

UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Serotonin 5HT-1A Receptor Density In The Brain Of The Spontaneously
Hypertensive Rats**

A thesis submitted in partial satisfaction of the requirements for the degree Master of
Science

in

Bioengineering

by

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Chair

University of California, San Diego

2010

Dedication

This thesis is dedicated to my loving husband Artemio Valdez Jr. I am grateful for his unconditional love, support, encouragement and humor. Thank you for believing in me when no one else did. I love you.

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ABSTRACT OF THE THESIS

**Serotonin 5HT-1A Receptor Density In The Brain Of The Spontaneously
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Professor Geert W. Schmid-Schönbein

Hypertension is associated with an increased risk for cardiovascular disease, renal failure and stroke. Recent evidence indicates a strong relationship between sleep and hypertension. Neuronal serotonin has been identified as a neurotransmitter that is involved in many processes necessary for the control of both sleep and waking. Serotonin

5HT-1A receptors have been implicated to be involved in the regulation of sleep and waking.

The objective of this study is to examine the extracellular domain receptor density for serotonin 5HT-1A. The extracellular domain density of the Serotonin 5HT-1A receptor was compared between control Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) in the hypothalamic region of the brain. Additionally, a group of animals were treated with an MMP inhibitor, doxycycline for 26 weeks. At the end of the treatment, systolic blood pressure, proteolytic activity and 5HT-1A receptors extracellular domain receptor density were measured.

Immunofluorescence results showed that 5HT-1A extracellular domain receptor density is significantly lower in SHR in the hypothalamic region of the brain compared to that in WKY ($p < 0.05$). After doxycycline treatment, the systolic blood pressure was suppressed in SHR and WKY. A significant decrease in systolic blood pressure was noted between the doxycycline treated and non-treated groups ($p < 0.05$). After doxycycline treatment, serotonin 5HT-1A extracellular domain receptor density was significantly increased in the treated SHRs ($p < 0.05$). These results suggest that doxycycline can increase extracellular domain receptor density.

Chapter 1: Introduction

1.1 Hypertension

Hypertension is defined as having a diastolic pressure equal or above 90 mmHg or a systolic pressure equal or above 140 mmHg (2). It has been identified as a multifactorial disease due to the effect of a combination of inherited, behavioral and environmental components (2,15). Chronic hypertension has been associated with an increased risk of heart disease, kidney disease, hardening of the arteries (arteriosclerosis), eye damage and stroke. These complications resulting from hypertension are referred to as end-organ damage.

Among different models of hypertension available today (13,21,31), the focus of my thesis will be on the genetic form of hypertension. Hence, I'll be studying the spontaneously hypertensive rat (SHR) and its normotensive control, the Wistar-Kyoto rat (WKY) (36). This model develops chronic (long duration) high blood pressure without any further intervention in our environment, analogous to essential hypertension in humans.

1.2 Chronic hypertension and inflammation

Recent evidence suggests a strong association between inflammation and hypertension. SHRs exhibit elevated levels of activated neutrophils, monocytes and other leukocytes in the circulation, as well as, the expression of iNOS and other vascular inflammatory markers than Wistar-Kyoto rats (WKYs) (15,17,38). In hypertensive's, leukocytes exhibit enhanced cytotoxicity and exhibit spontaneous degranulation of their neutrophils (33).

The presence of oxygen free radicals production and extensive apoptosis also serve as markers for inflammation. The SHRs have high levels of infiltration of lymphocytes and macrophages in the kidney and they play an important role in the development and maintenance of hypertension (21).

In contrast, acute inflammation can also cause a lowering of the blood pressure. In a few studies, Lyon hypertensive rats treated with silica (a selective in-vivo toxin to macrophages) exhibited lower blood pressure, as well as, decreased left ventricle weight (2).

1.3 Chronic hypertension and end-organ damage

There exists a need to understand the molecular mechanism that contributes to arterial hypertension because epidemiological evidence indicates that hypertensive patients are at a major risk of cardiovascular disease, such as kidney failure and stroke (17). For example, hypertensives exhibit cardiovascular lesions and various pathophysiological processes, including endothelial activation, inflammation and thrombogenesis, which contribute to the development of end-organ damage (31). In 1999, Luft et al. investigated a double transgenic rat (dTGR) model, in which rats transgenic for the human angiotensinogen and renin genes were crossed. These rats developed moderately severe hypertension but died of end-organ cardiac and renal damage by week 7. Evidence of end-organ damage was seen in sections of the heart and kidney, which exhibited necrosis and fibrosis (22).

1.4 Chronic hypertension and sleep

Sleep is often considered to be a restorative and refreshing process. It is characterized by complex activity of the cardiovascular autonomic mechanisms and by changes of arterial pressure and heart rate (20). Sleep studies show that fluctuations and variability of cortical and visceral activities involve different autonomic regulation of the cardiovascular system in relationship to the various sleep cycles (10).

For example, rapid-eye movement (REM) sleep is characterized by periods of relative hypertension and tachycardia. On the other hand, non-rapid eye movement (NREM) sleep, arterial pressure and heart rate tend to decrease. In regards to cardiovascular autonomic modulation, non-REM sleep is characterized by a decrease of sympathetic activity but an increase of parasympathetic activity. While, during REM sleep, a relative increase in sympathetic activity is demonstrated by an increased sympathetic outflow to muscle blood vessels (41,43). It has been shown that study subjects exhibiting higher vagal activity during sleep stage 0 reached sleep stage 4 (slow wave sleep) within 90 minutes, while those who had lower vagal activities did not reach stage 4 within the same period (23).

Other studies report that sleep disturbances and disorders (ie, sleep-related breathing disorders) may represent potential contributors to the initiation and progression of cardiovascular diseases. For example, sleep deprivation may cause an increase in blood pressures. Lusardi et al noted that in hypertensive patients, sleep deprivation induced increases in blood pressure, heart rate and urine norepinephrine on the morning after a night of inadequate sleep (16).

Sleep-wake regulating systems have been proposed to be located in various nuclei of the hypothalamus and the brain stem (35). These sleep-wake related nuclei largely overlap with the autonomic related nuclei. For example, the hindbrain sleep-wake regulating system is anatomically close to the autonomic nuclei in the brain stem. The parasympathetic center (dorsal motor nucleus and nucleus ambiguus) is directly regulated by the hindbrain sleep center, whereas the sympathetic center (rostral ventrolateral medulla) is modulated by the hindbrain wake center (18,19).

Since the autonomic nervous system is closely related to the regulation of blood pressure, Kuo et al (2003) explored whether spontaneous hypertension is associated with changes in sleep patterns. The study was able to confirm that the autonomic nervous system activity is related to the sleep stages, including active waking (AW), quiet sleep (QS or non-rapid eye movement) and paradoxical sleep (PS or rapid eye movement). Macrostructural and microstructural analysis of sleep patterns were carried out. Analysis of sleep revealed that when compared to WKY, SHR exhibit fewer QS and PS episodes and eventually shorter accumulation of QS and PS. In comparison to WKY, SHR may have less sleep time, poorer sleep quality and a greater tendency to wake up from QS. Such changes in sleep may be related to the cardiac autonomic changes (18,19).

Studies have provided evidence to support a strong correlation between sleep and cardiovascular diseases. Of particular interest is that the pathogenesis of hypertension is strikingly linked to sleep pathophysiology. For example, hypertensives patients whose nocturnal blood pressure falls may develop a higher degree of target organ damage, as well as, frequent cardiovascular events (6). Also, pathophysiological mechanisms (including sympathetic activation, endothelial dysfunction and oxidative stress) involved

in different sleep disturbances influence the development and progression of cardiac and vascular pathology (6,20).

1.5 Matrix Metalloproteinases (MMPs)

The matrix metalloproteinase (MMP) family consists of 24 members that are Zn^{2+} - and Ca^{2+} -dependent proteolytic enzymes that degrade extracellular matrix proteins. Based on their substrate specificity, cellular sources and transcriptional regulation, the MMP family have been classified into collagenase (eg, interstitial collagenase MMP-1 and MMP-13), gelatinase (MMP-2 and MMP-9), stromelysin (MMP-3), matrilysin (MMP-7) and membrane-type MMPs (MT1-MMP or MMP-14) (30,46).

In general, MMPs consist of a signal peptide, a propeptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths (also called the hinge region), a hemopexin (Hpx) domain of about 200 amino acids and in a few MMPs, a C-terminal domain (14,25,30). Some common structural features are the zinc-binding motif HEXXHXXGXXH in the catalytic domain and the cysteine switch motif PRCGXPD in the propeptide domain. This Cys- Zn^{2+} interaction maintains proMMP zymogen form by preventing a water molecule from binding to the zinc atom and causing catalysis (45).

MMPs are usually minimally expressed in normal physiological conditions, thus maintaining homeostasis. However, MMPs activities are specifically regulated by various factors, including hormones, growth factors, and cytokines (46). Endogenous MMP inhibitors (MMPIs) and tissue inhibitors of MMPs (TIMPs) control these enzymes. Over-expression or under-expression of MMPs results in an imbalance between activity of MMPs and TIMPS that can lead to a variety of pathological diseases, such as

cardiovascular disease, inflammation and cancer (45,46). Numerous studies have shown that MMPs play central roles in the progression of diseases such as atheroma, arthritis and cancer (45).

1.6 The link between hypertension and Matrix Metalloproteinases (MMPs)

The family of matrix metalloproteinase (MMP) and the natural tissue endogenous inhibitors (TIMPs) are involved in the regulation of the extracellular matrix (ECM) structure and metabolism. (46).

But the role of MMPs in hypertension is not entirely understood. Several studies have established an association between MMPs and hypertension, especially in the context of arterial wall hypertrophy (14). Some studies show that MMP-2 and -9 activities contribute to hypertensive remodeling and degradation of the blood-brain barrier during occlusion with reperfusion (14). Other studies show that diminished levels of MMP-2 and/or TIMP activity in the plasma of hypertensive people facilitates accumulation of types IV and V collagen and fibronectin on the vascular wall (25).

Recently our laboratory has shown that activation of select MMPs in the microcirculation cause diverse cell dysfunction by a receptor cleavage mechanism. Elevated levels of MMP-2, -9 and -7 in plasma of SHR cause cleavage of the extracellular, but not the intracellular domain of VEGFR-2 on endothelium, as well as, the insulin receptor (9,42) and are therefore responsible for capillary rarefaction and insulin resistance in the SHR, respectively.

1.6.1 Inhibition of MMPs using Doxycycline

Doxycycline is a broad – acting MMP inhibitor. Doxycycline is also a member of tetracycline family of antibiotics and has been used for the experimental treatment of many pathological conditions that involve MMPs (9). DeLano et al (2008) treated WKYs and SHRs with doxycycline (55 mg in drinking water, \approx 5.4 mg/kg per day) for 24 weeks. After doxycycline treatment, the blood pressure of the SHRs and WKYs was significantly reduced. The blood pressure of the doxycycline treated SHRs reached values similar to that of WKY rats prior to the treatment. The mechanism by which doxycycline reduces blood pressure may involve blockade of MMP activity that is involved in the cleavage of the beta-2 adrenergic receptor (32). Plasma proteolytic activity was significantly higher in the SHRs and was reduced by doxycycline treatment. Along with a reduction in proteolytic activity after doxycycline treatment, there was also significant enhancement in the average density of the insulin receptor label on leukocytes in both WKY rats and SHRs as well as other receptors involved in the pathophysiology of the SHR (9,42).

1.7 Serotonin and Sleep/Wake Cycle

Serotonin (5-hydroxytryptamine (5-HT)) has been implicated to play an important role in the sleep and wake cycle. Neuronal serotonin is involved in various physiological functions, including feeding, aggression, thermoregulation, motor activity, pain modulation, mood, learning and memory (10). The relationship between brain serotonin, sleep and wake is complex. Early studies indicate that serotonin (5-HT) is involved in the initiation and maintenance of sleep, while later studies associate the serotonergic neurons

to play a role in inhibiting sleep (27). As a neurotransmitter, it acts on many different receptors, therefore, it may be involved in many processes that are necessary for the control of both sleep and waking, which depends on the localization in the brain and the type of receptor it acts on (11,44). The intricate effects of 5-HT in the sleep-wake cycle is because 5-HT can act at different areas of the brain that have been associated with the control of sleep and wake and that different 5-HT receptors are selectively involved in the regulation of various sleep stages.

1.7.1 Serotonin as a sleep neurotransmitter

Early studies have shown that injecting low doses of 5-HT into the lateral ventricle in cats lead to drowsiness and sleep, after an initial period of arousal. Other studies have shown that lesions of serotonin containing cell bodies in the raphe nuclei of the brainstem (rostral group – the dorsal and medial raphe nuclei) in cats resulted in loss of sleep (41). Similarly, serotonin formation in the brain of cats was blocked by PCPA (*p*-chlorophenylalanine), which is reduced to abolished sleep in cats. Thus, serotonin seemed to be indeed a sleep substance, and high brain serotonin levels not only permit but also facilitate sleep and possibly are actually necessary for sleep to occur (43).

1.7.2 Serotonin as a wake neurotransmitter

Recent evidence made the serotonin-sleep relationship much less clear. Raphe lesions in rats were not as effective as lesions in cats in reducing sleep. In a study, serotonin formation in the brain was blocked with PCPA (*p*-chlorophenylalanine), which reduced and even abolished sleep in cats. However, following chronic PCPA treatment, sleep in cats reappeared, despite brain levels of serotonin being extremely low (27).

1.7.3 Multiple serotonergic receptors

Serotonin, as a neurotransmitter acts on many different receptors resulting in diverse serotonergic effects on sleep and waking dependent on receptor type. Serotonin receptors, also known as 5-hydroxytryptamine (5-HT) receptors are a group of G-protein coupled receptors and ligand-gated ion channels found in the central and peripheral nervous system (28). A few types of serotonin receptors are the 5HT-1A, 5HT-1B, 5HT-2 and 5HT-3. They mediate both excitatory and inhibitory neurotransmission. These receptors are activated by the neurotransmitter serotonin, which serves as their natural ligand (4).

These 5-HT receptors regulate the release of many neurotransmitters, including glutamate, GABA, dopamine, epinephrine/norepinephrine, and acetylcholine. Also, modulates the release of hormones, including oxytocin, prolactin, vasopressin, cortisol and corticotropin. These 5-HT receptors influence various biological and neurological processes including aggression, anxiety, appetite, cognition, learning, memory, mood, nausea, sleep and thermoregulation (10,16).

For the purpose of this thesis, I have chosen serotonin 5HT-1A receptor as a candidate receptor involved in sleep.

1.7.4 Characteristics and localization of 5HT-1A receptor

5HT-1A receptor is a G-protein coupled receptor and consist of seven transmembrane helices connected by intra- and extracellular loops. 5HT-1A receptors can be located on cell body (soma), dendrites, axons, as well as, both pre- and postsynaptically in nerve terminals (synapses) (1).

1.7.5 The function of 5HT-1A receptors

5HT-1A receptors regulate the release of many neurotransmitters, including glutamate, GABA, dopamine, epinephrine/norepinephrine, and acetylcholine. Also, they modulate the release of hormones, including oxytocin, prolactin, vasopressin, cortisol, and corticotropin. For example, 5HT-1A receptor activation has been shown to increase dopamine release in the medial prefrontal cortex, striatum, and hippocampus. It also influences various biological and neurological processes including aggression, anxiety, appetite, cognition, learning, memory, mood, nausea, sleep, and thermoregulation. For example, activation of 5HT-1A receptors has been demonstrated to impair cognition, learning, and memory by inhibiting the release of glutamate and acetylcholine in various areas of the brain (4,10,16).

The involvement of the 5HT-1A receptors in the regulation of sleep and waking is complex due to a multitude of pre- and postsynaptic actions that also involve other neurotransmitter systems. The dorsal raphe nucleus (DRN; a midbrain nucleus) represents the largest concentration of serotonergic neurons in the central nervous system (CNS) (26) and has been extensively studied for presynaptic function of the 5HT-1A, which act as autoreceptors. A study has shown that activation of these presynaptic autoreceptors produces a series of events leading to decreased serotonergic neurotransmission (feedback mechanism). On the other hand, postsynaptic 5HT-1A receptors are found on multiple serotonergic targets in various regions of the brain, including forebrain and brainstem (29,41,43). Therefore, the effects due to the activation of 5HT-1A receptors mainly depend on their localization. As a result, this receptor is involved in a variety of

functions including aggression, anxiety, depression, feeding, alcohol intake, hormone secretion, temperature regulation and state modulation.

1.7.6 Role of 5HT-1A receptors in sleep and wake state

Few studies have shown that the spontaneous firing of the DRN (dorsal raphe nucleus) serotonergic neurons, as well as, the local level of extracellular serotonin changes as a function of behavioral state (higher levels in wake (W), lower levels in slow-wave sleep (SWS) and lowest levels during rapid eye movement (REM) sleep) (6,41). Various studies have used selective 5HT-1A agonists and antagonists to study serotonin related state modulation. For example, application of 8-OH-DPAT into the DRN (dorsal raphe nucleus) results in a decrease in the local level of extracellular serotonin, as well as, in the levels of the main projection site in the hippocampus (11). On the other hand, application of a 5HT-1A antagonist (p-MPPI) causes an increase in the local levels of extracellular serotonin (16). What matters is that the effect of the agonist and antagonist differ mainly on the dose administered. For example, low doses have a local effect on the very sensitive autoreceptors, whereas high doses reach the widespread postsynaptic sites producing a succession of events (11,41).

The involvement of the 5HT-1A receptors in the regulation of sleep and waking is complex due to a multitude of pre- and postsynaptic actions that also involve other neurotransmitter systems. Low doses of the 5HT-1A agonists act predominantly presynaptically, whereas higher doses have wider reach producing extensive stimulation of postsynaptic 5HT-1A receptors. Monti and collaborators showed that a low dose (0.01 mg/kg s.c.) administration of 8-OH-DPAT decreases wakefulness and increase slow-wave sleep (SWS). On the other hand, at high dosage (0.1 and 0.375 mg/kg s.c.)

increases wakefulness and decreases SWS and rapid eye movement (REM) sleep (13,44). Similarly, De St. Hilaire-Kafi and collaborators showed that a high dose of 8-OH-DPAT (0.3 mg/kg s.c.) produced a significant increase in wakefulness and REM sleep latency and a decrease in SWS and REM sleep, whereas low doses (0.03 and 0.1 mg/kg s.c.) produced a tendency towards decrease in wakefulness and an increase in SWS (29).

During wakefulness and sleep deprivation, the release of serotonin is higher than during sleep, which causes continued or frequent stimulation of receptors resulting in a gradual diminish in their functional reactivity. Kreiss et al. showed that repeated injection of an agonist resulted in 5HT-1A receptors desensitization (22). In 2005, Roman et al. wanted to establish a relationship between chronic sleep loss and changes in 5HT-1A. Adult Wistar rats were subjected to a schedule of restricted sleep by allowing them only 4 hours of sleep per for eight days. They measured the sensitivity of the 5HT-1A receptor system by measuring the physiological response to a subcutaneous injection with the serotonin 1A agonist (8-OH-DPAT; 0.25 mg/kg body weight). This drug has been shown to cause a hypothermic response, which can be used as an indicator of the central 5HT-1A neurotransmission in rats and humans. Their results showed that, chronic sleep deprivation results in a gradual and persistent desensitization of the 5HT-1A receptor system. Even with unlimited recovery sleep the desensitization of the 5HT-1A system persisted for many days (32).

1.8 Goals of this Investigation

Kuo et al (2003) was successfully able to confirm that the autonomic nervous system activity is related to the sleep stages, including active waking (AW), quiet sleep (QS or non-rapid eye movement) and paradoxical sleep (PS or rapid eye movement).

Analysis of sleep revealed that when compared to WKY, SHR exhibit fewer QS and PS episodes and eventually shorter accumulation of QS and PS. In comparison to WKY rats, spontaneously hypertensive rats (SHRs) sleep is characterized by less sleep time, poorer sleep quality and a greater tendency to wake up from quiet sleep (QS) (18,19).

Serotonin (5-hydroxytryptamine (5-HT)) has been implicated to play an important role in the sleep and wake cycle. As a neurotransmitter, it acts on many different receptors, therefore, it may be involved in many processes that are necessary for the control of both sleep and waking, which depends on the localization in the brain and the type of receptor it acts on (10). One such receptor is the serotonin 5HT-1A receptors. Its involvement in the regulation of sleep and waking is complex due to a multitude of presynaptic and postsynaptic actions that also involve other neurotransmitter systems (1,28).

SHRs exhibit higher MMP activity when compared to WKY. Recent evidence from our lab, suggests that activation of select MMPs in the microcirculation cause diverse cell dysfunction by a receptor cleavage mechanism. Elevated levels of MMP-2, -9 and -7 in plasma of SHR cause cleavage of the extracellular, but not the intracellular domain of VEGFR-2 on endothelium, as well as, the insulin receptor (9,42).

Therefore, knowing that SHRs have altered sleep and wake cycle, as well as, exhibit elevated levels of proteolytic activity and that serotonin 5HT-1A receptors plays a major role in the sleep and wake cycle, my overall aim for this study is to determine the extracellular domain receptor density of serotonin 5HT-1A in the hypothalamus. In this study, immunofluorescence was performed to determine the difference in the serotonin 5HT-1A extracellular domain receptor densities in the SHRs compared to the WKY rats.

To analyze proteolytic activity, gelatin gel zymography was carried out. Another aim for this study is to study the effect of doxycycline treatment on blood pressure, proteolytic activity, as well as, serotonin 5HT-1A extracellular domain receptor density.

1.8.1 Objective

Examine the correlation between protease activity and receptor density in the brain of SHR and WKY rats.

1.8.2 Hypothesis

We hypothesize that due to elevated level of proteolytic activity in SHR brain, receptor cleavage of serotonin 5HT-1A receptor occurs. We also hypothesize that after the doxycycline treatment, serotonin 5HT-1A extracellular domain receptor density increases along with a decrease in proteolytic activity.

1.8.3 Specific Aims

1. Determine the Serotonin 5HT-1A extracellular domain receptor density in WKY and SHR rats.
 - a. Compare Serotonin 5HT-1A extracellular domain receptor density in SHR and WKY by immunofluorescence in the hypothalamus.
2. Determine the effect of doxycycline on blood pressure, serotonin 5HT-1A extracellular domain receptor density, as well as, protease activity in WKY and SHR brain.
 - a. Determine the systolic blood pressure in doxycycline treated and non-treated animal groups.
 - b. Determine by immunofluorescence Serotonin 5HT-1A receptor expression levels in SHR and WKY with and without doxycycline treatment.

c. Determine proteolytic activity in SHR and WKY with and without doxycycline treatment by gelatin gel zymography.

Chapter 2: Methods

2.1 Animals

The study was reviewed and approved by the University of California San Diego Animal Subjects Committee. Adult, Male Spontaneously Hypertensive Rats (SHR) at 34-38 weeks of age and their normotensive controls, Wistar-Kyoto Rats (WKY) (Charles River Laboratories, Wilmington, MA, USA) of comparable age were studied under general anesthesia (Nembutal, 50 mg/ml, 1 ml/kg bodyweight, i.m.) (Pentobarbital Sodium Injection, Ovation Pharmaceuticals, Inc., Deerfield, IL). After 20 minutes, reflex level was tested with a toe pinch. Animals which were responsive were given an additional waiting period of 5 -10 minutes and tested again. All of the rats utilized in this study were fully anesthetized and unresponsive to the toe pinch after this additional waiting period.

Following anesthesia, rats were secured to a cutting board resting atop a heating pad to maintain body temperature at 37°C. The fur was shaved and the left femoral artery and left femoral vein were cannulated. The mean arterial pressure and heart rate were recorded by a laboratory computer (Power Macintosh G3 with MacLab, Apple Computer Company, Cupertino, CA). Supplemental doses of anesthesia were administered intravenously at a dose of 5 mg/kg as needed after reflex testing. At the end of the study, the animals were euthanized (Fatal-Plus 120 mg/kg body weight i.v.).

2.2 Treatment Protocol

Subgroups of the WKY rats and SHRs were treated with doxycycline (55mg in drinking water, \approx 5.4 mg/kg per day for 11 weeks). At the end of 11 weeks, the

concentration of doxycycline was increased by 20% (from 55 mg to 66 mg in drinking water, \approx 6.5 mg/kg per day for 15 weeks). The drug was given in drinking water for a total time period of 26 weeks. Untreated group received standard chow and water.

2.3 Determination of Systolic Blood Pressure

Two animals from each group were measured by the tail-cuff method. The blood pressure was measured every week by the same investigator and at the same time of day.

2.4 Tissue Preparation and Embedding

At the end of surgery, the brain was removed from the animals and dissected into small portions for immunofluorescence and gelatin gel zymography. For immunofluorescence, a portion of the brain was embedded in Tissue-Tek O.C.T (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA) and snap frozen by immersion into 2-methylbutane chilled in liquid nitrogen. The frozen tissues were stored in -80°C prior to sectioning. For the gelatin gel zymography, small portions of the brain were placed in 1.5 ml tubes containing 500 μl CellLyticTM Mammalian Tissue Lysis/Extraction Buffer (Sigma) and frozen in liquid nitrogen. The frozen tubes were stored at -80°C .

2.5 Tissue Sectioning

Each brain sample was sectioned using a Leica CM 3500 cryostat. Brain sections with thickness of 5 μm were mounted on a microscope slide (Fisherbrand Superfrost Plus Microscope Slides, Fisher Scientific). Each slide carried one WKY sample and one SHR sample side by side to maintain standard conditions for all further analysis. Slides were then stored at -20°C until further use.

2.6 Immunofluorescence Labeling of 5HT-1A Receptor

Frozen 5 μm brain sections were fixed with acetone at -20°C for 10 minutes, air-dried and washed twice in PBS with 0.05% Triton X-100. Non-specific immunoadsorption was blocked by incubating sections with blocking solution (10% normal goat serum, 2% BSA, 0.05% Triton X-100 in PBS [pH 7.4]) and incubated at room temperature for 1 hour. Followed by 15 minutes avidin blocking and 15 minutes biotin blocking (kit SP-2001, Vector Laboratories Inc). Sections were washed twice in PBS with 0.05% Triton X-100. Rabbit polyclonal anti-5HT-1A, 1:500 (905-741-100, Assay Designs) antibody was diluted in blocking solution and applied overnight at 4°C . Controls for immunostaining included buffer alone without primary antibody or nonspecific purified rabbit immunoglobulin G (IgG).

The next day, sections were washed three times, 15 minutes each, in PBS with 0.05% Triton X-100. Secondary biotinylated anti-rabbit IgG (BA-1000, Vector Laboratories Inc) was added to a final dilution of 1:1000 and incubated for 1 hour at room temperature. Sections were washed twice in PBS with 0.05% Triton X-100. To detect biotin-labeled probes, the sections were incubated with Texas red avidin D (A-2006, Vector Laboratories Inc) at a dilution of 1:1000 in blocking solution for 1 hour at room temperature. Sections were then washed four times, 15 minutes each, and mounted with 4',6-diamidino-2-phenylindole (DAPI) containing Vectashield mounting medium (H-1200, Vector Laboratories Inc)

I also tried antibodies to various sleep and wake related neurotransmitters, including γ -aminobutyric acid (GABA), serotonin and hypocretin. However, I could only

get the serotonin 5HT-1A extracellular antibody (905-741-100, Assay Designs) to work on frozen sections.

2.7 Gelatin Gel Zymography Protocol

Frozen brain samples were homogenized, centrifuged and the supernatant collected and stored at -80°C . Protein content in the samples was measured by the BCA method with a commercial kit (Pierce Co). Protein concentrations for all four groups (control WKY, control SHR, doxycycline treated WKY and doxycycline treated SHR) were similar. Samples (40ug of total protein extracts) were separated on a 10% SDS gel with porcine gelatin (0.1g/1ml) at 100V (constant voltage) for 1.5 hours. The gels were washed four times, 15 minutes each, in renaturing buffer (2.5% v/v triton x -100) with gentle agitation at room temperature. Then, the gels were incubated in 30ml of buffer containing 50 mM Tris – HCl, pH 7.5, 10 mM CaCl_2 and 0.02% NaN_3 , for 24 hours at 37°C . Incubation in the presence of 20 mM EDTA was carried out as a control of the specificity of the gelatinolytic reaction. After incubation, gels were stained with Coomassie Blue R-250 for 2 hours and were unstained by immersing in acetic acid:methanol:water (1:5:4) until areas of gelatinolytic activity appeared as clear sharp bands against the blue background. Images of the gels were captured with a camera (Nikon) for band analysis.

2.8 Data Analysis

All immunofluorescence images were digitized and processed with Image J (NIH, <http://rsbweb.nih.gov/ij/>). Non-specific IgG image (served as the control image) was converted to 8-bit image resulting in a black image from which the threshold of the background was determined. Then I applied the above measured threshold to all the other

images of each animal groups. Applying the threshold, resulted in the elimination of the background and only the red spots (signifying positive signal) was visible (Fig 2-1). The fluorescence light intensity of the red label in the receptor clusters was digitally measured.

Statistical Analysis

All measurements are presented as mean \pm standard deviation. Comparisons of mean values between animal groups were carried out by two – tailed student's t-test. $p < 0.05$ was considered statistically significant.

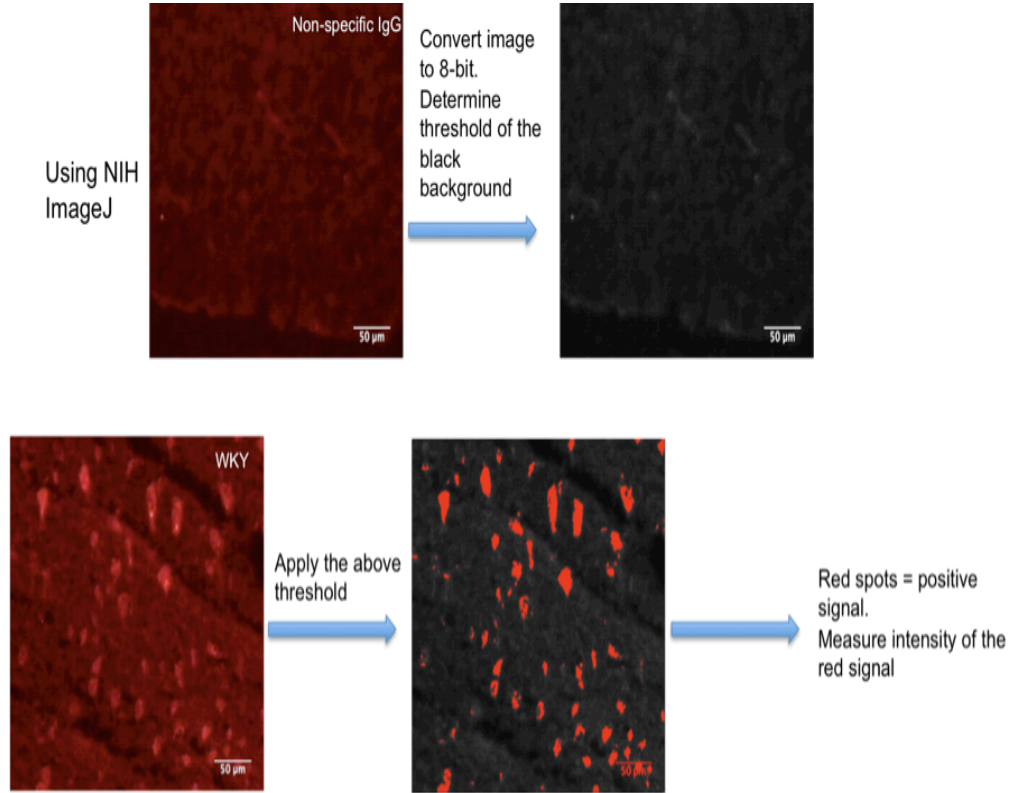


Figure 2-1. Immunofluorescence image analysis overview

Chapter 3: Results

3.1 Serotonin 5HT-1A extracellular domain receptor density

Serotonin 5HT-1A extracellular domain receptor density was detected using immunofluorescence with primary antibody against the extracellular domain in the brain of WKY and SHR. Compared with WKY rat, the SHR had on average a 17% lower receptor density ($p < 0.05$) in the hypothalamus region of the brain (Fig 3-1).

3.2 Suppression of blood pressure by doxycycline treatment

Chronic doxycycline treatment served to reduce the elevated blood pressure of SHRs (Fig 3-2). At the end of 26 weeks of treatment, the systolic blood pressure was significantly reduced to 156 ± 11 mmHg in doxycycline-treated SHRs compared to 205 ± 14 mmHg in non-treated SHRs ($p < 0.05$). Similarly, the systolic blood pressure for doxycycline-treated WKY was significantly reduced to 117 ± 9 mmHg compared to 169 ± 11 mmHg in non-treated WKYs ($p < 0.05$).

3.3 Serotonin 5HT-1A extracellular domain receptor density after doxycycline treatment

The extracellular domain density of the Serotonin 5HT-1A receptor in the hypothalamic region of the brain after doxycycline treatment was increased on average by 8% compared with non-treated SHR ($p < 0.05$) whereas no significant difference was detected between doxycycline-treated WKY and non-treated WKY (Fig 3-3).

Similarly, 5HT-1A receptor clusters/ 0.2mm^2 in the hypothalamic region of the brain after doxycycline treatment was increased on average by 23% compared with non-

treated SHR ($p < 0.05$) whereas no significant difference was detected between doxycycline-treated WKY and non-treated WKY (Fig 3-4).

3.4 Protease activity after doxycycline treatment

Proteolytic activities in brain homogenate were examined by gelatin gel zymography, which revealed a single proteolytic band at a molecular weight of about 60 kD. This ~60 kD proteolytic activity was significantly elevated ($p < 0.05$) in SHRs homogenate compared to WKY rats (Fig 3-5). However, doxycycline treatment did not attenuate the ~60 kD proteolytic activity in SHRs and WKY rats. As a control, ~60 kD proteolytic activity was blocked in-vitro by metal chelation (EDTA) and the MMP inhibitor, GM6001, but not with the serine protease blocker phenylmethylsulfonyl fluoride (PMSF).

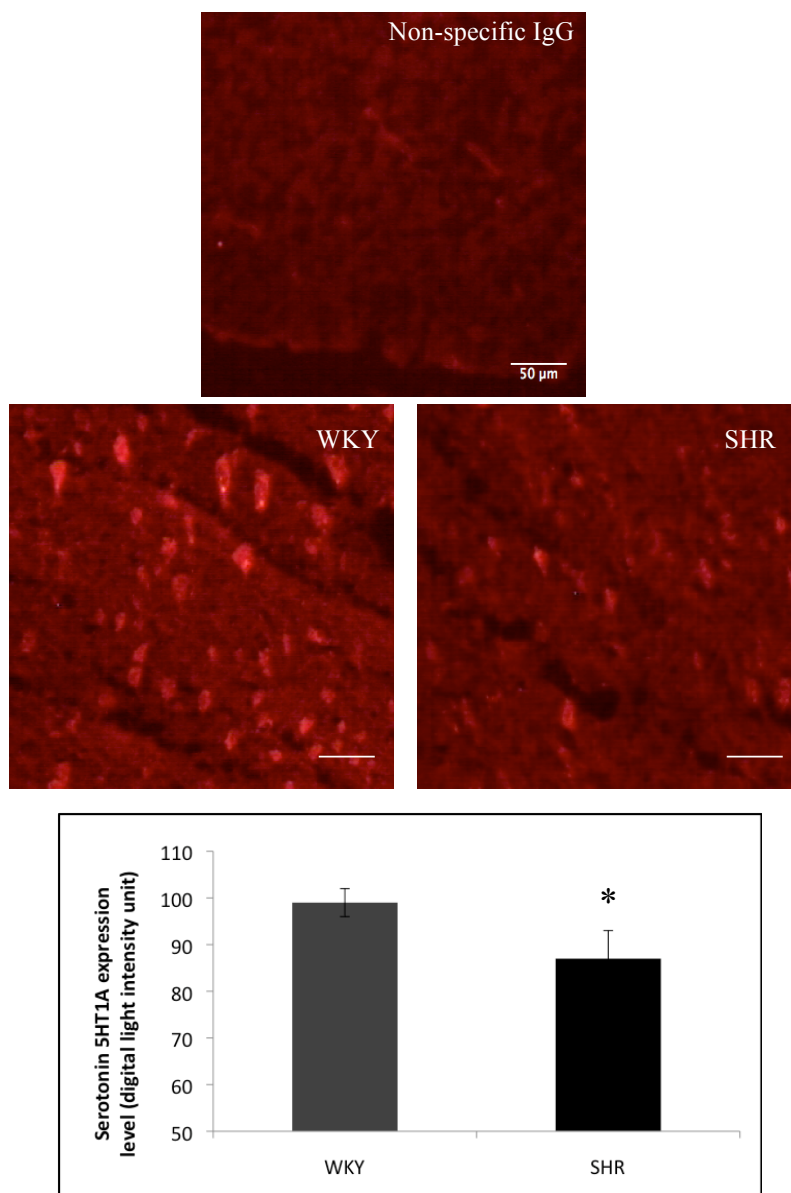


Figure 3-1. (Top panels) Micrographs of frozen immunohistochemical sections primary antibody against serotonin 5HT-1A extracellular domain as seen by Texas Red substrate in hypothalamus. *Non-specific IgG* is with an irrelevant primary antibody and *WKY* and *SHR* is for Wistar-Kyoto and Spontaneously Hypertensive Rat, respectively. Scale bar = 50 μm.

(Bottom bar graph) Light absorption measurements over whole tissue expressed as digital units. The SHR has lower expression of 5HT-1A receptors in hypothalamus. N=6 rats for each group. * $p < 0.05$ compared to WKY in student's t-test.

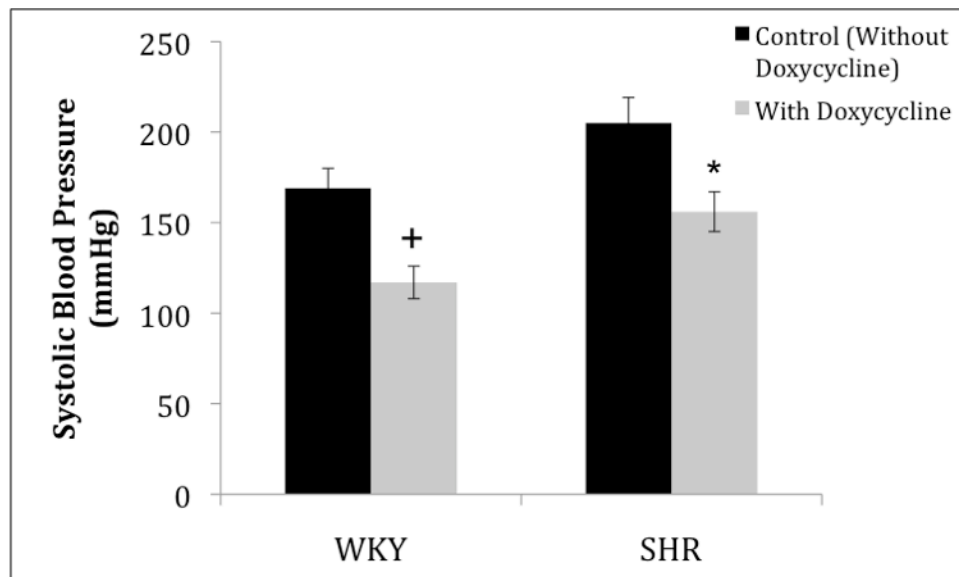


Figure 3-2. Systolic blood pressure measurement in conscious age-matched SHR and WKY with and without treatment with doxycycline. N=6 rats for each group. * $p < 0.05$ control SHR vs. doxycycline-treated SHR in student's t-test. + $p < 0.05$ control WKY vs. doxycycline-treated WKY in student's t-test.

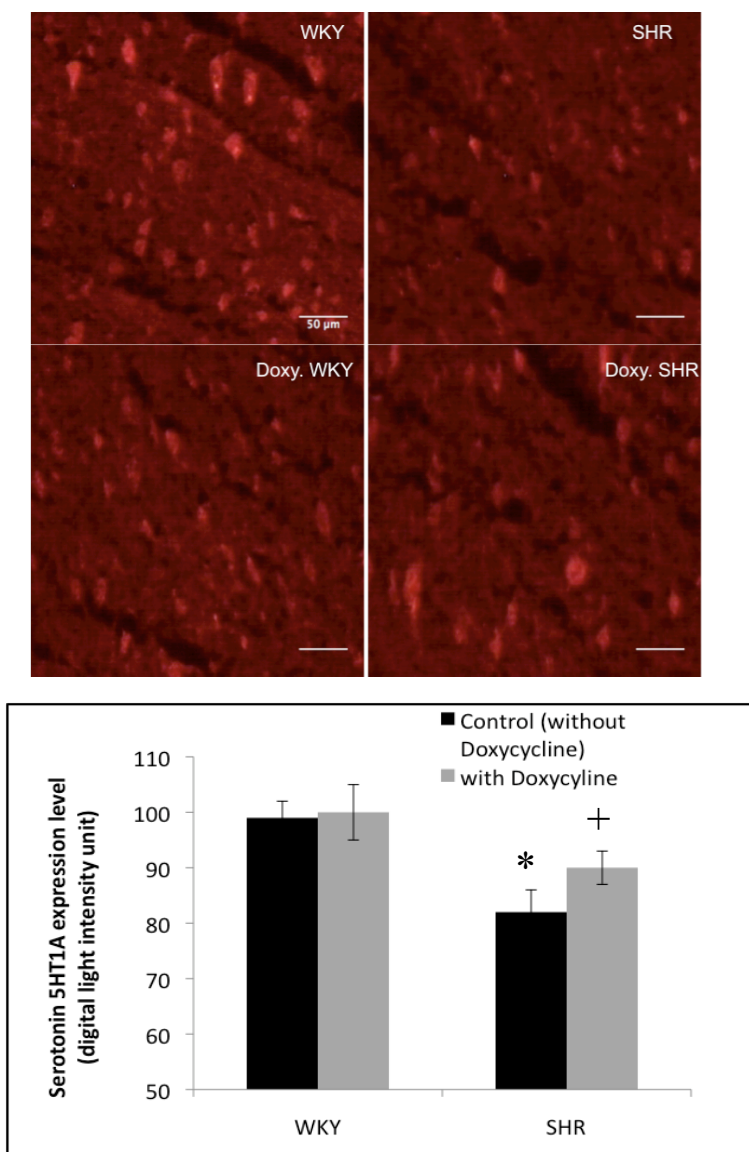


Figure 3-3. Micrographs (top panels) and light intensity measurements (bar graph below) of 5HT-1A expression level in the hypothalamus without (top images) and with (bottom images) doxycycline treatment. For explanation of cases, images and measurements please see Figure 3-1 legend. The doxycycline-treated SHR has higher expression level of 5HT-1A receptors compared to non-treated SHR. * $p < 0.05$ compared to WKY. + $p < 0.05$ compared to non-treated SHR in student's t-test. $N = 6$ rats for each group. Scale bar = 100 μm .

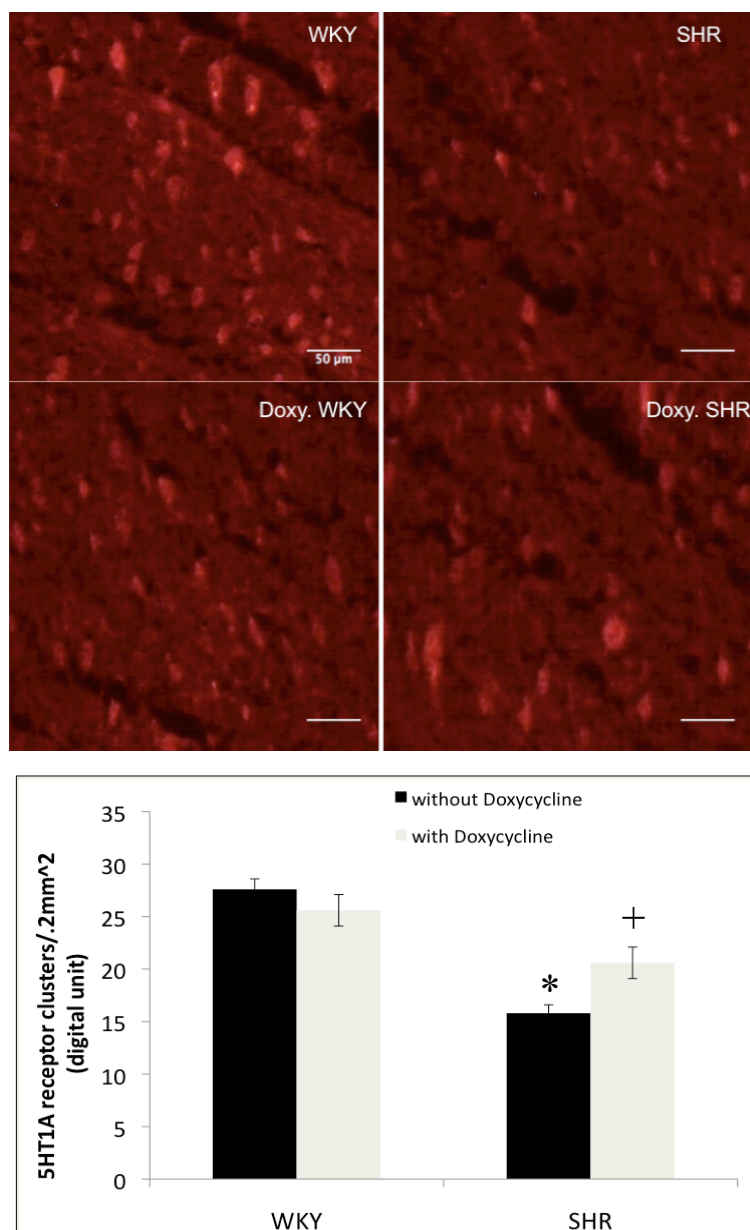


Figure 3-4. Micrographs (top panels) and # of receptor cluster/area (bar graph below) of 5HT-1A expression level in the hypothalamus without (top images) and with (bottom images) doxycycline treatment. For explanation of cases, images and measurements please see Figure 3-1 legend. The doxycycline-treated SHR has higher number of receptor cluster / .2 mm² of 5HT-1A receptors compared to non-treated SHR. *p<0.05 compared to WKY. +p<0.05 compared to non-treated SHR in student's t-test. N=6 rats for each group. Scale bar = 100 μm.

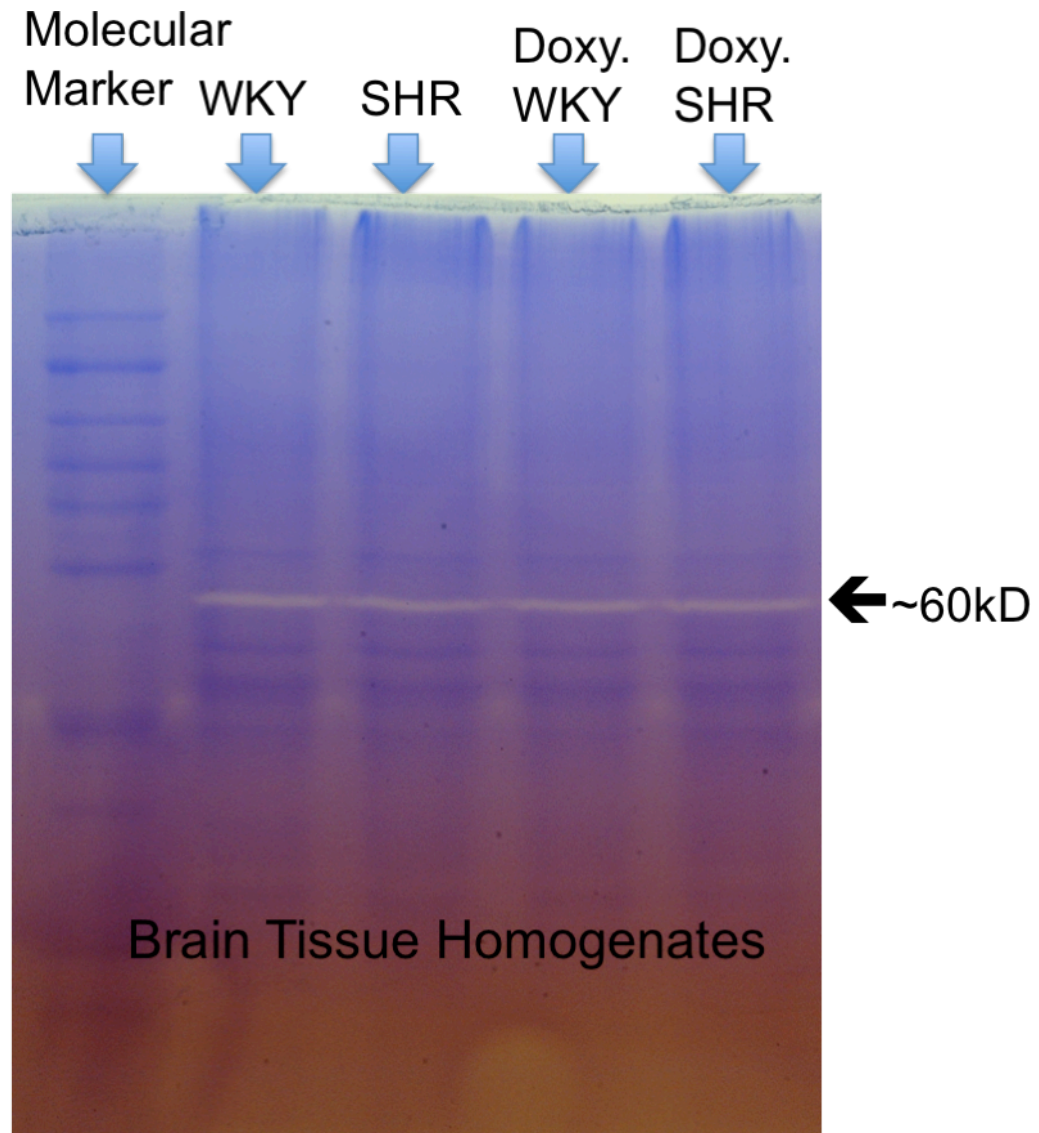


Figure 3-5. ~60kDa activity in WKY and SHR brain without and with doxycycline treatment determined by gelatin gel zymography. The protease activity in brain was confirmed with gel zymography using molecular weight standards for confirmation.

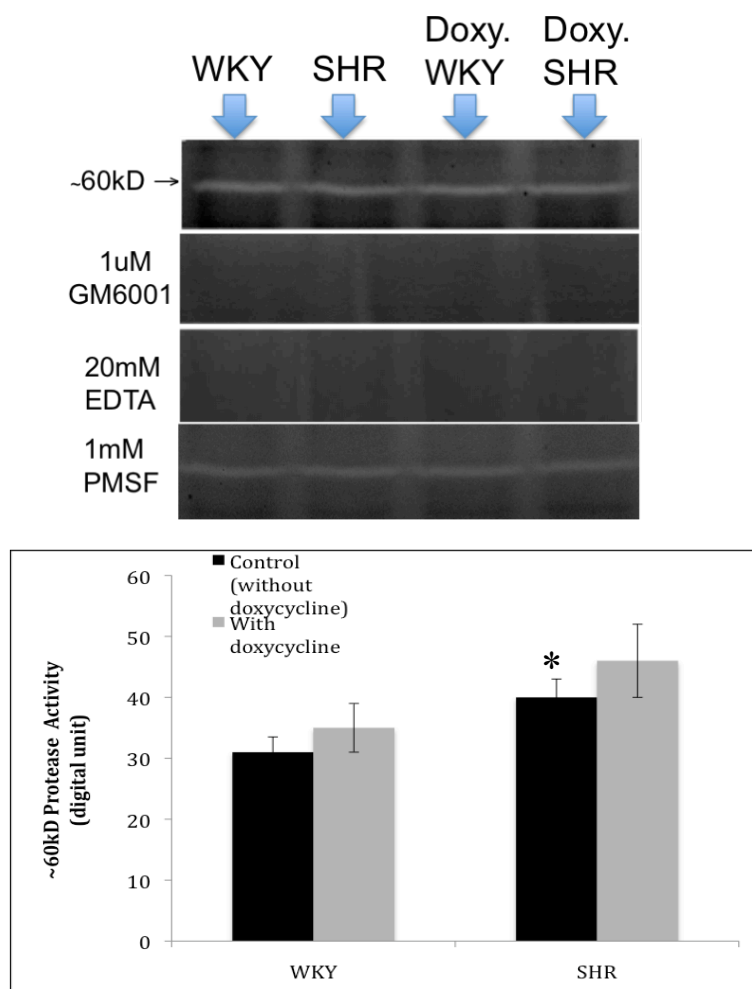


Figure 3-6. ~60kDa activity in WKY and SHR brain without and with doxycycline treatment determined by gelatin gel zymography. The protease activity in brain was confirmed with gel zymography using molecular weight standards for confirmation. As control, all protease activity is blocked in-vitro by metal chelation (EDTA) and general MMP inhibitor (GM6001) but not with PMSF. * $p < 0.05$ compared to non-treated WKY. N=6 for each group.

Chapter 4: Discussion

The current results indicate that the serotonin 5HT-1A extracellular domain receptor density is lower in SHR compared to the WKY. Chronic doxycycline treatment can significantly increase serotonin 5HT-1A extracellular domain receptor density and attenuate systolic blood pressure in the SHR.

In comparison to WKY rats, SHRs exhibit poorer sleep quality and quantity, elevated levels of proteolytic activity in brain tissue homogenates, as well as, reduced serotonin 5HT-1A extracellular domain receptor density in the hypothalamus.

4.1 Serotonin 5HT-1A extracellular domain receptor density

To examine serotonin 5HT-1A extracellular domain receptor density in this study, we used immunofluorescence with anti-5HT-1A antibody targeted at the extracellular receptor domain along with fluorescent light intensity measurements as a way to quantify their density. This approach allows us to detect the serotonin 5HT-1A receptors in the hypothalamic region of the brain, which is an alternative approach to western blot analysis that requires homogenization of the tissue.

I was interested in working with a few different antibodies, including antibodies targeted at the extracellular and intracellular domains of the serotonin 5HT-2A receptor, as well as the extracellular and intracellular domains of GABA(A). However, these antibodies required the tissue to be perfusion fixed with 4% paraformaldehyde, then sectioned on a freezing sliding microtome and stored as free floating sections with thickness of about 40 μm . Since I needed tissues for both immunofluorescence and gelatin gel zymography, I had to snap freeze them by immersion into 2-methylbutane

chilled in liquid nitrogen. I tried immunofluorescence with the above antibodies on 5 μm frozen sections on a slide but I did not get any labeling. The only antibody that worked with the 5 μm frozen sections is the extracellular anti-5HT-1A antibody from assay designs.

To study extracellular domain density of the serotonin 5HT-1A receptor in the brain, I focused on the hypothalamic region. The sleep – wake regulating system has been proposed to be located in various nuclei of the hypothalamus (35). The hypothalamus forms the ventral part of the diencephalon and is located below the thalamus. It is the center for homeostasis and responsible for various endocrine function (35). For example, the hypothalamus coordinates many hormonal and behavioral circadian rhythms, complex patterns of neuroendocrine outputs, complex homeostatic mechanisms and many important behaviors. Studies have suggested that the junction lesions between the posterior hypothalamus and midbrain, results in sleepiness (22,23). Inflammation of the anterior hypothalamic resulted in insomnia (24).

In this study, we found that the extracellular domain density of the serotonin 5HT-1A receptor is significantly lower in the SHR hypothalamus compared to the WKY hypothalamus. This evidence suggests that elevated levels of proteolytic activity in SHR may results in proteolytic cleavage of the extracellular domain of the serotonin 5HT-1A receptors.

Such level of receptor cleavage is in line with extracellular receptor cleavage observed for the insulin receptor, VEGFR-2 and others (9,43). Even though the number of receptors cleaved in vivo is on average relatively low, direct functional measurements suggest that they may have a considerable functional impact (10).

4.2 Effect of doxycycline treatment

Chronic doxycycline treatment in SHR_s has shown to attenuate systolic blood pressure, enhance sleep quality and quantity (consultation and collaboration with Dr. Terry B.J. Kuo, National Yang-Ming University, Taiwan) as well as enhances serotonin 5HT-1A extracellular domain receptor density in the hypothalamus. Prior studies have shown that chronic doxycycline treatment attenuates proteolytic activity in plasma and mesentery (9,42). However, I have at the moment no direct evidence for a reduced proteolytic activity in brain tissue homogenates after such treatment even though there is an increase in 5HT-1A extracellular domain receptor density in the SHR.

4.2.1 Systolic blood pressure measurement

Delano et al., (2008) treated two types of hypertension models, SHR_s and WKY rats, with doxycycline. At the end of 24 weeks of doxycycline treatment, the blood pressure of the SHR_s and WKY rats were significantly reduced. Plasma proteolytic activity was also significantly reduced. Along with a reduction in proteolytic activity, there was also significant enhancement in the average density of the insulin receptor label on leukocytes in both WKY rats and SHR_s. These results suggest that elevated MMP activity may be responsible for proteolytic cleavage of membrane receptors in SHR. After doxycycline treatment, proteolytic activity is reduced resulting in an enhancement in the average density of the insulin receptor (9).

In this study, I followed the treatment protocol in Delano et al., (2008) (9). For the first 11 weeks, doxycycline concentration was 55 mg in drinking water. However, there was no change in blood pressure as assessed by tail-cuff measurements of systolic

blood pressure. Therefore, I increased the dose to 66 mg in drinking water (a 20% increase). Blood pressure continued to stay the same, until around week 23 when there was a sharp drop in blood pressure and stabilized within a few weeks. At the end of 26 weeks of doxycycline treatment, the systolic blood pressure was significantly decreased in the treated compared to the non – treated animals. In fact, the blood pressure of doxycycline treated SHR had reached values similar to that of WKY rats prior to the start of treatment.

In order to confirm the tail – cuff measurements of systolic blood pressure with doxycycline treatment, we randomly chose two animals from each of the four groups (control WKY, control SHR, doxycycline – treated WKY and doxycycline – treated SHR) and cannulated the femoral artery under local anesthesia to directly measure systolic blood pressure at the end of the treatment. The systolic blood pressure of the non-treated SHR was 210 mmHg, compared to 160 mmHg in the doxycycline – treated SHR. These values are similar to the values we obtained via the tail – cuff measurements.

4.2.2 Serotonin 5HT-1A extracellular domain receptor density

In the hypothalamic region of the brain, only the SHR showed significant increase in the serotonin 5HT-1A extracellular domain receptor density after doxycycline treatment.

In comparison to WKY rats, SHRs have less sleep time, poorer sleep quality and a greater tendency to wake up from quiet sleep (QS) (18,19). Studies have shown serotonin 5HT-1A receptors to play an important role in sleep and waking cycle (4,28). Protease activity studies have shown SHRs to exhibit elevated levels of proteolytic activity (9,42). Since SHRs have sleep problems as well as elevated levels of proteolytic activity, it is

possible that these proteases cause cleavage of the serotonin 5HT-1A receptors.

Unpublished preliminary evidence by the research group of Prof. Kuo (Taiwan, direct conversation) shows that doxycycline treated SHR's have better quality and quantity of sleep compared to the non-treated SHR's.

4.2.3 Protease activity

The gelatin gel zymography conducted on brain homogenates was able to identify only one band at a molecular weight of around 60 kD. Currently, we do not know the identity of this protease. We were able to block the band in vitro by metal chelation (EDTA) and general metalloproteinase inhibitor (GM6001). However, we have not been able to identify any MMPs at this molecular weight. To identify the band, we plan to carry out mass spectrometry and in situ zymography.

The results from gelatin gel zymography show that SHR's have significantly elevated proteolytic activity compared to the WKY rats. However doxycycline treatment did not attenuate the proteolytic activity in the treated animals.

Chapter 5: Conclusion

In this study, we found that serotonin 5HT-1A extracellular domain receptor density is significantly lower in SHR's hypothalamus compared to that of WKY rats. This suggests that elevated levels of proteolytic activity in SHR resulted in proteolytic cleavage of the extracellular domain of the serotonin 5HT-1A receptors. The findings in this study are also consistent with our hypothesis that doxycycline can attenuate systolic blood pressure in hypertensive rats and increase serotonin 5HT-1A extracellular domain receptor density in SHR's hypothalamus. However, further investigation needs to be carried out to identify the ~60 kD proteolytic band, as well as serotonin 5HT-1A intracellular domain receptor density.

Chapter 6: Future Experiments

1. Identify ~60kD proteolytic activity via Mass spectrometry and in-situ zymography.
2. Determine intracellular domain receptor density for serotonin 5HT-1A.
3. Determine receptor density for other neurotransmitters involved in sleep/wake cycle, including GABA and hypocretin.

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