1-1-2013

Mechanisms Mediating Modulation Of The Cardiopulmonary Chemoreflex Control Of Regional Sympathetic Outputs By Adenosine A1 And A2a Receptors In The Nts

Zeljka Minic
Wayne State University,

MECHANISMS MEDIATING MODULATION OF CARDIOPULMONARY CHEMOREFLEX CONTROL OF REGIONAL SYMPATHETIC ACTIVITY BY ADENOSINE A_1 and A_{2a} RECEPTORS LOCATED IN THE NTS

by

ZELJKA MINIC

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2013

MAJOR: PHYSIOLOGY

Approved By:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

I would like to dedicate this dissertation to my family:

To my **mother and father** who have supported me throughout my schooling and encouraged me to strive while being far away from home. Thank you!

Drs. Scislo and O'Leary, thank you for being with me when times were tough, and mentoring me as I have been growing up. For that, I am a better person today!

Christian and Puch thanks for making me happy!

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to all of my committee members. **Dr. Scislo**: you have been my mentor and my advisor! You introduced me to science in a sense of writing, thinking, working, living… and you have truly given me unique perspective on a field of physiology through the eyes of your mentor and yourself. Your insights on lectures in cybernetics and a story about a little frog and its grandmother are only few of the memories that will always stay with me!

Thank you for helping me get better in tasks a graduate student needs to grasp. Consequently I feel I have become a young scientist who can professionally carry herself in this field.

I thank you for triple nerve recordings you taught me. I understand these skills are unique as they give truly integrative and yet regionally specific insight in overall state of the organism. I feel very privileged to be one of the few, next to you, who can do them. I will cherish this gift as I continue with my career.

Dr. O'Leary: you have been my co-mentor and as such you have had great impact on my life. Thank you for making my work couple of notches better whether that is paper, presentation or talk. You are a great teacher who taught me a lot.

Dr. Mueller: thank you for all your inputs to my work, always keeping open mind and helping me put my data into a bigger perspective. Thank you for being available to talk, troubleshoot, and borrow a rat!

Dr. Goshgarian: without your presence on my committee all additions to the project in regards to immunohistochemistry would not be possible. Thank you for helping me make my first steps into the field of neuroanatomy!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS; EXPERIMENTAL GROUPS; AND DRUGS

ASNA	adrenal sympathetic nerve activity
CCR	cardiopulmonary chemoreflex
CVLM	caudal ventrolateral medulla
GABA	gamma amino butyric acid
HR	heart rate
int-ASNA	intermediate adrenal sympathetic nerve activity
LSNA	lumbar sympathetic nerve activity
MAP	mean arterial pressure
NTS	nucleus of the solitary tract
PBG	phenylbiguanide
post-ASNA	postganglionic adrenal sympathetic nerve activity
pre-ASNA	preganglionic adrenal sympathetic nerve activity
RSNA	renal sympathetic nerve activity
RVLM	rostral ventrolateral medulla
TRPV1	transient receptor potential cation channel subfamily V member 1

Experimental groups:	
ACF	volume control
CPA 0.033 pmol	low level of A_1 adenosine receptor activation
CPA 3.3 pmol	medium level of A_1 adenosine receptor activation
CPA 330 pmol	high level of A_1 adenosine receptor activation
8-SPT	nonselective blockade of adenosine receptors
CGS 0.2 pmol	low level of A_{2a} adenosine receptor activation
CGS 2 pmol	medium level of A_{2a} adenosine receptor activation
CGS 20 pmol	high level of A_{2a} adenosine receptor activation
ZM 40 pmol	selective blockade of A_{2a} receptors
ACF+CGS	activation of A_{2a} receptors following volume control
$GABA_AX$+CGS	activation of A_{2a} receptors following $GABA_A$ receptors blockade
$GABA_BX$+CGS	activation of A_{2a} receptors following $GABA_B$ receptors blockade
$GABA_AX$	$GABA_A$ receptors blockade
$GABA_BX$	$GABA_B$ receptors blockade

Drug	Dose	Manufacturer
ACF artificial cerebrospinal fluid	Na 150.0, K 3.0, Ca 1.4, Mg 0.8 P 1.0, Cl 155.0 Mm ph= 7.2, solvent	prepared in the laboratory
	infusions	
α-chloralose	80 mg/kg, iv	Sigma
arfonad trimethaphan	2 mg/kg iv (10 μg in 1ml of 0.9% NaCl)	Hoffmann La Roche Ltd.
hexamethonium bromide	20 mg/kg iv	Sigma
phenylbiguanide	1-8 μg/kg (20 μg in 1ml of 0.9% NaCl), intra-atrial	Sigma
Urethane	500 mg/kg, iv	Sigma
	microinjections into the NTS	
8-(*p-sulfophenyl*) theophylline	1 nmol in 100 nl of ACF	Sigma
Bicuculline methiodide	10 pmol in100 nl of ACF	Tocris
CGS-21680	0.2, 2 or 20 pmol in 50 nl of ACF	Sigma
CPA, N^6-cyclopentlyadenosine	0.033, 3.3 or 330 pmol in 50 nl of ACF	Tocris
DiI, 1,1'-dioctadecyl-3,3,3'3'-tetramethyllindocarbocyanine dye	1% solution in 0.1% DMSO	Molecular probes
SCH-50911	1 nmol in 100 nl of ACF	Tocris
ZM-241385	40 pmol in 100 nl of ACF	Tocris

GENERAL INTRODUCTION

The general aim of this dissertation is to test the hypothesis that adenosine may act as a negative feedback regulator for cardiopulmonary chemoreflex (CCR). Powerful activation of the CCR results in severe hypotension which may lead to brainstem ischemia and cause release of adenosine into brainstem cardiovascular centers. Adenosine acting as a central neuromodulator may inhibit the reflex and prevent further deterioration of cardiovascular homeostasis observed following activation of the CCR.

Adenosine - neuromodulator acting in life or death situations

The role of adenosine as a neuromodulator in the central nervous system has been well established. Adenosine operating via A_1 or A_{2a} receptors may inhibit or activate, respectively, central neurons and the release of neurotransmitters from nerve terminals. Generally the inhibitory action of adenosine mediated via A_1 receptors prevails in most of the central nervous system (16; 91). For example, ingestion of caffeine, a nonselective antagonist of adenosine receptor subtypes, activates central processes by removing A_1 receptor mediated inhibition which prevails over A_{2a} receptor mediated facilitation. However, in the nucleus of the solitary tract (NTS), where primarily integration of cardiovascular and other autonomic reflexes occur, the facilitatory A_{2a} adenosine receptors play a dominant role over A_1 receptor mediated inhibition. This has been shown in classical studies by Barraco and Phillis who found that selective activation of NTS A_{2a} adenosine receptors and nonselective activation of adenosine receptor subtypes in via microinjections of adenosine yield similar effects, i.e. decreases in mean arterial pressure (MAP) and heart rate (HR) (1; 8; 10; 80; 81; 122). In contrast, selective activation of adenosine A_1 receptors located in the NTS evoked pressor responses, the effect opposite to that evoked by adenosine itself (1; 8; 10; 80;

81; 122). Adenosine levels in the central nervous system, including the NTS, increase during critical, life threatening situations such as ischemia, hypoxia, and severe hemorrhage as well as during stress/hypothalamic defense response (30; 87; 110; 116-118; 129; 138; 139). In this setting adenosine levels are increased via two different mechanisms. Under pathological (hypoxic) conditions intracellular ATP is catabolized to adenosine in ischemic neurons and glial cells and adenosine is released into the extracellular space. Under physiological conditions, as for example, during hypothalamic defense response ("flight or fight") ATP is neuronally released into the NTS and immediately catabolized to adenosine in the extracellular space by ectonucleotidases (118; 142). ATP may be also released from glial cells stimulated by active central neurons in their proximity and it is rapidly degraded to adenosine by ectonucleotidases (45; 51). Therefore, in all these pathological and physiological situations adenosine acts spatially, as a neuromodulator enveloping neural circuitry rather than acting as a neurotransmitter at specific synapses.

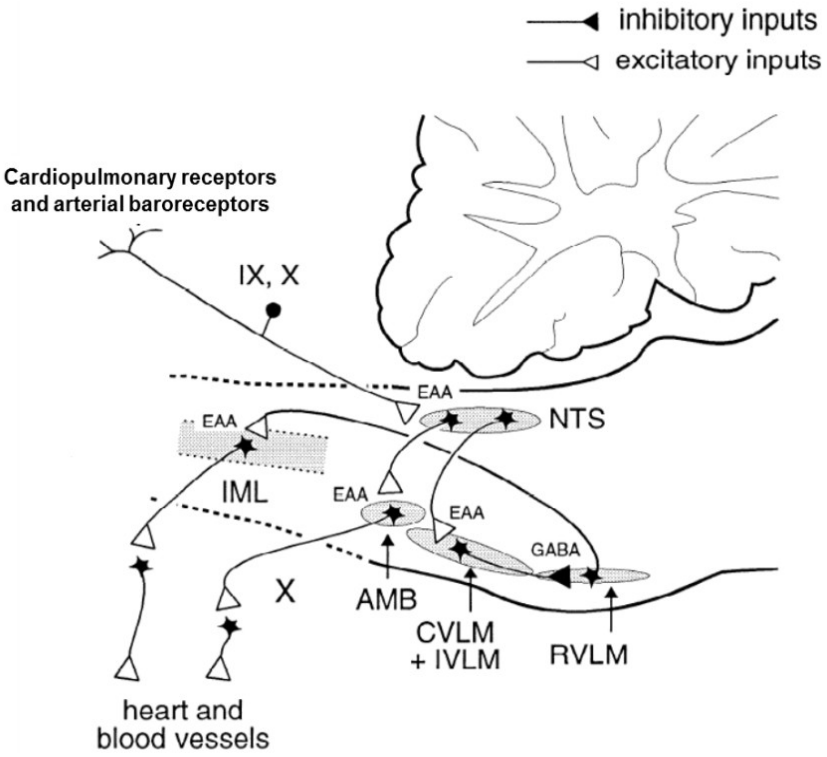

Figure 1. Medullary pathways of the cardiopulmonary and arterial baroreceptors. Abbreviations: GABA,γ-amino-butyric acid; GLU, glutamate; NTS, nucleus of the solitary tract; CVLM, caudal ventrolateral medulla; IVLM, intermediate ventro lateral medulla RVLM, rostral ventrolateral medulla; AMB, nucleus ambiguus; IML, intermediolateral column of spinal cord; SG, sympathetic ganglion; IX, glossopharyngeal nerve; X, vagal nerve. Modified from Dampney RAL 1994 (32).

Adenosine is released during large homeostatic imbalance between demand and delivery of oxygen to the central neurons and may act as a neuromodulator correcting the imbalance. Crucial reflexes responsible for cardio-respiratory homeostasis are primarily integrated in the NTS (Figure 1) (32). Therefore, adenosine may play an important role in regaining homeostasis by modulation of these reflexes at the level of the NTS. In support of this hypothesis, it has been reported that NTS contains the greatest density of adenosine uptake sites in the whole central nervous system (14).

The Bezold Jarisch reflex

The Bezold-Jarisch reflex, also known as the cardiopulmonary chemoreflex (CCR) is one of the homeostatic cardiovascular reflexes integrated in the NTS. This reflex was initially discovered as a powerful depressor, and cardiac slowing reflex activated by veratrum alkaloids (3; 18; 34; 62). Further studies showed that this reflex is evoked by activation of cardiopulmonary chemo-sensitive receptors and mechano-sensitive stretch receptors located on sensory vagal C fibers within the left ventricle and atria in intimate proximity to the coronary vasculature and also within the pulmonary circulation (5; 18; 27; 50; 68; 85). Chemo-sensitive and mechano-sensitive receptors are usually supplied by the same vagal afferent fibers and evoke similar reflex responses (100; 101). Cardiopulmonary chemo-sensitive receptors are naturally activated via prostaglandins, radical oxygen species and serotonin released during cardiac ischemia, infarct and reperfusion (68; 97). For example, serotonin is released from platelets, aggregating on damaged endothelium (83; 97) and increased serotonin levels activate $5HT_3$ serotonin receptors located on vagal cardiopulmonary afferent fibers (85; 136). Serotonin is also released from endothelial cells of coronary arteries during hypoxia (17) and catabolism of ATP to ADP may also contribute to this process

(83). The cardiopulmonary afferents may be also activated via other naturally released substances, for example, endogenous canabinoids operating via activation of vanilloid TRPV1 receptors, ATP operating via P2x receptors, nicotine, etc. (44; 67; 93). The CCR opposes the sympathoactivatory reflexes mediated by bradykinin released in the ischemic heart (4; 66; 127). This interaction between two opposite reflex effects may provide fine tuning of cardiovascular regulation during cardiac ischemia as suggested by Longhurst (66). The CCR pathway is similar to that mediating the arterial baroreflex and involves glutamatergic activation of NTS neurons, glutamatergic projection from the NTS to the caudal ventrolateral medulla (CVLM) and GABA-ergic projection from CVLM to the rostral ventrolateral medulla (RVLM) (132).

Activation of both mechano-sensitive and chemo-sensitive cardiopulmonary receptors evokes powerful reflex bradycardia, vasodilation and hypotension. These reflex responses are cardio-protective as they decrease afterload and heart rate, reducing oxygen demand in the hypoxic heart, therefore improving left ventricular function (37; 92; 97). This may help reduce heart muscle damage, prevent pulmonary edema, and restore cardiovascular balance despite existing coronary hypoperfusion (37). However, powerful activation of this reflex under ischemic conditions will cause apnea, exaggerate the decreases in cardiac output and total peripheral resistance causing profound hypotension thereby decreasing perfusion of vital organs resulting in cardiogenic shock (34; 48). Several studies showed that this reflex may be responsible for infarct related bradyarrhythmias, severe hypotension and sudden cardiac death especially in patients with infarct of the inferior and posterior wall of the heart (23; 68; 135). This reflex may also be responsible for vasovagal syncope, i.e. transient cerebral ischemia as a result of marked reflex hypotension (68). Sudden cardiac death in young

athletes is also often attributable to the Bezold-Jarisch reflex (13; 94). Summarizing, the cardiopulmonary chemoreflex (CCR) may help restore hemodynamic balance during myocardial ischemia, or it can further exaggerate the imbalance leading to cerebral ischemia and death. The final outcome may turn positive or negative depending on the fine tuning of this powerful reflex mechanism within the central nervous system. When powerful activation of the CCR leads to severe hypotension and brainstem ischemia adenosine may be released into the NTS and act as a neuromodulator restricting the reflex and preventing further deterioration of homeostatic imbalance and its deadly consequences.

Adenosine neuromodulation of cardiovascular control at the level of the NTS

Although adenosine acts spatially during severe hemodynamic imbalance leading to brainstem ischemia (87; 110; 129; 138; 139) it may create specific patterns of autonomic responses due to differential location of inhibitory A_1 and facilitatory A_{2a} receptors on NTS neurons integrating specific cardiovascular reflexes and projecting finally to specific regional sympathetic outputs (103; 108). Selective stimulation of these antagonistic receptor subtypes in the NTS results often, but not always, in reciprocal sympathetic and hemodynamic responses. Previous studies from our laboratory showed that selective stimulation of NTS adenosine A_1 receptors evokes mostly (2/3) pressor responses and differential regional sympathoactivation: preganglionic adrenal (pre-ASNA) > renal (RSNA) $\geq$ lumbar (LSNA) sympathetic nerve activity (107). These responses were mediated via inhibition glutamatergic transmission in the arterial baroreflex pathway as bilateral sinoaortic denervation + vagotomy as well as blockade of ionotropic glutamatergic transmission in the NTS abolished the pressor and sympathoactivatory responses (107). Relatively high variability of pressor/depressor

and vasoconstrictor/vasodilator responses evoked by stimulation of NTS A_1 adenosine receptors is a result of simultaneous triggering of 3 vasoactive responses: 1) sympathetic vasoconstriction; 2) preferential activation of the adrenal medulla, release of epinephrine into the circulation and β-adrenergic vasodilation; 3) release of vasopressin into the circulation due to inhibition of baroreflex pathway which normally restricts release of this peptide from hypothalamic nuclei (paraventricular and supraoptic) (32; 71-73). Direct assessment of the effect of NTS A_1 receptors on baroreflex control of HR and regional sympathetic outputs confirmed implications from previous studies that in fact A_1 adenosine receptors inhibit arterial baroreflex mechanisms at the level of the NTS (73; 107). This study showed that stimulation of NTS A_1 adenosine receptors uniformly shifted all baroreflex stimulus response function curves (for HR, RSNA, pre-ASNA and LSNA) over 50 mmHg toward higher MAP (102). Following the stimulation greater baroreceptor activation was required to overcome A_1 adenosine receptor-mediated inhibition of neurotransmission in the baroreflex pathway to evoke baroreflex responses. The baroreflex functions were shifted rightward along the MAP axis with only minor, regionally specific alterations of the range and gain of arterial baroreflex control. This was consistent with inhibition of neurotransmitter release from baroreceptor afferents terminating in the NTS.

Activation of NTS A_{2a} adenosine receptors, which functionally prevail over A_1 receptors in the NTS, evokes more contrasting regional sympathetic and vascular responses that those evoked by NTS A_1 receptor stimulation. Following selective stimulation of A_{2a} adenosine receptors marked decreases in MAP, HR and RSNA are accompanied with substantial increases in pre-ASNA, whereas no significant changes in LSNA were observed (104; 105). Note that NTS A_{2a} and A_1 receptors reciprocally

affect MAP and RSNA, have different effects on LSNA; however they both preferentially activate the adrenal medulla innervated by pre-ASNA. These two receptor subtypes have also reciprocal effects on regional vasculature: A_1 receptors evoke mostly vasoconstriction (sympathetic and vasopressinergic) (72), whereas A_{2a} receptors elicit differential regional vasodilation (iliac>>mesenteric>renal) which is mediated mainly by activation of the adrenal medulla and β-adrenergic vasodilation (9; 59). Although NTS A_{2a} adenosine receptors have highly diverse effects on regional sympathetic outputs and vascular beds, these receptors do not directly affect baroreflex mechanisms controlling the sympathetic outputs. The recent study from our laboratory showed that NTS A_{2a} receptors do not reset regional sympathetic baroreflex function curves along the MAP axis, therefore they do not alter the reflex reactivity to activation of arterial baroreceptors by changes in MAP (54). NTS A_{2a} adenosine receptor-mediated reciprocal shifts in baseline level of regional sympathetic activity (decreases in RSNA and increases in pre-ASNA) reciprocally altered the range and the gain of baroreflex control of RSNA (decreases) vs. pre-ASNA (increases). However the baseline shifts and consequent alterations of range and gain of baroreflex control of these sympathetic outputs were mediated via non-baroreflex mechanisms as the stimulus-response baroreflex functions were not shifted along the MAP axis.

Interestingly, adenosine operating in the NTS via A_{2a} receptors (which functionally prevail over A_1 receptors) was initially considered as a neuromodulator which facilitates glutamatergic neurotransmission in the arterial baroreflex pathway because "baroreflex like" global hemodynamic effects (decreases in MAP and HR) were observed following microinjections into the NTS of adenosine as well as of the selective agonist of A_{2a} adenosine receptors (CGS 21680) (1; 8; 10; 80; 81; 122). However, more

precise analysis of regional sympathetic responses evoked by selective stimulation of NTS A_{2a} adenosine receptors in our laboratory showed that this concept was very unlikely. Although MAP, HR and RSNA decreased upon stimulation of NTS A_{2a} receptors, which was consistent with facilitation of NTS baroreflex mechanisms, LSNA did not change and pre-ASNA increased in this setting, which was just opposite to decreases in pre-ASNA response evoked by activation of the arterial baroreflex (99; 104; 105). In addition, bilateral sinoaortic denervation + vagotomy or bilateral inhibition of glutamatergic transmission in the NTS did not significantly alter the hemodynamic and regional neural responses to stimulation of NTS A_{2a} receptors (105; 106). Finally, the direct assessment of baroreflex stimulus-response function curves confirmed that NTS A_{2a} adenosine receptors do not reset arterial baroreflex reactivity (54). Taken together these data indicate that activation of NTS A_{2a} receptors markedly alters baseline levels of regional sympathetic nerve activity and blood flow in regional vascular beds via non-baroreflex mechanisms as these cardiovascular responses are observed after deafferentation of the NTS. Therefore, it is likely that A_{2a} receptors may modulate activity of nerve fibers descending to the NTS from higher structures, most likely from the hypothalamus which have dense, reciprocal connection with the NTS (32; 64; 96). A_{2a} receptor-mediated modulation of this descending activity may interact with specific cardiovascular reflexes integrated in the NTS and finally evoke the diverse regional cardiovascular and neural responses.

Hypothetical adenosine neuromodulation of the CCR

As it was hypothesized above, adenosine may be released into the NTS during powerful reflex decreases in MAP evoked by activation of the CCR and inhibit this reflex preventing potential deadly consequences (fainting or even sudden death). This

hypothesis is consistent with inhibitory action of adenosine via A_1 receptors. Since both the CCR and arterial baroreflex pathways are mediated via the same medullary structures (NTS, CVLM and RVLM) and neurotransmitters (32; 131) and selective stimulation of NTS A_1 adenosine receptors does inhibit the arterial baroreflex pathway, it is likely that also the CCR pathway may be inhibited by this adenosine receptor subtype. However, the potential effect of NTS A_{2a} adenosine receptors, which are much more functionally relevant in the NTS than their A_1 counterparts, is not so clear**.** Although activation of NTS A_{2a} receptors evoked marked, contrasting regional sympathetic and hemodynamic responses these receptors did not inhibit the arterial baroreflex pathway at the level of the NTS (54; 104; 105). Interestingly, the pattern of regional sympathetic responses evoked by selective stimulation of NTS A_{2a} receptors resemble the pattern occasionally reported following stimulation of the CCR, i.e. decreases in RSNA, increases in pre-ASNA and no changes in LSNA (40; 52; 130), although other studies from our laboratory and by others reported uniform CCR-mediated inhibition of regional sympathetic outputs (19; 38; 57; 95; 100). The similarities between patterns of regional sympathetic responses triggered by the CCR and stimulation of NTS A_{2a} receptors could suggest that A_{2a} receptors may facilitate the CCR under some rare circumstances. This would be against our hypothesis, that adenosine serves as a negative feedback regulator for the CCR via both A_1 and A_{2a} receptors located in the NTS. However, our preliminary data showed that both A_1 and A_{2a} receptors may inhibit the regional sympathetic and hemodynamic responses evoked by stimulation of the CCR (78), which supports our hypothesis that adenosine indeed inhibits the CCR via both A_1 and A_{2a} receptor subtypes which mediate the inhibition via different mechanisms: A_1 receptors may directly inhibit glutamatergic transmission in

CCR pathway whereas A_{2a} receptors may facilitate release of an inhibitory neurotransmitter which in turn may inhibit the CCR pathway at the level of the NTS. Since stimulation of NTS A_{2a} receptors evokes decreases in RSNA, increases in pre-ASNA and no changes in LSNA it was reasonable to speculate that the A_{2a} receptor mediated inhibition of the CCR responses will be regionally specific: it may be greater for pre-ASNA as the effect of A_{2a} receptor stimulation is opposite to that evoked by the CCR and the smallest for RSNA as both NTS A_{2a} receptors and activation of the CCR inhibit this sympathetic output. The A_{2a} receptor-mediated inhibition of LSNA responses to stimulation of the CCR may be at intermediate level between those for pre-ASNA and RSNA.

Among possible inhibitory mechanisms which can be triggered in the NTS to inhibit the CCR pathway the most plausible choice is GABA released from intrinsic NTS GABA-ergic neurons or nerve terminals. Although there are no data which confirm the presence of GABA-ergic negative feedback in the CCR pathway at the level of the NTS, this GABA-ergic negative feedback is well documented in arterial baro- and chemoreflex pathways (55; 56; 65; 74; 119-121; 133; 140; 141). In addition, a recent review article by Zhang and Mifflin postulates that the negative GABA-ergic feedback is probably present in all cardiovascular reflexes integrated in the NTS (141).

It still remains undocumented if adenosine A_1 and A_{2a} adenosine receptors are present in the CCR pathway. It is likely that NTS A_1 receptors inhibit the CCR pathway similarly as they inhibit the arterial baroreflex pathway (102). However, the presence of A_{2a} receptors in the CCR pathways is not so certain as these receptors did not inhibit or facilitate arterial baroreflex control of the regional sympathetic outputs.

The studies performed in frame of this dissertation answered all the above

questions and positively proved the main hypothesis that adenosine operating via both A_1 and A_{2a} receptors in the NTS network serves as a negative feedback regulator for CCR control of regional sympathetic outputs and hemodynamic variables.

Experimental plan

Adenosine differentially modulates arterial baroreflex control of regional sympathetic outputs via A_1 and A_{2a} receptors located in the NTS circuitry. Based on previous studies and preliminary data I hypothesize that antagonistic A_1 and A_{2a} adenosine receptors may act in concert although via different mechanisms to inhibit the CCR. Adenosine is released into the NTS during hypoxia and severe post-hemorrhagic hypotension; it may be also released during profound reflex hypotension which occurs during powerful activation of the CCR. Therefore adenosine operating via both A_1 and A_{2a} receptors may serve as a negative feedback regulator for CCR mechanisms at the level of the NTS.

In addition, controversies in literature exist on regional sympathetic responses to activation of the CCR. Therefore, before addressing modulatory effect of adenosine receptor subtypes on CCR control of regional sympathetic outputs the differential regional reflex effects will be assessed using simultaneous recordings from three sympathetic nerves (renal, adrenal and lumbar).

Specific aims of dissertation

1. To test hypothesis that activation of A_1 adenosine receptors in the NTS differentially inhibits hemodynamic and regional sympathetic responses evoked by activation of the CCR.
2. To test hypothesis that activation of NTS A_{2a} receptors (acting in concert with A_1 receptors) differentially inhibits the CCR control of regional sympathetic outputs.

3. To test hypothesis that activation of NTS A_{2a} adenosine receptors mediates inhibition of hemodynamic and regional neural sympathetic responses evoked by activation of the CCR via facilitating of GABA release from NTS GABA-ergic neurons/terminals.

CHAPTER 1

Activation of NTS A_1 adenosine receptors inhibits regional sympathetic responses evoked by activation of cardiopulmonary chemoreflex

ABSTRACT

Previously we have shown that, adenosine operating via the A_1 receptor subtype may inhibit glutamatergic transmission in the baroreflex arc within the NTS and differentially increase renal (RSNA), preganglionic adrenal (pre-ASNA) and lumbar (LSNA) sympathetic nerve activity (ASNA>RSNA≥LSNA). Since the cardiopulmonary chemoreflex and the arterial baroreflex are mediated via similar medullary pathways and glutamate is a primary transmitter in both pathways, it is likely that adenosine operating via A_1 receptors in the NTS may differentially inhibit regional sympathetic responses evoked by activation of cardiopulmonary chemoreceptors. Therefore, in urethane/chloralose anesthetized rats (n=37) we compared regional sympathoinhibition evoked by the cardiopulmonary chemoreflex (activated with right atrial injections of serotonin $5HT_3$ receptor agonist, phenylbiguanide, PBG,1-8 μg/kg) before and after selective stimulation of NTS A_1 adenosine receptors (microinjections of N^6-cyclopentyl adenosine, CPA, 0.033-330 pmol/50 nl). Activation of cardiopulmonary chemoreceptors evoked differential, dose dependent sympathoinhibition (RSNA>ASNA>LSNA) and decreases in arterial pressure and heart rate. These differential sympathetic responses were uniformly attenuated in a dose dependent manner by microinjections of CPA into the NTS. Volume control (n=11) and blockade of adenosine receptor subtypes in the NTS via 8-(*p-sulfophenyl*) theophylline (8-SPT; 1 nmol in 100 nl) (n=9) did not affect the reflex responses. We conclude that activation of NTS A_1 adenosine receptors uniformly

inhibits neural and cardiovascular cardiopulmonary chemoreflex responses. A_1, adenosine receptors have no tonic modulatory effect on this reflex under normal conditions. However, when adenosine is released into the NTS (i.e. during stress or severe hypotension/ischemia) it may serve as negative feedback regulator for depressor and sympathoinhibitory reflexes integrated in the NTS.

INTRODUCTION

Numerous studies have shown that adenosine operates as a central neuromodulator of cardiovascular control at the level of the NTS (1; 9; 54; 59; 72; 81; 105; 107; 108; 110; 116). In the central nervous system adenosine serves as an inhibiting or stimulating neuromodulator operating via A_1 and A_{2a} receptors, respectively (90). The primary natural source of adenosine is ATP released from nerve terminals and glial cells which is subsequently catabolized to adenosine by ectonucleotidases (90; 114; 142). Importantly, adenosine is also released into the extracellular space during life threatening homeostatic imbalances such as hypoxia, ischemia and severe hemorrhage; in these pathological situations adenosine results from massive catabolism of intracellular ATP (87; 110; 129; 138; 139). Inasmuch as, the most basic, life supporting, reflexes (cardiovascular, respiratory and other autonomic reflexes) are primarily integrated in the NTS (32), it is not surprising that the greatest density of adenosine uptake sites in the entire central nervous system is located in the NTS (14).

The Bezold-Jarisch reflex (also termed the cardiopulmonary chemoreflex) was initially discovered as a powerful depressor and cardiac slowing response to veratridine alkaloids (62). It is mediated by polymodal receptors located on myelinated and unmyelinated vagal afferents from the cardiopulmonary region which are activated physiologically via mechanical and/or chemical stimuli as shown in extensive studies

performed on dogs and cats by Colleridges, Paintal, Dawes and Thoren (26; 27; 34; 84; 85; 126). In the rat the equivalent fibers are unmyelinated afferents which only exhibit low or high activation thresholds (124; 125). The majority of cardiac vagal afferents activated by mechanical stimuli in the rat are also activated by the serotonin $5HT_3$ receptor agonist, phenylbiguanide (PBG)(100; 101). The cardiopulmonary chemoreflex may be naturally activated via serotonin released from aggregated platelets during coronary clot formation or from ischemic endothelial cells (17; 43; 83) as well as via other naturally released substances, for example, endogenous canabinoids operating via activation of vanilloid TRPV1 receptors, ATP operating via P2x receptors, nicotine, etc. (44; 67; 93). Consistently, the cardiopulmonary chemoreflex is markedly enhanced during the acute phase of myocardial infarction (67; 93) which may be cardio-protective as it causes decreases in HR and afterload leading to decreased oxygen demand of the ischemic heart (37; 68; 97). However, powerful activation of this reflex may lead to severe hypotension, cerebral ischemia, bradyarrhythmia and even sudden cardiac death (13; 48; 68). If the reflex hypotension exceeds the capabilities of brainstem vascular autoregulation, adenosine may be released into the NTS from ischemic central neurons and glial cells. This increase in adenosine can have wide ranging, complex actions on the central integration of cardiovascular reflexes in these settings.

Adenosine operating via A_1 receptor subtype in the NTS may inhibit glutamatergic transmission in the baroreflex arc, reset arterial baroreflex control of heart rate (HR) and regional sympathetic nerve activity toward higher mean arterial pressure (MAP) and differentially affect baroreflex functions for renal (RSNA), preganglionic adrenal (pre-ASNA), postganglionic adrenal (post-ASNA) and lumbar (LSNA) sympathetic outputs (102; 107). Since the cardiopulmonary chemoreflex and arterial

baroreflex are mediated via similar medullary pathways and glutamate is a primary transmitter in both pathways at the level of the NTS (131), it is likely that adenosine operating via NTS A_1 receptors may differentially inhibit regional sympathetic and hemodynamic responses evoked by activation of cardiopulmonary chemoreceptors. This mechanism may serve as a negative feedback regulator for the cardiopulmonary chemoreflex, preventing the potentially deadly effects of over-excitation of the reflex which could result in life threatening hypotension and bradyarrhythmia. However, it remains unknown if A_1 adenosine receptors are present on those NTS neurons which mediate the cardiopulmonary chemoreflex. Our previous studies suggested differential localization of A_1 adenosine receptors on NTS neurons targeting different sympathetic outputs (103; 107). However, it remains unknown if these receptors are differentially located on those NTS neurons which mediate cardiopulmonary chemoreflex control of regional sympathetic outputs. Specifically, it is unknown if activation of NTS A_1 adenosine receptors differentially attenuates the inhibition of the pre-ASNA vs. RSNA vs. LSNA evoked by activation of cardiopulmonary chemoreceptors. The present study was designed to address the above hypotheses.

In addition, there are controversial reports on the responses of regional sympathetic outputs evoked by stimulation of cardiopulmonary afferents (19; 38; 40; 52; 57; 95; 100; 101; 130). For example, in some studies stimulation of cardiopulmonary $5HT_3$ receptors resulted in reflex increases in pre-ASNA, no change in LSNA and decreases in RSNA (40; 52; 130) whereas other studies showed uniform inhibition of these regional sympathetic outputs in this setting (19; 38; 95; 100). These discrepancies may result from comparisons of single nerve recordings performed in different animals under different experimental conditions in different laboratories. In the present study the

attempt was made to simultaneously record the reflex responses from all these sympathetic outputs (renal, adrenal and lumbar) in the same animal to allow quantifying and comparing the regionally specific differences in the reflex reactivity under identical experimental conditions.

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* endorsed by the American Physiological Society and published by the National Institutes of Health.

Design

Regional sympathetic (RSNA, pre-ASNA, post-ASNA and LSNA) and hemodynamic (MAP and HR) responses evoked by graded stimulation of cardiopulmonary chemoreceptors via injections into the right atrium of increasing doses of $5HT_3$ serotonin receptor agonist, PBG (1, 2, 4, and 8 μg/kg) were studied in 57 male Sprague-Dawley rats (380.0 ± 6.0 g). In 37 rats, A_1 adenosine receptors were activated in the caudal NTS via bilateral microinjections of three different doses of the selective A_1 receptor agonist, N^6-cyclopentyl adenosine, (CPA, 0.033, 3.0, and 330 pmol in 50 nl; numbers of animals in each group were 10, 13, and 14, respectively). In 9 rats, adenosine receptors were inhibited with water soluble, nonselective A_1 and A_{2a} receptor antagonist 8-(*p-sulfophenyl*) theophylline (8-SPT, 1 nmol in 100 nl). In 11 rats, the effect of bilateral microinjections of vehicle (artificial cerebrospinal fluid, ACF, 50 nl) on cardiopulmonary chemoreflex reactivity was evaluated. The cardiopulmonary chemoreflex stimulus-response curves obtained under control conditions were compared with the curves obtained approximately 5 min after bilateral stimulation or

inhibition of NTS A_1 adenosine receptors or following volume control. The cardiopulmonary chemoreflex reactivity was also assessed approximately one hour after the microinjections of A_1 adenosine receptor agonist into the NTS (recovery). The timeline of the protocols is presented in Figure 2.

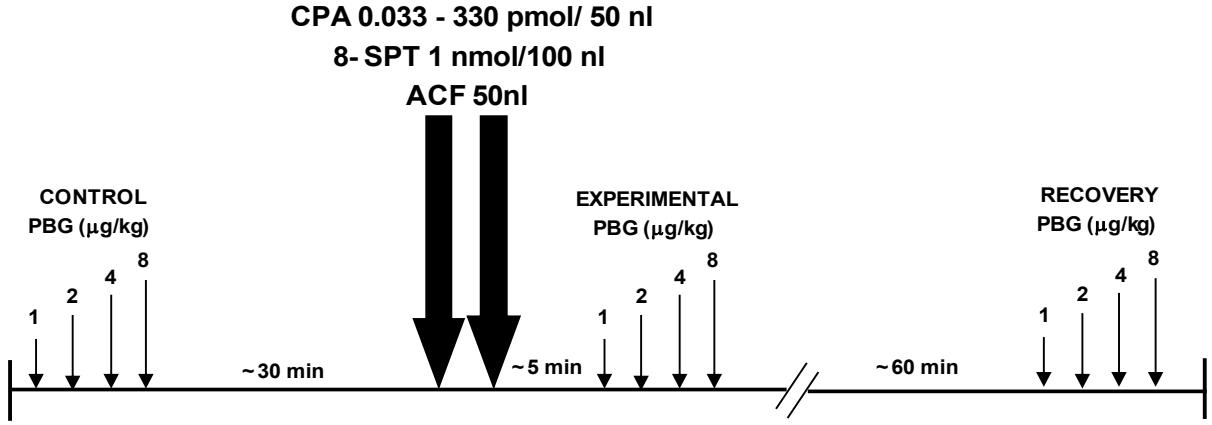

Figure 2. Timeline of experiments. Small arrows indicate injections of phenylbiguanide into the right atrium (1-8 μg/kg). Double large arrows indicate bilateral microinjections into the NTS of: A_1 adenosine receptor agonist CPA (0.033, 3.3 and 330 pmol in 50 nl), non-selective adenosine receptor antagonist, 8-SPT (1 nmol in 100 nl) or volume control (microinjections into the NTS of artificial cerebrospinal fluid, ACF, 50 nl).

Instrumentation and measurements

All procedures were described in detail previously (9; 54; 99; 100; 102; 105-107; 109-111). Briefly, male Sprague-Dawley rats (Charles River) were anesthetized with a mixture of α-chloralose (80 $mg \cdot kg^{-1}$) and urethane (500 $mg \cdot kg^{-1}$ i.p.), tracheotomized, connected to a small-animal respirator (SAR-830, CWE, Ardmore, PA), and artificially ventilated with 40% oxygen, 60% nitrogen mixture. Arterial blood gases were tested occasionally (Radiometer, ABL500, OSM3), and ventilation was adjusted to maintain Po_2, Pco_2, and pH within normal ranges. Average values measured at the end of each experiment were: Po_2 = 166.1 ± 5.1 Torr, Pco_2 = 35.3 ± 1.4 Torr and pH = 7.43 ± 0.01. The left femoral artery and vein were catheterized to monitor arterial blood pressure and infuse drugs and supplementary doses of anesthesia (12–21 $mg \cdot kg^{-1} \cdot h^{-1}$ of α-chloralose and 76–133 $mg \cdot kg^{-1} \cdot h^{-1}$ of urethane dissolved in 2.4–4.2 $ml \cdot kg^{-1} \cdot h^{-1}$ saline), respectively.

An additional catheter was inserted into the right atrium via right jugular vein for intra-atrial PBG injections.

Sinoaortic denervation was performed and tested similarly as it was described in previous papers from our laboratory (100; 105; 107). This procedure was performed to minimize baroreflex compensation for the decreases in MAP evoked by stimulation of cardiopulmonary receptors and also to eliminate possible activation of arterial chemoreceptors by PBG (15; 58). Importantly, unloading of arterial baroreceptors may differentially increase the regional sympathetic nerve activity thus it may obscure the regional differences in cardiopulmonary chemoreflex reactivity (99). Special attention was paid to preserve the integrity of the vagus nerve during stripping of the bifurcation of common carotid artery and cutting aortic depressor, superior laryngeal and sympathetic nerves. Experiments in which stimulation of the cardiopulmonary receptors with the maximal used dose of PBG (8μg/kg) evoked less than 20% decrease in MAP were not analyzed.

Neural recordings

In each experiment, simultaneous recordings from three (n=26) or two sympathetic outputs (n=31) were performed. Renal and adrenal nerves were exposed retroperitoneally form a flank incision, whereas lumbar sympathetic trunk was exposed from midabdominal incision. Neural recordings were accomplished as described previously (54; 99; 100; 102; 105-107; 109-111). Neural signals were initially amplified (2,000–20,000×) with bandwidth set at 100–1,000 Hz, digitized, rectified, and averaged in 1-s intervals. Background noise was determined 30–60 min after the animal was euthanized, yet still artificially ventilated to leave unaltered any potential movement artifacts. Determination of noise in pre-ASNA requires long post mortem recording as

this nerve may be active up to an hour after the euthanasia (21; 22). Resting nerve activity before each stimulation of the cardiopulmonary receptors was normalized to 100%.

The ratio between preganglionic and total nerve activity was initially tested with an intravenous bolus injection of the short-lasting (1-2 min) ganglionic blocker, Arfonad (trimethaphan, 2 $mg \cdot kg^{-1}$; Hoffmann-La Roche) and finally evaluated at the end of each experiment with hexamethonium (20 $mg \cdot kg^{-1}$ i.v.). RSNA was almost completely postganglionic; 3.8 ± 0.7 % (n = 56) of the activity persisted after the ganglionic blockade. LSNA was predominantly postganglionic; 33.1 ± 3.9 % (n = 40) of preganglionic activity remained after the blockade. According to the criteria established in our previous studies (54; 99; 102; 105-107; 109-111), pre-ASNA was considered as predominantly preganglionic if the activity remaining after ganglionic blockade at the end of each experiment was >75 % or predominantly postganglionic if the remaining activity after the blockade was <50%. For total of 47 adrenal nerve recordings 37 were found predominantly preganglionic, 4 predominantly postganglionic, and 6 had intermediate composition (int-ASNA) where 50-75% of activity remained after the blockade. Average activity measured after ganglionic blockade was 107.2 ± 3.3 %, 32.7±5.6 % and 65.0±2.2 % for pre-ASNA, post-ASNA and int-ASNA, respectively. Pre-ASNA increased over 100 % likely because of unloading of intact cardiopulmonary and the remaining peripheral baroreceptors during the decrease in MAP after the ganglionic blockade. The ganglionic blockade produces usually larger increases in pre-ASNA (17-28%) in rats with intact arterial baroreceptors (54; 102; 105). The arterial pressure and neural signals were digitized and recorded with a Hemodynamic and Neural Data Analyzer (Biotech Products, Greenwood, IN), averaged over 1-s intervals, and stored on hard disk for subsequent analysis.

Microinjections into the NTS

Bilateral microinjections of three different doses of the selective A_1 receptor agonist, CPA (0.033, 3.3 and 330 pmol in 50 nl of ACF) and the nonselective A_1-A_{2a} receptor antagonist, 8-SPT (1nmol in 100nl of ACF) were made with multibarrel glass micropipettes into the medial region of the caudal NTS as described previously (9; 54; 102; 105-107; 109-111). The doses and volumes of the agonist and antagonist were the same as used in our previous studies (54; 102; 106; 107; 109-111). The drugs were dissolved in ACF, and the pH was adjusted to 7.2 to unify pH across all our studies; in previous studies we used acidic A_{2a} adenosine receptor agonist (CGS 21680) which often precipitated in pH >7.2. In a separate group of animals bilateral microinjections of vehicle (50 nl of ACF at pH = 7.2) into the same site of the NTS were performed as a volume control. All microinjection sites were marked with DiI lypophilic dye (Molecular Probes, CA) and verified histologically as described previously (9; 54; 102; 105-107; 109-111). The microinjection sites are presented in Figure 3 using

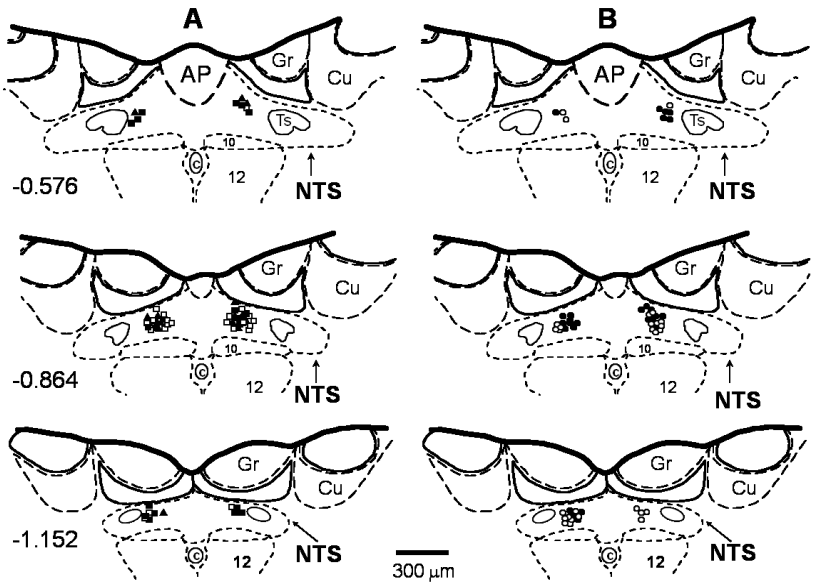

Figure 3. Microinjection sites in subpostremal NTS for all CPA experimental groups. Schematic diagrams show transverse sections of the medulla oblongata from a rat brain. AP, area postrema; c, central canal; 10, dorsal motor nucleus of vagus nerve; 12, nucleus of hypoglossal nerve; Ts, *tractus solitarius*; Gr, gracile nucleus; Cu, cuneate nucleus. Scale is shown at *bottom*; number on *left* schematic diagram denotes the rostrocaudal position in millimeters of the section relative to the obex according to the atlas of the rat subpostremal NTS. (7). Microinjection sites for different doses of A_1 adenosine receptor agonist [N^6-cyclopentyl adenosine (CPA)], nonselective adenosine receptor antagonist [8-sulfophenyl theophylline (8-STP)] & volume control [(microinjections of 50 nl of artificial cerebrospinal fluid (ACF)] were marked with fluorescent dye. A: ■, 0.033 pmol CPA; ▲, 1 nmol 8-SPT; ϒ, volume control. B: ●, 3.3 pmol CPA; ○, 330 pmol CPA.

diagrams based on the atlas of the rat subpostremal NTS by Barraco et al. (7).

Cardiopulmonary chemoreflex stimulus-response curves

The cardiopulmonary chemoreflex-responses were evoked using intra-atrial infusions of increasing doses (1-8 μg/kg) of the serotonin $5HT_3$ receptor agonist, phenylbiguanide (Sigma, 20 μg/1ml solution in 0.9% NaCl). This is a standard, experimentally well controlled method of activation of polymodal chemosensitive/mechanosensitive cardiopulmonary receptors used in most of the studies in the field (19; 38; 40; 52; 57; 95; 100; 101; 130). To minimize and unify the effects of receptor adaptation across the experimental conditions, the injections were always performed in increasing order of doses of PBG with 2.5 min intervals between the doses, similarly as it was done previously (100; 101). Total time to complete the stimulus-response curve was ~ 8 min. The stimulus-response curves were obtained under control conditions, then at least 30 min later, approximately 5 min after bilateral microinjections of drugs (CPA or 8-SPT) or vehicle into the caudal NTS, and again 60 min after the microinjections of CPA (recovery). Preliminary experiments showed that two stimulus-response curves obtained under control condition in half an hour interval revealed similar reactivity of the reflex. This approach allowed to assess cardiopulmonary chemoreflex reactivity during the most effective action of NTS adenosine A_1 receptor activation: the maximal hemodynamic and neural responses evoked by stimulation of A_1 adenosine receptors in the NTS with microinjection of CPA (330 pmol/50 nl) occur approximately 10-20 min after the microinjection (71; 107).

Data analysis

The cardiopulmonary reflex stimulus-response curves for MAP, HR and the regional sympathetic outputs under control conditions were compared with those

generated after bilateral stimulation of NTS A_1 adenosine receptors, nonselective blockade of A_1 + A_{2a} adenosine receptors, and microinjection of vehicle into the NTS. In a separate set of analysis control reflex stimulus-response curves obtained before microinjections of CPA were also compared with those generated ~70 min after the microinjections (recovery). The maximal reflex responses in all measured variables were compared between the experimental conditions. The level of inhibition of the reflex responses exerted by different levels of activation of NTS A_1 receptors (volume control and three doses of CPA) was calculated according to the equation: (control response - response after the microinjection into the NTS)/control response x 100%. A 100% inhibition reflected complete abolition of the response.

The analysis of variance and significance of differences between mean values were calculated using the statistical package SYSTAT version 11 (SYSTAT Software Inc., Richmond, CA, USA). Two-way ANOVA for repeated measures was used to compare the reflex responses of each variable across the experimental conditions (control vs. microinjection and control vs. recovery) and across four levels of activation of the cardiopulmonary reflex (four doses of PBG, 1-8μg/kg). Two-way ANOVA for repeated measures was also used to compare simultaneously recorded three nerve responses (RSNA vs. pre-ASNA vs. LSNA) evoked by four doses of PBG, whereas for comparison of different nerves and components of the adrenal nerve responses (RSNA vs. pre-ASNA vs. int-ASNA vs. post-ASNA vs. LSNA) and four doses of PBG a two-way ANOVA for independent measures was used. Two-way ANOVA for independent measures was also used to analyze the reflex responses evoked by volume control (ACF) and three doses of CPA (0.033-330 pmol) for the three sympathetic outputs (RSNA vs. LSNA vs. pre-ASNA) at each level of activation of the cardiopulmonary

chemoreflex. If significant interactions were found (nerve × experimental condition and nerve × dose), the differences between the means were calculated with a modified Bonferroni *t*-test. One-way ANOVA for independent measures followed by modified Bonferroni *t*-test was used for comparison of HR and MAP responses evoked by microinjections of ACF and the three doses of adenosine receptor antagonist, CPA. The changes in baseline levels of MAP, HR, RSNA, LSNA and pre-ASNA evoked by bilateral microinjections of three doses of CPA, the adenosine receptor antagonist, and the respective volume of vehicle were calculated as a difference between baseline values averaged during the last 30 s preceding the microinjections and the last 30 s of the 5 min period of the response to microinjections measured before generating the second cardiopulmonary chemoreflex stimulus response curve. An α-level of $P < 0.05$ was used to determine statistical significance.

RESULTS

Basal MAP and HR measured before the control cardiopulmonary chemoreflex stimulus-response curves in each animal ($n = 57$) were 88.0 ± 1.6 mmHg and 392.7 ± 6.8 beats·min^{-1}, respectively. Approximately 35 min after the control stimulus-response curves, the basal parameters, measured just before microinjections into the NTS of selective A_1 adenosine receptor agonist (CPA), nonselective A_1 + A_{2a} receptors antagonist (8-SPT) or vehicle (ACF) returned toward normal levels: 89.8 ± 1.7 mmHg and 383.8 ± 6.3 beats·min^{-1} for MAP and HR, respectively. Also, in those animals in which the third stimulus response curve was performed ~60 min after the microinjections of CPA (recovery, n = 35), basal MAP and HR were similar to those measured at the beginning of the experiment (85.9 ± 1.5 mmHg and 366.4 ± 8.1 beats·min^{-1}, respectively). There were no substantial differences between these three

basal levels. The effects of bilateral microinjections of three doses of selective A_1 adenosine receptor agonist (CPA, 0.033, 3.3 and 330 pmol in 50 nl, n=10, 13 and 14, respectively), nonselective A_1 + A_{2a} adenosine receptor antagonist, 8-SPT, 1 nmol in 100 nl, n=9), and vehicle (ACF, 50 nl, n=11) on resting hemodynamic and neural variables are presented in Table 1. Inasmuch as most of the effects of A_1 receptor

Table 1. Changes in baseline values following A_1 receptor agonist and nonselective adenosine antagonist. Baseline values of MAP, HR, RSNA, pre-ASNA and LSNA after bilateral microinjection of the selective NTS A_1 adenosine receptor agonist (CPA), respective volume control, ACF, and nonselective adenosine receptor antagonist 8-SPT.

		CPA pmol/50 nl			
	ACF	0.033	3.3	330	8-SPT 1
MAP, Δ mmHg	-9.1 ± 2.5	-0.8 ± 1.3*	-1.2 ± 2.4*	-2.0 ± 2.9	-19.5 ± 6.9
HR, Δ bpm	-25.9 ± 4.8	-13.4 ± 3.7	-24.8 ± 6.0	-50.3 ± 7.2*	-61.6 ± 17.3*
RSNA, Δ%	-17.4 ± 2.3	-3.9 ± 2.6*	-7.6 ± 4.9	-11.6 ± 3.7	-36.5 ± 5.2*
pre-ASNA, Δ%	3.6 ± 4.8#	13.5 ± 3.8#†	31.5 ± 7.7*#†	9.9 ± 4.0#†	-17.6 ± 5.4*#
LSNA, Δ%	-5.4 ± 2.2#	-0.3 ± 2.3	1.2 ± 2.6	-3.7 ± 2.0	-23.7 ± 6.8*

Values are means ± SE. *P < 0.05 vs. ACF; #, P<0.05 vs. RSNA; †, P<0.05 vs. LSNA.

activation are mediated via modulation of arterial baroreflex integration (102; 107) which is abolished by the sino-aortic baroreceptor denervation, the responses to CPA were limited similarly to what we previously observed after sino-aortic baroreceptor denervation plus vagotomy, i.e. increases in MAP, RSNA and LSNA were virtually abolished whereas the increases in pre-ASNA were attenuated (107). The hemodynamic and neural responses evoked by bilateral microinjections of adenosine receptor antagonist (8-SPT) and vehicle (ACF), performed in the present study in sinoaortic denervated animals, were accentuated compared to the responses observed in intact animals (54).

Cardiopulmonary chemoreflex differentially inhibits regional sympathetic outputs

An example of original recordings of hemodynamic and neural responses evoked by increasing activation of cardiopulmonary chemoreflex under control conditions is

presented in Figure 4 (left panels). Figure 5 presents averaged control cardio-pulmonary chemoreflex curves. Gradual activation of cardiopulmonary receptors via increasing doses of PBG (1-8 μg/kg) evoked dose dependent decreases in MAP and HR and differential decreases in regional sympathetic nerve activity (RSNA>pre-ASNA>LSNA). Figure 5A presents combined double and triple nerve recordings (RSNA, pre-ASNA, int-ASNA, post-ASNA and LSNA). Two way ANOVA for independent measures showed that the regional sympathetic outputs were inhibited to a different degree (nerve factor, $p<0.001$) and that the steepness of reflex stimulus response curves (Figure 5A) were different across the nerves (PBG dose vs. nerve interaction, $p=0.003$). Interestingly, no differences were observed between the responses of different components of the adrenal nerve: pre-ASNA, int-ASNA and post ASNA (PBG dose vs. nerve interaction $p=0.913$) although the responses of each of the components were significantly different from the responses in RSNA and LSNA. Therefore pre-ASNA (n=37) and int-ASNA (n=6) were combined for further calculations

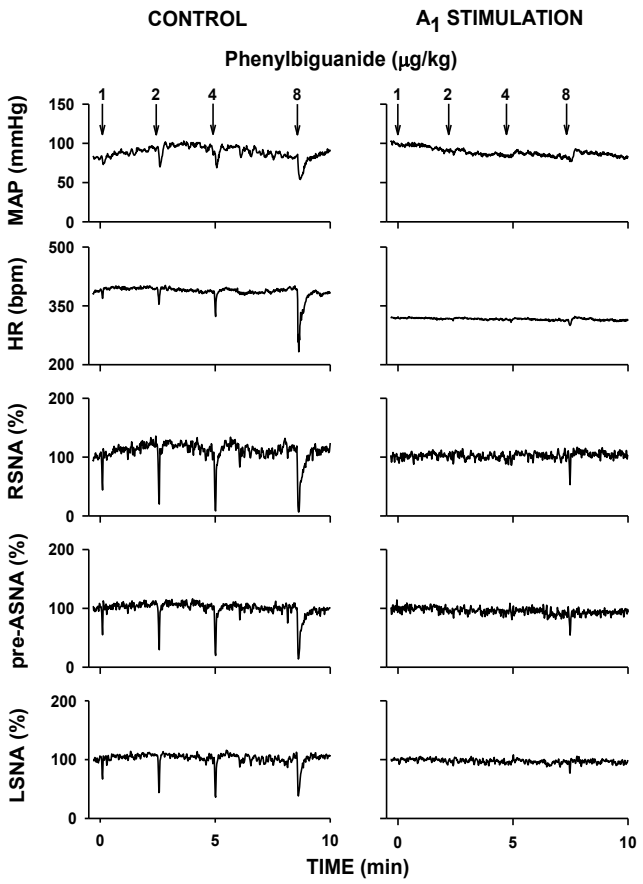

Figure 4. Example of the reflex responses under control conditions and following adenosine A_1 receptor activation. Cardiopulmonary chemoreflex responses of mean arterial pressure (MAP), heart rate (HR), renal (RSNA) and preganglionic adrenal (pre-ASNA) and lumbar (LSNA) sympathetic nerve activity evoked by increasing doses of phenylbiguanide into the right atrium under control conditions (left panels) and following stimulation of NTS A_1 adenosine receptors with the highest dose of the selective agonist, CPA (330 pmol in 50 nl). All recording were performed in one animal. The blockade of A_1 adenosine receptors in the NTS virtually abolished the reflex responses.

and considered as a predominantly preganglionic-ASNA (activity remaining after the ganglionic blockade >50%). Predominantly postganglionic ASNA (n=4) was excluded from further calculations.

The comparison of control neural responses recorded in all 57 animals (Figure 5A, double and triple nerve recordings combined) yielded very similar results to those where 3 sympathetic outputs (RSNA, pre-ASNA and LSNA) were recorded simultaneously (Figure 5B, n=21). The results of two way ANOVA for the triple nerve recordings only (repeated measures, nerve factor, p<0.001, PBG dose vs. nerve interaction p=0.001) (Figure 5B) were very similar to those obtained for all combined neural recordings (Figure 5A) (nerve factor, p<0.001, PBG dose vs. nerve interaction p=0.003). This attests that the reflex responses recorded from two or three sympathetic outputs under the same experimental conditions were fully comparable.

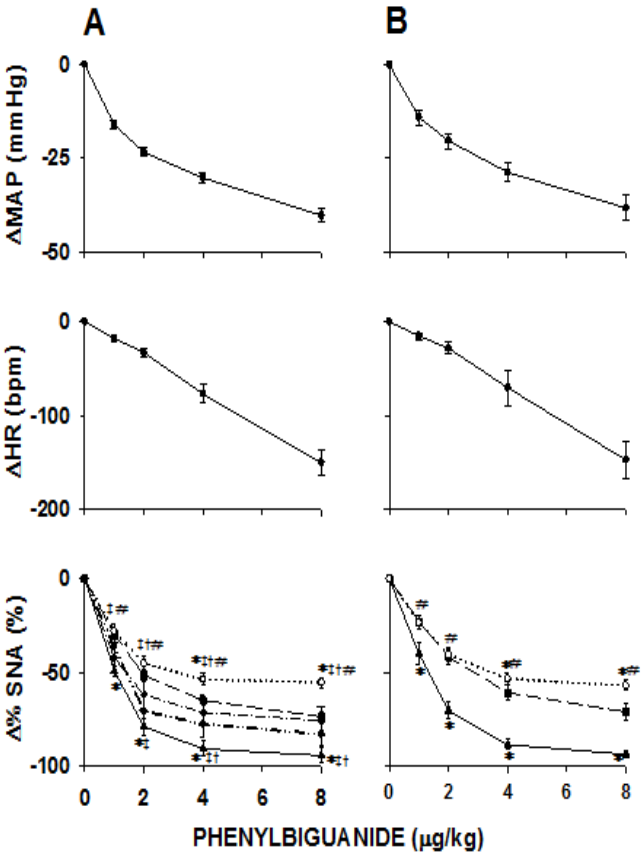

Figure 5. Averaged cardiopulmonary chemoreflex responses to PBG. Hemodynamic and regional neural responses evoked by gradual stimulation of cardiopulmonary receptors with increasing doses of phenylbiguanide. A: combined all neural recordings performed in 57 animals from lumbar ○ (39) preganglionic adrenal ■(37), intermediate adrenal ◆(6) postganglionic adrenal ●(4) and renal ▲ (56) sympathetic nerves; number of recordings from each nerve in parentheses. B: simultaneous neural recordings from renal, preganglionic adrenal and lumbar sympathetic nerves performed in 21 animals. MAP, mean arterial pressure; HR, heart rate; SNA, sympathetic nerve activity. Data are means ± standard error. Statistical differences, P<0.05: *, vs. preganglionic adrenal; ‡, vs. intermediate adrenal †, vs. postganglionic adrenal; #, lumbar vs. renal. There were no significant differences between the responses of different components of the adrenal nerve.

A_1 adenosine receptors inhibit cardiopulmonary chemoreflex mechanism at the level of the NTS

The example of original recordings (Figure 4) shows powerful inhibition of cardiopulmonary reflex responses following bilateral activation of NTS A_1 adenosine receptors with the maximal dose of CPA (330 pmol). This effect was dose dependent as shown in Figure 6. The stimulus response curves were gradually flattened as the

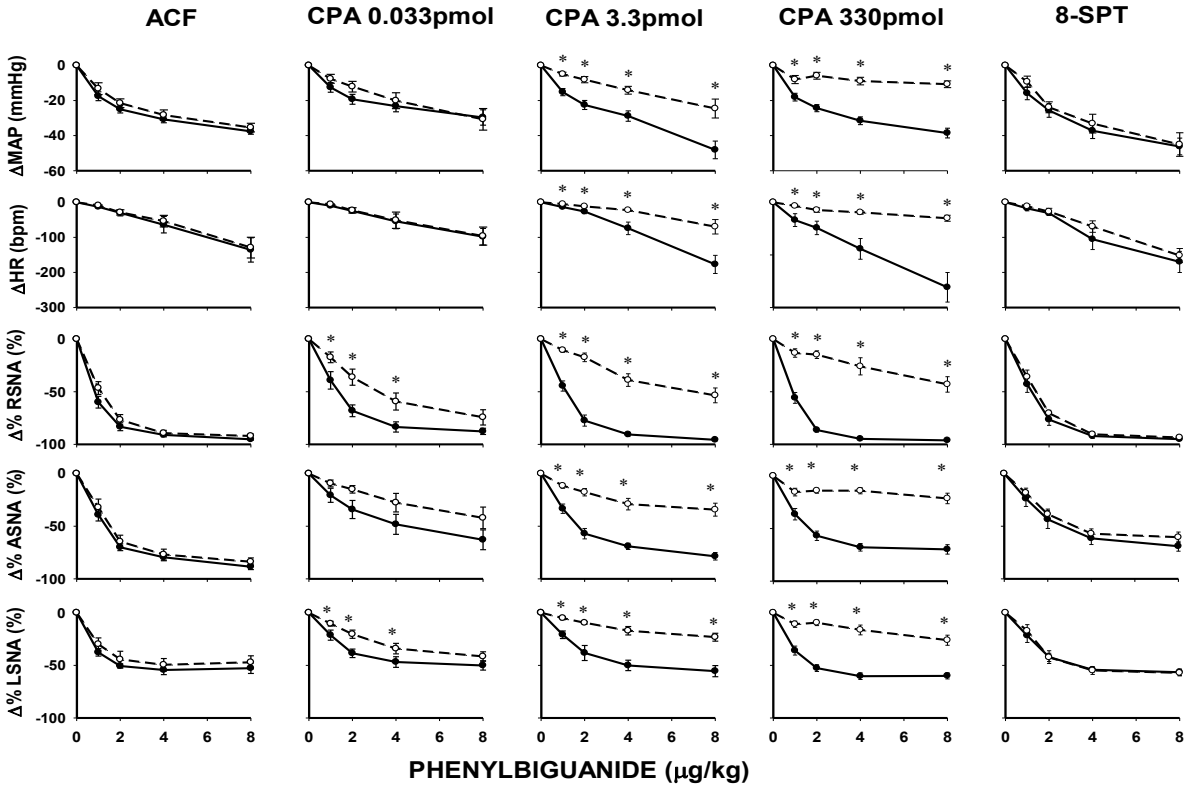

Figure 6. Comparisons of the reflex responses obtained under control and CPA conditions. Cardiopulmonary reflex responses obtained under control conditions ● vs. the responses obtained following volume control (ACF), increasing doses of A_1 adenosine receptor agonist (CPA) and adenosine receptor antagonist (8-SPT) ○. Data are means ± standard error. MAP, mean arterial pressure; HR, heart rate; RSNA, renal, ASNA, preganglionic adrenal, LSNA, lumbar sympathetic nerve activity. *, $P<0.05$ vs. control. Increasing activation of A_1 adenosine receptors gradually inhibited the reflex responses. Adenosine receptor antagonist and volume control did not alter the reflex responses.

dose of A_1 adenosine receptor agonist (CPA) increased. The smallest dose of the agonist (CPA, 0.033 pmol) significantly attenuated the reflex control of renal and lumbar sympathetic outputs and tended to inhibit pre-ASNA reflex responses (control vs.

experimental conditions effect, p=0.098). No attenuation of MAP and HR reflex responses were observed in this setting (control vs. experimental conditions effects, p=0.469 and p=0.889, respectively). Greater activation of NTS A_1 receptors (CPA, 3.3 and 330 pmol) evoked gradual and significant attenuation the reflex responses of all variables at all levels of activation of the cardiopulmonary chemoreflex. Comparisons across the nerves (renal, preganglionic adrenal and lumbar) and doses of CPA (from 0, volume control, to 330 pmol) showed that the nerves responded differentially to activation of the reflex (nerve effect, p<0.0001) and were inhibited by A_1 adenosine receptor agonist in a dose dependent manner (CPA dose effect, p<0.0001). However, the inhibition of the reflex responses in all sympathetic outputs was similar (no significant nerve vs. CPA dose interaction). Microinjections of vehicle (ACF) or adenosine receptor antagonist (8-SPT) did not affect the reflex responses (far left and right panels of Figure 6, respectively).

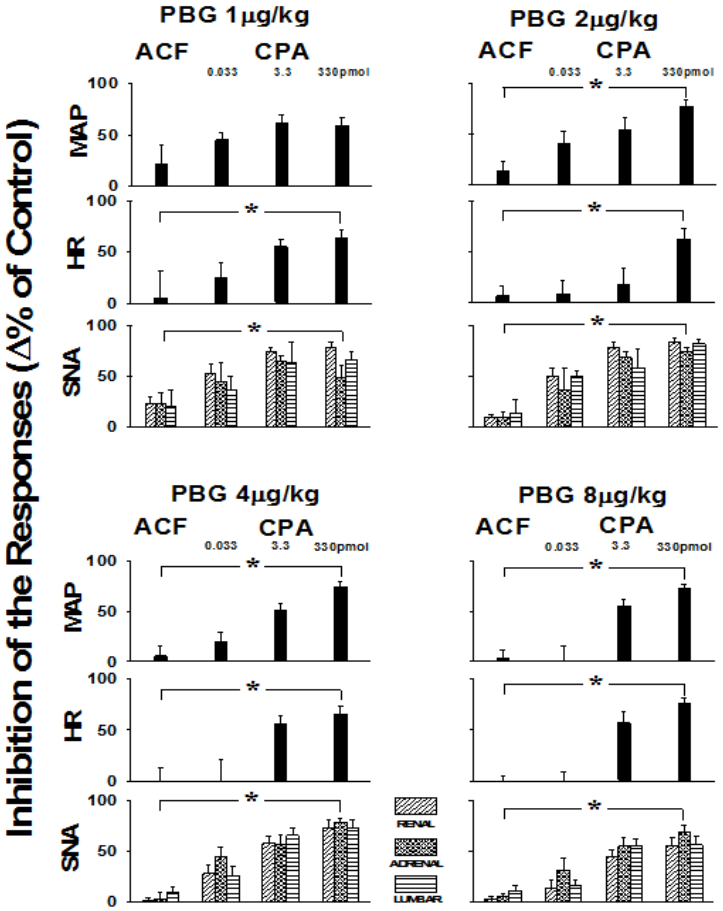

Figure 7. The inhibition of the control reflex responses evoked by A_1 receptor activation. Hemodynamic and regional sympathetic reflex responses evoked by volume control (ACF) and the increasing doses of adenosine A_1 receptor agonist, CPA microinjected into the NTS at four different levels of activation of the reflex (intra-atrial injections of phenylbiguanide, PBG, 1-8 µg/kg). Data are means ± standard error. SNA-sympathetic nerve activity of renal, preganglionic adrenal and lumbar nerves. CPA exerted significant, dose dependent inhibition of the reflex responses at all levels of activation of the reflex, *, P<0.05. No significant differences between the inhibition of regional sympathetic responses were found.

Figure 7 shows the level of inhibition of the cardiopulmonary chemoreflex after

activation of NTS A_1 adenosine receptors and respective volume control. Inhibition was quantified as the % reduction in the control response; therefore 100% inhibition corresponds to a completely abolished response. The cardiopulmonary chemoreflex responses of all recorded variables were inhibited in dose dependent manner at each level of activation of the reflex (highly significant CPA dose effects) (Figure 7). However, no significant differences in the inhibition of the three regional sympathetic outputs were found (nerve effects, $p>0.05$ for all comparisons). Thus, whereas

Table 2. Averaged cardiopulmonary chemoreflex responses to PBG evoked under control conditions and 1 hour after microinjection of A_1 adenosine receptor agonist CPA

			Control					Recovery				
CGS-21680 pmol/50 nl	n	PBG μg/kg	MAP Δ%	HR Δbpm	RSNA Δ%	pre-ASNA Δ%	LSNA Δ%	MAP Δ%	HR Δbpm	RSNA Δ%	pre- ASNA Δ%	LSNA Δ%
0.2	10	1	-8.7 ± 1.8	-7.2 ± 1.7	-18.9 ± 3.9	-12.4 ± 1.7	-13.2 ± 3.3	-9.8 ± 2.0	-7. ± 1.6	-24.1 ± 7.5	-18.9 ± 5.7	-14.5 ± 3.3
		2	-17.3 ± 2.5	-16.3 ± 3.4	-46.5 ± 6.4	-25.3 ± 5.2	-29.2 ± 5.5	-17.6 ± 3.2	-20.5 ± 8.3	-53.2 ± 8.3	l35.4 ± 8.4	-33.2 ± 5.5
		4	-24.5 ± 2.8	-28.5 ± 7.6	-67.7 ± 6.2	-43.1 ± 4.3	-42.7 ± 5.0	-22.8 ± 4.2	-31.3 ± 9.1	-73.2 ± 5.7	-53.7 ± 7.5	-46.9 ± 4.2
		8	-32.2 ± 4.2	-75.2 ± 25.4	-84.2 ± 2.7	-61.5 ± 2.8	-53.6 ± 4.2	-34.1 ± 6.0	-97.1 ± 34.9	-88.0 ± 2.5	-64.2 ± 5.2	-54.2 ± 3.4
2	10	1	-11.8 ± 1.8	-11.6 ± 3.1	-40.6 ± 4.9	-21.7 ± 2.7	-26.5 ± 3.1	-13.8 ± 2.3	-10.7 ± 1.3	-43.2 ± 6.1	-24.6 ± 3.6	-32.4 ± 6.6
		2	-21.5 ± 2.3	-20.7 ± 4.0	-76.2 ± 3.4	-51.0 ± 5.9	-56.2 ± 5.3	-17.4 ± 1.6*	-19.5 ± 3.9	-75.9 ± 4.6	-41.5 ± 5.8	-51.0 ± 5.9
		4	-28.2 ± 1.2	-53.3 ± 12.0	-83.8 ± 5.3	-65.2 ± 6.3	-60.7 ± 3.9	-24.8 ± 1.8*	-38.3 ± 6.1	-91.1 ± 2.0	-60.3 ± 5.8	-63.1 ± 5.7
		8	-38.3 ± 4.0	-125.8 ± 26.8	-94.7 ± 0.8	-77.1 ± 3.9	-66.6 ± 4.5	-33.7 ± 2.7	-130.6 ± 33.3	-94.0 ± 1.7	-70.9 ± 5.0	-69.5 ± 5.1
20	9	1	-10.5 ± 1.9	-11.2 ± 2.5	-36.7 ± 7.2	-27.8 ± 7.2	-23.4 ± 4.3	-10.3 ± 1.9	-14.3 ± 3.4	-36.8 ± 6.8	-31.3 ± 4.7	-27.1 ± 4.9
		2	-21.2 ± 1.6	-25.0 ± 5.4	-73.6 ± 4.1	-51.1 ± 7.6	-49.2 ± 4.6	-17.8 ± 2.1	-28.2 ± 6.3	-62.6 ± 3.7*	-42.4 ± 6.8	-44.0 ± 5.4
		4	-25.0 ± 1.5	-47.9 ± 9.0	-89.8 ± 3.0	-65.2 ± 5.5	-55.3 ± 3.8	-25.2 ± 2.3	-42.8 ± 8.1	-83.9 ± 2.6	-57.8 ± 6.7*	-60.0 ± 3.9
		8	-32.2 ± 2.0	-84.6 ± 19.0	-95.5 ± 1.4	-74.4 ± 6.4	-60.2 ± 4.0	-29.5 ± 2.5*	-84.6 ± 18.0	-86.5 ± 5.5	-65.4 ± 7.9	-62.6 ± 4.1

Values are means ± SE. *P<0.05 vs. control.

cardiopulmonary chemoreflex activation elicits markedly differential reductions in regional sympathetic nerve activity, NTS A_1 receptor stimulation inhibits these responses in a uniform fashion. This inhibition was reversible. Table 2 shows that the reflex responses completely recovered approximately 60 min after microinjections of the small and medium doses of CPA into the NTS and tended to recover after the highest dose (330 pmol) of the agonist (compare Table 2 and Figure 7).

DISCUSSION

The most important finding of the present study is that adenosine operating via A_1 receptors within the NTS powerfully inhibits the cardiopulmonary chemoreflex. In

addition, direct comparison of simultaneously recorded renal, adrenal, and lumbar sympathetic responses showed that activation of cardiopulmonary chemoreceptors exerts regionally different inhibition of these sympathetic outputs (RSNA>pre-ASNA>LSNA). Despite of the highly significant differences between cardiopulmonary chemoreflex inhibition of the regional sympathetic outputs, the attenuation of the cardiopulmonary chemoreflex responses by stimulation of NTS A_1 adenosine receptors was similar across the sympathetic outputs (RSNA=pre-ASNA=LSNA). Activation of the CCR evoked the greatest sympathoinhibition in RSNA but smaller in pre-ASNA and the smallest in LSNA. Stimulation of NTS A_1 adenosine receptors inhibited differential CCR responses in renal, adrenal and lumbar sympathetic outputs to a similar extent.

Potential mechanisms

Adenosine operates in the NTS under both physiological and pathological conditions. For example, during the hypothalamic defense response adenosine is released into the NTS (30) and contributes to the pressor component of this response via activation of A_1 receptors (35; 116; 118). Adenosine is also released into the central nervous system, including the NTS during ischemia, hypoxia or severe hemorrhage (87; 110; 129; 138; 139). Activation of presynaptic A_1 adenosine receptors in the central nervous system inhibits the release of transmitters from nerve terminals; central neurons may also be directly inhibited via postsynaptic A_1 adenosine receptors (90). In the NTS A_1 adenosine receptors are present on presynaptic afferent terminals and in somato-dendritic locations (63). The present study confirmed our hypothesis that adenosine operating via A_1 receptors may inhibit neurotransmission in cardiopulmonary chemoreflex pathway at the level of the NTS. These data are consistent with previous studies from our laboratory which showed that stimulation of NTS A_1 adenosine

receptors uniformly resets the baroreflex curves for renal, adrenal and lumbar sympathetic nerve activity toward higher arterial pressure most likely via inhibition of glutamatergic transmission in this reflex pathway (102; 107). The arterial baroreflex and cardiopulmonary chemoreflex are mediated via similar neural pathways and glutamate serves as a primarily neurotransmitter in both reflexes (131). Therefore, the powerful inhibition of cardiopulmonary chemoreflex responses due to activation of NTS A_1 adenosine receptors was mediated probably via inhibition of glutamate release in this reflex pathway at the level of the NTS. Interestingly, the attenuation of cardiopulmonary chemoreflex responses in renal and lumbar sympathetic outputs was already significant at the lowest level of activation of A_1 adenosine receptors (CPA, 0.033 pmol) whereas hemodynamic and adrenal responses were not yet significantly attenuated at this concentration of the agonist (Figure 6, second column of graphs). This lower threshold for adenosine A_1 receptor mediated inhibition of renal and lumbar reflex responses may be a result of differential location/expression of A_1 adenosine receptors on NTS neurons targeting sympathetic outputs directed to different vascular beds and organs.

Stimulation of NTS A_1 adenosine receptors differentially increases baseline sympathetic nerve activity: pre-ASNA>RSNA≥LSNA (107). The renal and lumbar sympathoexcitation was abolished via bilateral sinoaortic denervation combined with vagotomy; the adrenal sympathoexcitation was attenuated in this setting (107). However, it was unknown if arterial baroreflex afferents or vagal afferents or both contributed to the sympathoexcitation evoked by stimulation of A_1 adenosine receptors in the NTS. The present study showed that the sinoaortic denervation alone had very similar effect on baseline shifts of these sympathetic outputs compared to the effect of combined sinoaortic denervation plus vagotomy [compare Table 1 and ref (107)]. This

indicates that the sympathoexcitation produced by A_1 receptor stimulation, previously observed in animals with all afferents intact, is mediated via A_1 adenosine receptor inhibition of baroreflex but not cardiopulmonary chemoreflex pathway. Whereas, both sympathoinhibitory reflexes are powerfully inhibited by activation of NTS A_1 adenosine receptors the cardiopulmonary chemoreflex has little if any tonic activity thus inhibition of the arterial baroreflex is most likely responsible for the effects of NTS A_1 adenosine receptor stimulation in animals with all afferents intact. Collectively, the present and previous results would support the concept that during stress or the hypothalamic defense response, adenosine released within the NTS and operating via A_1 receptors (30; 35; 116; 118) may inhibit sympathoinhibitory reflexes (arterial baroreflex and cardiopulmonary chemoreflex).

It was not surprising that blockade of adenosine receptors in the NTS did not affect the cardiopulmonary chemoreflex responses. Adenosine is released into the NTS mostly in specific physiological or pathological situations (stress, hypoxia, ischemia, severe hemorrhage) (30; 87; 110; 118; 129; 138; 139). Therefore under normal conditions the tonic effects of adenosine are minimal if any. Our previous studies and reports from other laboratories showed very weak or no effects of blockade of adenosine receptors under resting, normal conditions. However, the blockade of A_{2a} or A_1+ A_{2a} receptors in the NTS markedly alters the pattern of autonomic responses to severe hemorrhage, a pathological condition during which adenosine levels increase in the central nervous system (110; 129). Summarizing, naturally released adenosine may have little or no effect on cardiovascular reflexes integrated in the NTS under normal conditions; however, adenosine neuromodulation may become important during pathological conditions when ischemia and hypoxia reach the NTS (87; 110; 129; 138;

139). This is consistent with the hypothesis that adenosine may serve as a negative feedback regulator for the Bezold Jarisch reflex only under pathological conditions, i.e. when the reflex causes life threatening hypotension which exceeds compensatory capability of brainstem autoregulation and adenosine is released into the NTS. It should be stressed that this reflex is naturally activated and enhanced during cardiac ischemia (17; 67; 83; 93) and the role of centrally released adenosine in modulation of cardiac ischemia is unknown .

Differential inhibition of regional sympathetic outputs by cardiopulmonary chemoreflex

The present study showed that under the same experimental conditions the Bezold-Jarisch reflex exerts a differential inhibition of regional sympathetic outputs (RSNA>pre-ASNA>LSNA). This differential pattern of regional sympathoinhibition was similar to that previously observed with stimulation of arterial baroreceptors under similar experimental conditions (54; 99; 102). This is consistent with the hypothesis that similar central pathways mediate both reflexes (131). The renal nerve is functionally consistent as it facilitates pressor responses via vasoconstriction, increase of sodium reabsorption and release of renin (39); all these components are inhibited by stimulation of arterial baroreceptors and perhaps cardiopulmonary chemoreceptors. In the adrenal nerve ~25% of fibers directed to norepinephrine containing chromaffin cells do not respond to cardiopulmonary chemoreflex stimulation (19). In addition to vasoconstricting fibers lumbar sympathetic nerve provides vasodilating fibers and also a substantial number of cutaneous fibers which do not respond strongly to the arterial baroreflex (33; 49) and likely cardiopulmonary chemoreflex stimuli, as suggested by the present study. Therefore the differential regional sympathoinhibition observed in the

present study may be a result of the differential composition of these three nerves. Interestingly, within the adrenal nerve there were no significant differences between the different components, although preganglionic and postganglionic outputs to the adrenal medulla often respond in a reciprocal manner to other stimuli (20; 21; 105). Although qualitatively similar regional sympathetic responses evoked by stimulation of cardiopulmonary receptors with PBG were reported previously (19; 38; 95; 100) this is the first study which allows for quantitative comparisons of the regional sympathetic responses recorded under identical experimental conditions (simultaneous triple nerve recordings).

There are only few controversial reports on cardiopulmonary chemoreflex control of pre-ASNA and LSNA. For example, increases in adrenal nerve activity were reported in response to intra pericardial infusions of phenylbiguanide (52). It is likely, that the activation of a subset of epicardiac serotonin $5HT_3$ receptors increases pre-ASNA (52); however, this effect is overwhelmed by activation of all cardiopulmonary chemoreflex receptors via intra-atrial injections of the agonist. In two other studies typical decreases in RSNA were accompanied with increases in pre-ASNA and no responses from LSNA (40; 130). In both these studies intravenous instead intra-atrial infusions of PBG were performed and experiments were conducted under methohexial instead of urethane/chloralose anesthesia. Since PBG injected into the femoral vein may be markedly diluted before reaching the cardiopulmonary area and taking into consideration that RSNA is inhibited to a much greater extent than LSNA, it is possible that the stimulation threshold for RSNA inhibition but not LSNA inhibition was reached in this study (130). It is more difficult to explain the increases in adrenal nerve activity observed in response to intravenous infusion of PBG (40); perhaps, the infusion of PBG

into the femoral vein and methohexial anesthesia could be responsible.

A recent study from McAllen's laboratory showed that in contrast to typical inhibition of RSNA, cardiac sympathetic activity increases in response to stimulation of cardiopulmonary receptors with PBG, although the reflex cardiac slowing prevail indicating simultaneous activation of vagal inhibition of the heart (95). Even though increase in parasympathetic control of the heart may be cardio-protective; simultaneous increase in sympathetic component increases the probability of cardiac arrhythmia, especially in the ischemic heart, a condition where the cardiopulmonary chemoreflex is naturally activated (67; 93). This potentially cardio-arrhythmic scenario is partially counteracted by inhibition of the adrenal medulla and decreased humoral activation of the heart as sympathetic outputs to both epinephrine and norepinephrine releasing chromafin cells are inhibited by the reflex (19).

Limitations of the method

The method of evoking the cardiopulmonary chemoreflex via intra-atrial infusion of PBG is widely accepted in the field (19; 38; 40; 52; 57; 95; 100; 101; 130) and allows for precise experimental control of the levels of activation of the reflex in dose dependent manner. It should be stressed that the cardiopulmonary chemoreflex is mediated via polymodal vagal afferent fibers. For example, the majority of cardiac vagal afferents responding to mechanical stimulation respond also to PBG (101). Many other substances may activate the cardiopulmonary chemoreceptors during cardiac ischemia and the early phase of infarct (for example, ATP via $P2_x$ receptors, amadamine via vanilloid TRPV1 receptors, nicotine, etc) (44; 67; 85; 93). Therefore the serotonin $5HT_3$ receptor agonist, PBG, may serve as a convenient tool for experimental dissection of the Bezold Jarisch reflex from other reflexes and humoral mechanisms triggered by

cardiac ischemia, for example, bradykinin activated sympathoexcitation mediated via sympathetic afferents from the heart (4; 66).

We used whole nerve adrenal recordings to make feasible simultaneous recordings from up to 3 sympathetic outputs. Therefore our recordings did not allow us to distinguish between sympathetic outputs directed to epinephrine vs. norepinephrine containing chromafin cells. However, this is a minor disadvantage as Cao and Morrison showed that the cardiopulmonary chemoreflex inhibits both sympathetic outputs; it inhibits virtually all spinal neurons projecting to the epinephrinergic chromafin cells and ~ ¾ neurons supplying norepinephrinergic chromafin cells (19).

Similarly as in our previous studies we did not use a selective A_1 adenosine receptor antagonist, as the selective antagonists can be dissolved in dimethyl sulfoxide (DMSO) but not in water (54; 110). Microinjections of DMSO into the NTS evoke profound and long lasting decreases in MAP, HR and all the regional sympathetic nerve activity (preliminary studies). Therefore we chose a water soluble, nonselective antagonist of adenosine receptor subtypes (8-SPT). This antagonist effectively blocked A_1 (and A_{2a}) adenosine receptors in the NTS in a previous study from our laboratory (110). The blockade of adenosine receptor subtypes, performed in the present study under normal, physiological conditions, did not have significant effects on responses evoked by stimulation of the cardiopulmonary chemoreflex in all recorded hemodynamic and neural variables. Therefore, further quantification of the effect of selective blockade of A_1 adenosine receptors in the NTS was not necessary.

Perspectives

The cardiopulmonary chemoreflex provides regionally specific inhibition of non-cardiac sympathetic outputs as well as stimulation of parasympathetic activity to the

heart (19; 38; 95; 100). This may be cardio-protective by decreasing cardiac afterload and oxygen consumption but may also lead to a dangerous hypotension and bradyarrhythmias, fainting and even sudden cardiac death when the cardiopulmonary chemoreflex (Bezold Jarisch reflex) is highly activated (68). Adenosine operating via A_1 receptors in the NTS powerfully inhibits these reflex responses. Since adenosine is released into the central nervous system during hypotensive phase of severe hemorrhage (110; 129) it may serve as a negative feedback regulator for the cardiopulmonary chemoreflex. Fine tuning of sympathoinhibitory and sympathoexcitatory reflexes originating from vagal cardiopulmonary afferents and cardiac sympathetic afferents, respectively, has been previously proposed by Longhurst (66). This parallel tuning of simultaneously activated vagal and sympathetic afferents (for example, with serotonin released from aggregating platelets during clot formation in coronary arteries) occurs via reciprocal effects of both reflexes on sympathetic outflow and arterial pressure as well as via reciprocal inhibition between neuronal pools mediating both reflexes in the NTS (66). The present study demonstrates that this parallel tuning mechanism may be supplemented by "the last chance mechanism" of negative feedback mediated by adenosine which powerfully inhibits the sympathoinhibitory cardiopulmonary chemoreflex preventing life threatening reflex hypotension. Our previous study showed that another sympathoinhibitory reflex, i.e. arterial baroreflex, is also inhibited by activation of A_1 (but not A_{2a}) adenosine receptors in the NTS (54; 102). Taken together, this suggests that adenosine may play a role of universal negative feedback regulator preventing severe hypotension mediated by sympathoinhibitory, reflexes primarily integrated in the NTS. Further studies may show if adenosine inhibits or facilitates sympathoexcitatory reflexes integrated in the NTS (for

example arterial chemoreflex or sympathetic afferent reflexes).

CHAPTER 2

Paradoxical inhibition of cardiopulmonary chemoreflex control of regional sympathetic outputs evoked by activation of NTS A_{2a} adenosine receptors

ABSTRACT

Previously we have shown that stimulation of inhibitory NTS A_1 adenosine receptors attenuates cardiopulmonary chemoreflex (CCR) sympatho-inhibition of renal (RSNA), preganglionic adrenal (pre-ASNA) and lumbar (LSNA) sympathetic nerve activity and decreases in arterial pressure and heart rate. We have also shown that activation of the facilitatory NTS A_{2a} adenosine receptors has differential effects on the baseline levels of these sympathetic outputs; decreasing RSNA, increasing pre-ASNA and not changing LSNA. Therefore, we hypothesized that stimulation of A_{2a} receptors will facilitate the CCR evoked decreases in RSNA, but it will not change the CCR responses in LSNA and will attenuate the CCR responses in pre-ASNA. In urethane/chloralose anesthetized rats (n=38) we compared regional sympathetic responses evoked by stimulation of the CCR with right atrial injections of the serotonin $5HT_3$ receptor agonist, phenylbiguanide, (PBG 1-8 μg/kg) before and after selective stimulation or blockade of NTS A_{2a} adenosine receptors (microinjections into the NTS of CGS-21680 0.2-20 pmol/50 nl or ZM-241385 40 pmol/100 nl, respectively). Surprisingly, we found that the facilitatory A_{2a} adenosine receptors paradoxically inhibited the hemodynamic and regional sympathetic reflex responses. The reflex sympathetic responses were inhibited uniformly despite of the contrasting shifts in baseline sympathetic activity suggesting that these two effects are mediated via different mechanisms. Blockade of NTS A_{2a} receptors virtually did not affect the CCR responses indicating little tonic A_{2a} receptor activity. These data suggest that stimulation of NTS

A_{2a} receptors triggers unknown inhibitory mechanism(s) which in turn inhibit transmission in the CCR pathway at the level of the NTS.

INTRODUCTION

Adenosine operating via A_1 and A_{2a} receptors is a powerful central modulator of cardiovascular reflexes primarily integrated in the nucleus of the solitary tract (NTS) (1; 81; 103; 108; 123). The importance of adenosine neuromodulation occurring in the NTS is stressed by the fact that the NTS contains the greatest density of adenosine uptake sites in the entire central nervous system (14). Adenosine is released into the NTS during life threatening situations. During the stress/hypothalamic defense response the source of endogenous adenosine is extracellular ATP released from descending hypothalamic terminals or activated glial cells and catabolized to adenosine by ectonucleotidases (30; 116-118; 142). In contrast, during severe hemodynamic imbalance (ischemia, hypoxia or severe hemorrhage) intracellular ATP is catabolized to adenosine inside hypoxic neurons and glial cells and then adenosine is released into the extracellular space (87; 129; 138; 139). Despite this global action, adenosine evokes contrasting effects on regional sympathetic outputs and blood pressure control via two antagonistic A_1 and A_{2a} receptor subtypes which inhibit and activate neurotransmitter release and central neurons, respectively (90). Our previous studies showed that selective stimulation of NTS A_1 receptors evokes pressor responses and differential increases in renal (RSNA), preganglionic adrenal (pre-ASNA), and lumbar (LSNA) sympathetic nerve activity (107) mostly via inhibition of baroreflex restraint of these sympathetic outputs (102). In contrast, selective stimulation of NTS A_{2a} receptors evokes depressor responses and greatly diverse regional sympathetic responses: decreases in RSNA, increases in pre-ASNA, and no changes in LSNA (104; 105).

These highly contrasting effects of adenosine receptor subtypes on regional cardiovascular control most likely are the result of differential localization of A_1 and A_{2a} receptors on NTS interneurons which finally target different sympathetic outputs as we proposed previously (103; 108).

Our most recent paper showed that stimulation of NTS A_1 adenosine receptors inhibited the cardiopulmonary chemoreflex (CCR)-mediated regional sympathetic responses in a uniform way, although this reflex (also known as the Bezold Jarisch reflex) evoked differential regional sympathoinhibition (RSNA>pre-ASNA>LSNA) (53). The Bezold-Jarisch reflex is a powerful depressor, cardiac slowing and sympathoinhibitory reflex activated by polymodal mechano- and chemoreceptors which transmit information from the cardiopulmonary area to the NTS via unmyelinated vagal C fibers in the rat (26; 84; 85; 124-126). Powerful activation of this reflex may contribute to fainting and is even blamed for some episodes of sudden cardiac death in young athletes (13; 48; 68).

Since A_{2a} adenosine receptors, which dominate in the NTS, usually exert opposite effects to those evoked by A_1 receptors we anticipated that selective activation of NTS A_{2a} receptors may differentially facilitate the CCR. This was likely because the same pattern of changes in regional sympathetic outputs that are observed with stimulation of A_{2a} receptors (104; 105) has also been reported occasionally with activation of the CCR; e.g., increases in pre-ASNA, decreases in RSNA and no changes in LSNA (40; 52; 130). However, the majority of previous reports and our most recent study indicated that CCR differentially inhibits the regional sympathetic nerve activity (RSNA>ASNA≥LSNA) (19; 38; 53; 95; 100; 131). Therefore, considering the diverse pattern of regional sympathetic responses to stimulation of NTS A_{2a} receptors,

we hypothesized that stimulation of these receptors may facilitate CCR responses to a greater extent in RSNA than LSNA. In contrast, pre-ASNA reflex responses may be even attenuated as activation of A_{2a} adenosine receptors and CCR afferents exerts the opposite effects on this sympathetic output.

We compared the regional sympathetic responses evoked by gradual activation of CCR afferents before and after stimulation of NTS A_{2a} adenosine receptors with increasing doses of the selective agonist (CGS-21680). We also investigated the effect of inhibition of these receptors with the selective antagonist (ZM-241385) to test whether A_{2a} receptors are tonically active in the CCR pathway. To our surprise, instead of differential facilitation of CCR control of regional sympathetic outputs we observed paradoxical, uniform inhibition of all regional sympathetic reflex responses similar to that observed previously with stimulation of antagonistic A_1 adenosine receptors in the NTS (53).

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* endorsed by the American Physiological Society and published by the National Institutes of Health.

Design

We compared regional sympathetic (renal, adrenal, and lumbar) and hemodynamic (heart rate, HR, and mean arterial pressure, MAP) responses evoked by graded stimulation of the cardiopulmonary chemoreceptors before and after selective activation or inhibition of NTS A_{2a} adenosine receptors in a total of 38 male Sprague-Dawley rats, weight = 367.4 ± 6.3 g. In 29 rats, the comparisons were made between

CCR stimulus response function curves obtained under control conditions and approximately 5 min after microinjections into the NTS of three different doses of the selective A_{2a} adenosine receptor agonist, CGS-21680, (0.2, 2.0, and 20 pmol in 50 nl; numbers of animals in each group were 10, 10 and 9, respectively). In an additional 9 rats the CCR function curves were compared before and ~ 5 min after inhibition of NTS A_{2a} adenosine receptors with a water soluble, selective A_{2a} receptor antagonist; ZM-241385 (40 pmol in 100 nl). The CCR reactivity was also assessed approximately one hour after the pharmacological manipulations in the NTS (recovery) in 36 rats; in two of them the evaluation of the recovery reflex curves was impossible for technical reasons. The timeline of the protocol is presented in Figure 8.

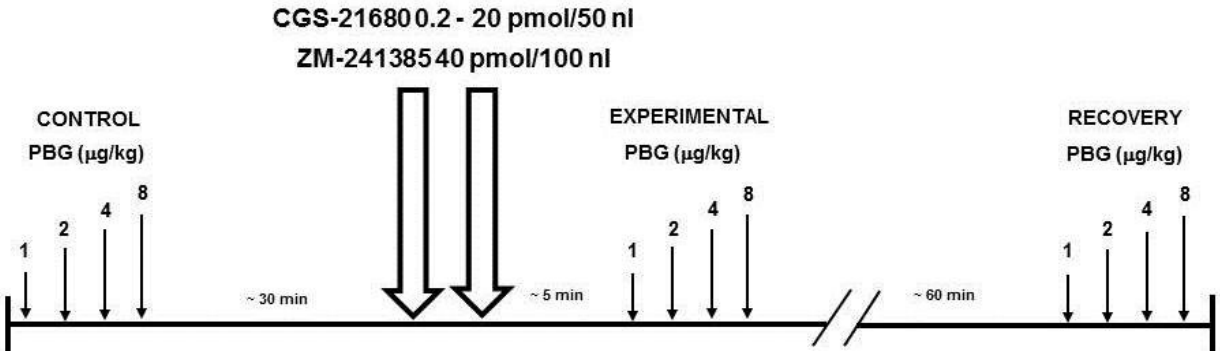

Figure 8. Timeline of CSG-21680 experiments. Small arrows indicate bolus injections of PBG in increasing doses (1-8 μg/kg) into the right atrium in 2.5 min intervals. Double large arrows represent bilateral microinjections into the NTS of: A_{2a} adenosine receptor agonist CGS-21680 (0.2, 2 and 20 pmol in 50 nl), and selective A_{2a} adenosine receptor antagonist, ZM-241385 (40 pmol in 100 nl).

Instrumentation and measurements

All procedures were described in detail previously (53; 100; 105-107). Briefly, male Sprague-Dawley rats (Charles River) were anesthetized with a mixture of α-chloralose (80 $mg \cdot kg^{-1}$) and urethane (500 $mg \cdot kg^{-1}$ i.p.), tracheotomized, connected to a small-animal respirator (SAR-830, CWE, Ardmore, PA), and artificially ventilated with 40% oxygen, 60% nitrogen mixture. Arterial blood gases were tested occasionally (Radiometer, ABL500, OSM3), and ventilation was adjusted to maintain Po_2, Pco_2, and

pH within normal ranges. Average values measured at the end of each experiment were: Po_2 = 153.1 ± 8.1 Torr, Pco_2 = 33.7 ± 1.2 Torr and pH = 7.4 ± 0.01. The left femoral artery and vein were catheterized to monitor arterial blood pressure; infuse drugs and to supplement anesthesia (12–21 $mg \cdot kg^{-1} \cdot h^{-1}$ of α-chloralose and 76–133 $mg \cdot kg^{-1} \cdot h^{-1}$ of urethane dissolved in 2.4–4.2 $ml \cdot kg^{-1} \cdot h^{-1}$ saline), respectively. An additional catheter was inserted into the right atrium via the right jugular vein for intra atrial PBG injections. The appropriate position of the catheter in the right atrium was confirmed post mortem. Sinoaortic denervation was performed and tested similarly as in the previous studies from our laboratory; special attention was given to preserve the vagus nerve intact (53; 100; 105; 107).

Neural recordings

In each experiment, simultaneous recordings from three (n=35) or two sympathetic outputs (n=3) were performed. Renal and adrenal nerves were exposed retroperitoneally form a flank incision, whereas lumbar sympathetic trunk was exposed from midabdominal incision. Neural recordings were accomplished as described previously (53; 100; 105-107). Neural signals were initially amplified (2,000–20,000×) with bandwidth set at 100–1,000 Hz, digitized, rectified, and averaged in 1-s intervals. Background noise was determined after the animal was euthanized yet artificially ventilated to leave unaltered any potential movement artifacts. Resting level of the nerve activity was normalized to 100% before each stimulation of the cardiopulmonary receptors.

The ratio between preganglionic and total nerve activity was initially tested with an intravenous bolus injection of the short-lasting (1-2 min) ganglionic blocker, Arfonad (trimethaphan, 2 $mg \cdot kg^{-1}$; Hoffmann-La Roche), and finally evaluated at the end of each

experiment with hexamethonium (20 mg·kg^{-1} i.v.). RSNA was almost completely postganglionic; 9.2 ± 2.6% (n = 38) of the activity persisted after the ganglionic blockade; LSNA was predominantly postganglionic; 41.4 ± 5.1% (n = 37) of preganglionic activity remained after the blockade. Of total of 36 adrenal nerves recorded 22 were predominantly preganglionic pre-ASNA (>75% of activity remained after the ganglionic blockade), 4 predominantly postganglionic post-ASNA (<50% of activity remained after the ganglionic blockade) and 10 had intermediate composition, int-ASNA (50%-75% of activity remained after the ganglionic blockade). Average levels of preganglionic, intermediate, and postganglionic adrenal nerve activity after ganglionic blockade were: 103.8 ± 8.4, 65.4 ± 3.1, 23.3 ± 5.9%, respectively. Similarly as in our previous study predominantly postganglionic ASNA was excluded from further calculations whereas pre-ASNA (n=22) and int-ASNA (n=10) were combined as predominantly preganglionic ASNA (>50% of activity remained after the ganglionic blockade) (53). Nevertheless, a separate comparison of the effects of adenosine A_{2a} receptor agonist and antagonist on all components of the adrenal nerve was also performed (n=36). The arterial pressure and neural signals were digitized and recorded with a Hemodynamic and Neural Data Analyzer (Biotech Products, Greenwood, IN), averaged over 1-s intervals, and stored on the hard disk for subsequent analysis.

Microinjections into the NTS

Bilateral microinjections of three different doses of the selective A_{2a} receptor agonist, CGS-21680 (0.2, 2 and 20 pmol in 50 nl of ACF), and the antagonist, ZM-241385 (40 pmol in 100 nl of ACF), were made with multibarrel glass micropipettes into the medial region of the caudal NTS as described previously (9; 53; 105; 107). The doses and volumes of the agonist and antagonist were the same as used in our

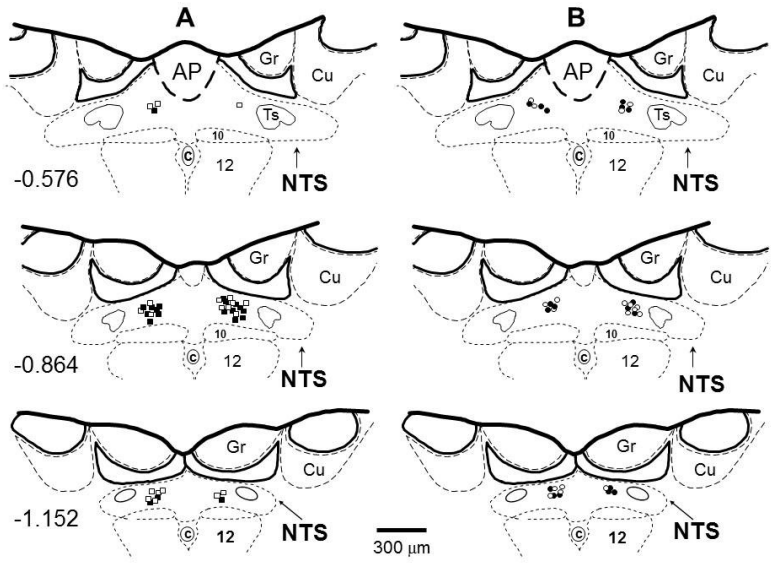

Figure 9. Microinjection sites in the subpostremal NTS for all CGS-21680 experimental groups. AP, area postrema; c, central canal; 10, dorsal motor nucleus of vagus nerve; 12, nucleus of hypoglossal nerve; Ts, tractus solitarius; Gr, gracile nucleus; Cu, cuneate nucleus. Scale is shown at the *bottom*; number on the *left* of schematic diagram denotes the rostrocaudal position in millimeters of the section relative to the obex according to the atlas of the rat subpostremal NTS (7). Bilateral microinjection sites for different doses of A_{2a} receptor agonist, CGS-21680 and A2a receptor antagonist, ZM-241385 were marked with fluorescent dye. A: ■, 0.2 pmol CGS-21680; □, 2 pmol CGS-21680; B: ●, 20 pmol CGS-21680, ○, 40 pmol ZM-241385.

previous studies (53; 54; 102; 106; 107; 110). The drugs were dissolved in ACF with pH adjusted to 7.2; the selective A_{2a} adenosine receptor agonist CGS-21680 often precipitates in a pH >7.2. All microinjection sites were marked with DiI lypophilic dye (Molecular Probes, CA) and verified histologically as described previously (9; 53; 105; 107). The microinjection sites are presented in Figure 9 using diagrams based on the atlas of the rat subpostremal NTS by Barraco et al. (7).

Cardiopulmonary chemoreflex stimulus-response curves

The CCR stimulus-response curves were generated as described in our previous studies (53; 100; 101). Briefly, the cardiopulmonary receptors were stimulated using bolus injections into the right atrium of increasing doses (1-8 μg/kg) of serotonin $5HT_3$ receptor agonist, phenylbiguanide (PBG, Sigma, 20 μg/1ml solution in 0.9% NaCl) in 2.5 min intervals between the injections. Total time to complete the stimulus-response curve was ~ 8 min. The curves were obtained under control conditions; 30 min later, approximately 5 min after bilateral microinjections of drugs (CGS-21680 or ZM-241385) into the caudal NTS, and again 60 min after the microinjections, recovery (Figure 8).

This approach allowed assessment of the CCR reactivity during the most effective action of the NTS adenosine A_{2a} receptor activation which occurs ~ 5-15 min after the microinjection of the agonist (105; 106).

Data analysis

The cardiopulmonary reflex stimulus-response curves constructed for MAP, HR and the regional sympathetic outputs under control conditions were compared with those generated after bilateral stimulation/inhibition of NTS A_{2a} adenosine receptors with microinjections of selective agonist and antagonist, CGS-21680 and ZM-241385, respectively. Control reflex stimulus-response curves obtained before microinjections of CGS-21680 or ZM-241385 were also compared with the functions generated ~60 min after the microinjections (recovery). The maximal reflex responses in all measured variables were compared between the experimental conditions. The effects exerted by different levels of activation of NTS A_{2a} receptors (three doses of CGS-21680, 0.2, 2.0, and 20 pmol) on the reflex responses were calculated according to the equation: [(control response - response after the microinjection into the NTS)/control response] x 100%. 100% inhibition reflects complete abolition of the response, whereas negative values represent facilitation of the reflex responses. The same equation was used to calculate the effect of inhibition of NTS A_{2a} receptors (ZM-214385) on the reflex responses.

The data were analyzed using the statistical package SYSTAT version 11 (SYSTAT Software Inc., Richmond, CA, USA). Two-way ANOVA for repeated measures was used to compare the reflex responses of each variable across the experimental conditions (control vs. microinjection and control vs. recovery) and across four levels of activation of the CCR (four doses of PBG, 1-8 μg/kg). Two-way ANOVA

for independent measures was used to compare control renal (n=38), preganglionic adrenal (n=32) and lumbar (n=37) sympathetic responses to four doses of PBG. The effects of three doses of CGS-21680 (0.2-20 pmol) on the reflex responses of the three sympathetic outputs (RSNA vs. pre-ASNA vs. LSNA) at each level of activation of the CCR (comparisons of reflex response curves obtained after gradual activation of NTS A_{2a} adenosine receptors and for changes of the reflex responses vs. control conditions, Δ%) were also evaluated using two-way ANOVA for independent measures. If significant interactions were found (nerve × dose), the differences between the means were calculated with a modified Bonferroni *t*-test. One-way ANOVA for independent measures followed by modified Bonferroni *t*-test was used for comparison of HR and MAP reflex response curves obtained after microinjections of the three doses of adenosine receptor agonist, CGS-21680. The changes in baseline levels of MAP, HR, RSNA, pre-ASNA and LSNA evoked by bilateral microinjections of CGS-21680 and ZM-241385 were calculated as a difference between baseline values averaged during the last 30 s preceding the microinjections and the last 30 s of the 5 min period of the response to microinjections measured before generating the second cardiopulmonary chemoreflex function-curve. An α-level of $P < 0.05$ was used to determine statistical significance.

RESULTS

Average resting MAP and HR values measured before the control CCR stimulus-response curves (n=38) were: 83.5 ± 1.5 mmHg and 411.0 ± 6.1 bpm, respectively. Basal parameters measured just before NTS microinjections of A_{2a} adenosine receptor agonist, CGS-21680, or antagonist, ZM-241385, returned to normal resting values: 82.4 ± 1.6 mmHg and 405.3 ± 6.3 bpm for MAP and HR, respectively. Also, in those animals

in which the third reflex curve was generated ~60 min after the microinjections (recovery, n=36), baseline MAP and HR were similar to those measured at the beginning of experiment (81.7 ± 1.2 mmHg and 398.2 ± 6.4 bpm, respectively). There were no differences between these three baseline levels of MAP and HR measured during three stages of the experiments: control, stimulation or blockade of NTS A_{2a} adenosine receptors, and recovery (P>0.05 for all comparisons).

The effect of NTS A_{2a} adenosine receptors on baseline values

The effects of bilateral microinjections of the selective A_{2a} adenosine receptor agonist, CGS-21680 (0.2, 2, and 20 pmol in 50 nl, n = 10, 10, and 9, respectively), and the selective A_{2a} receptor antagonist, ZM-241385 (40 pmol in 100 nl, n = 9) on resting hemodynamic and neural variables are presented in Table 3. The effects of the smallest

Table 3. Changes in baseline values following A_{2a} receptor agonist and antagonist. Baseline values of MAP, HR, RSNA, pre-ASNA, and LSNA following bilateral NTS microinjections of selective A_{2a} adenosine receptor agonist, CGS-21680, and antagonist ZM-241385

	CGS-21680 pmol/ 50 nl			ZM-241385 40 pmol/ 100 nl
	0.2	2	20	
MAP, Δ mmHg	-6.1 ± 4.2	-6.4 ± 3.3	-9.2 ± 3.6#	-2.4 ± 3.8
HR, Δ bpm	-30.5 ± 5.0#	-29.9 ± 8.4#	-54.5 ± 11.3#	-10.7 ± 9.6
RSNA, Δ%	-15.3 ± 4.1 #	-17.7 ± 4.9‡#	-33.3 ± 6.5∗‡#	-3.4 ± 7.2
pre-ASNA, Δ%	2.6 ± 4.1†#	21.1 ± 6.0∗†‡#	14.6 ± 8.3†‡#	-6.0 ± 4.1
LSNA, Δ%	-6.1 ± 3.0†	-8.6 ± 3.3†#	-15.4 ± 4.3†#	-3.5 ± 3.3

Values are means ± SE. ∗P < 0.05 vs. 0.2 pmol CGS; †P<0.05 vs. RSNA; ‡P<0.05 ASNA vs. LSNA; # vs. 0

dose of CGS-21680 (0.2 pmol/ 50 nl) on baseline values were similar to the effects of bilateral microinjections of 50 nl of ACF (volume control) reported in our previous study (53). Graded levels of A_{2a} receptor activation (CGS-21680; 2, 20 pmol/ 50 nl) evoked similar patterns of changes in baseline levels of hemodynamic and regional sympathetic variables as observed previously: decreases in MAP, HR, RSNA and post-ASNA, increases in pre-ASNA and much smaller changes in LSNA. (104-106). Selective blockade of the A_{2a} adenosine receptors with microinjections of ZM-241385, did not

alter baseline levels of any variables (P>0.05).

Activation of NTS A_{2a} adenosine receptors inhibits cardiopulmonary chemoreflex responses

An example of original recordings of hemodynamic and neural responses to activation of the CCR under control and experimental conditions is presented in Figure 10 (left and right panels, respectively). Under control conditions gradual activation of the cardiopulmonary receptors by increasing doses of PBG (1–8 μg/kg) evoked dose-dependent decreases in MAP and HR and differential decreases in regional sympathetic nerve activity (RSNA > pre-ASNA ≥ LSNA) (nerve effect P<0.0001; nerve vs. dose interaction P=0.007). These differential neural responses were similar to those observed in our previous study (53). The hemodynamic and neural reflex responses were powerfully inhibited following bilateral activation of NTS A_{2a} adenosine receptors with the maximal dose of CGS-21680 (20 pmol/50 nl) (Figure 10, right panels). This

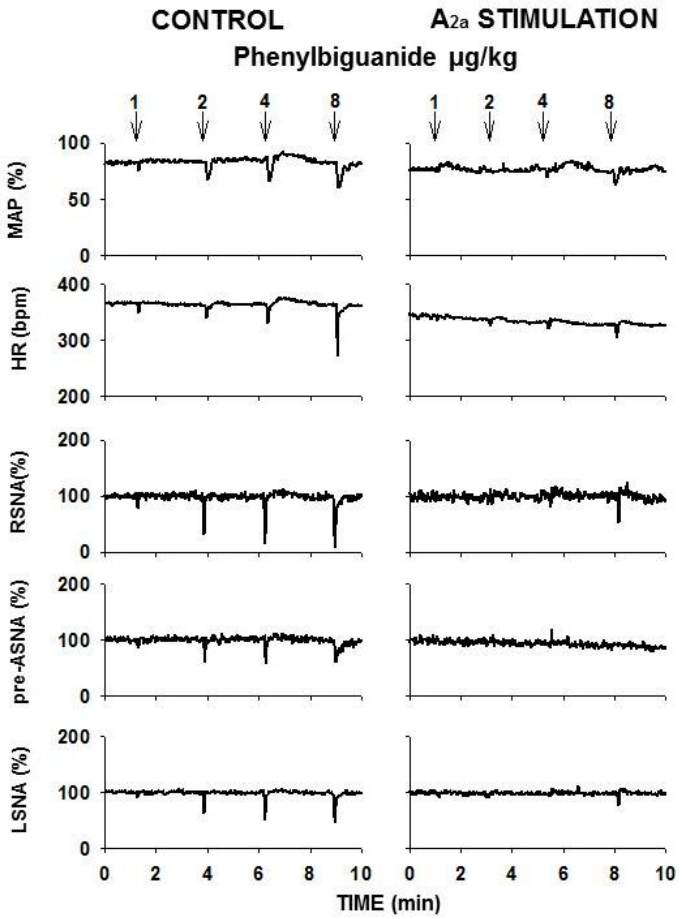

Figure 10. An example of cardiopulmonary chemoreflex responses in mean arterial pressure (MAP), heart rate (HR), renal (RSNA), pre-adrenal (pre-ASNA) and lumbar (LSNA) sympathetic nerve activity evoked by intra-atrial injections of phenylbiguanide (1-8 μg/kg) under control conditions (left panels) and after stimulation of the NTS A_{2a} adenosine receptors (right panels) with the highest dose of the A_{2a} agonist, CGS-21680 (20 pmol in 50 nl). All recordings were performed in one animal. Stimulation of the A_{2a} receptors in the NTS virtually abolished the cardiopulmonary chemoreflex responses.

effect was dose dependent as shown in Figure 11. The lowest level of A_{2a} receptor

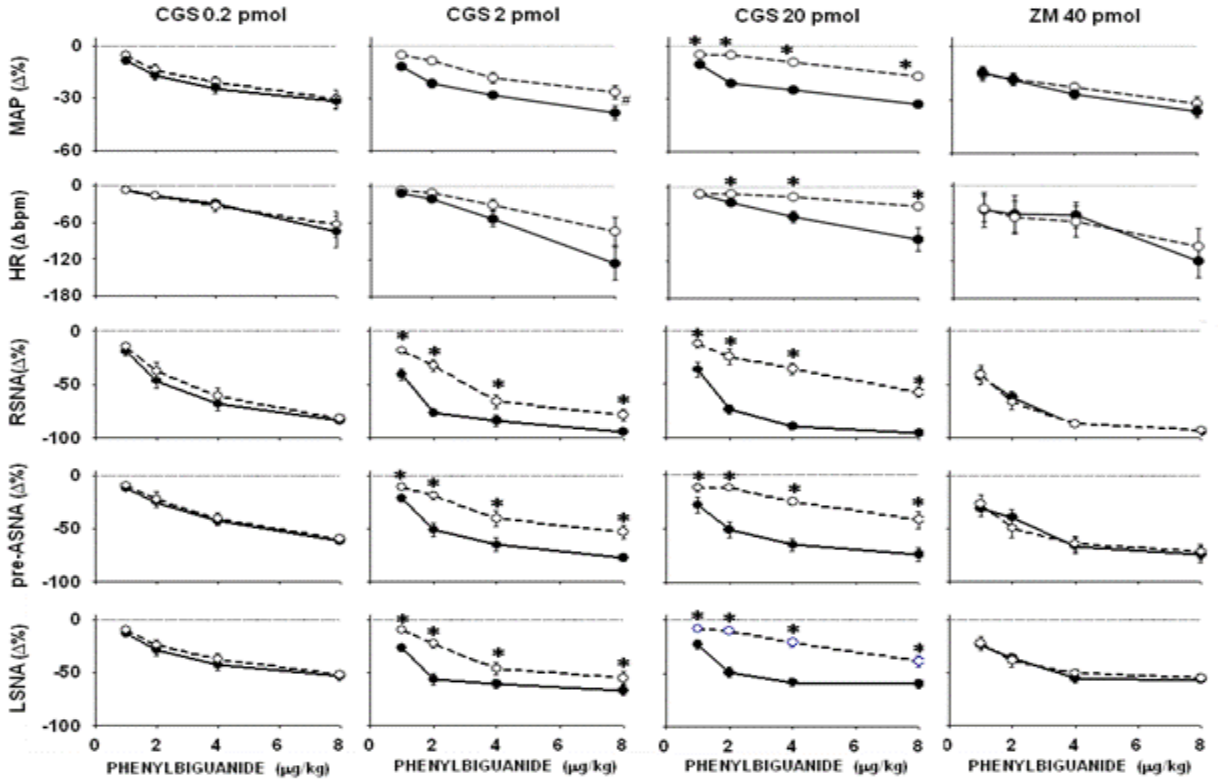

Figure 11. Comparisons of cardiopulmonary chemoreflex responses obtained under control conditions (●) vs. the responses obtained after microinjections of A_{2a} receptor agonist CGS-21680, in increasing doses 0.2, 2 or 20 pmol/ 50 nl or the selective A_{2a} antagonist, ZM-241385, 40 pmol/ 100 nl (○). Data are means ± SE. ∗, P<0.05 vs. control; **#,** P<0.05 significant parallel shift of the experimental vs. control curves without significant PBG dose vs. experimental condition interaction. Progressive activation of A_{2a} adenosine receptors gradually shifted the control reflex function upward indicating dose dependent inhibition of the reflex responses. The A_{2a} receptor blockade had no effect on the reflex responses.

activation (CGS-21680, 0.2 pmol/50 nl) did not alter the control reflex responses (control vs. experimental condition effect P>0.05 for all variables). Therefore this group may be considered as a volume control containing a physiologically insignificant amount of the agonist. Increasing activation of A_{2a} receptors (CGS-21680 2 and 20 pmol) gradually shifted all neural and hemodynamic reflex response curves upward and flattened them. At the highest dose of the agonist the inhibitory effect was highly significant (control vs. experimental condition effect, P<0.0001 for MAP and all the neural outputs and P=0.012 for HR). The slope of all reflex response function curves was significantly alerted vs.

control conditions (experimental effect vs. PBG dose interactions, $P<0.02$ for all variables).

Comparisons across the nerves (renal, pre-adrenal, and lumbar) and doses of CGS-21680 (0.2-20 pmol/ 50 nl) showed that the nerves responded differentially at each level of activation of the reflex (nerve effect, $P<0.05$) and were inhibited in dose-dependent manner by A_{2a} receptor agonist (CGS dose effect, $P<0.05$). However, no differences were found between the inhibition of the CCR responses in RSNA, vs. pre-ASNA, vs. LSNA; slopes of the curves were similar (no significant nerve x CGS dose interactions were found). This suggests that despite the contrasting shifts in the baseline levels of regional sympathetic nerve activity, the stimulation of NTS A_{2a} adenosine receptors similarly inhibited the reflex responses of all sympathetic outputs. Blockade of A_{2a} adenosine receptors with ZM-241385, did not alter the CCR reactivity of all variables (far right panels of Figure 11); control vs. blockade condition effect, $P>0.05$ for all variables.

Interestingly, although stimulation of NTS adenosine A_{2a} receptors reciprocally affects baseline pre-ASNA vs. post-ASNA there were no differences between the inhibitions of different components of the adrenal nerve. Comparisons across pre-ASNA, int-ASNA and post-ASNA vs. dose of CGS 21680 (0.2-20 pmol) with two-way ANOVA showed no nerve component effect ($P>0.05$ at each level of activation of the reflex).

Figure 12 shows to what extent the reflex responses were inhibited after activation of NTS A_{2a} adenosine receptors compared to the control reflex responses (Δ% of inhibition). A 100% inhibition corresponds to completely abolished CCR responses. Downward deflections of some bars (which may represent a tendency to

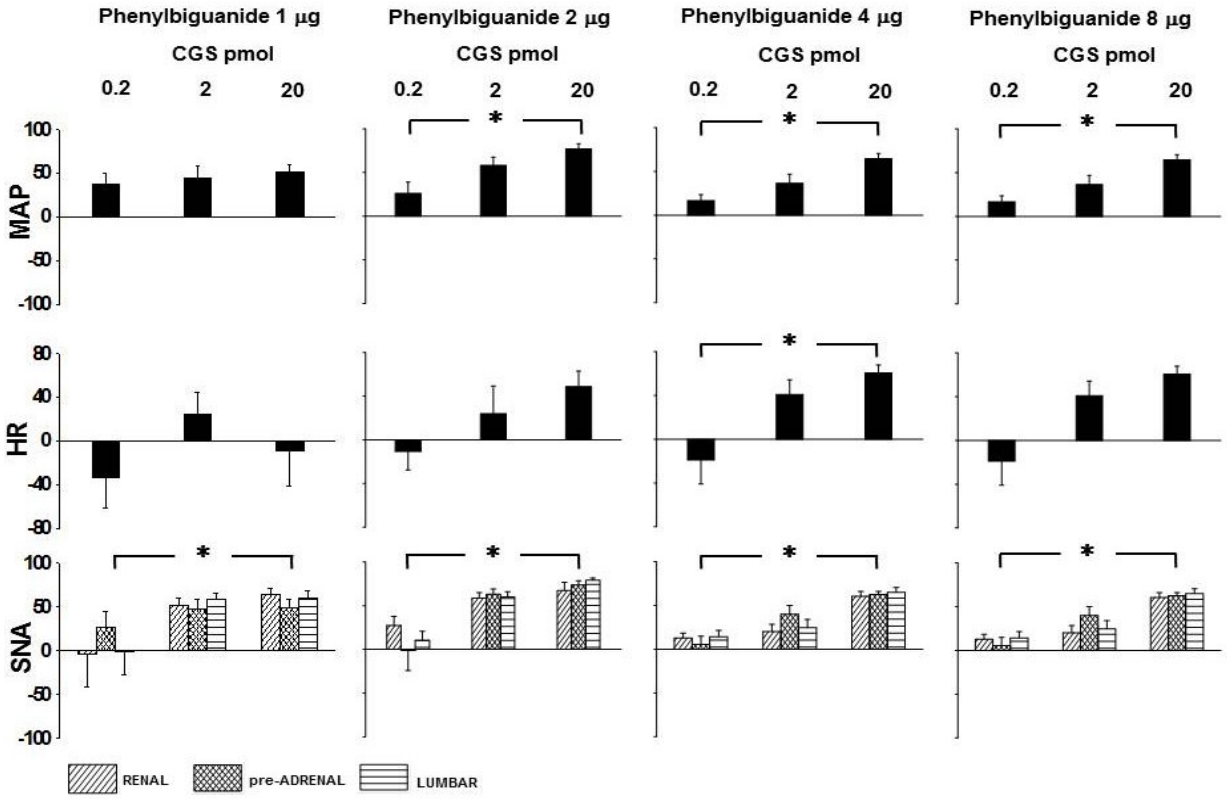

Figure 12. The inhibition of hemodynamic and regional sympathetic control reflex responses evoked by increasing doses of the A_{2a} receptor agonist, CGS-21680 (0.2-20 pmol) at four different levels of activation of the reflex (phenylbiguanide 1-8 μg/kg). Data are means ± SE. SNA - renal, preganglionic adrenal and lumbar sympathetic nerve activity. CGS-21680 exerted significant, dose dependent inhibition of the reflex responses at all levels of activation of the reflex. No differences between the inhibition of the reflex responses in all regional sympathetic outputs were found. The negative deflections of the bars may correspond to potential facilitation of the reflex; however, all the negative bars were not significantly different from zero.

facilitation of the reflex) were not significantly different from zero. The regional sympathetic outputs were inhibited by microinjections of the A_{2a} receptor agonist (CGS-21680) in a dose dependent manner (highly significant dose effect $P=0.002$ for the lowest level of activation of the CCR and $P<0.0001$ for all higher levels of the activation); however, no differences between the inhibition of the sympathetic outputs were found (nerve effect, $P>0.05$ for each level of activation of the CCR). Also the inhibition of all adrenal nerve components (pre-ASNA, int-ASNA and post-ASNA) was uniform (nerve component effect, $P>0.05$ for each level of activation of the CCR). MAP reflex responses were also inhibited in a dose dependent manner ($P<0.02$) at all but the

lowest level of activation of the CCR. However, the inhibition of the HR responses was more variable reaching significance only for the moderate activation of the CCR (4 μg of PBG).

Although the antagonist and the smallest dose of the agonist of A_{2a} adenosine receptors had no significant effect on the CCR responses (Figure 11, the far right and left panels, respectively) the comparison of these two experimental conditions may suggest that the antagonist may show some tendency to facilitate the reflex at the moderate level of its activation (Table 4). However, the drug effect reached significance

Table 4. Inhibition of the reflex responses after small dose of A2a agonist and antagonist. Cardiopulmonary chemoreflex responses, PBG (1-8 μg/kg), after microinjections of selective A_{2a} agonist (CGS-21680) or A_{2a} antagonist (ZM-241385).

DRUGS	PBG μg/kg	MAP Δ%	HR Δ bpm	RSNA Δ%	pre-ASNA Δ%	LSNA Δ%
CGS-21680 0.2 pmol/ 50 nl	1	36.8 ± 13.5	-33.8 ± 28.2	-4.4 ± 37.4	26.4 ± 18.2	-1.3 ± 26.9
	2	26.0 ± 12.8	-10.9 ± 16.8	27.4 ± 10.3	-1.3 ± 22.9	10.4 ± 10.4
	4	16.6 ± 6.8	-18.9 ± 22.0	12.8 ± 5.5	5.4 ± 9.7	14.2 ± 7.2
	8	9.0 ± 8.2	5.6 ± 10.3	2.3 ± 3.0	1.5 ± 5.7	3.0 ± 4.2
ZM-241385 40 pmol/ 100 nl	1	-23.8 ± 36.1	-43.9 ± 30.2	5.6 ± 14.3	16.7 ± 10.0	9.9 ± 20.3
	2	-2.7 ± 18.3	-53.2 ± 26.3	-9.9 ± 10.2	-36.6 ± 26.0	-5.0 ± 12.0
	4	14.0 ± 6.0	-25.1 ± 23.1	1.4 ± 2.6	1.0 ± 5.5	8.7 ± 3.1
	8	13.0 ± 3.8	23.1 ± 10.4	1.9 ± 1.6	2.6 ± 4.6	3.8 ± 4.4

Data are expressed as percent inhibition from control responses which are standardized to zero level. Positive values represent inhibition of the reflex while negative numbers suggest facilitation of the responses. Values are means ± SE. Two-way ANOVA showed a significant drug effect (P=0.022) at moderate activation of the reflex (PBG, 2 μg/kg) but no differences across the nerves (P>0.05 for all doses of PBG).

only for regional sympathetic responses evoked by moderate activation of the CCR (2μg

Table 5. Averaged cardiopulmonary chemoreflex responses to PBG evoked under control conditions and 1 hour after microinjection of A_{2a} adenosine receptor agonist CGS-21680.

			Control					Recovery				
CGS-21680 pmol/50 nl	n	PBG μg/kg	MAP Δ%	HR Δbpm	RSNA Δ%	pre-ASNA Δ%	LSNA Δ%	MAP Δ%	HR Δbpm	RSNA Δ%	pre- ASNA Δ%	LSNA Δ%
0.2	10	1	-8.7 ± 1.8	-7.2 ± 1.7	-18.9 ± 3.9	-12.4 ± 1.7	-13.2 ± 3.3	-9.8 ± 2.0	-7. ± 1.6	-24.1 ± 7.5	-18.9 ± 5.7	-14.5 ± 3.3
		2	-17.3 ± 2.5	-16.3 ± 3.4	-46.5 ± 6.4	-25.3 ± 5.2	-29.2 ± 5.5	-17.6 ± 3.2	-20.5 ± 8.3	-53.2 ± 8.3	I35.4 ± 8.4	-33.2 ± 5.5
		4	-24.5 ± 2.8	-28.5 ± 7.6	-67.7 ± 6.2	-43.1 ± 4.3	-42.7 ± 5.0	-22.8 ± 4.2	-31.3 ± 9.1	-73.2 ± 5.7	-53.7 ± 7.5	-46.9 ± 4.2
		8	-32.2 ± 4.2	-75.2 ± 25.4	-84.2 ± 2.7	-61.5 ± 2.8	-53.6 ± 4.2	-34.1 ± 6.0	-97.1 ± 34.9	-88.0 ± 2.5	-64.2 ± 5.2	-54.2 ± 3.4
2	10	1	-11.8 ± 1.8	-11.6 ± 3.1	-40.6 ± 4.9	-21.7 ± 2.7	-26.5 ± 3.1	-13.8 ± 2.3	-10.7 ± 1.3	-43.2 ± 6.1	-24.6 ± 3.6	-32.4 ± 6.6
		2	-21.5 ± 2.3	-20.7 ± 4.0	-76.2 ± 3.4	-51.0 ± 5.9	-56.2 ± 5.3	-17.4 ± 1.6*	-19.5 ± 3.9	-75.9 ± 4.6	-41.5 ± 5.8	-51.0 ± 5.9
		4	-28.2 ± 1.2	-53.3 ± 12.0	-83.8 ± 5.3	-65.2 ± 6.3	-60.7 ± 3.9	-24.8 ± 1.8*	-38.3 ± 6.1	-91.1 ± 2.0	-60.3 ± 5.8	-63.1 ± 5.7
		8	-38.3 ± 4.0	-125.8 ± 26.8	-94.7 ± 0.8	-77.1 ± 3.9	-66.6 ± 4.5	-33.7 ± 2.7	-130.6 ± 33.3	-94.0 ± 1.7	-70.9 ± 5.0	-69.5 ± 5.1
20	9	1	-10.5 ± 1.9	-11.2 ± 2.5	-36.7 ± 7.2	-27.8 ± 7.2	-23.4 ± 4.3	-10.3 ± 1.9	-14.3 ± 3.4	-36.8 ± 6.8	-31.3 ± 4.7	-27.1 ± 4.9
		2	-21.2 ± 1.6	-25.0 ± 5.4	-73.6 ± 4.1	-51.1 ± 7.6	-49.2 ± 4.6	-17.8 ± 2.1	-28.2 ± 6.3	-62.6 ± 3.7*	-42.4 ± 6.8	-44.0 ± 5.4
		4	-25.0 ± 1.5	-47.9 ± 9.0	-89.8 ± 3.0	-65.2 ± 5.5	-55.3 ± 3.8	-25.2 ± 2.3	-42.8 ± 8.1	-83.9 ± 2.6	-57.8 ± 6.7*	-60.0 ± 3.9
		8	-32.2 ± 2.0	-84.6 ± 19.0	-95.5 ± 1.4	-74.4 ± 6.4	-60.2 ± 4.0	-29.5 ± 2.5*	-84.6 ± 18.0	-86.5 ± 5.5	-65.4 ± 7.9	-62.6 ± 4.1

Values are means ± SE. *P<0.05 vs. control

of PBG) without significant drug vs. nerve interaction.

The A_{2a}-mediated inhibition of the reflex responses was reversible. The CCR responses almost completely recovered ~60 min following NTS microinjections of A_{2a} receptor agonists (Table 5).

DISCUSSION

The present study showed that activation of facilitory A_{2a} adenosine receptors in the NTS paradoxically inhibited CCR hemodynamic and regional sympathetic responses. The NTS A_{2a} adenosine receptors stimulate neurotransmitter release so it was surprising that their activation inhibited the CCR. Although stimulation of NTS A_{2a} receptors evokes contrasting effects on regional sympathetic outputs (it decreases RSNA, increases pre-ASNA and does not markedly alter LSNA) the inhibition of the reflex responses of these sympathetic outputs was similar. This study indicated that NTS A_{2a} receptors are indeed present on NTS neurons/nerve terminals participating in CCR neurotransmission and inhibit this neurotransmission similarly as the inhibitory A_1 adenosine receptors did in our previous study (53). Although it seems that NTS A_{2a} receptors do not exert a significant, tonic inhibition of the reflex they may be activated upon release of adenosine into the NTS, for example during hypotensive stage of severe hemorrhage as we observed it previously (75; 110).

Differential modulation of sympathoinhibitory reflexes by adenosine receptor subtypes

Selective stimulation of NTS A_{2a} adenosine receptors evokes contrasting changes in the baseline levels of efferent sympathetic nerve activity: decreases in RSNA and post-ASNA, increases in pre-ASNA, and no changes in LSNA were observed (104-106). Therefore, we initially expected that activation of these receptors

may differentially alter CCR responses of these sympathetic outputs (it could facilitate RSNA reflex responses, attenuate pre-ASNA responses and less affect LSNA responses). To our surprise the CCR responses of all these sympathetic outputs were inhibited similarly. In addition there were no differences in A_{2a} receptor mediated inhibition of different components of the adrenal nerve (pre-ASNA, int-ASNA and post-ASNA). This indicates that the diverse shifts in baseline levels of regional sympathetic nerve activity and the inhibition of CCR-evoked responses in these sympathetic outputs are mediated by two different, most likely independent mechanisms. The inhibition of the reflex responses was probably mediated via facilitation of inhibitory mechanisms closely linked to the CCR pathway in the NTS, whereas contrasting shifts of baseline levels of regional sympathetic activity, mediated via NTS A_{2a} adenosine receptors, seem independent of reflex mechanisms and glutamatergic transmission in the NTS. We showed previously that these baseline shifts persist after sinoaortic denervation combined with vagotomy as well as after bilateral ionotropic glutamatergic blockade of the NTS (105; 106). The independent effects on baseline sympathetic activity and the reflex sympathetic responses were similar to those observed previously where we assessed the modulatory effects of NTS A_{2a} receptors on arterial baroreflex control of the regional sympathetic outputs (54). In that study despite the typical contrasting shifts of baseline levels of pre-ASNA vs. RSNA, no resetting of the stimulus-response baroreflex functions along MAP axis was observed. We proposed that the shifts of baseline levels of regional sympathetic nerve activity may be mediated by A_{2a} adenosine receptors located on non-glutamatergic projections to the NTS from hypothalamic nuclei which have dense, reciprocal connections with the NTS (for example, dorsomedial and paraventricular hypothalamic nuclei) (46; 96; 108).

Consistent with the uniform inhibition of the CCR responses in regional sympathetic outputs by NTS A_{2a} adenosine receptors was also a dose dependent attenuation of MAP responses in this setting. HR responses were also attenuated, although this effect was accompanied with much greater variability. This variability most likely arises from counteracting effect of the CCR on parasympathetic and sympathetic control of the heart. Although the CCR triggers powerful vagal bradycardia, this reflex also simultaneously activates cardiac sympathetic nerves which oppose the dominating parasympathetic response, as it has been shown very recently by McAllen's group (25; 31; 95). In addition, NTS A_{2a} adenosine receptors may differentially affect these two counteracting mechanisms.

Our previous studies showed that NTS A_1 adenosine receptors inhibit glutamatergic transmission in the arterial baroreflex arc and in the CCR, which is consistent with direct inhibition of glutamate release from nerve terminals or central neurons by presynaptic and postsynaptic A_1 receptors, respectively (53; 102; 107). In contrast, activation of NTS A_{2a} receptors did not alter arterial baroreflex responses however it inhibited the CCR responses similarly as A_1 adenosine receptors did; however, via a different, likely indirect mechanism.

Taken together, the present and previous data show that there are contrasting functional differences between A_1 vs. A_{2a}-adenosine receptors in neuromodulation of the CCR and arterial baroreflex mechanisms located in the NTS: 1) direct inhibition of neurotransmission in both reflex pathways is mediated by A_1 receptors, 2) selective, indirect inhibition of CCR, but not arterial baroreflex, pathway is provided by A_{2a} receptors, and 3) contrasting shifts in baseline levels of sympathetic activity mediated by A_{2a} adenosine receptors are independent of the reflex mechanisms. These

functional differences most likely reflect differential localization of adenosine receptor subtypes on NTS neurons/nerve terminals mediating both sympathoinhibitory reflexes and targeting different regional sympathetic outputs. It is worth noticing that the differences (both functional and neuroanatomical) appear to be greater for A_{2a} adenosine receptors, which dominate in the NTS, than for A_1 receptors which exert uniform, direct inhibition of both reflexes (9; 53; 54; 102; 104-107).

Although stimulation of NTS A_{2a} receptors evoked powerful inhibition of the hemodynamic and regional sympathetic CCR responses, the blockade of these receptors exerted only a very small, if any, facilitation of the reflex responses indicating that A_{2a} receptors do not exert a significant tonic inhibition on this reflex at the level of the NTS. Lack of marked, tonic adenosine modulation of mechanisms of cardiovascular control at the level of the NTS and in other medullary cardiovascular centers was usually observed in our laboratory and by others (53; 54; 110; 123). This is not surprising, as adenosine release into the NTS during normal, physiological conditions is negligible. However, adenosine is released into the central nervous system, including the NTS, during stress/hypothalamic defense response, severe hemorrhage, ischemia and hypoxia (30; 87; 110; 116-118; 129; 138; 139). During the severe hemodynamic imbalance adenosine levels increase several fold in the central nervous system and then adenosine does modulate neural control of the cardiovascular system (87; 110; 129; 138; 139). In the present study we used relatively small, short lasting activation of only one subset of receptors participating in the reflex to construct the most specific, dose dependent hemodynamic and regional neural stimulus-response function curves. Therefore the CCR depressor responses observed in the present study were too small and too short lasting by design to expect release of adenosine between CCR

stimulations. However, in a preliminary report, we showed that during severe hemorrhage naturally released adenosine does inhibit neural and hemodynamic CCR responses and this inhibition is removed by microinjections into the NTS of nonselective A_1/A_{2a} adenosine receptor antagonist, 8-(p-sulfophenyl)theophylline (75).

Potential mechanisms

In contrast to A_1 adenosine receptors which exert uniform inhibition of glutamatergic transmission in both CCR and arterial baroreflex pathways via decrease in Ca^{++} influx into the synaptic terminals or postsynaptic hyperpolarization of NTS neurons (90), the facilitory A_{2a} receptors may exert inhibition of the CCR via more complex mechanisms. The paradoxical inhibition of the CCR by A_{2a} adenosine receptors may be mediated, for example, via facilitation of GABA release from NTS GABA-ergic neurons and/or terminals via postsynaptic and/or presynaptic A_{2a} receptors, respectively. A_{2a} receptors may also disinhibit GABA release by removing dopaminergic inhibition of GABA release via direct A_{2a}-D_2 receptor interactions as it was suggested for basal ganglia (36; 47). Since dopamine itself has been reported to inhibit afferent activation of NTS neurons (60; 61) it is also possible that A_{2a} receptors may facilitate this inhibitory mechanism. A_{2a} receptors may also facilitate a vasoactive intestinal peptide dependent release of GABA as it has been shown in hippocampal nerve terminals (29). Since NTS contains glycinergic terminals/receptors, given that glycine inhibits arterial chemoreflex at the level of the NTS in the rat, and considering that A_{2a} adenosine receptors may activate glycine release from central neurons, it is possible that A_{2a} adenosine receptors may inhibit the CCR via facilitation of glycine release which then would inhibit transmission in this reflex pathway (6; 42; 88). It is also possible that adenosine A_{2a} receptors facilitate both GABA and glycine release as co-

localization of glycinergic and GABA-ergic mechanisms in the NTS has been reported (11); both GABA and glycine mediate CCR responses at the level of the rostral ventrolateral medulla (131). Which of those potential mechanisms is indeed responsible for A_{2a} receptor mediated inhibition of the CCR pathway remains to be elucidated in future studies.

Limitation of the method

Major limitations of the method were described in detail in our previous study concerning NTS A_1 adenosine receptor mediated inhibition of the CCR (53). Briefly, we believe that this method of evoking the CCR with increasing doses of PBG, which activate only a subset of CCR afferents, is a good, well controlled experimental tool widely accepted in the field (19; 38; 40; 52; 57; 95; 100; 101; 130). Selective activation of serotonin $5HT_3$ receptors on polymodal vagal afferents from cardiopulmonary area dissects the activation of CCR from other reflexes and humoral mechanisms which may be triggered by cardiac ischemia which is known to naturally activate the CCR (17; 50; 67; 83; 93; 97). Although we could not distinguish between adrenal nerve fibers supplying adrenergic vs. noradrenergic chromafin cells in the adrenal medulla it has been shown previously that both these types of adrenal nerve fibers are similarly inhibited by activation of the CCR (19).

In the present paper we did not provide the microinjection volume control for the hemodynamic and regional neural responses evoked by the CCR. However, this volume control was completed in our previous study and did not show any effects on the hemodynamic and regional neural reflex responses evoked by activation of the CCR and the arterial baroreflex (53; 54; 102). In addition, the lowest dose of A_{2a} receptor agonist (CGS-21680, 0.2 pmol) did not alter the reflex reactivity in all recorded

variables; therefore it may serve well as a volume control containing physiologically negligible amount of the agonist.

Perspectives and significance

We reported previously that activation of adenosine A_1 receptors inhibits glutamatergic transmission in arterial baroreflex and CCR pathways at the level of the NTS (53; 102; 107). These studies suggested that adenosine, operating via inhibitory A_1 receptors, may attenuate hypotension mediated by sympathoinhibitory reflexes, which are primarily integrated in the NTS. This mechanism may contribute to fine tuning of these reflexes in preventing excessive depressor and consequently ischemic effects. The present study showed that activation of facilitatory A_{2a} receptors in the NTS paradoxically inhibits CCR control of regional sympathetic outputs although it does not inhibit baroreflex control of these outputs (54). This indicates that both A_1 and A_{2a} adenosine receptor subtypes act in concert by powerfully inhibiting the CCR; however, via two different mechanisms: direct and indirect inhibition of neurotransmission in this reflex arc mediated by A_1 and A_{2a} receptors, respectively. The inhibitory effects of NTS A_{2a} receptors were nearly as powerful as those exerted by NTS A_1 receptors (53). This shows that adenosinergic inhibition of the CCR at the level of the NTS is much more potent than the inhibition of baroreflex pathway which is mediated only by NTS A_1 adenosine receptor subtype. This difference may have a physiologic basis because the cardiopulmonary chemoreflex (or Bezold-Jarisch reflex) may cause more severe bradycardia and hypotension than that normally evoked by activation of the arterial baroreflex. Therefore, the CCR needs a stronger negative feedback system compared to the arterial baroreflex. Powerful activation of the depressor Bezold-Jarisch reflex may lead to severe hemodynamic imbalance, ischemia and consequently to the release

of adenosine into the NTS. Then, adenosine may act as especially powerful negative feedback regulator for this reflex, via both A_1 and A_{2a} adenosine receptor subtypes. The above mechanism may prevent potentially dangerous consequences, for example, fainting or life threatening bradyarrhythmias, arising from simultaneous activation of vagal and sympathetic control of the heart, which may lead even to sudden cardiac death of young athletes (13; 48; 68; 95).

CHAPTER 3

NTS A_{2a} adenosine receptors inhibit the cardiopulmonary chemoreflex control of regional sympathetic outputs via GABA-ergic mechanism

ABSTRACT

Adenosine is a powerful central neuromodulator acting via opposite A_1 (inhibitory) and A_{2a} (excitatory) receptors. However, in the NTS both adenosine receptor subtypes attenuate cardiopulmonary chemoreflex (CCR) sympatho-inhibition of renal, adrenal and lumbar sympathetic nerve activity and reflex decreases in arterial pressure and heart rate. A_1 receptors inhibit glutamatergic transmission in the CCR pathway whereas A_{2a} receptors most likely facilitate release of an unknown inhibitory neurotransmitter which in turn inhibits the CCR. We hypothesized that A_{2a} adenosine receptors inhibit the CCR via facilitation of GABA release in the NTS. In urethane/chloralose anesthetized rats (n=51) we compared regional sympathetic responses evoked by stimulation of the CCR with right atrial injections of serotonin $5HT_3$ receptor agonist, phenylbiguanide, (PBG 1-8 μg/kg) before and after selective stimulation of NTS A_{2a} adenosine receptors (microinjections into the NTS of CGS-21680 20 pmol/50 nl) preceded by blockade of $GABA_A$ or $GABA_B$ receptors in the NTS (bicuculline 10 pmol /100 nl, or SCH-50911, 1 nmol/100 nl). We found that stimulation of NTS A_{2a} adenosine receptors inhibits CCR evoked hemodynamic and regional sympathetic reflex responses via a GABA-ergic mechanism. Blockade of $GABA_A$ receptors virtually abolished A_{2a} mediated inhibition of the CCR and had tonic effects on the reflex mechanism. $GABA_B$ receptors had much weaker but significant effects. These effects were similar for the different sympathetic outputs. We conclude that adenosine operating in the NTS inhibits CCR integration by both A_1 and A_{2a} receptor

subtypes, although via very different mechanisms: direct inhibition of glutamate release and facilitation of GABA release, respectively.

INTRODUCTION

Adenosine modulates cardiovascular reflexes primarily integrated in the nucleus of the solitary tract via inhibitory A_1 receptors and facilitory A_{2a} receptors linked to Gi and Gs proteins, respectively (1; 16; 81; 90; 103; 108; 115; 123). Activation of adenosine A_1 and A_{2a} receptors inhibits and facilitates neurotransmitter release and central neurons via pre- and postsynaptic mechanisms, respectively. Under physiological conditions a natural source of adenosine in the NTS is ATP released from nerve terminals and glial cells and then catabolized by ectonucleotidases; this mechanism may be triggered by stress/hypothalamic defense response (30; 116-118; 142). Under pathological conditions such as ischemia, hypoxia and severe hemorrhage intracellular ATP is catabolized to adenosine and adenosine is globally released from hypoxic neurons and glial cells (87; 129; 138; 138; 139). Therefore adenosine acts spatially reaching both A_1 and A_{2a} receptors in NTS neuronal network. However, this neuromodulator produces specific patterns of autonomic responses most likely due to the differential location of these two antagonistic receptors on NTS neurons/terminals which participate in different reflexes and finally target different sympathetic outputs (98; 103; 104; 107; 108). Selective activation of NTS A_1 and A_{2a} receptors usually, but not always yields reciprocal results. For example, activation of A_{2a} receptors results in depressor responses accompanied with contrasting regional sympathetic responses: decreases in renal (RSNA), increases in preganglionic adrenal (pre-ASNA) and no changes in lumbar (LSNA) sympathetic nerve activity (98; 104). In contrast, selective activation of NTS A_1 adenosine receptors evokes mostly pressor responses

accompanied with differential increases in regional sympathetic activity (pre-ASNA>RSNA≥LSNA) (107). Selective stimulation of NTS A_1 and A_{2a} receptors has also different effects on integration of baroreflex control of regional sympathetic outputs: A_1 receptors inhibit glutamatergic transmission which uniformly shifts the baroreflex stimulus response functions for RSNA, pre-ASNA and LSNA toward higher mean arterial pressure (MAP) (102; 107); in contrast A_{2a} receptors do not alter the baroreflex set point although they decrease baseline level of neural activity as well as the gain and range of the reflex for RSNA and increase all these variables for pre-ASNA via non-baroreflex mechanisms (54).

These two antagonistic receptors may also act in concert to inhibit the cardiopulmonary chemoreflex (CCR) control of regional sympathetic outputs acting via different mechanisms within the NTS. The CCR, also known as Bezold-Jarisch reflex, is triggered by polymodal mechano- and chemoreceptors and mediated by afferent vagal C fibers (26; 84; 85; 97; 124-126). This reflex evokes profound depressor, cardiac slowing and sympathoinhibitory responses which can lead to fainting and has been occasionally attributed to sudden cardiac death in young athletes (13; 68; 97). Inasmuch as activation of A_1 receptors inhibits neurotransmitter release, these receptors likely inhibit the CCR within the NTS by inhibiting glutamatergic transmission in the CCR pathway similarly as occurs in the arterial baroreflex network (53). In contrast, the facilitatory A_{2a} receptors may inhibit the CCR reflex pathway via release of an inhibitory neurotransmitter (78). The most likely candidate is GABA as stimulation of central A_{2a} adenosine receptors does facilitate GABA release in different neuronal networks including respiratory reflexes integrated in the NTS (2; 28; 36; 41; 47; 69; 70; 86; 113; 137). Negative GABA-ergic feedback operating at the level of the NTS in

arterial baro- and chemoreflex pathways is well documented (55; 56; 65; 74; 119; 120; 128; 133; 140; 141). There is extensive evidence that both $GABA_A$ and $GABA_B$ receptors operate synergistically inhibiting these reflexes at the level of the NTS (55; 120; 128; 140; 141). Both $GABA_A$ and $GABA_B$ receptors are tonically active in modulation of the baroreflex network at the level of the NTS (55; 119; 141). It is unknown if GABA-ergic negative feedback also operates in the CCR pathway although Zhang and Mifflin speculated that GABA may be involved in the regulation of all cardiovascular reflexes integrated in the NTS (141). Therefore, since activation of A_{2a} receptors in the NTS inhibits the CCR and these receptors facilitate neurotransmitter release, we hypothesize that these receptors facilitate GABA release which thereby inhibits CCR control of regional sympathetic outputs. The present study was designed to answer the following specific questions: 1) Do NTS A_{2a} adenosine receptors inhibit the CCR control of regional sympathetic outputs via facilitation of GABA release in the NTS network? 2) Are both $GABA_A$ and $GABA_B$ receptors involved? 3) Does GABA tonically inhibit the CCR pathway under normal conditions?

METHODS

All protocols and surgical procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* endorsed by the American Physiological Society and published by the National Institutes of Health.

Design

The experiments were performed using 51 male Sprague-Dawley rats, weighing 373.5 g (mean ± SE = 373.5 ± 6.9 g). In 27 rats the contribution of GABA-ergic mechanisms to A_{2a} adenosine receptor mediated inhibition of cardiopulmonary

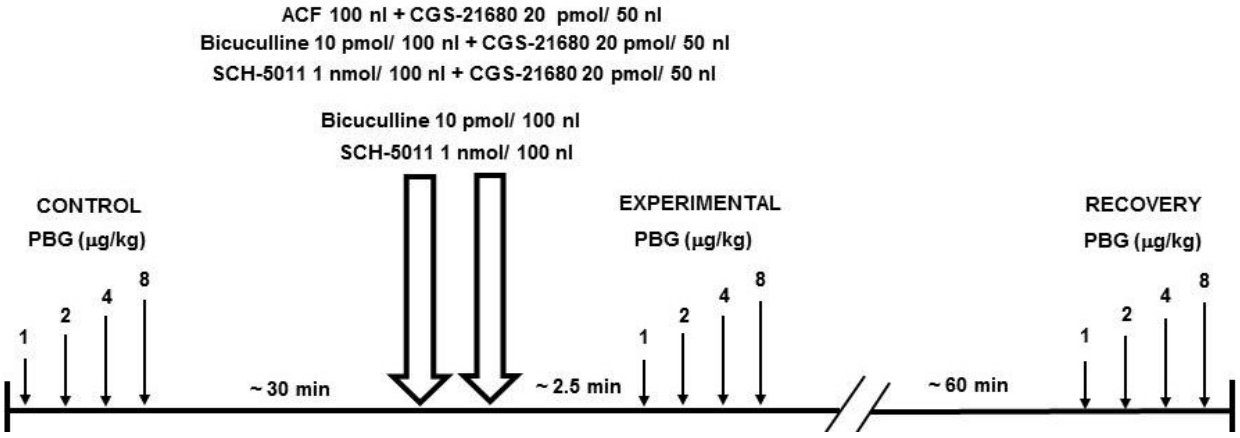

Figure 13. Timeline of experiments. Small arrows indicate bolus injections of phenylbiguanide (PBG) in increasing doses (1-8 μg/kg) into the right atrium in ~2.5 min intervals. Double large arrows represent bilateral microinjections into the NTS of: a) artificial cerebrospinal fluid, ACF (100 nl) or $GABA_A$ receptor antagonist, Bicuculline (10 pmol/ 100 nl) or $GABA_B$ receptor antagonist, SCH-5011 (1 nmol/100 nl) followed by A_{2a} adenosine receptor agonist CGS-21680 (20 pmol in 50 nl), or b) $GABA_A$ receptors antagonist alone (Bicuculline 10 pmol/ 100 nl) or $GABA_B$ receptors antagonist alone (SCH-5011 1 nmol/ 100 nl).

chemoreflex responses of regional neural and hemodynamic variables was assessed; CCR stimulus response function curves obtained under control conditions were compared with the curves obtained after bilateral microinjections into the NTS of A_{2a} adenosine receptor agonist (CGS-21680, 20 pmol/50 nl) following pretreatment with: vehicle control (microinjections of 100 nl artificial cerebrospinal fluid, ACF) or blockade of NTS $GABA_A$ or $GABA_B$ receptors (microinjections of Bicuculline, 10 pmol/100 nl, or SCH-50911, 1 nmol/100 nl, respectively). To asses potential tonic effects of NTS GABA-ergic mechanisms on CCR control of regional neural and hemodynamic variables, in 24 rats the CCR function curves were compared before and after the $GABA_A$ or $GABA_B$ receptor blockade alone. CCR reactivity was also assessed approximately one hour after the pharmacological manipulation in the NTS (recovery). The timeline of the protocol is presented in Figure 13.

Instrumentation and measurements

All procedures were described in detail previously (53; 100; 105-107). Briefly,

male Sprague-Dawley rats (Charles River) were anesthetized with a mixture of α-chloralose (80 $mg \cdot kg^{-1}$) and urethane (500 $mg \cdot kg^{-1}$ i.p.), tracheotomized, connected to a small-animal respirator (SAR-830, CWE, Ardmore, PA), and artificially ventilated with 40% oxygen, 60% nitrogen mixture. Arterial blood gases were tested occasionally (Radiometer, ABL500, OSM3), and ventilation was adjusted to maintain Po_2, Pco_2, and pH within normal ranges. Average values measured at the end of each experiment were: Po_2 = 156.8 ± 6.2 Torr, Pco_2 = 34.3 ± 0.8 Torr and pH = 7.41 ± 0.0. The left femoral artery and vein were catheterized to monitor arterial blood pressure; infuse drugs and to supplement anesthesia (12–21 $mg \cdot kg^{-1} \cdot h^{-1}$ of α-chloralose and 76–133 $mg \cdot kg^{-1} \cdot h^{-1}$ of urethane dissolved in 2.4–4.2 $ml \cdot kg^{-1} \cdot h^{-1}$ saline), respectively. An additional catheter was inserted via the right jugular vein for PBG injections into the right atrium. The appropriate position of the catheter in the right atrium was confirmed post mortem. Sinoaortic denervation was performed and tested similarly as in the previous studies from our laboratory; special attention was given to preserve the vagus nerve (53; 100; 105; 107).

Neural recording

In each experiment, simultaneous recordings from three (n=41) or two sympathetic outputs (n=10) were performed. Renal and adrenal nerves were exposed retroperitoneally form a flank incision, whereas lumbar sympathetic trunk was exposed from midabdominal incision. Neural recordings were accomplished as described previously (53; 100; 105-107). Neural signals were initially amplified (2,000–20,000×) with bandwidth set at 100–1,000 Hz, digitized, rectified, and averaged in 1-s intervals. Background noise was determined after the animal was euthanized yet artificially ventilated to leave unaltered any potential movement artifacts. Resting level of the

nerve activity was normalized to 100% before each activation of the CCR.

The ratio between preganglionic and total nerve activity was initially tested with an intravenous bolus injection of the short-lasting (1-2 min) ganglionic blocker, Arfonad (trimethaphan, 2 $mg \cdot kg^{-1}$; Hoffmann-La Roche), and finally evaluated at the end of each experiment with hexamethonium (20 $mg \cdot kg^{-1}$ i.v.). RSNA was virtually completely postganglionic; 7.3 ± 2.4% (n = 48) of the activity persisted after the ganglionic blockade; LSNA was mostly postganglionic; 34.9 ± 4.3% (n = 51) of preganglionic activity remained after the blockade. Total of 43 adrenal nerves were recorded with 28 being predominantly preganglionic pre-ASNA (>75% of activity remained after the ganglionic blockade), 7 were predominantly postganglionic post-ASNA (<50% of activity remained after the ganglionic blockade) and 8 had intermediate composition, int-ASNA (50%-75% of activity remained after the ganglionic blockade). Average levels of preganglionic, intermediate, and postganglionic adrenal nerve activity after ganglionic blockade were: 90.2 ± 2.2, 62.4 ± 3.0, 34.7 ± 7.3%, respectively. Stimulation of the CCR similarly inhibits all three components of the adrenal nerve, although this inhibition is significantly different than the inhibition of RSNA and LSNA (RSNA>ASNA>LSNA) (53; 78). Also activation of A_{2a} adenosine receptors similarly inhibits all three components of the adrenal nerve (78). Therefore, in the present study all components of the adrenal nerve were combined for further analysis and referred as ASNA (n=43). The arterial pressure and neural signals were digitized and recorded with a Hemodynamic and Neural Data Analyzer (Biotech Products, Greenwood, IN), averaged over 1-s intervals, and stored on the hard disk for subsequent analysis.

Microinjections into the NTS

Bilateral microinjections of the volume control (ACF, 100 nl), $GABA_A$ or $GABA_B$

receptor blockade (Bicuculline, 10 pmol/ 100 nl or SCH-50911, 1 nmol/ 100 nl, respectively) followed ~2-3 min later with selective A_{2a} receptor agonist, CGS-21680 (20 pmol in 50 nl of ACF) were made with multibarrel glass micropipettes into the medial region of the caudal NTS as described previously (9; 53; 105; 107). The doses and volumes of the agonist and antagonist were the same as used in previous studies (53; 54; 55; 102; 106; 107; 110; 133). The drugs were dissolved in ACF with pH adjusted to 7.2 since the selective A_{2a} adenosine receptor agonist CGS-21680 often precipitates in a pH >7.2. All microinjection sites were marked with DiI lypophilic dye (Molecular Probes, CA) and verified histologically as described previously (9; 53; 105; 107). The microinjection sites are presented in Figure 14 using diagrams based on the atlas of the rat subpostremal NTS by Barraco et al. (7).

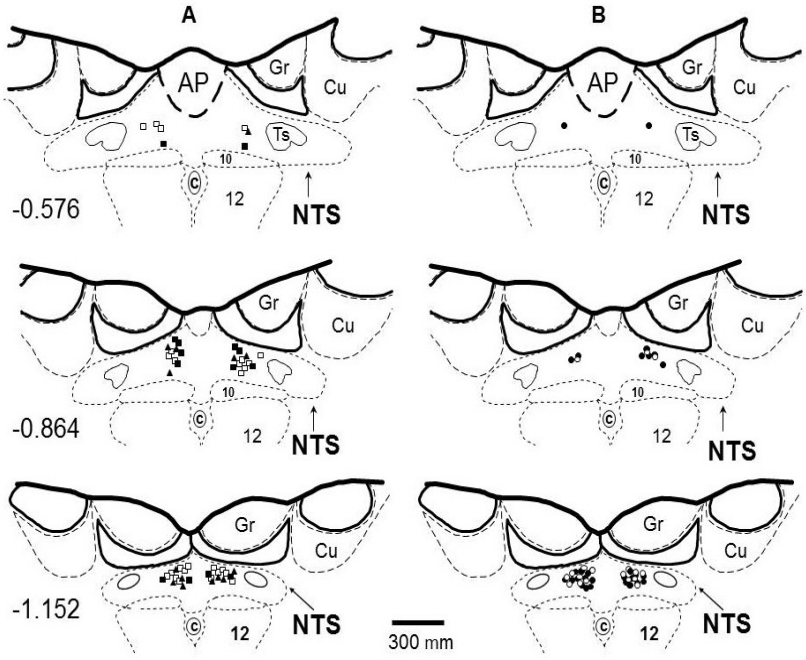

Figure 14. The microinjection sites for all experiments were located within subpostremal nucleus tractus solitarii (NTS) as shown in schematic diagrams of transverse sections of the medulla oblongata from a rat brain. AP, area postrema; c, central canal; 10, dorsal motor nucleus of vagus nerve; 12, nucleus of hypoglossal nerve; Ts, tractus solitarius; Gr, gracile nucleus; Cu, cuneate nucleus. Scale is shown at the *bottom*; number on the *left* of schematic diagram denotes the rostrocaudal position in millimeters of the section relative to the obex according to the atlas of the rat subpostremal NTS by Barraco et al. (7). Bilateral microinjection sites for different pharmacological agents were marked with fluorescent dye. A: ■, ACF (100 nl)+CGS-21680(20 pmol/50 nl); □, Bicuculline (10 pmol/ 100 nl)+CGS-21680 (20 pmol/50 nl); ▲ SCH-50911 (1 nmol/ 100 nl)+CGS-21680 (20 pmol/ 50 nl) B: ●, Bicuculline (10 pmol/ 100 nl); ○, SCH 50911 (1 nmol/ 100 nl).

Cardiopulmonary chemoreflex stimulus-response curves

The cardiopulmonary receptors were stimulated using bolus injections into the

right atrium of increasing doses (1-8 μg/kg) of serotonin $5HT_3$ receptor agonist, phenylbiguanide (PBG, Sigma, 20 μg/1 ml solution in 0.9% NaCl) in 2.5 min intervals between the injections (53; 100; 101). Total time to complete the stimulus-response curve was ~ 8 min. The curves obtained under control conditions were compared with those obtained 30 min later, approximately 2.5 min after bilateral microinjections of pharmacological agents into the caudal NTS (either ACF 100 nl or Bicuculline 10 pmol/100 nl or SCH-50911 1 nmol/ 100 nl followed with CGS-21680 20 pmol/50 nl, or Bicuculline 10 pmol/100 nl or SCH-50911 1 nmol/ 100 nl alone) and again with curves obtained 60 min after the microinjections, recovery (Figure 13). This approach allowed assessment of the CCR reactivity during the most effective action of the adenosine A_{2a} receptor activation which occurs ~ 5-15 min after the microinjection of the agonist (CGS-21680) (105; 106).

Data analysis

The cardiopulmonary reflex stimulus-response curves constructed for MAP, HR, RSNA, ASNA and LSNA under control conditions were compared with those generated after bilateral stimulation of NTS A_{2a} adenosine receptors (CGS-21680, 20 pmol/ 50 nl) preceded by either volume control (ACF, 100 nl) or, $GABA_A$ (bicuculline, 10 pmol/ 100 nl) or $GABA_B$ (SCH-50911, 1 nmol/ 100 nl) receptors blockade. The CCR function curves were also compared before and after blockade of either $GABA_A$ or $GABA_B$ receptors alone. Additionally, the control CCR stimulus-response curves were compared with those generated ~60 min after the microinjections (recovery). The maximal reflex responses in all measured variables were compared between the experimental conditions: ACF+CGS-2160 vs. Bicuculline+CGS-21680 or SCH-50911+CGS-21680 and bicuculline alone vs. SCH-50911 alone. The effects exerted by

activation of A_{2a} receptors preceded by volume control or GABA-ergic blockade on the reflex responses were calculated according to the equation: [(control response - response after the microinjection into the NTS)/control response] x 100%. 100% inhibition reflects completely abolished CCR response, whereas negative values represent facilitation of the reflex. The same equation was used to calculate the effects of blockade of $GABA_A$ or $GABA_B$ receptors alone on the reflex responses.

The data were analyzed using the statistical package SYSTAT version 11 (SYSTAT Software Inc., Richmond, CA, USA). Two-way ANOVA for repeated measures was used to compare the reflex responses of each variable across the experimental conditions (control vs. microinjection and control vs. recovery) and across four levels of activation of the CCR (four doses of PBG, 1-8 μg/kg). Two-way ANOVA for independent measures was used to compare control renal (n=48), adrenal (n=43) and lumbar (n=51) sympathetic CCR responses to four doses of PBG (1-8 μg/kg). Two-way ANOVA for independent measures was used to analyze the relative changes in reactivity for: ACF+CGS-2160 vs. Bicuculline+CGS-21680 and ACF+CGS-2160 vs. SCH-50911+CGS-21680. Two-way ANOVA for independent measures was also used for comparisons of relative changes in the reflex responses of three sympathetic outputs (RSNA vs. ASNA vs. LSNA) evoked by $GABA_A$ vs. $GABA_B$ receptor blockades (NTS microinjections of Bicuculline, 10 pmol/100 nl, vs. SCH-50911, 1 nmol/ 100 nl, respectively) at each level of activation of the CCR (PBG 1-8 μg). If significant interactions (nerve × drug) were found, the differences between the means were calculated with a modified Bonferroni *t*-test. One-way ANOVA for independent measures followed by modified Bonferroni *t*-test was used for comparison of HR and MAP reflex response curves. The changes in baseline levels of MAP, HR, RSNA,

ASNA and LSNA evoked by bilateral microinjections of either ACF or Bicuculline or SCH-50911 followed by CGS-21680 or effects of microinjections of Bicuculline or SCH-50911 alone were calculated as a difference between baseline values averaged during the last 30 s preceding the microinjections and the last 30 s of the 2.5 min period of the response to microinjections measured before generating the second cardiopulmonary chemoreflex function-curve. An α-level of $P< 0.05$ was used to determine statistical significance.

RESULTS

Average baseline MAP and HR values measured before the control CCR stimulus-response curves (n=51) were: 84.8 ± 1.4 mmHg and 398.9 ± 7.2 bpm, respectively. The baseline values measured just before NTS microinjections of drugs into the NTS returned to control levels: 82.6 ± 1.5 mmHg and 398.2 ± 4.6 bpm for MAP and HR, respectively. Also, baseline MAP and HR values measured ~60 min after the microinjections (recovery, n=51), were not markedly different from those measured at the beginning of experiment (79.4 ± 1.5 mmHg and 379.1 ± 7.4 bpm, respectively).

Effects of GABA-ergic blockade on NTS A_{2a} adenosine receptor mediated changes in baseline values

The effects of bilateral microinjections of the selective A_{2a} adenosine receptor agonist, CGS-21680 (20 pmol in 50 nl) following volume control (ACF, 100 nl, n=7), $GABA_A$ receptors blockade (Bicuculline, 10 pmol/ 100 nl, n=13), or GABAB receptors blockade (SCH-50911, 1 nmol/ 100 nl, n=7) on resting hemodynamic and neural variables are presented in Table 6. Selective activation of A_{2a} adenosine receptors following volume control evoked a typical pattern of changes in baseline levels of hemodynamic and regional sympathetic variables as observed in our previous studies

Table 6. Changes in baseline values under different experimental conditions. Values of MAP, HR, RSNA, ASNA, and LSNA after bilateral NTS microinjections of selective A_{2a} adenosine receptor agonist CGS-21680, following respective volume control (ACF), GABAA blockade (Bicuculline) or GABAB receptors blockade (SCH-50911)

	ACF (100 nl) + CGS-21680 (20 pmol/ 50 nl) n=7	Bicuculline (10 pmol/100 nl) + CGS-21680 (20 pmol/ 50 nl) n=13	SCH-50911 (1 nmol/100 nl) + CGS-21680 (20 pmol/ 50 nl) n=7
MAP, Δ mmHg	-15.0 ± 3.9	-0.1 ± 4.1*	-19.1 ± 3.1
HR, Δ bpm	-56.4 ± 12.8	-44.0 ± 8.6	-67.3 ± 16.5
RSNA, Δ%	-37.6 ± 7.7	-24.6 ± 5.2	-34.4 ± 3.0
ASNA, Δ%	11.4 ± 10.8 † ‡	0.3 ± 5.0 †	-7.6 ± 1.9 † ‡
LSNA, Δ%	-16.3 ± 3.5 †	-10.3 ± 5.4 †	-16.7 ± 3.1 †

Values are means ± SE. ⁎P < 0.05 vs. ACF 100 nl + CGS-21680 (20 pmol/ 50 nl); †P<0.05 vs. RSNA; ‡P<0.05 ASNA vs. LSNA.

during activation of A_{2a} receptors alone: decreases in MAP, HR, and RSNA, increases in ASNA and much smaller changes in LSNA. Pretreatment with $GABA_A$ receptor antagonist attenuated A_{2a} receptor mediated decreases in MAP and tended to decrease changes in all other baseline values; however this effect did not reach statistical significance. Pretreatment with $GABA_B$ receptor antagonist did not significantly change A_{2a} receptor-evoked alterations in baseline values although it tended to reverse the responses in baseline ASNA. Blockade of $GABA_A$ and $GABA_B$ receptors alone did not markedly affect the baseline values although it tended to decrease them slightly (Table 7).

Table 7. Changes in baseline values following GABA-erigc blockade. Values of MAP, HR, RSNA, ASNA, and LSNA following bilateral NTS microinjections of Bicuculline or SCH-50911 receptors blockade

	Bicuculline (10 pmol/ 100 nl) n=15	SCH-50911 (1 nmol/100 nl) n=9
MAP, Δ mmHg	-1.4 ± 3.3	-5.0 ± 3.0
HR, Δ bpm	-9.4 ± 8.1	-26.9 ± 6.1
RSNA, Δ%	-12.5 ± 5.8	-9.4 ± 3.2
ASNA, Δ%	-4.8 ± 8.6	-5.1 ± 4.1
LSNA, Δ%	-3.9 ± 5.0	-6.9 ± 2.7

Values are means ± SE. ⁎P < 0.05 vs. SCH-50911; †P<0.05 vs. RSNA; ‡P<0.05 ASNA vs. LSNA.

The effects of GABA-ergic blockade on NTS A_{2a} adenosine receptor mediated inhibition of cardiopulmonary chemoreflex responses

An example of original recordings and the averaged data for hemodynamic and neural responses evoked by activation of the CCR under control and experimental conditions are presented in Figures 15 and 16. Under control conditions (Figure 15, left panels, Figure 16 solid lines) gradual activation of the cardiopulmonary receptors by

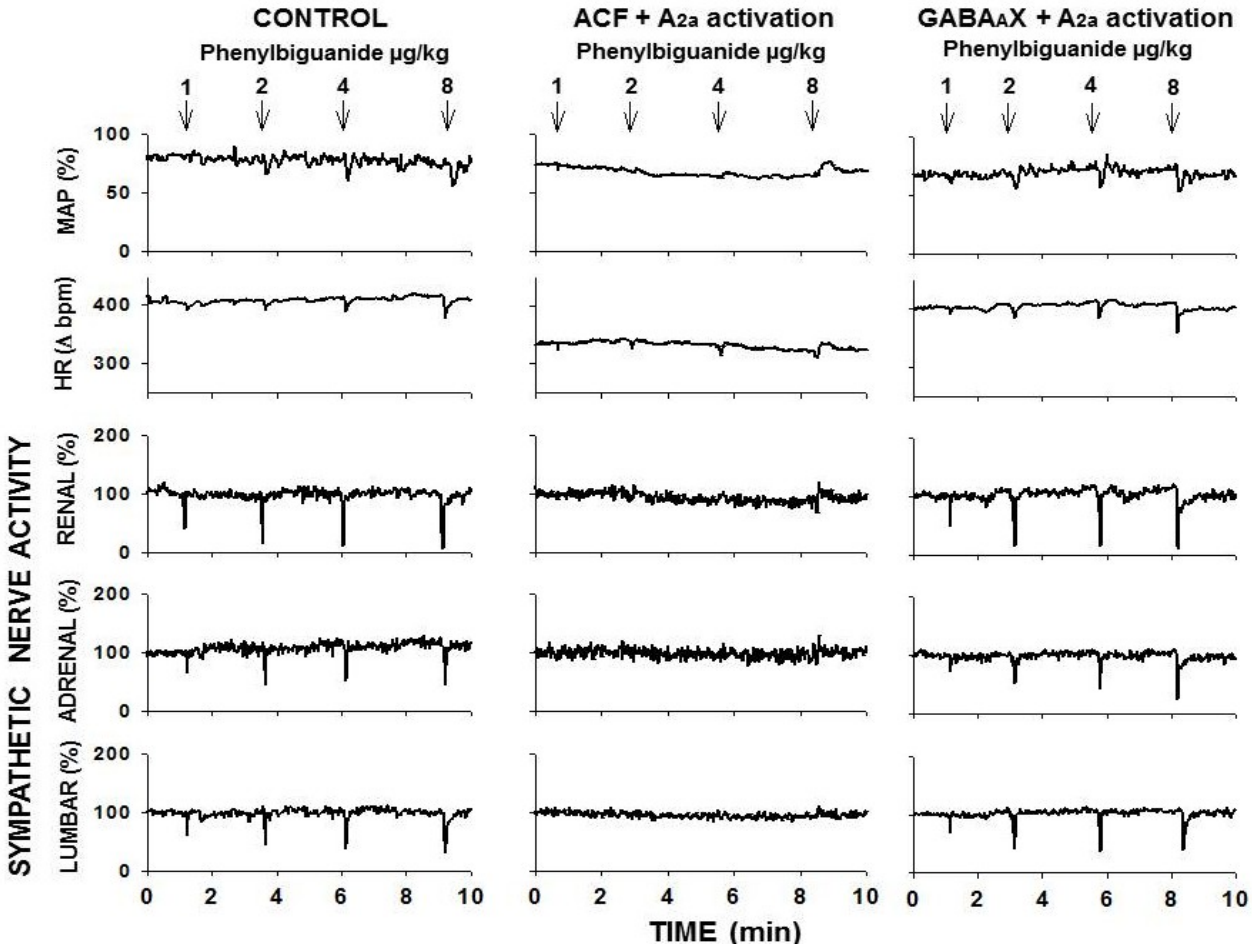

Figure 15. An example of cardiopulmonary chemoreflex responses in mean arterial pressure (MAP), heart rate (HR), renal (RSNA), adrenal (ASNA) and lumbar (LSNA) sympathetic nerve activity evoked by intra-atrial injections of phenylbiguanide (1-8 μg/kg) under control conditions (left panels), and after stimulation of the NTS A_{2a} adenosine receptors preceded by volume control (CGS-21680 20 pmol/ 50 nl+ACF 100 nl, middle panels) or following stimulation of the A_{2a} adenosine receptors preceded by $GABA_A$ receptor blockade (Bicuculline, 10 pmol/ 100 nl + CGS-21680, 20 pmol/ 50 nl, in left panels). The left and right panels are recordings preformed in one animal while the middle panels show powerful inhibition of the CCR responses following activation of A_{2a} receptors recorded in the other animal (ACF + CGS-21680). $GABA_A$ receptor blockade markedly attenuated A_{2a} mediated inhibition of the CCR responses.

increasing doses of PBG (1-8 μg/kg) evoked dose-dependent decreases in MAP and HR and uniform, yet differential inhibition in regional sympathetic nerve activity (RSNA > ASNA > LSNA) similarly as previously observed (53). Two-way ANOVA for all control data and all levels of the reflex activation showed a significant nerve effect ($p < 0.0001$) and nerve vs. dose interaction ($p = 0.001$). Bilateral activation of NTS A_{2a} adenosine receptors following pretreatment with volume control powerfully inhibited the CCR hemodynamic and neural reflex responses (Figure 15, middle panels, Figure 16 left panels). This inhibition was similar to that observed previously when NTS A_{2a} adenosine receptors were activated without pretreatment with ACF (78). Blockade of NTS $GABA_A$ receptors abolished the inhibitory effect of adenosine A_{2a} receptor stimulation on the CCR (Figure 15, right panels, Figure 16, middle panels). Two-way ANOVA showed no significant differences between the experimental conditions for all comparisons; however, slopes for HR and RSNA remained slightly altered after the blockade (experimental condition vs. dose interactions were significant for these two variables, $P<0.05$). The blockade of $GABA_B$ receptors decreased NTS A_{2a} receptor-mediated inhibition of the CCR responses to a lesser extent. The difference between control and experimental reflex function curves was smaller after $GABA_B$ receptor blockade (Figure 16, right panels) compared to the effect of volume control (Figure 16, left panels). However, some of A_{2a} receptor mediated inhibition of the reflex responses persisted after $GABA_B$ blockade; two-way ANOVA showed significant experimental condition effects ($P<0.05$) for all variables except for HR (Figure 16, right panels).

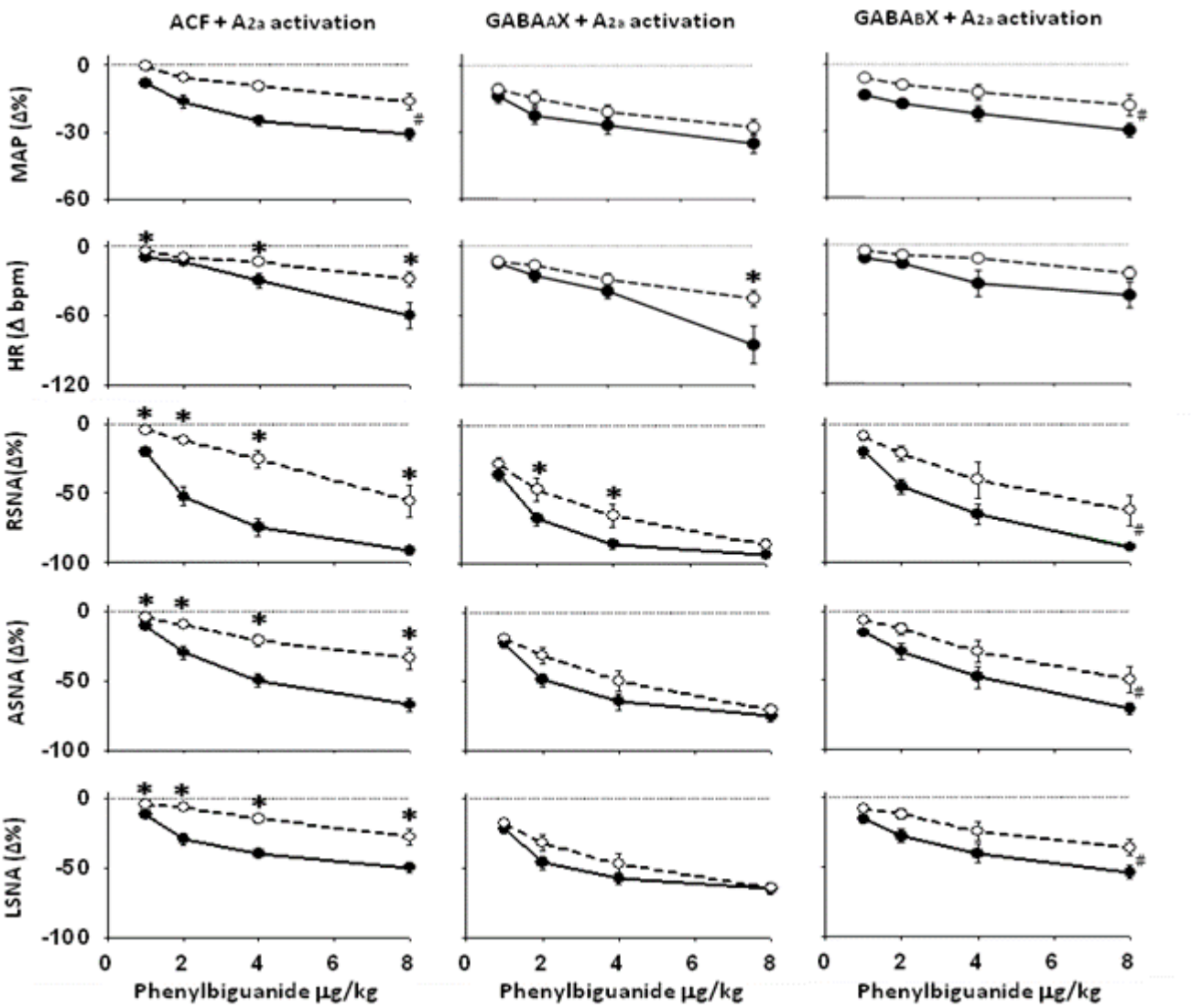

Figure 16. Comparisons of cardiopulmonary chemoreflex responses obtained under control conditions (●) vs. the responses obtained after microinjections of either volume control (ACF, 100 nl), $GABA_A$ (Bicuculline 10 pmol/ 100 nl), or $GABA_B$ (SCH 50911, 1 nmol/ 100 nl), receptors antagonists followed by A_{2a} receptor agonist (CGS-21680, 20 pmol/ 50 nl), (○). Data are means ± SE. ∗, $P<0.05$ vs. control; #, $P<0.05$ significant parallel shift of the experimental vs. control curves without significant PBG dose vs. experimental condition interaction. Activation of A_{2a} receptors following volume control showed powerful inhibition of the hemodynamic and regional sympathetic CCR responses. $GABA_A$ receptor blockade attenuated adenosine A_{2a} receptor mediated inhibition of the CCR responses, while $GABA_B$ receptors had smaller effect.

Figure 17 shows the extent of A_{2a} mediated-inhibition of the CCR reflex responses under each experimental condition (pretreatment with volume control vs. $GABA_A$ or $GABA_B$ receptor blockade). A 100% inhibition corresponds to a completely abolished reflex response, while 0% change represents no inhibition of the reflex reactivity. Selective activation of A_{2a} receptors following volume control (ACF) markedly

inhibited the CCR responses. This inhibition was virtually abolished by NTS $GABA_A$ blockade; the remaining inhibition was not different from zero for HR and all neural responses except for RSNA at 4 μg of PBG. In contrast, after $GABA_B$ receptor blockade

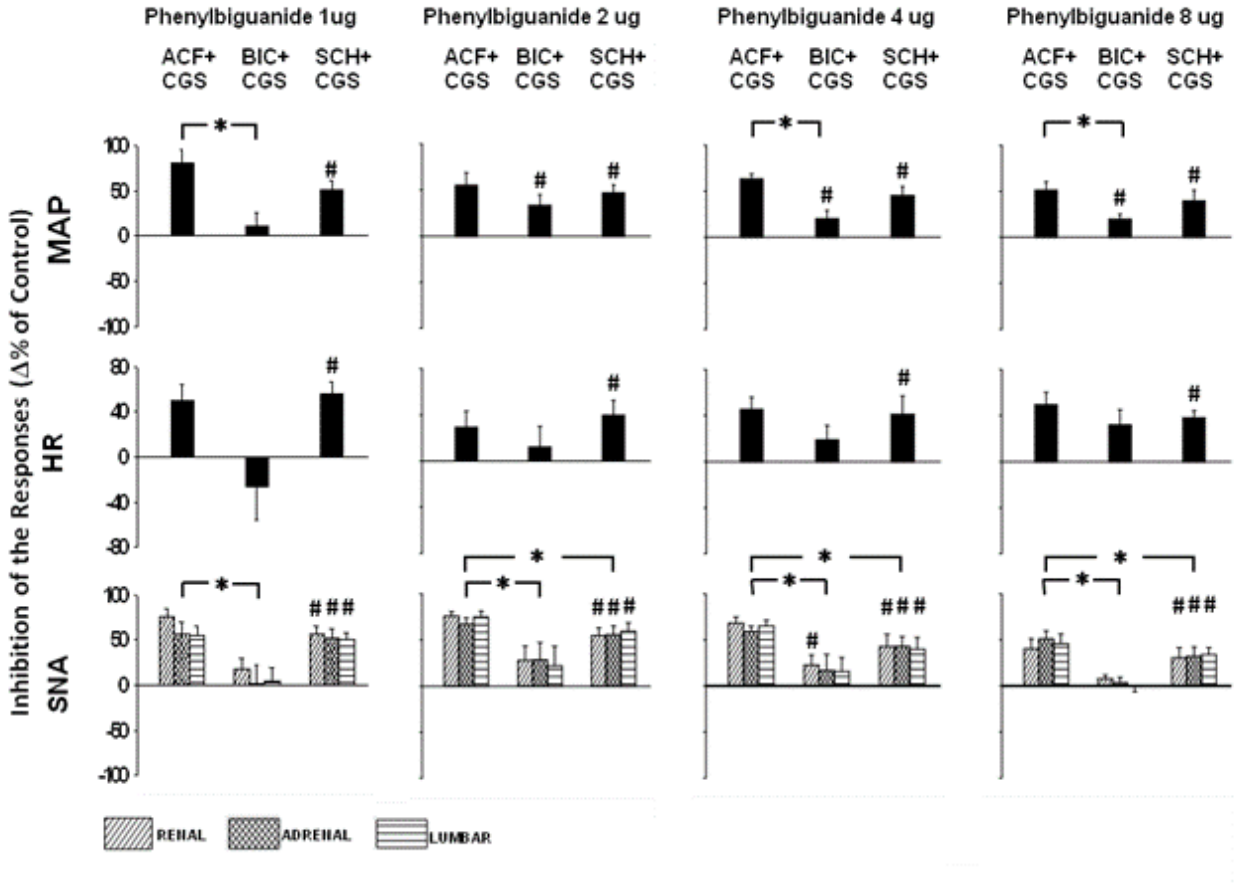

Figure 17. The relative inhibition of hemodynamic and regional sympathetic reflex evoked by stimulation of NTS A_{2a} adenosine receptors (CGS-21680, 20 pmol/ 50 nl) after pretreatment with volume control (ACF+CGS), $GABA_A$ receptor blockade (microinjections of Bicuculline, 10 pmol/ 100 nl, BIC+CGS) or $GABA_B$ receptor blockade (microinjections of SCH-50911, 1 nmol/100 nl, SCH+CGS) at four different levels of activation of the reflex (phenylbiguanide, PBG 1-8 μg/kg). Data are means ± SE. SNA - sympathetic nerve activity (renal, adrenal and lumbar). $GABA_A$ receptor blockade removed A_{2a} receptor mediated inhibition of reflex responses to much greater extent than $GABA_B$ receptor blockade.

the inhibition of reflex responses tended to decrease but A_{2a} receptor activation still caused significant attenuation of the CCR responses. Two-way ANOVA showed significant ACF vs. $GABA_A$ blockade effect ($P<0.05$ for all levels of activation of the reflex) without significant nerve effects and experimental condition vs. nerve

interactions. This showed that following $GABA_A$ blockade all nerve responses were similarly restored towards control levels. The $GABA_B$ blockade had a much smaller effect. Two-way ANOVA showed a significant ACF vs. $GABA_B$ blockade effect only for moderate activation of the reflex (PBG 2 and 4 μg).

The reflex reactivity returned to normal levels ~ 60 min after GABA receptor blockade. No significant differences between the experimental conditions were found for neural and hemodynamic variables (Table 8).

Table 8. Averaged cardiopulmonary chemoreflex responses to PBG (1-8 μg/kg) evoked under control conditions (control) and 1 hour after microinjection of drugs (recovery).

			Control					Recovery				
DRUGS	n	PBG μg/kg	MAP Δ%	HR Δbpm	RSNA Δ%	ASNA Δ%	LSNA Δ%	MAP Δ%	HR Δbpm	RSNA Δ%	ASNA Δ%	LSNA Δ%
ACF+CGS	7	1	-7.9 ± 1.2	-9.2 ± 1.5	-20.3 ± 3.4	-10.8 ± 1.8	-11.0 ± 2.3	-8.4 ± 2.0	-10.4 ± 2.2	-24.1± 5.8	-16.2 ± 3.8	-13.7 ± 3.7
		2	-16.3 ± 2.8	-13.5 ± 2.9	-52.4 ± 6.9	-29.9 ± 4.4	-29.0 ± 4.2	-14.7 ± 2.7	-15.2 ± 3.3	-44.9 ± 9.1	-28.9 ± 5.8	-23.6 ± 5.2
		4	-24.8 ± 2.3	-29.7 ± 6.8	-74.5 ± 6.5	-49.8 ± 5.1	-39.4 ± 2.7	-21.3 ± 3.5	-27.4 ± 5.8	-75.9 ± 8.1	-52.2 ± 6.7	-40.9 ± 5.9
		8	-30.9 ± 2.7	-59.5 ± 11.5	-91.6 ± 2.6	-67.5 ± 5.0	-49.6 ± 3.6	-29.9 ± 3.6	-58.4 ± 8.2	-87.9 ± 4.3	-59.9 ± 3.7	-49.8 ± 4.3
BIC+CGS	13	1	-13.8 ± 3.1	-14.9 ± 4.0	-35.7 ± 4.9	-22.5 ± 3.0	-21.8 ± 3.4	-12.9 ± 3.7	-10.4 ± 2.9	-31.7 ± 6.2	-22.9 ± 4.7	-22.8 ± 4.3
		2	-22.6 ± 3.5	-25.5 ± 5.6	-67.8 ± 4.7	-48.9 ± 4.9	-46.0 ± 4.9	-22.2 ± 3.4	-24.2 ± 4.7	-66.5 ± 4.2	-49.2 ± 3.3	-47.1 ± 4.7
		4	-27.0 ± 3.8	-39.0 ± 6.3	-85.8 ± 3.3	-64.8 ± 5.8	-57.1 ± 4.5	-27.6 ± 3.9	-44.3 ± 6.3	-85.5 ± 4.9	-68.3 ± 3.7	-61.5 ± 5.5
		8	-35.1 ± 4.2	- 85.3 ± 16.1	-93.3 ± 1.8	-74.6 ± 4.7	-64.2 ± 3.5	-33.9 ± 3.5	-86.8 ± 18.0	-94.7 ± 1.3	-78.5 ± 3.3	-67.8 ± 4.4
SCH+CGS	7	1	-13.9 ± 1.6	-11.4 ± 2.3	-20.7 ± 3.3	-15.6 ± 2.9	-15.5 ± 2.4	-9.6 ± 1.6	-7.0 ± 1.6	-16.2 ± 2.8	-14.0 ± 5.4	-9.1 ± 2.3
		2	-17.8 ± 2.1	-16.2 ± 3.2	-45.7 ± 5.5	-29.4 ± 5.4	-27.5 ± 5.1	-12.3 ± 3.9	-9.0 ± 2.1	-37.5 ± 9.9	-19.8 ± 6.0	-24.1 ± 7.4
		4	-22.3 ± 3.4	-33.5 ± 11.3	-65.1 ± 7.4	-47.8 ± 7.9	-40.0 ± 6.8	-22.8 ± 2.8	-15.6 ± 4.6	-62.7 ± 7.1	-42.6 ± 8.2	-38.0 ± 4.1
		8	-29.8 ± 3.1	-43.5 ± 10.9	-88.9 ± 2.2	-70.5 ± 3.9	-53.7 ± 4.8	-27.5 ± 3.4	-31.4 ± 7.4	-85.3 ± 2.9	-64.4 ± 5.4	-55.0 ± 2.2

Values are means ± SE. PBG, phenylbiguanide; HR, heart rate, MAP, mean arterial pressure, RSNA, renal sympathetic nerve activity, ASNA, adrenal sympathetic nerve activity; LSNA, lumbar sympathetic nerve activity.

Figure 18 shows effects of NTS GABA-ergic blockade alone on the CCR responses. Blockade of $GABA_A$ receptors in the NTS tended to exaggerate the reflex responses (Figure 18, left panels). However, differences between control and experimental reflex function curves did not reach statistical significance. Nevertheless, when more specific comparisons of changes in reflex reactivity were measured at each level of activation of the reflex they revealed facilitation of the reflex (Figure 19). Figure 19 shows Δ% change of the reflex responses obtained after $GABA_A$ and $GABA_B$ blockade compared to the respective control responses. Data were computed similarly as in Figure 17; upward deflections represent inhibition and downward deflections

represent facilitation of the reflex responses. The blockade of $GABA_A$ receptors significantly facilitated CCR reflex responses of all neural and hemodynamic variables at moderate levels of CCR activation (PBG, 2 and 4 μg/kg). In contrast, $GABA_B$ receptor blockade did not alter the reflex reactivity (virtually no significant differences vs. zero were found). Two-way ANOVA confirmed significant drug effect (Bicuculline vs. SCH-50911, $P<0.05$ for all variables at all levels of activation of the reflex). These data suggest that endogenous GABA operating mostly via $GABA_A$ receptors may provide some tonic inhibition of the CCR pathway at the level of the NTS.

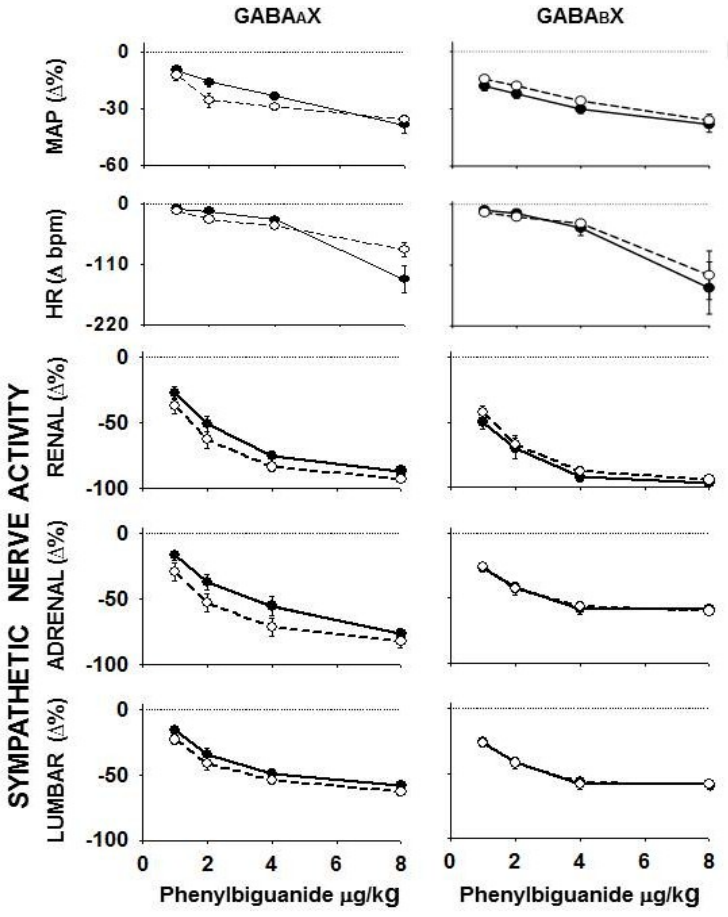

Figure 18. Comparisons of cardiopulmonary chemoreflex responses obtained under control conditions (●) vs. the responses obtained NTS $GABA_A$ or $GABA_B$ receptor blockades (microinjections of Bicuculline, 10 pmol/ 100 nl or SCH-50911, 1 nmol/100 nl, respectively) (○). Data are means ± SE. $GABA_A$ receptor blockade tended to facilitate the reflex responses (downward shifts of the reflex function curves obtained after the blockade).

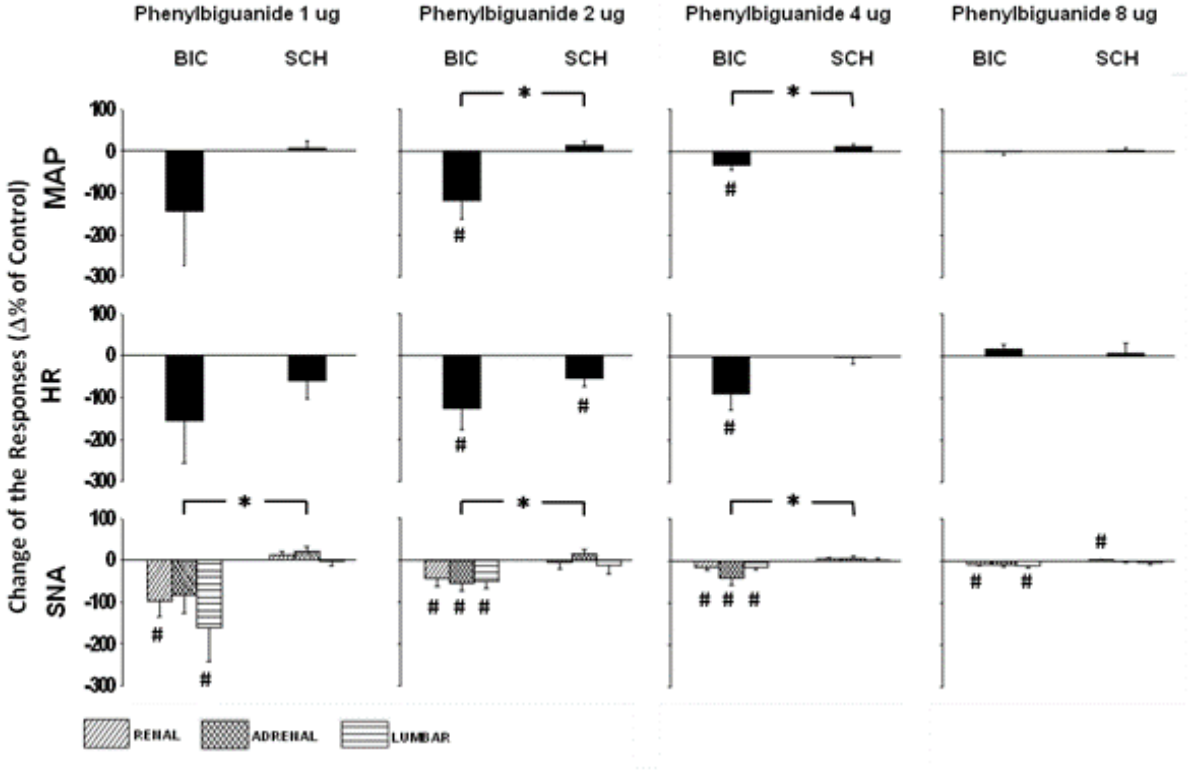

Figure 19. The relative changes of hemodynamic and regional sympathetic reflex responses evoked by $GABA_A$ or $GABA_B$ receptor blockade in the NTS (microinjections of Bicuculline, 10 pmol/ 100 nl, or SCH-50911, 1 nmol/100 nl, respectively) at four different levels of activation of the cardiopulmonary chemoreflex (intra-atrial phenylbiguanide, PBG 1-8 μg/kg). Data are means ± SE. SNA, sympathetic nerve activity (renal, adrenal and

DISCUSSION

The present study revealed the mechanism by which A_{2a} adenosine receptors exert powerful inhibition of hemodynamic and regional sympathetic CCR responses. In contrast to A_1 adenosine receptors, which directly inhibit glutamatergic transmission in arterial baro- and cardiopulmonary chemoreflex pathways (53; 102; 107), A_{2a} receptors facilitate release of GABA which in turn inhibits the CCR network. Although A_{2a} adenosine receptors do not inhibit the arterial baroreflex pathway (54) they provide powerful GABA-ergic inhibition of the CCR. This GABA-ergic inhibition is mediated mostly via $GABA_A$ receptors with a much smaller contribution of $GABA_B$ receptors. The

inhibition of the reflex responses was similar for different regional sympathetic outputs despite the marked regional differences in response to stimulation of NTS A_{2a} adenosine receptors themselves as well as the responses to the CCR. Importantly, the present study showed for the first time that GABA-ergic feedback operates in the CCR mechanisms at the level of the NTS and provides slight, although significant, tonic inhibition of the reflex.

A_{2a} adenosine receptor mediated GABA-ergic inhibition of the CCR

Adenosine A_{2a} receptor–mediated facilitation of GABA release has been shown in the cerebral cortex, basal ganglia, and hippocampus (2; 28; 36; 41; 47; 69; 70; 86; 113; 137). Further, A_{2a} adenosine receptor mediated facilitation of GABA release in medullary respiratory centers has been reported as injections of A_{2a} receptor agonist CGS-21680 into the fourth ventricle of piglets and rats inhibited respiratory drive and this effect was abolished by bicuculline (69; 137). Also apnea evoked by stimulation of the superior laryngeal nerve in piglets was facilitated by A_{2a} adenosine receptor stimulation and this inhibition was removed by bicuculline (2). In contrast, selective stimulation of NTS A_{2a} adenosine receptors does not inhibit arterial baroreflex control of HR and regional sympathetic outputs (54), although presence of GABA-ergic negative feedback is well documented in this reflex pathway (55; 56; 65; 74; 119; 120; 128; 133; 140; 141). NTS A_{2a} receptors do modulate baroreflex control of regional sympathetic outputs; however, only via reciprocal alterations of the baseline levels of renal vs. adrenal nerve activity which occur via non-baroreflex mechanisms as no resetting of baroreflex function curves toward higher MAP was observed (54). Although the arterial baroreflex and the CCR are mediated via similar medullary pathways (131), there was a significant difference in A_{2a} receptor modulation of these two reflexes, powerful inhibition

of the CCR observed in the present study and lack of inhibition of arterial baroreflex reactivity observed in a previous study (54). These differences support the hypothesis that adenosine receptor subtypes are differentially located in different reflex pathways at the level of the NTS.

It should be stressed that in the NTS adenosine operates mostly via A_{2a} receptors with a much smaller contribution of A_1 receptors. This remains in contrast to action of adenosine in most of the central nervous system where A_1 receptor inhibitory action prevails (16; 91). Microinjections of adenosine and selective A_{2a} receptor agonist, CGS-21680, in to the NTS evoke similar responses, i.e. decreases in MAP whereas stimulation of NTS A_1 adenosine receptors evokes the opposite effect to those evoked by adenosine and the A_{2a} receptor agonist (8; 10). The less potent A_1 receptors inhibit both arterial baroreflex and CCR mechanisms in the NTS via direct inhibition of glutamatergic transmission in both pathways. In contrast the dominant A_{2a} adenosine receptors selectively inhibit the CCR reactivity via facilitation of GABA release which in turn inhibits transmission in this reflex arc.

The A_{2a} mediated GABA-ergic inhibition of CCR reactivity was similar across the regional sympathetic outputs despite both the differential regional sympathetic responses to CCR activation (RSNA>ASNA>LSNA) (53; 78) as well as the reciprocal changes in basal levels of regional sympathetic activity evoked by activation of NTS A_{2a} adenosine receptors. Therefore, the triggering of GABA-ergic negative feedback by NTS A_{2a} receptors occurs via mechanisms independent of those responsible for the contrasting baseline shifts in regional sympathetic activity. The inhibitory mechanisms seem also independent from those producing differential inhibition of regional sympathetic outputs by the CCR.

Contribution of $GABA_A$ and $GABA_B$ receptors to A_{2a}-mediated inhibition of the CCR

The present study showed that activation of adenosine A_{2a} receptors inhibits the CCR responses mainly via postsynaptic $GABA_A$ receptors with a much smaller contribution of $GABA_B$ receptors which are located on both post and presynaptic sites in the NTS circuitry (55; 119; 140; 141). A_{2a} receptors are located mostly presynaptically in the NTS (24). Therefore their activation most likely facilitates GABA release from synaptic terminals. However, it does not exclude the possibility that A_{2a} receptors may also activate NTS GABA-ergic neurons via a postsynaptic mechanism. Both these mechanisms may facilitate the release of GABA which then acts mostly via postsynaptic $GABA_A$ receptors with much lesser effect on the presynaptic action of $GABA_B$ receptors.

Both $GABA_A$ and $GABA_B$ receptors inhibit baroreflex activated neurons in the NTS (55; 119; 133; 140; 141). Activation of $GABA_A$ receptors inhibits responses of NTS neurons activated from the aortic depressor or vagus nerves to a greater extent than activation of $GABA_B$ receptors (134; 140). The increases in MAP evoked by selective stimulation of NTS $GABA_B$ receptors were reduced ~ 50% following sinoaortic denervation suggesting presynaptic location of these receptors on arterial baroreflex afferents (133). Since the present study was performed on sinoaortic denervated animals this may partially explain why we observed the predominate role of $GABA_A$ receptors with much smaller contribution of $GABA_B$ receptors to A_{2a} adenosine receptor mediated inhibition of the CCR.

To our knowledge this is the first report demonstrating GABA-ergic inhibition of the CCR at the level of the NTS. Previous studies confirmed NTS GABA-ergic inhibition only in arterial baro- and chemoreflex networks (141). The present study showed that

$GABA_A$ but not $GABA_B$ receptors exert some tonic inhibitory effect on the CCR. This is consistent with previous observations that blockade of $GABA_A$ receptors increases spontaneous activity of NTS neurons whereas blockade of $GABA_B$ receptors did not alter activity of these neurons (12; 56; 134).

Other potential inhibitory mechanisms which may be triggered by NTS A_{2a} receptors

The present study confirmed our hypothesis that NTS A_{2a} adenosine receptors inhibit the CCR reactivity via GABA-ergic mechanism with a dominant role of $GABA_A$ compared to $GABA_B$ receptors. This effect was most likely mediated via direct facilitation of GABA release from nerve terminals or activation of NTS GABA-ergic neurons by A_{2a} presynaptic and postsynaptic receptors, respectively. However indirect facilitation of GABA release by NTS A_{2a} adenosine receptors should be also considered. For example, activation of A_{2a} receptors may disinhibit GABA release via direct A_{2a}-D2 interactions or via facilitation of release of vasoactive intestinal peptide as it has been shown in basal ganglia and hippocampal nerve terminals, respectively (29; 36; 47).

A_{2a} adenosine receptors may also facilitate dopamine release and dopamine in turn may inhibit afferent activation of NTS neurons (60; 61; 112). It is also possible that A_{2a} receptor mediated facilitation of glycine release may contribute to the inhibition of the CCR (6; 42; 65). However, this concept is less likely, because glycinergic NTS neurons were found in the lateral portions of the NTS which are areas not involved in integration of the CCR (11). In addition, inhibitory potentials triggered in NTS neurons via afferent vagal stimulation were reduced by blockade of GABA but not glycine (134).

It should be stressed, that A_{2a} receptor-mediated inhibition of the CCR was virtually abolished by blockade of $GABA_A$ receptors and significantly attenuated by

blockade of $GABA_B$ receptors (Figure 16 and 17). These data strongly suggest that the majority of inhibition of the CCR exerted by A_{2a} adenosine receptors is mediated via GABA-ergic mechanism with smaller if any contribution of other inhibitory mechanisms which may be triggered in the NTS by activation of A_{2a} adenosine receptors. Nevertheless, all the above potential possibilities should be considered in future studies addressing mechanisms of inhibitory action of A_{2a} receptors in the NTS.

Limitations of the method

The limitations concerning the method of evoking the CCR responses and whole nerve recordings were discussed in detail in our previous study (53). Here we focus on the limitations of blockade of GABA receptor subtypes in the NTS.

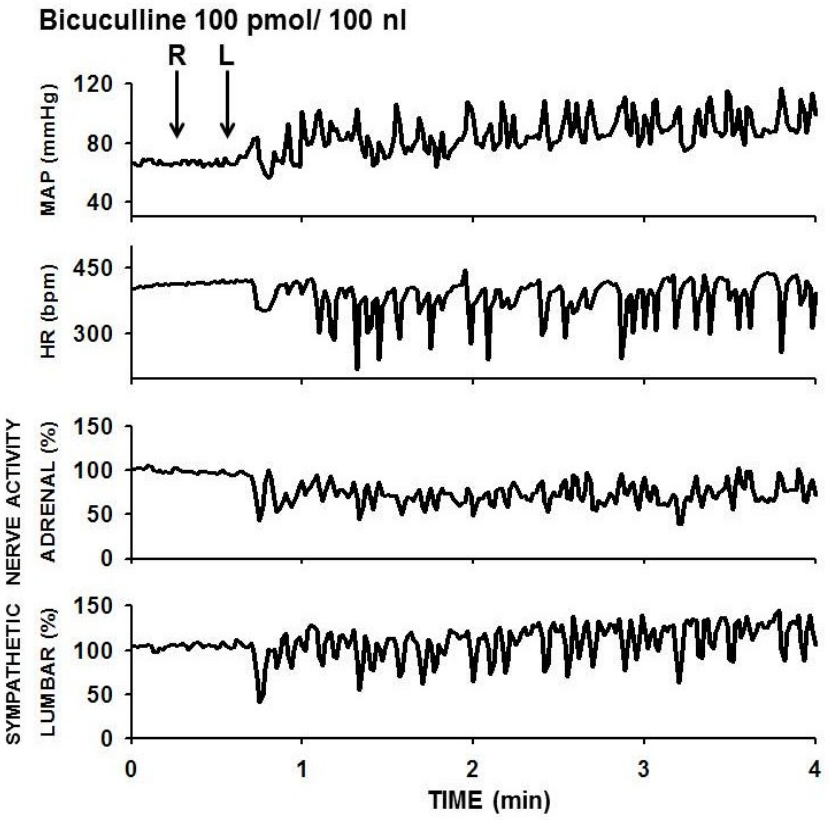

Figure 20. An example of effect of bilateral microinjections of high dose of Bicuculline (100 pmol/100 nl) into the right (R) and left (L) sides of the NTS on baseline values of mean arterial pressure (MAP), heart rate (HR), adrenal and lumbar sympathetic nerve activity recorded in one animal. Approximately 10 sec following the microinjections all baselines become markedly unstable.

Usually blockade of $GABA_A$ receptors in medullary nuclei is achieved with up to 500 pmol/ 100 nl of bicuculline (79; 82; 89). However this level of complete blockade of $GABA_A$ receptors in the NTS was not possible as removal of negative feedback control of all autonomic reflexes integrated in the NTS results in great instability of hemodynamic and sympathetic variables as observed during

preliminary studies. Figure 20 shows the effect of bilateral microinjection of 100 pmol of bicuculline in 100 nl of ACF on baseline levels of hemodynamic variables and regional sympathetic nerve activity. High amplitude flickering of baseline values occurred in all variables. Therefore in preliminary studies we assessed the optimal dose of the antagonist which will effectively inhibit NTS GABA-ergic mechanisms, yet will not destabilize the resting baseline values so that the reflex responses may be analyzed and compared with control conditions. We achieved this effect with microinjections of 10 pmol of bicuculline in 100 nl volume. We also attempted to combine $GABA_A$ and $GABA_B$ receptors blockade; however, removal of virtually all GABA-ergic tone in the NTS yielded in marked decreases in baseline levels of MAP, HR and sympathetic nerve activity which made the comparisons with control conditions very difficult and perhaps unreliable. Therefore, we abandoned the double blockade.

Perspectives

The present study revealed the presence of GABA-ergic inhibition of the CCR which may be triggered by stimulation of NTS A_{2a} adenosine receptors. NTS A_1 adenosine receptors exert similarly powerful inhibition of the CCR (53). Although A_1 adenosine receptors inhibit also arterial baroreflex pathway at the level of the NTS, A_{2a} receptors selectively inhibit the CCR but not arterial baroreflex pathway (54; 102). Taken together these data show that adenosine operating in the NTS powerfully inhibits the CCR mechanisms via synergistic action of both antagonistic receptor subtypes (dominant A_{2a} and secondary A_1 receptors) whereas arterial baroreflex is inhibited to much lesser extent as this occurs only via A_1 adenosine receptors. Why then does adenosine exert such powerful inhibition of the CCR pathway compared to a much smaller inhibition of the arterial baroreflex pathway? It is likely that this powerful

modulation of the CCR via both adenosine receptor subtypes has physiological implications. The CCR (also known as the Bezold-Jarisch reflex) evokes life threatening decreases in MAP via marked sympatho-inhibition, peripheral vasodilation and severe bradycardia which may result in fainting (13; 68; 97). This reflex is even implicated in mediating sudden cardiac death in young athletes (13). The powerful reflex decreases in MAP may lead to brainstem hypoxia and release of adenosine. Therefore adenosine may work as a negative feedback regulator of this particular sympathoinhibitory reflex as shown in Figure 21. During cardiac ischemia moderate

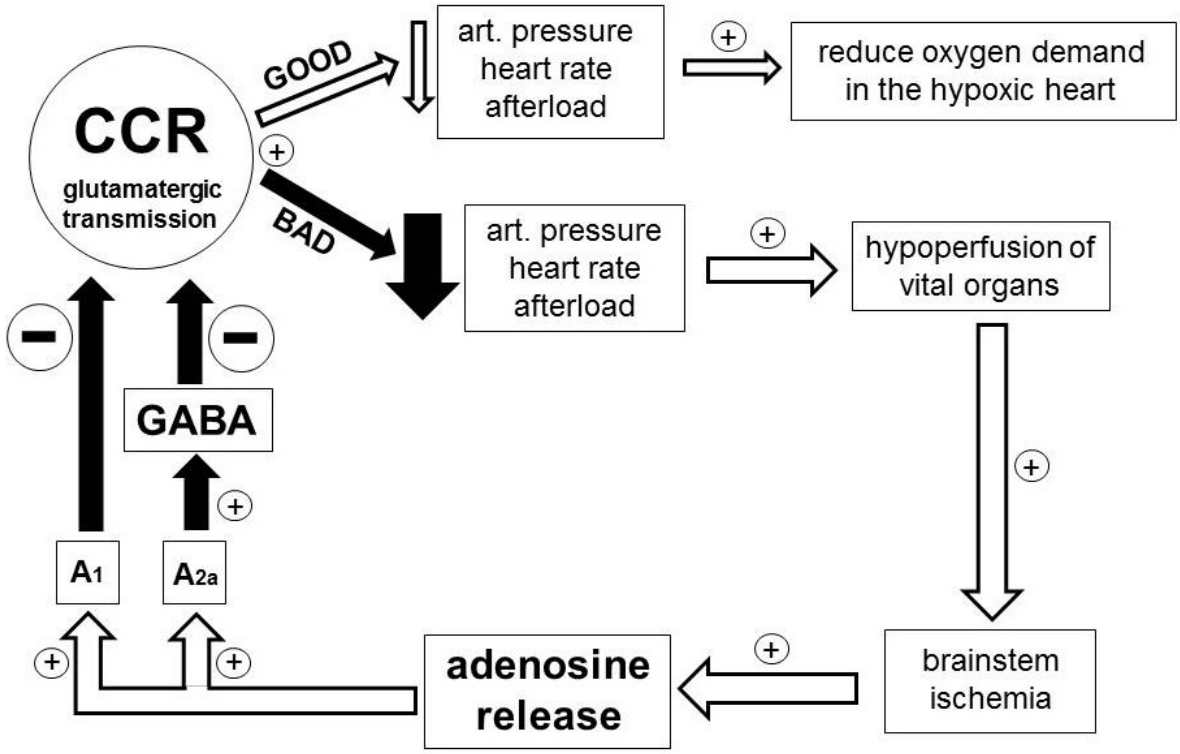

Figure 21. Potential fine tuning of the CCR by adenosine released into the NTS. Diagram shows direct and indirect adenosinergic inhibition of cardiopulmonary chemoreflex (CCR) via A_1 (inhibitory) and A_{2a} (facilitatory) receptors under conditions when activation of the CCR is becoming detrimental for the organism (BAD) and may lead to release of adenosine into the NTS. A_1 adenosine receptors directly inhibit glutamatergic transmission in the CCR pathway whereas A_{2a} receptors facilitate the release of GABA which in turn inhibits transmission in the reflex pathway. Note that moderate activation of the CCR is cardioprotective (GOOD) and does not lead to release of adenosine into the NTS.

activation of the CCR, buffered by simultaneous activation of sympathetic afferents, may be helpful as it would reduce afterload and decrease oxygen demand in the

hypoxic heart. However, powerful activation of this reflex may be detrimental as amplification of the hypotension might lead to death. During this severe hypotension adenosine may be released into the NTS and powerfully inhibit the CCR mechanism via both receptor subtypes. This occurs via different mechanisms: direct inhibition of glutamatergic neurotransmission in the CCR pathway and facilitation of GABA release via A_1 and A_{2a} receptors, respectively. This synergistic inhibitory action of both adenosine receptor subtypes may act as a strong negative feedback regulator for the CCR preventing life threatening effects of powerful activation of this reflex.

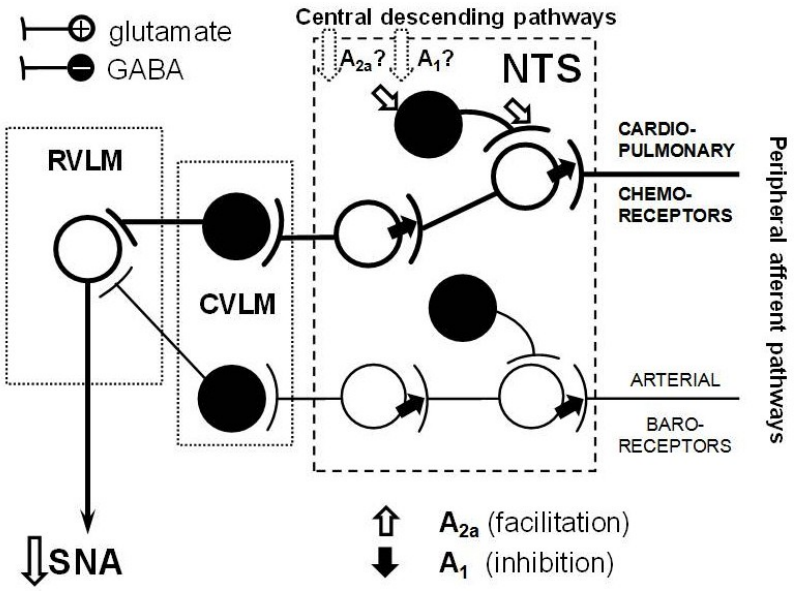

Figure 22. Hypothetical sites of action of A_1 and A_{2a} adenosine receptor subtypes on NTS neurons/terminals mediating the CCR and arterial baroreflex within NTS network. RVLM and CVLM are rostral and caudal ventrolateral medulla, respectively. Dotted arrows represent nerve terminals descending to the NTS from higher structures via which adenosine may modulate baseline levels of hemodynamic and neural variables independently of modulation of the reflex mechanisms. A_1 adenosine receptors (filled arrows) may directly inhibit the CCR and arterial baroreflex pathways while A_{2a} receptors (open arrows) may inhibit the CCR selectively by activation of GABA-ergic neurons and/or terminals linked to the CCR pathway in the NTS. Note that the CCR pathway is inhibited by both adenosine receptor subtypes (A_{2a} receptors which dominate in the NTS and A_1 receptors which are less potent in the NTS) whereas the arterial baroreflex pathway is inhibited to much lesser extent via the weaker A_1 adenosine receptors only.

This and our previous studies (53; 54; 78; 102) implicate specific, differential sites of action for adenosine receptor subtypes in sympathoinhibitory cardiovascular reflexes integrated in the NTS (Figure 22). Adenosine operating via the less powerful A_1 receptors in the NTS inhibits both the arterial baroreflex and the CCR via inhibition of glutamate release. However the dominant A_{2a} receptors selectively inhibit the CCR via presynaptic facilitation of GABA

release or postsynaptic activation of NTS GABA-ergic neurons. Specific location of A_1 and A_{2a} receptors in NTS circuitry require further investigation. Our preliminary data are consistent with the implications from our functional studies presented in Figure 22 (76; 77).

APPENDIX

WAYNE STATE UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 02-02-11

Protocol Effective Period: March 21, 2011 – February 28, 2014

Year 2 Annual Review Date: March 1, 2012
Year 3 Annual Review Date: March 1, 2013

TO: Dr. Donal S. O'Leary
Department of Physiology
4126 Scott Hall

FROM: Lisa Anne Polin, Ph.D.
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 02-02-11

"NTS Adenosine receptors in Cardiovascular control"

DATE: March 01, 2013

The Annual Review of your animal research protocol and any applicable grant applications has been conducted and approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC). The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	Cat.
RATS	Sprague-Dawley, Male, 300-400g	1900	D

Species Amendments	Strain	Qty.	Cat.
NONE			

Be advised that any change in the procedures used, a change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

REFERENCES

1. Abdel-Rahman AA and Tao S. Differential alteration of neuronal and cardiovascular responses to adenosine microinjected into the nucleus tractus solitarius of spontaneously hypertensive rats. *Hypertension* 27: 939-948, 1996.
2. Abu-Shaweesh JM. Activation of central adenosine A_{2a} receptors enhances superior laryngeal nerve stimulation-induced apnea in piglets via a GABAergic pathway. *J Appl Physiol* 103: 1205-1211, 2007.
3. Aviado DM and Guevara AD. The Bezold-Jarisch reflex. A historical perspective of cardiopulmonary reflexes. *Ann N Y Acad Sci* 940: 48-58, 2001.
4. Baker DG, Coleridge HM, Coleridge JC and Nerdrum T. Search for a cardiac nociceptor: stimulation by bradykinin of sympathetic afferent nerve endings in the heart of the cat. *J Physiol* 306: 519-536, 1980.
5. Baker DG and Coleridge HMCJCG. Vagal afferent C-fibers from the ventricle. In: Cardiac Receptors, edited by Hainsworth R, Kidd C and Linden RJ. Cambridge, UK: Camridge University Press, 1979, p. 117-137.
6. Baptista V and Varanda WA. Glycine binding site of the synaptic NMDA receptor in subpostremal NTS neurons. *J Neurophysiol* 94: 147-152, 2005.
7. Barraco R, el Ridi M, Ergene E, Parizon M and Bradley D. An atlas of the rat subpostremal nucleus tractus solitarius. *Brain Res Bull* 29: 703-765, 1992.
8. Barraco RA, el Ridi MR, Ergene E and Phillis JW. Adenosine receptor subtypes in the brainstem mediate distinct cardiovascular response patterns. *Brain Res Bull* 26: 59-84, 1991.
9. Barraco RA, O'Leary DS, Ergene E and Scislo TJ. Activation of purinergic receptor subtypes in the nucleus tractus solitarius elicits specific regional

vascular response patterns. *J Auton Nerv Syst* 59: 113-124, 1996.

10. Barraco RA and Phillis JW. Subtypes of adenosine receptors in the brainstem mediate opposite blood pressure responses. *Neuropharmacology* 30: 403-407, 1991.
11. Batten TF, Pow DV and Saha S. Co-localisation of markers for glycinergic and GABAergic neurones in rat nucleus of the solitary tract: implications for co-transmission. *J Chem Neuroanat* 40: 160-176, 2010.
12. Bennett JA, McWilliam PN and Shepheard SL. A gamma-aminobutyric-acid-mediated inhibition of neurones in the nucleus tractus solitarius of the cat. *J Physiol* 392: 417-430, 1987.
13. Benton Ng and Maginot KR. Sudden Cardiac Death in Young Athletes:Trying to Find the Needle in the Haystack. *Wisconsin Medical Journal* 106: 335-342, 2007.
14. Bisserbe JC, Patel J and Marangos PJ. Autoradiographic localization of adenosine uptake sites in rat brain using [^{3}H]nitrobenzylthioinosine. *J Neurosci* 5: 544-550, 1985.
15. Brophy S, Ford TW, Carey M and Jones JF. Activity of aortic chemoreceptors in the anaesthetized rat. *J Physiol* 514 (Pt 3): 821-828, 1999.
16. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* 87: 659-797, 2007.
17. Burnstock G, Lincoln J, Feher E, Hopwood AM, Kirkpatrick K, Milner P and Ralevic V. Serotonin is localized in endothelial cells of coronary arteries and released during hypoxia: a possible new mechanism for hypoxia-induced vasodilatation of the rat heart. *Experientia* 44: 705-707, 1988.
18. Campagna JA and Carter C. Clinical relevance of the Bezold-Jarisch reflex.

Anesthesiology 98: 1250-1260, 2003.

19. Cao WH and Morrison SF. Responses of adrenal sympathetic preganglionic neurons to stimulation of cardiopulmonary receptors. *Brain Res* 887: 46-52, 2000.

20. Carlsson S, Skarphedinsson JO, Delle M, Hoffman P and Thoren P. Differential responses in post- and pre-ganglionic adrenal sympathetic nerve activity and renal sympathetic nerve activity after injection of 2-deoxy-D-glucose and insulin in rats. *Acta Physiol Scand* 145: 169-175, 1992.

21. Carlsson S, Skarphedinsson JO, Delle M, Hoffman P and Thoren P. Reflex changes in post- and preganglionic sympathetic adrenal nerve activity and postganglionic sympathetic renal nerve activity upon arterial baroreceptor activation and during severe haemorrhage in the rat. *Acta Physiol Scand* 144: 317-323, 1992.

22. Carlsson S, Skarphedinsson JO, Jennische E, Delle M and Thoren P. Neurophysiological evidence for and characterization of the post-ganglionic innervation of the adrenal gland in the rat. *Acta Physiol Scand* 140: 491-499, 1990.

23. Carter T and Ellis K. Right-ventricular infarction. *Crit Care Nurse* 25: 52-8, 60, 2005.

24. Castillo-Melendez M, Krstew E, Lawrence AJ and Jarrott B. Presynaptic adenosine A_{2a} receptors on soma and central terminals of rat vagal afferent neurons. *Brain Res* 652: 137-144, 1994.

25. Chianca DA, Jr. and Machado BH. Microinjection of NMDA antagonist into the NTS of conscious rats blocks the Bezold-Jarisch reflex. *Brain Res* 718: 185-188,

1996.

26. Coleridge HM, Coleridge JC, Dangel A, Kidd C, Luck JC and Sleight P. Impulses in slowly conducting vagal fibers from afferent endings in the veins, atria, and arteries of dogs and cats. *Circ Res* 33: 87-97, 1973.

27. Coleridge HM, Coleridge JC and Kidd C. Cardiac Receptors in the dog, with particular reference to two types of afferent ending in the ventricular wall. *J Physiol* 174: 323-339, 1964.

28. Cunha RA and Ribeiro JA. Purinergic modulation of [^{3}H]GABA release from rat hippocampal nerve terminals. *Neuropharmacology* 39: 1156-1167, 2000.

29. Cunha-Reis D, Ribeiro JA and Sebastiao AM. A_1 and A_{2a} receptor activation by endogenous adenosine is required for VIP enhancement of K+ -evoked [^{3}H]-GABA release from rat hippocampal nerve terminals. *Neurosci Lett* 430: 207-212, 2008.

30. Dale N, Gourine AV, Llaudet E, Bulmer D, Thomas T and Spyer KM. Rapid adenosine release in the nucleus tractus solitarii during defence response in rats: real-time measurement in vivo. *J Physiol* 544: 149-160, 2002.

31. Daly MD and Kirkman E. Cardiovascular responses to stimulation of pulmonary C fibres in the cat: their modulation by changes in respiration. *J Physiol* 402: 43-63, 1988.

32. Dampney RA. Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 74: 323-364, 1994.

33. Davisson RL, Possas OS, Murphy SP and Lewis SJ. Neurogenically derived nitrosyl factors mediate sympathetic vasodilation in the hindlimb of the rat. *Am J Physiol Heart Circ Physiol* 272: H2369-H2376, 1997.

34. Dawes GS and COMROE JH, Jr. Chemoreflexes from the heart and lungs. *Physiol Rev* 34: 167-201, 1954.

35. Dawid-Milner MS, Silva-Carvalho L, Goldsmith GR and Spyer KM. A potential role of central A_1 adenosine receptors in the responses to hypothalamic stimulation in the anaesthetized cat. *J Auton Nerv Syst* 49: 15-19, 1994.

36. Dayne MR, Larson G, Orona RA and Zahniser NR. Opposing actions of adenosine A_{2a} and dopamine D2 receptor activation on GABA release in the basal ganglia: evidence for an A_{2a}/D2 receptor interaction in globus pallidus. *Synapse* 22: 132-138, 1996.

37. De Ferrari GM, Vanoli E, Stramba-Badiale M, Hull SS, Jr., Foreman RD and Schwartz PJ. Vagal reflexes and survival during acute myocardial ischemia in conscious dogs with healed myocardial infarction. *Am J Physiol Heart Circ Physiol* 261: H63-H69, 1991.

38. Dias AC, Vitela M, Colombari E and Mifflin SW. Nitric oxide modulation of glutamatergic, baroreflex, and cardiopulmonary transmission in the nucleus of the solitary tract. *Am J Physiol Heart Circ Physiol* 288: H256-H262, 2005.

39. DiBona GF. Neural control of the kidney: functionally specific renal sympathetic nerve fibers. *Am J Physiol Regul Integr Comp Physiol* 279: R1517-R1524, 2000.

40. Ditting T, Hilgers KF, Scrogin KE, Linz P and Veelken R. Influence of short-term versus prolonged cardiopulmonary receptor stimulation on renal and preganglionic adrenal sympathetic nerve activity in rats. *Basic Res Cardiol* 101: 223-234, 2006.

41. Duy PM, Xia L, Bartlett D, Jr. and Leiter JC. An adenosine A_{2a} agonist injected in the nucleus of the solitary tract prolongs the laryngeal chemoreflex by a

GABAergic mechanism in decerebrate piglets. *Exp Physiol* 95: 774-787, 2010.

42. Edwards FA and Robertson SJ. The function of A_2 adenosine receptors in the mammalian brain: evidence for inhibition vs. enhancement of voltage gated calcium channels and neurotransmitter release. *Prog Brain Res* 120: 265-273, 1999.

43. Evans RG, Ludbrook J and Michalicek J. Characteristics of cardiovascular reflexes originating from 5-HT_3 receptors in the heart and lungs of unanaesthetized rabbits. *Clin Exp Pharmacol Physiol* 17: 665-679, 1990.

44. Evans RG, Ludbrook J and Michalicek J. Use of nicotine, bradykinin and veratridine to elicit cardiovascular chemoreflexes in unanaesthetized rabbits. *Clin Exp Pharmacol Physiol* 18: 245-254, 1991.

45. Fields RD and Burnstock G. Purinergic signalling in neuron-glia interactions. *Nat Rev Neurosci* 7: 423-436, 2006.

46. Fontes MA, Tagawa T, Polson JW, Cavanagh SJ and Dampney RA. Descending pathways mediating cardiovascular response from dorsomedial hypothalamic nucleus. *Am J Physiol Heart Circ Physiol* 280: H2891-H2901, 2001.

47. Fuxe K, Ferre S, Genedani S, Franco R and Agnati LF. Adenosine receptor-dopamine receptor interactions in the basal ganglia and their relevance for brain function. *Physiol Behav* 92: 210-217, 2007.

48. Greenberg HM. Bradycardia at onset of sudden death: potential mechanisms. *Ann N Y Acad Sci* 427: 241-252, 1984.

49. Habler HJ, Janig W, Krummel M and Peters OA. Reflex patterns in postganglionic neurons supplying skin and skeletal muscle of the rat hindlimb. *J Neurophysiol* 72: 2222-2236, 1994.

50. Hainsworth R. Reflexes from the heart. *Physiol Rev* 71: 617-658, 1991.

51. Haydon PG and Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev* 86: 1009-1031, 2006.

52. Higuchi S, Morgan DA and Mark AL. Contrasting reflex effects of chemosensitive and mechanosensitive vagal afferents. *Hypertension* 11: 674-679, 1988.

53. Ichinose TK, Minic Z, Li C, O'Leary DS and Scislo TJ. Activation of NTS A_1 adenosine receptors inhibits regional sympathetic responses evoked by activation of cardiopulmonary chemoreflex. *Am J Physiol Regul Integr Comp Physiol* 303: R539-R550, 2012.

54. Ichinose TK, O'Leary DS and Scislo TJ. Activation of NTS A_{2a} adenosine receptors differentially resets baroreflex control of renal vs. adrenal sympathetic nerve activity. *Am J Physiol Heart Circ Physiol* 296: H1058-H1068, 2009.

55. Ito S and Sved AF. Influence of GABA in the nucleus of the solitary tract on blood pressure in baroreceptor-denervated rats. *Am J Physiol Regul Integr Comp Physiol* 273: R1657-R1662, 1997.

56. Jordan D, Mifflin SW and Spyer KM. Hypothalamic inhibition of neurons in the nucleus tractus solitarius of the cat is GABA mediated. *J Physiol* 399: 389-404, 1988.

57. Kashihara K, Kawada T, Yanagiya Y, Uemura K, Inagaki M, Takaki H, Sugimachi M and Sunagawa K. Bezold-Jarisch reflex attenuates dynamic gain of baroreflex neural arc. *Am J Physiol Heart Circ Physiol* 285: H833-H840, 2003.

58. Kirby GC and McQueen DS. Effects of the antagonists MDL 72222 and ketanserin on responses of cat carotid body chemoreceptors to 5-hydroxytryptamine. *Br J Pharmacol* 83: 259-269, 1984.

59. Kitchen AM, Scislo TJ and O'Leary DS. NTS A_{2a} purinoceptor activation elicits hindlimb vasodilation primarily via a beta-adrenergic mechanism. *Am J Physiol Heart Circ Physiol* 278: H1775-H1782, 2000.

60. Kline DD, Hendricks G, Hermann G, Rogers RC and Kunze DL. Dopamine inhibits N-type channels in visceral afferents to reduce synaptic transmitter release under normoxic and chronic intermittent hypoxic conditions. *J Neurophysiol* 101: 2270-2278, 2009.

61. Kline DD, Takacs KN, Ficker E and Kunze DL. Dopamine modulates synaptic transmission in the nucleus of the solitary tract. *J Neurophysiol* 88: 2736-2744, 2002.

62. Krayer O. The history of the Bezold-Jarisch effect. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 240: 361-368, 1961.

63. Krstew E, Jarrott B and Lawrence AJ. Autoradiographic visualisation of axonal transport of adenosine A_1 receptors along the rat vagus nerve and characterisation of adenosine A_1 receptor binding in the dorsal vagal complex of hypertensive and normotensive rats. *Brain Res* 802: 61-68, 1998.

64. Krukoff TL, Mactavish D and Jhamandas JH. Activation by hypotension of neurons in the hypothalamic paraventricular nucleus that project to the brainstem. *J Comp Neurol* 385: 285-296, 1997.

65. Kubo T and Kihara M. Evidence for the presence of GABAergic and glycine-like systems responsible for cardiovascular control in the nucleus tractus solitarii of the rat. *Neurosci Lett* 74: 331-336, 1987.

66. Longhurst JC, Tjen ALS and Fu LW. Cardiac sympathetic afferent activation provoked by myocardial ischemia and reperfusion. Mechanisms and reflexes.

Ann N Y Acad Sci 940: 74-95, 2001.

67. Lupinski SL, Schlicker E, Pedzinska-Betiuk A and Malinowska B. Acute myocardial ischemia enhances the vanilloid TRPV1 and serotonin 5-HT_3 receptor-mediated Bezold-Jarisch reflex in rats. *Pharmacol Rep* 63: 1450-1459, 2011.

68. Mark AL. The Bezold-Jarisch reflex revisited: clinical implications of inhibitory reflexes originating in the heart. *J Am Coll Cardiol* 1: 90-102, 1983.

69. Mayer CA, Haxhiu MA, Martin RJ and Wilson CG. Adenosine A_{2a} receptors mediate GABAergic inhibition of respiration in immature rats. *J Appl Physiol* 100: 91-97, 2006.

70. Mayfield RD, Suzuki F and Zahniser NR. Adenosine A_{2a} receptor modulation of electrically evoked endogenous GABA release from slices of rat globus pallidus. *J Neurochem* 60: 2334-2337, 1993.

71. McClure JM, O'Leary DS and Scislo TJ. Stimulation of NTS A_1 adenosine receptors evokes counteracting effects on hindlimb vasculature. *Am J Physiol Heart Circ Physiol* 289: H2536-H2542, 2005.

72. McClure JM, O'Leary DS and Scislo TJ. Neural and humoral control of regional vascular beds via A_1 adenosine receptors located in the nucleus tractus solitarii. *Am J Physiol Regul Integr Comp Physiol* 300: R744-R755, 2011.

73. McClure JM, Rossi NF, Chen H, O'Leary DS and Scislo TJ. Vasopressin is a major vasoconstrictor involved in hindlimb vascular responses to stimulation of adenosine A_1 receptors in the nucleus of the solitary tract. *Am J Physiol Heart Circ Physiol* 297: H1661-H1672, 2009.

74. Mifflin SW, Spyer KM and Withington-Wray DJ. Baroreceptor inputs to the

nucleus tractus solitarius in the cat: modulation by the hypothalamus. *J Physiol* 399: 369-387, 1988.

75. Minic Z, Li C, O'Leary DS and Scislo TJ. Severe hemorrhage attenuates cardiopulmonary chemoreflex (CCR) control of renal and adrenal sympathetic nerves via adenosine operating in the nucleus of the solitary tract (NTS). *FASEB J* 27: 1118.13, 2013.

76. Minic Z, O'Leary DS, Goshgarian HG and Scislo TJ. Immunohistochemistry confirms the functional evidence that A_1 adenosine receptors directly inhibit the cardiopulmonary chemoreflex (CCR) pathway whereas A_{2a} receptors inhibit the reflex via facilitation of GABA release in the caudal nucleus of the solitary tract (cNTS) in the rat. *FASEB J* 27: 2013.

77. Minic Z, Llewellyn-Smith IJ, O'Leary DS and Scislo TJ. A_{2a} adenosine receptors modulate cardiopulmonary chemoreflex control of regional sympathetic outputs via activation of GABAergic neurons within the caudal portion of the nucleus of the solitary tract (cNTS): functional and anatomical evidence. *FASEB J* 26: 1091.28, 2012.

78. Minic Z, O'Leary DS and Scislo TJ. Activation of NTS A_{2a} adenosine receptors impairs cardiopulmonary chemoreflex responses of adrenal (ASNA), renal (RSNA), and lumbar (LSNA) sympathetic nerve activity. *FASEB J* 24: 624.11, 2010.

79. Moffitt JA, Heesch CM and Hasser EM. Increased $GABA_A$ inhibition of the RVLM after hindlimb unloading in rats. *Am J Physiol Regul Integr Comp Physiol* 283: R604-R614, 2002.

80. Mosqueda-Garcia R, Tseng CJ, Appalsamy M, Beck C and Robertson D.

Cardiovascular excitatory effects of adenosine in the nucleus of the solitary tract. *Hypertension* 18: 494-502, 1991.

81. Mosqueda-Garcia R, Tseng CJ, Appalsamy M and Robertson D. Modulatory effects of adenosine on baroreflex activation in the brainstem of normotensive rats. *Eur J Pharmacol* 174: 119-122, 1989.

82. Mueller PJ and Mischel NA. Selective enhancement of glutamate-mediated pressor responses after $GABA_A$ receptor blockade in the RVLM of sedentary versus spontaneous wheel running rats. *Front Physiol* 3: 447, 2012.

83. Oei HH, Hughes WE, Schaffer SW, Longenecker GL and Glenn TM. Platelet serotonin uptake during myocardial ischemia. *Am Heart J* 106: 1077-1081, 1983.

84. Paintal AS. Vagal sensory receptors and their reflex effects. *Physiol Rev* 53: 159-227, 1973.

85. Paintal AS. Effects of drugs on chemoreceptors, pulmonary and cardiovascular receptors. *Pharmacol Ther B* 3: 41-63, 1977.

86. Phillis JW. Inhibitory action of CGS 21680 on cerebral cortical neurons is antagonized by bicuculline and picrotoxin-is GABA involved? *Brain Res* 807: 193-198, 1998.

87. Phillis JW, Walter GA, O'Regan MH and Stair RE. Increases in cerebral cortical perfusate adenosine and inosine concentrations during hypoxia and ischemia. *J Cereb Blood Flow Metab* 7: 679-686, 1987.

88. Pimentel FF, Bonagamba LG and Machado BH. Pressor response to chemoreflex activation before and after microinjection of glycine into the NTS of awake rats. *Am J Physiol Regul Integr Comp Physiol* 284: R1000-R1009, 2003.

89. Possas OS, Lopes OU and Cravo SL. Glutamatergic and GABAergic inputs to

the RVL mediate cardiovascular adjustments to noxious stimulation. *Am J Physiol Regul Integr Comp Physiol* 280: R434-R440, 2001.

90. Ralevic V and Burnstock G. Receptors for purines and pyrimidines. *Pharmacol Rev* 50: 413-492, 1998.

91. Ribeiro JA, Sebastiao AM and de Mendonca A. Adenosine receptors in the nervous system: pathophysiological implications. *Prog Neurobiol* 68: 377-392, 2002.

92. Robertson D, Hollister AS, Forman MB and Robertson RM. Reflexes unique to myocardial ischemia and infarction. *J Am Coll Cardiol* 5: 99B-104B, 1985.

93. Rocha I, Rosario LB, de Oliveira EI, Barros MA and Silva-Carvallho L. Enhancement of carotid chemoreceptor reflex and cardiac chemosensitive reflex in the acute phase of myocardial infarction of the anesthetized rabbit. *Basic Res Cardiol* 98: 175-180, 2003.

94. Rossi L. Structural and non-structural disease underlying high-risk cardiac arrhythmias relevant to sports medicine. *J Sports Med Phys Fitness* 35: 79-86, 1995.

95. Salo LM, Woods RL, Anderson CR and McAllen RM. Nonuniformity in the von Bezold-Jarisch reflex. *Am J Physiol Regul Integr Comp Physiol* 293: R714-R720, 2007.

96. Sawchenko PE and Swanson LW. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205: 260-272, 1982.

97. Schultz HD. Cardiac vagal chemosensory afferents. Function in pathophysiological states. *Ann N Y Acad Sci* 940: 59-73, 2001.

98. Scislo TJ, Augustyniak RA, Barraco RA, Woodbury DJ and O'Leary DS. Activation of P2x-purinoceptors in the nucleus tractus solitarius elicits differential inhibition of lumbar and renal sympathetic nerve activity. *J Auton Nerv Syst* 62: 103-110, 1997.

99. Scislo TJ, Augustyniak RA and O'Leary DS. Differential arterial baroreflex regulation of renal, lumbar, and adrenal sympathetic nerve activity in the rat. *Am J Physiol Regul Integr Comp Physiol* 275: R995-R1002, 1998.

100. Scislo TJ and DiCarlo SE. Gender difference in cardiopulmonary reflex inhibition of sympathetic nerve activity. *Am J Physiol Heart Circ Physiol* 267: H1537-H1543, 1994.

101. Scislo TJ, DiCarlo SE and Collins HL. Daily spontaneous running did not alter vagal afferent reactivity. *Am J Physiol Heart Circ Physiol* 265: H1564-H1570, 1993.

102. Scislo TJ, Ichinose TK and O'Leary DS. Stimulation of NTS A_1 adenosine receptors differentially resets baroreflex control of regional sympathetic outputs. *Am J Physiol Heart Circ Physiol* 294: H172-H182, 2008.

103. Scislo TJ, Kitchen AM, Augustyniak RA and O'Leary DS. Differential patterns of sympathetic responses to selective stimulation of nucleus tractus solitarius purinergic receptor subtypes. *Clin Exp Pharmacol Physiol* 28: 120-124, 2001.

104. Scislo TJ and O'Leary DS. Activation of A_{2a} adenosine receptors in the nucleus tractus solitarius inhibits renal but not lumbar sympathetic nerve activity. *J Auton Nerv Syst* 68: 145-152, 1998.

105. Scislo TJ and O'Leary DS. Differential control of renal vs. adrenal sympathetic nerve activity by NTS A_{2a} and P2x purinoceptors. *Am J Physiol Heart Circ*

Physiol 275: H2130-H2139, 1998.

106. Scislo TJ and O'Leary DS. Differential role of ionotropic glutamatergic mechanisms in responses to NTS P2x and A_{2a} receptor stimulation. *Am J Physiol Heart Circ Physiol* 278: H2057-H2068, 2000.

107. Scislo TJ and O'Leary DS. Mechanisms mediating regional sympathoactivatory responses to stimulation of NTS A_1 adenosine receptors. *Am J Physiol Heart Circ Physiol* 283: H1588-H1599, 2002.

108. Scislo TJ and O'Leary DS. Purinergic mechanisms of the nucleus of the solitary tract and neural cardiovascular control. *Neurol Res* 27: 182-194, 2005.

109. Scislo TJ and O'Leary DS. Vasopressin V_1 receptors contribute to hemodynamic and sympathoinhibitory responses evoked by stimulation of adenosine A_{2a} receptors in NTS. *Am J Physiol Heart Circ Physiol* 290: H1889-H1898, 2006.

110. Scislo TJ and O'Leary DS. Adenosine receptors located in the NTS contribute to renal sympathoinhibition during hypotensive phase of severe hemorrhage in anesthetized rats. *Am J Physiol Heart Circ Physiol* 291: H2453-H2461, 2006.

111. Scislo TJ, Tan N and O'Leary DS. Differential role of nitric oxide in regional sympathetic responses to stimulation of NTS A_{2a} adenosine receptors. *Am J Physiol Heart Circ Physiol* 288: H638-H649, 2005.

112. Sebastiao AM and Ribeiro JA. Adenosine A_2 receptor-mediated excitatory actions on the nervous system. *Prog Neurobiol* 48: 167-189, 1996.

113. Shindou T, Richardson PJ, Mori A, Kase H and Ichimura M. Adenosine modulates the striatal GABAergic inputs to the globus pallidus via adenosine A_{2a} receptors in rats. *Neurosci Lett* 352: 167-170, 2003.

114. Spyer KM, Lambert JH and Thomas T. Central nervous system control of

cardiovascular function: neural mechanisms and novel modulators. *Clin Exp Pharmacol Physiol* 24: 743-747, 1997.

115. Spyer KM and Thomas T. A role for adenosine in modulating cardio-respiratory responses: a mini-review. *Brain Res Bull* 53: 121-124, 2000.

116. St Lambert JH, Dashwood MR and Spyer KM. Role of brainstem adenosine A_1 receptors in the cardiovascular response to hypothalamic defence area stimulation in the anaesthetized rat. *Br J Pharmacol* 117: 277-282, 1996.

117. St Lambert JH, Dawid-Milner MS, Silva-Carvalho L and Spyer KM. Action of adenosine receptor antagonists on the cardiovascular response to defence area stimulation in the rat. *Br J Pharmacol* 113: 159-164, 1994.

118. St Lambert JH, Thomas T, Burnstock G and Spyer KM. A source of adenosine involved in cardiovascular responses to defense area stimulation. *Am J Physiol Regul Integr Comp Physiol* 272: R195-R200, 1997.

119. Sved AF and Tsukamoto K. Tonic stimulation of $GABA_B$ receptors in the nucleus tractus solitarius modulates the baroreceptor reflex. *Brain Res* 592: 37-43, 1992.

120. Sved AF and Tsukamoto K. Tonic stimulation of $GABA_B$ receptors in the nucleus tractus solitarius modulates the baroreceptor reflex. *Brain Res* 592: 37-43, 1992.

121. Sved JC and Sved AF. Cardiovascular responses elicited by gamma-aminobutyric acid in the nucleus tractus solitarius: evidence for action at the GABAB receptor. *Neuropharmacology* 28: 515-520, 1989.

122. Tao S and Abdel-Rahman AA. Neuronal and cardiovascular responses to adenosine microinjection into the nucleus tractus solitarius. *Brain Res Bull* 32: 407-417, 1993.

123. Thomas T, St Lambert JH, Dashwood MR and Spyer KM. Localization and action

of adenosine A_{2a} receptors in regions of the brainstem important in cardiovascular control. *Neuroscience* 95: 513-518, 2000.

124. Thoren P, Noresson E and Ricksten SE. Cardiac receptors with non-medullated vagal afferents in the rat. *Acta Physiol Scand* 105: 295-303, 1979.

125. Thoren P, Noresson E and Ricksten SE. Resetting of cardiac C-fiber endings in the spontaneously hypertensive rat. *Acta Physiol Scand* 107: 13-18, 1979.

126. Thoren PN. Characteristics of left ventricular receptors with nonmedullated vagal afferents in cats. *Circ Res* 40: 415-421, 1977.

127. Tjen ALS, Pan HL and Longhurst JC. Endogenous bradykinin activates ischaemically sensitive cardiac visceral afferents through kinin B2 receptors in cats. *J Physiol* 510 (Pt 2): 633-641, 1998.

128. Tsukamoto K and Sved AF. Enhanced gamma-aminobutyric acid-mediated responses in nucleus tractus solitarius of hypertensive rats. *Hypertension* 22: 819-825, 1993.

129. Van Wylen DG, Park TS, Rubio R and Berne RM. Cerebral blood flow and interstitial fluid adenosine during hemorrhagic hypotension. *Am J Physiol Heart Circ Physiol* 255: H1211-H1218, 1988.

130. Veelken R, Hilgers KF, Leonard M, Scrogin K, Ruhe J, Mann JF and Luft FC. A highly selective cardiorenal serotonergic 5-HT_3-mediated reflex in rats. *Am J Physiol Heart Circ Physiol* 264: H1871-H1877, 1993.

131. Verberne AJ and Guyenet PG. Medullary pathway of the Bezold-Jarisch reflex in the rat. *Am J Physiol Regul Integr Comp Physiol* 263: R1195-R1202, 1992.

132. Verberne AJ and Guyenet PG. Midbrain central gray: influence on medullary sympathoexcitatory neurons and the baroreflex in rats. *Am J Physiol Regul Integr*

Comp Physiol 263: R24-R33, 1992.

133. Vitela M and Mifflin SW. gamma-Aminobutyric acid(B) receptor-mediated responses in the nucleus tractus solitarius are altered in acute and chronic hypertension. *Hypertension* 37: 619-622, 2001.

134. Wang Y, Jordan D and Ramage AG. Both $GABA_A$ and $GABA_B$ receptors mediate vagal inhibition in nucleus tractus solitarii neurones in anaesthetized rats. *Auton Neurosci* 152: 75-83, 2010.

135. Webb SW, Adgey AA and Pantridge JF. Autonomic disturbance at onset of acute myocardial infarction. *Br Med J* 3: 89-92, 1972.

136. Whalen EJ, Johnson AK and Lewis SJ. Functional evidence for the rapid desensitization of 5-HT_3 receptors on vagal afferents mediating the Bezold-Jarisch reflex. *Brain Res* 873: 302-305, 2000.

137. Wilson CG, Martin RJ, Jaber M, Abu-Shaweesh J, Jafri A, Haxhiu MA and Zaidi S. Adenosine A_{2a} receptors interact with GABAergic pathways to modulate respiration in neonatal piglets. *Respir Physiol Neurobiol* 141: 201-211, 2004.

138. Winn HR, Rubio R and Berne RM. Brain adenosine production in the rat during 60 seconds of ischemia. *Circ Res* 45: 486-492, 1979.

139. Yan S, Laferriere A, Zhang C and Moss IR. Microdialyzed adenosine in nucleus tractus solitarii and ventilatory response to hypoxia in piglets. *J Appl Physiol* 79: 405-410, 1995.

140. Zhang J and Mifflin SW. Receptor subtype specific effects of GABA agonists on neurons receiving aortic depressor nerve inputs within the nucleus of the solitary tract. *J Auton Nerv Syst* 73: 170-181, 1998.

141. Zhang W and Mifflin S. Plasticity of GABAergic mechanisms within the nucleus of the solitary tract in hypertension. *Hypertension* 55: 201-206, 2010.

142. Zimmermann H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. *Prog Neurobiol* 49: 589-618, 1996.

ABSTRACT

MECHANISMS MEDIATING MODULATION OF CARDIOPULMONARY CHEMOREFLEX CONTROL OF REGIONAL SYMPATHETIC ACTIVITY BY ADENOSINE A_1 and A_{2a} RECEPTORS LOCATED IN THE NTS

by

ZELJKA MINIC

August 2013

Advisor: Dr. Tadeusz J. Scislo

Major: Physiology

Degree: Doctor of Philosophy

Adenosine is an important neuromodulator of cardiovascular control at the level of the nucleus of the solitary tract (NTS) where cardiovascular and other autonomic reflexes are primarily integrated. Levels of adenosine increase in the NTS during life threatening hypotension, ischemia and hypoxia. Adenosine may modulate cardiovascular reflexes to correct hemodynamic imbalance, acting via A_1 and A_{2a} receptors which inhibit and facilitate neurotransmitter release, respectively. Previous studies from our laboratory showed that NTS A_1 adenosine receptors inhibit the arterial baroreflex whereas A_{2a} receptors do not reset baroreflex control of regional sympathetic outputs, although stimulation of these receptors decreases renal (RSNA) increases preganglionic adrenal (pre-ASNA) and does not alter lumbar (LSNA) sympathetic nerve activity. Cardiopulmonary chemoreflex (CCR), also known as Bezold-Jarisch reflex, triggered by various chemicals inhaled or released during myocardial ischemia, may have beneficial effects when it decreases afterload and heart rate thus decreasing oxygen demand. However, strong activation of the CCR may cause severe bradycardia and hypotension leading to hypoperfusion of vital organs, fainting and even death.

During this severe reflex hypotension adenosine may be released into NTS. Then adenosine may inhibit the reflex to regain hemodynamic balance. A_1 adenosine receptors likely inhibit glutamatergic transmission in the CCR pathway as they inhibit the arterial baroreflex mediated via similar medullary pathways and transmitters. Interestingly, preliminary data showed that NTS A_{2a} facilitatory receptors also inhibit the CCR. Therefore I hypothesized that adenosine operating via both receptor subtypes may act as negative feedback regulator for CCR control of regional sympathetic outputs acting via two different mechanisms: A_1 receptors mediated direct inhibition and A_{2a} receptor mediated facilitation of release of an inhibitory neurotransmitter (most likely GABA) which in turn inhibits the CCR pathway. Since both NTS A_1 and A_{2a} adenosine receptors exert differential effects on regional sympathetic outputs I anticipated that the inhibition of CCR control of regional sympathetic outputs will be also regionally specific.

In urethane-chloralose-anesthetized rats I compared regional reflex sympathoinhibition evoked by activation of cardiopulmonary receptors with intra-atrial injections of phenylbiguanide (1-8 μg/kg) before and after stimulation of NTS A_1 and A_{2a} adenosine receptors via microinjections into the NTS of N^6cyclopentyl adenosine (0.33-330 pmol/50 nl) and CGS 21680 (0.2-20 pmol/50 nl), respectively. The comparisons were made also following nonselective blockade of A_1 and A_{2a} receptors ((8-(p-sulfofenyl) theophyline, 1 nmol/100 nl)) and selective blockade of A_{2a} receptors (ZM-2143285, 40 pmol/ 100 nl) and following respective volume controls. Gradual stimulation of both A_1 and A_{2a} adenosine receptors located in the NTS caused similar dose dependent inhibition of hemodynamic and regional sympathetic responses. The inhibition was independent of differential effects of adenosine receptor subtypes in regional sympathetic activity suggesting that these two effects are mediated via different

mechanisms in the NTS. Blockade of adenosine receptor subtypes did not affect the CCR showing that under normal conditions adenosine levels are too low to affect the reflex. Blockade of $GABA_A$ receptors in the NTS (Bicuculline, 10 pmol/100 nl) removed A_{2a} receptor mediated inhibition of the reflex whereas blockade of $GABA_B$ receptors (SCH-50911, 1 nmol/100 nl) attenuated the A_{2a} receptor mediated inhibition to a lesser extent. I found also that GABA operating via $GABA_A$ but not $GABA_B$ receptors exerts small, but significant tonic inhibition of the CCR. In addition, simultaneous recordings from the three sympathetic outputs showed that CCR differentially inhibits regional sympathetic outputs (RSNA>pre-ASNA>LSNA); this observation clarified inconsistent reports on CCR control of these sympathetic outputs.

I conclude that adenosine may act as a negative feedback regulator of the CCR control of neural and hemodynamic variables. NTS A_1 receptors directly inhibit glutamatergic transmission in the reflex pathway, whereas A_{2a} receptors facilitate release of GABA which inhibits the CCR pathway mostly via $GABA_A$ receptors.

AUTOBIOGRAPHICAL STATEMENT

ZELJKA MINIC

Education:

PhD: Wayne State University, Detroit, MI (8/10 – Present)
Degree: Physiology
B.S. University of Detroit Mercy, Detroit, MI – Biochemistry (2005-2009)

Experience:

- Graduate Research Assistant – Wayne State University, Detroit, MI (2010 – Present)

Awards:

- Wayne State Univ. Interdisciplinary Biomedical Sciences Fellowship (2010 –2013)
- WSU/UM Joint Physiology Symposium, data presentation award, 2011
- Wayne State Univ., Graduate School Exhibition, Poster Presentation Award 2nd place, 2012

Publications:

1. Ichinose TK, **Minic Z**, Li C, O'Leary DS, Scislo TJ. Activation of A_1 adenosine receptors inhibits regional sympathetic responses evoked y activation of cardiopulmonary chemoreflex. *Am J Physiol Regul Integr Comp Physiol* 2012 Sep; 303(5):R539-R550. Epub 2012 Jul 18.

Abstracts:

1. **Minic Z**, O'Leary DS, and Scislo TJ. Activation of NTS A_{2a} adenosine receptors impairs cardiopulmonary chemoreflex control of renal (RSNA), adrenal (ASNA) and lumbar (LSNA) sympathetic nerve activity. *FASEB J* 24: 624.11, 2010.
2. McClure JM, **Minic Z**, Li C, O'Leary DS, and Scislo TJ. Mechanisms mediating heart rate (HR) responses evoked by activation of NTS A_1 adenosine receptors. *FASEB J* 24: 624.6 2010.
3. **Minic Z**, O'Leary DS, and Scislo TJ. Activation of NTS A_{2a} adenosine receptors impairs cardiopulmonary chemoreflex (CCR) responses of renal (RSNA), adrenal (ASNA), and lumbar (LSNA) sympathetic nerve activity via activation of GABA-ergic neurons. *FASEB J* 25: 844.3, 2011.
4. **Minic Z**, O'Leary DS, and Scislo TJ. A_{2a} adenosine receptors modulate cardiopulmonary chemoreflex control of regional sympathetic outputs via activation of GABAergic neurons within the caudal portion of the nucleus of the solitary tract (cNTS): functional and anatomical evidence. *FASEB J* 26: 1091.28, 2012.
5. **Minic Z**, Goshgarian HG, O'Leary DS, and Scislo TJ. Immunohistochemistry confirms the functional evidence that A_1 adenosine receptors directly inhibit the cardiopulmonary chemoreflex (CCR) pathway whereas A_{2a} receptors inhibit the reflex via facilitation of GABA release in the caudal nucleus of the solitary tract (cNTS) in the rat. *FASEB J* 26: 1118.15, 2013.
6. **Minic Z**, Li C, O'Leary DS and Scislo TJ. Severe hemorrhage attenuates cardiopulmonary chemoreflex (CCR) control of renal and adrenal sympathetic nerves via adenosine operating in the nucleus of the solitary tract (NTS). *FASEB J* 26: 1118.13, 2013.