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BAROREFLEX ADAPTATIONS IN PREGNANCY:
THE EFFECT OF OVARIAN HORMONE METABOLITES
AND CENTRAL NERVOUS SYSTEM CONTROL

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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ABSTRACT

Pregnancy, a condition characterized by increased circulating levels of ovarian hormones, is associated with numerous cardiovascular changes including alterations in baroreflex function. The presence of elevated levels of 3α-hydroxy-dihydroprogesterone (3α-OH-DHP), the primary metabolite of progesterone and the most potent endogenous positive modulator of central nervous system (CNS) GABA$_A$ receptors (90, 93), may be one mechanism mediating the effect of pregnancy on baroreflex function. Results from previous studies in our laboratory evaluating the effect of pregnancy on efferent baroreflex function, were suggestive of a CNS mechanism. However, it is possible that the alterations in baroreflex function observed in pregnant animals may actually reflect changes at an earlier point in the baroreflex pathway. The purpose of this dissertation was to evaluate the effect of pregnancy on the afferent and CNS components of the baroreflex pathway and to further elucidate CNS mechanisms involved in control of baroreflex function.

The effect of pregnancy and 3α-OH-DHP on baroreceptor reflex afferents were evaluated by recording changes in aortic depressor nerve discharge in response to changes in arterial blood pressure in virgin and pregnant rats. A significantly lower baseline mean arterial pressure (MAP) and a leftward shift in the baroreceptor discharge curve to a lower
operating pressure range was observed in pregnant animals. Baroreceptor sensitivity to increments in MAP, however, was not effected by pregnancy. Administration of 3α-OH-DHP to virgin animals had no effect on baroreceptor function indicating that the effect of pregnancy on baroreceptor function was not due to elevated levels of this hormone during pregnancy. These results are consistent with a pressure dependent resetting of the baroreceptors and suggest that the potentiated baroreflex sympathoinhibition and attenuated sympathoexcitation of pregnancy, and the responses to 3α-OH-DHP previously observed in virgin rats, are most likely mediated through CNS mechanisms rather than afferent mechanisms. Thus, the effect of circulating 3α-OH-DHP on responsiveness of identified pressure sensitive, sympathoexcitatory neurons in the rostral ventrolateral medulla (RVLM) to endogenously released γ-amino-butyric acid (GABA) was evaluated in the second study. Intravenous administration of 3α-OH-DHP significantly reduced both threshold and saturation MAP of sympathoexcitatory neurons in the RVLM and, at higher concentrations, also prolonged the time to recovery. These results are consistent with the potentiation of baroreflex sympathoinhibitory responses at high MAP during pregnancy and therefore suggest that the RVLM may be one CNS site of action mediating the effects of 3α-OH-DHP on control of sympathetic outflow. However, the RVLM receives inhibitory and excitatory drive from several areas in the CNS and alterations in the balance of neural inputs to the RVLM may also contribute to alterations in baroreflex function during pregnancy. Tonic neural inputs from the caudal ventrolateral medulla (CVLM) to the RVLM were evaluated in virgin female rats in the final study in this dissertation. Pressor and sympathoexcitatory responses to inhibition of CVLM were still
evident during RVLM GABA_A receptor blockade and were no different during combined RVLM GABA_A and GABA_B receptor block. This suggests first, that GABAergic influences from the CVLM to the RVLM are mediated primarily by GABA_A receptors and second, that there is a tonic non-GABAergic inhibitory input from the CVLM to the RVLM. Meanwhile, decreases in MAP and renal sympathetic nerve activity (RSNA) due to activation of the CVLM with glutamate were reversed to an excitatory pressor response in the presence of RVLM GABA_A receptor blockade, indicating that there is an excitatory input from the CVLM to the RVLM which is masked by tonic inhibitory inputs.
DEDICATED TO MY PARENTS,

JIRAPUN AND PAWANA LAIPRASERT,

FOR THEIR LOVING SUPPORT OF ALL MY ENDEAVORS
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ABSTRACTS

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FIELDS OF STUDY

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marked with Chicago sky blue dye (50 nl) and identified histologically (closed circles). *Right = CVLM:* The site for maximum pressor response to microinjected GABA was functionally defined as the CVLM, marked with Chicago sky blue dye (50 nl) and identified histologically (closed squares). Brainstem sections were adapted from *The Rat Brain in Stereotaxic Coordinates* (94). Sol, nucleus of the solitary tract; Cu, cuneate nucleus; Ecu, external cuneate nucleus; Amb, ambiguus nucleus; Sp, spinal trigeminal tract.
2.1 Effects of pregnancy on baroreceptor discharge function: A significant decrease in baseline MAP and a corresponding decrease in curve midpoint (A3) were observed in pregnant animals. However, there were no significant differences in any of the remaining parameters used to evaluate baroreceptor discharge function. Values are means ± SEM; NA, nerve activity; NTP, nitroprusside; PE, phenylephrine; MAP, mean arterial pressure; * p < 0.05

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3.1 Effects of treatments on baseline mean arterial pressure (MAP) and unit activity (UA): Values are means ± SEM. Neither baseline MAP nor baseline UA was significantly affected by i.v. administration of vehicle (40% β-cyclodextrin) or 3α-OH-dihydroprogesterone (3α-OH-DHP). pps, pulses per second.
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3.4 Recovery parameters for unit activity (UA): Values are mean ± SEM. Neither vehicle nor 3α-OH-DHP (1.12 μg/Kg) affected t ½ UA.

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4.3 Functional Identification of CVLM & RVLM: The CVLM and RVLM were functionally identified as the site of maximum pressor and depressor responses to microinjection of GABA (500 pmol, 50 nl) respectively. As expected, significant increase in both arterial pressure (MAP) and nerve activity (RSNA) were observed following injection of GABA in the CVLM. Microinjection of GABA in the RVLM produced significant decreases in MAP and RSNA. There were no differences between BIC and BIC+ CGP35348 groups. Values = mean ± SEM; * P ≤ 0.05
CHAPTER 1

INTRODUCTION

CARDIOVASCULAR ADAPTATIONS IN PREGNANCY:

Several hemodynamic adaptations occur early in normal human pregnancy a condition associated with increased levels of circulating estrogen and progesterone. Although progesterone levels are elevated early on in rats, estradiol levels are not significantly increased compared to virgin controls until near term (34) and similar changes in hemodynamic profile can be observed in rats at near term pregnancy. In humans, blood and plasma volume increase by about 40% during pregnancy (29, 30). Similarly, cardiac output increases 40% due to increases in both heart rate and stroke volume (29, 30). Although these changes would tend to increase arterial blood pressure, a decrease in total peripheral resistance (TPR), and therefore a decreased mean arterial pressure (MAP), is observed by the end of the first trimester in humans (29, 30).

While the exact mechanisms producing the decreased TPR have not been determined, several possibilities have been suggested. Previously, it was suggested that an increase in blood flow to the uterus may be one mechanism that contributes to the decrease in TPR during pregnancy. However, the hemodynamic changes associated with pregnancy
are apparent early in pregnancy, at which time uterine blood flow is still low. In fact, even at near term pregnancy, more than half of the decreased vascular resistance cannot be accounted for by an increase in uterine blood flow (30).

The changes that occur in arterial baroreflex function during pregnancy may be one important mechanism contributing to the hemodynamic profile of pregnancy. In 1974, Humphreys and Joels evaluated the baroreflex control of MAP in anesthetized rabbits by altering pressure within the vascularly isolated carotid sinus and observing blood pressure responses (51). In comparison to non-pregnant animals, the range of blood pressure responses that could be elicited in pregnant animals was found to be significantly diminished. The minimum arterial blood pressures achieved at high carotid sinus pressures (baroreceptors loaded) remained similar between pregnant and non-pregnant animals. However, when the baroreceptors were unloaded (low isolated carotid sinus pressures), significantly lower maximum arterial blood pressures were observed in pregnant animals (51). In subsequent studies, the contribution of changes in cardiac output and resistance in the hind limb to the diminished baroreflex pressor response were also evaluated using the isolated carotid sinus preparation (52, 53). These studies demonstrated that the attenuated baroreflex pressor response in pregnancy was most likely due to the lower hind limb vascular resistance observed in pregnant animals rather than to attenuated changes in cardiac output (52, 53).

The results of these studies which evaluated the effect of pregnancy by measuring arterial pressure responses, however, may be confounded by alterations in vascular responsiveness which occur in pregnant animals. Several possible mechanisms that
contribute to the changes in vascular responsiveness observed in pregnancy have been demonstrated. These possibilities include a change in levels of circulating vasoactive substances and/or a change in the responsiveness of vascular smooth muscle to circulating vasoactive substances (37). Increased levels of circulating vasodilators, such as prostaglandins, have been demonstrated during normal pregnancy and may be one mechanism contributing to the decrease in TPR (37). In addition to vasodilatory effects however, prostaglandins have also been implicated in altering the response of the vasculature to vasoconstrictors during pregnancy. Pregnant animals have decreased vascular responsiveness to vasoconstrictors, such as angiotensin II (ANGII) (91). The decrease in vasoconstrictor responsiveness to ANGII is not attributed to a decrease in either receptor number or in receptor affinity (91). Instead, inhibition of prostaglandin synthesis was found to restore pressor responses to ANGII towards levels observed in non pregnant animals (91). Recovery of pressor responses to norepinephrine (NE) and vasopressin was also observed following prostaglandin synthesis inhibition (91). Changes in smooth muscle characteristics in the vasculature may also contribute to the change in responsiveness observed during pregnancy. Meyer et al (84) have demonstrated that not only are arteries from pregnant rats less sensitive to membrane depolarization by K+ than virgin rats, but a decrease in arterial basal tone and myogenic responses to pressure stimuli are decreased as well. Differences between arteries from pregnant and virgin animals were eliminated by removal of the endothelium, suggesting that the decreased vascular responsiveness may be due to alterations in the relaxing influence of the endothelium (84). Several laboratories have demonstrated that levels of endothelium-derived relaxing factor
(EDRF), also known as nitric oxide, an endogenous vasodilator, are elevated during pregnancy (37, 92).

Our laboratory has evaluated the effect of pregnancy on baroreflex function in a more direct manner by measuring sympathetic outflow in rats. In addition to a significantly lower baseline MAP, sympathoexcitatory responses to decreases in arterial blood pressure were significantly attenuated in pregnant animals. Consistent with the diminished sympathoexcitatory response, the slope of the nitroprusside (NTP) end of the baroreflex function curve was decreased in pregnant animals indicating that reflex sensitivity to decreasing pressure was reduced. Sympathoinhibitory responses to increases in blood pressure, meanwhile, were potentiated in pregnant animals (23, 47, 80). These results suggest a central nervous system (CNS) mechanism. Recent reports in the literature also support the possibility of a neural mechanism in the decreased vascular sensitivity of pregnancy. Hines et al recently demonstrated that pressor responses to infusion of the vasoconstrictor, ANGII, in pregnant rats was restored following total autonomic blockade (50). Similarly, autonomic blockade also was found to restore pressor responses to infusion of arginine vasopressin (49).

Although the mediator for altered control of sympathetic outflow during pregnancy is not known, the ovarian hormones and their metabolites, in particular the primary metabolite of progesterone, 3α-OH-dihydroprogesterone (3α-OH-DHP), are likely candidates. The presence of elevated levels of 3α-OH-DHP, the most potent endogenous positive modulator of CNS GABA_\text{A} receptors, may contribute to the changes in baroreflex function observed during pregnancy. In studies evaluating the effect of 3α-OH-DHP on
baroreflex function, acute intravenous administration of 3α-OH-DHP to virgin female rats was demonstrated to attenuate sympathoexcitatory responses to decreasing pressure (47, 80). This response is qualitatively similar to the effects of pregnancy and indicates that 3α-OH-DHP may contribute to the changes in baroreflex function.

It is important to note, however, that, although an effect of pregnancy has previously been described in the effector mechanisms of baroreflex function, the baroreceptor reflex is made up of several components and changes due to pregnancy may actually occur at any point in the baroreflex arc. Changes in baroreceptor afferent discharge, within the CNS pathway, or in sympathetic efferent outflow may be transmitted through the remainder of the pathway and be reflected as an alteration in vascular responses. In this work we are interested in the further elucidation of the possible mechanisms underlying the alterations in baroreflex function in pregnancy.

**ARTERIAL BARORECEPTORS**

Arterial baroreceptors are slowly adapting, sensory stretch receptors important in the rapid buffering of blood pressure around a set point. The nerve terminal endings of the baroreceptors are located within the vessel walls of the aortic arch and carotid sinus. A sustained elevation in pressure results in the classic phenomenon, chronic arterial baroreceptor resetting (83). In established hypertension, the operating pressure range of the baroreceptor reflex curve shifts towards higher operating pressures and a decrease in baroreceptor sensitivity is observed. Pathological changes in vessel wall structure, such as increased vascular wall thickness and a corresponding decrease in distensibility may also
occur in chronic baroreceptor resetting, and contribute to the diminished baroreceptor sensitivity (13, 101). A second type of baroreceptor resetting, acute resetting, has also been described (64, 67). Unlike chronic resetting which can take weeks to develop, acute resetting develops within a few (3-5) minutes of an acute change in pressure and is reversible upon the return to the original arterial pressure (65, 67). During acute hypertensive resetting, the baroreceptor reflex curve is also shifted to the right towards a higher operating pressure range (33, 66, 67). However, pathological changes in vascular wall structure which contribute to changes in baroreceptor responsiveness do not have time to develop during acute resetting and thus no alteration in baroreceptor sensitivity is observed in acute resetting. During acute hypotension the baroreceptor reflex curve is shifted toward lower MAP with no change in sensitivity to increments in pressure. Similar responses have also been demonstrated in both aortic and carotid sinus baroreceptor afferents during chronic hypotension (54, 103). Pressure dependent resetting, elicited by sustained depression of MAP, results in a significant leftward shift of the baroreceptor function curve to a lower operating pressure range with no accompanying changes in the vessel wall or in baroreceptor sensitivity (54, 100). In previous studies, in our and other laboratories, pregnancy has been shown to be associated with a significantly lower baseline MAP and a leftward shift of the efferent baroreflex curve consistent with pressure dependent hypotensive resetting of the baroreflex (12, 23, 47, 80). However, significant changes in baroreflex responsiveness were also observed and suggest that there is an additional effect of pregnancy which is not pressure dependent and may involve the CNS.
CNS BAROREFLEX PATHWAY:

Primary afferent fibers carrying signals from baroreceptors located in the aortic arch have been shown to travel via the aortic nerve to terminate on neurons in the nucleus tractus solitarius (NTS) (Figure 1.1). Retrograde labeling studies have demonstrated that these fibers project to and terminate on the ipsilateral NTS converging with afferents from other cardiovascular sensory receptors. While in most animal models the aortic depressor nerve contains both baroreceptor and chemoreceptor afferents, this is not the case in the rat. In the rat, a paucity of glomus tissues, an accessory structure associated with chemoreceptor afferents, has been shown in the aortic nerve (8). Functional studies have demonstrated that stimulation of the rat aortic nerve produces reflex sympathoinhibitory responses in renal and cardiac nerves consistent with baroreceptor afferent activation (89). Phrenic and laryngeal nerve activity, however, is likewise inhibited during aortic nerve stimulation suggesting that chemoreceptor afferents are not present in the aortic nerve (102). Mapping studies have demonstrated that the aortic nerve terminates in the dorsomedial and lateral subnuclei of the NTS, rostral to the obex (21, 28) and termination sites of afferents are also confined to the area of the NTS rostral to the obex (32). Chemoreceptor afferents however, are found to terminate caudal to the obex (31). These results suggest that there are few functional chemoreceptor afferents traveling within the aortic. As a result of the relative absence of chemoreceptors in the aortic nerve in the rat, these nerves are often used when evaluating arterial baroreceptor reflex afferents.

A major site of convergence for incoming cardiovascular information, in addition to afferent projections from the arterial baroreceptors in the great arteries, the NTS also receives
Figure 1.1. **Medullary baroreflex pathway schematic**: Components of medullary baroreflex pathway are shown. Increase in blood pressure activate baroreceptors which then stimulate neurons in the NTS. Neurons in the NTS project to and excite baroreflex driven neurons in the CVLM. Excited CVLM neurons provide inhibitory influences to the RVLM. Tonically active, baroreflex independent neurons from the CVLM also inhibit RVLM neurons. RVLM neurons provide sympathoexcitatory output to sympathetic preganglionic neurons in the IML. NTS = nucleus of the solitary tract; CVLM = caudal ventrolateral medulla; RVLM = rostral ventrolateral medulla; IML = intermediolateral cell column; EAA = excitatory amino acid.
and integrates afferent information from arterial chemoreceptors, cardiac baroreceptors, and cardiac mechanoreceptors. Excitatory and inhibitory projections from various nuclei in the brain involved in cardiovascular control of blood pressure, have also been shown to reciprocally project to, and may modulate, baroreflex function at the level of the NTS. The precise function of these secondary afferents however, have not been elucidated. Although a wide variety of neurotransmitters have been identified in the NTS, there is evidence to suggest that the neurotransmitter involved in the baroreflex activation of neurons in the NTS is most likely the excitatory amino acid (EAA), L-glutamate (38). Blockade of glutamate receptors, for instance, with kynurenic acid, a nonspecific L-glutamate receptor antagonist, in the NTS abolishes responses to baroreceptor stimulation (38). Administration of exogenous L-glutamate in the NTS, on the other hand, produces depressor and sympathoinhibitory responses similar to activation of the baroreceptor reflex.

In the next step of the medullary baroreflex pathway, neurons in the NTS project to and excite neurons in the ipsilateral caudal ventral lateral medulla (CVLM) (Figure 1.1) (28). Also known as the depressor area, the CVLM is thought to be an obligatory synapse in the CNS baroreflex pathway (26) since inhibition of CVLM neurons with kainic acid, a neurotoxin, has been shown to abolish the baroreflex (28). Inhibition of the CVLM has been shown to elicit a large increase in MAP and sympathetic nerve activity. The pressor response produced following inhibition of the CVLM, suggests that neurons in the CVLM provide tonic inhibition of sympathetic outflow.

Recent studies have demonstrated that there are two functionally distinct populations of neurons in the CVLM, baroreflex dependent neurons and baroreflex independent neurons
Earlier studies by Cravo et al suggested that these two populations of neurons may be segregated, with baroreflex driven neurons located in the more rostral aspect of the CVLM and tonically active baroreflex independent neurons towards the caudal portion of the CVLM (24). However later reports by this group have indicated that this anatomical distinction may not be as precise as was previously thought (25). The primary neurotransmitter activating baroreflex dependent neurons in the CVLM is thought to be endogenously released glutamate (28).

Administration of exogenous glutamate has been shown to produce depressor and sympathoinhibitory responses indicating that receptors for glutamate are present in the CVLM (28). Blockade of glutamate receptors in the CVLM with kynurenic acid, a nonspecific glutamate receptor blocker, abolishes responses to baroreflex stimulation suggesting that the baroreflex sensitive neurons in the CVLM are activated by glutamate released from NTS neurons (38).

The neurotransmitter activating tonically active baroreflex independent neurons in the CVLM, however, is not known.

CVLM neurons, in turn, project to and synapse on tonically active neurons in the ipsilateral rostral ventrolateral medulla (RVLM) (Figure 1.1) (28). Commonly accepted as the final site for tonic sympathoexcitatory drive to preganglionic sympathetic neurons in the intermediolateral cell column (IML) of the spinal cord, the RVLM is an integral component in the central control of cardiovascular function (39). Although the precise role of RVLM in the generation of sympathetic output, either as the central site of origin of sympathetic output (110) or as one component of a network oscillator (35), is unclear, it is well accepted that stimulation of RVLM neurons produces an increase in blood pressure. This pressor response is associated with an increased total peripheral resistance through activation of
sympathetic output. Meanwhile inhibition produces the opposite results. The profound depressor response observed following inhibition of the RVLM suggest that neurons in the RVLM are a major source of tonic sympathetic drive (28, 35, 39, 110).

The RVLM has also been shown to be important in the integration of cardiovascular input from supramedullary sites. Pressor effects can be elicited by stimulation of a number of areas, including the following sites: the hypothalamic defense area (perifornical area), midbrain periaqueductal gray (PAG), Kolliker-Fuse nucleus, area postrema, medullary lateral tegmental field, or paraventricular nucleus (PVN) of the hypothalamus (18, 27, 28, 98). Excitatory influences from these areas have been demonstrated to be mediated, at least partially, through the RVLM (61). Inhibition of RVLM neurons, likewise, abolishes or attenuates the pressor effects produced by stimulation of these areas. Stimulation of the locus coeruleus and lateral hypothalamus has been demonstrated to produce depressor effects which are mediated through the RVLM (4, 112).

There is a large amount of evidence indicating that the CVLM is a major source of inhibitory influence on the tonically active RVLM (26, 28, 81, 111). The primary inhibitory neurotransmitter candidate thought to be released by CVLM neurons at the RVLM is \( \gamma \)-aminobutyric acid (GABA) (26, 28, 81, 111). Blockade of GABA\(_A\) receptors with bicuculline injected into the RVLM, for instance, has been shown to abolish baroreflex responses and results in substantial increases in MAP and RSNA (26, 109). The pressor and sympathoexcitatory responses following GABA\(_A\) receptor blockade suggests that the tonic inhibition of RVLM neurons is mediated by GABA (26, 109). Furthermore, elimination of the
baroreflex by inhibition of GABA<sub>A</sub> receptors suggests that GABA<sub>A</sub> receptors in the RVLM represent the primary mechanism for arterial baroreflex inhibition of tonic excitatory drive to preganglionic sympathetic neurons in the IML.

Although there is strong evidence to suggest that arterial baroreflex initiated GABAergic inhibition of the RVLM by the CVLM is mediated through GABA<sub>A</sub> receptors (26, 39, 109) there are a growing number of reports suggesting that GABA<sub>B</sub> receptors are also involved in mediating GABAergic inhibition of the RVLM (5, 6, 71). Tonically active, baroreflex independent, GABAergic projections from the CVLM to the RVLM have been described recently (24-26). Disruption of neuronal activity in the CVLM has been shown to elicit a larger increase in MAP than interruption of baroreflex afferent input alone, suggesting a baroreflex independent component (24, 38). However, the post-synaptic GABA receptor type in the RVLM which mediates the tonic baroreflex independent inhibition is not known.

While the CVLM is accepted as the primary source of inhibitory influences on the tonically active RVLM, less is known about tonic excitatory inputs to the RVLM. As mentioned above, several central nervous system sites have been shown to provide excitatory projections to the RVLM. These projections, however, have not been shown to be tonically active. Until recently only one area, the pontine reticular formation, has been identified as a potential source of tonic excitatory drive to the RVLM (42). In 1997, Ito & Sved (55) proposed that the CVLM may also be a source of tonic excitatory drive to the RVLM. In this study, blockade of excitatory amino acid (EAA) receptors in the RVLM elicited a significant decrease in MAP only after neuronal activity in the CVLM was
inhibited with muscimol. The lack of MAP response to EAA receptor block in the RVLM of intact animals suggests that there is a tonic non-EAA mediated excitatory signal to the RVLM which maintains sympathoexcitatory tone despite blockade of EAA receptors.

**GABA:**

The inhibitory amino acid neurotransmitter, GABA is prevalent throughout the brain. GABA activity has been demonstrated in the NTS, CVLM, RVLM, IML, and numerous other sites involved in cardiovascular control (28, 39). While modulation of GABAergic influences in any of these areas may significantly affect sympathetic output, increased inhibition of tonically active neurons in the RVLM would most directly explain the alterations in baroreflex control of sympathetic output observed during pregnancy.

The GABAₐ receptor is a ligand gated chloride anion channel (93). Activation of GABAₐ receptors by the binding of GABA, opens the ion channel and increases chloride conductance (93). Thus, through hyperpolarization, neuronal activity of the cells is inhibited. In addition to GABA, it has been shown that several other classes of compounds have unique binding sites on the GABAₐ receptor, including benzodiazepines, barbiturates, convulsants, and neurosteroids (79, 93). Binding of any of these compounds allosterically modulates activity of the chloride channel.

The receptor itself is considered to be a heteroligomer composed of four to five membrane spanning subunits forming the integral chloride ion channel. Five different types of GABAₐ receptor subunits have been identified including α, β, γ, δ, ρ (79, 93). Multiple isoforms of the α, β, and γ subtypes have also be identified including (α₁-₆, β₁-₄,
and have been shown to be differentially expressed in the various regions of the CNS. Binding and affinity of the receptor to GABA or other ligands is dependent on the subunit composition of the GABA$_A$ receptor. Benzodiazepine binding and allosteric modulation of the GABA receptor, for instance, requires the presence of the $\gamma_2$ subunit in the receptor complex (93). Likewise, studies have shown that positive modulation of GABA$_A$ receptor function by the neuroactive steroid, 3$\alpha$-OH-DHP, is also dependent on the composition of the GABA$_A$ receptor, requiring the $\gamma_1$ subunit in combination with an $\alpha$ subunit (93, 96). Efficacy of 3$\alpha$-OH-DHP binding and allosteric modulation of the GABA$_A$ receptor complex, varies depending on the $\alpha$ subunit isoform present in the receptor complex. It is unclear, however, which $\alpha$ isoform is required to produce the greatest modulation of GABA$_A$ receptor function. The largest potentiation of GABA mediated chloride conductance has been demonstrated with the $\alpha_1$ subunit, followed by lesser potentiation with the $\alpha_2$, and $\alpha_3$ isoforms (105). Flunitrazepam binding studies, however, have demonstrated that the greatest potentiation of binding occurs when the $\alpha_3$ isoform is present in the receptor complex (70). The least modulation of GABA$_A$ receptor function is observed when the $\alpha_6$ isoform is present (70).

The ovarian hormones, estrogen and progesterone, have been demonstrated to play an important role in regulating central GABA$_A$ receptor synthesis, composition, and affinity (48, 78). Progesterone and estrogen pretreatment has been shown to increase the number of GABA binding sites in many brain regions (78). Estrogen in particular has been demonstrated to promote the synthesis of $\alpha$ and $\gamma$ subunits, important in neurosteroid binding (see above) (48). Estrogen has also been shown to affect GABA synthesis, and
the rate of GABA turnover (77, 104). Estradiol has also been demonstrated to reduce GABA_A receptor binding and lower levels of GABA are found in the hypothalamus during proestrus, when estrogen is high, representing an alteration in GABA turnover in response to high estrogen (77). Decreased binding of muscimol, a GABA_A agonist, has also been demonstrated in ovariectomized rats treated with estradiol (104). It has been demonstrated that the presence of both estrogen and progesterone is important and an interaction between the hormones may explain the apparent inconsistencies (104). Progesterone treatment in estrogen primed animals, for instance, increases muscimol binding suggesting that interaction of the hormones is necessary (104).

3α-OH-DHP:

The 5α-reduced, 3α hydroxylated metabolite of progesterone, 3α-OH-DHP, is formed by metabolism of progesterone to an intermediate product 5α-

dihydroprogesterone (5α-DHP), by the enzyme 5α-reductase (59, 93). This intermediate is then hydroxylated by 3α-hydroxysteroid oxidoreductase to the neuroactive metabolite, 3α-OH-DHP (59, 93). The enzymes involved in the CNS formation of 3α-OH-DHP from progesterone have been shown to be present primarily in glial cells (5α-reductase) and astrocytes (3α-hydroxysteroid oxidoreductase) (59, 93). Plasma and brain levels of the 3α-OH-DHP have been shown to follow those of progesterone.

Recently the primary metabolite of progesterone, 3α-hydroxy-
dihydroprogesterone (3α-OH-DHP) has been demonstrated to be the most potent endogenous positive modulator of central nervous system GABA_A receptor function (79,
3α-OH-DHP belongs to a class of compounds called neurosteroids (79, 93). The rapid (seconds to minutes) alterations in central nervous system excitability by neurosteroids does not fit in the classical genomic mechanism of steroid hormones action which requires de novo protein synthesis (79, 93). Additionally, inhibition of protein synthesis has no effect on neurosteroid activity (93). In studies in which progestagens were bound to the large protein molecule albumin, thus preventing intracellular access through diffusion, unique and stereospecific neurosteroid binding sites on membrane receptors have been demonstrated (60). When administered in 10-30 nM concentrations, neurosteroids are potent modulators of GABA_\text{A} receptor function (79). In hippocampal neurons, for instance, neurosteroids have been shown to increase the duration of inhibitory post-synaptic currents (79). At higher concentrations (μM range), neurosteroids have been shown to directly open the chloride channel (93). The results of previous studies in our laboratory are consistent with an increased GABAergic influence in the RVLM of pregnant animals (23, 80). Increased GABAergic inhibition of tonically active neurons in the RVLM would produce the attenuated sympathoexcitatory responses observed during pregnancy. The mechanisms involved in mediating an increased GABAergic influence are not known, however, elevated levels of 3α-OH-DHP in pregnancy is one possibility. Plasma levels of 3α-OH-DHP are elevated (20-30 ng/ml) (93) during pregnancy to concentrations which have been demonstrated to potentiate GABA mediated inhibition.
SPECIFIC GOALS:

The purpose of this dissertation was to evaluate the effect of pregnancy on afferent and CNS components of the baroreflex pathway. CNS mechanisms involved in the control of baroreflex function were also investigated. In order to determine whether changes in efferent baroreflex function may actually be due to changes occurring in the afferent portion of the reflex pathway, the effect of pregnancy on baroreceptor reflex afferent function was assessed and results are described in Chapter 2. The effect of exogenous 3α-OH-DHP was also evaluated in these studies to determine if elevated levels of the metabolite during pregnancy may alter afferent mechanisms and thus contribute to the changes observed in baroreceptor reflex function.

An effect of pregnancy and 3α-OH-DHP on CNS control of the baroreflex may also contribute to the differences in baroreflex function between virgin and pregnant animals, and has been suggested by earlier studies. Several lines of evidence provided in the literature point to the RVLM as the most likely site of action for 3α-OH-DHP. However, this has not been previously evaluated. Thus, Chapter 3, investigates the effect of circulating 3α-OH-DHP, administered in concentrations similar to those found in pregnancy, on arterial pressure sensitivity of spinally projecting neurons in the RVLM to endogenously released GABA.

There are, however, several other possible CNS mechanisms that could contribute to altered baroreflex function in pregnancy. One such mechanism is an alteration in the balance between inhibitory and excitatory influences within the RVLM. Chapter 4 evaluates the involvement of RVLM GABA<sub>A</sub> and GABA<sub>B</sub> receptors in mediating tonic
inhibition from the caudal ventrolateral medulla (CVLM). Recent literature has suggested that the CVLM may also have a role in providing tonic excitatory influences to the RVLM and thus, this possibility is also evaluated in the experiments described in Chapter 4.
CHAPTER 2

AFFERENT BARORECEPTOR DISCHARGE IN PREGNANT RATS

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ABSTRACT

The baroreflex function curve is shifted to lower operating pressures, efferent sympathoexcitatory responses are attenuated and sympathoinhibitory responses are potentiated in pregnant (P) compared to virgin (V) rats (23, 47, 80). It has been proposed that during pregnancy elevated levels of 3α-hydroxy-dihydroprogesterone (3α-OH-DHP), a major metabolite of progesterone, may contribute to this difference, since acute intravenous administration of 3α-OH-DHP to virgin female rats mimics the effects of pregnancy on the baroreflex (47, 80). The current experiments evaluated the effect of pregnancy and 3α-OH-DHP on aortic depressor nerve activity. Baroreceptor discharge curves were obtained in Inactin anesthetized rats by recording aortic NA during ramp
increases and decreases in mean arterial pressure (MAP) [i.v. phenylephrine and nitroprusside infusion] before (Control, C), 15 minutes (E1), and 30 minutes (E2) following 3α-OH-DHP (220 μg/Kg bolus + 25 μg/Kg/min infusion, i.v.). Baseline blood pressure was significantly lower in pregnant (109 ± 4.4 mmHg) compared to virgin (122 ± 2.8 mmHg) rats. The only significant difference in the baroreceptor discharge curves was a decrease in curve midpoint in pregnant rats (V = 140 ± 2.7 vs. P = 124 ± 3.6 mmHg). 3α-OH-DHP had no effect on afferent baroreceptor discharge curves in either virgin or pregnant groups. These results suggest that pressure dependent baroreceptor resetting may contribute to a shift in the baroreflex curve to lower operating pressures, but cannot completely explained differences in baroreflex function between virgin and pregnant animals.

INTRODUCTION

Many cardiovascular adaptations associated with pregnancy have been reported in the literature. For example, blood volume and cardiac output are increased, arterial pressure is decreased and heart rate is elevated in pregnant compared to virgin animals (29, 30, 52, 73). Among the adaptations to pregnancy are changes in arterial baroreflex function. Sympathoexcitatory responses to decreases in arterial blood pressure are attenuated and sympathoinhibitory responses to increases in blood pressure are potentiated in pregnant animals (23, 47, 80). In addition, baseline arterial pressure is lower in pregnant animals and the baroreflex function curve is shifted to a lower operating pressure range (12, 23, 47, 80).
The presence of elevated levels of the primary metabolite of progesterone, 3α-hydroxy-dihydroprogesterone (3α-OH-DHP), may be one mechanism involved in producing the changes in baroreflex function during pregnancy. Previous studies in our laboratory have demonstrated that acute intravenous administration of 3α-OH-DHP to virgin female rats attenuates sympathoexcitatory responses to increases in arterial blood pressure, a response also seen in pregnancy (47, 80). The 3α-OH-DHP metabolite of progesterone belongs to a class of compounds known as neurosteroids. It has been demonstrated that 3α-OH-DHP is the most potent endogenous positive modulator of central nervous system (CNS) GABA_A receptors (90, 93). The changes in baroreflex function observed during pregnancy are consistent with a potentiation of GABA_A receptors in CNS sites responsible for sympathetic outflow and preliminary experiments in our laboratory suggest that direct application of 3α-OH-DHP to the rostral ventrolateral medulla produces changes in baroreflex function similar to those observed following intravenous administration (45). These results indicate that CNS effects of 3α-OH-DHP may contribute to the effects of pregnancy on arterial baroreflex function. However, effects on baroreflex function could occur through effects on afferent mechanisms as well. Whether pregnancy and the primary progesterone metabolite, 3α-OH-DHP, have an effect on baroreceptor afferent discharge that could participate in baroreflex adaptations has not been addressed. Therefore, the current experiments were designed to evaluate the effects of pregnancy and of 3α-OH-DHP on the afferent limb of the baroreflex.
METHODS

Experiments were performed in 51 female Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) at 3 to 5 months of age. Prior to the experiment, daily vaginal smear cytology was used to determine stage of the estrus cycle and each rat was followed through at least 2 full estrus cycles. Animals to be included in the pregnant groups (n = 26) were placed with a fertile male and day 1 of pregnancy was determined when sperm were observed in vaginal smears. Experiments were then performed on pregnant rats on days 20-21. Experiments in rats included in the virgin groups (n = 25) were performed in animals in the estrous stage of the cycle.

Surgical preparation:

Rats were anesthetized with Inactin (100 mg/Kg, i.p.). The trachea was cannulated and the rat artificially ventilated with room air supplemented with oxygen (Harvard Small Animal Ventilator: Harvard Apparatus, Edenbridge, Kent.). Body temperature was monitored and maintained at 37°C. The rat was then instrumented with a left femoral arterial and venous catheter to monitor arterial blood pressure and for intravenous administration of supplemental anesthetic (0.1 mg/Kg, i.v. as needed) respectively. Three catheters were also implanted in the left jugular vein for intravenous drug infusion.

To verify the effect of pregnancy on efferent baroreflex function in Inactin anesthetized rats, the left renal nerve was isolated retroperitoneally and placed on a bipolar platinum recording electrode in virgin (n = 13) and pregnant (n=14) rats. The renal nerve
was secured on the electrode with dental impression material (Coltene President’s dental acrylic) and the wound sutured closed.

Afferent baroreceptor responses were evaluated in a separate set of experiments by recording from the aortic nerve. The aortic nerve in 12 virgin and 12 pregnant rats, was isolated through a ventral approach. Once isolated, the nerve was placed on a bipolar platinum recording electrode and secured in place with dental impression material (Coltene President’s: Mahwah, NJ).

**Drugs and Solution:**

The anesthetic Inactin, was obtained from Research Biochemical International (Natick, MA) and dissolved in sterile water. Phenylephrine (PE) and nitroprusside (NTP) were purchased from Sigma Chemical Co. (St. Louis, MO) and diluted in isotonic saline. The primary metabolite of progesterone, 3α-hydroxy-dihydroprogesterone, was also obtained from Sigma Chemical Co. (St. Louis, MO) and was dissolved in 40% β-cyclodextran.

**Experimental Protocol:**

**Efferent Nerve Activity Baroreflex Experiments:** While the effect of pregnancy on baroreflex function has been previously evaluated in our laboratory in both urethane anesthetized (47) and in conscious (80) rats, the attenuation in sympathoexcitatory responses to low blood pressures in pregnant urethane anesthetized rats is relatively small compared to the conscious preparation. To evaluate whether this may be due to the type
of anesthetic used, preliminary experiments using the anesthetic, Inactin, a long lasting
rodent anesthetic, were performed in 13 virgin and 14 pregnant animals.

Similar to previous protocols (47, 80) baroreflex function was evaluated by
recording renal sympathetic nerve activity (RSNA) responses to slow ramp (approximately
2 mmHg/sec) decreases and increases in mean arterial pressure (MAP) [i.v. NTP and PE
infusion respectively]. Nerve activity responses were obtained as MAP was lowered to 50
mmHg and raised to 200 mmHg from baseline. MAP and RSNA were allowed to recover
between pressure ramps. Any nerve activity signal remaining 30 minutes after an overdose
of anesthetic was defined as electrical noise and subtracted from the nerve activity values.

**Afferent Nerve Activity Baroreceptor Experiments:** Changes in baroreceptor
afferent nerve discharge were evaluated by recording aortic nerve activity (AONA)
responses to slow ramp decreases and increases in MAP [i.v. NTP and PE infusion
respectively]. Baroreceptor discharge responses were obtained as MAP was lowered to
50 mmHg and raised to 200 mmHg from baseline. MAP and AONA were allowed to
recover between pressure ramps. Baroreceptor discharge curves were obtained before
(Control, C), 15 minutes following 3α-hydroxy-dihydroprogesterone (3α-OH-DHP) (220
µg/Kg bolus + 25 µg/Kg infusion, i.v., E1), and again 15 minutes following a second dose
of 3α-OH-DHP (220 µg/Kg bolus + 25 µg/Kg infusion, i.v., E2). Since arterial
baroreceptors are sensitive to the rate of change of pressure, care was taken to ensure that
the rate of decreases and increases in MAP was consistent between curves. The animals
were then sacrificed at the end of the experiment via an overdose of anesthetic and any
nerve activity signal remaining after 30 minutes was defined as electrical noise. The pups
of pregnant animals were counted and weighed to ensure that pup weight was normal for the day of pregnancy. Pregnant rats had $12 \pm 1$ fetuses, weighing an average of $4.1 \pm 0.1$ g each.

Data was collected using the Biopac Data Acquisition System. The neural signal was amplified 50,000X and recorded on a Dell Pentium II 333 mHz computer. The signal was simultaneously monitored on a loudspeaker and on the computer screen as data was collected. Collected data was analyzed using Acknowledge v3.0 software. Data points for mean arterial pressure (MAP), heart rate (HR), and nerve activity (NA) were obtained at a rate of 1 Hz. The Acknowledge system was used to limit raw NA signal to activity that exceeded a selected voltage set above noise level.

**Data Analysis:**

As absolute multiunit nerve activity values are dependent on recording conditions, nerve activity was standardized as a percent of the initial baseline of the control curve (C).

Data obtained in the efferent nerve activity baroreflex experiments were fit to the following nonlinear logistic curve equation:

$$y = \frac{A1}{1 + \exp(A2(X-A3)))} + A4$$

Where $A1 = \text{NA Range}$, $A2 = \text{Slope coefficient}$, $A3 = \text{MAP at curve midpoint}$, and $A4 = \text{Minimum NA}$. The maximum NA and maximum slope of each curve were then calculated using the following equations:
Maximum NA = A1 + A4

Maximum slope = -(A2 * A1)/A4

Data obtained in experiments evaluating the afferent limb of the baroreflex by measuring AONA responses were fit to a similar nonlinear logistic curve equation:

\[ y = -\frac{A1}{1+\exp(A2(X-A3))} + A4 \]

Where A1 - A3 are the same as above and A4 = Maximum NA. The minimum NA and maximum slope of each baroreceptor discharge curve were then calculated using the following two equations:

Minimum NA = A4 - A1

Maximum slope = (A2 * A1)/A4

In all experiments, values for baseline MAP, baseline raw NA, and maximum raw NA were also determined for each curve. Baseline HR and HR range were also evaluated.

Linear regressions on the linear portion of the RSNA or AONA discharge response to NTP and also to PE were performed in order to separately evaluate the sensitivity to hypotensive and hypertensive challenges.
Unpaired-t tests were used for comparisons of parameters between virgin and pregnant rats and one way analysis of variance (ANOVA) with repeated measures followed by Student Newman Keuls post test was used to evaluate the response to 3α-OH-DHP for a given parameter within each group. Data are presented as mean ± SEM. p ≤ 0.05 was considered significant.

RESULTS

Effects of pregnancy on RSNA baroreflex curves.

Prior studies in our laboratory have evaluated the effects of pregnancy on baroreflex function in urethane anesthetized (47) and in conscious (80) rats. While evidence for attenuated sympathoexcitatory responses was observed in pregnant urethane anesthetized rats, the sympathoexcitatory response was relatively small in the anesthetized compared to the conscious preparation. Preliminary experiments using the anesthetic, Inactin, were performed in 13 virgin (V) and 14 pregnant (P) animals in order to evaluate whether this may be due to the type of anesthetic used. The results indicated that under Inactin anesthesia, responses similar to those obtained in urethane anesthetized animals were observed and at high MAP were more similar to responses in conscious animals (Figure 2.1). In pregnant rats, the baroreflex function curve midpoint (A3) tended to be lower (P =118 ± 2.4, V =126 ± 2.8 mmHg, p < 0.056) indicating a shift in the curve to a lower operating pressure range. Consistent with this shift, baseline MAP was significantly lower in pregnant (104 ± 3.1 mmHg) compared to virgin (121 ± 3.3 mmHg) animals. Slope of the NTP portion of the curve tended (p <0.08) to be decreased in pregnant (-1.9
Figure 2.1 Effect of pregnancy on baroreflex curves: Mean renal sympathetic nerve activity (RSNA) baroreflex curves in Inactin anesthetized rats are shown. Pregnancy was associated with a decrease in baseline mean arterial pressure (MAP) (closed circles) and a decrease in the baroreflex curve midpoint (arrow). Additionally, the sympathoinhibitory response to high MAP was potentiated in pregnant animals and sensitivity to decreases in MAP tended (p<0.08) to be less in pregnant rats (see text). (*, p<0.05)
± 0.27) compared to virgin (-2.6 ± 0.32) rats suggesting that during pregnancy sympathoexcitatory responses to low MAP are attenuated. Meanwhile, a significantly lower minimum NA (A4) in pregnant rats (P = 11 ± 2.8, V = 22 ± 3.2 % baseline) indicated that sympathoinhibitory responses were potentiated at high MAP. Thus, although not as pronounced as in conscious rats, potentiated sympathoinhibition and attenuated sympathoexcitation are evident in pregnant Inactin anesthetized rats.

The effect of pregnancy on baseline HR and HR range in these Inactin anesthetized rats was also evaluated in these experiments. HR data was obtained in 13 pregnant and 9 virgin animals. Baseline HR was significantly higher in pregnant compared to virgin animals (P = 408 ± 8.0 bpm, V = 359 ± 7.5 bpm). However, no difference was observed in HR range between pregnant and virgin groups (P = 46 ± 7.4 bpm, V = 53 ± 10.1 bpm).

Effects of pregnancy on aortic nerve discharge curves.

The effect of pregnancy on aortic depressor nerve activity was evaluated in 12 virgin and 12 pregnant rats and the results are summarized in table 2.1. Baseline MAP, but not baseline raw NA, was found to be significantly lower in pregnant compared to virgin rats. A significant decrease in the baroreceptor discharge curve midpoint (A3) was also observed indicating a leftward shift of the curve during pregnancy (Figure 2.2). There were, however, no significant difference in any of the other curve coefficients: NA range (A1), slope coefficient (A2), or maximum NA (A4). Similarly, the calculated parameters, minimum NA and maximum slope were not significantly different between virgin and pregnant animals. Further comparison of the slopes of the baroreceptor
Figure 2.2 Effect of pregnancy on baroreceptor discharge curves: Mean baroreceptor discharge curves for 12 pregnant and 12 virgin rats are shown. Pregnancy was associated with a significant decrease in baseline mean arterial pressure (MAP) (closed circles) and a parallel leftward shift in the baroreceptor discharge curve (arrow). AONA = aortic nerve activity.
**Table 2.1: Effects of pregnancy on baroreceptor discharge function.** A significant decrease in baseline MAP and a corresponding decrease in curve midpoint (A3) were observed in pregnant animals. However, there were no significant differences in any of the remaining parameters used to evaluate baroreceptor discharge function. Values are means ± SEM; NA, nerve activity; NTP, nitroprusside; PE, phenylephrine; MAP, mean arterial pressure; * p < 0.05

<table>
<thead>
<tr>
<th>Baroreceptor discharge curve parameters</th>
<th>Virgin (n = 12)</th>
<th>Pregnant (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 – NA Range (% baseline)</td>
<td>229 ± 21.8</td>
<td>205 ± 17.5</td>
</tr>
<tr>
<td>A2 – Slope Coefficient</td>
<td>0.050 ± 0.003</td>
<td>0.055 ± 0.004</td>
</tr>
<tr>
<td>A3 – Curve Midpoint (mmHg)</td>
<td>140 ± 2.7</td>
<td>124 ± 3.6 *</td>
</tr>
<tr>
<td>A4 – Max. NA (% baseline)</td>
<td>258 ± 19.7</td>
<td>231 ± 11.9</td>
</tr>
<tr>
<td>Raw Max. NA (i/sec)</td>
<td>451 ± 57.4</td>
<td>450 ± 58.8</td>
</tr>
<tr>
<td>Min. NA (% baseline)</td>
<td>29 ± 7.1</td>
<td>26 ± 8.3</td>
</tr>
<tr>
<td>Max. Slope (% baseline/mmHg)</td>
<td>2.9 ± 0.30</td>
<td>2.8 ± 0.28</td>
</tr>
<tr>
<td>NTP slope (% baseline/mmHg)</td>
<td>2.1 ± 0.27</td>
<td>1.8 ± 0.20</td>
</tr>
<tr>
<td>PE slope (% baseline/mmHg)</td>
<td>2.6 ± 0.32</td>
<td>2.0 ± 0.17</td>
</tr>
<tr>
<td>Raw Baseline NA (imp/sec)</td>
<td>195 ± 32.8</td>
<td>189 ± 22.5</td>
</tr>
<tr>
<td>Baseline MAP (mmHg)</td>
<td>122 ± 2.8</td>
<td>109 ± 4.4 *</td>
</tr>
</tbody>
</table>

response to NTP and PE were performed using linear regression analysis of the linear portion of the NTP and PE ends of the curve and neither NTP nor PE slopes were found to be different between the two groups.

The effect of pregnancy on baseline HR and HR range was also evaluated. As expected, pregnancy was associated with a significantly higher baseline HR (P = 431 ± 8.8 bpm, V = 378 ± 7.1 bpm). HR range, however, was not different between pregnant and
Table 2.2. Effect of 3α-OH-DHP on baroreceptor afferent nerve discharge in virgin rats. Responses of aortic depressor nerve discharge to changes in arterial blood pressures were obtained at control (C), 15 minutes following administration of 3α-OH-DHP (E1), and 15 minutes following a second administration of 3α-OH-DHP (E2). In virgin rats, administration of 3α-OH-DHP had no effect on measured baroreceptor discharge curve parameters. Additionally, no effect was observed on either baseline mean arterial pressure (MAP) or baseline raw nerve activity (NA). Values = means ± SEM.

<table>
<thead>
<tr>
<th>Baroreceptor discharge curve parameters</th>
<th>C</th>
<th>E1</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1 - NA Range (% baseline)</td>
<td>229 ± 21.8</td>
<td>234 ± 22.6</td>
<td>207 ± 21.4</td>
</tr>
<tr>
<td>A2 – Slope Coefficient</td>
<td>0.050 ± 0.003</td>
<td>0.052 ± 0.003</td>
<td>0.058 ± 0.004</td>
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<tr>
<td>A3 – Curve Midpoint (mmHg)</td>
<td>140 ± 2.7</td>
<td>141 ± 2.5</td>
<td>136 ± 2.9</td>
</tr>
<tr>
<td>A4 – Max. NA (% baseline)</td>
<td>258 ± 19.7</td>
<td>264 ± 19.7</td>
<td>246 ± 21.2</td>
</tr>
<tr>
<td>Raw Max. NA (i/sec)</td>
<td>451 ± 57.4</td>
<td>465 ± 56.2</td>
<td>468 ± 57.2</td>
</tr>
<tr>
<td>Min. NA (% baseline)</td>
<td>29 ± 7.1</td>
<td>31 ± 7.0</td>
<td>39 ± 8.5</td>
</tr>
<tr>
<td>Max. Slope (%baseline/mmHg)</td>
<td>2.9 ± 0.30</td>
<td>3.0 ± 0.03</td>
<td>3.1 ± 0.41</td>
</tr>
<tr>
<td>NTP slope (% baseline/mmHg)</td>
<td>2.0 ± 0.28</td>
<td>2.3 ± 0.32</td>
<td>2.3 ± 0.29</td>
</tr>
<tr>
<td>PE slope (% baseline/mmHg)</td>
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<td>2.6 ± 0.33</td>
<td>2.8 ± 0.34</td>
</tr>
<tr>
<td>Raw Baseline NA (imp/sec)</td>
<td>195 ± 32.8</td>
<td>192 ± 33.6</td>
<td>193 ± 31.7</td>
</tr>
<tr>
<td>Baseline MAP (mmHg)</td>
<td>122 ± 2.9</td>
<td>120 ± 3.5</td>
<td>121 ± 3.5</td>
</tr>
</tbody>
</table>

virgin groups (P = 59 ± 9.9 bpm, V = 45 ± 7.4 bpm). Since baroreflex induced changes in HR were minimal in anesthetized rats, baroreflex HR responses were not evaluated further.
Table 2.3. *Effect of 3α-OH-DHP on baroreceptor afferent nerve discharge in pregnant rats.* Aortic depressor nerve discharge responses were obtained at control (C), 15 minutes following intravenous administration of 3α-OH-DHP (E1), and again 15 following a second administration of 3α-OH-DHP (E2). Comparison of measured parameters (A1-A4) demonstrated that 3α-OH-DHP had no effect on the baroreceptor afferent nerve discharge curve. Likewise, there was no effect of 3α-OH-DHP on raw maximum NA, minimum NA, slopes, or baseline parameters. Values = means ± SEM.

**Effect of 3α-OH-DHP**

The effect of exogenous administration of the primary metabolite of progesterone, 3α-OH-DHP, on baroreceptor discharge was evaluated in 12 virgin and 12 pregnant rats and the results are summarized in Tables 2.2 and 2.3.
Figure 2.3 Effect of 3α-OH-DHP on baroreceptor discharge curves in virgin rats: Mean baroreceptor discharge curves for 12 virgin rats are shown. Baroreceptor discharge curves in virgin rats at either 15 minutes (E1) or 30 minutes (E2) following administration of 3α-OH-DHP were no different from the control curve (C). MAP = mean arterial pressure; AONA = aortic nerve activity. ● = baseline MAP.
Figure 2.4 Effect of 3α-OH-DHP on baroreceptor discharge curve in pregnant rats: Mean baroreceptor discharge curves for 12 pregnant rats are shown. Similar to results observed in virgin animals, 3α-OH-DHP had no significant effect on the baroreceptor discharge curve in pregnant rats at either 15 minutes (E1) or 30 minutes (E2) following administration. C = control curve; MAP = mean arterial pressure; AONA = aortic nerve activity. • = baseline MAP.
Intravenous administration of 3α-OH-DHP to virgin animals had no effect on baseline MAP or baseline raw NA compared to control. No significant effect on the baroreceptor discharge curve coefficients (A1 - A4) was observed following administration of 3α-OH-DHP (Table 2.2, Figure 2.3). Likewise, the calculated parameters, minimum NA and maximum slope, were not significantly different from control. Analysis of the NTP and PE slopes showed that there was no effect of 3α-OH-DHP on sensitivities at either end of the curve (Table 2.2). Baseline HR and HR range were also unaffected by administration of 3α-OH-DHP (not shown).

The effect of exogenous 3α-OH-DHP administration in pregnant animals was also evaluated. As in the virgin group, no effect of 3α-OH-DHP on baseline MAP or baseline raw NA was observed. Baroreceptor discharge curve coefficients (A1-A4) following administration of 3α-OH-DHP, were not different from control (Table 2.3, Figure 2.4). Similarly, minimum NA, maximum slope, and NTP and PE slopes were not different (Table 2.3). Baseline HR and HR range were also unaffected by 3α-OH-DHP in pregnant animals (not shown).

These results indicate that, unlike the response of baroreflex efferents observed in previous studies in our laboratory (47, 80), 3α-OH-DHP has no effect on afferent discharge curves in either virgin or pregnant animals.

**DISCUSSION**

There are a number of cardiovascular adaptations that are associated with pregnancy including increases in blood volume, cardiac output, and heart rate and a
decrease in arterial pressure (29, 30, 52, 73). Similar to other reports, a relative decrease in baseline arterial pressure and tachycardia were observed in the pregnant rats in the current study. Among the adaptations to pregnancy are changes in arterial baroreflex function. Previous studies in our laboratory have demonstrated that the lower baseline blood pressure during pregnancy is associated with a decrease in the midpoint of the baroreflex function curve to a lower operating pressure range. In addition, sympathoexcitatory responses to decreases in arterial blood pressure are attenuated and an increased sympathoinhibition occurs at high blood pressures in pregnant compared to virgin rats (23, 47, 80). The effects of pregnancy on baroreflex function were verified in Inactin anesthetized rats in the current study.

The changes in baroreflex function observed during pregnancy may be due, in part, to the presence of elevated levels of the primary metabolite of progesterone, 3α-OH-DHP, the most potent positive modulator of central nervous system (CNS) GABA_A receptors. Previous studies evaluating the effect of 3α-OH-DHP on baroreflex function have demonstrated that acute intravenous administration of 3α-OH-DHP to virgin female rats attenuates efferent baroreflex sympathoexcitatory responses (47, 80). This response mimics an effect of pregnancy on baroreflex function and is consistent with a CNS mechanism of action, but could also be due to effects on afferent baroreceptor discharge. Thus, the current experiments were designed to evaluate the effects of pregnancy and 3α-OH-DHP on the afferent limb of the baroreflex.

The effect of pregnancy on the afferent limb of the baroreflex was evaluated by recording aortic nerve activity responses to changes in pressure. A significant decrease in
baseline mean arterial blood pressure (MAP) and a shift in the baroreceptor discharge curve midpoint to the left was observed in pregnant animals. However, the other curve parameters (baroreceptor discharge curve range (A1), slope coefficient (A2), maximum NA (A4), minimum NA) and responsiveness to hypotensive and hypertensive challenges (maximum slope, NTP slope, and PE slope) were not different. These results are consistent with a pressure-dependent hypotensive resetting of the baroreceptors in pregnant rats to a lower operating pressure range, whereby the baroreceptor function curve undergoes a parallel shift in the direction of the prevailing pressure with no change in sensitivity to increments in pressure (22, 46, 54). This leftward shift of the baroreceptor afferent nerve discharge curve likely contributes to the leftward shift of the baroreflex curve that occurs in pregnancy (23, 80). However, pressure dependent downward resetting of baroreceptors is characterized by a parallel leftward shift of the baroreceptor and baroreflex function curve along the pressure axis with no effect on sensitivity to increments in pressure (22, 46, 54). Thus, mechanisms other than pressure dependent baroreceptor resetting must account for the attenuated sympathoexcitatory and the potentiated sympathoinhibitory baroreflex responses seen in pregnant animals.

In contrast to the effects of 3α-OH-DHP on baroreflex control of efferent sympathetic outflow (47, 80), acute administration of 3α-OH-DHP to virgin rats did not have an effect on the baroreceptor afferent nerve discharge curve. Heart rate was also unaffected by the presence of 3α-OH-DHP. Similarly, administration of 3α-OH-DHP to pregnant rats did not affect either the baroreceptor afferent nerve discharge curve or HR parameters. These results indicate that 3α-OH-DHP does not have a direct effect on
baroreceptor discharge and that previous observations on the effect of intravenous 3α-OH-DHP on baroreflex function likely occur at the level of the CNS.

A CNS site of action for the effects of 3α-OH-DHP on baroreflex function are supported by other studies from our laboratory. Extracellular recordings were made from presumed pre-sympathetic, spinally projecting, baroreflex sensitive neurons in the rostral ventrolateral medulla. Intravenous administration of 3α-OH-DHP decreased the arterial pressure threshold for inhibition of these neurons (69), which is consistent with the potentiated baroreflex sympathoinhibition seen in pregnancy (23, 80). In addition, preliminary experiments demonstrated that local microinjection of 3α-OH-DHP into the rostral ventrolateral medulla resulted in attenuated baroreflex sympathoexcitatory responses (45).

PERSPECTIVES

Previous studies in our laboratory have demonstrated that pregnancy is associated with a decreased mean arterial pressure (MAP), a shift in the baroreflex function curve to a lower operating pressure range, potentiated baroreflex sympathoinhibition, and attenuated baroreflex sympathoexcitation (23, 47, 80). Acute intravenous administration of 3α-OH-DHP, the primary metabolite of progesterone and the most potent endogenous positive modulator of central nervous system GABA<sub>A</sub> receptor function (90, 93), attenuates efferent baroreflex sympathoexcitatory responses, similar to the effect of pregnancy. These results are consistent with increased GABAergic influence in CNS sites controlling sympathetic outflow in pregnant animals. Recent studies evaluating the effect of 3α-OH-DHP on the
rostral ventrolateral medulla, have demonstrated changes in neuronal discharge (69) and baroreflex function (45) consistent with effects observed during pregnancy (23, 47, 80). The current experiments were designed to evaluate the effects of pregnancy and 3α-OH-DHP on the afferent limb of the baroreflex. In this study a significant parallel leftward shift of the baroreceptor discharge curve to a lower operating pressure in pregnant animals was observed, which is consistent with a pressure dependent resetting. This downward resetting of the baroreceptor function curve likely contributes to the shift of the baroreflex curve to a lower operating pressure range in pregnant animals. Administration of 3α-OH-DHP, however, did not have an effect on the afferent nerve discharge in either virgin or pregnant animals. These results indicate that the previously observed effects of 3α-OH-DHP on the baroreflex are most likely due to a CNS mechanism of action and may contribute to the attenuated sympathoexcitation seen in pregnant compared to virgin animals.
CHAPTER 3

NEUROSTEROID MODULATION OF ARTERIAL BAROREFLEX SENSITIVE NEURONS IN ROSTRAL VENTROLATERAL MEDULLA OF THE RAT.

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Columbus, Ohio 43210-1218

ABSTRACT

The major metabolite of progesterone, 3α-OH-dihydroprogesterone (3α-OH-DHP), is the most potent endogenous positive modulator of central nervous system (CNS) GABA<sub>A</sub> receptors. Acute intravenous administration of 3α-OH-DHP to virgin female rats potentiates arterial baroreflex sympathoinhibitory responses. The current experiments tested the possibility that circulating 3α-OH-DHP potentiates central GABAergic influences in the rostral ventrolateral medulla (RVLM). The unit activity of spontaneously active, spinally projecting, and arterial pressure sensitive neurons was recorded in the RVLM of urethane anesthetized rats. Arterial pressure sensitivity of RVLM neurons was tested before (control) and 10 minutes after bolus injection (44μl,
i.v.) of 3α-OH-DHP (1.12 μg/Kg, n= 19) or vehicle (40% β-cyclodextrin, n=8). Both threshold pressure and saturation pressure for inhibition of RVLM neurons were decreased following acute administration of a physiological dose of 3α-OH-DHP (1.12 μg/Kg, i.v.), which produces plasma concentrations similar to those seen during pregnancy (20-30 ng/ml), suggesting potentiated responsiveness to endogenously released GABA. Following suppression by 3α-OH-DHP, high doses of the inactive stereoisomer, 3β-OH-DHP (112-224 μg/Kg, i.v.; n = 8), restored unit activity, presumably by displacing 3α-OH-DHP from the neurosteroid binding site on GABA\textsubscript{A} receptors.

INTRODUCTION

The rostral ventrolateral medulla (RVLM) is an essential component in the central nervous system (CNS) pathway for regulation of sympathetic outflow. Commonly accepted as the final site for tonic sympathoexcitatory drive to preganglionic sympathetic neurons in the intermediolateral cell column (IML) of the spinal cord, neurons in the RVLM receive and integrate cardiovascular input from multiple CNS sites (28). In particular, as the final step in the medullary baroreflex pathway, the RVLM receives direct GABAergic inhibitory input from the caudal ventral lateral medulla (CVLM) (25, 81). GABAergic influence on RVLM neurons represents the primary mechanism for arterial baroreflex inhibition of tonic drive to sympathetic preganglionic neurons in the IML.

Previous studies in our laboratory have demonstrated that pregnancy is associated with decreased mean arterial pressure (MAP), potentiated baroreflex sympathoinhibition

42
and attenuated baroreflex sympathoexcitation (23, 80). These results are consistent with an increased GABAergic influence in the RVLM of pregnant animals. Although the mediator for altered control of sympathetic outflow during pregnancy is not known, the ovarian hormones and/or their metabolites, particularly the primary metabolite of progesterone, 3α-OH-dihydroprogesterone (3α-OH-DHP), are likely candidates.

The progesterone metabolite, 3α-OH-DHP, is the most potent endogenous positive modulator of CNS GABA_A receptor function (90, 93). Plasma levels of 3α-OH-DHP are elevated during pregnancy to concentrations that have been demonstrated to potentiate GABA mediated inhibition in vitro (79). The mechanism of action of 3α-OH-DHP is thought to be nongenomic and due to immediate membrane effects produced by the binding of 3α-OH-DHP to a unique neurosteroid binding site on the GABA_A receptor complex (79, 93).

Previously our laboratory reported that acute administration of 3α-OH-DHP to virgin rats produced an attenuated baroreflex sympathoexcitation and potentiated baroreflex sympathoinhibition (47, 80), an effect which is qualitatively similar to the effects of pregnancy (23, 80). These results suggest a CNS mechanism. Thus, the purpose of the current study was to determine if circulating 3α-OH-DHP, in concentrations similar to those found in pregnancy, acutely altered the arterial pressure sensitivity of spinally projecting neurons in the RVLM to endogenously released GABA.
MATERIALS AND METHODS

Surgical Preparation

Experiments were performed in 21 virgin female Sprague Dawley rats (3-5 months old; Harlan Sprague-Dawley, Indianapolis, IN) weighing 225 - 270 g. Rats were anesthetized with intraperitoneal urethane (1.5 g/Kg) and supplemented (0.15 g/Kg, i.v.) as needed. A subcutaneous injection of dexamethasone (1.2 mg/Kg) was also given in order to limit nervous tissue swelling. The trachea was cannulated, and the rat artificially ventilated (CWE SAR830 Ventilator) with O₂ enriched room air. Body temperature was monitored and maintained at 37°C. An arterial catheter was placed into either the right carotid or right femoral artery to monitor arterial blood pressure and three jugular catheters were implanted for subsequent systemic drug administration. The rat was then placed in a stereotaxic apparatus and an occipital craniotomy performed. The occipital parietal membrane and dura were cut and folded laterally to expose the brainstem.

A laminectomy was performed to expose the spinal cord between C₂ and T₂, and the spinal cord was stabilized on the same plane as the brainstem by means of a rigid clamp on the dorsal vertebral process of T₂. The head was tilted forward until the calamus scriptorius was located 2.4 mm posterior to interaural zero (62). Tubocurarine (0.1 mg/Kg, i.v.) was administered to paralyze the rat, and a tungsten monopolar stimulating electrode (tip diameter 0.1 mm) was advanced into the dorsal lateral funiculus on the left side of the spinal cord at the level of C₂ (immediately medial to the dorsolateral sulcus and 0.3 mm ventral to the dorsal surface). This region contains descending axonal projections from the RVLM to spinal preganglionic sympathetic neurons in the IML (99). A pressor
response (20 - 40 mm Hg) during brief electrical stimulation of the spinal cord (5 mA, <1 ms, 5 Hz) verified the location of the electrode tip in the dorsolateral funiculus.

**Drugs and Solution**

Urethane ethyl carbamate (99%) and phenylephrine (PE) were purchased from Sigma Chemical Co, (St. Louis, MO). Tubocurarine chloride was obtained from Bristol Myers Squibb (Princeton, NJ). The urethane, tubocurarine, and PE were each diluted in isotonic saline. Dexamethasone sodium phosphate was purchased from Steris Laboratories (Phoenix, AZ). 2-Hydroxypropyl-β-cyclodextrin was purchased from Research Biochemicals International (Natick, MA) and dissolved in a 50:50 solution of distilled water and isotonic saline to make 40% β-cyclodextrin. The progesterone metabolites, 5α-pregnan-3α-ol-20-one (3α-OH-dihydroprogesterone, 3α-OH-DHP) and 5α-pregnan-3β-ol-20-one (3β-OH-dihydroprogesterone, 3β-OH-DHP), also obtained from Sigma Chemical Co. (St. Louis, MO), were dissolved in 40% β-cyclodextrin. Chicago Sky Blue 6B 80% was obtained from Aldrich (Milwaukee, WI), and neutral red was purchased from National Diagnostics (Highland Park, NJ).

**Single Unit Recordings**

Extracellular unit recording from cells in the RVLM was performed using glass microelectrodes (outer tip diameter ≈ 1 μm; resistance 1-2 MΩ) filled with 1% Chicago Sky Blue dye dissolved in 1M NaCl. The electrode was advanced into the left side of the brainstem using a hydraulic microdrive (David Kopf). Spontaneously active neurons
within anterioposterior coordinates of 0.5 - 0.8 mm rostral to calamus scriptorius, 1.7 - 2.2 mm lateral to midline, and 2.7 - 3.8 mm ventral to the dorsal surface were identified. The signal was amplified 10,000 times using two Grass P15 preamplifiers and monitored on a loudspeaker as well as on a dual beam storage oscilloscope (Tektroniks, R5031). A rate meter/window discriminator (Winston Electronics, RAD-IIA) was used to determine the firing frequency of the unit. Unit activity (UA), heart rate (HR), and MAP were monitored on a polygraph (79D, Grass Instruments) and stored on videotape (DR-886, Neuro Data Instrument Corp.) for later analysis using data acquisition software (Computerscope, RC Electronics).

Identification of spontaneously firing units as spinally projecting neurons involved in the regulation of cardiovascular function was determined by means of two different tests. First, spontaneous units were tested for antidromic spike production in response to electrical stimulation of the dorsal lateral funiculus in the spinal cord (0.5 Hz, 5 mA, < 1 ms). Neurons demonstrating a constant latency from the stimulus to the evoked spike and observation of collision with a spontaneous action potential suggested that the neuron projected to the spinal cord (Fig. 3.1). During antidromic activation of spontaneously active neurons, slight variability in latencies may occur due to the effect of different levels of membrane polarization on the delay between antidromic invasion of the initial segment and the soma-dendritic region. Therefore, neurons with latency variation of less than 0.2 ms during repeated spinal cord stimuli were considered antidromically activated (74, 107). Second, the baroreflex sensitivity of the cell was tested. Slow ramp increases in MAP were elicited by graded intravenous infusions (Razel infusion pump) of PE (9 - 300
µg/min.) while monitoring the UA of the neuron. Arterial baroreceptors are rate sensitive and thus, for a given cell, care was taken to ensure that the rate of ramp increases in MAP was similar each time pressure sensitivity was tested. A progressive decrease in UA as MAP was elevated indicated arterial baroreflex sensitivity of the cell. Neurons that were both antidromically activated by dorsal lateral funiculus stimulation and greatly inhibited by elevations in MAP were presumed to be presympathetic cardiovascular neurons of the RVLM (39) and were included in this study.

**Experimental Protocol**

The relationship between MAP and UA was determined in RVLM neurons that met the above criteria during a gradual increase in MAP (control response). After MAP and UA returned to baseline values, a bolus intravenous injection of 3α-OH-DHP (1.12 µg/Kg, n=19) was administered. This dose was estimated to produce a maximum plasma concentration (≈ 22 ng/ml) within the reported range of plasma concentrations seen during pregnancy (20-30 ng/ml) (93). Ten minutes following drug administration, the pressure sensitivity of the neuron was retested. The effect of vehicle (40% β-cyclodextrin, i.v.) was evaluated in eight experiments using a similar protocol, except that an equivalent volume of 40% β-cyclodextrin (44 µl), instead of 3α-OH-DHP, was administered.

Preliminary experiments evaluating the effects of a higher dose of 3α-OH-DHP were also performed. In seven cells that had received 1.12 µg/Kg 3α-OH-DHP (i.v.), the effect of subsequent administration of a higher dose of 3α-OH-DHP (11.2 µg/Kg, i.v.) was also tested.
The response of identified RVLM neurons to elevation of MAP was quantified by four parameters: MAP and UA values recorded at threshold and at saturation. After the experiments, taped data of MAP and instantaneous unit discharge were digitized (RC Electronics Computerscope) and 5 msec averages were obtained (Microsoft Excel; Seattle, WA). Maximum and minimum UA were identified during a 1 min period immediately preceding the PE-induced pressure ramp. During a slow pressure ramp (≈ 5 mm Hg/sec), the pressure at which action potential firing frequency decreased below minimum baseline firing rate, and continued to decrease as pressure increased, was defined in the current study as the threshold pressure for inhibition of the unit. This method of threshold determination served to standardize measurements both within and between animals. However, it should be noted that this experimentally defined threshold is most likely higher than the physiological threshold of RVLM neurons. The pressure at which the neuron ceased firing or reached a minimum firing rate was defined as the saturation pressure (Fig. 3.3). The rate of recovery for MAP and UA was also evaluated in this study by measuring four parameters. The half time for MAP recovery (t ½ MAP) and the half time for recovery of UA (t ½ UA) were both determined. Additionally, values for UA at t ½ MAP and the MAP at t ½ UA were obtained (Fig 3.3).

The relatively long half-life of 3α-OH-DHP (~ 1 hour in humans) (17) prohibited reliable assessment of recovery from the drug effect. However, high concentrations of 3β-OH-DHP have been shown to compete with 3α-OH-DHP for binding sites on the GABA_A receptor complex in vitro (95). In some experiments (n = 8) it was noted that UA did not completely return to pre-3α-OH-DHP baseline levels after the last pressure ramp. In these
experiments (1.12 μg/Kg 3α-OH-DHP, n=5; 11.2 μg/Kg 3α-OH-DHP, n=3), a high concentration of the inactive stereoisomer, 3β-OH-DHP (112 - 224 μg/Kg, i.v.), was administered to test for reversal of the effects of 3α-OH-DHP. MAP and UA prior to and after administration of 3β-OH-DHP were compared.

Once a neuron was accepted for inclusion in the study, the experimental protocol lasted approximately 30-60 minutes. A neuronal recording was considered stable only if shape and height of the spike were consistent during the entire recording period. At the end of the experiment, the location of the tip of the recording electrode in the brain stem was marked by ejecting Chicago Sky Blue dye from the pipette by iontophoresis (-25 μA for 20 min). Standard histological techniques were used to fix and section the brainstem (50μm sections, neutral red stain). The recording site was estimated by comparison with a rat brain atlas (94).

**Statistical Analysis**

A paired t-test was used to compare control and treatment values. Data obtained in cells that received more than one dose of 3α-OH-DHP were analyzed using a one way ANOVA for repeated measures followed by Student Newman Keuls post hoc test. P < 0.05 was considered significant. Values are means ± SEM.
RESULTS

Identification and Characterization of Presympathetic RVLM Pressure Sensitive Neurons.

For inclusion in the study, spontaneously firing neurons recorded in the RVLM were tested for antidromic activation that would indicate that these neurons were spinally projecting neurons. Figure 3.1 shows sequential oscilloscope traces of an RVLM unit that was antidromically activated by electrical stimulation of the dorsal lateral funiculus in the spinal cord (0.5 Hz, 5 mA, 0.5 ms). A constant latency between the stimulus artifact and the evoked action potential (Fig. 3.1, A-C) and observation of collision of the stimulus with a spontaneous action potential (Fig. 3.1, D) suggests that the neuron projected directly to the spinal cord. Baroreflex sensitivity of each neuron was also tested. Substantial inhibition of a neuron in response to increased MAP suggested that the neuron was part of the central baroreflex pathway.

Although many spontaneously firing neurons were recorded in the area of the RVLM, protocols were performed only on those neurons meeting the criteria described above. A total of 22 spontaneously active neurons were both antidromically activated from the dorsal lateral funiculus in the spinal cord and inhibited by elevations in MAP. Elevated arterial pressure resulted in complete cessation of unit discharge in 15 neurons and discharge was inhibited to $35.9 \pm 9.0\%$ of baseline in the remaining 7 neurons. These neurons were assumed likely to be presympathetic RVLM neurons involved in the baroreflex regulation of sympathetic outflow. Antidromic latencies ranging between 2 to 13 msec (mean: $6.15 \pm 0.8$ msec) were observed in these neurons. Calculated conduction
Figure 3.1. Identification of a spinally projecting RVLM neuron: Antidromic evoked potentials in an RVLM neuron due to electrical stimulation of the ipsilateral spinal dorsal lateral column. Stimulus artifact is seen on far left. A-C demonstrate a constant latency for evoked potential, which is indicative of direct connection between RVLM neuron and the spinal cord. Latency for this neuron was 4.92 ms. Collision, shown in D, occurred when a spontaneous action potential (*) rendered the neuron refractory.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Baseline MAP (mm Hg)</th>
<th>Baseline UA (pps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>93.07 ± 7.5</td>
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</tr>
<tr>
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<tr>
<td>Control</td>
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<td>94.17 ± 2.8</td>
<td>13.64 ± 2.0</td>
</tr>
<tr>
<td>3α-OH-DHP (1.12 μg/Kg)</td>
<td>19</td>
<td>93.11 ± 3.4</td>
<td>13.01 ± 2.3</td>
</tr>
</tbody>
</table>

Table 3.1. Effects of treatments on baseline mean arterial pressure (MAP) and unit activity (UA): Values are mean ± SEM. Neither baseline MAP nor baseline UA were significantly affected by i.v. administration of vehicle (40% β-cyclodextrin) or 3α-OH-DHP. pps, pulses per second.

Velocities, assuming a linear conduction pathway and an estimated distance of 2.5 cm between stimulus and recording site, ranged from 1.92 to 12.5 m/sec (mean: 5.9 ± 0.80 m/sec). Baseline firing frequencies of identified neurons ranged between 3.2 to 32.4 pulses per second (pps). Of the 22 cells studied, pulse synchronous activity was evident in 11 cells (Figure 3.2). Although not stringently evaluated, several of the neurons appeared to also demonstrate a respiratory-like rhythm (n=9).

Figure 3.3 shows a typical response of an RVLM neuron to a ramp increase in MAP. Parameters used to characterize responses are indicated on the figure.
Figure 3.2. Pulse synchronous discharge of an RVLM neuron: Approximately 60 superimposed traces are shown. Action potentials are correlated with early diastolic phase of arterial pressure pulse. UA = unit activity; AP = arterial pressure.
Figure 3.3. Identification of response and recovery parameters: Ramp increase in mean arterial pressure (MAP) produced inhibition of unit activity (UA) of a spinally projecting RVLM neuron. MAP and UA at threshold (T), point at which firing frequency decreased below baseline, and at saturation (S), point at which cell discharge reached minimum frequency, were measured. Recovery parameters were obtained after removal of phenylephrine stimulus. Point at which MAP and UA were half recovered (*) was determined, and half time to recovery for MAP (t ½ MAP) and UA (t ½ UA) was calculated. pps = pulses per second.
**Effect of 3α-OH-DHP on Baseline, Threshold, and Saturation Parameters.**

The effects of vehicle and 3α-OH-DHP on presumed presympathetic, baroreflex sensitive RVLM neurons were evaluated, and the results are summarized in Tables 3.1 and 3.2. Baseline values of MAP and UA measured immediately before the pressure ramps were not affected by either vehicle or 3α-OH-DHP (1.12 μg/Kg, i.v., Table 3.1). Vehicle alone did not have an effect on threshold or saturation values in the eight neurons tested (Table 3.2, Fig 3.4). Responses to intravenous administration of 3α-OH-DHP (1.12 μg/Kg, i.v.) were determined in a total of 19 neurons. The maximum plasma concentration that could be achieved with this dose (∼22 ng/ml) was calculated to be within the range of concentrations seen in pregnancy (20 - 30 ng/ml) (93). Both the threshold MAP for inhibition of the unit and the saturation MAP were decreased by 3α-OH-DHP (1.12 μg/Kg, i.v.), indicating an increased sensitivity of RVLM neurons to increases in arterial blood pressure. In five of these neurons, responses to intravenous administration of vehicle (44 μl, 40% β-cyclodextrin) had been tested before 3α-OH-DHP. Statistical analysis of the data with and without these five neurons revealed the same significant differences and therefore data from all 19 neurons are shown (Table 3.2, Fig. 3.5).

Preliminary data evaluating the effect of subsequent administration of a higher dose of 3α-OH-DHP (11.2 μg/Kg) was obtained in 7 of these 19 cells. Repeated measures ANOVA on the subset of seven neurons exposed to both 1.12 μg/Kg and 11.2 μg/Kg 3α-OH-DHP revealed that subsequent administration of the higher dose of 3α-OH-DHP did
Figure 3.4. Effects of vehicle: Administration of vehicle (40% β-cyclodextrin) had no effect on baseline mean arterial pressure (MAP), threshold pressure, or saturation pressure of RVLM neurons (n=8). Bars = values ± SEM.
Table 3.2. Effects of treatments on threshold and saturation parameters: Values are mean ± SEM. Administration of the primary dose of 3α-OH-DHP (1.12 pg/Kg) used in this study produced significant decreases in MAP at both threshold and saturation. Vehicle alone had no effect. (* P < 0.05)

<table>
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<tr>
<th></th>
<th>n</th>
<th>Threshold MAP</th>
<th>UA @ Threshold</th>
<th>Saturation MAP</th>
<th>UA @ Saturation</th>
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<td>Control</td>
<td>8</td>
<td>120.9 ± 7.7</td>
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<td>3α-OH-DHP (1.12 μg/Kg)</td>
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<td>117.2 ± 5.2*</td>
<td>11.39 ± 2.1</td>
<td>146.0 ± 5.4*</td>
<td>1.35 ± 0.6</td>
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</tbody>
</table>

not produce any further decrease in either threshold MAP (117 ± 6.1 mm Hg) or saturation MAP (152 ± 4.9 mm Hg).

Effect of 3α-OH-DHP on Recovery Parameters.

Recovery parameters for MAP and UA following the ramp increase in arterial pressure are summarized in Tables 3.3 and 3.4. Because of technical limitations or a prolonged time for recovery (> 10 minutes), it was not possible to quantitate recovery parameters in all neurons. Recovery after PE induced elevations in pressure was evaluated in 15 of 19 neurons exposed to 3α-OH-DHP (1.12 μg/Kg). The t ½ MAP (Table 3.3) and t ½ UA (Table 3.4) were unaffected by vehicle or 3α-OH-DHP (1.12 μg/Kg), indicating similar rates of recovery for both blood pressure and UA between control and treatment responses in these groups.
Table 3.3. Recovery parameters for mean arterial pressure (MAP): Values are mean ± SEM. Neither vehicle nor 3α-OH-DHP at a physiological dose affected half time for recovery of MAP (t ½) or unit activity (UA) at t ½ MAP.

Recovery parameters were obtained for five of seven neurons exposed to the highest dose of 3α-OH-DHP (11.2 μg/Kg, i.v.) after administration of the physiological dose of 3α-OH-DHP (1.12 μg/Kg, i.v.). Repeated measures ANOVA on control, 1.12 μg/Kg, and 11.2 μg/Kg 3α-OH-DHP values in these five cells revealed that t ½ UA was increased (C = 84.2 ± 41.2; After 3α-OH-DHP (11.2 μg/Kg) = 136.5 ± 55.5 sec), and MAP at t ½ UA was less (Control = 117.8 ± 7.8; After 3α-OH-DHP (11.2 μg/Kg) = 105.3 ± 4.0 mm Hg), at the highest dose of 3α-OH-DHP. Therefore, in the presence of 11.2 μg/Kg 3α-OH-DHP, recovery of UA was prolonged and thus MAP was lower at t ½ UA.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>t ½ MAP (sec)</th>
<th>UA @ t ½ MAP (pps)</th>
<th>UA @ t ½ MAP (%baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>70.73 ± 11.6</td>
<td>3.69 ± 1.2</td>
<td>32.0 ± 11.9</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>74.93 ± 11.9</td>
<td>4.33 ± 1.2</td>
<td>40.5 ± 9.4</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>50.08 ± 8.1</td>
<td>3.26 ± 0.8</td>
<td>35.8 ± 8.1</td>
</tr>
<tr>
<td>3α-OH-DHP (1.12 μg/Kg)</td>
<td>15</td>
<td>62.39 ± 19.4</td>
<td>3.58 ± 1.4</td>
<td>43.73 ± 10.9</td>
</tr>
</tbody>
</table>
Table 3.4. Recovery parameters for unit activity (UA): Values are mean ± SEM. Neither vehicle nor 3α-OH-DHP (1.12 μg/Kg) affected t ½ UA.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>t ½ UA (sec)</th>
<th>MAP @ t ½ UA (mm Hg)</th>
<th>MAP @ t ½ UA (% baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>65.68 ± 12.3</td>
<td>129.93 ± 8.5</td>
<td>140.1 ± 10.5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>65.24 ± 18.72</td>
<td>125.89 ± 8.6</td>
<td>138.3 ± 11.7</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>107.69 ± 20.6</td>
<td>118.27 ± 4.6</td>
<td>121.7 ± 4.0</td>
</tr>
<tr>
<td>3α-OH-DHP (1.12 μg/Kg)</td>
<td>15</td>
<td>98.18 ± 26.9</td>
<td>115.22 ± 5.1</td>
<td>123.3 ± 4.3</td>
</tr>
</tbody>
</table>

**Effect of 3β-OH-DHP**

Baseline values of MAP and UA obtained before (control) and 10 minutes after administration of 1.12 μg/Kg 3α-OH-DHP (i.v.) were not different (Table 3.1). During the experiment, final MAP and UA values were also noted following the last PE-induced pressure ramp in the presence of 3α-OH-DHP.

The effect of administration of a high concentration of the inactive stereoisomer, 3β-OH-DHP, was evaluated in eight neurons that, at the time of the experiment, did not appear to fully recover following the final pressure ramp in the presence of 3α-OH-DHP (1.12 μg/Kg, n=5; 11.2 μg/Kg, n=3). MAP and UA for these eight neurons, were compared immediately before and 1 min after bolus administration of high concentrations
Table 3.5. Effect of 3β-OH-DHP on MAP and UA: Values are means ± SEM. In a subset of neurons that were inhibited by 3α-OH-DHP, administration of the stereoisomer 3β-OH-DHP (maximum dose of 224 ng/kg) produced a significant increase in MAP and UA (*p<0.05).

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Baseline MAP (mm Hg)</th>
<th>Baseline UA (pps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 3β-OH-DHP</td>
<td>8</td>
<td>77.7 ± 7.09</td>
<td>7.7 ± 2.41</td>
</tr>
<tr>
<td>After 3β-OH-DHP</td>
<td>8</td>
<td>83.5 ± 7.16 *</td>
<td>10.5 ± 3.14 *</td>
</tr>
</tbody>
</table>

of 3β-OH-DHP (112-224 μg/Kg). Despite a slight but significant increase in baseline MAP following administration of 3β-OH-DHP, a significant increase in UA was observed (Table 3.5). This is consistent with reversal of the effects of 3α-OH-DHP due to competition at the binding site by the inactive stereoisomer, 3β-OH-DHP.

**Histology**

Post hoc histological examination of the recording sites verified that the neurons were distributed within an area previously described as the RVLM (Fig 3.6) (10, 36, 39, 116).

**DISCUSSION**

The RVLM is an integral component in the central pathway for control of cardiovascular function and is considered to be the final site for tonic sympathoexcitatory
Figure 3.5. Effects of 3α-OH-DHP: There was no significant effect of 3α-OH-DHP (1.12 μg/Kg i.v., n=19) on baseline (MAP). However, 3α-OH-DHP significantly decreased threshold pressure and saturation pressure of RVLM neurons. This indicates that 3α-OH-DHP had a potentiating effect on baroreflex mediated sympathoinhibition. Bars = values ± SEM; * p ≤ 0.05.
Figure 3.6. Post hoc histochemical analysis of recording sites: Extracellular recording sites were marked with 1% Chicago Sky Blue dye in 1 M NaCl at the end of each experiment by passing a 25 μA cathodal current through the electrode for 20 minutes. Serial sections through brainstem at the level of the RVLM show that neurons from which recordings were taken were indeed located in the area of the RVLM. ●, Recording sites (21 neurons). Sol, nucleus of the solitary tract; Cu, cuneate nucleus; Ecu, external cuneate nucleus; IO, inferior olive; Amb, ambiguus nucleus; Sp, spinal trigeminal tract.
drive to preganglionic sympathetic neurons in the spinal cord (39). Neurons in the RVLM receive and integrate cardiovascular input from both supra-medullary and medullary nuclei (28). When arterial pressure increases, the medullary baroreflex pathway is activated. Increased discharge of afferent fibers from arterial baroreceptors results in excitation of neurons in the nucleus tractus solitarius (NTS), followed by excitation of neurons in the CVLM (28, 39). A monosynaptic projection from the CVLM to the RVLM (25, 81) inhibits tonically active neurons in the RVLM, which results in decreased discharge of preganglionic sympathetic neurons in the IML of the spinal cord (57). Inhibition of neurons in the RVLM by the CVLM is mediated by release of the amino acid neurotransmitter GABA (111). GABAergic influences represent the primary mechanism for arterial baroreflex inhibition of tonic sympathetic drive.

Earlier studies in our laboratory, evaluating the effects of pregnancy on baroreflex function showed that pregnancy potentiated sympathoinhibitory and attenuated sympathoexcitatory baroreflex responses (23, 80). These effects are consistent with an increased GABAergic inhibition in the RVLM of pregnant animals. The mediator for these pregnancy-associated adaptations in control of sympathetic outflow is not known, but likely candidates are the ovarian hormones and/or their metabolites which are elevated during pregnancy. It has been recently demonstrated that the primary metabolite of progesterone, 3α-OH-DHP, is the most potent endogenous positive modulator of CNS GABA_A receptor function (90, 93). Plasma concentrations of 3α-OH-DHP are elevated during pregnancy to levels which have been demonstrated to potentiate GABA mediated inhibition (79, 93).
3α-OH-DHP belongs to a class of compounds known as neurosteroids whose primary action appears to be positive modulation of GABA<sub>A</sub> receptor function. The mechanism of action is not thought to be genomic, because the effects are rapid (seconds to minutes) (79) and inhibition of protein synthesis does not alter the effect of neurosteroids (93). The neurosteroids bind to a unique and stereospecific site on the GABA<sub>A</sub> receptor complex. Administered in 10-30 nM concentrations, neurosteroids are potent modulators of GABA<sub>A</sub> receptor function, prolonging the duration of chloride channel opening (79). Neurosteroids have been shown to produce an increased duration of inhibitory postsynaptic currents in hippocampal neurons (40). At higher concentrations (μM range) neurosteroids have been shown to directly open the chloride channel (79).

Previous studies in our laboratory evaluated the effect of acute administration of 3α-OH-DHP on baroreflex function in both anesthetized (47) and conscious rats (80). Acute administration of 3α-OH-DHP to virgin rats resulted in attenuated sympathoexcitatory responses and potentiated sympathoinhibitory responses. In other words, the acute response to exogenously administered 3α-OH-DHP in virgin rats was qualitatively similar to the effects of pregnancy. Although a CNS mechanism was implied by these previous studies, direct evidence was not provided. The purpose of this study was to determine if circulating levels of 3α-OH-DHP, administered in concentrations similar to those found in pregnancy, altered the sensitivity of sympathoexcitatory neurons in the RVLM, to endogenously released GABA.
Characterization of presympathetic RVLM cardiovascular neurons

In the current study, spinally projecting spontaneously firing neurons in the RVLM which were inhibited by elevations in arterial pressure, were presumed to be presympathetic neurons. Baseline unit activity (UA) of RVLM neurons included in this study also exhibited a wide range of firing frequencies, varying between 3.2 and 32.4 pps, which is consistent with the baseline firing rates reported by others (0.5 - 40 pps) (2, 3, 14, 36). Antidromic latencies between 2 and 13 msec (mean: 6.15 ± 0.8 msec) and conduction velocities between 1.9 and 12.5 m/sec were observed in this study. Conduction velocities ranging between 0.4 and 11 m/sec have been reported (3, 14, 36, 58, 76, 86). The wide distribution of conduction velocities reported in the literature suggests involvement of both myelinated (> 1 m/sec) and unmyelinated (< 1 m/sec) fiber types. None of the neurons included in this study had conduction velocities < 1 m/sec, indicating that the axons of neurons in this study were probably myelinated. The mean conduction velocity of neurons included in this study (5.9 ± 0.8 m/sec) is similar to that reported for presympathetic RVLM neurons by Granata and Kitai (5.5 ± 2.6 m/sec), Kanjhan et al (4.9 ± 2.7 m/sec), and Lipski et al (5.2 ± 2.3 m/sec) (36, 58, 76). Mean conduction velocities reported by Morrison et al are somewhat lower (3.1 ± 0.1 m/sec) (86). However, almost one-half the RVLM neurons characterized in that study were silent (49%) (86). Compared to conduction velocities of silent RVLM neurons, the conduction velocities of spontaneously active neurons tend to be higher (86). In our experiments, only spontaneously active RVLM neurons were studied, and thus we may have selected for neurons with myelinated axons and higher conduction velocities. Additionally, the use of relatively low resistance
electrodes (1-2 MΩ) in the current experiments would bias the recording toward larger cells with myelinated axons.

Although not used as a criterion in the positive identification of RVLM neurons, a correlation between neuronal firing pattern and cardiac cycle was observed in many units included in the study. As has been reported by others (14, 58), correlation between the cardiac cycle and unit discharge was evident primarily at elevated MAP, especially in those units exhibiting high baseline firing frequencies. Pulse synchrony was defined as a pattern of discharge that correlated with no more than one half the cardiac cycle. With use of this criterion, evidence of pulse synchronous discharge was observed in 11 of 22 neurons. The number of pulse synchronous neurons may have been underestimated in this study. Because of the nature of the protocol, arterial pressure was not elevated for a prolonged period of time which would have been necessary for definitive characterization of a neuron as pulse synchronous. It is likely that had enough data at high pressures been obtained, more neurons would have been characterized as pulse synchronous.

Although a direct correlation between UA and respiratory activity was not possible in this study because phrenic nerve activity was not recorded, a respiratory-like rhythm was noted in nine identified RVLM baroreflex sensitive neurons. Of the nine neurons, five were additionally found to exhibit a pulse synchronous pattern which was unmasked at high MAP. Recent reports have demonstrated an effect of central respiratory drive on the baroreflex at the level of the RVLM. Brown and Guyenet (14) demonstrated that spinally projecting barosensitive RVLM cells showing acute sensitivity to plasma CO₂ levels have a prominent respiratory related rhythm. A direct correlation between phrenic nerve
discharge and UA of baroreflex sensitive RVLM neurons has been shown by Miyawaki et al (85). However, Granata and Kitai (36) reported that antidromically activated RVLM neurons with respiratory related activity demonstrated no baroreceptor modulated activity. This apparent inconsistency may be due to the differences in recording sites between the studies. Compared to the study by Granata and Kitai (36) baroreflex sensitive RVLM neurons with respiratory related rhythm in the study by Miyawaki (85) were located more dorsally in the RVLM. Although a respiratory modulation was evident in approximately one third of the neurons in the current experiments, these neurons did not exhibit the characteristic on-off firing pattern of respiratory neurons and were greatly inhibited by elevated arterial pressure, suggesting that they were primarily involved in the arterial baroreflex (14, 58).

Post hoc histological verification of the recording sites for 21 neurons recorded in this study were obtained and revealed that the neurons were within the area described in the literature as the RVLM (39, 58, 85). In the current study only 3 of the 21 neurons were located within 400 μm of the ventral surface. The majority of recorded neurons were located more dorsally (Fig. 6) and this may account for the relatively large number of neurons exhibiting respiratory like rhythm. This distribution is consistent with reports by Brown and Guyenet (14) and Miyawaki et al (85) demonstrating that presynaptic neurons 600-700 μm dorsal to the ventral surface of the medulla and 300-400 μm caudal to the caudal tip of the facial nucleus show a prominent respiratory rhythm.

The large number of dorsally located neurons may be a peculiarity of the experimental protocol of the current study. The recording electrode was advanced from

67
the dorsal surface of the brain stem, and as soon as a spinally projecting pressure sensitive neuron was isolated, the experiment was begun. Thus, more ventral sites were frequently not explored.

**Effect of 3α-OH-DHP on identified RVLM neurons**

The majority of results in this study were obtained at a physiological dose of 3α-OH-DHP (1.12 μg/Kg, i.v.). This dose of 3α-OH-DHP was chosen to produce circulating levels of 3α-OH-DHP comparable to those seen during pregnancy. Maximal plasma concentrations achieved at this dose were calculated to be ≈ 22 ng/ml, and normal plasma concentrations of 3α-OH-DHP during pregnancy are 20-30 ng/ml (93). At this dose, acute administration of 3α-OH-DHP produced a significant decrease in both threshold MAP and saturation MAP. This decrease in threshold and saturation pressures is consistent with an increased sensitivity of identified RVLM neurons to endogenously released GABA. The effects of 3α-OH-DHP were subtle (approximately 10 % change) as might be expected with a substance that modulates the response to an endogenous transmitter.

Preliminary studies in which a higher dose of 3α-OH-DHP (11.2 μg/Kg, n=9) was administered revealed no further effect on threshold or saturation, indicating that near maximal effects of 3α-OH-DHP are seen at physiologically relevant circulating levels.
**Effect of 3α-OH-DHP on recovery parameters of RVLM neurons**

3α-OH-DHP was also found to have an effect on recovery after inhibition in response to elevations in pressure. Half time to recovery for MAP was not different between control and treatment for any of the groups (vehicle or 3α-OH-DHP). This indicates that, once the PE stimulus was removed, MAP recovered at the same rate, thus eliminating the possibly confounding factor of different MAP recovery rates, which could affect recovery of the neuron. An effect of 3α-OH-DHP on recovery of UA was observed only in the presence of the highest dose of 3α-OH-DHP (11.2 μg/Kg, i.v.; n= 5) used in this study. At this dose, the t½ UA was significantly prolonged (Control = 84.2 ± 41.2; After 3α-OH-DHP (11.2 μg/Kg) = 136.5 ± 55.5 sec). Also, as expected with a longer time period over which to recover, MAP at t ½ UA was significantly lower (Control = 117.8 ± 7.8; After 3α-OH-DHP (11.2 μg/Kg) = 105.3 ± 4.0 mm Hg). This effect on recovery is consistent with positive modulation of GABA<sub>A</sub> receptors by 3α-OH-DHP.

For a given level of GABA present, inhibition of neurons would be greater in the presence of 3α-OH-DHP compared with control. In the presence of 3α-OH-DHP, actual levels of endogenously released GABA would have to decrease further before UA could recover, and thus time to recovery would be prolonged.

Although plasma concentrations achieved after administration of the highest dose of 3α-OH-DHP (11.2 μg/Kg, i.v.) likely exceed plasma levels during pregnancy, the results are still potentially significant. The enzymes responsible for converting progesterone to neuroactive metabolites are located both peripherally and within the CNS. Both circulating and centrally synthesized progesterone are converted to 3α-OH-DHP in
the brain, and CNS concentrations of 3α-OH-DHP may exceed plasma concentrations by 100 fold (97). Thus, acute intravenous administration of the higher dose of 3α-OH-DHP in the current experiments would result in acute exposure of the brain to concentrations that might well be within the physiologically relevant range for the central nervous system.

Effect of 3β-OH-DHP on RVLM neurons

Currently, specific antagonists for the neurosteroid binding site are not available. However, high concentrations of the inactive stereoisomer, 3β-OH-DHP, have been shown to compete with 3α-OH-DHP for binding sites and thereby reverse the positive modulation of GABA_A receptors by 3α-OH-DHP (95). In the current experiments, any modulatory effect of 3α-OH-DHP on baseline firing rate would be most evident after a manipulation whereby endogenous GABA is elevated (i.e. after increased MAP). In 8 of 19 RVLM neurons, incomplete recovery of both MAP and UA was observed after the final pressure ramp in the presence of 3α-OH-DHP (11.2 μg/Kg, n = 3; 1.12 μg/Kg, n = 5) suggesting an inhibitory effect. In these neurons, the effect of the inactive stereoisomer 3β-OH-DHP was evaluated. The relatively high dose of 3β-OH-DHP (112-224 μg/Kg) used in this study was chosen in an effort to produce maximal competition at the neurosteroid binding site on GABA_A receptors. Baseline levels of MAP increased slightly with administration of 3β-OH-DHP. However, despite the slight increase in pressure, 3β-OH-DHP produced a significant increase in UA within 1 min of administration, indicating reversal of the inhibitory effect of 3α-OH-DHP. Additionally, the rapid effect of 3β-OH-DHP further suggests that the mechanism of action of the neuroactive metabolite of
progesterone, 3α-OH-DHP, was through a stereospecific nongenomic action at a unique binding site on the GABA$_A$ receptor complex.

Although the results of this study show that 3α-OH-DHP, administered to achieve plasma concentrations similar to those seen in pregnancy, has an effect on neurons in the RVLM, it should be recognized that the actual site of action of 3α-OH-DHP remains uncertain. 3α-OH-DHP is a highly lipid soluble molecule and therefore has access to all CNS sites following intravenous administration. GABAergic influences have been demonstrated in other medullary nuclei in the baroreflex pathway, including the NTS and the CVLM (28). However, potentiation of GABAergic responses in the NTS or the CVLM might be expected to produce sympathoexcitation and attenuation of baroreflex sympathoinhibition (39), an opposite effect from that observed in both this and previous baroreflex studies (47, 62, 80). As the final site for sympathoinhibitory influences, the RVLM is the most likely site in the medullary baroreflex pathway where an increase in GABAergic influences would produce a potentiation of sympathoinhibition.

One potential mechanism for the preferential effect of 3α-OH-DHP in the RVLM, would be a greater affinity for 3α-OH-DHP by RVLM neurons compared to other regions involved in the central baroreflex pathway. Affinity of 3α-OH-DHP for the GABA$_A$ receptor is dependent on the subunit composition of the receptor. Studies have demonstrated that although the β subunit of the GABA$_A$ receptor complex has no effect on modulation of GABA induced chloride current, different α and γ subunit isoforms (41, 70) may significantly affect the efficacy of 3α-OH-DHP to modulate GABA$_A$ receptor binding and function (93). Although it has not been determined in the medulla,
heterogeneity of GABA\textsubscript{A} receptors in other areas of the CNS has been proposed to account for regional differences in neurosteroid responsiveness (93). Thus, it is possible that GABA\textsubscript{A} receptors are distributed such that more receptors in the RVLM contain the appropriate subunits to maximize modulation of the GABA\textsubscript{A} receptor complex by 3\alpha-OH-DHP.

Lastly, although this study demonstrated an effect of intravenous 3\alpha-OH-DHP on arterial pressure sensitivity of RVLM neurons, the CNS site of action for the effects of 3\alpha-OH-DHP on control of sympathetic outflow may not necessarily be restricted to the RVLM. The RVLM receives tonic excitatory drive from several supra-medullary structures, (28, 39) and it is possible that potentiation of GABAergic inhibition at one of these sites could have contributed to the results of the current experiments.

**PERSPECTIVES**

The current study demonstrated that acute increases in circulating levels of the neuroactive metabolite of progesterone, 3\alpha-OH-DHP, result in potentiation of baroreflex inhibition of brain stem RVLM neurons. The fact that near maximal effects were observed after a dose calculated to produce plasma concentrations within the range seen during pregnancy was administered suggests that these results may be physiologically relevant. Thus, variations in levels of ovarian hormones and their metabolites, as occur during the estrus cycle and during pregnancy, may affect CNS regulation of sympathetic outflow and cardiovascular function. Although acute administration of the progesterone metabolite to virgin female animals produced effects qualitatively similar to the effects of pregnancy, the
effects of long-term exposure to elevated levels of 3α-OH-DHP, as would occur in pregnancy, remain to be evaluated. In addition, preliminary experiments in which baroreflex control of efferent sympathetic nerve activity has been evaluated in male (43) and ovariectomized female rats (68) suggest that prior exposure to ovarian hormones is necessary for the acute effects of 3α-OH-DHP to be fully evident. Thus, it is likely that modulation of sympathetic outflow by ovarian hormones and their metabolites is the result of an interaction between genomic and nongenomic actions within the central nervous system.
ABSTRACT

Recent reports suggest that there are tonic inhibitory and excitatory inputs from the caudal ventrolateral medulla (CVLM) to the rostral ventrolateral medulla (RVLM). These experiments evaluated \( \gamma \)-amino butyric acid (GABA) receptor type mediating CVLM inhibition of the RVLM. In Inactin anesthetized (100 mg/kg, i.p.) female rats, the CVLM and RVLM were defined as sites of maximum pressor and depressor responses to microinjected GABA (500 pmol, 50 nl). Inhibition of the CVLM (GABA) increased mean arterial pressure (MAP, 29± 3.4 mmHg) and efferent renal sympathetic nerve activity (RSNA, 49± 9.4%). Although reduced (MAP, 20± 3.5 mmHg; RSNA, 26± 9.7%), excitatory responses persisted following GABA\( \alpha \) receptor blockade of the RVLM.
with bicuculline (BIC, 400 pmol, 100 nl; n=12). In the presence of combined GABA_A and GABA_B receptor blockade (400 pmol BIC + 400 pmol CGP35348, 100 nl) of the RVLM (n=8), inhibition of the CVLM still resulted in an increase in MAP (17± 3.2 mmHg) and RSNA (13± 5.1%). The decrease in MAP (-17± 4.1 mmHg) and RSNA (-34± 6.1%) due to activation of the CVLM with glutamate (500 pmol, 50 nl) was reversed to an increase in MAP (19± 7.9 mmHg) and RSNA (18± 8.1%) in the presence of RVLM GABA_A receptor blockade (BIC, n=7). These results suggest a tonic non-GABAergic inhibitory influence from the CVLM on the RVLM and an excitatory influence from the CVLM on the RVLM which is masked by tonic inhibitory inputs.

INTRODUCTION

The rostral ventrolateral medulla (RVLM) is a critical brainstem site involved in the regulation of cardiovascular function. Excitation of the RVLM produces an increase in sympathetic outflow and arterial blood pressure (28, 39, 55). Inhibition of neurons in the RVLM results in a decrease in sympathetic outflow and arterial pressure demonstrating that this region is tonically active (28, 39, 55). As a final common site for modulation of sympathoexcitatory drive to preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord, the RVLM receives and integrates neural inputs from several areas in the central nervous system. One area in particular, the caudal ventrolateral medulla (CVLM), has repeatedly been shown to have an important role in the modulation of RVLM sympathetic premotor neurons (24, 25, 28, 39, 57).
Many studies have demonstrated that the CVLM supplies a major inhibitory influence on the RVLM (24, 25, 28, 39, 57, 82) and that GABA is the primary neurotransmitter mediating inhibition of sympathetic premotor neurons in the RVLM (26, 63, 111). Although there is strong evidence to suggest that arterial baroreflex initiated GABAergic inhibition of the RVLM by the CVLM is mediated through GABA_A receptors (39) there are a growing number of reports suggesting that GABA_B receptors are also involved in mediating GABAergic inhibition of the RVLM (5, 6, 71). Inhibitory GABAergic projections from the CVLM to the RVLM that are tonically active and independent of the baroreflex have been described (24-26). However, the post-synaptic GABA receptor type in the RVLM which mediates the baroreflex independent inhibition is not known. Thus, the relative involvement of RVLM GABA_A and GABA_B receptors in mediating GABAergic influences from the CVLM to the RVLM remains unclear.

Most of the reports in the literature have concentrated on elucidating the sources and nature of the inhibitory influences on the tonically active RVLM. In comparison, less is known about tonic excitatory inputs to the RVLM. While stimulation of several central nervous system sites has been shown to excite the RVLM, these areas have not been shown to provide tonic excitation to the RVLM (28). The pontine reticular formation, however, is one area which has been identified as a potential source of tonic excitatory drive to the RVLM (42). Recently a study by Ito et al (55) has suggested that the CVLM may also be a source of tonic excitatory drive to the RVLM. Thus, the current study was designed to evaluate the role of RVLM GABA_A and GABA_B receptors in mediating inhibition from the
CVLM to the RVLM and the role of the CVLM in providing tonic excitation to the RVLM.

**MATERIALS AND METHODS**

**Surgical Preparation**

Experiments were performed in 20 virgin female Sprague Dawley rats (3-5 months old; Harlan Sprague-Dawley, Indianapolis, IN) weighing 225 - 280g. Two complete estrus cycles were documented by daily vaginal smear cytology in all rats and experiments were performed on the day of estrous to minimize the effect of varying hormone levels. The estrous stage of the cycle is easily identified and is characterized by low circulating levels of estrogen and progesterone (15). Rats were anesthetized with intraperitoneal Inactin (100 mg/Kg) and supplemented (0.1mg/Kg, i.v.) as needed. The trachea was cannulated and the rat artificially ventilated (CWE SAR830 Ventilator) with O₂ enriched room air. Body temperature was monitored (Yellow Springs Instrument Co) and maintained at 37°C. The rat was then instrumented with a left femoral arterial catheter (Microline tubing with 28 gauge Teflon tip) to monitor arterial blood pressure and a left femoral venous catheter (polyethylene tubing 50) for subsequent systemic drug administration. The left renal nerve was isolated retroperitoneally, placed on a bipolar platinum recording electrode, and secured in place with a dental impression material (Coltene President's dental acrylic). All wounds were sutured closed.

The rat was then placed in a stereotaxic apparatus and an occipital craniotomy performed. The occipital parietal membrane and dura were cut and folded laterally to
expose the brainstem. The right nucleus tractus solitarius (ML = 0.5, AP = 0.5, DV = 0.5) was electrolytically lesioned (1 mA anodal current, 10 s) using a Teflon insulated tungsten electrode. The head was then tilted forward until the calamus scriptorius was located 2.4 mm posterior to interaural zero (61) and all subsequent microinjections were performed on the left side of the brainstem. Tubocurarine (0.1 mg/Kg, i.v.) was administered to paralyze the rat.

**Drugs and Solution**

Inactin was obtained from Research Biochemicals International (Natick, MA) and dissolved in sterile water. Tubocurarine chloride was obtained from Bristol Myers Squibb (Princeton, NJ). Phenylephrine was purchased from Sigma Chemical Co, (St. Louis, MO) and diluted in isotonic saline. L-glutamic acid, γ-amino-butyric acid, muscimol, and bicuculline methiodide were obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in phosphate buffered saline. CGP-35348, a GABA<sub>B</sub> antagonist, was generously provided by Novartis (Basel, Switzerland). Chicago Sky Blue 6B (80%) was obtained from Aldrich Chemical Company (Milwaukee, WI) and neutral red was purchased from National Diagnostics (Highland Park, NJ).

**Central Microinjection**

Microinjections in the CVLM were performed using a triple barrelled glass micropipette, filled with γ-amino-butyric acid (GABA, 10 mM), l-glutamic acid (GLU, 10 mM), and muscimol (MUSC, 2 mM), in separate barrels and mounted on a
micromanipulator (David Kopf). A double barreled glass micropipette, filled with GABA (10 mM) and bicuculline methiodide (BIC, 4 mM) in separate barrels was mounted on another micromanipulator, and used for microinjections into the RVLM. In experiments evaluating combined GABA\textsubscript{A} and GABA\textsubscript{B} receptor blockade, a double barreled glass micropipette containing GABA (10 mM) in one barrel and the combination of BIC + CGP36348 (BIC 4mM + CGP35348 4mM) in a separate barrel was used for RVLM microinjections. The CVLM (ML = 1.9 - 2.0, AP = -0.2 - -0.4, DV = -2.4 - -2.6 relative to calamus scriptorius) was functionally identified as the site producing the maximum increase in mean arterial blood pressure (MAP) and renal sympathetic nerve activity (RSNA) following microinjection of GABA (500 pmol, 50 nl). Microinjection of GABA (500 pmol, 50 nl) which produced maximum decreases in MAP and RSNA was used to identify the RVLM (ML = 1.9 - 2.0, AP =0.5 - 0.7, DV = -3.5 - -3.8 relative to calamus scriptorius).

The RSNA signal was amplified 100,000 - 200,000 times using a Grass P511 amplifier with a band pass filter (high frequency cutoff = 10,000 Hz; low frequency cutoff = 30-100 Hz). The signal was monitored on a loudspeaker as well as on a dual beam storage oscilloscope (Tektronix, R5113). A rate meter/window discriminator (Winston Electronics, RAD-IIA) was set to count multiunit nerve activity exceeding a selected voltage, just above noise level. Since the absolute value of multiunit nerve activity is dependent on recording conditions, nerve activity was standardized as a percent of the initial baseline value immediately preceding recorded experimental values. Renal sympathetic nerve activity (RSNA), heart rate (HR), and mean arterial blood pressure
(MAP) were monitored on a polygraph (MFE Instruments Corp., MFE 1800) and stored on videotape (Neuro Data Instrument Corp., DR-886) for later analysis.

**Experimental Protocol**

Reflex inhibition of RSNA following elevated arterial pressure (phenylephrine, PE, bolus, 5μg/Kg, i.v.) was obtained at the beginning of the protocol (baroreflex control). Changes in MAP and RSNA due to microinjection of GABA (500 pmol, 50 nl) in the CVLM were then obtained before (control) and after blockade of GABA_A receptors in the RVLM with BIC (400 pmol, 100 nl; n=12). As determined by preliminary experiments, BIC was supplemented (200 pmol, 50 nl) at 5 minute intervals throughout the protocol to maintain blockade of GABA_A receptors in the RVLM. Blockade of GABA_A receptors in the RVLM was verified by elimination of the reflex sympathoinhibitory response to elevations of MAP with PE (n=7). In five animals, MAP was greater than 175 mmHg following BIC and therefore reflex responses to further elevations of MAP were not tested. In seven of the 12 animals which received BIC in the RVLM, the effect of GLU (500 pmol, 50 nl) in the CVLM was also obtained before and after GABA_A receptor blockade in the RVLM.

In a second group of animals the effect of combined GABA_A and GABA_B receptor block in the RVLM was evaluated. In these experiments, MAP and RSNA responses to microinjection of GABA in the CVLM were determined before and after microinjection of the combined antagonists (400 pmol BIC + 400 pmol CGP-35348; 100 nl; n=8) in the RVLM. Similar to the BIC experiments, GABA receptor blockade was maintained with
supplements of the antagonist solution (50 nl) at 5 minute intervals and blockade was tested by elimination of the baroreflex response to increased MAP. Additionally, GABA receptor blockade was verified by testing the response to microinjection of GABA in the RVLM at the end of the experiment.

In both the BIC and combined blockade experiments, the response to the long term GABA A agonist, muscimol (MUSC, 100 pmol, 50 nl), in the CVLM was also evaluated in the presence of GABA receptor blockade of the RVLM.

In 17 of the 20 animals used in this study, the right NTS lesion was verified at the end of the experiment by testing for elimination of the baroreflex response following either inhibition of left CVLM with muscimol (n=15) or left NTS lesion (n=2).

At the end of the experiment the RVLM and CVLM were marked by injection of Chicago Sky Blue dye (1%, 25 nl). Standard histological techniques were used to fix and section the brainstem (60μm sections, neutral red stain). The microinjection site was estimated by comparison with a rat brain atlas (94).

Statistical Analysis

Data sets were analyzed using a one way ANOVA for repeated measures followed by Student Newman Keuls post hoc test. Paired t-tests were used to compare changes in MAP and RSNA before and after GABA blockade within a group. Unpaired t-tests were used to compare changes between the BIC and combined antagonists groups. P < 0.05 was considered significant. Values are expressed as mean ± SEM.
RESULTS

Preliminary experiments were performed to determine a protocol that would effectively block GABA receptors in the RVLM over a 45 minute period. Efficacy of receptor blockade was evaluated either by testing for elimination of baroreflex sympathoinhibitory responses following injection of BIC in the RVLM or loss of MAP and RSNA responses to microinjection of GABA (1 mM) into the RVLM following administration of BIC + CGP35348. The concentrations of BIC (4 mM) and BIC + CGP35348 (4 mM) used in this study were based on those previously reported in the literature (5, 106, 113). The results of preliminary experiments indicated that a single microinjection of either BIC or BIC + CGP35348 was inadequate for maintenance of a complete GABA receptor blockade over a 45 minute period. Therefore, in order to ensure that data obtained following injection of the antagonist(s) was not confounded by partial recovery of the RVLM from GABA receptor blockade, a regimen of antagonist administration was developed. A single injection (400 pmol, 100 nl) of the antagonist(s) followed by supplement injection (200 pmol, 50 nl) at regular 5 minute intervals was found to reliably establish and maintain GABA receptor blockade for one hour. Using this regimen, supplemental injections of the GABA antagonists did not produce any additional effects on MAP or RSNA. Nevertheless, efficacy of GABA receptor blockade was evaluated periodically during the protocol and at the end of each experiment included in this study.
Before NTS Lesion | After (15 min) NTS lesion
---|---
BIC (n=12)  |  |
MAP (mm Hg) | 123 ± 4.2 | 119 ± 2.9 |
RSNA (% Control) | 100 | 100 ± 6.3 |
BIC + CGP35348 (n=8) |  |
MAP (mm Hg) | 125 ± 5.8 | 116 ± 4.7 |
RSNA (% Control) | 100 | 143 ± 29.4 |

Table 4.1. Effect of Right NTS Lesion on Baseline Values. Electrolytic lesion of the right NTS had no effect on either baseline MAP or RSNA. There was no significant difference between the two groups before or after right NTS lesion. Values = mean ± SEM.

Nucleus tractus solitarius (NTS) lesion

In all experiments included in this study microinjections were performed unilaterally on the left side of the brainstem. To prevent contralateral baroreflex compensation of responses to microinjection into the left RVLM and CVLM, the right NTS was electrolytically lesioned at the beginning of each experiment. Right NTS lesion in both BIC (n=12) and BIC + CGP35348 (n=8) groups, had no lasting effect on either baseline mean arterial pressure (MAP) or renal sympathetic nerve activity (RSNA) (Table 4.1). In 17 of 20 animals, sympathoinhibitory responses following elevation of MAP with a bolus injection of PE were tested 15 -25 minutes following the right side lesion and again at the end of the experiment following interruption of baroreflex responses from the left side, using either a left NTS lesion (n=2) or MUSC in the left CVLM (n=15). A ratio

83
After right NTS lesion & left BX Disruption

<table>
<thead>
<tr>
<th></th>
<th>Baseline MAP (mm Hg)</th>
<th>∆RSNA/∆MAP (%C/mm Hg)</th>
<th>Baseline MAP (mm Hg)</th>
<th>∆RSNA/∆MAP (%C/mm Hg)</th>
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<tr>
<td>BIC (n=11)</td>
<td>123 ± 4.6</td>
<td>-2.1 ± 1.9</td>
<td>162 ± 3.8</td>
<td>-0.03 ± 0.04 *</td>
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<tr>
<td>BIC+CGP3 5348 (n=6)</td>
<td>126 ± 7.6</td>
<td>-1.7 ± 0.11</td>
<td>152 ± 5.5</td>
<td>-0.07 ± 0.06 *</td>
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Table 4.2. Verification of right NTS lesion. In 17 of 20 animals, response to baroreflex activation was obtained after destruction of only the right NTS and again following the additional disruption of the left side of the baroreflex. Left side of the baroreflex was eliminated either through injection of MUSC in the CVLM (n=15) or through left NTS lesion (n=2) at the end of the experiment in the presence of GABA receptor blockade in the left RVLM. Baroreflex responses were essentially eliminated following removal of left side of the baroreflex, indicating that the initial right NTS lesion was complete. Values = mean ± SEM; * P < 0.05.

of change in RSNA to change in MAP (ΔRSNA/ΔMAP) was used to estimate baroreflex gain. In the animals tested in both BIC (n=11) and BIC+CGP35348 (n=6) groups, interruption of both left and right sides of the baroreflex pathway resulted in a profound reduction in the absolute value for ΔRSNA/ΔMAP (Table 4.2). Elimination of the baroreflex, through interruption of the pathway on the left side at the end of the experiment, indicated that the initial right NTS lesion had been complete.

**Functional Identification of CVLM & RVLM**

The CVLM was functionally identified as the site at which the maximum increase in MAP and RSNA was observed in response to microinjection of GABA (500 pmol, 50
TABLE 4.3. Functional Identification of CVLM & RVLM. The CVLM and RVLM were functionally identified as the site of maximum pressor and depressor responses to microinjection of GABA (500 pmol, 50 nl) respectively. As expected, significant increase in both arterial pressure (MAP) and nerve activity (RSNA) were observed following injection of GABA in the CVLM. Microinjection of GABA in the RVLM produced significant decreases in MAP and RSNA. There were no differences between BIC and BIC+ CGP35348 groups. Values = mean ± SEM; * P ≤ 0.05

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<tr>
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<th>CVLM</th>
<th>RVLM</th>
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<td></td>
<td>Baseline</td>
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<tr>
<td></td>
<td>MAP (mmHg)</td>
<td>MAP (mmHg)</td>
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<tr>
<td></td>
<td>ΔMAP (%C)</td>
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<td></td>
<td>ΔRSNA (%C)</td>
<td>ΔRSNA (%C)</td>
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<tr>
<td>BIC (n=12)</td>
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<tr>
<td>MAP</td>
<td>114 ± 3.5</td>
<td>135 ± 3.8</td>
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<tr>
<td>ΔMAP</td>
<td>28.9 ± 3.4*</td>
<td>-20.5 ± 3.5*</td>
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<tr>
<td>ΔRSNA</td>
<td>49.01 ± 9.4*</td>
<td>-25 ± 5.1*</td>
</tr>
<tr>
<td>CVLM</td>
<td>117 ± 6.1</td>
<td>142 ± 5.0</td>
</tr>
<tr>
<td>ΔMAP</td>
<td>24.8 ± 3.9*</td>
<td>-17.4 ± 2.9*</td>
</tr>
<tr>
<td>ΔRSNA</td>
<td>40.5 ± 4.9*</td>
<td>-24.6 ± 2.5*</td>
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BIC+CGP35348 (n=8)

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<th>Baseline</th>
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<tr>
<td></td>
<td>MAP (mmHg)</td>
<td>MAP (mmHg)</td>
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<td></td>
<td>ΔMAP (%C)</td>
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<td>ΔRSNA (%C)</td>
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<tr>
<td>CVLM</td>
<td>117 ± 6.1</td>
<td>117 ± 6.1</td>
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<tr>
<td>ΔMAP</td>
<td>24.8 ± 3.9*</td>
<td>24.8 ± 3.9*</td>
</tr>
<tr>
<td>ΔRSNA</td>
<td>40.5 ± 4.9*</td>
<td>40.5 ± 4.9*</td>
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Both MAP and RSNA increased significantly following microinjection of GABA in the CVLM, consistent with removal of tonic inhibitory influences from the CVLM to the RVLM (Table 4.3) and were within the range of values reported in the literature (115). Peak responses were observed within 20 seconds and returned to baseline within 2 minutes of microinjection. There were no statistical differences between responses in the two groups indicating that initial responsiveness of the CVLM to GABA was similar in both the BIC and BIC+CGP35348 groups.

The RVLM was functionally identified as the site producing the maximum decrease in MAP and RSNA due to microinjection of GABA (500 pmol, 50 nl). As expected, in both groups, microinjection of GABA in the RVLM produced a significant decrease in MAP and RSNA, consistent with inhibition of tonic sympathoexcitatory output (Table 4.3). Responsiveness of the RVLM to GABA was similar between BIC and BIC + CGP35348 groups and MAP responses were similar to those previously reported (5, 106).

**Effect of BIC in RVLM**

The effect of inhibiting the CVLM was evaluated before and during RVLM GABA receptor block. Microinjection of GABA (500 pmol, 50 nl) in the CVLM prior to GABA receptor block in the RVLM elicited a significant increase in both MAP and RSNA, both of which recovered to control levels within two minutes (Fig 4.1). Microinjection of the GABA receptor antagonist, BIC, in the RVLM produced an increase in MAP and RSNA. Following BIC in the RVLM, baroreflex sympathoinhibition
Figure 4.1. Inhibition of CVLM during \( \text{GABA}_A \) receptor blockade in RVLM: Inhibition of the CVLM with \( \text{GABA} \) (G) reversibly increased mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA). Blockade of \( \text{GABA}_A \) receptors in the RVLM with bicuculine (B) increased MAP and RSNA. During \( \text{GABA}_A \) receptor blockade in the RVLM, inhibition of the CVLM (B+G) resulted in further increases in MAP and RSNA. C = control; G = \( \text{GABA} \) in CVLM; R = recovery; B = bicuculine in RVLM; B + G = bicuculine in RVLM + \( \text{GABA} \) in CVLM. * different from C; # different from B alone; \( P < 0.05 \).
Figure 4.2. Changes due to blockade of CVLM: The increases in MAP and RSNA due to inhibition of the CVLM with GABA were reduced in the presence of GABA<sub>A</sub> receptor blockade in the RVLM. Bic = bicuculline; * different from change before Bic; P < 0.05.
due to elevated MAP (PE bolus) was eliminated in the seven rats tested, suggesting that GABA_A receptor blockade in the RVLM was complete (26, 109). In the presence of GABA_A receptor blockade of the RVLM, microinjection of GABA in the CVLM produced a further increase in MAP and RSNA (Figure 4.1). However, compared to responses to GABA in the CVLM prior to GABA_A receptor blockade in the RVLM, the change in MAP and RSNA due to inhibition of the CVLM with GABA were smaller in the presence of BIC in the RVLM (Figure 4.2).

The response to microinjection of the long acting GABA_A agonist, muscimol (MUSC), in the CVLM was also evaluated. As expected, during GABA_A receptor blockade of the RVLM, MUSC in the CVLM elicited long-lasting increases in MAP and RSNA of similar magnitude as those seen with injection of GABA in the CVLM. In the presence of BIC in the RVLM, microinjection of MUSC in the CVLM (n=12) resulted in a significant increase in MAP (C(BIC)= 163 ± 4.9 mmHg; MUSC = 179 ± 5.6 mmHg). RSNA also tended (P=0.06) to increase (C(BIC)= 143 ± 6.3 %C; MUSC = 156 ± 11.7 %C) with microinjection of MUSC in the CVLM.

These results confirm previous reports in the literature that there is a tonic baroreflex dependent GABA_A receptor mediated inhibition from the CVLM to the RVLM. However these results also indicate that there is a component of the tonic inhibitory influence from the CVLM on the RVLM which is not mediated by GABA_A receptors.
Effect of Combined GABA\textsubscript{A} and GABA\textsubscript{B} receptor blockade in the RVLM

To determine if GABA\textsubscript{B} receptors in the RVLM play a role in mediating GABAergic inhibition from CVLM to RVLM, in eight separate experiments, a combination of BIC and CGP35348, a specific GABA\textsubscript{B} receptor antagonist, was microinjected into the RVLM. In order to verify that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the RVLM were blocked, the responses to GABA (50 pmol, 50 nl) microinjected into the RVLM were evaluated before and after microinjection of the antagonists. Prior to administration of the blockers, microinjection of GABA (50 pmol, 50 nl) into the RVLM resulted in significant decreases in MAP and RSNA. In the presence of BIC+CGP35348 in the RVLM, MAP and RSNA responses to microinjection of GABA (50 pmol,50 nl) into the RVLM were eliminated, indicating that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the RVLM were blocked (Figure 4.3).

Microinjection of GABA (500 pmol, 50 nl) in the CVLM prior to injection of BIC + CGP35348 in the RVLM produced a significant increase in both MAP and RSNA similar to the group receiving BIC alone (Table 4.3, Figure 4.4). Microinjection of the combination of GABA antagonists in the RVLM produced a significant increase in MAP. Although an initial increase in RSNA was also observed, unlike the increase in MAP, the RSNA response was not statistically significant. Following the initial response, both MAP and RSNA tended to recover towards original baseline levels over several minutes (GX in Fig. 4.4). In order to verify that GABA receptors remained blocked despite the partial recovery, the effect of microinjected GABA (50 pmol, 50 nl) was tested periodically. No response to GABA in the RVLM was observed during combined GABA\textsubscript{A} and GABA\textsubscript{B}
Figure 4.3. Blockade of GABA$_A$ and GABA$_B$ receptors in RVLM: Microinjection of GABA (G, 50 pmol, 50 nl) into the RVLM decreased MAP and RSNA. Following combined GABA$_A$ and GABA$_B$ receptor blockade (GX = Bicuculline + CGP-35348), responses to GABA in the RVLM were eliminated (GX + G). * different from change with G alone. P< 0.05.
Figure 4.4. Inhibition of CVLM during blockade of GABA_A and GABA_B receptors in RVLM: Inhibition of the CVLM with GABA reversibly increased MAP and RSNA (G). Blockade of GABA_A and GABA_B receptors in the RVLM (GX) increased MAP and tended to increase RSNA. During combined GABA_A and GABA_B receptor blockade in the RVLM, inhibition of the CVLM resulted in further increases in MAP and RSNA (GX+G). C = control; R = recovery; * different from C; # different from GX; P< 0.05.
receptor blockade. In experiments examining the effects of GABA<sub>B</sub> antagonism in the NTS, Sved et al. (113) made similar observations using this GABA<sub>B</sub> antagonist and demonstrated that, although MAP tended to recover, responses to baclofen, a specific GABA<sub>B</sub> agonist, remained blocked.

Once MAP and RSNA had stabilized following GABA<sub>A</sub> and GABA<sub>B</sub> receptor blockade in the RVLM, responses to inhibition of the CVLM with GABA were evaluated. As with BIC alone, microinjection of GABA (500 pmol, 50 nl) in the CVLM in the presence of BIC+CGP35348 in the RVLM, continued to elicit a further significant increase in MAP. This increase (17 ± 3.2 mmHg) tended (P = 0.12) to be smaller than the response to GABA in the CVLM observed prior to injection of BIC+CGP35348 in the RVLM (25 ± 4.1 mmHg). In the presence of BIC+CGP35348 in the RVLM, RSNA increased following GABA in the CVLM (13 ± 5.2 %C). However, this increase was significantly less than the RSNA response prior to GABA<sub>A</sub> and GABA<sub>B</sub> receptor blockade (41 ± 5.0%C). The MAP and RSNA responses to inhibition of the CVLM in the presence of GABA<sub>A</sub> and GABA<sub>B</sub> receptor blockade of the RVLM were no different from responses during GABA<sub>A</sub> receptor block of the RVLM suggesting that the GABAergic influence from the CVLM is mediated primarily by GABA<sub>A</sub> receptors. The continued increase in MAP and RSNA suggests that there is a sympathoinhibitory influence from the CVLM which is not GABAergic in the RVLM.

In six rats, the effects of inhibition of the CVLM with MUSC, a long acting GABA<sub>A</sub> agonist, were also evaluated in the presence of combined GABA<sub>A</sub> and GABA<sub>B</sub>
blockade in the RVLM. Microinjection of MUSC in the CVLM, during GABA A and GABA B receptor blockade in the RVLM, resulted in a significant increase in MAP (C(BIC+CGP35348) = 161 ± 3.6 mmHg; MUSC = 182 ± 4.3 mmHg). However, microinjection of MUSC in the CVLM, had no significant effect on RSNA (C(BIC+CGP35348) = 120.1 ± 8.7% C; MUSC = 126.2 ± 6.0% C).

**Effect of GLU in CVLM**

Totonically active excitatory projections from the CVLM to the RVLM have been proposed by others but have not been directly assessed (28, 55, 75). In the current experiments, pressor and sympathoexcitatory responses to blockade of the CVLM were attenuated by GABA receptor blockade in the RVLM. However, evidence of a tonically active excitatory projection from the CVLM to the RVLM was not demonstrated.

To test for the presence of an excitatory projection from the CVLM to the RVLM, in a subset of seven animals, the responses to inhibition (GABA: 500 pmol, 50 nl) and excitation (GLU: 500 pmol, 50 nl) of the CVLM were tested before and after GABA A receptor blockade (BIC) in the RVLM. Similar to previous results, inhibition of the CVLM (GABA) in this subset of rats continued to result in an increase in MAP (20 ± 6.3 mmHg; P< 0.06) and RSNA (19 ± 10.5 %C; P<0.05) during GABA A blockade in the RVLM, suggesting removal of a tonic inhibitory influence from the CVLM. Excitation of the CVLM with GLU prior to GABA A receptor block resulted in significant depressor (-17 ± 4.1 mmHg) and sympathoinhibitory (-34 ± 6.1 %C) responses. Following blockade of GABA A receptors in the RVLM (BIC) however, the response to GLU in the CVLM
Figure 4.5. Changes due to activation of CVLM during GABA_A receptor blockade in RVLM: Microinjection of l-glutamate (l-glu, 500 pmol, 50 nl) into the CVLM decreased MAP and RSNA (Control). Following blockade of GABA_A receptors in the RVLM with bicuculline, microinjection of l-glu in the CVLM resulted in significant increases in MAP and RSNA (Bic). * different from Control. P< 0.05.
Figure 4.6. Microinjection sites: **Left = RVLM**: The site for maximum depressor response to microinjected GABA was functionally defined as the RVLM, marked with Chicago sky blue dye (50 nl) and identified histologically (closed circles). **Right = CVLM**: The site for maximum pressor response to microinjected GABA was functionally defined as the CVLM, marked with Chicago sky blue dye (50 nl) and identified histologically (closed squares). Brainstem sections were adapted from *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 1998). Sol, nucleus of the solitary tract; Cu, cuneate nucleus; Ecu, external cuneate nucleus; Amb, ambiguus nucleus; Sp, spinal trigeminal tract.
was reversed to significant pressor ($19 \pm 7.9 \text{ mmHg}$) and sympathoexcitatory ($18 \pm 8.1\%$) responses (Figure 4.5) suggesting that there is an excitatory pathway from the CVLM to the RVLM.

**Histology**

Post hoc histological examination of the microinjection sites verified that they were within areas previously described as the RVLM and CVLM (Figure 4.6) (39).

**DISCUSSION**

As a final common site for tonic sympathoexcitatory drive to preganglionic sympathetic neurons in the spinal cord, the rostral ventrolateral medulla (RVLM) receives and integrates cardiovascular input from many areas in the central nervous system (28, 39). In the medullary baroreflex pathway, the caudal ventrolateral medulla (CVLM) has been shown to provide the inhibitory influences in the RVLM (24, 25, 28, 39, 57). Several laboratories (1, 24, 28, 57) have demonstrated that CVLM neurons are antidromically activated from the RVLM. In addition, retrograde labeling from the RVLM of GABAergic CVLM neurons, which also express the c-fos proto-oncogene in response to an increase in arterial pressure, suggests that there is a direct GABAergic projection from the CVLM to the RVLM (19). It has also been repeatedly shown that activation of the arterial baroreflex increases activity in the CVLM and results in inhibition of the RVLM, thus decreasing sympathoexcitatory drive from the RVLM (28). Similarly, interruption of
neural transmission in the CVLM blocks arterial baroreflex responses and produces an
increase in RVLM neuronal activity which is consistent with removal of tonic baroreflex
inhibitory influences to the RVLM (28). Tonic inhibitory influences from CVLM to
RVLM which are independent of the baroreflex, have also been described (24, 25, 38).
Disruption of neuronal activity in the CVLM has been shown to elicit a larger increase in
MAP than interruption of baroreflex afferent input alone, suggesting a baroreflex
independent component (24, 38). Cravo et al demonstrated the existence of both
baroreflex dependent and baroreflex independent sympathoinhibitory influences from the
CVLM (24).

It has been demonstrated that arterial baroreflex inhibition of RVLM neurons is
due to activation of GABA$_A$ receptors in the RVLM (109). Blockade of GABA$_A$ receptors
in the RVLM with the GABA$_A$ specific antagonist, bicuculline, increases neuronal activity
in the RVLM and eliminates sympathoinhibitory responses to activation of the arterial
baroreflex (26, 109, 111). The increased sympathoexcitatory activity in the RVLM is
consistent with removal of a tonic GABAergic inhibition of RVLM neurons. Sun et al
(111) demonstrated that iontophoretic application of BIC on identified presympathetic
RVLM neurons eliminates baroreflex responsiveness of arterial pressure sensitive neurons.
From these studies, it is clear that GABAergic projections from CVLM to RVLM
participate in CVLM inhibition of sympathetic outflow.

Less is known about regulation of the pathway mediating tonic baroreflex
independent inhibitory influences from the CVLM. Interestingly, several studies have
suggested that GABA$_B$ receptors are present and may contribute to tonic inhibition of
neurons in the RVLM (5, 6, 71). In 1995, Li et al reported that microinjection of the GABA_B receptor agonist, baclofen, in the RVLM produced significant hypotension in rats, suggesting that GABA_B receptors are present in the RVLM (71). In preliminary experiments in three animals, microinjection of CGP55845, a GABA_B receptor antagonist, in the RVLM did not elicit an increase in arterial pressure, suggesting that these receptors may not be tonically active (71). In contrast, Amano et al found that administration of the GABA_B antagonist, 2-hydroxysaclofen, in the RVLM produced pressor responses (5). Likewise, Avanzino et al has demonstrated that administration of CGP35348, a potent and GABA_B receptor specific antagonist, in the RVLM elicited increases in MAP and HR consistent with removal of a tonic inhibition mediated by GABA_B receptors (6). Differences in GABA_B receptor antagonist efficacy and potency may have also contributed to the apparent conflicting results. The current experiments were designed to evaluate the relative involvement of RVLM GABA_A and GABA_B receptors in mediating GABAergic influences from the CVLM.

**Evaluation of Inhibitory Influences**

Microinjection of the GABA_A receptor antagonist, BIC, in the RVLM elicited an increase in both MAP and RSNA consistent with the removal of tonic GABAergic inhibitory input to the RVLM. Peak MAP was achieved 1 - 3 minutes after BIC injection and stabilized at the new higher level within 5 minutes. These results are consistent with results for microinjection of BIC in the RVLM previously reported in the literature (109, 114). An increase in RSNA (C = 100; BIC = 146 ± 8.1 %C) was also observed following
microinjection of BIC in the RVLM which is consistent with an increase in sympathetic outflow due to disinhibition of excitatory neurons in the RVLM. However, while a significant increase in RSNA was observed following GABA_A receptor blockade in the RVLM, this increase was not as great as might be expected. Previous experiments from our laboratory demonstrated that bilateral microinjection of BIC in the RVLM produced increases in RSNA to more than 250% of baseline levels (44). The more moderate sympathoexcitatory responses seen in the current experiments were likely due to the nature of the preparation. Although NTS lesion on the side contralateral to the unilateral RVLM microinjection would eliminate baroreflex mediated compensation from that side, it is possible that tonic baroreflex independent inhibitory input was still intact. In other words, destruction of the NTS may not remove tonic baroreflex independent inhibition to the RVLM on the lesioned side, whereas bilateral microinjection of antagonists directly into both left and right RVLM, as in our previous experiment, would remove inhibitory influences on both sides, thus allowing greater disinhibition. Despite the smaller response of MAP and RSNA to unilateral BIC administration, repeated testing indicated that unilateral GABA receptor blockade in the RVLM was complete in each animal included in these studies.

The MAP and RSNA responses following blockade of GABA_A receptors in the RVLM confirm previous reports that GABA_A receptors mediate tonic inhibition of the RVLM (5, 26, 109). In order to evaluate the extent of GABA_A receptor involvement in mediating inhibitory influences from the CVLM to the RVLM, the effect of inhibiting the CVLM was tested during GABA_A receptor blockade in the RVLM. If GABAergic
inhibition from the CVLM was mediated solely by GABA_A receptors in the RVLM, then prior injection of BIC would eliminate responses to blocking the CVLM. In the current experiments, however, microinjection of GABA to inhibit activity in the CVLM during RVLM GABA_A receptor blockade, continued to evoke an increase in both MAP and RSNA. This increase was smaller than the response observed prior to BIC in the RVLM, indicating that GABA_A receptors are at least partially responsible for mediating tonic inhibition of the RVLM. However, the continued increase in MAP and RSNA observed in these experiments suggests that there is a source of inhibitory influence from the CVLM which is not mediated by GABA_A receptors in the RVLM. Recently, Natarajan and Morrison (88) reported similar findings, whereby bilateral inhibition of the CVLM with muscimol produced increases in splanchnic nerve activity above those produced by prior microinjection of bicuculline into the RVLM.

Other reports have suggested that GABA_B receptors may also play a role in mediating tonic GABAergic influences in the RVLM (5, 6, 71). It is possible that the tonic inhibition from CVLM to RVLM that remained following GABA_A blockade in the RVLM was mediated by GABA_B receptors. To evaluate this possibility experiments were repeated in a separate group of animals using a combination of GABA_A (BIC) and GABA_B (CGP35348) receptor antagonists in the RVLM. Similar to microinjection of BIC alone, microinjection of the combination of antagonists in the RVLM increased MAP and tended to increase RSNA which peaked within 1-3 minutes. However, unlike the experiments with BIC alone, MAP and RSNA recovered towards original baseline levels within 10 minutes of microinjection. These observations raised the possibility that GABA_A and
GABA\textsubscript{B} receptors may not have been completely blocked by the combination of antagonists or were recovering from blockade rapidly despite the regimen of administration. However, the arterial baroreflex response to increased arterial pressure was eliminated when the combined antagonists were present in the RVLM indicating that at least GABA\textsubscript{A} receptors in the RVLM were blocked (26, 109). In order to test whether GABA\textsubscript{B} receptors were also blocked, the response to a low dose of GABA in the RVLM was compared before and after microinjection of BIC+ CGP35348 into the RVLM. While injection of a low dose of GABA in the RVLM elicited significant pressor and sympathoexcitatory responses prior to GABA\textsubscript{A} and GABA\textsubscript{B} receptor block, no response was observed following antagonist microinjection, suggesting that both GABA\textsubscript{A} and GABA\textsubscript{B} receptors in the RVLM were completely blocked despite the partial recovery of MAP towards baseline levels and the relative unresponsiveness of RSNA to microinjection of the combined antagonists in the RVLM. Although the mechanism for partial recovery is not known, similar observations have been reported by Sved et al. following microinjection of CGP35348 in the NTS (113). Even though the depressor response elicited by microinjection of CGP35348 in the NTS was transient, responses to the GABA\textsubscript{B} agonist, baclofen, were blocked.

Once GABA\textsubscript{A} and GABA\textsubscript{B} receptor block was verified in the RVLM, the effect of inhibiting the CVLM with GABA was evaluated in the presence of BIC + CGP35348 in the RVLM. As with the experiments with only GABA\textsubscript{A} receptor block in the RVLM, microinjection of GABA in the CVLM with BIC + CGP35348 in the RVLM, continued to produce an increase in both MAP and RSNA. Similar results were also observed when the
long acting GABA$_A$ agonist, MUSC, was microinjected in the CVLM. Thus, although the pressor response to inhibition of the CVLM was attenuated with GABA$_A$ and GABA$_B$ receptors blocked in the RVLM, MAP and RSNA responses persisted, suggesting that the CVLM continued to provide inhibitory influences on the RVLM in the presence of complete GABA receptor blockade. Additionally, since there was no significant difference in the response to inhibiting the CVLM in the presence of BIC versus BIC+CGP35348, it appears that GABAergic inhibition from the CVLM to the RVLM is mediated primarily by GABA$_A$ receptors and that there is a tonically active non-GABAergic inhibitory influence from the CVLM on the RVLM.

It is important to note, however, that the CVLM is heterogeneous and lacks clear anatomical boundaries. Anatomical studies have shown that CVLM neurons retrogradely labeled from the RVLM are extensively mingled with neurons of the A1 (CVLM region) and C1 (RVLM region) cell groups (19). The microinjection sites were chosen in the current experiments based on functional criteria of maximal pressor and depressor responses to GABA. However, this technique does not eliminate the possibility that drugs injected into the functionally defined CVLM did not have effects on more caudally located sympathoexcitatory neurons. Additionally, all the projections of CVLM neurons have not yet been identified. A projection from the CVLM to the pontine reticular formation (PRF), an area which has been shown to provide tonic excitatory influences to the RVLM, has been anatomically demonstrated in the cat (108). Although the nature of the projection is not known, inhibition of CVLM neurons which project to PRF neurons could possibly result in increased excitatory influences from the PRF to the RVLM. Thus, although
inhibition or excitation of CVLM neurons had effects on sympathetic outflow in the current experiments, this would not necessarily indicate that the effects were mediated through a direct pathway from the CVLM to the RVLM.

Although the general consensus is that tonic inhibition of the RVLM is largely GABAergic (11, 28, 111), other inhibitory neurotransmitters have been identified in the RVLM (11, 28) and may contribute to the residual inhibitory influence from the CVLM to the RVLM during GABA\(_A\) and GABA\(_B\) blockade of the RVLM. Glycine, for example, is a possible neurotransmitter candidate which may be involved in mediating inhibitory influences in the RVLM. Receptors for glycine have been described in the RVLM (72) and Blessing et al (11) reported that in rabbits blockade of glycine receptors in the RVLM with strychnine resulted in small but reproducible increases in MAP consistent with removal of tonic inhibition of RVLM neurons by glycine. Although found in less abundance than GABAergic neurons, glycinergic neurons have been demonstrated in the rat RVLM through labeling studies (19). Tonic glycinergic influence, however, at the RVLM has not been demonstrated. Experiments evaluating the effect of bilateral injection of strychnine in rat RVLM showed no noticeable effect of blocking glycine receptors on arterial pressure (5). However, if tonic glycinergic influences are normally modest, this may be masked by other tonic influences at the RVLM.

**Evaluation of Excitatory Influences**

Tonic inhibitory influences from the CVLM to the RVLM act to restrain tonic activity in neurons in the RVLM. However, the origin of tonic excitatory influences in the RVLM is not as well understood. In a recent study by Ito et al., blockade of excitatory
amino acid (EAA) receptors in the RVLM elicited a significant decrease in MAP only after neuronal activity in the CVLM was inhibited with muscimol (55). The lack of MAP response to EAA receptor block in the RVLM of intact animals suggested that there is a tonic non-EAA mediated excitatory signal from the CVLM to the RVLM which maintains sympathoexcitatory tone despite blockade of EAA receptors in the RVLM (55). Thus, the model proposed by this group suggests that the CVLM may be one source of non-EAA excitation to the RVLM. In the current study, the role of the CVLM in providing excitatory influences to the RVLM was evaluated.

Initially, it was expected that if a tonic excitatory influence from the CVLM to the RVLM existed, then inhibition of the CVLM in the presence of combined GABA_A and GABA_B receptor blockade in the RVLM, should produce a decrease in MAP and RSNA. However, MAP and RSNA increased when the CVLM was inhibited during RVLM GABA receptor blockade, indicating that blocking GABA receptors in the RVLM did not eliminate all inhibitory influences from the CVLM. Thus, non-GABAergic inhibition from the CVLM continued to mask any excitatory influence, or if an excitatory pathway existed then it was not tonically active. Therefore, in order to directly evaluate an excitatory influence on the RVLM from the CVLM, the effect of GLU in the CVLM was tested before and after microinjection of BIC in the RVLM. As expected, microinjection of GLU in the CVLM prior to BIC in the RVLM produced a decrease in MAP and RSNA (28). This is consistent with the activation of CVLM neurons which provide inhibitory influences to the RVLM. With GABA_A receptors blocked in the RVLM, microinjection of GLU in the CVLM reversed the MAP and RSNA response and a significant increase in both MAP
and RSNA was observed. The implications of this sympatoexcitatory response is twofold. First, a pressor response to activation of the CVLM indicates that there is an excitatory projection from the CVLM to the RVLM. Second, this excitatory projection is either balanced by inhibitory non-GABA\textsubscript{A} influences from the CVLM to the RVLM, or this pathway is not tonically active. It is important to note that excitation of the CVLM by microinjection of GLU may activate other neuronal pathways involved in cardiovascular regulation which are not normally active.

**PERSPECTIVES**

The RVLM has been shown to receive and integrate neural inputs, both inhibitory and excitatory, from many areas in the central nervous system. Until recently the primary focus of research has concentrated on the tonic inhibitory influences to the RVLM. While several inhibitory neurotransmitters have been reported in the RVLM, the general consensus is that the tonic inhibition of the RVLM is primarily GABAergic and the CVLM has been identified as the main source of this inhibition. The continuous development of new and more effective antagonists, in this case CGP-35348, a potent GABA\textsubscript{B} receptor antagonist, has allowed for a more careful evaluation of the tonic inhibitory influences from the CVLM. The current study demonstrated that the CVLM provides not only a tonic GABAergic influence to the RVLM but a tonic non-GABAergic inhibitory influence as well. Additionally, the GABAergic influence provided by the CVLM is likely mediated primarily by GABA\textsubscript{A} receptors in the RVLM, since responses to blockade of the CVLM during combined blockade of GABA\textsubscript{A} and GABA\textsubscript{B} receptors were no different than
responses during GABA_A receptor blockade alone. While less is understood about the nature of the excitatory influences balancing the inhibitory influences on the RVLM, recent studies have suggested that the CVLM may be a source of excitatory influences to the RVLM (28, 55, 75). The data from the present study confirms that there is an excitatory projection from the CVLM to the RVLM, but the influences of tonic GABAergic and non-GABAergic inhibitory projections from the CVLM to the RVLM predominate.
CHAPTER 5

SUMMARY

This dissertation is directed towards evaluating the effect of pregnancy on afferent and central nervous system (CNS) components of the baroreflex pathway and the further elucidation of CNS mechanisms involved in control of baroreflex function. The role of the primary metabolite of progesterone, 3α-OH-DHP, in mediating the effects of pregnancy on the baroreceptor reflex is also assessed in this work. Earlier studies in our laboratory have shown that among the many cardiovascular changes that occur during pregnancy are alterations in efferent baroreflex function (23, 47, 80). In pregnant animals, a significantly lower baseline mean arterial pressure (MAP) and a leftward shift in the efferent baroreflex function curve to lower operating pressure ranges can be observed. Sympathoexcitatory responses at low arterial blood pressures are significantly attenuated during pregnancy. These studies also showed that sympathoinhibition is potentiated at high MAP in pregnant animals (23, 47, 80). It has been proposed that the presence of elevated levels of the primary metabolite of progesterone, 3α-OH-DHP, during pregnancy, is one mechanism that contributes to the differences in efferent baroreflex function. Administration of 3α-OH-DHP to virgin rats, for example, has been shown to significantly attenuate the sympathoexcitatory response to low MAP, an effect quantitatively similar to the effects of pregnancy on baroreflex function (47, 80).
Although a CNS mechanism of action is suggested by these studies, alterations in efferent baroreflex function could reflect alterations which have occurred at earlier points in the baroreflex pathway. The effect of pregnancy on afferent and CNS components of the baroreflex pathway, however, has not been previously addressed. The contribution of alterations in afferent mechanisms to baroreflex adaptations during pregnancy was evaluated in the first study in this dissertation. Baroreceptor afferent nerve discharge response to changes in arterial pressure was measured by recording multiunit activity from the aortic depressor nerve, a nerve which has been demonstrated to contain almost exclusively baroreceptor afferents in the rat (8, 89, 102). In the current study, baseline MAP was significantly lower in pregnant animals. Consistent with pressure-dependent resetting of baroreceptors to the prevailing pressure, the baroreceptor discharge curve in pregnant animals was shifted significantly to the left towards a lower operating pressure range with no change in slope. It is most likely that this parallel leftward shift in the baroreceptor afferent discharge curve contributes to the shift of efferent baroreflex function observed during pregnancy (23, 80). The shift in the efferent baroreflex function curve, however, was also accompanied by significant changes in baroreflex sensitivity (47, 80). This was not evident in the afferent component of the baroreflex. Baroreceptor sensitivity to increments in pressure did not differ between pregnant and virgin animals, consistent with the characteristics of pressure dependent downward resetting (22, 46, 54). Thus, mechanisms other than pressure dependent baroreceptor resetting must contribute to adaptations in baroreflex sensitivity during pregnancy.
The effect of administration of 3α-OH-DHP on afferent baroreceptor discharge was also evaluated in order to determine whether elevated levels during pregnancy may play a role in altered baroreceptor function. However, baroreceptor discharge curves obtained after administration of 3α-OH-DHP were no different from control curves in either virgin or pregnant groups. Thus, elevated levels of the progesterone metabolite during pregnancy does not have a direct effect on baroreceptor afferent nerve discharge.

While 3α-OH-DHP was not found to contribute to alterations in afferent baroreflex mechanisms during pregnancy, it has been shown to have an effect on baroreflex function, thus the effect of 3α-OH-DHP must occur at the level of the CNS. Alterations in the CNS component of the baroreflex may account for the attenuated sympathoexcitatory and the potentiated sympathoinhibitory responses seen in pregnant animals. During pregnancy, 3α-OH-DHP is present in concentrations which have been shown to potentiate chloride conductance of CNS GABA_A receptors and the differences in baroreflex function between virgin and pregnant animals are consistent with a potentiation of GABA receptors in CNS sites responsible for modulating sympathetic outflow. Although GABA_A receptors have been demonstrated in several sites in the medullary baroreflex pathway, increased GABAergic inhibition at many of these sites would not result in attenuated sympathoexcitatory and potentiated sympathoinhibitory baroreflex responses. Increased GABAergic inhibition in the NTS or the CVLM, for instance, would produce the opposite results. An increase in GABAergic influence in the RVLM, however, the final site in the baroreflex pathway for modulation of sympathetic outflow to
the peripheral vasculature, would produce alterations in sympathetic outflow, consistent with the results of the earlier baroreflex function studies.

There are several possible mechanisms which may result in an increase in GABAergic influence preferentially in the RVLM. One possibility is the presence of a greater number of GABA<sub>A</sub> receptors in RVLM. Elevated levels of estrogen and progesterone during pregnancy, may increase GABA<sub>A</sub> receptor levels (16) since these ovarian hormones have been shown to be involved in regulating central GABA<sub>A</sub> receptor synthesis, composition, and affinity (48, 78). A preferential effect of 3α-OH-DHP in the RVLM compared to other regions involved in the central baroreflex pathway may be another mechanism contributing to the increase in GABAergic influence in the RVLM.

Affinity of 3α-OH-DHP for the GABA<sub>A</sub> receptor is dependent on the subunit composition of the receptor (77, 104) and estrogen in particular has been demonstrated to promote the synthesis of α and γ subunits, important in neurosteroid binding (48). Thus during pregnancy, it is possible that GABA<sub>A</sub> receptors in the RVLM preferentially contain the subunits to maximize affinity of the GABA<sub>A</sub> receptor to 3α-OH-DHP. The effect of 3α-OH-DHP on neurons in the RVLM, however, has not been previously addressed. The focus of the second study in this dissertation was to determine the effect of intravenous administration of 3α-OH-DHP, in concentrations similar to those found in pregnancy, on arterial pressure sensitivity of spinally projecting neurons in the RVLM to endogenously released GABA.

The effects of 3α-OH-DHP were evaluated while recording from spontaneously firing baroreflex sensitive neurons in the RVLM. Baseline values of MAP and unit
activity (UA) were not affected by 3α-OH-DHP (1.12 μg/Kg, i.v.). However, both threshold MAP and saturation MAP for inhibition of RVLM neurons were significantly decreased following administration of 3α-OH-DHP. This indicates that sensitivity of RVLM neurons to elevated arterial blood pressure, and therefore endogenous inhibitory influences, was increased in the presence of 3α-OH-DHP. This result is consistent with potentiation of baroreflex sympathoinhibition to elevated MAP during pregnancy.

Recovery of UA (t½ UA) and MAP (t½ MAP) from a pressure ramp was also evaluated and while recovery was unaffected by the primary dose of 3α-OH-DHP, at higher concentrations of 3α-OH-DHP (11.2 μg/Kg, i.v.), recovery of UA from elevated blood pressure was delayed. The longer recovery time suggests that levels of GABA would have to decrease further before UA could recover, consistent with a potentiated GABAergic influence. In cells which did not appear to fully recover, the effect of administration of the inactive stereoisomer, 3β-OH-DHP, a competitor with 3α-OH-DHP for binding site on the GABA A receptors, was also evaluated. Despite a slight increase in pressure, a rapid increase in UA was observed following 3β-OH-DHP, indicating that 3β-OH-DHP reversed the inhibitory effect of 3α-OH-DHP, presumably by displacing 3α-OH-DHP from its binding site on the GABA A receptor. The results of this study demonstrate that acute increases in circulating levels of the neuroactive metabolite, 3α-OH-DHP, to levels which are similar to those found in pregnancy, potentiates baroreflex inhibition of baroreflex sensitive, spinally projecting neurons in the RVLM through a stereospecific nongenomic mechanism of action at the GABA A receptor.
Although this study demonstrated that intravenous administration of 3α-OH-DHP has an effect on neurons in the RVLM, the specific site of action of 3α-OH-DHP remains uncertain. Additionally, the functional significance of modulation by 3α-OH-DHP in the RVLM was not addressed by this study and remains to be evaluated. Preliminary studies in our laboratory have examined the effect of direct application of 3α-OH-DHP in the RVLM on baroreflex function. Similar to studies in which 3α-OH-DHP was administered peripherally, microinjection of 3α-OH-DHP in the RVLM attenuated sympathoexcitatory responses to low MAP (45) suggesting that the effects of 3α-OH-DHP in the RVLM may indeed be one mechanism of action.

Additionally, the effects of long-term exposure to elevated levels of 3α-OH-DHP, as would occur in pregnancy, remain to be evaluated. Variations in levels of ovarian hormones and their metabolites, as occur during the estrus cycle and during pregnancy, may affect central nervous system regulation of sympathetic outflow and cardiovascular function. Preliminary experiments have also suggested that prior exposure to ovarian hormones is necessary for the acute effects of 3α-OH-DHP to be fully evident (68).

While the changes in baroreflex function observed during pregnancy are consistent with potentiation of GABA_A receptors in the RVLM, there are other possible mechanisms which may also account for the adaptations in baroreflex function. Changes in the balance between inhibitory and excitatory influences within brainstem areas involved in regulation of cardiovascular function may also affect baroreflex function. Increased levels of inhibitory influences and/or a decrease in excitatory influences at the RVLM, for instance, would decrease the total amount of sympathoexcitatory output from the RVLM.
and baroreflex function would be altered in a manner similar to that observed during pregnancy. However, the sources, as well as the mediators, of the inhibitory and excitatory influences in the RVLM are not fully understood. In order to begin evaluating these possibilities, tonic inhibitory and excitatory influences from the CVLM to the RVLM were addressed in the final study of this dissertation.

The CVLM has been shown to be one major source of tonic inhibitory influence on the RVLM and the neurotransmitter mediating this inhibitory influence has been repeatedly demonstrated to be GABA (28, 109, 111). Arterial baroreflex initiated GABAergic inhibition from the CVLM is thought to be mediated primarily by GABA_A receptors in the RVLM (39, 109, 111). Recently, however, a second population of tonically active neurons in the CVLM, independent of the baroreflex, has been identified (24, 25, 28) and the post-synaptic receptor type which mediates the baroreflex independent inhibition is not known. Several studies have shown that GABA_B receptors in the RVLM are also active in mediating GABAergic influences (5, 6, 71) and thus RVLM GABA_B receptors may be one possible subtype involved in mediating baroreflex independent inhibition from the CVLM.

In order to evaluate the role of RVLM GABA_A and GABA_B receptors in mediating CVLM inhibition, the effect of inhibiting the CVLM was evaluated before and during either GABA_A receptor blockade or combined GABA_A and GABA_B receptor blockade in the RVLM. As expected, inhibition of the CVLM with GABA elicited significant pressor and excitatory responses prior to GABA receptor blockade in the RVLM, consistent with removal of inhibitory influences to the RVLM. Microinjection of
GABA in the CVLM during GABA\textsubscript{A} receptor blockade of the RVLM, however, continued to produce an increase in MAP and RSNA indicating that inhibitory influences from the CVLM to the RVLM were still active despite GABA\textsubscript{A} receptor block. While, this response was significantly smaller compared to responses prior to GABA\textsubscript{A} receptor block, the continued increase in MAP and RSNA observed in these experiments suggests that there is a source of inhibitory influence from the CVLM which is not mediated by GABA\textsubscript{A} receptors in the RVLM and thus the role of GABA\textsubscript{B} receptors was evaluated. Yet, pressor responses to inhibition of the CVLM continued to persist even in the presence of combined GABA\textsubscript{A} and GABA\textsubscript{B} receptor blockade and when compared, the response to inhibition of the CVLM during combined GABA\textsubscript{A} and GABA\textsubscript{B} receptor block were no different from responses during GABA\textsubscript{A} receptor block in the RVLM, suggesting that the GABAergic influence from the CVLM is mediated primarily by GABA\textsubscript{A} receptors. The continued increase in MAP and RSNA suggests that there is an inhibitory influence from the CVLM which is not GABAergic. One neurotransmitter, glycine, has been suggested as a likely candidate (11, 72). Although relatively less abundant than GABAergic neurons, glycineric neurons have been demonstrated in the rat RVLM (19), however, tonic glycineric influence at the RVLM has not been demonstrated. Future experiments in which glycine receptors in the RVLM are blocked with strychnine would test for the role of glycine.

Although tonic GABAergic influences from the CVLM to the RVLM in virgin animals was evaluated, inhibitory influences in pregnant animals was not examined in this study. Differences in modulation of GABAergic influences in the RVLM between virgin
and pregnant animals may be one factor contributing to the adaptations in baroreflex responses during pregnancy. However, differences in amount of GABAergic or non-GABAergic inhibition may also contribute to the baroreflex adaptations observed during pregnancy and thus need to be evaluated in future studies.

Recent reports in the literature have suggested that the CVLM may also be a source of tonic excitatory input to the RVLM (55). The presence of an excitatory projection from the CVLM to the RVLM was evaluated by testing responses to excitation of the CVLM before and after GABA_A receptor blockade (BIC) in the RVLM. While excitation of the CVLM with GLU prior to GABA_A receptor block resulted in significant depressor and sympathoinhibitory responses, following blockade of GABA_A receptors in the RVLM (BIC), the response to excitation of the CVLM was reversed to pressor and sympathoexcitatory responses, suggesting that there is an excitatory projection from the CVLM to the RVLM. However, this excitatory projection is either balanced by inhibitory non-GABA_A influences from the CVLM to the RVLM since a pressor and sympathoexcitatory response was elicited after GABA_A receptor block in the RVLM, or this pathway is not tonically active.

While the neurotransmitter involved in the excitatory projection from CVLM to RVLM was not addressed in this study, one possible candidate is angiotensin II (AII). Endogenous AII is tonically released from several central cardiovascular sites including RVLM and CVLM (20, 56, 87, 117). Sved et al has demonstrated that tonic AII in the RVLM accounts for a large portion of the sympathetic output from the RVLM (56). AII directly infused into the RVLM has also been shown to potentiate sympathoexcitatory
responses of the baroreflex (7, 9, 56, 87). Preliminary studies in our laboratory have demonstrated decreased pressor and excitatory responses of RVLM to All in pregnant animals (unpublished results).

This thesis has demonstrated that alterations in afferent baroreceptor reflex function contribute in part to the changes observed in previous efferent baroreflex function studies through pressure dependent resetting of the baroreceptors. Pregnancy, however, is also associated with changes in baroreflex sensitivity which cannot be attributed to an effect on afferent discharge, or the 3α-OH-DHP progesterone metabolite on afferent baroreceptor function. An effect of pregnancy and 3α-OH-DHP on CNS control of the baroreflex may contribute to the differences in baroreflex function responsiveness between virgin and pregnant animals, and has been suggested by earlier studies. In the current studies, evidence supporting this proposed mechanism was described. Elevated levels of circulating 3α-OH-DHP, in these studies, was found to have an effect on CNS mechanisms, in particular barosensitive neurons in the RVLM. There are, however, several other possible factors. For instance alterations in the balance between inhibitory and excitatory influences within the RVLM, which can contribute to the differences observed in baroreflex function could contribute to altered baroreflex function in pregnancy. While the last study in this dissertation provided data on the regulation of excitatory and inhibitory influences in the RVLM, a further understanding of neural connections within the baroreflex pathway is needed. Future experiments would be
designed towards the further evaluation of these connections and extending these experiments to pregnant rats.
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