-induced tachycardia.**

In the next set of experiments (n=8), the study addressed the question of which autonomic components mediate restraint-induced tachycardia. For this purpose, rats were subjected to the restraint 15 min after injection of either atenolol, methyl-scopolamine or vehicle (Fig. 21; data values for HR are also presented in this figure; for the differences in HR values see Table 3). After vehicle, the restraint provoked tachycardic responses similar to those described in the previous sections. In animals with sympathetic blockade, restraint provoked only a small transient tachycardia, and during the second half of restraint, HR did not differ from pre-restraint or basal values.

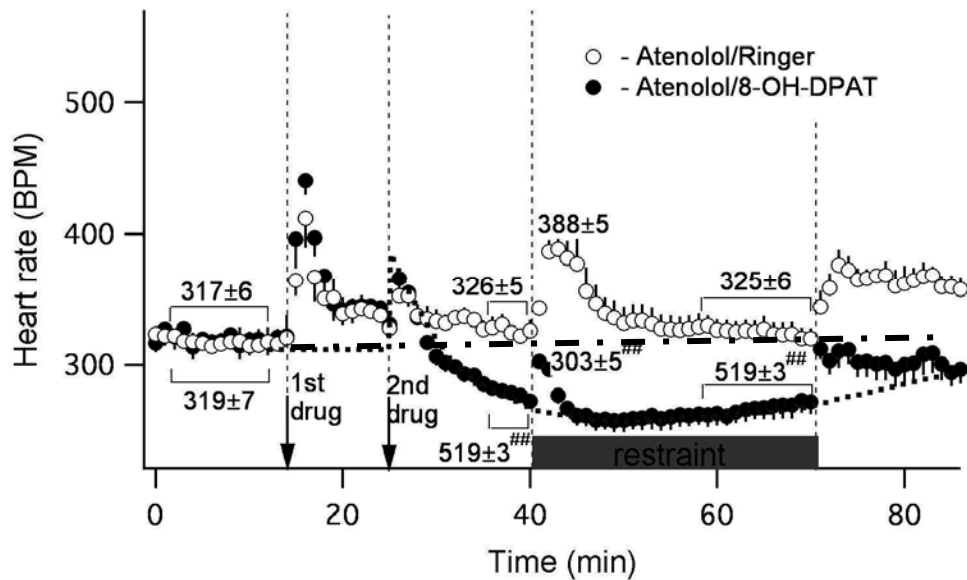
Administration of methyl-scopolamine caused a rapid rise in the HR and remained elevated. Subjecting rats to the restraint after vagal blockade caused initial tachycardia, with HR values significantly higher compared to post-vehicle restraint. After vagal blockade, the increase in the HR for the “steady-state” component (vs. pre-restraint values) was significantly higher compared to Ringer (Table 3). For both vehicle and methyl-scopolamine conditions, “steady-state” values were significantly different compared to pre-restraint ( $p < 0.01$ ).



**Fig. 21** Autonomic blockade reveals components of restraint-elicited tachycardic responses. Traces show changes in heart rate in animals pre-treated, on different days, with vehicle (Ringer),  $\beta$ -adrenergic blocker atenolol (2mg/kg s/c) or muscarinic holinergic blocker methylscopolamine (50 $\mu$ g/kg s/c); n=8. Time of injection and the restraint period are indicated on the time axis. Mean data values are presented near corresponding traces. \* and \*\* - significantly different from the corresponding pre-restraint level,  $p < 0.05$  and  $p < 0.01$ , respectively. The horizontal dashed line highlights pre-restraint values of the HR. Note substantial difference during the second half of the restraint between the three conditions.

## **2.6 Effects of systemic 8-OH-DPAT on restraint-induced tachycardia after sympathetic blockade.**

In this experiment (n=6), after sympathetic blockade with atenolol, either 8-OH-DPAT or vehicle was administered prior to the restraint (see Fig. 22 for traces and data values). The vehicle injection provoked a small and short-lasting tachycardia, so that prior to the restraint, HR did not differ from the basal level. In vehicle-treated animals, the restraint elicited only an initial transient tachycardic component of moderate amplitude. Injection of 8-OH-DPAT caused slow and long lasting bradycardia, so that before the restraint, HR was significantly different from both the basal level and from the pre-restraint value in the Vehicle condition. After 8-OH-DPAT, the restraint provoked a small transient tachycardic response ( $+25\pm 3$  BPM) that was significantly different compared to vehicle ( $+62\pm 5$  BPM,  $p < 0.01$ , n=6). The time course of 8-OH-DPAT-elicited bradycardia was similar to that occurring in the first experiment of this study (ie. the drug alone).

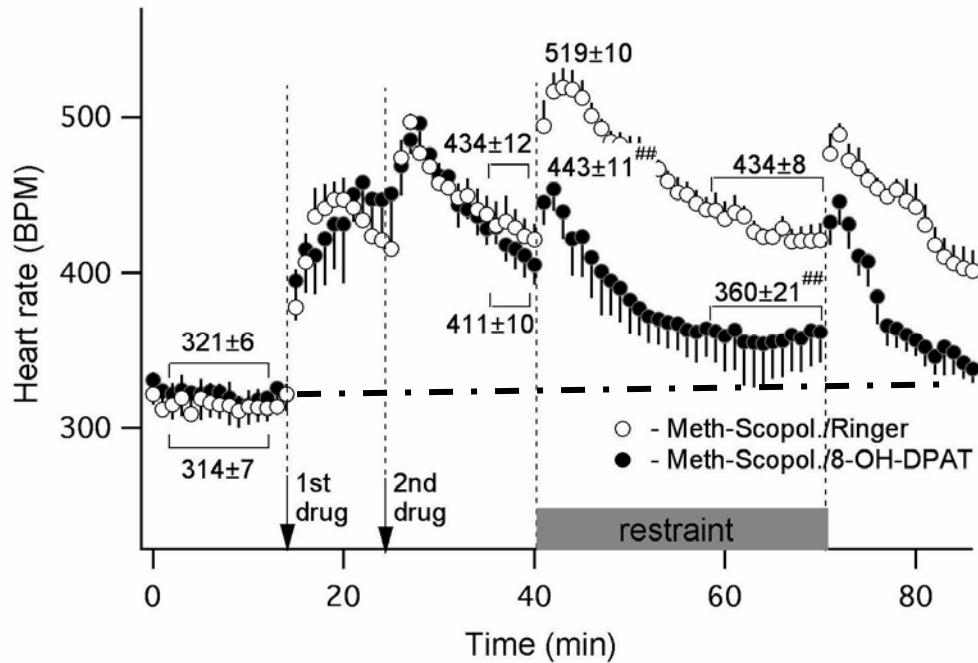


**Fig. 22** Systemically administered 8-OH-DPAT attenuates transient stress-induced tachycardia persisting after  $\beta$ -adrenergic blockade with atenolol (2mg/kg). Traces show changes in heart rate in animals pre-treated, on different days, with either Atenolol/Ringer (O) or Atenolol/8-OHDPAT (●) combination; n=6. Drugs were administered subcutaneously. Time of injection and the restraint period are indicated on the time axis. Mean data values are presented near corresponding traces.

### - significantly different from vehicle for the same time point,  $p < 0.01$ .

## **2.7 Effects of systemic 8-OH-DPAT on restraint-induced tachycardia after vagal blockade.**

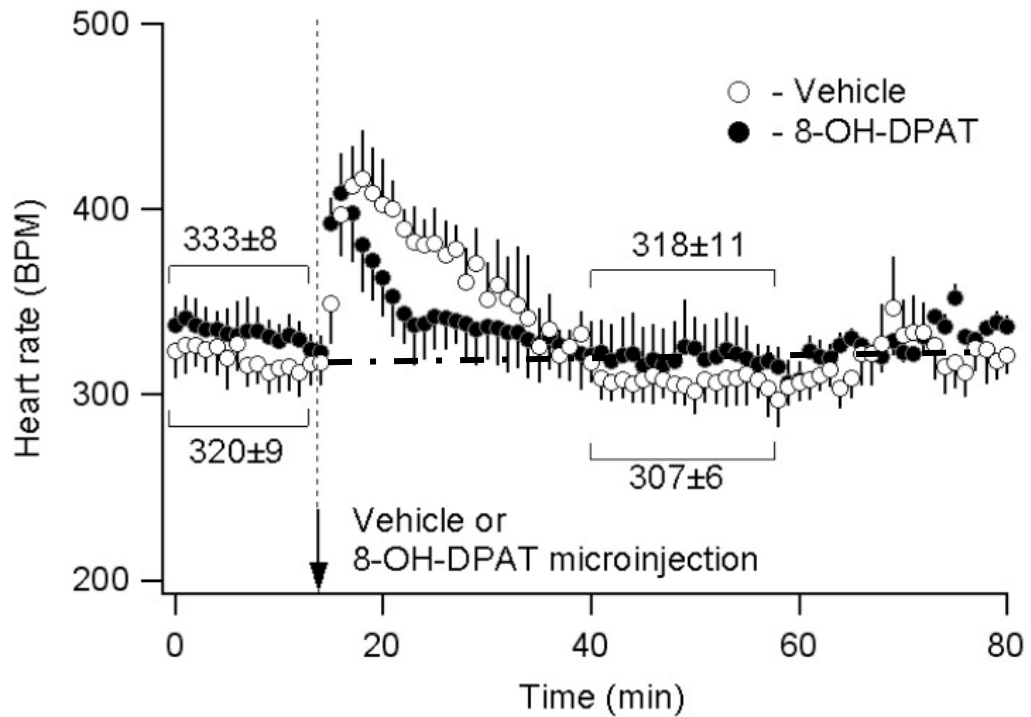
In 6 animals tested, administration of methyl-scopolamine caused a rapid increase in HR. Effects of a subsequent injection of 8-OH-DPAT did not differ from those of vehicle, so that the pre-restraint values for both conditions were not different (see Fig. 23 for traces and data values). Restraint-induced tachycardia was substantially and significantly attenuated in 8-OH-DPAT treated animals, in terms of both absolute values (see Fig. 24 ) and the magnitude of increase ( $+85 \pm 19$  BPM after vehicle vs.  $+32 \pm 9$  BPM after the drug,  $p < 0.01$ ,  $n = 6$ ). After reaching peak values, HR began to fall, with a time course similar for both conditions, so that “steady-state” values were also significantly different from each other, but not different from corresponding pre-restraint values (although there was a tendency for a fall for the drug condition, with  $p = 0.062$ ).



**Fig. 23** Systemically administered 8-OH-DPAT attenuates stress-induced tachycardia persisting after muscarinic cholinergic blockade with methyl-scopolamine (50 $\mu$ g/kg). Traces show changes in heart rate in animals pre-treated, on different days, with either methyl-scopolamine/Ringer (O) or methyl-scopolamine/8-OH-DPAT (●) combinations; n=6. Drugs were administered subcutaneously. Time of injection and the restraint periods are indicated on the time axis. Mean data values are presented near corresponding traces. ## - significantly different from vehicle for the same time point,  $p < 0.01$ .

## **2.9 Effects of intra-medullary microinjection of 8-OH-DPAT on the basal heart rate.**

Apart from a short-lasting tachycardia associated with handling, microinjection of either Ringer's solution or 8-OH-DPAT into the medullary raphe had no effect on the basal heart rate (see Fig. 24 for illustrations and data values). Handling-related increase in HR was shorter after 8-OH-DPAT ( $T_{1/2}=4.2\pm 0.4$  min) compared to Ringer ( $T_{1/2}=11\pm 0.9$  min,  $p<0.05$ ,  $n=6$ ).

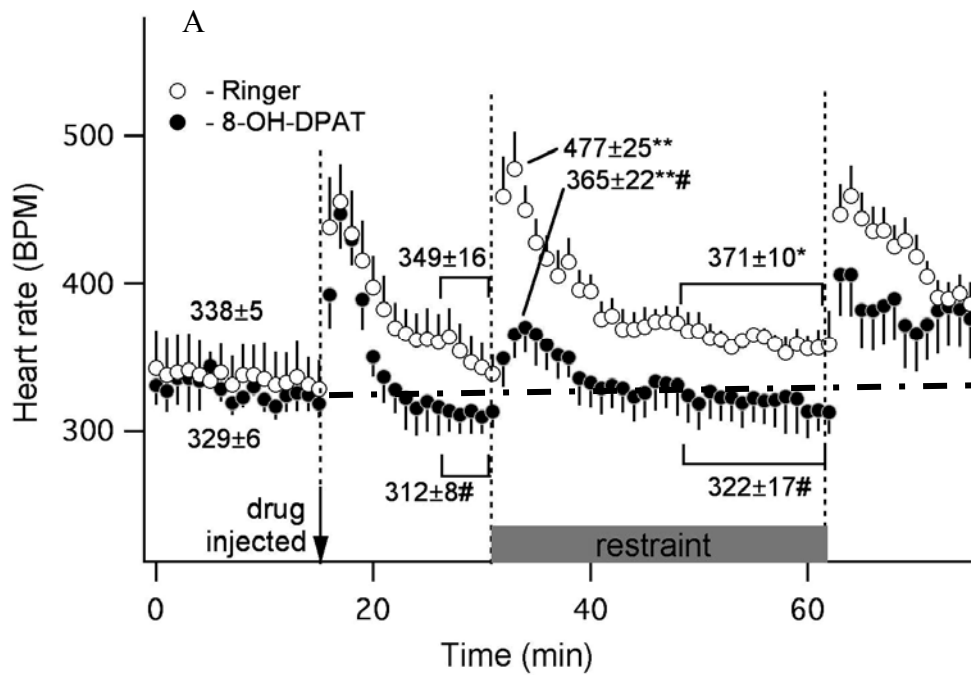


**Fig. 24** Intra-raphé microinjection of 8-OH-DPAT does not affect basal heart rate. Traces show changes in heart rate in animals treated, on different days, with either vehicle (O) or 8-OH-DPAT (1nmol in 100nl; ●); n=6. Note faster return to the baseline after the drug. Time of injection is indicated on the time axis.

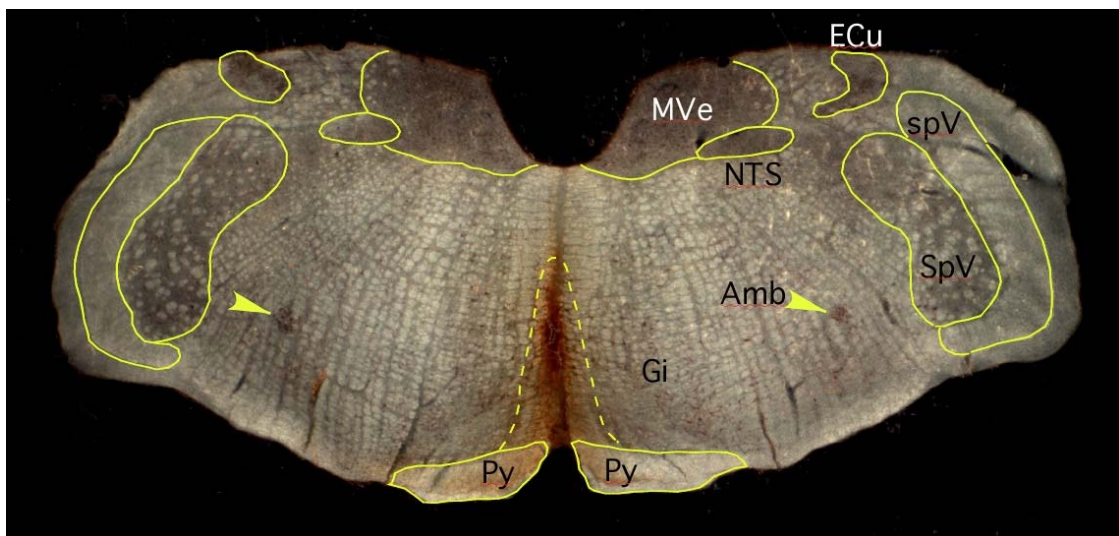
### **2.10 Effects of intra-medullary microinjection of 8-OH-DPAT on restraint-induced tachycardia.**

To identify the potential location of 5-HT<sub>1A</sub> receptors responsible for the described above anti-tachycardic effects of 8-OH-DPAT, drug or vehicle was microinjected into the raphe/parapyramidal area of the lower brainstem (n=8). The procedure of animal handling during injection caused transient tachycardia of similar magnitude, but the return to the basal level (or, in some animals, even slightly below this level) was faster after 8-OH-DPAT, so that pre-restraint HR values were significantly different (see Fig. 25 for graphs and data values).

After 8-OHDPAT, restraint-induced tachycardia was attenuated, both in terms of absolute HR values for the transient and steady-state components (Fig. 25A) and in terms of differences between the pre-restraint and the restraint values. For the peak tachycardia, the latter were reduced from  $+144 \pm 11$  to  $+59 \pm 7$  BPM (n=8,  $p < 0.05$ ), and for the steady-state component from  $+31 \pm 4$  to  $8 \pm 5$  BPM. An example of a histologically confirmed injection site is presented in Fig. 24B. In another 5 animals, control microinjections were performed 2.5 mm more dorsally. The results did not find any effects of 8-OH-DPAT in this control group (data not shown).



(B)

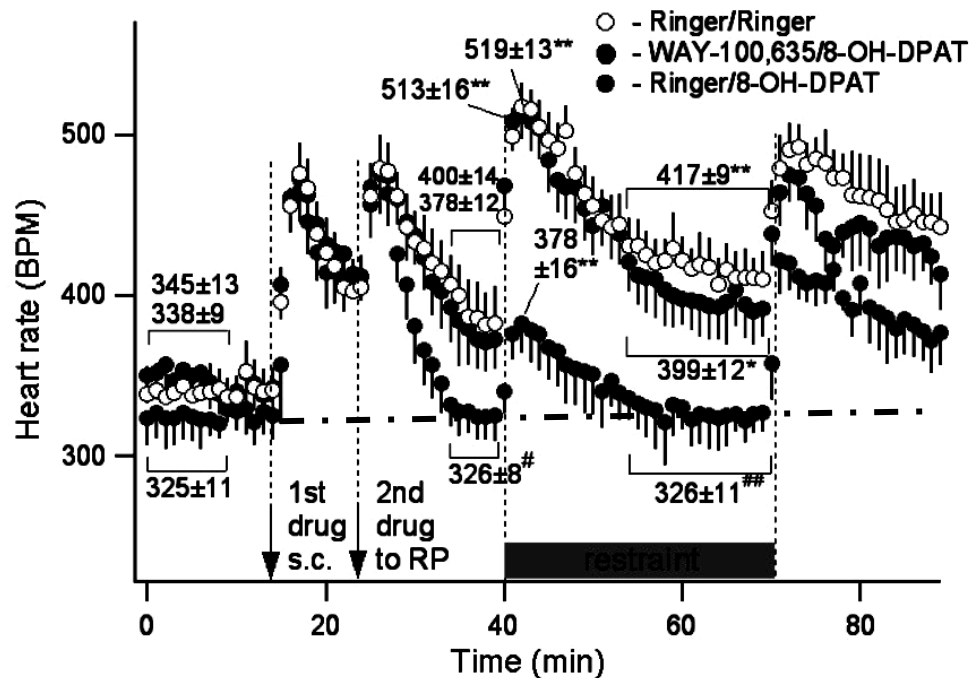


**Fig 25** Intra-raphe microinjection of 8-OH-DPAT attenuated tachycardia elicited by restraint stress. (A) - traces show changes in heart rate in animals pre-treated, on different days, with either vehicle or 8-OH-DPAT (1nmol in 100nl; n=8). Time of injection and the restraint period are indicated on the time axis. Mean data values are presented near corresponding traces \* and \*\* -significantly different from the pre-injection basal level,  $p < 0.05$  and  $p < 0.01$ , respectively; # -significantly different from

vehicle for the same time point,  $p < 0.05$ . (B) – Illustration of the intra-medullary injection site (dark-field photograph of the coronal section cut through the lower brainstem). Brown area surrounded by the dashed line contains the HRP reaction product (see Methods). Abbreviations: Amb – nucleus ambiguus; ECu – external cuneate nucleus; Gi –gigantocellular reticular nucleus; NTS – nucleus of the solitary tract; Py – pyramid; spV – spinal tract of the trigeminal nerve; SpV – spinal nucleus of the trigeminal nerve.

### **2.11 Effects of systemic WAY-100,635 on anti-tachycardic action of intra-medullary administered 8-OH-DPAT.**

In the experiment described above, 8-OH-DPAT was microinjected into the raphe/parapyramidal area at relatively high concentration. In order to prove that the anti-tachycardic effect of the drug represented a specific effect mediated by its interaction with 5-HT<sub>1A</sub> receptors, in another 6 rats intra-medullary microinjections of 8-OH-DPAT were preceded by systemic administration of either WAY-100,635 (100µg/kg) or vehicle; systemic vehicle followed by intra-medullary vehicle served as a control. As illustrated in Fig.26, if the drug was given after the vehicle, it substantially and significantly attenuated restraint-induced tachycardia, similar to the previous experiment. Pre-treatment with WAY-100,635 completely abolished the effect of 8-OH-DPAT, so that the magnitude of the tachycardia did not differ from that following intra-medullary administration of the vehicle. Data values are presented in Fig.26.



**Fig. 26** Systemically administered WAY-100,635 (100µg/kg) prevents anti-tachycardic effects of intra-medullary microinjected 8-OH-DPAT. Traces show changes of heart rate in animals pre-treated, on different days, with the following combination of drugs: (O) - Ringer (s/c) / Ringer (brain microinjection, 100nl); (●)-Ringer (s/c) / 8-OH-DPAT (brain microinjection, 1nmol in 100nl); (●) - WAY-100,635 (s/c, 100µg/kg) / 8-OH-DPAT (brain microinjection, 1nmol in 100nl). Time of injections and the restraint period are indicated on the time axis. Mean data values are presented near corresponding traces \* and \*\* - significantly different from the pre-injection basal level,  $p < 0.05$  and  $p < 0.01$ , respectively; # and ## - significantly different from two other conditions (Ringer/Ringer and WAY-100,635/8-OH-DPAT) for the same time point,  $p < 0.05$  and  $p < 0.01$ , respectively.

## CHAPTER VI

### DISCUSSION

#### **PART I: Effects of inhibition of the amygdala on stress-induced tachycardia in rats.**

This study is the first investigating the effect of pharmacological inhibition of the amygdala on stress-induced tachycardiac and HRV responses. The study have demonstrated that activation of inhibitory GABA<sub>A</sub> receptors in the amygdala prior to restraint stress reduces the duration, but not the magnitude, of tachycardia and prevents the increase of the LF/HF ratio of the spectral power. Another novel finding is that restraint stress modifies spectral parameters of the HRV in a manner believed to reflect sympathetic activation and vagal inhibition.

The major aim of this study was to investigate the role of the amygdala in cardiac responses to unconditioned psychological stressors. Tachycardia during restraint stress in rats is well-documented (Barron and Van Loon 1989; Chen and Herbert 1995; McDougall, Widdop et al. 2005). It occurs predominantly due to an increase in cardiac sympathetic nerve activity (Barron and Van Loon 1989). Substantial, but indirect evidence suggests that the amygdala could be the source of this elevated cardiac sympathetic drive. Many animal studies report activation of amygdala neurones by stressful situations Substantial, but indirect evidence suggests that the amygdala could be the source of this elevated cardiac sympathetic drive. Many animal studies report activation of amygdala neurones by stressful situations (Dayas, Buller et al. 1999; Chen, Joaquim et al. 2005; Crane, French et al. 2005; Trneckova, Armario et al. 2006). Electrical or chemical stimulation of the amygdala evoked increases in heart rate (Galeno and Brody 1983; Gelsema, McKittrick et al. 1987; al Maskati and Zbrozyna 1989; Soltis and DiMicco 1991). Lesion of the

amygdala consistently attenuated pressor responses to conditioned and unconditioned stressful stimuli, but produced controversial results with regard to the stress-induced cardiac changes, most often being without effect (Galeno and Brody 1983; Iwata, LeDoux et al. 1986; Roozendaal, Koolhaas et al. 1991; Sanders and Shekhar 1991; Carter, Pinnock et al. 2004). In the now classical study by LeDoux and his colleagues (1986), authors specifically emphasized that amygdala lesion did not affect tachycardic response to conditioned stimuli, in contrast to pressor and locomotor responses. In humans, stress-induced amygdala activation correlates with the magnitude of associated tachycardia (Critchley, Rotshtein et al. 2005). All these findings indicate that the amygdala neurons responsible for tachycardia *may be* activated during stress; my findings provide first direct evidence that they are indeed activated. Using similar experimental strategy, Kubo et al. (2004) recently demonstrated that activation of the amygdala neurons is also essential for the restraint-induced pressor responses.

The experiment examined amygdala effects immediately after pharmacological inhibition of its neurons, and I thus suggest that this condition is essential for revealing cardiac effects elicited by this brain area during stress. The experiments found that tachycardia provoked by restraint stress consists of two components – early amygdala-independent and late amygdala-dependent. Amygdala blockade did not affect the magnitude of the initial tachycardia, suggesting that it is initiated from a different brain region. In contrast, the sustained component of tachycardia was totally abolished after the blockade. The results thus do not contradict previous lesion studies (which used different stress paradigms) where only early tachycardic component was present and unaffected by the lesion (Iwata, LeDoux et al. 1986; LeDoux, Sakaguchi et al. 1986). The initial tachycardia likely reflects a general arousal reaction, not necessarily associated with emotional coloration, whereas sustained long-lasting increase of heart rate may be the result of perceiving the situation as unpleasant or threatening. This interpretation is in agreement with the established role of the amygdala in emotional processing.

This study is also the first where cardiac effects of a direct intervention in a defined brain area were examined using heart rate variability analysis, an empirical

tool for assessing changes in vagal and sympathetic cardiac autonomic neural activity. The experiments found that restraint causes increase in the LF component and decrease in HF component of the spectral power. According to the accepted interpretation of HRV data (1996), this suggests increased sympathetic and reduced parasympathetic outflow to the heart, as HF component predominantly reflects vagal activity, whereas both autonomic branches contribute to the LF component. HRV data are thus in agreement with previous reports, both in humans and rodents, showing that psychological stressors modify heart rate variability, leading to an increase of the LF/HF ratio (Sgoifo, Stilli et al. 1996; Inagaki, Kuwahara et al. 2004; Lucini, Di Fede et al. 2005). As amygdala blockade prevented changes in both high-frequency and low-frequency components of the power spectrum (and changes in their ratio), we speculate that sustained tachycardia during restraint was a result of amygdala-induced reciprocal activation of cardiac presympathetic and inhibition of cardiac vagal neurons.

Based on the finding that muscimol injections did not affect basal heart rate, and that its bradycardic effect was directly proportional to the stress-evoked response, these results conclude that the amygdala does not contribute to the control of heart rate at rest. The results are in good accord with the current understanding of central mechanisms that regulate the cardiovascular system during stress (Dampney, Coleman et al. 2002). The dorsomedial hypothalamus is a critical component of the pathway mediating stress-induced cardiac effects (DiMicco, Gale et al. 1979); inactivation of this area almost completely suppresses rise in heart rate during stress (Stotz-Potter, Willis et al. 1996). The dorsomedial hypothalamus is the principal target of the amygdala excitatory efferent neurons whose activation causes tachycardia (Soltis and DiMicco 1991) and it is likely that the sustained amygdala-dependent component of stress-induced tachycardia was mediated by this link in our rats. Descending projections from the dorsomedial hypothalamus then cause activation of spinal cardiac sympathetic neurons – either directly or after relaying in the brainstem (Dampney, Coleman et al. 2002).

The traditional limitation of studies based on brain microinjection of a pharmacologically active substance is the difficulty in assessing its spread. To

overcome this problem, at least in part, I performed muscimol microinjections in the adjacent area (ventral striatum), with the location of the injection cannula tip 2 mm dorsal to the intra-amygdala site. The experiments did not find any effect of muscimol on stress-induced tachycardic and HRV responses in this case, thus the location consider 2 mm radius as a conservative estimate for the drug spread from the injection site. In reality it must be smaller as I did not observe any inhibitory effects from the three “missed” sites located just outside the amygdala (Fig.13). Control experiment also demonstrates that the described effects are not a general response to the infusion of muscimol into the brain.

This study intended to inactivate the principal output region of the amygdala – the central nucleus. However, as the volume of the injectate was quite large in the experiment, it is likely that the medial amygdala was also affected, as documented by our histological observations. Interestingly, neurons in the medial amygdala are activated en masse by the restraint (Crane, French et al. 2005), and their inhibition attenuates restraint-induced pressor responses (Kubo, Okatani et al. 2004). Whether the medial amygdala also contributes to the stress-induced tachycardia remains an open question, and requires a separate study.

Attempting to conduct the experiments in the least invasive manner, they did not assess the restraint-induced effects on the arterial pressure. Pressor responses during restraint are well documented in rats (Barron and Van Loon 1989; Irvine, White et al. 1997; McDougall, Widdop et al. 2005). Recently, Kubo et al. (2004) have demonstrated that injection of muscimol into the medial amygdala attenuates these pressor responses, and thus it could well be that they were also reduced in our rats following muscimol administration. As arterial pressure - heart rate baroreflex still operates during stress (though with a right- and upward shift of the baroreflex curve as shown by Hatton et al., (1997) (Hatton, Brooks et al. 1997), this might have led to an *increase* of stress-induced tachycardia compared to controls. As the experiments found a *reduction* of this tachycardia, it is highly unlikely that baroreflex-related mechanisms compromised our results. If anything, it may be that without baroreflex engagement, anti-tachycardic effects of amygdala inhibition might be even more prominent.

The results suggest that activation of the amygdala neurons during psychological stresses necessary for a sustained elevation of the heart rate and for the modulation of cardiac autonomic nerve activity leading in the increase in LF/HF ratio of the spectral power of the heart rate. These effects could be mediated via direct or indirect connections from amygdala to the downstream-located brain areas involved in cardiac control.

## **PART II: Effects of activation of 5-HT<sub>1A</sub> receptors on stress induced tachycardia in rats.**

### **1. Effects of 8-OH-DPAT on the basal heart rate.**

Bradycardic effects of 8-OH-DPAT observed in our rats are in full agreement with previous reports comprehensively reviewed by (McCall and Clement 1994; Ramage 2001). In earlier studies, it was clearly demonstrated that the action of the drug is not peripheral (Fozard, Mir et al. 1987). Traditional interpretation of cardiac effects induced by 5-HT<sub>1A</sub> agonists is that these are caused by sympathetic withdrawal and/or vagal activation (McCall and Clement 1994; Ramage 2001), where terms “sympathetic” and “vagal” are used as synonyms for “adrenergic” and “cholinergic”. Our HRV analysis results - specifically the rise in the RMSSD (root-mean-square of the beat-to-beat differences, an index that reflects increased difference between adjacent RR interval) and in the high-frequency power (reflecting vagally-mediated respiratory sinus arrhythmia) - indicate that indeed 8-OH-DPAT modified the activity of vagal neurons. However, the fact that the drug still caused a substantial fall in HR after combined  $\beta$ -adrenergic and muscarinic receptor blockade indicates that activity of some other cardiac receptors must be involved in the genesis of 8-OH-DPAT-elicited bradycardia.

It is now well recognized that cardiac sympathetic and cardiac vagal nerve terminals, in addition to noradrenaline and acetylcholine, contain numerous other neurotransmitters, defined usually as “NANC” – “non-adrenergic non-cholinergic” (Rubino A, 1996). Acting centrally, 8-OH-DPAT modifies the activity of cardiac autonomic nerves (McCall and Clement 1994) and this must lead to the alteration of cardiac release of both classical and NANC neurotransmitters. This study consider that this is an alternative plausible explanation of the 8-OH-DPAT-induced bradycardia reported here. As 8-OH-DPAT is a well known hypothermic agent, it may be that fall in body temperature (not measured in our study) directly contributed to the overall bradycardic effect of the drug. At present it cannot define whether observed bradycardia occurred due to the reduction in the excitatory or due the increase in the inhibitory cardiac drive; clarification of the underlying

pharmacological mechanisms and evaluation of potential hypothermia-induced fall in heart rate requires further studies.

## 2. Site and mechanism of action of 8-OH-DPAT

8-OH-DPAT completely abolished the steady-state component of the restraint-induced tachycardia and substantially suppressed the initial transient component. These effects were dose-dependent, with a concentration range similar to those reported previously (McCall and Clement 1994; Ramage 2001). The effect of the drug was mediated via 5-HT<sub>1A</sub> receptors as confirmed by the sensitivity of the effect to WAY-100,635, a selective antagonist of these receptors. While the experiments cannot entirely exclude a NANC-dependent bradycardic action of 8-OH-DPAT in reducing stress-induced tachycardia, its dominant effect appears to be due to a central sympatholytic action. This follows from the finding that the major component of the stress-induced tachycardia is sympathetically mediated.

5-HT<sub>1A</sub> receptors are widely expressed in the brain, including areas involved in cardiac control during stress (Wright, Seroogy et al. 1995; Kia, Miquel et al. 1996). Of those, the medullary raphe/parapyramidal area is of major interest as this is a putative location of presympathetic cardiomotor neurons activated during stress. Evidence for this was initially presented by Zaretsky *et al.* (Zaretsky, Zaretskaia et al. 2003) who observed substantial attenuation of tachycardia after intra-raphé injection of muscimol in stressed rats. The recent study in rabbits, Naliviko E et al. found that microinjection of 8-OH-DPAT in this area attenuated tachycardic responses to the air-jet stress (Nalivaiko, Ootsuka et al. 2005). The microinjection results support this finding and confirm that the effect of the drug was indeed due to the activation of 5-HT<sub>1A</sub> receptors, as it was sensitive to the selective 5-HT<sub>1A</sub> receptor antagonist. Both systemic administration and intra-medullary microinjection of 8-OH-DPAT also caused more a rapid return of HR to the basal level after handling-related tachycardia compared to vehicle. This is not surprising as handling is also a stressful event, and likely activates the same brain areas as does restraint. While the antitachycardic action of 8-OH-DPAT during stress could be mediated by limbic structures involved in emotional processing, our microinjection experiments indicate that, at least in part, the

drug's action may be realized in the medullary raphe, via auto-inhibitory 5-HT<sub>1A</sub> receptors (Helke, Capuano et al. 1997). Intra-medullary microinjection of 8-OH-DPAT had no effect on the basal HR, in contrast to the systemic administration of the drug that caused bradycardia. This is in good accord with the already cited study by Zaretsky *et al.* (Zaretsky, Zaretskaia et al. 2003) who did not observe any changes in the basal HR after pharmacological inhibition of the raphe region by muscimol. The finding suggests that the bradycardic effect of 8-OH-DPAT (as opposed to its anti-tachycardic effect) is mediated at some other location. Additional studies are required to identify this location.

Intriguingly, pre-treatment with WAY-100,635 alone did not affect basal HR or stress-induced tachycardia, similar to a number of other studies where the antagonist prevented effects of agonists, but was without effect when given alone (eg. Stanhope et al. 1996 (Stanhope and Dourish 1996)). This suggests that during stress, there is no intrinsic release of 5-HT in the vicinity of 5-HT<sub>1A</sub> receptors involved in cardiac control. Thus the functional significance of these receptors remains unclear.

### **3. Autonomic mechanisms involved in cardiac control during stress, and modulation of these mechanisms by 8-OH-DPAT.**

This is the first pharmacological dissection of cardiac responses during restraint, a widely used stress paradigm. Restraint-induced tachycardia is well documented (Barron and Van Loon 1989; Chen and Herbert 1995; McDougall, Widdop et al. 2005) but effects of autonomic blockade on cardiac changes during restraint have not been assessed. The study found that  $\beta$ -adrenergic blockade completely abolished the steady-state tachycardia and substantially reduced the initial transient tachycardic component. As adrenals do not contribute to the rise in the HR during restraint (Barron and Van Loon 1989) the findings indicate that increase in the cardiac sympathetic nerve activity, with subsequent activation of  $\beta$ -adrenoreceptors, is the predominant mechanism mediating restraint-induced tachycardia. The sustained tachycardic component was reduced by 8-OHDPAT, and thus the drug effect could be defined as cardiac sympatholytic. Such an effect is in agreement with previous studies

in anaesthetized animals (McCall and Clement 1994; Ramage 2001), extends the previous knowledge to the conscious state and, most importantly, indicates that 5-HT<sub>1A</sub> receptor activation efficiently suppresses the *stress-induced* rise in cardiac sympathetic activity.

8-OH-DPAT strongly suppressed the stress-induced tachycardic component that persisted after muscarinic receptor blockade. As discussed above, restraint-related tachycardia (and especially its steady-state component) is sympathetically mediated, and thus, provided that the drug action is central, it must conclude that it attenuated stress-induced elevation of noradrenaline release from cardiac sympathetic nerves. These experiments cannot define here the 8-OH-DPAT-sensitive component as “steady-state” because, surprisingly, in this experiment HR was not maintained at a high level for the whole duration of the restraint. The results do not have a satisfactory explanation for why this occurred; it may be that the second drug injection in this experiment affected adaptation during restraint. What is most important here is that during restraint, 8-OH-DPAT caused a near-parallel downward shift in HR compared to the control (post-vehicle) condition, indicating that the drug sensitive component was sustained, at least compared to control. The data suggest that a central sympatholytic effect is not the only mechanism responsible for the anti-tachycardic action of 8-OH-DPAT during restraint stress. Transient vagal withdrawal is the likely mechanism underlying the initial short-lasting restraint-induced tachycardic component, as this component virtually disappeared following the muscarinic receptor blockade. It is unlikely that non-adrenergic transmitters released from the sympathetic nerves contribute to this component, as in this case the time course of their effect must be similar to that of the noradrenaline effect (which is sustained as we have demonstrated here using  $\beta$ -adrenoreceptor blockade). The fact that 8-OH-DPAT substantially reduced the atenolol-insensitive transient component indicates that activation of 5-HT<sub>1A</sub> receptors in the brain may also modify activity of cardiac vagal neurons, counteracting their inhibition during stress. This idea is supported by previous experiments in anaesthetized animals (Ramage 2001). The potential underlying mechanism could be the same as described for the gastric vagal neurons, namely their disinhibition by 8-OHDPAT- induced presynaptic suppression of GABA release (Browning and Travagli 1999).

A comparison of stress-induced responses after autonomic blockade revealed another interesting phenomenon, namely that after methyl-scopolamine, the sustained component of the tachycardia was substantially larger than in control. Clearly, the methyl-scopolamine-resistant sustained component was sympathetically-mediated, as it was completely suppressed by atenolol. It must be then that in the control situation, this steady-state component was gradually reduced by some mechanism. One potential explanation is that in the course of restraint, the initial vagal withdrawal may change to a gradual restitution of vagal activity; such sympatho-vagal coactivation has been recently reported in rats during conditioned fear (Carrive 2006). The lack of slowly developing bradycardia after  $\beta$ -adrenergic blockade does not necessarily contradict this suggestion as vagal effects could be presynaptic and thus would not be observed when noradrenaline effects are suppressed. This hypothesis is certainly speculative, and requires more direct evidence.

The anti-tachycardic effect of 8-OH-DPAT reported in my thesis is in full agreement with the previous study in rabbits of Dr. Nalivaiko et al., where they have demonstrated that the drug suppresses tachycardia elicited by air jet stress (Nalivaiko, Ootsuka et al. 2005). The tachycardic responses in rabbits were quite modest (<50 BPM), and thus the value of the current study is not only in extending our previous observation to a new species – the rat - but also in demonstrating that activation of 5-HT<sub>1A</sub> receptors may counteract a quite vigorous rise in the heart rate. Van den Buuse and Wegener (van den Buuse and Wegener 2005) recently reported anti-tachycardic effects of 5-HT<sub>1A</sub> receptor agonists during another stress paradigm, the open field. The authors found that both tachycardic effects of stress and anti-tachycardic effects of 5-HT<sub>1A</sub> agonists were strain-dependent. The results are in good accord with this report in terms of similar sensitivity to 8-OH-DPAT of two lines derived from the Wistar strain (my Hooded Wistars vs. Wistar Kyoto rats from van den Buuse).

#### **4. Methodological issues.**

The certainly acknowledge that subcutaneous administration of drugs is a stressful procedure; it consistently evoked tachycardic responses due to handling and injection-induced pain. These effects are, however, relatively short-term, and the study provided enough time between injection and stress onset, so that heart rate returned to near-basal level. Additionally, the experiments always paired drug with vehicle, thus excluding any effect of injection-related stress. Both autonomic blockers used in the study (atenolol and methyl-scopolamine) poorly penetrate the blood-brain barrier, and thus it is likely that their effects were predominantly peripheral. Aiming to conduct the study in the least invasive manner and focussing on cardiac effects, the experiments did not measure arterial pressure in our rats. The study believe that lack of the pressure data does not diminish the value of or compromise our results. Systemic administration of 8-OH-DPAT at doses similar to ours reduced arterial pressure in conscious rats at rest (Buisson-Defferier and Van den Buuse 1992), and therefore bradycardic effects of the drug observed in this study at resting state (without stress) were clearly not the consequence of baroreflex. Restraint stress causes sustained pressor response in rats (Barron and Van Loon 1989; Chen and Herbert 1995; McDougall, Widdop et al. 2005). While effects of 8-OH-DPAT on the restraint-induced pressor responses have not been studied, the drug consistently suppressed rises in the arterial pressure elicited by open field and air-jet stresses (Nalivaiko, Ootsuka et al. 2005; van den Buuse and Wegener 2005). It is therefore also unlikely that baroreflex made any contribution to the anti-tachycardic effects of 8-OH-DPAT during restraint; if anything, the negative chronotropic action of the drug could counteract – and overcome – the potential tachycardic effect of the baroreflex.

The results indicate that: i) tachycardia induced by restraint stress occurs due to sustained increase in cardiac sympathetic activity associated with a transient vagal withdrawal; ii) activation of central 5-HT<sub>1A</sub> receptors attenuates this tachycardia, by suppressing both autonomic effects; iii) some relevant receptors are located in the medullary raphe/parapyramidal area.

While I did not find any evidence of activation of these receptors by intrinsically released serotonin during psychological stress, we believe that our results represent both theoretical and clinical interest. Firstly, 5-HT<sub>1A</sub> receptors could serve as a marker (though non-specific) for the identification of the raphe-spinal presympathetic cardiomotor neurons. Secondly, presented here evidence for the central cardioprotective effect of 5-HT<sub>1A</sub> receptor agonists may be clinically relevant, as suppression of excessive cardiac sympathetic tone at its origin, in the brain, could represent an alternative therapeutic strategy for those cardiac patients who do not tolerate or who are insensitive to beta-blockers.

## CHAPTER VII

### CONCLUSIONS

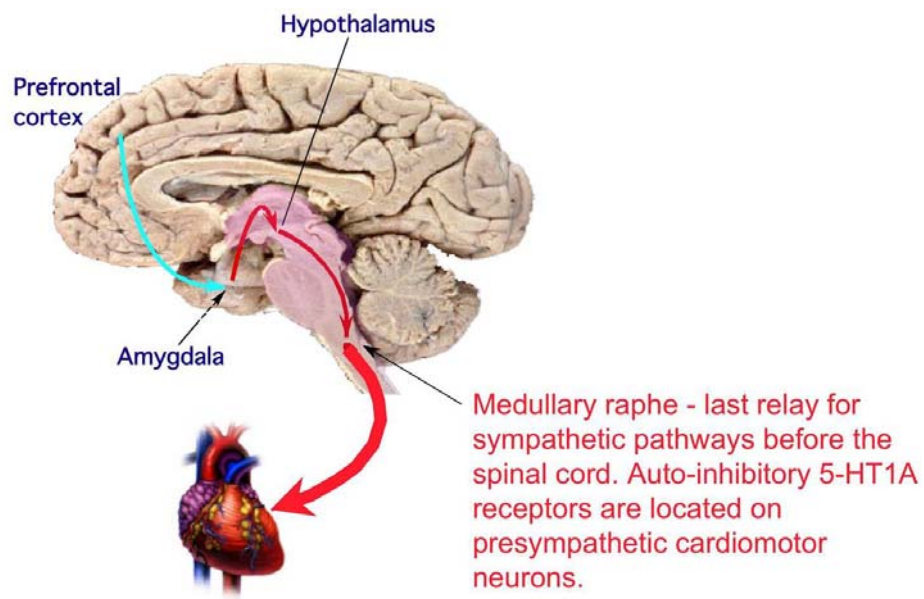
This study has demonstrated that the activation of inhibitory GABA<sub>A</sub> receptors in amygdala reduces tachycardia induced by restraint stress. Furthermore, the activation of amygdala neurons during psychological stress is necessary for a sustained elevation in heart rate. It is likely that amygdala does not participate in the control of heart rate at rest (baseline).

The studies of the activation of 5-HT<sub>1A</sub> receptors on stress-induced tachycardia in rats are as follows:

- i) Activation of central 5-HT<sub>1A</sub> receptors evokes bradycardia mediated by cardiac non- $\beta$ -adrenergic, noncholinergic neurotransmitter mechanisms.
- ii) A sustained component of tachycardia elicited by the restraint stress in rats is mainly due to sympathetic activation, whereas vagal withdrawal contributes to initial larger transient component.
- iii) 8-OH-DPAT substantially attenuates both these autonomic components, in a dose dependent manner and acting via 5-HT<sub>1A</sub> receptors.
- iv) Activation of 5-HT<sub>1A</sub> receptors somewhere else reduces basal heart rate by NANC mechanisms.
- v) At least some of these receptors must be located in the medullary raphe-parapyramidal area.
- vi) No evidence for the activation of the same receptors either at rest or during psychological stress.
- vii) 5-HT<sub>1A</sub> agonists could be potential candidates for centrally acting cardioprotective drugs.

In summary, the studies indicated the following for the first time:

- i) The activation of amygdala neurons during psychological stress is necessary for a sustained elevation of the heart rate levels.
- ii) Amygdala neurons mediated cardiac autonomic nerve activity via direct or indirect connections starting from amygdala to hypothalamus, the medulla, spinal cord, cervical ganglion to the heart.
- iii) Medullary raphe was the last relay for sympathetic pathway before the spinal cord.
- iv) The reduced density of 5-HT<sub>1A</sub> receptors in the medullary raphe led to increase cardiac sympathetic tone (Fig. 27).



**Fig.27** The summary of the activation of amygdala and medullary raphe.

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