Studies on the Role of Group II Metabotropic Glutamate Receptors in Medulla Oblongata on Blood Pressure Regulation

（血圧調節機構における延髄のグループ II 代謝型グルタミン酸受容体の役割に関する研究）

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Chapter 1

General Introduction

1.1 Pathogenesis of Hypertension

Hypertension is defined as a systolic blood pressure (SBP) ≥140 mmHg and/or a diastolic blood pressure (DBP) ≥90 mmHg (Bolívar, 2013; Lamprea-Montealegre *et al.*, 2018). It is a leading risk factor for cardiovascular diseases worldwide (World Health Organization, 2013) and requires more attention at present. Currently, the life expectancy is increasing, but it also has a positive correlation with rising hypertension cases considering that blood pressure is known to increase with age (Franco *et al.*, 2005). The pathogenesis of hypertension is multifactorial and complex, which includes dietary factors, genetics, and the neurohormonal system (Bolívar, 2013). A diet with high sodium or low potassium content increases the risk of developing hypertension. A high-sodium diet combined with low potassium increases the extracellular fluid volume (Meneely and Battarbee, 1976). When extracellular fluid is overloaded, vasoconstriction will occur, resulting in hypertension (Blaustein *et al.*, 2006). The mutations of several genes (e.g., those that cause glucocorticoid remediable aldosteronism [GRA]) have been identified as Mendelian forms of hypertension (Lifton *et al.*, 2001). In GRA, the hypersecretion of aldosterone results in high salt concentrations in the blood, leading to hypertension (Luft, 2003). In the neurohormonal system, the activation of angiotensin II may result in hypertension (Carlson and Wyss, 2008). Angiotensin II not only induces the activity of the sympathetic nervous system, but also produces reactive oxygen species, which can cause atherosclerosis (Weiss *et al.*, 2001; Carlson and Wyss, 2008). Regardless of the cause of hypertension, blood pressure control is important in reducing the probability of a severe illness such as stroke or heart failure (Lamprea-Montealegre *et al.*, 2018).

1.2 Clinical Treatments for Hypertension

The clinical treatments for hypertension are divided into nonpharmacological and pharmacological treatment (Weber *et al.*, 2014). Nonpharmacological treatment includes regular exercise and diet management (World Health Organization, 2013). Patients with hypertension are advised to reduce the salt intake, control alcohol consumption, and stop cigarette smoking (World Health Organization, 2013; Weber *et al.*, 2014). On the other hand, pharmacological treatment, which includes the use of antihypertensive medications, is offered particularly when nonpharmacological treatment alone fails to treat hypertension (Weber *et al.*, 2014). Various antihypertensive drugs exist, including alpha- and beta-adrenergic blockers, diuretics, renin-angiotensin system inhibitors, vasodilators, and/or calcium-channel blockers (Weber *et al.*, 2014; Fares *et al.*, 2016). Alpha-adrenergic blockers prevent norepinephrine from tightening the vascular walls (Nash, 1990; Weber *et al.*, 2014). Beta-adrenergic blockers inhibit epinephrine to decrease the heart rate and dilate the vessels (Frishman, 2003; Weber *et al.*, 2014). Labetalol is a combined alpha–beta-adrenergic blocker that is commonly used to treat hypertensive disorders of pregnancy (Rosenthal and Oparil, 2002). Diuretics make the kidneys excrete more sodium in urine, and are divided into three types according to the part that they act on (Shah *et al.*, 2004; Weber *et al.*, 2014). Hydrochlorothiazide is a thiazide-type diuretic that has been used in hypertension treatment for at least half a century due to its safety (Messerli and Bangalore, 2011). Renin-angiotensin system inhibitors block the activation of angiotensin II or the conversion of angiotensin I to achieve vasodilation and prevent vasoconstriction (Weber *et al.*, 2014). Captopril with a short half-life is widely used in the treatment of hypertension (Duchin *et al.*, 1988). Hydralazine, a

direct vasodilator, is prescribed in developing countries due to its low cost (Kandler *et al.*, 2011). Calcium-channel blockers decrease calcium ions in cardiac muscle and vascular smooth muscle, thereby inhibiting the contraction of these muscles and lowering blood pressure (Weber *et al.*, 2014; Fares *et al.*, 2016). Amlodipine, a calcium-channel blocker, has been approved to be an effective and safe antihypertensive medication in the past two decades (Fares *et al.*, 2016). Antihypertensive treatment has been widely studied, but it only offers a temporary solution.

1.3 Overall Control of Blood Pressure and Heart Rate

Blood pressure is mainly modified by two factors: cardiac output and total peripheral resistance (Mayet and Hughes, 2003; Joyner and Limberg, 2014). Heart rate and stroke volume affect cardiac output (Mayet and Hughes, 2003); thus, blood pressure is closely associated with heart rate. Controlling blood pressure is extremely complicated, which involves local control, global neural control, and renal-endocrine system (Chopra *et al.*, 2011). For instance, endothelin-1 locally regulates tonus of blood vessels, and may increase blood pressure along with aging (Goel *et al.*, 2010; Chopra *et al.*, 2011). Increased (circulating) endothelin-1 may cause vascular inflammation and structural reformation, which promotes vasoconstriction (Goel *et al.*, 2010). The vascular endothelium produces nitric oxide, which exerts various physiological actions, including vasodilation, vasoconstrictor inhibition, anti-thrombosis, anti-inflammation, and anti-proliferation (Chen *et al.*, 2008; Chopra *et al.*, 2011). The renal-endocrine system is composed of renin, angiotensin II, and aldosterone (Chopra *et al.*, 2011). When blood pressure decreases, the juxtaglomerular cells

in the kidneys release renin (Castrop *et al.*, 2010). Angiotensin I, a precursor of angiotensin II, is cleaved by renin (Castrop *et al.*, 2010; Chopra *et al.*, 2011). Then, angiotensin I is converted into angiotensin II by the angiotensin-converting enzyme, which exists in the vascular endothelium, lungs, and kidneys (Castrop *et al.*, 2010; Chopra *et al.*, 2011; Weber *et al.*, 2014). Angiotensin II not only acts as a vasoconstriction hormone, but also stimulates the release of aldosterone from the adrenal cortex (Castrop *et al.*, 2010). Aldosterone is capable of preserving sodium and increasing blood pressure (Castrop *et al.*, 2010; Chopra *et al.*, 2011). The sympathetic nervous system modulates blood pressure, especially during short-term changes (Chopra *et al.*, 2011). When the sympathetic nerve is activated, the blood vessels constrict, leading to high blood pressure (Joyner and Limberg, 2014). The baroreceptors in the carotid sinus and aortic arch detect increasing blood pressure and transmit this information to the central nervous system via the aortic nerves, which are a part of vagal nerves (Cheng *et al.*, 1997; Chopra *et al.*, 2011). The vagal nerves have a parasympathetic innervation to the heart, playing a role in lowering the blood pressure and suppressing the output of the sympathetic nerve (Breit *et al.*, 2018). The autonomic nervous system is divided into the sympathetic and parasympathetic nervous systems, which have diverse functions (McCorry, 2007). The cardiovascular center in the brainstem is a part of the autonomic nervous system that controls blood pressure (Joyner and Limberg, 2014).

1.4 Central Regulation of Blood Pressure and Heart Rate

The brainstem is extremely important in blood pressure control; it includes the medulla oblongata, a region known as the cardiovascular center (Colombari *et al.*, 2001; Joyner and

Limberg, 2014). The schematic view of neural pathways of the medulla oblongata in blood pressure regulation is presented in Figure 1-1. The central nervous system (brainstem), subsequently autonomic nervous system, receives the sensory impulses when blood pressure changes (McCorry, 2007). Baroreceptors, sensors of blood pressure alteration, relay this sensory information to the brainstem via vagal nerves to regulate blood pressure (Cheng *et al.*, 1997; McCorry, 2007). The central terminal of baroreceptor afferent fibers is a neuron of the nucleus tractus solitarius (NTS), the first cardiovascular site in the dorsal medulla oblongata (Talman, 1997; Colombari *et al.*, 2001). The primary neurotransmitter released from sensory afferents is glutamate, which gives NTS neurons the information primarily via the binding of excitatory postsynaptic non-N-methyl-D-aspartate receptors (Kline, 2008). NTS neurons send glutamatergic projections to the neurons located in the ipsilateral caudal ventrolateral medullary depressor area (CVLM) (Sved *et al.*, 2000; Viard and Sapru, 2002). Thereafter, some CVLM neurons, including gamma-aminobutyric acid (GABA) fibers, send inhibitory projections to the presympathetic neurons located in the ipsilateral ventrolateral medullary pressor area (RVLM) (Viard and Sapru, 2002; Mandel and Schreihofer, 2008). The RVLM is the first site for regulating the sympathetic nervous system with catecholaminergic projection (Granata, 2003). The activation of second- and higher-order baroreceptor NTS neurons excites the CVLM neurons with glutamatergic inputs. Consequently, CVLM neurons suppress the RVLM via the release of GABA. Silencing of RVLM neurons reduces the sympathetic vasomotor tone, which causes bradycardia and depressor effects (Mandel and Schreihofer, 2008; Joyner and Limberg, 2014). As an initial site for handling cardiovascular input, the modulation of the NTS is important for blood pressure regulation.

1.5 Metabotropic Glutamate Receptors

Glutamate serves as a primary excitatory neurotransmitter in mammalian central nervous system and also functions as a neuromodulator to start a second messenger signaling cascade in some cases (Niswender and Conn, 2010). These actions are mediated through ionotropic and metabotropic glutamate receptors (mGluRs) (Crupi *et al.*, 2019). mGluR is a G-protein-coupled receptor, and has eight subtypes or is classified into three groups; group I mGluR (mGluR1/5) is Gq protein, and group II and III mGluR (mGluR2/3 and mGluR4/6/7/8) are Gi protein (Blackshaw *et al.*, 2011). These groups have different functions. For example, mGluR1/5 (group I) stimulates phospholipase-C signaling cascades, but mGluR2/3 (group II) and mGluR4/6/7/8 (group III) inhibit the adenylyl cyclase pathway (Niswender and Conn, 2010; Blackshaw *et al.*, 2011). All mGluRs initiate intracellular signaling cascades (Blackshaw *et al.*, 2011), which provide short- and long-term modulation of glutamatergic transmission in various neural networks (Anwyl, 1999; Anwyl, 2009). They also exist in the medulla oblongata, particularly in the NTS (Hay *et al.*, 1999; Chen *et al.*, 2002). The expression of mGluRs is quite varied in the postnatal stage, and each subtype exhibits different changes according to brain development (Catania *et al.*, 1994). mGluR was found to be involved in modulating glutamatergic transmission in the autonomic nervous system (Chen *et al.*, 2002). In early studies, the microinjection of various mGluR modulators, particularly group II mGluRs, into the dorsal area of the medulla oblongata in Sprague-Dawley or Wistar rats greatly changed blood pressure with short-term operation (Tables 1-1 and 1-2). However, these studies have discrepancies; for instance, both agonists and antagonists had similar effects on blood pressure. Thus, the role of mGluR in the

brainstem, especially NTS, on blood pressure regulation remains obscure. To the best of my knowledge, no study has investigated the contribution of mGluR modulators in animal models of impaired blood pressure regulation (e.g., hypertension). mGluR1/5 depolarizes neurons to excite cells, and has the potential to cause neuronal death (Dodd, 2002; Sekizawa *et al.*, 2009). On the contrary, mGluR2/3 hyperpolarizes neurons to inhibit cells (Sekizawa *et al.*, 2009). Thus, it has been used for further investigations.

1.6 Animal Model of Primary Hypertension

Spontaneously hypertensive rats (SHRs) have been widely used as an animal model of essential hypertension to study its mechanism (Pinto *et al.*, 1998). SHRs develop hypertension at 6 weeks of age, and their blood pressure reaches a plateau of about 200 mmHg at 12 weeks of age (Okamoto and Aoki, 1963; Anishchenko *et al.*, 2015). The blood pressure and heart rate of SHRs and Wistar-Kyoto rats (WKYs), a normotensive model, are nearly the same until 2 weeks of age (Dickhout and Lee, 1998). Thereafter, the heart rate of SHRs increases at 3 weeks of age and is slightly higher than that of WKYs at 4 weeks of age (Kuwahara *et al.*, 1996). A remarkable distinction of the SBP between SHRs and WKYs can be observed at 6 weeks of age (Dickhout and Lee, 1998). The firing rates of the extracellular units of RVLM neurons are higher in adult SHRs than in WKYs *in vivo* (Chan *et al.*, 1991). The electrophysiological properties of RVLM neurons and their responses to angiotensin II differ between SHRs and WKYs, even in the neonatal stage (Matsuura *et al.*, 2002). Therefore, postnatal development seems to be highly crucial, which forms the diversity between SHRs and WKYs on blood pressure regulation.

1.7 Objective

With the increase of the aging population and numerous refined diets, the prevalence of hypertension is increasing. Thus, adequate blood pressure regulation becomes more imperative at this moment. The medulla oblongata is important in regulating blood pressure because it is the cardiovascular center. Glutamatergic transmission is the origin of activating the signals in blood pressure regulation, and mGluRs are one of the critical contributors in this process. Therefore, the mGluRs in the dorsal medulla oblongata play an important role in blood pressure regulation (Figure 1-2).

In this work, mGluR modulators were applied into the extracellular cerebrospinal fluid of the brainstem in juvenile SHRs and WKYs for 6 weeks (long-term) by using a mini-osmotic pump to perform chronic modulation of mGluRs at the dorsal medulla oblongata. Long-term modification constructs the basis of memory and learning more sufficiently than short-term modification (Deperrois and Graupner, 2020). The alleviation or exacerbation of hypertension development indicates the effect of mGluRs on blood pressure regulation. The blood pressure and heart rate were measured throughout the entire experiment. Moreover, the autonomic nervous activity and baroreflex function were evaluated to determine the mechanism of mGluRs in modulating the blood pressure and heart rate.

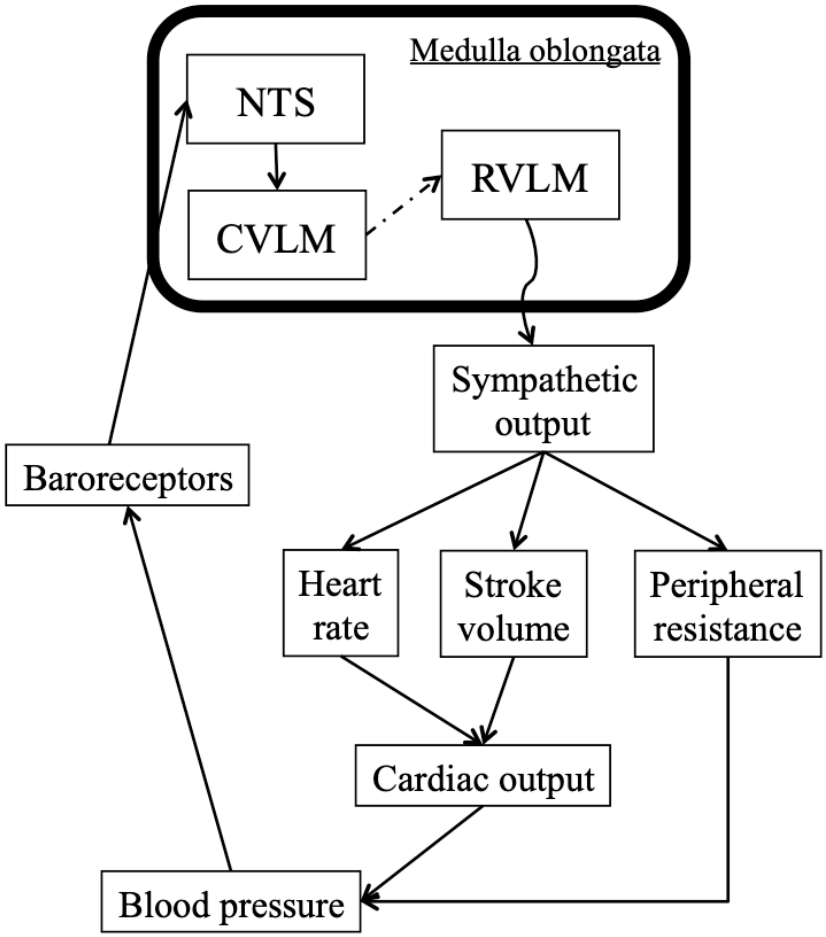


Figure 1-1. Conceptual scheme for the blood pressure regulation via the central nervous system. The solid lines indicate excitation, and the dashed line indicates inhibition. NTS, nucleus tractus solitarius; CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla.

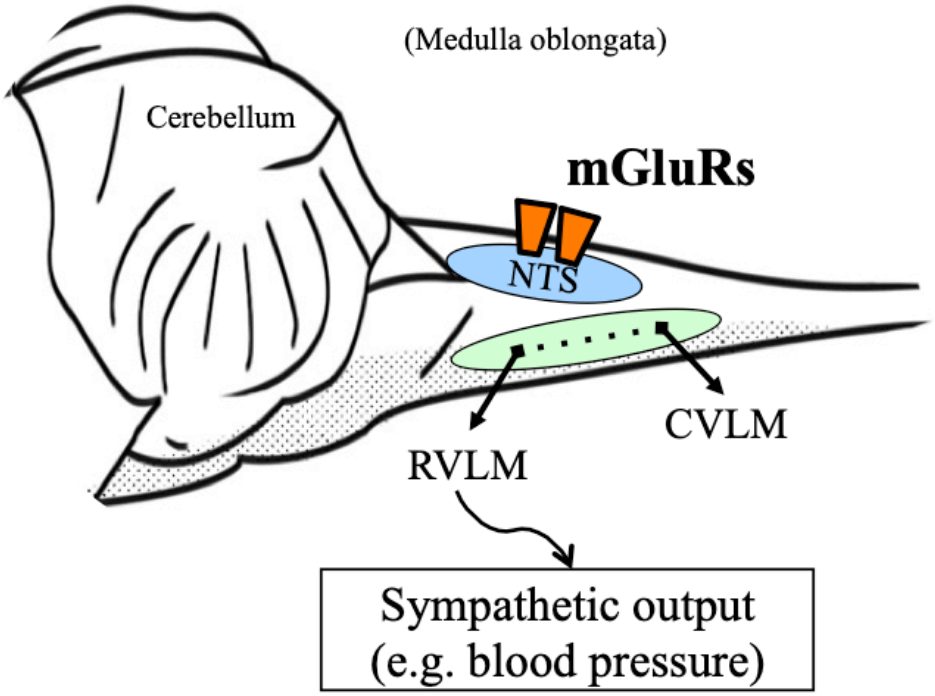


Figure 1-2. Scheme for the possible connection between blood pressure regulation and metabotropic glutamate receptors (mGluRs) in medulla oblongata. mGluRs was discovered to be involved in modulating glutamatergic transmission in the anatomic nervous system, and might change blood pressure via NTS-CVLM-RVLM projections. mGluRs, metabotropic glutamate receptors; NTS, nucleus tractus solitarius; CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla.

Table 1-1. Effects of mGluR modulator microinjection on blood pressure and heart rate in early studies.

mGluR modulator	Rat	Location	BP	HR	Author
Group I					
agonist					
3,5-DHPG	Wistar	NTS	↓	↓	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
	Wistar	NTS	↓	↓	Viard and Sapru, 2002
Group II					
agonist					
ACPD	SD	NTS	↓	↓	Foley *et al.*, 1998
	Wistar	NTS	↓	↓	Antunes *et al.*, 2003
4C3HPG	Wistar	NTS	↓	↓	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
APDC	Wistar	NTS	↓	↓	Viard and Sapru, 2002
Group III					
agonist					
L-AP4	Wistar	NTS	↓	↓	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
	Wistar	NTS	↓	↓	Viard and Sapru, 2002
R,S-PPG	Wistar	NTS	↓	↓	Viard and Sapru, 2002
Group I					
antagonist					
AIDA	Wistar	NTS	↓	↓	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
	Wistar	NTS	-	-	Viard and Sapru, 2002
Group II					
antagonist					
MCPG	SD	NTS	-	-	Foley *et al.*, 1998
	Wistar	NTS	-	-	Antunes *et al.*, 2003
EGLU	Wistar	NTS	↓	↓	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
	Wistar	NTS	↓	↓	Viard and Sapru, 2002
LY341495	Wistar	NTS	↓	↓	Viard and Sapru, 2002
APICA	Wistar	NTS	↓	↓	Viard and Sapru, 2002
MCCG	Wistar	NTS	↓	↓	Viard and Sapru, 2002
Group III					
antagonist					
CPPG	Wistar	NTS	-	-	Matsumura *et al.*, 1999
	Wistar	RVLM	↑	↑	Tsuchihashi *et al.*, 2000
	Wistar	NTS	↓	↓	Viard and Sapru, 2002
MSOP	Wistar	NTS	↓	↓	Viard and Sapru, 2002

BP, blood pressure; HR, heart rate; SD, Sprague-Dawley; NTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla; ↓, decrease; ↑, increase; -, constant.

Table 1-2. Compound of mGluR modulators in Table 1-1.

Modulator	Compound
3,5-DHPG	3,5-dihydroxy-phenylglycine
ACPD	1-amino-1,3-dicarboxycyclopentane
4C3HPG	RS-4-carboxy-3-hydroxyphenylglycine
APDC	(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid
L-AP4	L-2-amino-4-phosphonobutyric acid
R,S-PPG	(R,S)-4-phosphonophenylglycine
AIDA	(RS)-1-aminoindan-1,5-dicarboxylic acid
MCPG	alpha-methyl-4-carboxyphenylglycine
EGLU	(2S)-alpha-ethylglutamic acid
LY341495	2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid
APICA	(RS)-1-amino-5-phosphonoindan-1-carboxylic acid
MCCG	(2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine
CPPG	(RS)-alpha-cyclopropyl-4-phosphono-phenyl-glycine
MSOP	(RS)-alpha-methylserine-o-phosphate

Chapter 2

Effects of the Central Administration of LY379268 (mGluR2/3 Agonist) or LY341495 (mGluR2/3 Antagonist) on Blood Pressure and Heart Rate in Spontaneously Hypertensive Rats (SHRs) and Wistar-Kyoto Rats (WKYs)

2.1 Introduction

Blood pressure regulation is important in reducing the risk of developing cardiovascular diseases (Lamprea-Montealegre *et al.*, 2018). Aside from dietary management and regular exercise, antihypertensive medicines are also prescribed to patients with hypertension (World Health Organization, 2013). Antihypertensive medicines include alpha- and beta-adrenergic blockers, angiotensin receptor blockers, and calcium-channel blockers (Fares *et al.*, 2016). The dorsal part of the medulla oblongata is known to be a crucial site associated with cardiovascular regulation (Colombari *et al.*, 2001). It neurologically controls blood pressure through a baroreflex mechanism by sending glutamatergic projections (Sved *et al.*, 2000; Chopra *et al.*, 2011). Various modulators of mGluR, especially groups I and II, have been studied to achieve blood pressure control (Tables 1-1 and 1-2). However, the role of mGluRs in the brainstem still remains unclear. Group I mGluR (mGluR1/5) depolarizes neurons to excite cells (Sekizawa and Bonham, 2006), while group II mGluR (mGluR2/3) hyperpolarizes to inhibit cells (Sekizawa *et al.*, 2009). Considering that glutamate-mediated excitotoxicity can cause neuronal death (Dodd, 2002), mGluR2/3 has been used for further investigations. The microinjection of mGluR2/3 modulators into the dorsal area of the medulla oblongata elicits depressor effects with agonists and even antagonists (Foley *et al.*, 1998; Matsumura *et al.*, 1999; Viard *et al.*, 2002; Antunes and Machado, 2003), which is contradictory. Given that mGluR2/3 in the dorsal medulla oblongata exerts depressor effects, efforts should be made to clarify the relationship between mGluR2/3 and blood pressure regulation in the dorsal medulla oblongata of SHRs and WKYs.

2.2 Materials and Methods

1. Animals and Rearing Condition

The experimental protocols on the use of SHRs and WKYs were approved by the Animal Care and Use Committee of the University of Tokyo (No.: P17-033). All animals were managed according to the Guidelines for the Care and Use of Laboratory Animals established by the Graduate School of Agriculture and Life Sciences at the University of Tokyo.

Four-week-old male SHRs (n = 15) and WKYs (n = 9) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were housed in a temperature-controlled room (24 ± 1 °C) under automatic controlled lighting (lights on: 0800-2000 h) and given food (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*.

The body weight was monitored once per week. The amount of food intake was measured and recorded daily.

2. Dorsal Hindbrain Treatment of mGluR2/3 modulators, LY379268 and LY341945

Six-week-old SHRs and WKYs underwent surgery to implant a mini-osmotic pump, which delivered solutions continuously for 6 weeks. The animals were anesthetized with isoflurane (Pfizer Japan Inc., Tokyo, Japan), and the following surgical procedures were performed under sufficient anesthesia (i.e., the absence of withdrawal reflex). The lateral cervical space of the animals was opened, and a catheter (external diameter 0.61 mm, internal diameter 0.28 mm) was inserted into the cranial cavity through the foramen magnum with its

tip located near the caudal end of the medulla oblongata (Figure 2-1A). The other end of the catheter was connected to the mini-osmotic pump (ALZET Model 2006, DURECT Corporation, Cupertino, CA, USA), which was implanted in the subcutaneous space (Figure 2-1B). The mini-osmotic pumps had 200-μl reservoir volume were filled with LY379268 (0.40 μg/day) or LY341495 (0.40 μg/day) at least 60 h before implantation as described in the instruction manual, and ran a pumping rate of 0.15 μl/h. Bilateral intracranial injection of LY379268 (1.0 μg/side) on the amygdala of male Long-Evans rat weighing 400 g was utilized in an early study (Lu *et al.*, 2007), so 0.40 μg/day was used in the present study according to the 0.4-time difference in body weight. Sham surgery was performed in animals without the mini-osmotic pump attached as a control. The modulator treatments were conducted for 6 weeks.

3. Reagents

(1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid, also known as LY379268 (Tocris Bioscience, Bristol, UK), is a potent and selective mGluR2/3 agonist.

(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid, also known as LY341495 (Tocris Bioscience, Bristol, UK), is a potent and selective mGluR2/3 antagonist.

4. Monitoring of Blood Pressure and Heart Rate

The blood pressure (BP) and heart rate (HR) of conscious animals were measured with a tail-cuff apparatus (BP-98AL, Softron Co., Ltd., Tokyo, Japan) 3 days before surgery and every week at 6 to 12 weeks of age. The rats were placed in a net holder ringed with a

temperature controller (35 °C) outside and then wrapped with a cloth (Figure 2-2A). A cuff connecting to the apparatus was set at the ridge of the tail to measure BP and HR via tail vasculature (Figure 2-2B). Five minutes after fixation, six sets of recordings were sequentially taken, and the latter three sets were averaged and used as data.

5. Statistical Analysis

Data are mostly expressed as mean ± SEM. The difference between all groups during LY379268 or LY341495 treatment was evaluated through two-way ANOVA followed by Tukey's test for multiple comparisons. Significant differences were considered at $P < 0.05$.

2.3 Results

1. Adaptability during LY379268 or LY341495 Treatment

In terms of food intake, the sham control group of WKYs had lower average daily feed intake compared with other groups (Figure 2-3A). No difference was observed in the body weight of different groups during treatment (Figure 2-3B). After the treatment was completed, similar body weight and body weight gain were observed in all rats (Figure 2-4). Therefore, these data indicate that LY379268 or LY341495 treatment did not affect nor worsen the rats' basal metabolism and function.

2. Effect of LY379268 or LY341495 on BP and HR

The systolic BP (SBP) and diastolic BP (DBP) increased with age in all groups of SHRs, but did not change in all groups of WKYs (Figures 2-5A, 2-5B), which indeed represent as

the normotensive model. Under LY341495 treatment, SHRs showed greater SBP than sham control SHRs (sham control vs. antagonist, 174.0 ± 3.7 vs. 200.6 ± 2.2 mmHg at 10 weeks of age, $P < 0.001$) (Figure 2-5A), and a significant increase was still observed when the treatment was completed at 12 weeks of age (Figure 2-6A). On the contrary, LY379278-treated SHRs showed lower SBP than sham control SHRs (sham control vs. agonist, 174.0 ± 3.7 vs. 155.4 ± 2.4 mmHg at 10 weeks of age, $P < 0.001$) (Figure 2-5A), and a significant difference was observed even after the treatment was completed (Figure 2-6A). Nevertheless, these BP changes were not observed in WKYs, which showed no difference between experimental groups and sham control group, even after the treatment was completed (Figure 2-6A).

In both SHRs and WKYs, the DBP was substantially altered by LY341495, especially with ages (Figures 2-5B, 2-6B). LY379268 treatment did not show a depressor effect in DBP as observed in the SBP among SHRs (Figures 2-5B, 2-6B).

Moreover, the HRs of SHRs and WKYs treated with LY379268 or LY341495 for 6 weeks were not different from those of rats without treatment (Figures 2-5C, 2-7). SHRs had higher HRs than WKYs from the beginning of the experiment (Figures 2-5C, 2-7).

2.4 Discussion

To the best of my knowledge, this work is the first to perform dorsal hindbrain treatment using a mini-osmotic pump for 6 weeks, making the observations of body weight changes and amount of food intake of paramount important. Although the experimental groups displayed variations in food intake (Figure 2-3A), weight loss was not observed when

comparing with the sham control groups (Figure 2-3B). For this reason, the changes of physiological parameters, such as BP and HR, were affected by the pharmacological action of LY379268 or LY341495, not toxic reaction.

SHRs start to develop hypertension at 6 to 12 weeks of age (Okamoto and Aoki, 1963; Anishchenko *et al.*, 2015). Given that the 6-week treatment of LY379268 suppressed the SBP gain in young SHRs, mGluR2/3 might be a possible important contributor for this condition. The involvement of mGluR in BP regulation has been previously reported, but the specific subtypes of mGluR responsible for this action remain unknown. The decreased SBP response induced by mGluR agonist was verified with 1-amino-1,3-dicarboxycyclopentane (ACPD), RS-4-carboxy-3-hydroxyphenylglycine (4C3HPG), and (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid (APDC) (Foley *et al.*, 1998; Matsumura *et al.*, 1999; Viard *et al.*, 2002; Antunes and Machado, 2003). ACPD is a non-selective mGluR agonist that affects all subtypes of mGluR (Conn and Pin, 1997; Foley *et al.*, 1998; Antunes and Machado, 2003; Niswender and Conn, 2010). 4C3HPG is a weak mGluR2/3 agonist but a potent antagonist of mGluR1 (Flor *et al.*, 2002; Niswender and Conn, 2010); thus, 4C3HPG may increase BP (Tsuchihashi *et al.*, 2000). This is because 4C3HPG may also be an mGluR1 antagonist and block N-methyl-D-aspartate, resulting in high BP with weakened baroreflex (Zhang and Mifflin, 1998; Viard *et al.*, 2002). APDC was one of the first selective mGluR2/3 agonists, but it has been substituted with LY354740 or LY379268 at present due to its lower potency and specificity for mGluR2/3 (Flor *et al.*, 2002; Niswender and Conn, 2010). Furthermore, LY354740 is believed to be an mGluR2 agonist and mGluR3 antagonist (Imre, 2007; Niswender and Conn, 2010).

In the present study, the SBP of WKYs was not affected by LY341495 treatment

compared with the sham control group (Figures 2-5A, 2-6A), which is inconsistent with earlier studies that have shown lower SBP in the experimental group than in the sham control group (Viard *et al.*, 2002). Nevertheless, no changes in SBP were observed in earlier studies that utilized alpha-methyl-4-carboxyphenylglycine (MCPG), an mGluR antagonist, on normotensive models (Foley *et al.*, 1998; Antunes and Machado, 2003). MCPG is a nonselective group I/II mGluR antagonist (Kim *et al.*, 2002), which has limitation on pointing out the critical subtypes of mGluR. In addition, the SBP of SHRs treated with LY341495 significantly increased compared with that of the sham control group (Figures 2-5A, 2-6A), which is consistent with a earlier study that used (2S)-alpha-ethylglutamic acid (EGLU) (Tsuchihashi *et al.*, 2000). Although EGLU is a selective mGluR2/3 antagonist, it cannot easily cross the blood-brain barrier due to its low potency (Balazs *et al.*, 2006; Barker *et al.*, 2006). For this reason, the effect of microinjected EGLU on SBP was unsatisfactory (Matsumura *et al.*, 1999). The contradictory results of LY341495 treatment in the present study and the study of Viard and Sapru (2002) may be due to the duration of treatment on rats.

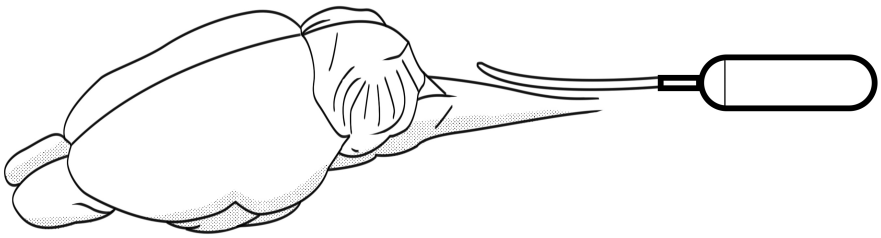
HR was not altered significantly in the present study, which is inconsistent with the results of early studies, wherein HR changes were positively related to BP changes (Foley *et al.*, 1998; Matsumura *et al.*, 1999; Tsuchihashi *et al.*, 2000; Viard *et al.*, 2002; Antunes and Machado, 2003). However, the advantage of the present study is that LY379268 or LY341495 treatment was directly administered to the dorsal medulla oblongata; hence, the response of BP and HR might originate directly from the nucleus tractus solitaries…, the crucial site in modulating baroreflex function (Talman, 1997; Sved *et al.*, 2000; Colombari *et al.*, 2001). In addition, the 6-week chronic mGluR2/3 treatment could affect the onset of

hypertension. mGluR2/3 treatment was successfully administered to the dorsal medulla oblongata, but the optimal dose for this treatment via dorsal hindbrain still needs to be determined. The development of hypertension was intensified by LY341495, which may rapidly cause physical dysfunction with myocardial hypertrophy (Shan *et al.*, 1999). Therefore, LY379268 seems to be a better option for confirming the acceptable dose of dorsal hindbrain mGluR2/3 treatment. Although specific and precise targets were not determined for the application, this method might be more clinically relevant than microinjection.

2.5 Conclusion

After 6-week chronic treatment, hypertension development was suppressed with LY379268 and the progression of hypertension was accelerated with LY341495 in SHRs. No significant changes were observed in the normotensive model while providing the same mGluR2/3 treatments. These findings indicate that mGluR2/3 treatment in the dorsal medulla oblongata can be used to regulate blood pressure in hypertensive subjects, which may easily cause dysfunctional blood pressure control.

A



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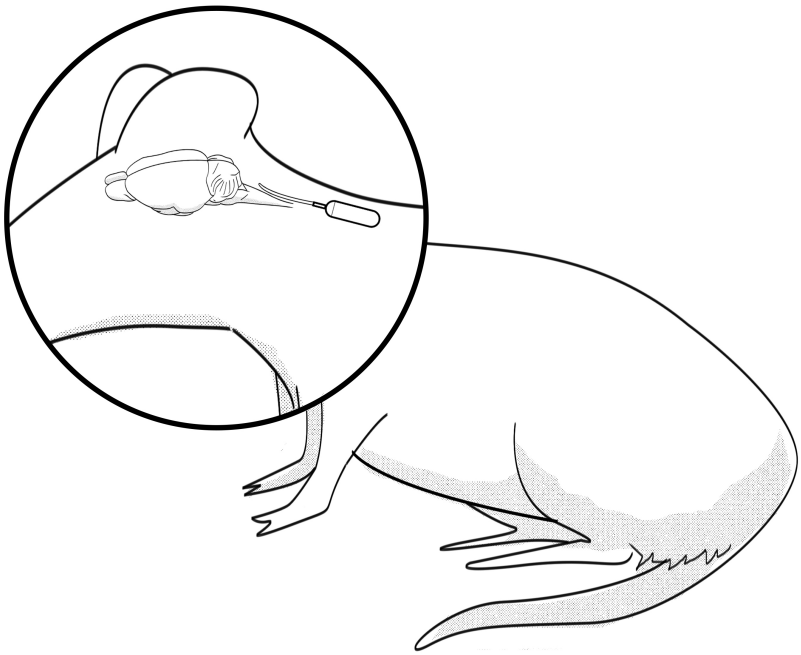
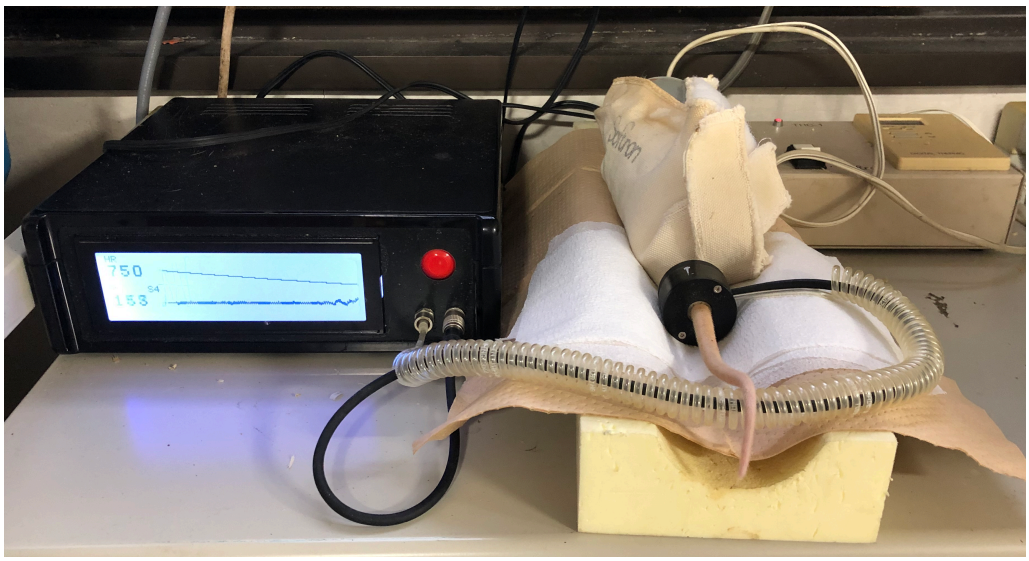


Figure 2-1. Schematic of the surgical catheterization for dorsal hindbrain treatment and mini-osmotic pump implantation. In panel A, the catheter was inserted to the dorsal part of the medulla oblongata, which is near the location of the nucleus tractus solitarius. Then, the catheter was connected to a mini-osmotic pump. Panel B shows the position of the device inside the rat, which was placed in the subcutaneous area on its back.

A



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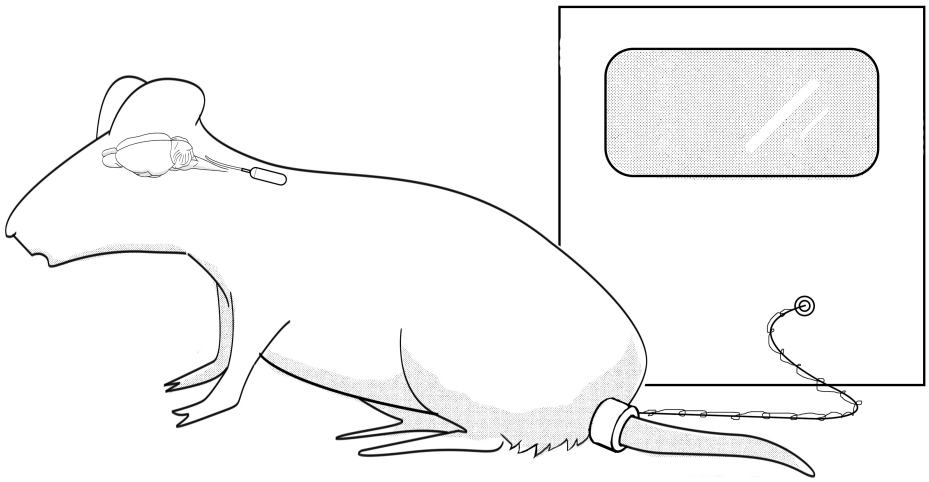


Figure 2-2. Schematic of the tail-cuff method for measuring blood pressure (BP) and heart rate (HR). Panel A shows the actual setup of the BP and HR measurement. Panel B demonstrates the connection between the rat with dorsal hindbrain treatment and tail-cuff apparatus, which was placed at the ridge of the tail.

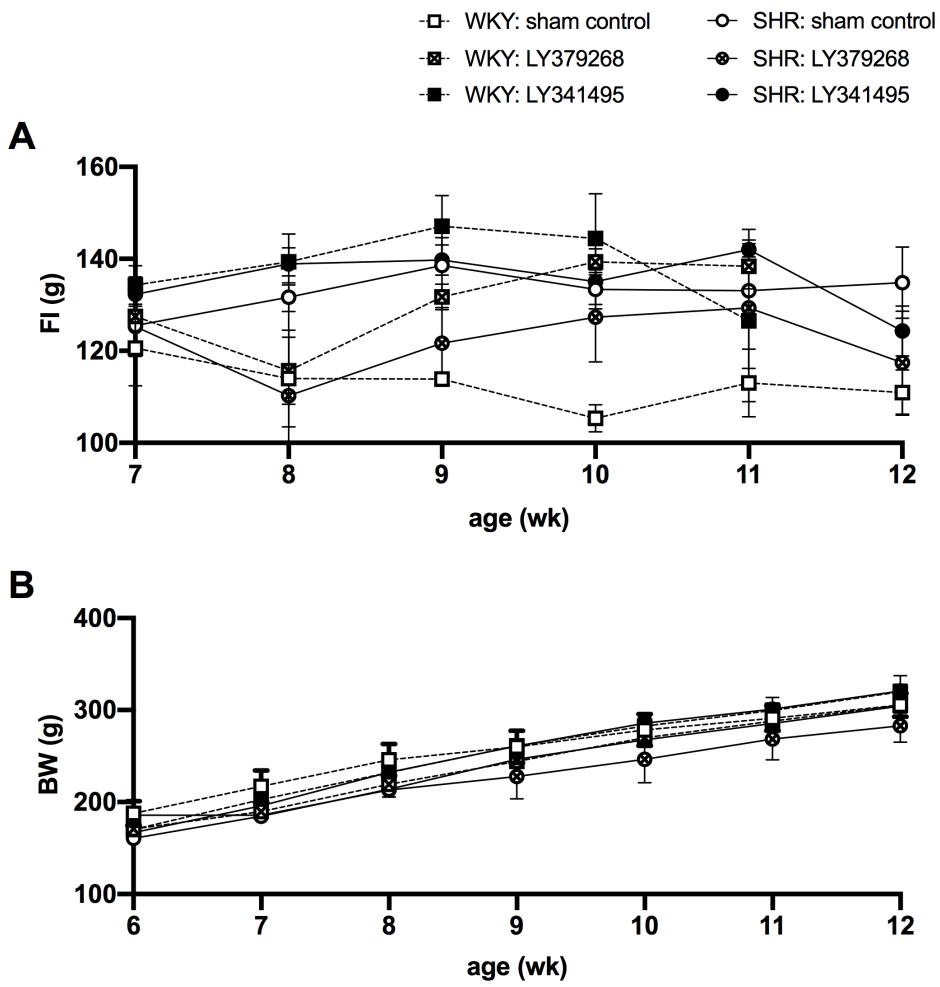


Figure 2-3. Changes of food intake (FI) and body weight (BW) during 6-week treatment of LY379268 or LY341495 on SHRs and WKYs. The amount of FI showed little difference between groups (A), but similarity was observed in all rats' BW gain (B). The data of the LY341495-treated WKYs are the average of two rats, and the rest of the groups are mean ± SEM from three to five rats. FI, food intake; BW, body weight; wk, week.

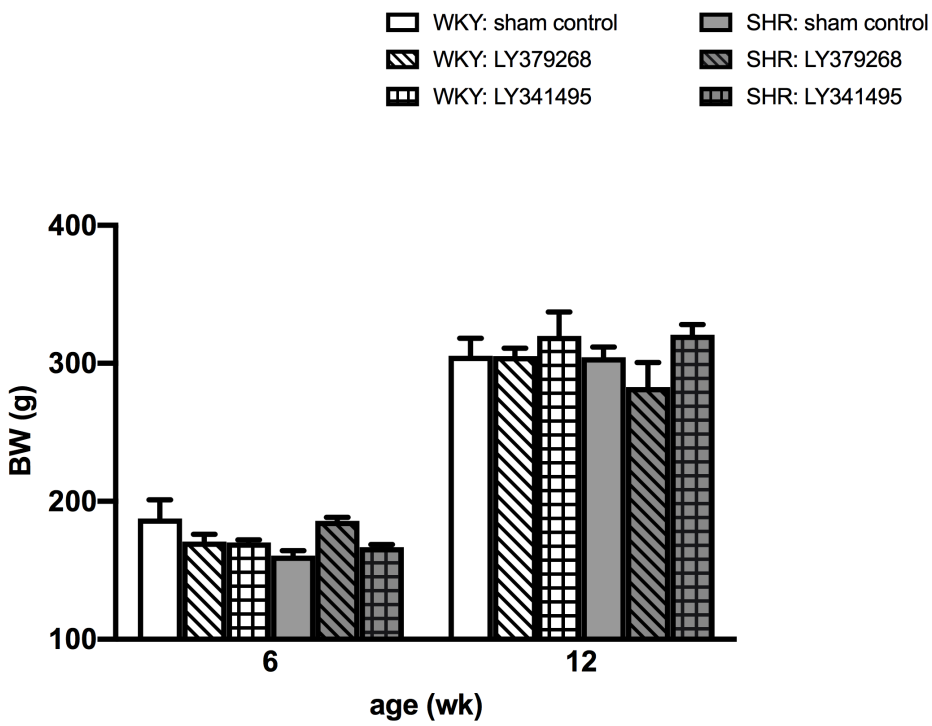


Figure 2-4. Comparisons of body weight before and after 6-week treatment of LY379268 or LY341495 on SHRs and WKYs. No difference was found between any two groups in the same age. Statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data of the LY341495-treated WKYs are the average of two rats, and the rest of the groups are mean ± SEM from three to five rats. BW, body weight; wk, week.

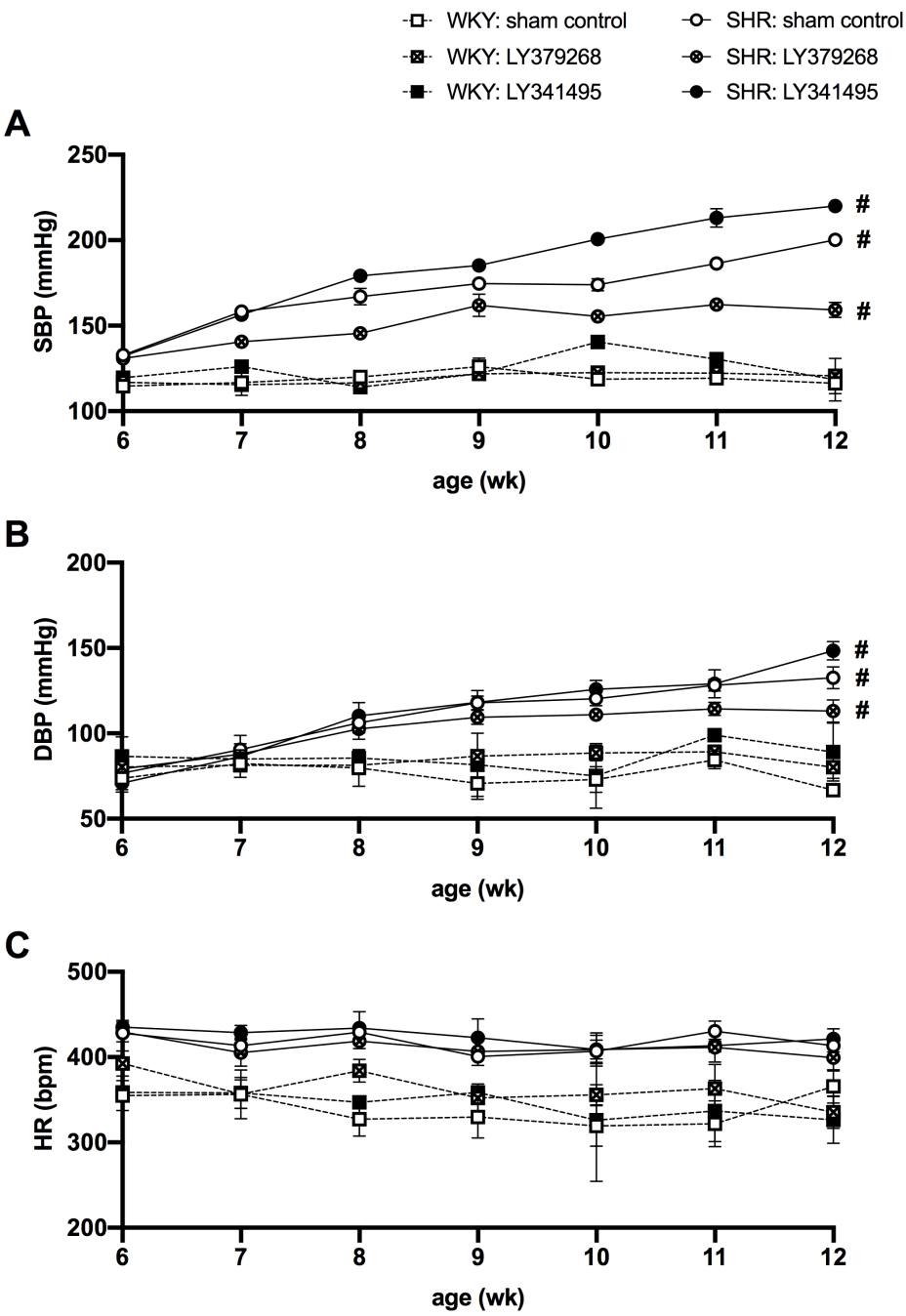


Figure 2-5. Development of blood pressure and heart rate during 6-week treatment of LY379268 or LY341495 on SHRs and WKYs. Under both treatments, the greatest alteration was observed in the SBP of SHRs. #$P < 0.05$; considering the time effect, statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data of the LY341495-treated WKYs are the average of two rats, and the rest of the groups are mean ± SEM from three to five rats. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; wk, week.

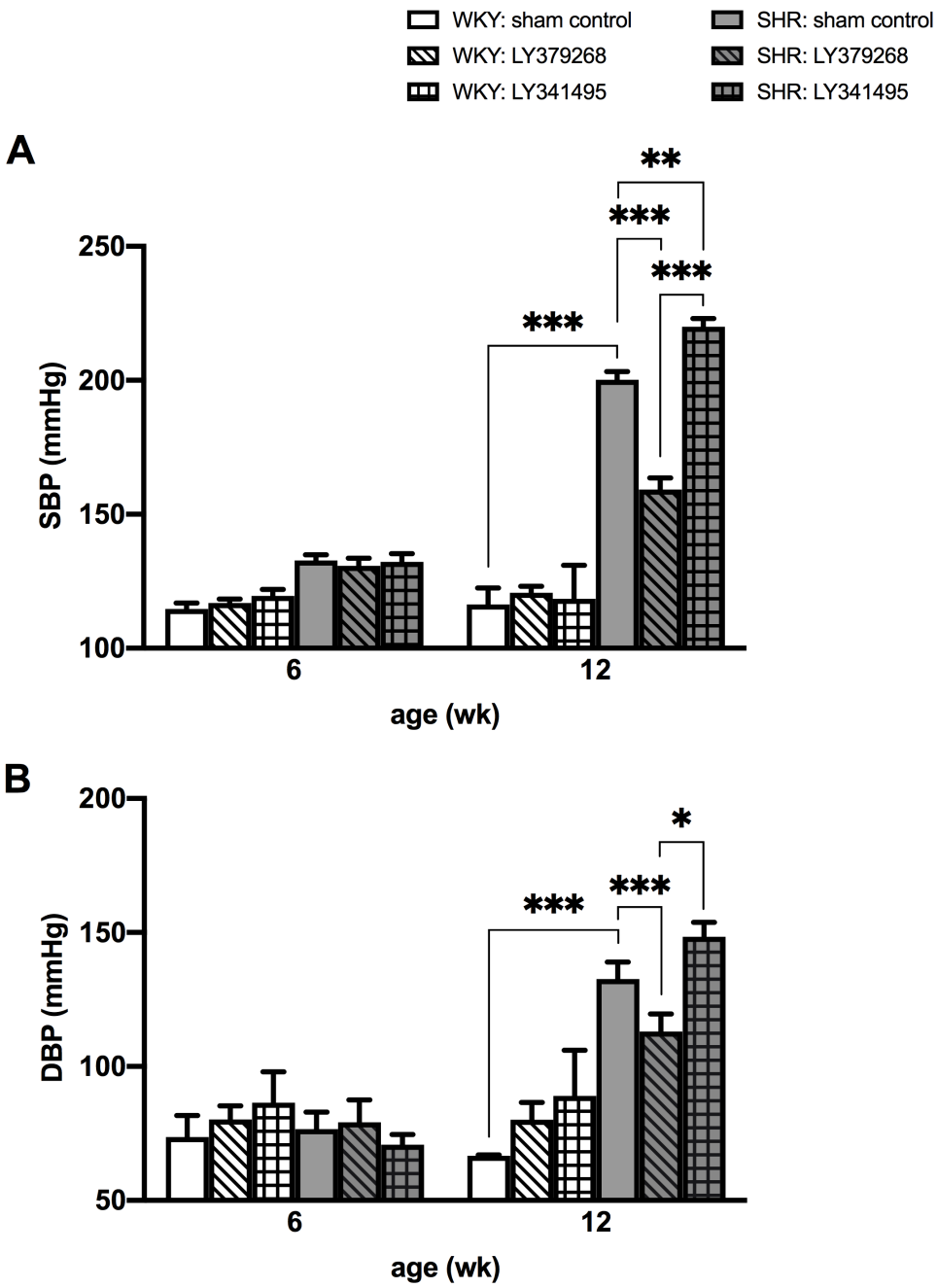


Figure 2-6. Comparisons of blood pressure (BP) before and after 6-week treatment of LY379268 or LY341495 on SHRs and WKYs. Under the same treatments, significant changes in BP were observed in SHRs but not in WKYs. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data of the LY341495-treated WKYs are the average of two rats, and the rest of the groups are mean ± SEM from three to five rats. SBP, systolic blood pressure; DBP, diastolic blood pressure; wk, week.

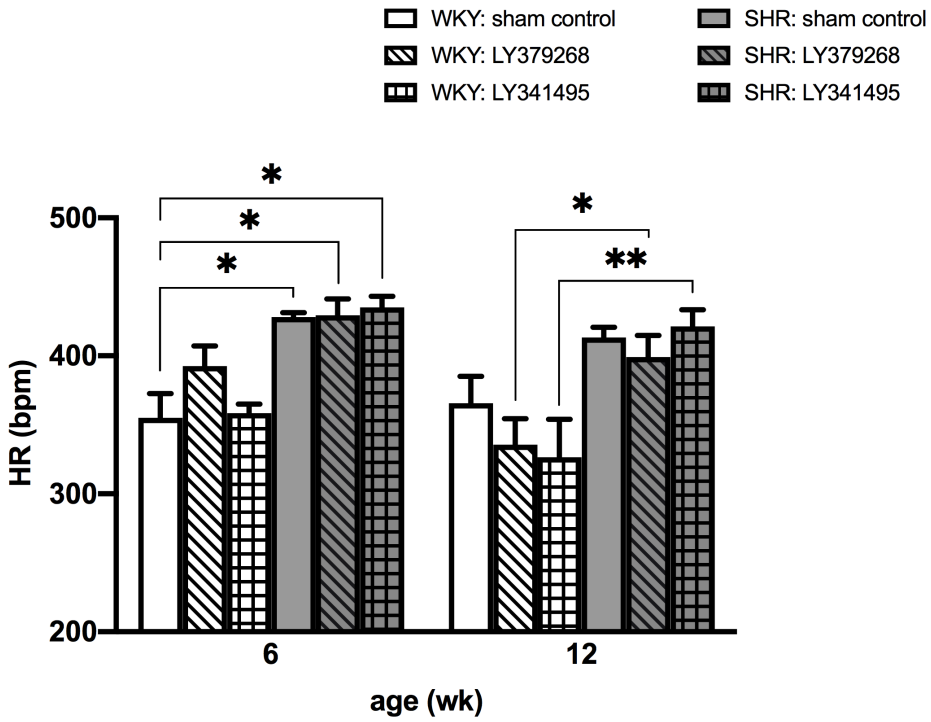


Figure 2-7. Comparisons of heart rate before and after 6-week treatment of LY379268 or LY341495 on SHRs and WKYs. Significant differences were observed between SHRs and WKYs at the same age with the same operation. *$P < 0.05$ and **$P < 0.01$; statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data of the LY341495-treated WKYs are the average of two rats, and the rest of the groups are mean ± SEM from three to five rats. HR, heart rate; wk, week.

Chapter 3

Comparisons between Various Doses of LY379268 on Blood Pressure and Heart Rate during and after Central Administration in SHRs

3.1 Introduction

In the previous chapter, LY379268 (mGluR2/3 agonist) and LY341495 (mGluR2/3 antagonist) treatment caused blood pressure changes in SHRs, which showed that mGluR2/3 is a potential site for blood pressure regulation. In fact, LY379268 showed therapeutic potential for hypertension. The advantage of dorsal hindbrain treatment is bypassing the blood-brain barrier adequately, so as to achieve direct effect to the medulla oblongata (Glascock *et al.*, 2011; Huang *et al.*, 2019). The most challenging and risky problem is that high concentrations of mGluR2/3 might induce neurotoxicity (Huang *et al.*, 2019). Given that dorsal hindbrain treatment with mGluR2/3 modulates the glutamatergic neurotransmission, inappropriate dose may induce abnormal neuronal cell death and cognitive dysfunction (Rahn *et al.*, 2012; Vaarmann *et al.*, 2013). The toxicity of LY379268 via the dorsal hindbrain method requires further evaluation to confirm whether the altered blood pressure is a result of the drug's efficacy and not systemic toxicity. Therefore, in this chapter the optimal dose of dorsal hindbrain mGluR2/3 treatment was investigated to determine the beneficial effect and safe concentrations.

3.2 Materials and Methods

1. Animal Preparation

- Animals and Rearing Condition

Four-week-old male SHRs (n = 26) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The rearing and housing of all animals were in accordance with the methods described in Chapter 2.

Daily observation was conducted, and the body weight was measured twice per week.

- Surgical Operation of Dorsal Hindbrain LY379268 Treatment

Six-week-old SHRs underwent surgery to implant a mini-osmotic pump for delivering solution continuously for 6 weeks, and the procedure for the operation was the same as that described in Chapter 2. Mini-osmotic pumps were filled with LY379268 at least 60 h before implantation as described in the instruction manual. The dosage was selected from 0.40 μg/day to 40 μg/day. Sham surgery was performed in animals without mini-osmotic pump attached as a control.

2. Reagents

LY379268 is a potent and selective mGluR2/3 agonist.

3. Toxicity Measurement of Dorsal Hindbrain LY379268 Treatment

Three or five SHRs were used per dose as a group for investigation. When the animals did not show enough eating and drinking capability, were very inactive, or lost their weight, they were euthanized. The body weight gain was also considered as a supportive mark (endpoint) for sacrificing the animals. The dose and number of deaths of LY379268 treatment were used to calculate the 50% lethal dose (LD_{50}) (Quest Graph™ LD_{50} Calculator, AAT Bioquest, Inc., Sunnyvale, CA, USA).

4. Monitoring of Blood Pressure and Heart Rate

The blood pressure (BP) and heart rate (HR) of conscious animals were measured with a tail-cuff apparatus, and the handling method was described in Chapter 2.

The monitoring of BP and HR was conducted 3 days before surgery and twice per week at 6 to 15 weeks of age.

5. Statistical Analysis

All data are expressed as mean ± SEM. The Kaplan-Meier curve and log-rank test were used to examine the difference of the SHR mortality rate among different doses of LY379268 treatment. The dose-dependent hypertensive effect within a week after surgical operation was evaluated by one-way ANOVA followed by Tukey's test. The correlation among each dose of LY379268 treatment was evaluated through one-way ANOVA followed by Tukey's test for multiple comparisons. Significant differences were considered at $P < 0.05$.

3.3 Results

1. Systemic Toxicity of LY379268 Treatment

Six-week-old SHRs received five different doses of LY379268 (Table 3-1). Within a week after surgical operation, the systolic BP (SBP) seemed to show a dose-dependent hypertensive effect, but no significant difference was observed between any two doses ($P = 0.30$) (Figure 3-1). LY379268 exhibited systemic toxicity (LD_{50} = 4.63 μg/day) when administered to the dorsal medulla oblongata of SHRs (Figure 3-3). The Kaplan-Meier survival curves with a follow-up period revealed that the SHRs receiving 0.40 μg/day of LY379268 showed the best survival rate as confirmed by the log-rank test (Figure 3-2, $P < 0.05$). Moreover, the SHRs receiving 0.40 μg/day of LY379268 showed very similar body weight gain curve with the sham control group did (Figure 3-4). Therefore, 0.40 μg/day was

selected for the following studies to achieve the same effect level on BP.

2. Effect of Different Doses of LY379268 on BP and HR

The SBP of the sham control group gradually increased, and reached 200 mmHg at 13 weeks of age, indicating that SHRs are indeed a hypertensive model (Figure 3-5A). The 40 μg/day dose of LY379268 resulted in the lowest SBP among all groups (115.5 mmHg) (Figure 3-5A). Three weeks of LY379268 treatment significantly decreased the SBP of 9-week-old rats treated with 40 and 4.0 μg/day doses (127.0 and 127.2 mmHg, respectively) (Figure 3-5A). After the 6-week LY379268 treatment was completed, the SBP of all dosing groups was around 160 mmHg, which was significantly different from that of the sham control group (187.4 mmHg) (Figure 3-5A). The low SBP observed in the experimental groups still remained at 15 weeks of age even after the treatment was completed (Figure 3-5A). In the dosing groups, the diastolic BP (DBP) between each dose had no remarkable difference except the 40 μg/day dosing group (Figure 3-5B), wherein the treatment decreased the DBP (i.e., 53.5 mmHg at 8 weeks of age, 61.0 mmHg at 10 weeks of age, etc.) (Figure 3-5B). After the treatment was completed, the lowest DBP was observed in the 40 μg/day dosing group, while the DBP in the other dosing groups was similar (Figure 3-5B). When LY379268 treatment was started, the HR of the 4.0 μg/day, 10 μg/day, and 40 μg/day dosing groups decreased dramatically, and the decrease was dose dependent (13%, 18%, and 22%, respectively) (Figure 3-5C). The effect was continuous at least midway during the treatment period. The HR of the 0.40 μg/day and 1.3 μg/day dosing groups remained the same as that of the sham control group (Figure 3-5C). After the treatment was completed, no difference was observed in the HR between any two groups with respect to the dose (Figure 3-5C).

3.4 Discussion

Although several acute experiments have performed bolus microinjection of mGluR modulators into the dorsal area of the medulla oblongata (Foley *et al.*, 1998; Matsumura *et al.*, 1999; Tsuchihashi *et al.*, 2000; Viard and Sapru, 2002; Antunes and Machado, 2003), no study has investigated the long-term effects of mGluR2/3 stimulation in this brain area. Therefore, the long-term effects of mGluR2/3 stimulation on BP and the toxic effects were preliminary examined in the present study to determine the appropriate dose of the agonist. Dose escalation was performed in this study to determine the best and safest dose of LY379268 via dorsal hindbrain treatment (Le Tourneau *et al.*, 2009). As suggested by earlier studies (Motulsky and Christopoulos, 2003), at least five doses of agonist were used for the dose-response experiments, which makes our results more credible. The five doses were in a tripled row (Motulsky and Christopoulos, 2003), and our results showed that the BP at 1-week post-administration was not substantially different among various concentrations (Figure 3-1).

Determining the LD_{50}, which is defined as the dose that kills 50% of the test animals, is the first step for evaluating the toxicity of a substance (Akhila *et al.*, 2007). The LD_{50} test was established since 1927 and is beneficial for standardized medication while facing a dangerous drug (Zbinden and Flury-Roversi, 1981). According to the dose-response curve (Figure 3-3), the LD_{50} was calculated to be 4.63 μg/day, and no adverse effects were observed at a dose of 0.40 μg/day (Abal *et al.*, 2017), which also has the best survival rate (Table 3-1, Figure 3-2). One-tenth of LD_{50} is the most recommended dose, with no adverse

effects (Liu *et al.*, 2017). Considering the same effect on BP, a lower dose is highly recommended due to fewer side effects and less inadvertent hazardous exposure (Daughton and Ruhoy, 2013). Furthermore, 0.40 μg/day is suggested as the potential and suitable concentration for long-term mGluR2/3 application to suppress the development of hypertension.

DBP indicates now much pressure the blood exerts on the artery walls between beats when the heart is resting (Widmaier *et al.*, 2011). While the heart is resting between beats, the coronary arteries accept and provide blood with oxygen to the heart (Ferro *et al.*, 1995). Lower DBP was observed under LY379268 treatment at a dose of 40 μg/day (Figure 3-5B). This indicates that the heart did not receive the necessary amount of oxygen and blood, which may weaken the heart. Thus, the decrease HR in the 40 μg/day dosing group was understandable. The fluctuation of the HR curve was a result of the high dose of LY379268, which leads to impairment of the heart's hemodynamic function and eventually heart failure (Mladěnka *et al.*, 2018).

Five different doses of LY379268 suppressed the development of hypertension in SHRs, which correlates with the result in Chapter 2. The reason for this response is still unclear; thus, further research is needed.

3.5 Conclusion

LY379268 treatment in the dorsal medulla oblongata of SHRs can alleviate the progression of hypertension, which indicates that mGluR2/3 in the medulla oblongata is essential in blood pressure regulation. For dorsal hindbrain mGluR2/3 treatment, a dose of

0.40 μg/day is highly recommended, which can be used for further assessments.

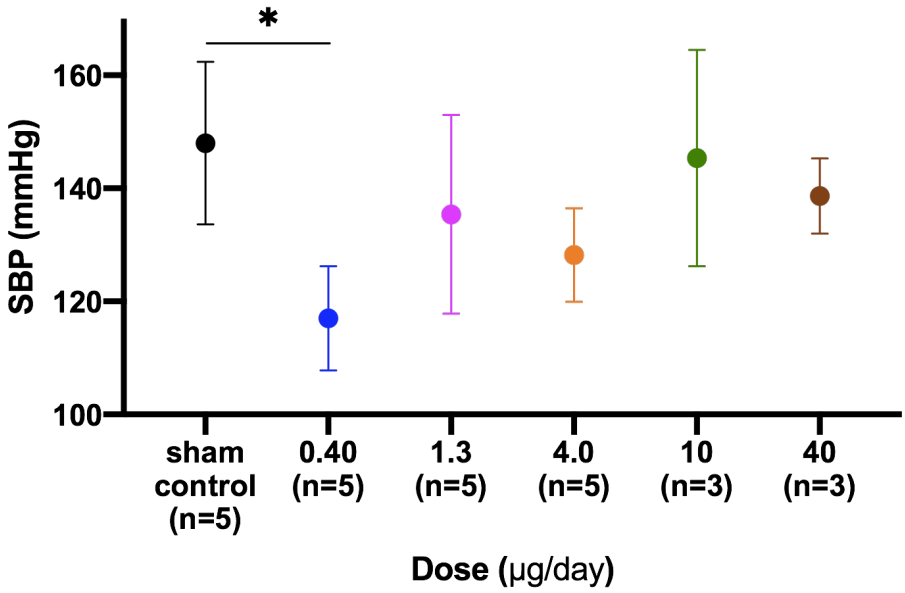


Figure 3-1. Systolic blood pressure at 1-week post-implantation surgery in response to various LY379268 doses in SHRs. The dose of 0.40 µg/day showed less postoperative effects. *$P < 0.05$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from three or five rats. SBP, systolic blood pressure.

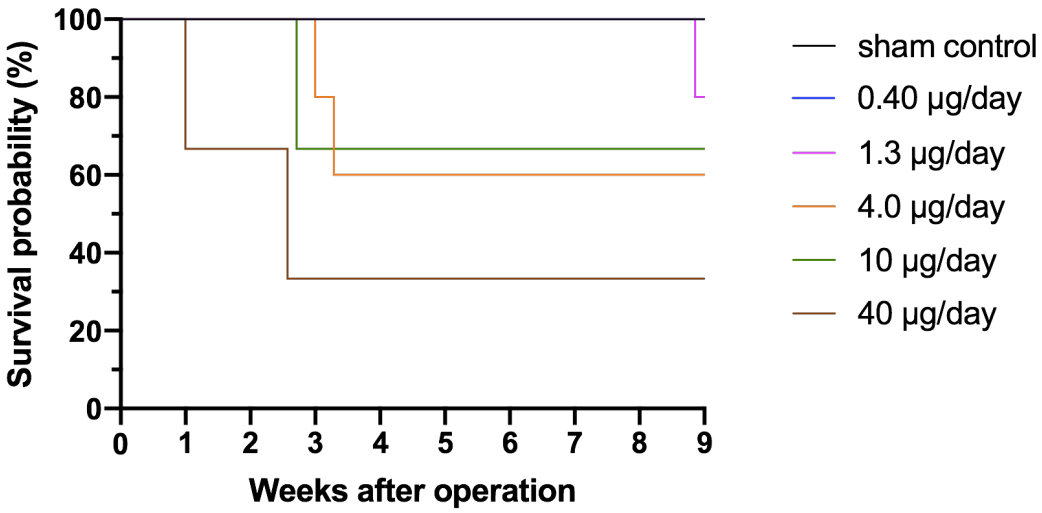


Figure 3-2. Kaplan-Meier survival estimate in response to various LY379268 doses in SHRs. The dose of 0.40 μg/day showed the best survival ($P < 0.05$) among all experimental groups. The data in each group are mean ± SEM from three or five rats.

- LD_{50} = 4.6341
- Equation：

$$Y = -0.9465 + \frac{34.4344 - 0.9465}{1 + (\frac{X}{4.6341})^{-1.7403}}$$

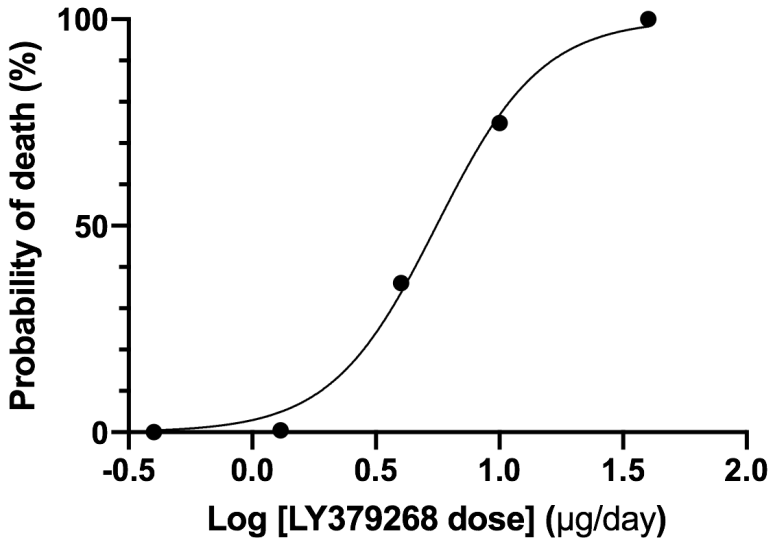


Figure 3-3. Probability of death at 1-week post-operation was plotted against each dose of LY379268 on a logarithmic scale. The fitted curve as a standard sigmoidal function indicating LD_{50} is approximately 4.63 μg/day. The data in each group are mean ± SEM from three or five rats.

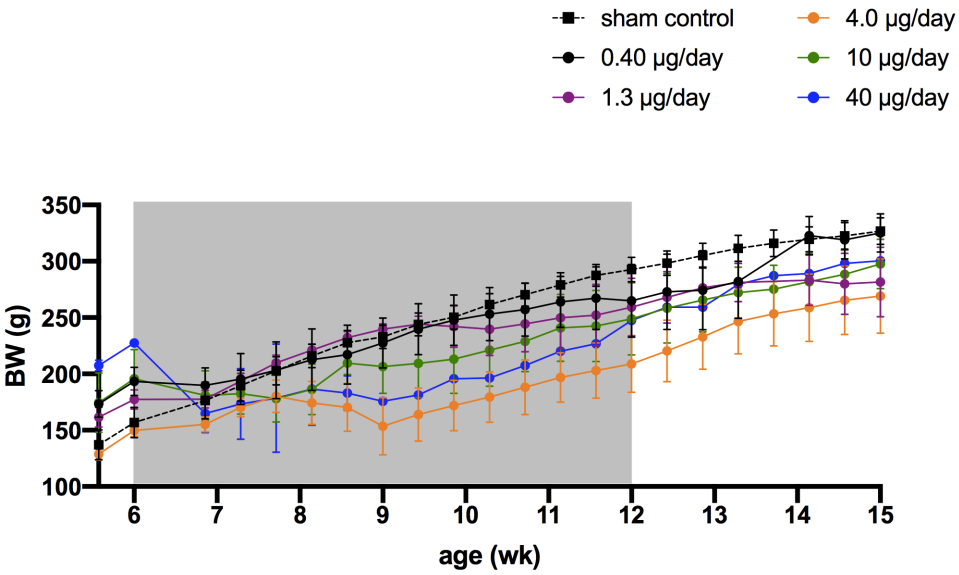


Figure 3-4. Body weight measured during and after various doses of LY379268 on SHRs aged 6 to 15 weeks. The LY379268 treatment was given to rats at 6 to 12 weeks of age. The shaded area indicates the period of drug application. Under LY379268 treatment, lesser weight gain was observed in higher doses (4.0-40 μg/day), especially at the age of 6 to 9 weeks. The data in each group are mean ± SEM from three to five rats. BW, body weight; wk, week.

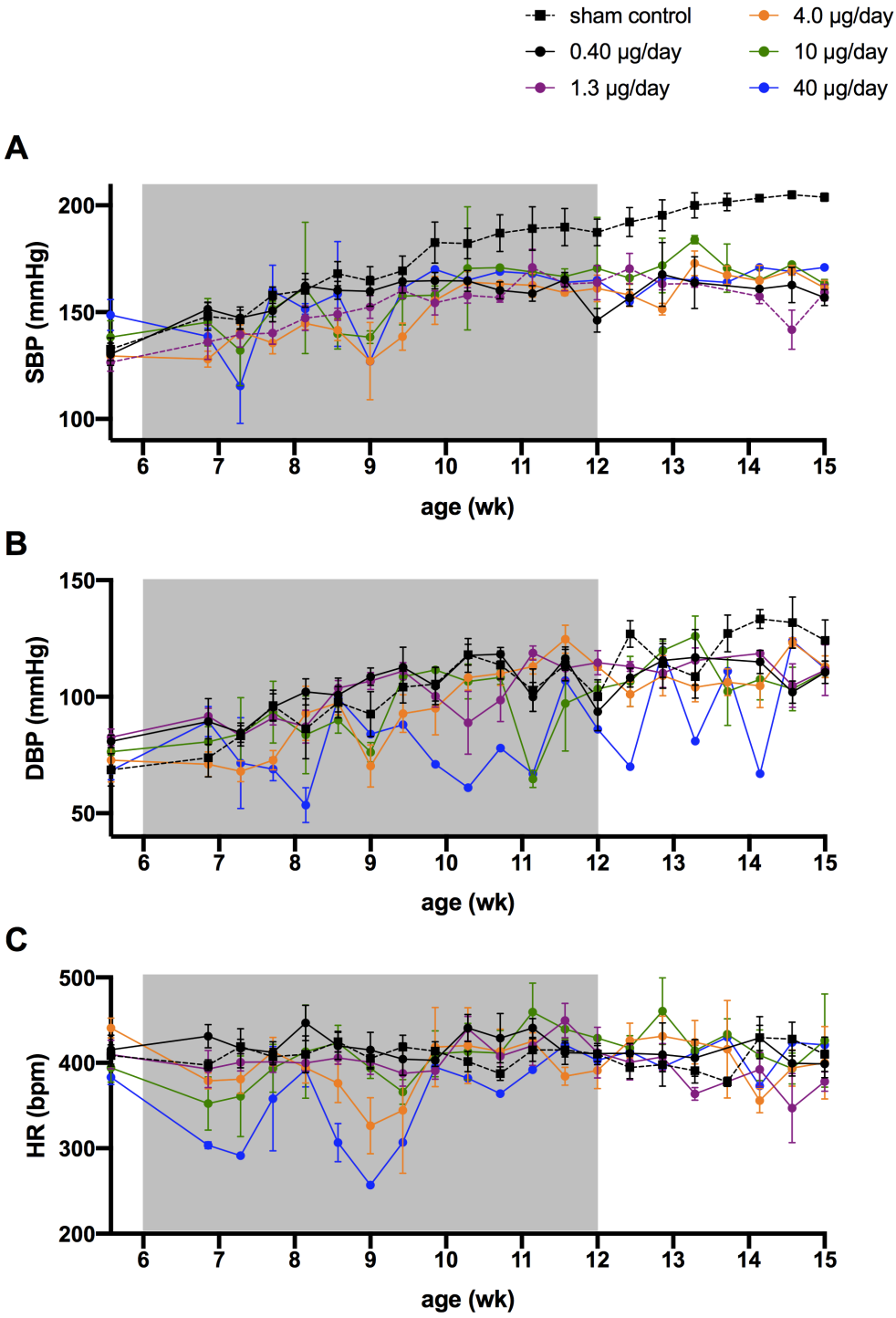


Figure 3-5. Blood pressure and heart rate measured during and after various doses of LY379268 on SHRs aged 6 to 15 weeks. The LY379268 treatment was given to rats at 6 to 12 weeks of age. The shaded area indicates the period of drug application. When LY379268 treatment was finished at 12 weeks of age, over 200 mmHg of SBP was shown in the sham control group, and the SBP still remained around 160 mmHg in all experimental groups. The data in each group are mean ± SEM from three or five rats. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; wk, week.

Table 3-1. Mortality rate and survival time of operated SHRs in each dose of dorsal hindbrain LY379268 treatment.

Dose (μg/day)	Numbers of operation	Numbers of death / euthanization	Mortality (%)	Survival time (day)
0 (sham control)	5	0	0	-
0.40	5	0	0	-
1.3	5	1	20	63
4.0	5	2	40	22
10	3	1	33	19
40	3	2	67	12.5

Chapter 4

Effects after Central Administration of mGluR2/3 Treatment on Blood Pressure and mGluR2/3 Expression in SHRs and WKYs

4.1 Introduction

Modulation of glutamatergic signaling via mGluR2/3 in the medulla oblongata was able to change the blood pressure, and the changes still remained 3 weeks after the treatment was completed. In controlling abnormal blood pressure, the heart and kidneys are the primary organs that bear the brunt of work (Meneely and Battarbee, 1976; Blaustein *et al.*, 2006). Many regions in the medulla oblongata play an important role in cardiovascular regulation, such as the nucleus tractus solitarius (NTS) (Colombari *et al.*, 2001). The NTS is known as the first central site to control the autonomic nervous function with its reflex, transmitting signals by releasing glutamate (Talman, 1997; Colombari *et al.*, 2001; Chen *et al.*, 2002). mGluR2 and mGluR3, which are classified as group II mGluR, inhibit adenylyl cyclase to reduce the cell cyclic adenosine monophosphate cascades and modulate calcium ion channels subsequently (Conn and Pin, 1997; Niswender and Conn, 2010; Blackshaw *et al.*, 2011; Crupi *et al.*, 2019). Their effects are quite different in cognitive psychiatric disorders (Jin *et al.*, 2018; Wood *et al.*, 2018). In cognitive disorders, mGluR2 may be destructive, but mGluR3 may be protective (Jin *et al.*, 2018). mGluR2 and mGluR3 have unequal contribution in sleep and wake cortical oscillations during the assessment of psychiatric disorders (Wood *et al.*, 2018). Therefore, the individual contributions of mGluR2 and mGluR3 in modulating blood pressure and their distribution in the medulla oblongata still need to be further investigated. In this work, an observational study was conducted aiming to compare the blood pressure and heart rate of SHRs and WKYs more than 6 weeks after mGluR2/3 treatment and to determine the role of mGluR2 and mGluR3 in blood pressure regulation and their distribution in the medulla oblongata.

4.2 Materials and Methods

1. Animal Preparation

- Animals and Rearing Condition

Four-week-old male SHRs (n = 15) and WKYs (n = 15) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The housing and rearing of animals were in accordance with the methods described in Chapter 2.

The body weight was recorded once per week. The amount of food intake was weighed and documented daily.

- Surgical Operation of Dorsal Hindbrain mGluR2/3 Treatment

At 6 weeks of age, the rats were subjected to dorsal hindbrain mGluR2/3 treatment with implantation surgery of mini-osmotic pump, which delivered solutions of LY379268 (0.40 μg/day; mGluR2/3 agonist) or LY341495 (0.40 μg/day; mGluR2/3 antagonist) continuously for 6 weeks. The procedure for the operation was the same as that described in Chapter 2. Sham surgery was performed in animals without mini-osmotic pump attached as a control.

2. Reagents

LY379268 and LY341495 are potent and selective mGluR2/3 agonist and antagonist, respectively.

3. Monitoring of Blood Pressure and Heart Rate

The blood pressure (BP) and heart rate (HR) of conscious animals were recorded with a tail-cuff apparatus, and the handling method was described in Chapter 2.

The monitoring of BP and HR was conducted 3 days before surgery and every week at 6 to 21 weeks of age in order to monitor the situation after the treatment was completed at 12 weeks of age.

4. Preparation of Medulla Oblongata Tissue

When the animals reached 12 or 21 weeks of age, they were decapitated under isoflurane anesthesia. The entire medulla oblongata was removed immediately. The right medulla oblongata was stored in RNA stabilization solution (RNAlater™, Invitrogen, Carlsbad, CA, USA) at -80 ˚C for later analysis of gene expression levels. The left medulla oblongata was soaked and stored in 4% paraformaldehyde (PFA) solution containing phosphate buffered saline (PBS) at 4 ˚C for 24 to 48 h in order to perform fixation.

5. Measurement of Gene Expression Levels

1. RNA Isolation

Total RNA was isolated from the medulla oblongata by using 1 ml of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and a cell destructive equipment (Shake Master Auto; Hirata Corporation, Kumamoto, Japan) for 3 min. Then, the medulla oblongata sample was centrifuged at 12,000 × g for 5 min at 4 °C, and the upper aqueous phase was transferred. The RNA was extracted from the upper aqueous phase by 200 µl of chloroform per 1 ml of TRIzol reagent with 15 s vortex and centrifugation at 12,000 × g for 15 min at 4 °C. About 400 µl of supernatant of the fluid was obtained, and the same volume of isopropyl alcohol was added for initial homogenization. The entire fluid was mixed for 15 s and incubated at room temperature for 10 min. Thereafter, the fluid was centrifuged at 12,000 × g

for 15 min at 4 °C. The supernatant was removed completely, and the RNA pellet was washed with 500 µl of 70% ethanol. The sample was centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was removed completely, and the RNA pellet was air-dried for 15 min and then dissolved with 10 µl of diethyl pyrocarbonate-treated water. The RNA concentration was detected through spectrophotometric analysis (NanoDrop One, Thermo Fisher Scientific, Waltham, MA, USA).

2. cDNA Synthesis

The first-strand cDNA was synthesized according to the protocol of SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, Carlsbad, CA, USA).

3. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was conducted for mGluR2 and mGluR3 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control. The mGluR2, mGluR3, and GAPDH primers were obtained from FASMAC (Kanagawa, Japan). The nucleotide sequences of the forward and reverse primers were as follows:

GAPDH	5′-TCACCACCATGGAGAAGG-3′	5′-GCTAAGCAGTTGGTGGTGCA-3′
mGluR2	5′-CGTGAGTTCTGGGAGGAGAG-3′	5′-GCGGACCTCATCGTCAGTAT-3′
mGluR3	5′-GTGGTCTTGGGCTGTTTGTT-3′	5′-GCAGCATGTGAGCACTTTGT-3′.

Ultrapure water was produced with a Milli-Q ultrapure water system (Millipore, Bedford, MA, USA). The ultrapure water was added to each cDNA sample for 100× dilution

in order to perform qPCR. The standard curve was assessed by triplicates of 10×, 100×, and 1000× serial dilutions of a sample's cDNA. The ultrapure water was used as a substitute for cDNA sample in the negative control. The composition of 100× diluted cDNA, 0.5 mM primer, and THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) was as follows:

100× diluted cDNA	5 µl
ultrapure water	4 µl
forward primer	0.5 µl
reverse primer	0.5 µl
THUNDERBIRD SYBR qPCR Mix	10 µl
Total volume	20 µl

The qPCR amplification was performed on LightCycler (Roche, Mannheim, Germany) with the following condition.

95 °C	30 s	
95 °C	10 s	45 cycles
58 °C	10 s	
72 °C	12 s	

For the quantitative evaluation of gene expression, the mGluR2 and mGluR3 mRNA levels were normalized to the GAPDH mRNA.

6. Immunohistochemical (IHC) Assessment and Quantification

1. Tissue Cryopreservation

After fixation, the left medulla oblongata tissues were moved to PBS for washing. Sucrose infiltration was performed on these tissues, and in order of 10%, 20%, and 30% sucrose in PBS at least 12 h per concentration for cryoprotection. Thereafter, the tissues were embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA, USA) at -80 °C for cryopreservation.

2. Tissue Slice Preparation

Medulla oblongata tissues were cut into 40-μm coronal slices with a sliding microtome apparatus (REM-710, Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and a heat exchanger (MC-802C, Yamato Kohki Industrial Co., Ltd., Saitama, Japan). The tissue slices were stored with anti-freezing solution containing ethylene glycol and glycerol in a 24-well cell culture plate at 4 °C.

3. IHC Staining

The detailed protocol for IHC staining is presented in Figure 4-1. First, the tissue slices were washed with PBS to remove the anti-freezing solution. Then, they were blocked with blocking reagent (Block Ace powder, DS Pharma Biomedical Co., Ltd., Osaka, Japan), which was dissolved in PBS (4 mg/ml) as a stocking solution, to prevent nonspecific binding. Rabbit monoclonal antibody to mGluR2 (#ab150387, Abcam, Cambridge, UK) was used as a primary antibody, and goat anti-rabbit IgG antibody with Alexa Fluor 488 (#A11008,

Invitrogen, Carlsbad, CA, USA) was selected as secondary antibody. Both antibodies were diluted with 0.3% PBS-Triton (Triton™ X-100, Sigma-Aldrich, Saint Louis, MO, USA). The tissue slices were washed with 0.03% PBS-Triton after reactions from both antibodies. After the tissue slices were well stained, they were adhered onto a MAS-coated glass slide (MAS-01, Matsunami Glass Ind., Ltd., Osaka, Japan), and then mounted with aqueous mounting medium (Fluoromount™, Diagnostic BioSystems, Pleasanton, CA, USA).

4. Quantification of the Positive Area of mGluR2

The positive areas were identified by the deposition of green dye with light microscopy, and the general anatomical structures of the medulla oblongata were determined based on textbook reference (Paxinos and Watson, 2014). The positive areas are around -10.5 mm to the bregma and were taken with a fluorescent microscope digital camera (DP73, Olympus, Tokyo, Japan) with 958.4 ms as exposure time. Then the pictures including the positive areas were evaluated with ImageJ v1.53 (National Institutes of Health, Bethesda, MD, USA).

7. Measurement of Echocardiography and Renal Ultrasonography

Echocardiography and renal ultrasonography were performed on 18-week-old SHRs and sham control WKYs under 2% isoflurane anesthesia in air with a flow rate of 1 l/min by using a preclinical imaging system (Vevo 3100, FujiFilm VisualSonics, Toronto, ON, Canada) and a linear array transducer (MS-550S, FujiFilm VisualSonics, Toronto, ON, Canada). Echocardiography was recorded using the preclinical imaging system as early described (Tochinai *et al.*, 2018). Image analysis was performed for the left ventricular short axis, left ventricular inflow waveform, and mitral valve septal tissue waveform. Cardiac function

parameters such as HR, ejection fraction, and cardiac output were calculated using an analysis software (Vevo LAB, FujiFilm VisualSonics, Toronto, ON, Canada). The parameters of the renal artery on both sides, including the resistive index (RI) and pulsatility index (PI), which indicate the severity of renal diseases, were also examined and calculated (Petersen *et al.*, 1997). The examination results from the sham control (WKY) were used as a normotensive baseline.

8. Statistical Analysis

All data are expressed as mean ± SEM. The body weight, BP, and HR between SHRs and WKYs during mGluR2/3 treatment were compared through two-way ANOVA followed by Tukey's test for multiple comparisons. Statistical analyses were performed using one-way ANOVA followed by Tukey's test for multiple comparisons to examine the remaining data. Significant differences were considered at $P < 0.05$.

4.3 Results

1. Adaptability during mGluR2/3 Treatment

Comparing the amount of food intake between groups, SHRs had lower average daily food intake while undergoing LY379268 treatment (Figure 4-2A). During mGluR2/3 treatment, no difference was observed in the body weight of both groups, especially in WKYs (Figure 4-2B). Similar body weight gain was observed in all rats after mGluR2/3 treatment was completed (Figure 4-3). Thus, the rats' basal metabolism and function were not changed obviously by mGluR2/3 treatment.

2. The Effect of mGluR2/3 Treatment on BP and HR of SHRs and WKYs

Figure 4-4 shows the time course changes of BP and HR in 6- to 21-week-old SHRs and WKYs. The systolic BP (SBP) and diastolic BP (DBP) increased with age in SHRs but not in WKYs regardless of treatment (Figures 4-4A, 4-4B, 4-5A, 4-5B). After LY379268 (mGluR2/3 agonist) treatment ended at 12 weeks of age, the agonist effect on SBP was evident in SHRs (sham control vs. agonist, 204.0 ± 3.2 vs. 160.4 ± 7.3 mmHg; $P < 0.001$), but not in WKYs (sham control vs. agonist, 123.6 ± 1.6 vs. 111.6 ± 2.6 mmHg; $P = 0.847$) (Figures 4-4A, 4-5A). Meanwhile, LY341495 (mGluR2/3 antagonist) treatment slightly increased the SBP in SHRs (sham control vs. antagonist, 204.0 ± 3.2 vs. 224.0 ± 2.8 mmHg; $P = 0.095$), but not in WKYs (sham control vs. antagonist, 123.6 ± 1.6 vs. 140.0 ± 2.3 mmHg; $P = 0.355$) (Figures 4-4A, 4-5A). The antagonist effect was observed at 21 weeks of age, 9 weeks after the treatment ended (sham control vs. antagonist, 214.6 ± 6.9 vs. 241.8 ± 5.2 mmHg; $P < 0.01$) (Figure 4-4A). At the same time, the response to LY379268 treatment still prevailed (sham control vs. agonist, 214.6 ± 6.9 vs. 164.4 ± 2.5 mmHg; $P < 0.001$) (Figure 4-4A). When mGluR2/3 treatments was completed, no changes were observed in the SBP of WKYs (Figures 4-4A, 4-5A).

Both antagonist and agonist effects on DBP were not evident in SHRs even at 12 weeks of age (sham control vs. agonist vs. antagonist, 125.2 ± 10.9 vs. 116.4 ± 7.0 vs. 135.6 ± 9.2 mmHg; $P = 0.999$) (Figures 4-4B, 4-5B). DBP was decreased or increased in SHRs at 21 weeks of age, 9 weeks after the treatment ended, but the difference was not statistically significant (sham control vs. agonist vs. antagonist, 141.6 ± 8.2 vs. 119.6 ± 4.6 vs. 173.0 ± 8.1 mmHg; $P = 0.0732$) (Figures 4-4B, 4-5B). Similar to that on SBP, mGluR2/3 had no

significant effect on DBP in WKYs at any age.

HR was similar for SHRs and WKYs during the treatment period. After the treatment, HR was significantly higher in SHRs than in WKYs regardless of treatments (Figures 4-4C, 4-5C). The effect of mGluR2/3 treatment on HR was not observed in either SHRs or WKYs even at 21 weeks of age (Figures 4-4C, 4-5C).

3. mGluR2/3 Expression in the Medulla Oblongata of SHRs and WKYs

When mGluR2/3 treatment was completed at 12 weeks of age, the relative gene expression levels of mGluR2 were significantly higher in SHRs treated with LY341495 (Figure 4-6A). Meanwhile, the mRNA expression levels of mGluR2 in WKYs were not altered (Figure 4-6C). No significant difference in mGluR3 mRNA expression was found in SHRs and WKYs (Figures 4-6B, 4-6D).

LY341495-treated SHRs still had higher gene expression levels of mGluR2 at 21 weeks of age, 9 weeks after mGluR2/3 treatment ended (Figure 4-7A). At the same time, LY341495-treated WKYs also showed higher relative gene expression levels of mGluR2 (Figure 4-7C). LY379268 or LY341495 treatment did not significantly affect the response of mGluR3 expression levels among all groups (Figures 4-7B, 4-7D).

4. Positive area of mGluR2 in the Medulla Oblongata of SHRs and WKYs

With mGluR2/3 treatment, significant alteration of gene expression levels of mGluR2 was detected; thus, the distribution of mGluR2 needs to be further investigated. Upon examination of the whole medulla oblongata of SHRs and WKYs, the positive area of mGluR2 was found at around -10.5 mm to the bregma and the dorsolateral aspect of the

medulla oblongata: cochlear nucleus (Figure 4-8). When mGluR2/3 treatment was completed at 12 weeks of age, greater positive area of mGluR2 was observed and calculated in SHRs treated with LY341495 (Figure 4-9). At the same time, no alteration was found in all WKYs (Figure 4-9). Drastic changes occurred 9 weeks after mGluR2/3 treatment ended. The positive area of mGluR2 was increased in LY379268-treated WKYs (Figure 4-10), while no variation was observed in all SHRs.

5. Effect of mGluR2/3 Treatment on Cardiac and Renal Function

The representative echocardiography and renal ultrasonography recordings are shown in Figure 4-11, and their parameters are summarized in Table 4-1. LY379268 treatment did not change the HR and stroke volume of SHRs as indicated by the similar cardiac output among groups. SHRs treated with LY341495 had higher HR and stroke volume, so greater cardiac output was calculated. The ejection fraction and fractional shortening of SHRs and WKYs were almost similar after LY379268 treatment. The left renal artery peak systolic velocity of SHRs was almost identical to that of WKYs after LY379268 treatment, while no alteration was observed in LY341495-treated SHRs. The left and right renal artery lowest diastolic velocity of experimental groups was elevated to a similar degree in WKYs. Other parameters such as renal artery resistive index (RA-RI) and renal artery pulsatility index (RA-PI) were not affected by LY379268 treatment in either sides of the kidneys. LY341495 caused RA-RI and RA-PI to diverge from the normotensive baseline, especially in the left kidney. Overall, no significant effect on the heart or kidneys was observed after mGluR2/3 treatment.

4.4 Discussion

In WKYs (normotensive rats), all parameters were not affected by mGluR2/3 treatment even after the treatment was completed. This finding suggests that mGluR2/3 is a silent mechanism in normal physiological condition, particularly in blood pressure regulation at the dose used in this study. Unlike WKYs, the parameters of SHRs showed great alterations after mGluR2/3 treatment, especially SBP (Figure 4-4A). LY379268 decreased the SBP, while LY341495 showed the opposite effect (Figure 4-5A). Different outcomes were observed between SHRs and WKYs, which should be evaluated at an earlier age. SHRs have significantly greater heart rate and pulse pressure than WKYs at 4 weeks of age (Dickhout *et al.*, 1998), which indicates that the cardiovascular-related neural outflow may be different in both rats at a young age. Thus, cardiac hypertrophy starts to appear in 4-week-old SHRs (Kokubo *et al.*, 2005).

Based on the results, LY379268 attenuated hypertension development, while LY341495 exacerbated the progression of hypertension. LY379268, a mGluR2/3 agonist, only reduced 40 mmHg of SBP, failing to completely prevent the development of hypertension. The application of the agonist to mGluR2/3 in the medulla oblongata evokes the activation of baroreceptor reflex and changes the baroreceptor-heart rate response (Head and Adams, 1992; Hay *et al.*, 1999; Imre, 2007). The non-excludable SBP value might result from the condition of the heart, but was not observed in echocardiogram significantly. Adjusting the process of glutamatergic signals can evoke an augmented response via regulating gamma-aminobutyric acid (GABA)ergic input in the medulla oblongata, which is an evidence of neuroplasticity (Mischel *et al.*, 2015). Changing the neuronal activity of the NTS in the medulla oblongata contributes to the impaired cardiorespiratory function according to the degree of

neuroplasticity (Zoccal *et al.*, 2014). Both mGluR2 and mGluR3 are known to have the ability to modulate plasticity, especially within the long-term depression of excitatory neurotransmission in the central nervous system (Crupi *et al.*, 2019). Therefore, in this study, the alteration of SBP still remained even after mGluR2/3 treatment on the medulla oblongata was stopped, which may result from the ability of neuroplasticity in the brainstem.

The control of hypertension development by mGluR2/3 treatment can be attributed to the following: mGluR2 could have regulated the blood pressure. mGluR2/3 is a G-protein-coupled receptor (Gi alpha subunit, Giα) that negatively couples to adenylyl cyclase to reduce the cell cyclic adenosine monophosphate cascades and subsequently modulates sodium and potassium currents (Sekizawa *et al.*, 2009; Blackshaw *et al.*, 2011). An earlier study reported elevated Giα protein expression in the heart and aorta of SHRs compared with WKYs (Marcil *et al.*, 1997); this increase may also occur in the baroreflex pathway and could modulate the development of hypertension. Quantitative analysis revealed that the increase in the Gi protein levels in the heart was higher in SHRs than in WKYs starting at the age of 2 weeks (Anand-Srivastava, 1996). Moreover, inactivated Giα proteins have been used as a predictive factor for amending the development of hypertension (Anand-Srivastava, 1992; Böhm *et al.*, 1992). Pertussis toxin was used to inactivate the expression of Giα proteins; it successfully postponed the starting time of hypertension development but still has no effect on WKYs (Kost *et al.*, 1999; Li and Anand-Srivastava, 2002). Consistent with the present findings, mGluR2 might have a specific role in blood pressure regulation.

Peripheral vascular resistance and cardiac output can easily manipulate blood pressure (Mayet and Hughes, 2003; Joyner and Limberg, 2014). No variation was observed in

peripheral resistance in the present study, including renal circulation. This finding is in good agreement with an early study, which found that the high BP level in the hypertensive brain could be maintained by increasing vascular responsiveness or sympathetic firing (Julius and Nesbitt, 1996). Cardiac output is mainly associated with HR and pulse pressure, which indicates the difference between SBP and DBP (Mayet and Hughes, 2003; Koenig *et al.*, 2015). The mGluR2/3-treated SHRs had lower or higher SBP, depending on agonists/antagonists, than the sham control but did not show substantial alterations in their DBP and HR. Although LY341495 slightly increased the cardiac output probably due to the sympathetic nervous system (Rubinger *et al.*, 2012), a less rigorous activation of the cardiac output does not qualify as a research result. Vascular resistance is determined by the vascular structure and its elastic fibers (Mayet and Hughes, 2003; Bruno *et al.*, 2012). Genetic defects on the vascular structure or elastic fibers could induce congenital hypertension, and the critical timing is at the fetal and early postnatal stages (Mayet and Hughes, 2003; Arribas *et al.*, 2006). Therefore, this mechanism may not be involved in the observed effects of mGluR2/3 treatment. The hypertensive model *per se* may have left ventricular hypertrophy at 17 weeks of age (Alvarez *et al.*, 2008), but it was not significantly indicated in the present study (until 21 weeks of age).

mGluR2-mediated blood pressure regulation was found to be distributed in the dorsolateral aspect of the medulla oblongata, which is the location of the cochlear nucleus (Figure 4-8). The NTS, the primary site of the cardiovascular system in the medulla oblongata (Talman, 1997; Colombari *et al.*, 2001), is not involved in the positive area. The cochlear nucleus, which integrates auditory resource and the neurotransmission of excitatory amino acid, is the particular location that showed a positive area in the present study (Lee and

Godfrey, 2014). The action of mGluR2 in the cochlear nucleus includes providing a response when the adjacent mossy fiber terminal releases glutamate and causes neurotransmission of GABA from the Golgi terminal to granule cell dendrites (Petralia *et al.*, 1996), which may indirectly affect the NTS via the amygdala (Watanabe *et al.*, 2015). However, the specific mechanism behind this process remains obscure.

4.5 Conclusion

The reaction to dorsal hindbrain mGluR2/3 treatment is completely different between SHRs and WKYs. Nevertheless, mGluR2/3 in the medulla oblongata seems to have an important role in blood pressure regulation, especially in the progression of dysfunction. Moreover, mGluR2 is a more important target than mGluR3 during the modulation of blood pressure via the medulla oblongata.

Washing	PBS Room temperature (shake), 10 min
↓	
Blocking	Blocking reagent's stocking solution / PBS (1: 10) Room temperature (shake), 2 h
↓	
Primary Antibody	Primary antibody solution★1 4 °C (shake), at least 60 h
↓	
Washing	0.03% PBS-Triton Room temperature (shake), 10 min, 3 times
↓	
Secondary Antibody	Secondary antibody solution★2 Room temperature (shake), 2 h, dark place
↓	
Washing	0.03% PBS-Triton Room temperature (shake), 10 min, 3 times, dark place
↓	
Adhered	MAS-coated glass slide
↓	
Mounted	Aqueous mounting medium

★1 Rabbit Anti-mGluR2 (Abcam #ab150387) / 0.3% PBS-Triton (1: 5000)

★2 Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen #A11008) / 0.3% PBS-Triton (1: 1000)

Figure 4-1. Protocol for immunohistochemical staining of mGluR2 in the medulla oblongata.

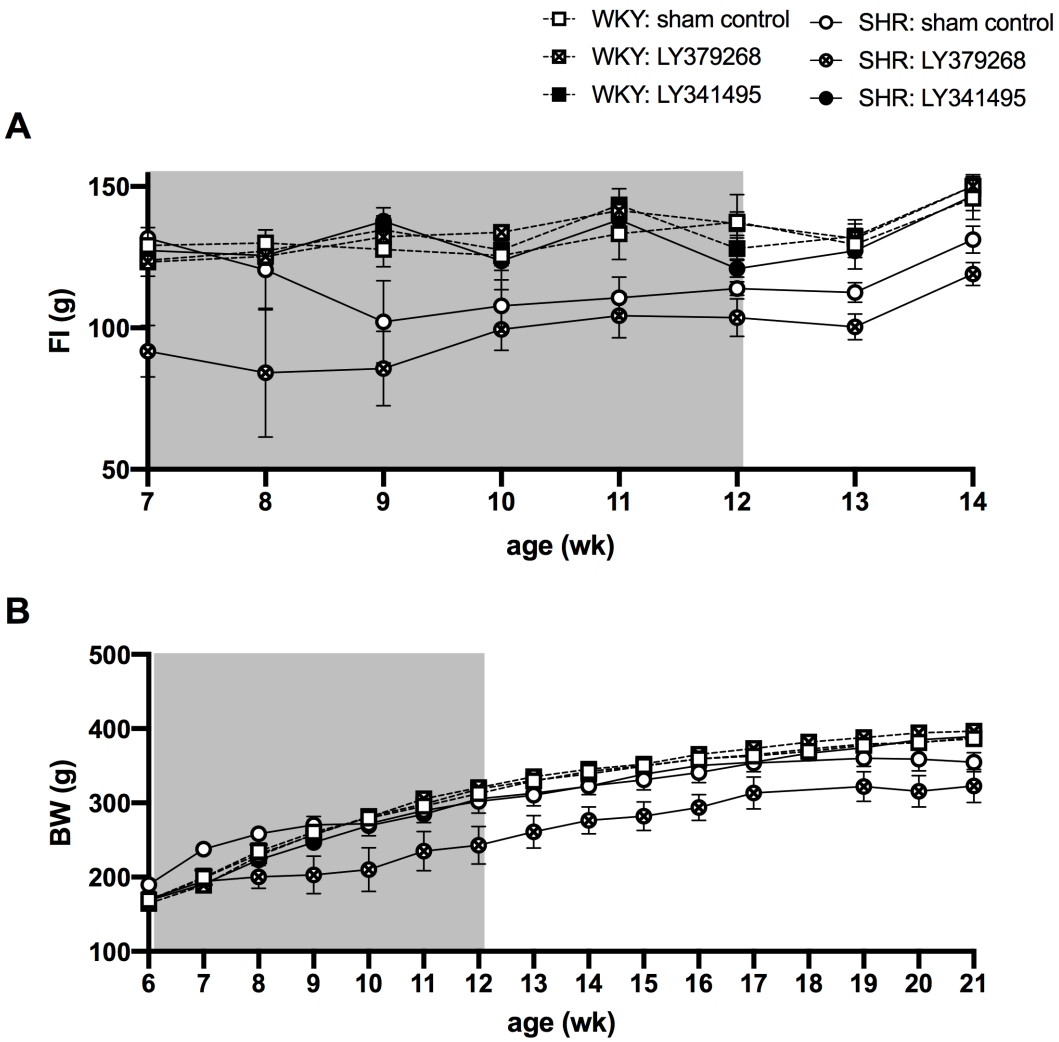


Figure 4-2. Food intake (FI) and body weight (BW) measured in various groups of rats aged 6 to 21 weeks. The mGluR2/3 treatment was given to rats at 6 to 12 weeks of age. The shaded area indicates the period of drug application. The variation of BW (B) resulted from the amount of FI (A), which was not significantly affected by mGluR2/3 treatment. The data in each group are mean ± SEM from five rats. FI, food intake; BW, body weight; wk, week.

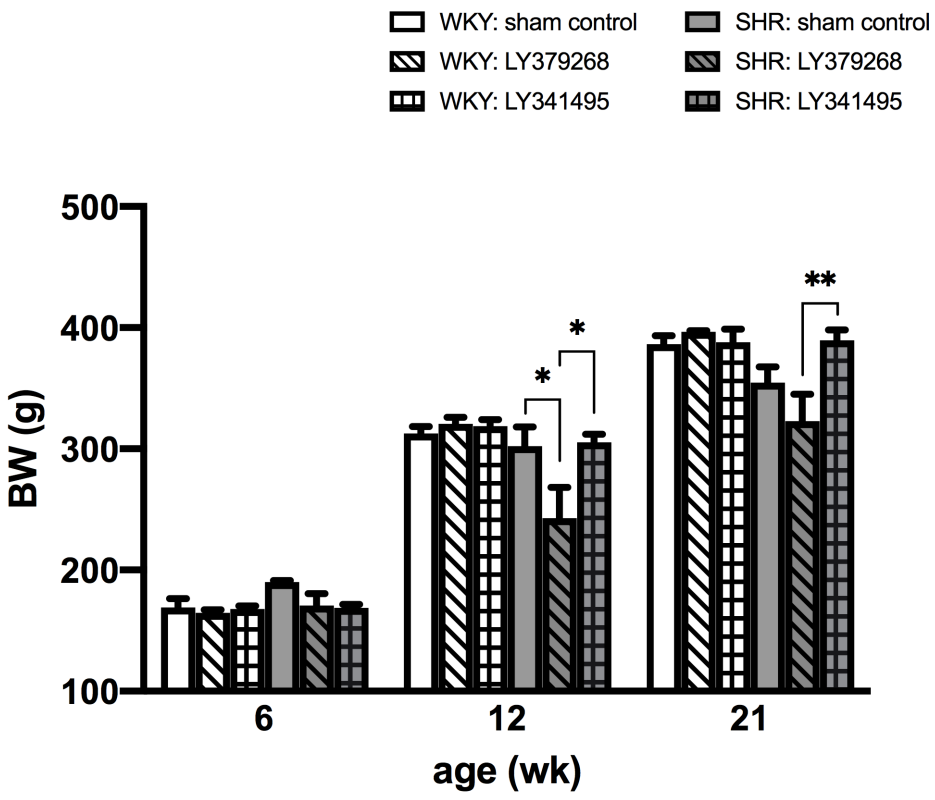


Figure 4-3. Body weight measured in SHRs and WKYs aged 6 (before treatment), 12 (during treatment), and 21 (after treatment) weeks. Significant difference was observed between LY379268- and LY341495-treated SHRs. *$P < 0.05$ and **$P < 0.01$; statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from five rats. BW, body weight; wk, week.

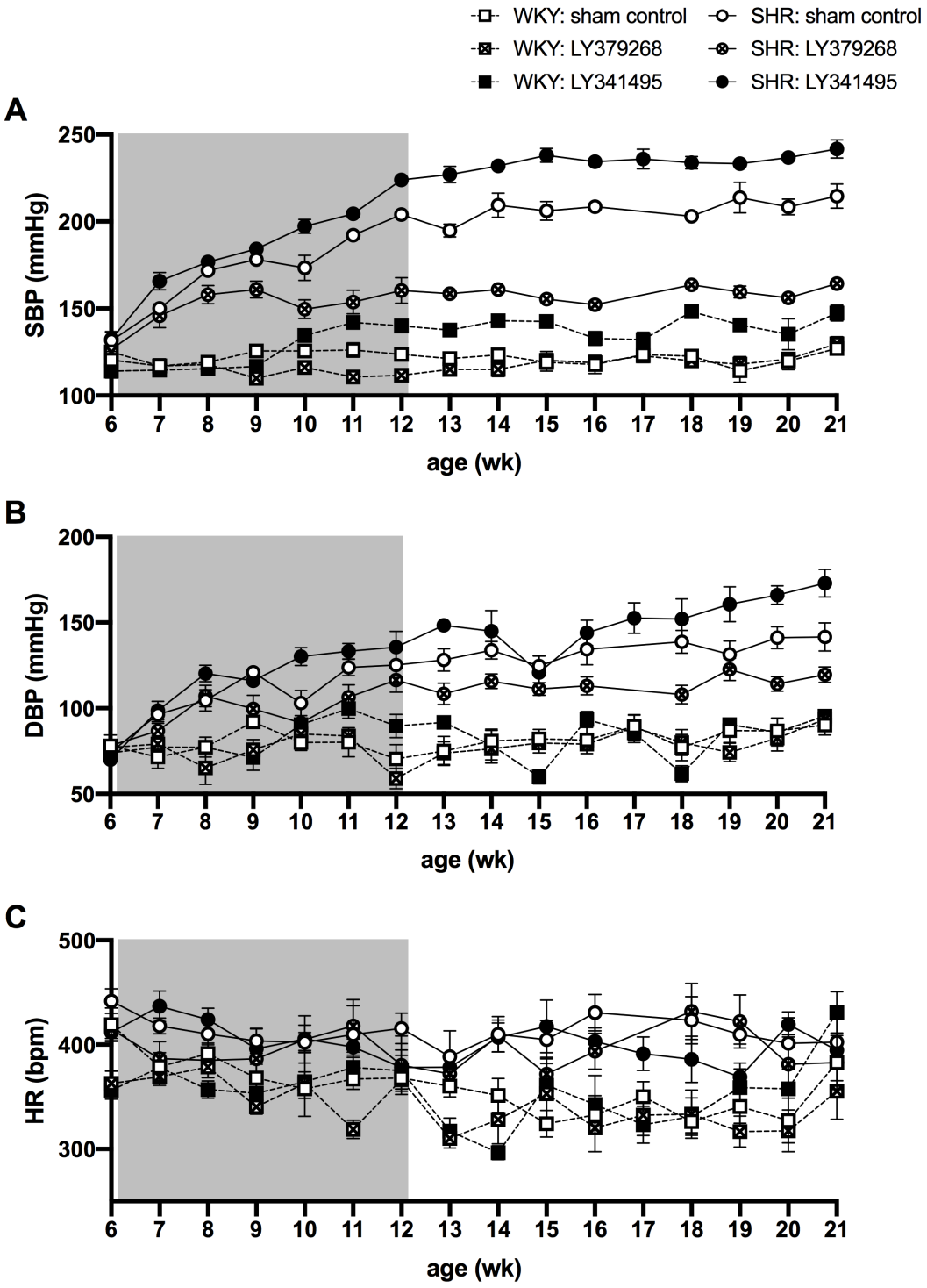
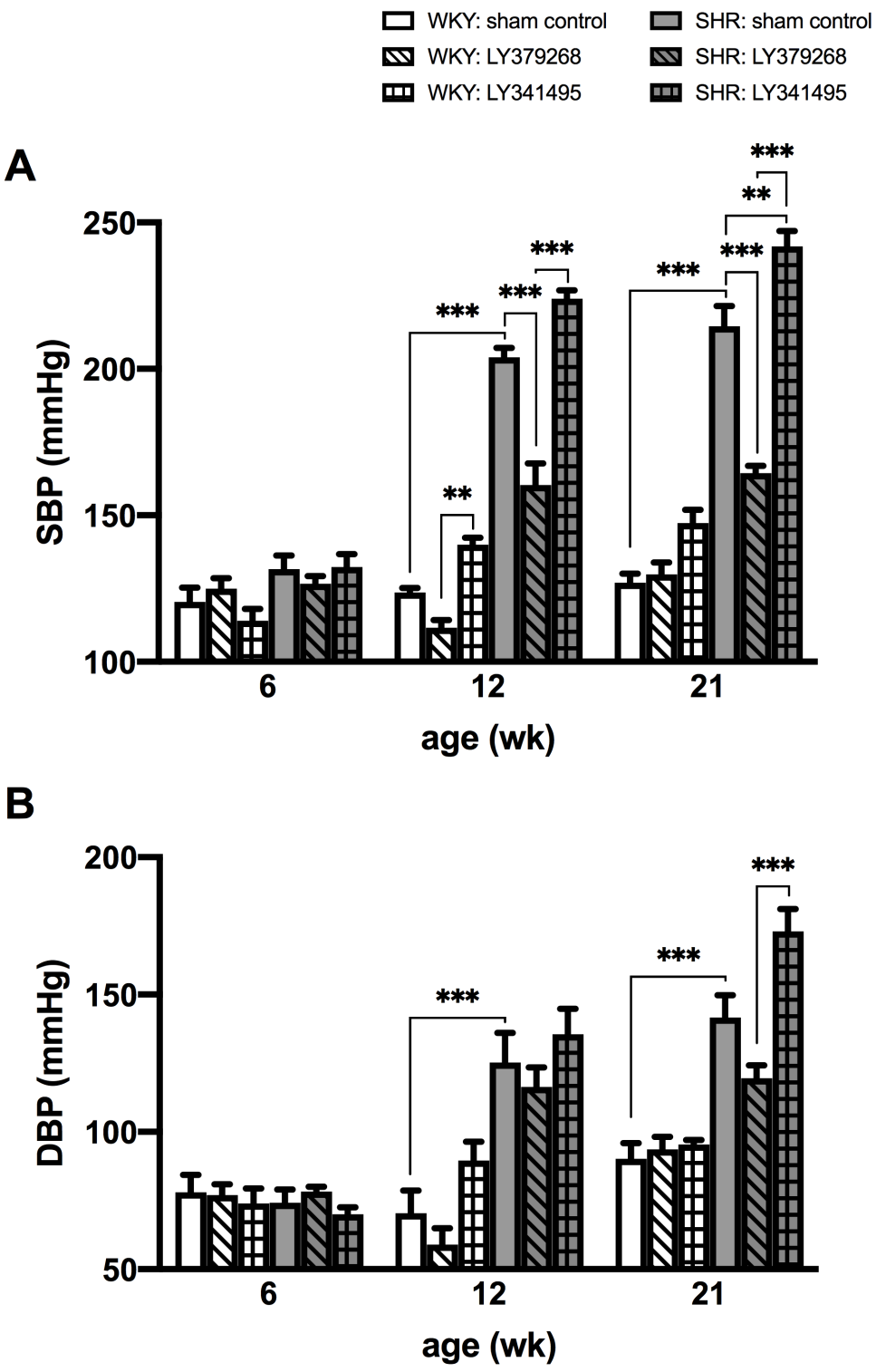


Figure 4-4. Blood pressure and heart rate measured in various groups of rats aged 6 to 21 weeks. The mGluR2/3 treatment was given to rats at 6 to 12 weeks of age. The shaded area indicates the period of drug application. The effect of mGluR2/3 treatment was greater in SHRs than in WKYs, especially on SBP. The data in each group are mean ± SEM from five rats. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; wk, week.



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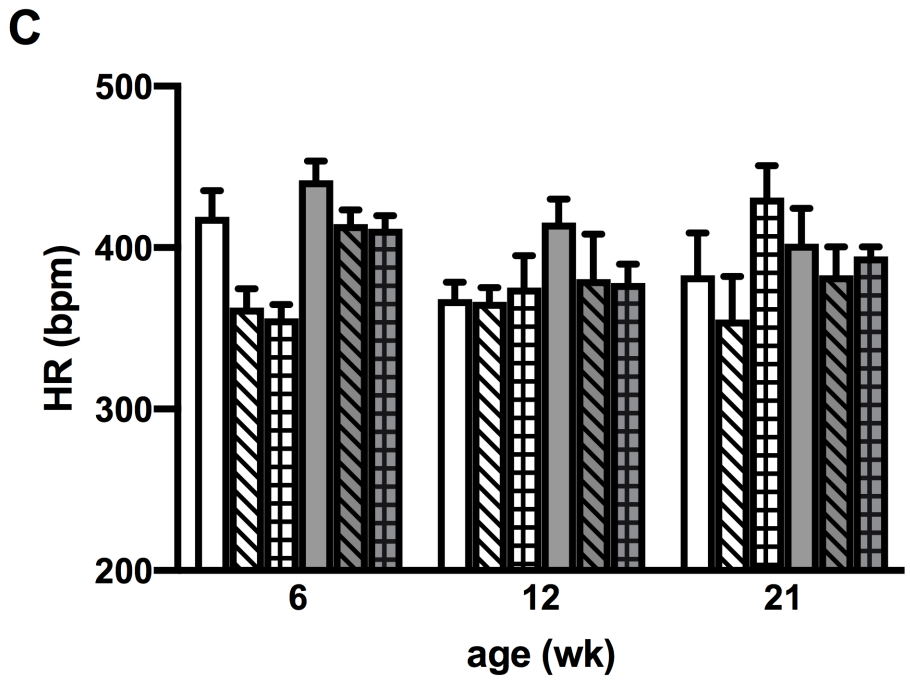


Figure 4-5. Blood pressure and heart rate measured in SHRs and WKYs aged 6 (before treatment), 12 (during treatment), and 21 (after treatment) weeks. The suppressed effect of LY379268 and the intensified effect of LY341495 were observed, especially on SBP. $^{**}P < 0.01$ and $^{***}P < 0.001$; statistical evaluations were performed using two-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from five rats. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; wk, week.

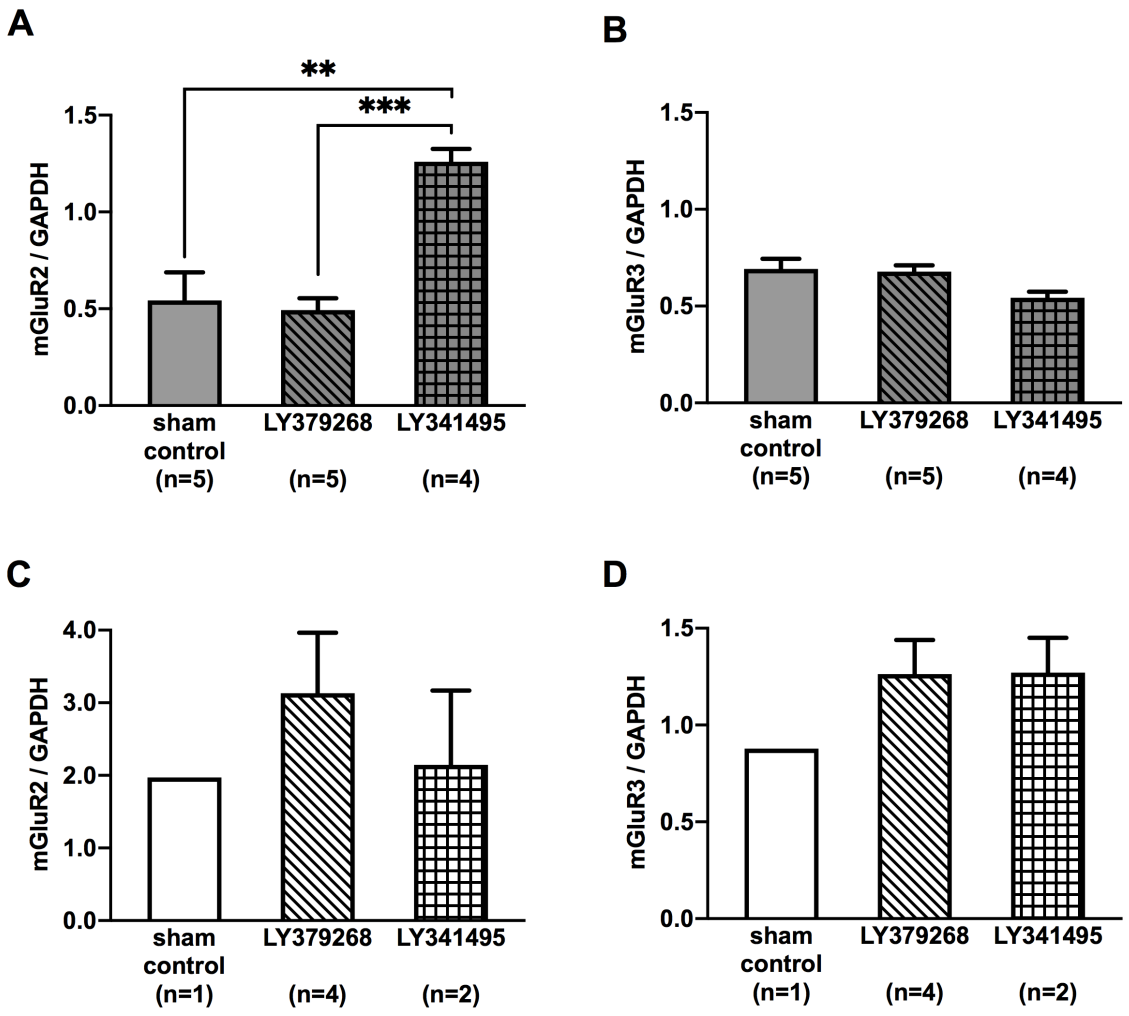


Figure 4-6. Relative mRNA expression levels of mGluR2 and mGluR3 in 12-week-old medulla oblongata samples of SHRs (A, B) and WKYs (C, D). Each mRNA expression was estimated by real-time PCR using GAPDH housekeeping gene expression. Among all groups, SHRs treated with LY341495 had the greatest increase in the expression level of mGluR2. $^{**}P < 0.01$, $^{***}P < 0.001$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from one to five rats.

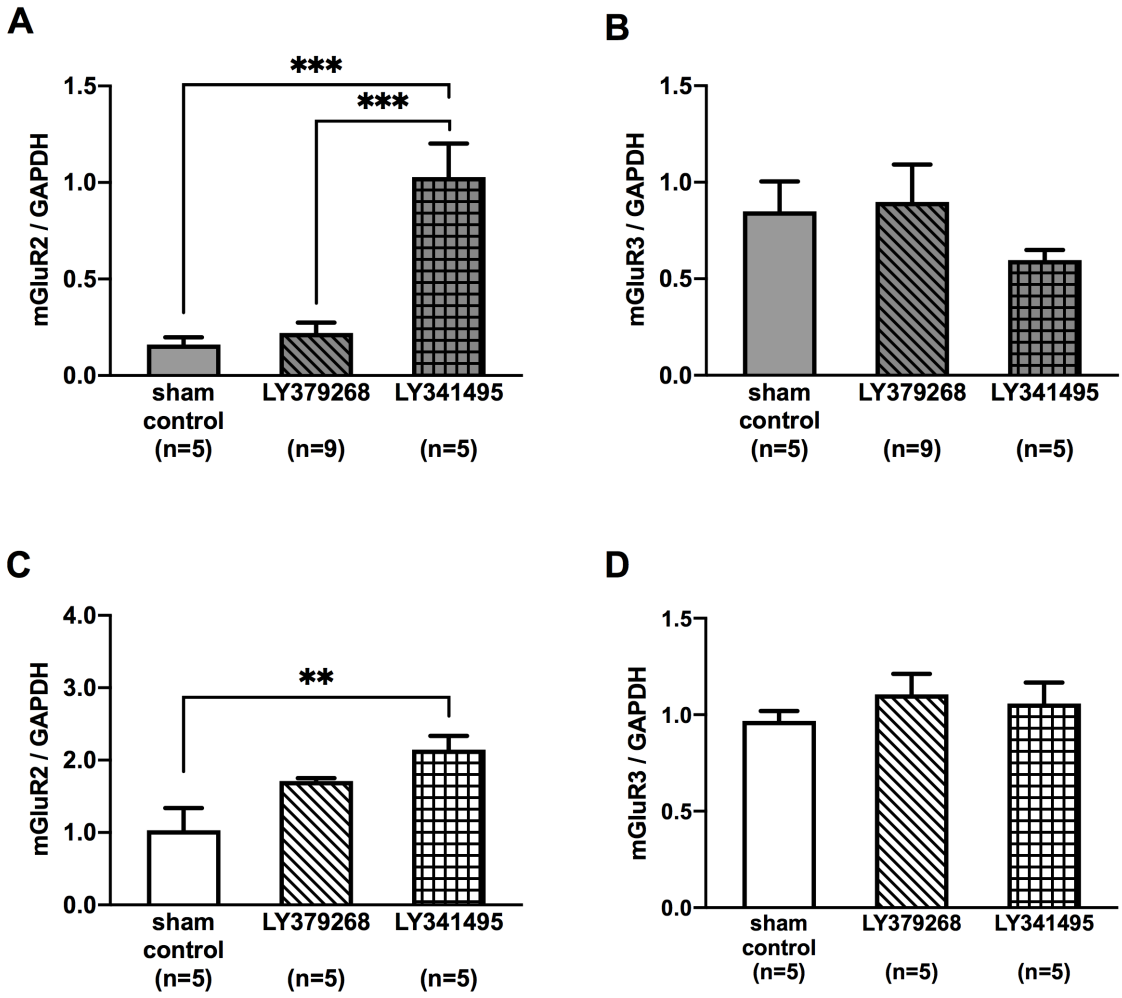


Figure 4-7. Relative mRNA expression levels of mGluR2 and mGluR3 in 21-week-old medulla oblongata samples of SHRs (A, B) and WKYs (C, D). Each mRNA expression was estimated by real-time PCR using GAPDH house-keeping gene expression. Greater expression of mRNA was observed in mGluR2, and no difference in mGLuR3 mRNA was observed between either groups. $^{**}P < 0.01$, $^{***}P < 0.001$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from five or nine rats.

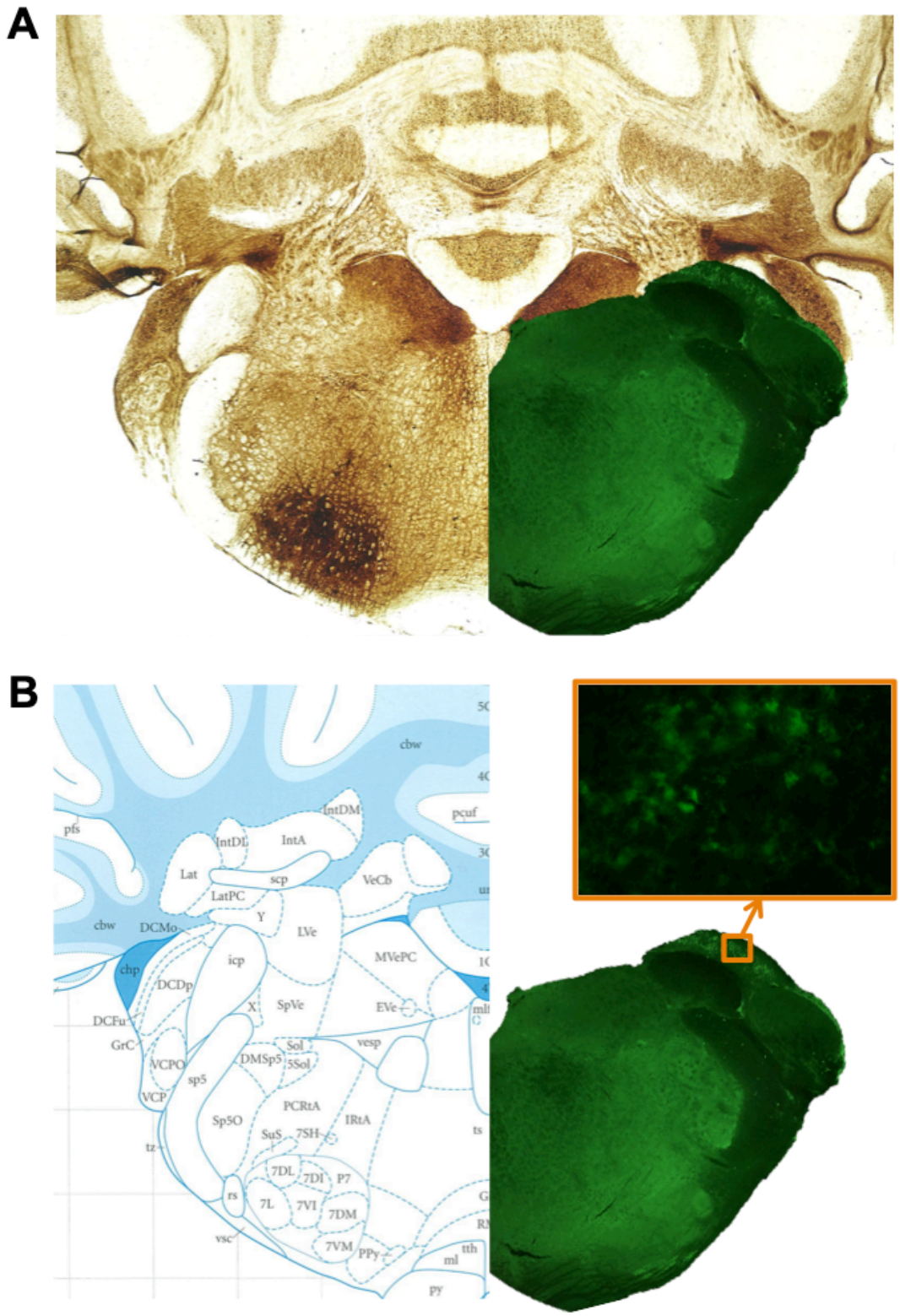


Figure 4-8. Immunohistochemical staining of mGluR2 in the medulla oblongata of rats. The 40-µm of coronal slice was distinguished with brain atlas (Paxinos and Watson, 2014). (A) Stained slice was paralleled with acetylcholinesterase (AChE) staining (bregma -11.04 mm). (B) The location of the positive area on the stained slice was identified as the cochlear nucleus in the parallel with the schematic image (bregma -11.04 mm). DC, dorsal cochlear nucleus; Sol, solitary tract.

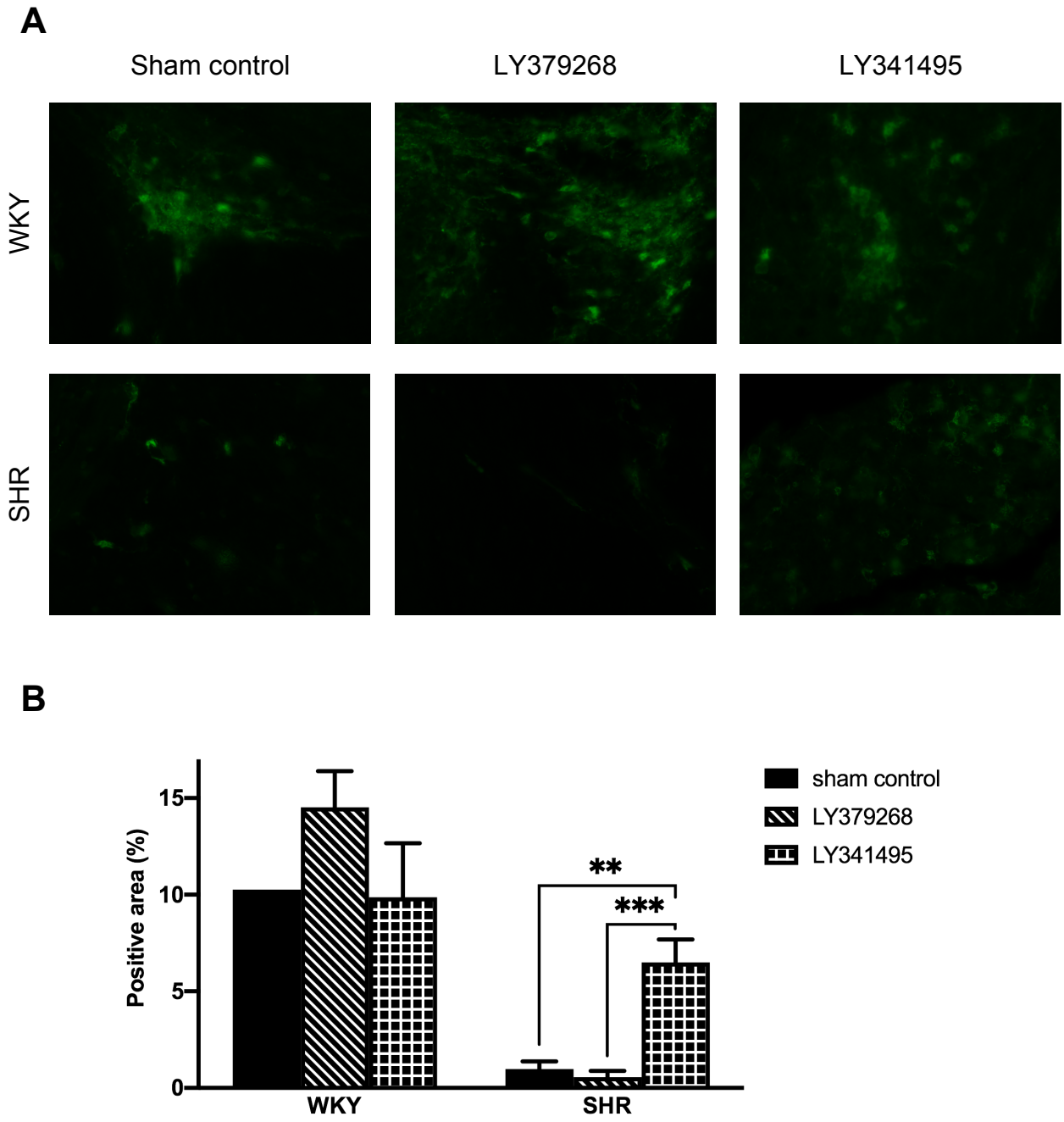


Figure 4-9. LY341495 increased the positive area of mGluR2 in 12 week-old SHRs. (A) The representative images show immunohistochemical staining with a mGluR2 antibody using medulla oblongata obtained from 12-week-old SHRs and WKYs. (B) The positive area of mGluR2 in medulla oblongata tissues obtained from SHRs and WKYs was calculated. The magnification is 200× for each image in panel A. Note that mGluR2 expression is indicated by the green color. $^{**}P < 0.01$, $^{***}P < 0.001$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from four rats.

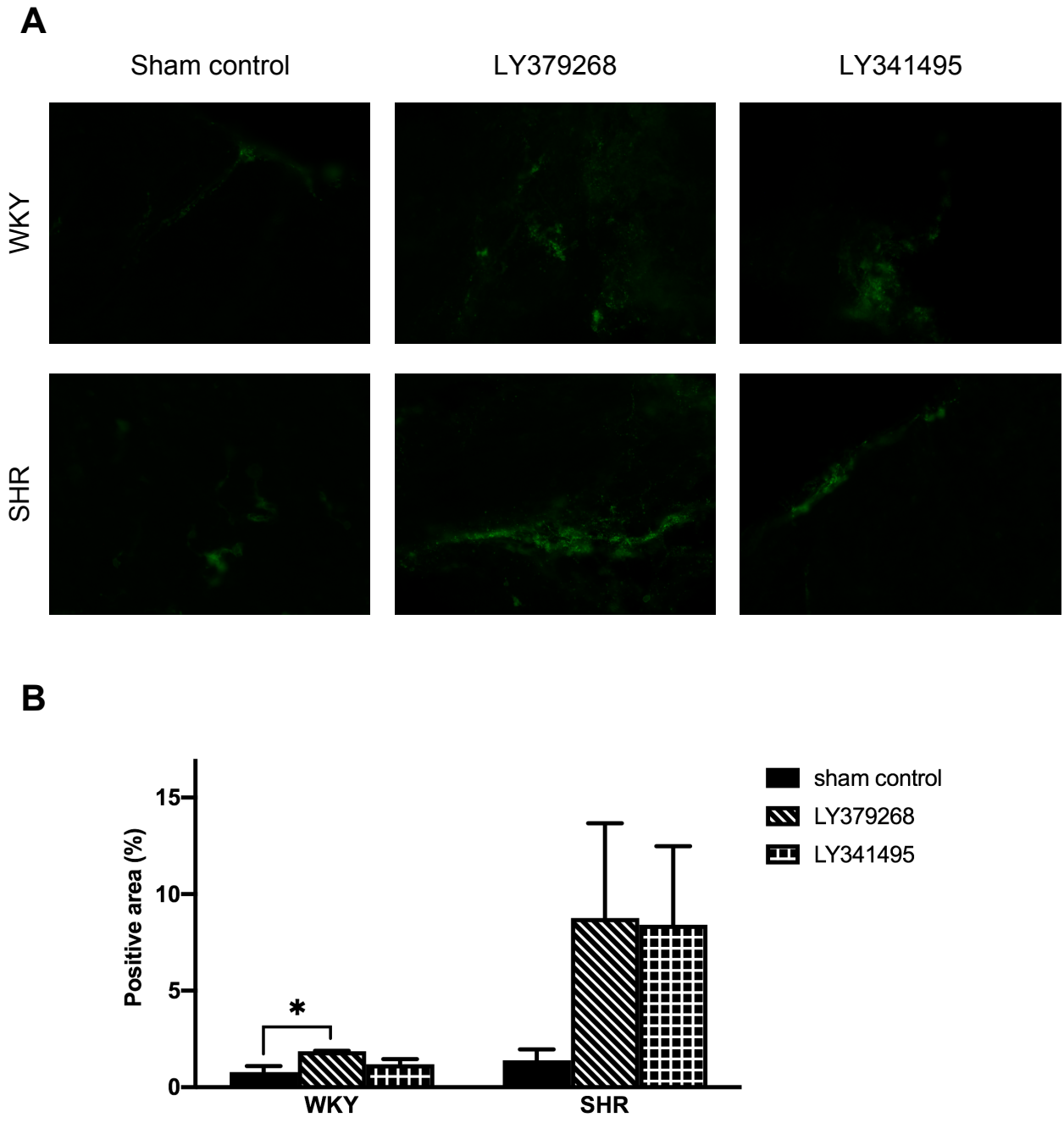


Figure 4-10. Augmented positive area of mGluR2 was revealed only in LY379268-treated WKYs, 9 weeks after the treatment ended. (A) The representative images of immunohistochemical staining were presented on the medulla oblongata sections obtained from 21-week-old rats. (B) The positive area of mGluR2 on the medulla oblongata obtained from SHRs and WKYs was measured and showed greater increase in LY379268-treated WKYs. The magnification is 200× for each image in panel A. Note that mGluR2 expression is indicated by the green color. **P* < 0.05; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from four rats.

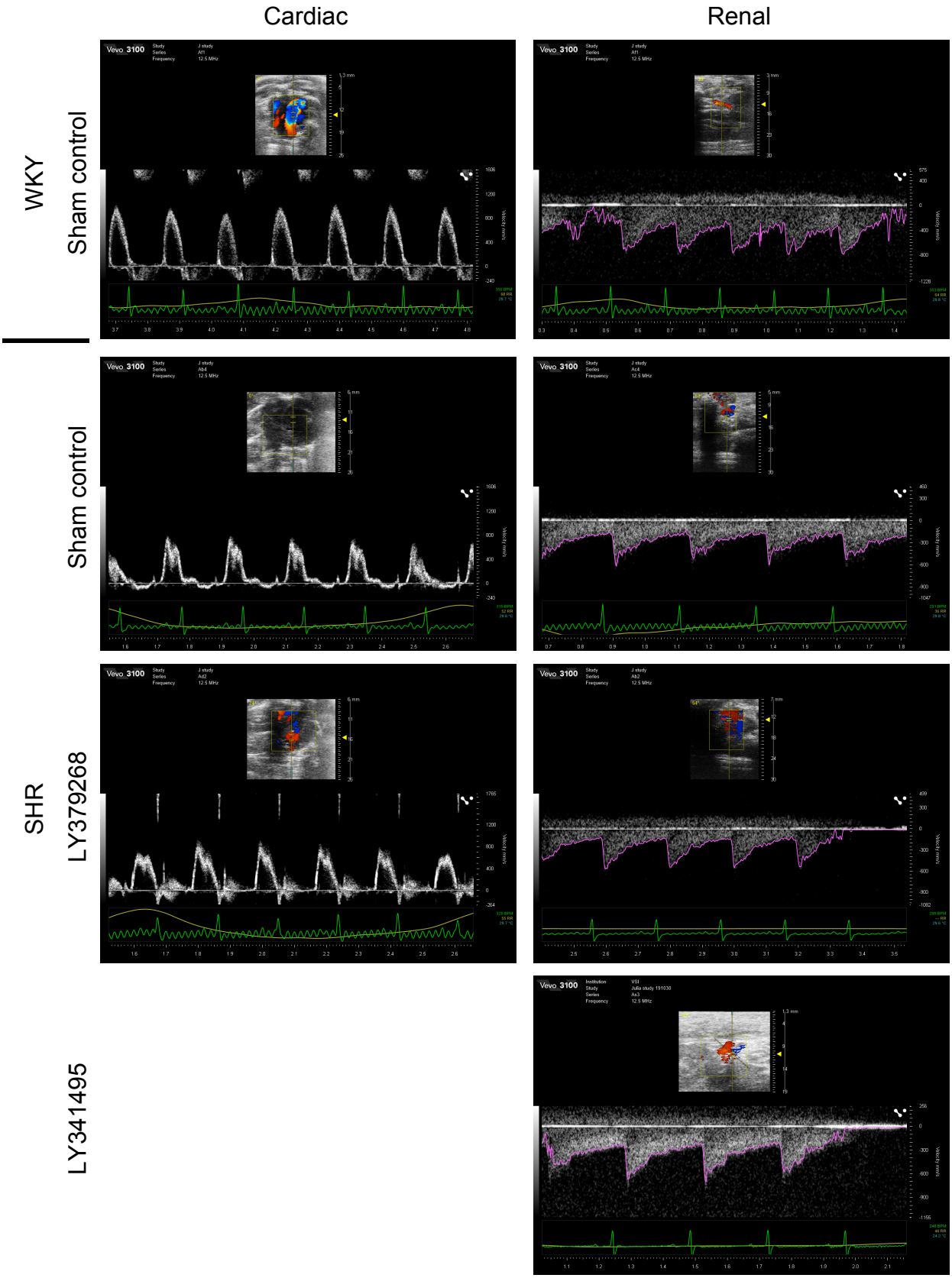


Figure 4-11. mGluR2/3 treatment did not affect the heart and kidneys. The representative images of echocardiography and renal ultrasonography are presented, which assessed cardiac and renal function of 18-week-old rats. Multiple heartbeats and respiration curves are shown in the lower part of each image.

Table 4-1. Effects of LY379268 and LY341495 in the brainstem of 18-week-old rats on the cardiac and renal parameters of ultrasonography.

Parameter	Unit	Wistar Kyoto rat	Spontaneously hypertensive rat		
			Sham control	LY379268	LY341495
Cardiac					
Heart Rate	bpm	329.5±28.1	284.0±16.0	308.1±48.3	343.4±30.4
Stroke Volume	μl	213.4±19.0	217.6±10.2	206.2±30.0	241.0±41.8
Ejection Fraction	%	69.1±5.6	58.1±8.4	65.1±5.9	61.6±7.2
Fractional Shortening	%	40.3±4.4	32.3±5.9	37.1±4.6	34.8±5.3
Cardiac Output	ml/min	70.3±8.3	61.7±3.5	64.3±15.9	81.8±10.2
Renal					
RRA-PSV	mm/s	786.3±131.0	555.9±111.4	813.2±447.6	544.2±147.9
LRA-PSV	mm/s	780.6±172.6	430.4±149.9	490.3±170.5	440.8±201.1
RRA-LDV	mm/s	231.7±83.5	143.9±22.5	223.6±56.0	215.4±51.3
LRA-LDV	mm/s	237.4±68.0	158.1±65.2	191.2±102.9	207.9±92.3
RRA-RI	-	0.70±0.10	0.74±0.04	0.68±0.13	0.60±0.04
LRA-RI	-	0.67±0.12	0.63±0.08	0.63±0.09	0.53±0.05
RRA-PI	-	1.38±0.40	1.48±0.16	1.28±0.46	0.91±0.14
LRA-PI	-	1.30±0.55	1.11±0.35	1.07±0.34	0.75±0.08

No difference was observed between either groups. Statistical evaluations were performed using one-way ANOVA followed Tukey's test. Data in each group are mean ± SEM from four or five rats. RRA-PSV, right renal artery-peak systolic velocity; LRA-PSV, left renal artery-peak systolic velocity; RRA-LDV, right renal artery-lowest diastolic velocity; LRA-LDV, left renal artery-lowest diastolic velocity; RRA-RI, right renal artery-renal arterial resistive index; LRA-RI, left renal artery-renal arterial resistive index; RRA-PI, right renal artery-pulsatility index; LRA-PI, left renal artery-pulsatility index.

Chapter 5

Responses of Autonomic Nervous Control and Baroreflex Function with the Central Administration of mGluR2/3 Treatment in SHRs

5.1 Introduction

mGluR2/3-related blood pressure regulation was observed in the dorsal medulla oblongata, but the mechanism of the cardiovascular system was not entirely elucidated via the examination of molecular diagnosis and cardiorenal function. The analysis of heart rate variability is considered to be an early diagnostic tool for detecting autonomic impairment (Kuwahara *et al.*, 1996; Nguyen *et al.*, 2019). By virtue of heart rate variability assessment, the distinction of the autonomic nervous system between SHRs and WKYs has been detected as early as 4 weeks of age (Kuwahara *et al.*, 1996). The adrenaline content in the medulla oblongata is also higher in SHRs than in WKYs at 4 weeks of age (Wijnen *et al.*, 1978). SHRs begin to develop hypertension at 6 weeks of age (Okamoto and Aoki, 1963; Anishchenko *et al.*, 2015). SHRs and WKYs have different mean arterial pressure (MAP)-heart rate (HR) response at 6 weeks of age (Head and Adams, 1992). Since 1859, the relationship between MAP and HR has been considered to demonstrate the baroreflex function (Farah *et al.*, 1999). Baroreflex is a mechanism that supports the maintenance of blood pressure at a steady level all the time (Head, 1995); it is affected by the status of the sympathetic nervous system and cardiac vagal response (Furlan *et al.*, 2019). The present study was designed to examine the activity of the autonomic nervous system during mGluR2/3 treatment in the medulla oblongata of SHRs with heart rate variability analysis, a supportive method for the early diagnosis of proven sepsis in neonatal medicine (Nguyen *et al.*, 2019). Catecholamine levels in the blood and baroreflex function were also investigated after the 6-week mGluR2/3 treatment was completed to determine the contribution of mGluR2/3 modulation on blood pressure regulation.

5.2 Materials and Methods

1. Animal Preparation

- Animals and Rearing Condition

Four-week-old male SHRs (n = 27) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The housing and rearing of animals were in accordance with the methods described in Chapter 2.

- Surgical Operation of Dorsal Hindbrain mGluR2/3 Treatment

At 6 weeks of age, the SHRs were subjected to dorsal hindbrain treatment of mGluR2/3 with implantation surgery of mini-osmotic pump, which delivered solutions of LY379268 (0.40 μg/day; mGluR2/3 agonist) or LY341495 (0.40 μg/day; mGluR2/3 antagonist) continuously for 6 weeks, and the procedure for the operation was the same as that described in Chapter 2. Sham surgery was performed in animals without mini-osmotic pump as a control.

2. Indication of Autonomic Nervous System Activity

- Implantation of Telemetry Device for Electrocardiogram Recording

During the surgery of dorsal hindbrain treatment on SHRs, an electrocardiogram (ECG) telemetry device (ATE-01S, Softron Co., Ltd., Tokyo, Japan) was also implanted under isoflurane anesthesia. Paired wire electrodes of the transmitter were placed under the skin of the dorsal and ventral thorax for recording the apex-base lead ECG. Recordings were started at 1 week after surgery with a signal receiving board (ATR-1001, Softron Co., Ltd., Tokyo, Japan) placed underneath each cage in the chamber (MIR-554, PHC Holdings Corporation,

Tokyo, Japan) under standardized condition of temperature (24 ± 1 °C) and lighting (8:00-20:00 h). An ECG processor system (EP95U, Softron Co., Ltd., Tokyo, Japan) was used to record ECG signals continuously. The recording period lasted for 24 consecutive hours uninterrupted for 6 weeks at the age of 7 to 12 weeks. Then, the data were analyzed after removing interference.

- Analysis of Heart Rate Variability

Power spectral analysis of heart rate variability (HRV) was performed using the SRV-2W software (Softron Co., Ltd., Tokyo, Japan), and both time domain and frequency domain methods were used. The time domain analysis was based on R-R intervals for calculating the standard deviation (SD) and coefficient of variation (CV). In the frequency domain method, power spectral components were primarily classified into the low frequency range (LF; 0.04 to 1.0 Hz) and the high frequency range (HF; 1.0 to 3.0 Hz) (Kuwahara *et al.*, 1994). The normalized power spectral components of low frequency (LFnu) and high frequency (HFnu) were also calculated in order to diminish the alteration in very low frequency, highlighting the interaction between sympathetic and parasympathetic nerves (Burr, 2007).

3. Measurement of Catecholamine Concentration

When the dorsal hindbrain mGluR2/3 treatment of SHRs was completed at 12 weeks of age, the rats were decapitated under isoflurane anesthesia. Blood samples were then collected from the left superior vena cava, and centrifuged at 480 × g for 15 min at 4 °C after 4 °C overnight. The supernatant of the blood sample (blood serum) was stored at -80 °C for later analysis of catecholamine concentration.

The catecholamine concentration in the blood serum was assessed with an ELISA kit (Cat Combi ELISA RUO EIA-4309R, DRG Instruments GmbH, Marburg, Germany) in accordance with the manufacturer's instructions.

4. Assessment of Reflex Bradycardia and Reflex Tachycardia

- Surgical Preparation for Recording

After dorsal hindbrain mGluR2/3 treatment was completed at 12 weeks of age, treatment was removed, and SHRs were subjected to invasive catheterization (Figure 5-1). They were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg; Sigma-Aldrich, Saint Louis, MO, USA) dissolved in the distilled water. The animals were placed in the supine position, and then the following surgical procedures were performed under abolition of pain reflexes induced by pinching the animal's paws. A polyethylene catheter was inserted into the left femoral vein for intravenous administration of pharmacological agents. The left femoral artery was also cannulated to measure arterial blood pressure (BP).

- Evaluation of Baroreflex Activity

Arterial BP was tracked via the catheterized femoral artery with a catheter-transducer system (Nihon Kohden, Tokyo, Japan) connected to a computer acquisition system (Softron Co., Ltd., Tokyo, Japan) during the entire measurement. The MAP was calculated from measured systolic and diastolic BP. The lead II electrocardiography recording was achieved with needle electrodes. After 5 min of basal control recording, intravascular injections of phenylephrine (PE; 21 μg/kg) or sodium nitroprusside (SNP; 50 μg/kg) were performed through the catheter of the femoral vein (Almeida *et al.*, 2019; Lai *et al.*, 2019). Then, the cardiovascular waveforms were recorded for 15 min. After eliminating interference, the

waveforms of HR and MAP were made with averaged of each recording minute.

The baroreflex sensitivity was evaluated using the value of altered MAP (ΔMAP) and corresponding reflex of HR (ΔHR) at the peak responses to PE and SNP application (Thaeomor *et al.*, 2010; Almeida *et al.*, 2019). The baseline data for comparing peak responses were calculated as the average values over the 5-min period of basal value recording. PE and SNP increase or decrease MAP accordingly, so the responses from HR are defined as bradycardia and tachycardia reflex, respectively (Crestani *et al.*, 2010; Almeida *et al.*, 2019).

- Reagents

Phenylephrine hydrochloride (#163-11791; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in distilled water and stored as a stock solution with a concentration of 1 mg/ml. Sodium nitroprusside dihydrate (#199-02602; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was also dissolved in the distilled water and stored as a stock solution with a concentration of 3 mg/ml.

5. Statistical Analysis

All data are expressed as mean ± SEM. The comparison between groups was evaluated through one-way ANOVA followed by Tukey's test for multiple comparisons. Significant differences were considered at $P < 0.05$.

5.3 Results

1. Response of Autonomic Nervous System to mGluR2/3 Treatment on SHR

HRV analysis revealed the effect of mGluR2/3 treatment on autonomic nervous function in conscious SHRs (Figure 5-2). HR decreased with age (Figure 5-2A), whereas the HRV indices of time and frequency domain analyses, namely, SD, CV, and LF, time-dependently increased regardless of mGluR2/3 treatments (Figures 5-2B, 5-2C, 5-2D). Compared with sham control, the LY379268-treated group showed progressive increase in LF (Figure 5-2D) and HF (Figure 5-2E) but decreased in LF/HF ratio (Figure 5-2F) during the treatment period. On the other hand, the LY341495-treated group displayed adverse effects especially at 10 weeks of age, including decreased HF (Figure 5-2E) and increased LF/HF ratio (Figure 5-2F), while showing gradual increase in LF (Figure 5-2D).

The results of HRV analysis at 12 weeks of age in light phase, dark phase, and light and dark phases combined (total) are presented in Figure 5-3. All animal groups showed higher HR in the dark phase than in the light phase (Figure 5-3A). LY341495 treatment decreased HR, which was significant in the light phase (Figure 5-3A). Meanwhile, photoperiodic differences were not evident in HRV parameters obtained from the time domain analysis (Figures 5-3B, 5-3C). LY379268 treatment had no effect on the time domain data at 12 weeks of age (Figures 5-3B, 5-3C). On the other hand, LY341495 treatment increased the SD significantly, but the effect was not dependent on the photoperiod (Figure 5-3B). For the parameters from the frequency domain analyses, the altered level of LF/HF ratio (Figure 5-3F) was dependent on changes in HF (Figure 5-3E), particularly in the light phase. The agonist effect on LF was completely opposite when the data were normalized by total power. LY379268 substantially increased LF (Figure 5-3D) but significantly decreased LFnu, especially in the light phase (Figure 5-3G). Given that the spectral power in a given range can be influenced by power in other ranges, such as the extremely low frequency range in this

study, the latter result could be legitimate.

2. Effect of mGluR2/3 Treatment on Catecholamine Concentration

LY379268 treatment did not change blood catecholamine levels in SHRs: adrenaline (sham control vs. agonist, 1.07 ± 0.11 vs. 1.15 ± 0.16 ng/ml; n = 4 or 5 each; $P = 0.92$) (Figure 5-4A), and noradrenaline (sham control vs. agonist, 0.83 ± 0.14 vs. 1.16 ± 0.21 ng/ml; n = 4 or 5 each; $P = 0.79$) (Figure 5-4B). On the contrary, LY341495 treatment significantly increased adrenaline (sham control vs. antagonist, 1.07 ± 0.11 vs. 2.48 ± 0.15 ng/ml; n = 4 each; $P < 0.001$) (Figure 5-4A), and noradrenaline levels (sham control vs. antagonist, 0.83 ± 0.14 vs. 2.64 ± 0.61 ng/ml; n = 4 each; $P < 0.05$) (Figure 5-4B).

3. Effects of Dorsal Hindbrain mGluR2/3 Treatment on Baroreflex Function

The recording of HR and BP over time revealed that responses to PE or SNP exhibited a unimodal pattern (Figures 5-5, 5-6).

The initial phase had an increase in MAP (Figure 5-5B) and a related decrease in HR (Figure 5-5A) within 20 s after PE injection. The reformed phase had a sustained decrease in MAP and correlative varied HR reflex during the remaining 15-min observation period (Figure 5-5). After PE injection, the MAP of all groups almost returned to the 5-min basal level, but not HR. Compared with the HR in the 5-min basal control recording, the HR of the LY379268 group was lower, while that of the other two groups was higher.

After SNP injection in 20 s, the primary phase had a decreased MAP (Figure 5-6B) and increased HR (Figure 5-6A). During the remaining 15-min observation period, the reformed phase had steady MAP and HR (Figure 5-6). All groups had higher level of MAP after SNP

injection. The HR of the sham control group was not altered after SNP injection; the HR of the LY379268 group showed a slight decrease, whereas that of the LY341495 group increased substantially.

In the evaluation of baroreflex function, the LY379268 group displayed better bradycardia reflex in response to PE injection than other groups (Figure 5-7A), which means that the baroreflex function of SHRs was largely augmented by LY379268 treatment. Nevertheless, all groups displayed similar levels of tachycardia reflex after SNP injection (Figure 5-7B).

5.4 Discussion

In the time-domain analysis, mGluR2/3 treatment decreased HR (Figure 5-2A) and increased SD and CV (Figures 5-2B, 5-2C), which indicates that the impact from surgical operation has faded in accordance with the decline in physical pressure to the regulation system (Hunt *et al.*, 2018). Higher risk of hypertension has association with lower SD (Hemingway *et al.*, 2005) is indicated in the sham control group and LY379268 group, but not in LY341495 group (Figure 5-2B). Moreover, increases in SD and CV are likely used as indicators of better baroreflex sensitivity (Chang *et al.*, 2013), so the improvement of baroreflex function is enhanced by LY379268. The occurrence of autonomic nervous function during LY341495 treatment is hard to be interpreted with time domain analysis; therefore, frequency domain analysis can be used for reinforcement.

Substantial increase in LF was observed in all experimental groups, particularly in the LY341495 group, compared with the sham control group (Figure 5-2D). The distinction

between groups can be augmented clearly with LFnu, which increased in the LY341495 group and decreased in the LY379268 group after the treatment (Figures 5-2G, 5-3G). LF reflects both sympathetic and parasympathetic nervous activities (Gehrmann *et al.*, 2000); hence, it is quite complicated to decide the main source. Nevertheless, higher LF is related to hypertension, which is consistent with an early study (Kuwahara *et al.*, 1996). Furthermore, greater level of LFnu might have resulted from the restriction of sinus node responsiveness while facing high cardiac sympathetic activation and downregulation of beta-adrenoceptor (van de Borne *et al.*, 1997). Compared with the sham control group, increases and decreases in HF were caused by LY379268 and LY341495 treatment, respectively (Figure 5-2E). The same outcome was also shown in HFnu (Figure 5-2H). HF represents the parasympathetic cardiac control (Kuwahara *et al.*, 1994), so LY379268 or LY341495 could activate or suppress parasympathetic nerve activity, respectively. The LF/HF ratio was decreased by LY379268 and increased by LY341495 (Figure 5-2F). The LF/HF ratio is an index of interaction between sympathetic and parasympathetic nerves (Kuwahara *et al.*, 1994). Collectively, in the medulla oblongata, LY379268 activates the parasympathetic nervous system and simultaneously decreases the sympathetic nerve activity, whereas LY341495 reduces the parasympathetic nerve activity and triggers the activation of the sympathetic nerve system at the same time.

The level of catecholamine in the blood was not affected by the parasympathetic dominant condition found in the LY379268 group (Figure 5-4), suggesting that mGluR2/3 activation in the medulla oblongata did not change the impaired aspects in peripheral areas (Sata *et al.*, 2018). The release of catecholamine from the kidneys may have additive effects to the development of hypertension, increasing peripheral resistance (Galloway and Westfall,

1982). In this regard, catecholamine level was unaffected by LY379268. On the other hand, LY341495 treatment on the medulla oblongata brought sympathetic dominance and also resulted in high level of catecholamine (Figure 5-4). LY341495's antagonist effect on the medulla oblongata might increase the activation of phenylethanolamine N-methyltransferase (an enzyme for synthesizing adrenaline from noradrenaline) in the "A2 region," where the nucleus tractus solitarius (NTS) is located; subsequently, the adrenaline content in the NTS would be elevated, which increases blood pressure (Wijnen *et al.*, 1978). Adrenaline is released to the bloodstream by the activation of chromaffin cells on the adrenal medulla (Sala *et al.*, 2008; Dünser and Hasibeder, 2009), which receives acetylcholine signal from the splanchnic nerve of the sympathetic nervous system (Sala *et al.*, 2008; Breit *et al.*, 2018). Instead of adrenaline being secreted by the adrenal glands with the acetylcholinergic sympathetic nervous system (Sala *et al.*, 2018), the sympathetic nerve induces the production of adrenaline, which makes adrenaline act as a neurotransmitter, similar to noradrenaline (Brown *et al.*, 1984). Moreover, 6-week LY341495 treatment made dominance of sympathetic nerve system continues a duration, which exceeded the production of adrenaline evoked by sympathetic overstimulation (Dünser and Hasibeder, 2009). The overactivity of the sympathetic nervous system may exacerbate the hypertensive condition, and results in early end-organ damage, such as ventricular hypertrophy with thickening of myocardial fibers (Fisher and Paton, 2012; Shan *et al.*, 1999).

In terms of the baroreflex response, SHRs had better reflex bradycardia with LY379268 treatment, but no alteration of reflex tachycardia in all mGluR2/3 treatment. LY379268 treatment on SHRs resulted in parasympathetic dominance, which stimulated the release of acetylcholine from the vagal nerve in cardiomyocytes (Manabe *et al.*, 1991). Thus,

acetylcholine can bind with M2 muscarinic receptors to decrease the heart rate (LaCroix *et al.*, 2008). This reaction was observed in the present study, which is called reflex bradycardia. In reality, rilmenidine, a recognized antihypertensive prescription, reduces sympathetic baroreflex response and increases cardiac vagal baroreflex sensitivity (Head *et al.*, 1995). No differentiation in reflex tachycardia of all SHRs is understandable. Reflex tachycardia usually occurs in hypotension, which is activated during low blood pressure (Dünser and Hasibeder, 2009). SHRs, a hypertensive model, already have the risk of developing tachycardia (Palatini *et al.*, 1997). LY379268 did not change the genetic essence of SHR but decreased hypertension development. As a hypertensive model, SHRs have a potential impaired set point for blood pressure regulation (Takahashi *et al.*, 2011). LY341495 accelerated the progression of hypertension and caused baroreflex dysfunction is totally confirmed. Abnormal baroreflex function results in cardiac vagal suppression and neural sympathetic continuous existence (Furlan *et al.*, 2019), which was also demonstrated in LY341495-treated SHRs via HRV analysis.

The result of HRV analysis can explain the reaction of blood pressure during mGluR2/3 treatment. This notion is based on present and early findings, in which SHRs had higher LF level and LF/HF ratio but the same HF level than WKYs (Kuwahara *et al.*, 1996), suggesting the minor or major activity of sympathetic nerves with LY379268 or LY341495, respectively (Kuwahara *et al.*, 1994). An increase in BP is followed by increasing sympathetic output and lower parasympathetic tone (Fisher and Paton, 2012) because sympathetic dominance can increase blood pressure and parasympathetic dominance can decrease blood pressure (Widmaier *et al.*, 2011). During LY341495 treatment, the response from the sympathetic division was substantial, which was also evidenced with catecholamine level in the blood.

LY341495 increased the LF and LF/HF ratio, demonstrating that tachycardia is a result of increased activity of the autonomic nervous system on the heart (Hammoud *et al.*, 2018). Thus, reflex tachycardia was not significant with SNP injection. Although LY379268 did not change the blood's catecholamine level, it improved baroreflex function as indicated by SD and CV in HRV analysis and improved baroreflex response in reflex bradycardia.

5.5 Conclusion

The alteration of blood pressure from mGluR2/3 treatment in the medulla oblongata is fundamentally ascribed to the activity of the autonomic nervous system. The inhibition of mGluR2/3 activated the sympathetic nervous system, leading to the excessive production of catecholamine, which causes abnormal blood pressure regulation, such as hypertension. Compensating mGluR2/3 attenuated hypertension development through parasympathetic dominance without reforming the set point of blood pressure regulation, which displayed better baroreflex response in reflex bradycardia, but not reflex tachycardia.

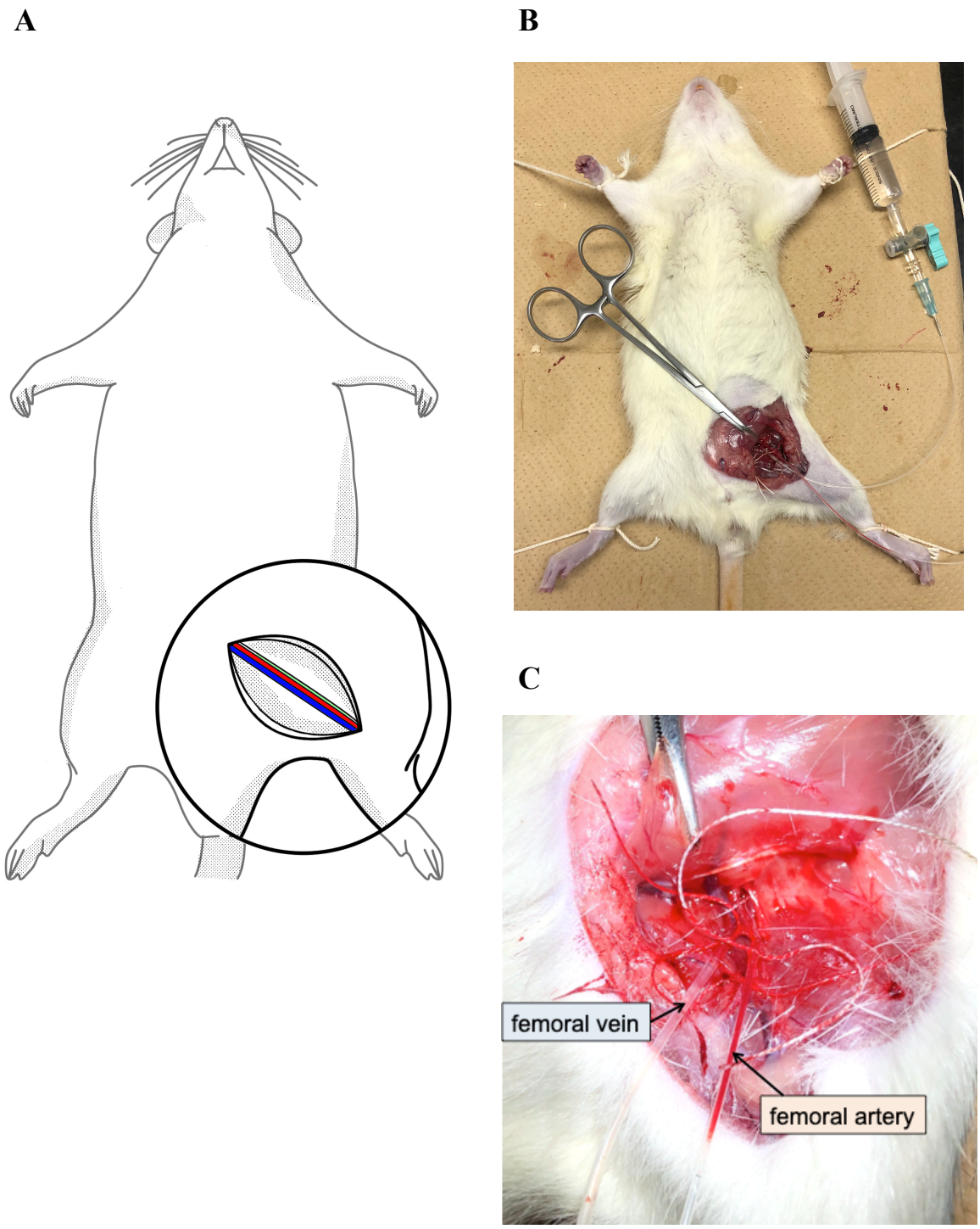
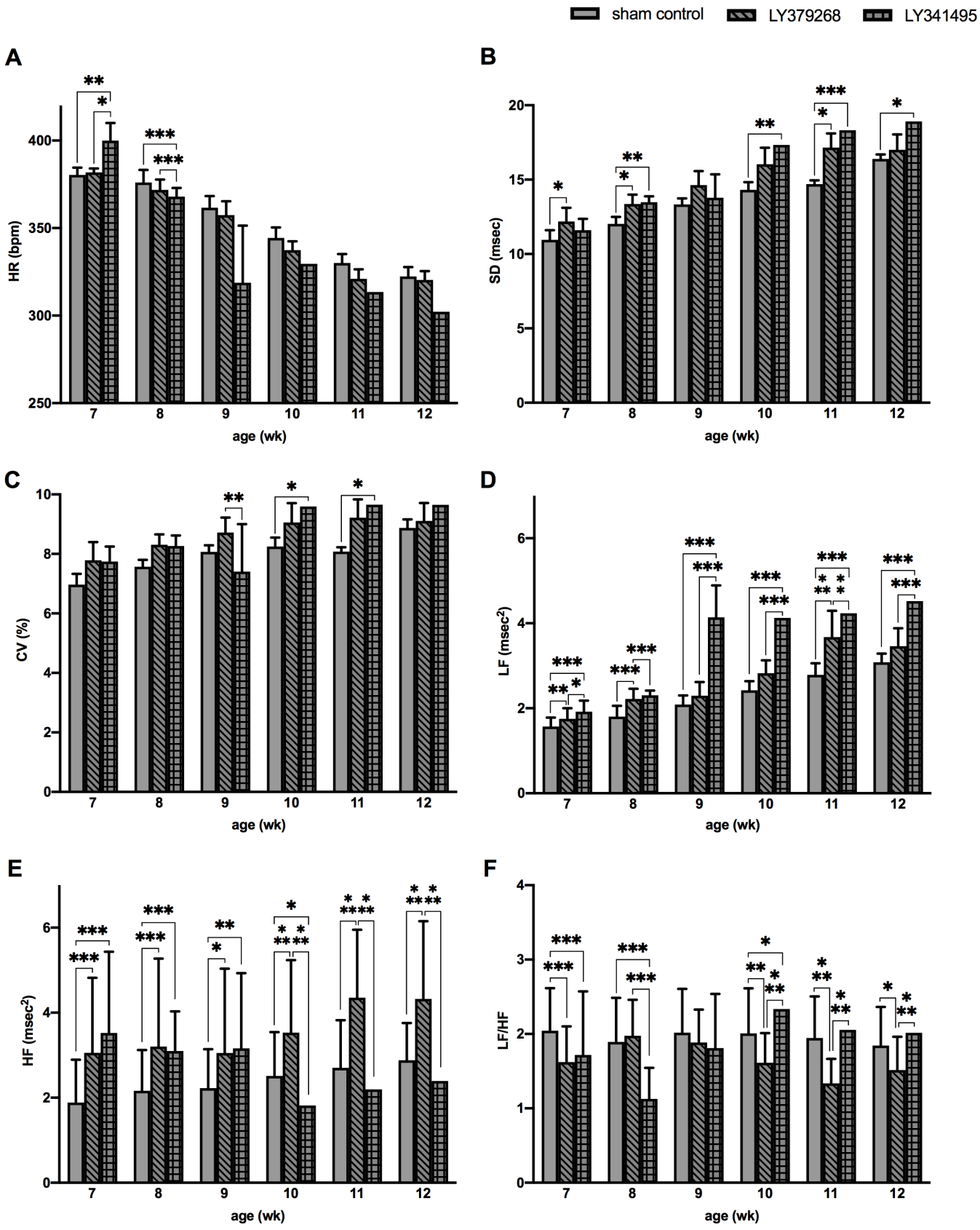


Figure 5-1. Surgical preparation for recording arterial blood pressure and drug administration. Panel A demonstrates the schematic of the structures, such as femoral vein (blue) and femoral artery (red). Panel B displays the actual surgical operation. Panel C shows the enlarged view of the catheterized area with the cannulated vessels.



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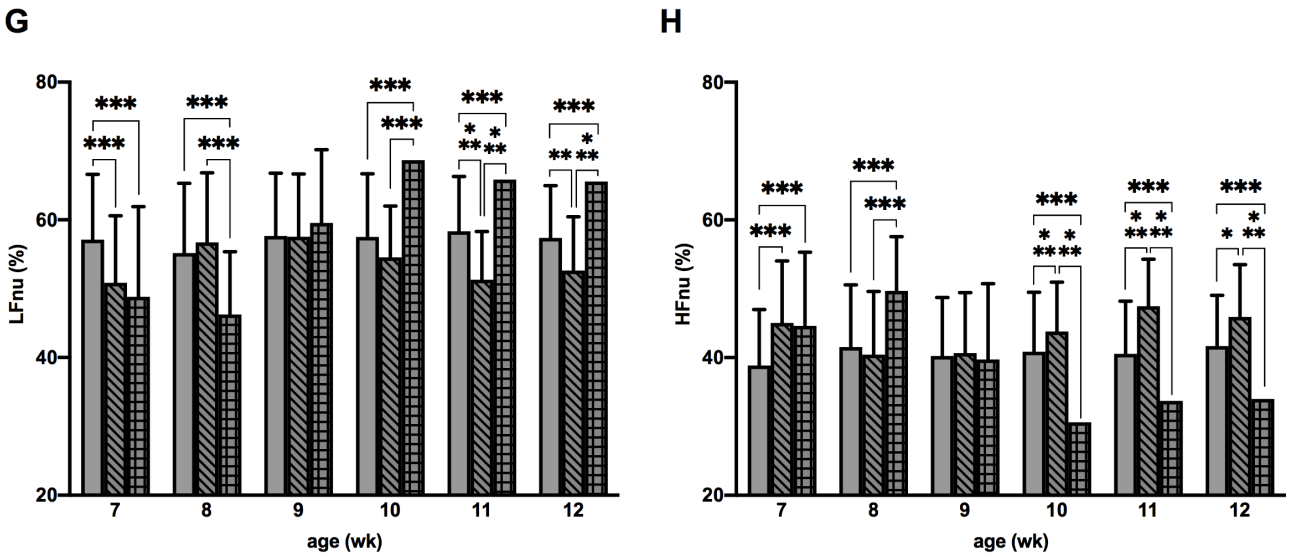
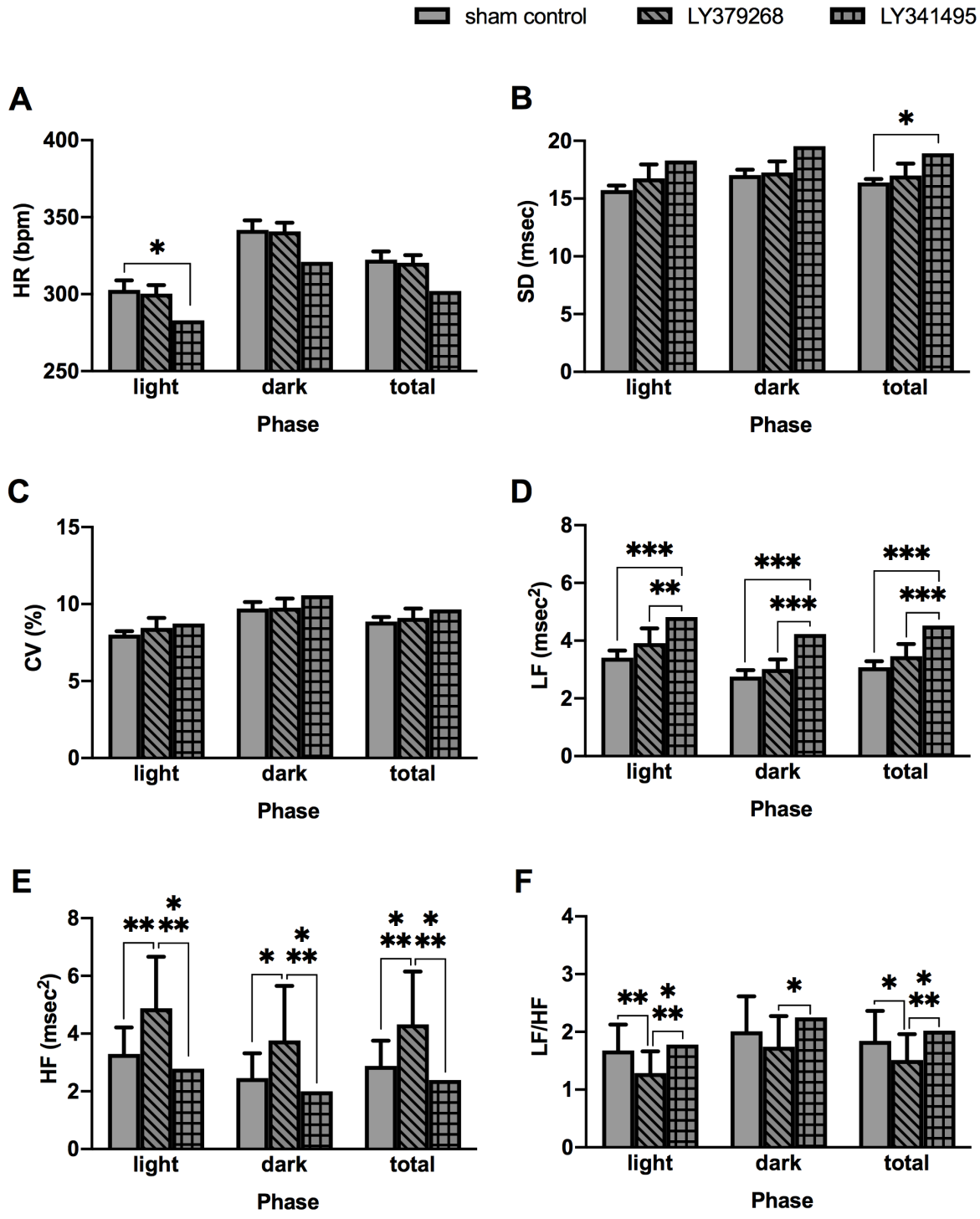


Figure 5-2. Variation of heart rate variability in 7- to 12-week-old in SHRs in 24 h per week. The mGluR2/3 treatment was given to rats at 6 to 12 weeks of age. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from one to five rats. HR, heart rate; SD, standard deviation; CV, coefficient of variation; LF, low frequency power; HF, high frequency power; LF/HF ratio, LF to HF ratio; LFnu, normalized low frequency power; HFnu, normalized high frequency power; wk, week.



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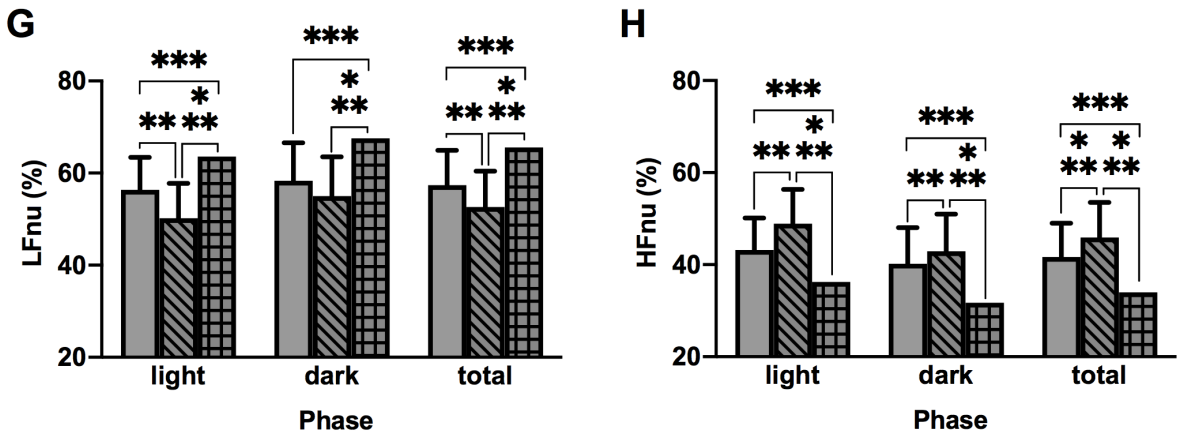

Figure 5-3. Specific photoperiod data of 12-week-old SHRs when the mGluR2/3 treatment was completed. The mGluR2/3 treatment was given to rats at 6 to 12 weeks of age. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from one and five rats. HR, heart rate; SD, standard deviation; CV, coefficient of variation; LF, low frequency power; HF, high frequency power; LF/HF ratio, LF to HF ratio; LFnu, normalized low frequency power; HFnu, normalized high frequency power.

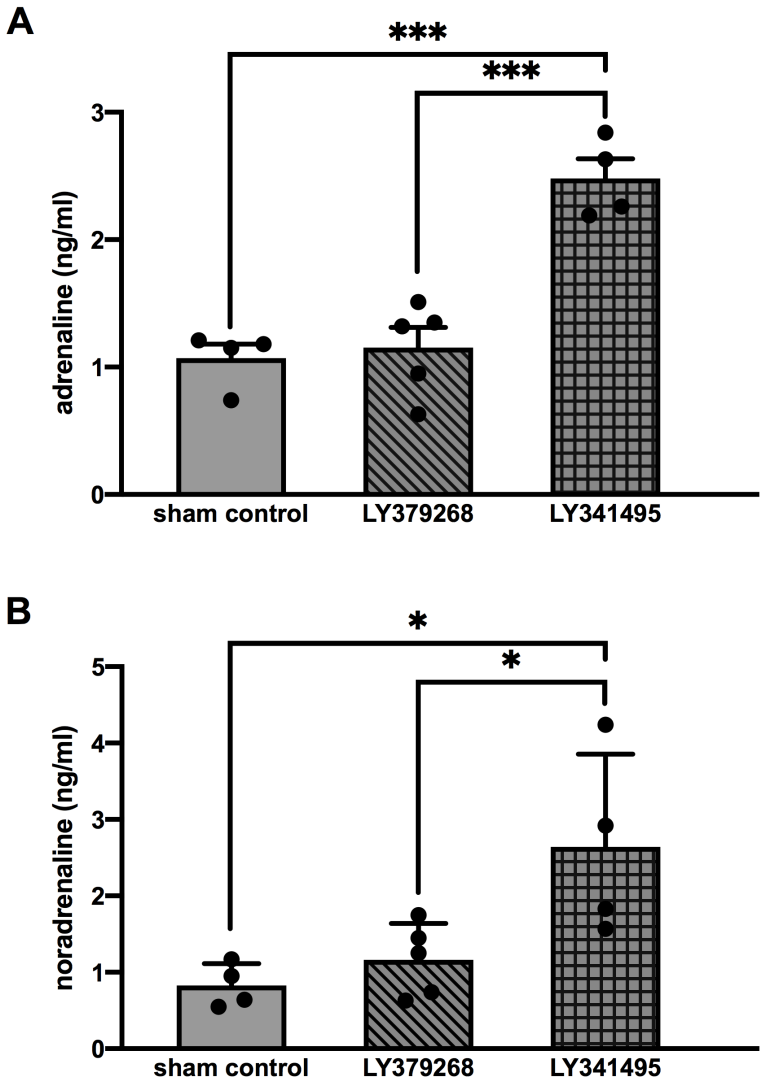

Figure 5-4. Catecholamine levels in the serum sample of SHRs from each group at the age of 12 weeks when the mGluR2/3 treatment was completed. Significantly higher concentrations of adrenaline and noradrenaline were detected after LY341495 treatment. No significant difference was observed between the sham control group and the LY379268 group. *$P < 0.05$ and ***$P < 0.001$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from four to five rats.

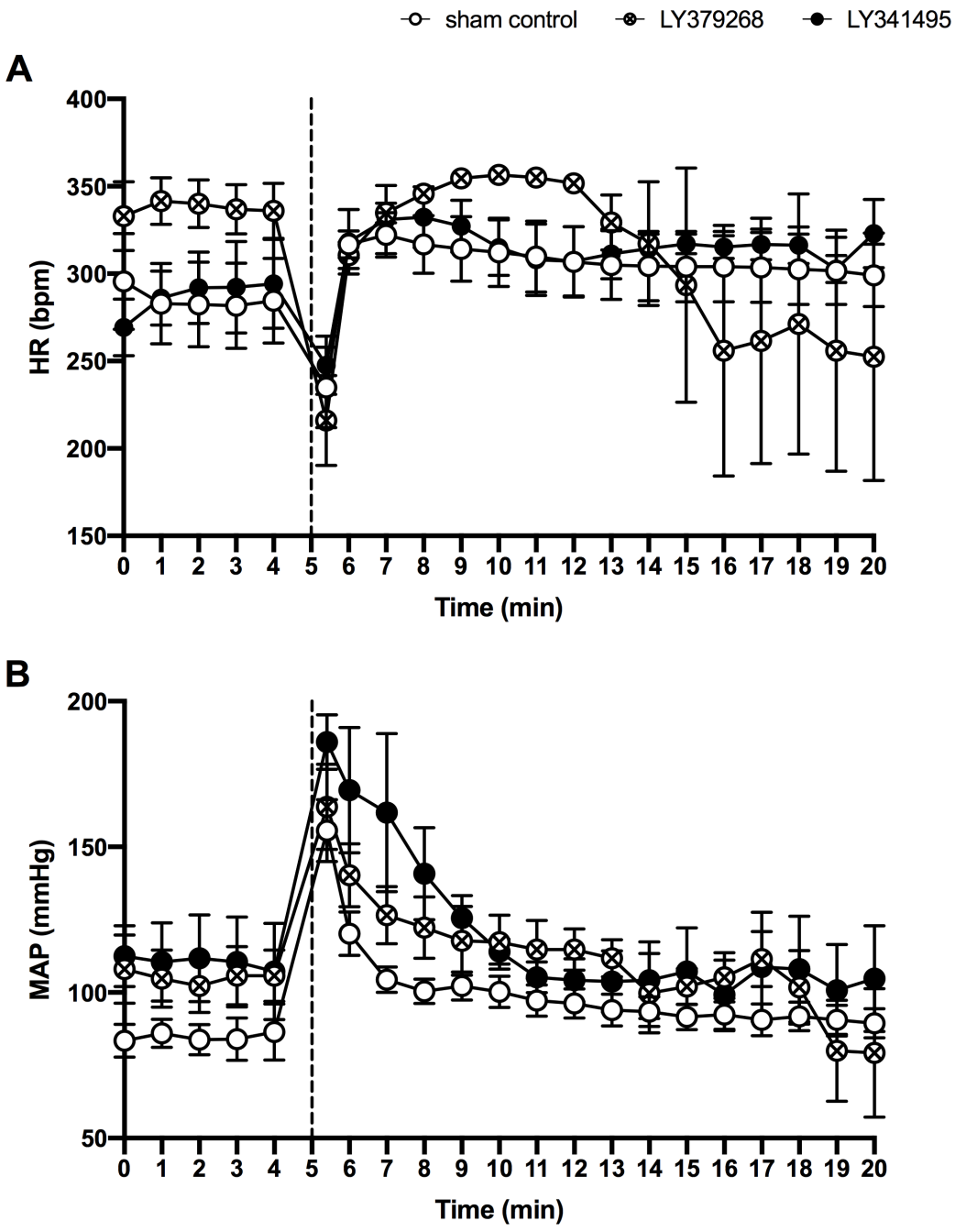

Figure 5-5. Response of heart rate (A) and mean arterial pressure (B) to intravenous injection of phenylephrine (21 μg/kg) in three groups of SHRs treated with mGluR2/3. The vertical dashed lines indicate the onset time of phenylephrine injection. The data in each group are mean ± SEM from four to five rats. HR, heart rate; MAP, mean arterial pressure.

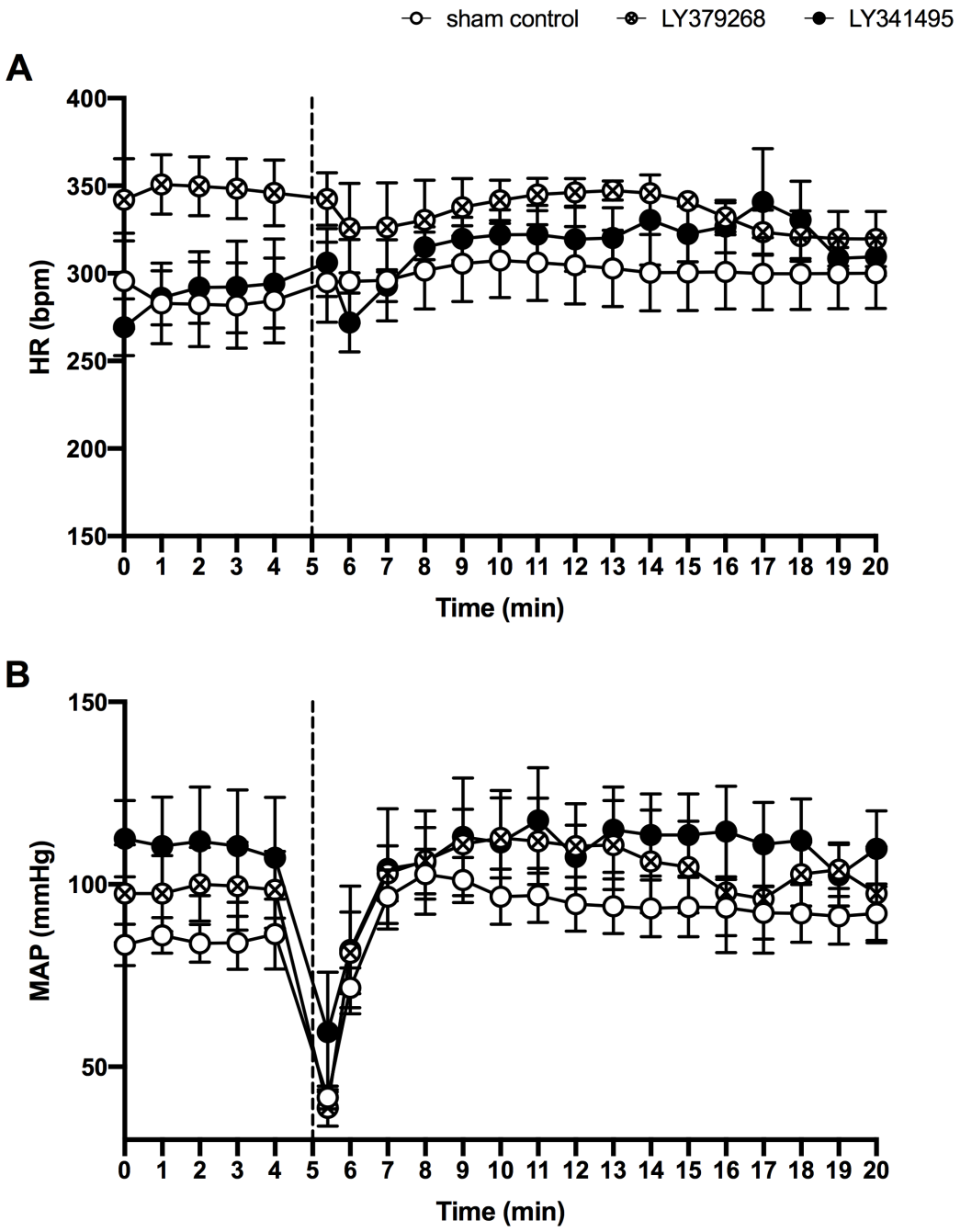

Figure 5-6. Response of heart rate (A) and mean arterial pressure (B) to intravenous injection of sodium nitroprusside (50 μg/kg) in three groups of SHRs treated with mGluR2/3. The vertical dashed lines indicate the onset time of sodium nitroprusside injection. The data in each group are mean ± SEM from four to five rats. HR, heart rate; MAP, mean arterial pressure.

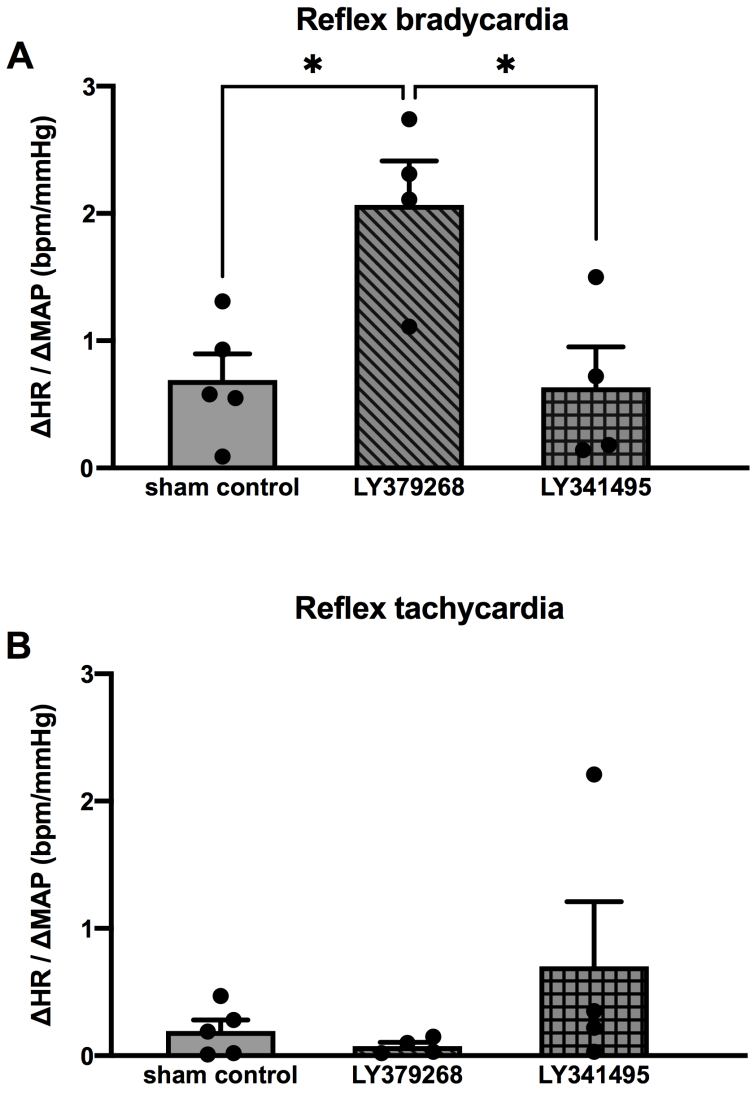

Figure 5-7. Effect of mGluR2/3 treatment on baroreflex sensitivity. Baroreflex function is assessed with altered mean arterial pressure (ΔMAP) and corresponding heart rate reflex (ΔHR) to intravenous injection of phenylephrine (21 μg/kg) and sodium nitroprusside (50 μg/kg), respectively, in three groups of mGluR2/3-treated SHRs. The initial response was defined as the peak response occurring within 20 s after injections. LY379268-treated SHRs had much better reflex bradycardia than others. *$P < 0.05$; statistical evaluations were performed using one-way ANOVA followed by Tukey's test. The data in each group are mean ± SEM from four to five rats.

Chapter 6

General Discussion and Conclusion

6.1 Discussion

This study showed that 6-week (chronic) dorsal hindbrain mGluR2/3 treatment with LY379268 (mGluR2/3 agonist) and LY341495 (mGluR2/3 antagonist) on juvenile rats changed blood pressure (BP) regulation, especially in SHRs. By virtue of LY379268, the mGluR2/3-altered value of systolic BP (SBP) was similar in various doses. For dorsal hindbrain mGluR2/3 treatment, 0.40 μg/day is the most satisfactory and reliable dose. The predominance of the autonomic nervous system activity may explain the decrease or increase in the BP of SHRs during and after the 6-week administration of mGluR2/3 agonist or antagonist in the brainstem, respectively. The critical target might be mGluR2 in the dorsal medulla oblongata. In HRV analysis, an increase in parasympathetic nervous activity from agonist treatment was confirmed by the relatively high HF and low LF/HF ratio. Increased sympathetic nervous activity from antagonist treatment was also confirmed by the comparably low HF and high LF/HF ratio in HRV analysis, as well as an increased catecholamine level in the blood. LY379268 improved the baroreflex function of SHRs, particularly in reflex bradycardia. On the other hand, echocardiography and renal ultrasonography data showed no considerable changes in cardiac function, thus failing to support the effect of mGluR2/3 treatment in SHRs. All these results suggest that the balance of parasympathetic and sympathetic nervous activity via mGluR2/3-mediated compensation or inhibition may modulate the regulation of BP.

mGluR2/3 is only located in the dorsal part of the medulla oblongata, such as the nucleus tractus solitarius (NTS), area postrema, and dorsal motor nucleus of vagus nerve (Hay *et al.*, 1999). In the present study, during the regulation of BP via modulating mGluR2/3 in the medulla oblongata, the mGluR2 subtypes have an important role, and the

significant distribution is probably located at the cochlear nucleus. The cochlear nucleus is the first structure of the central auditory system, which sends projection to amygdala (Paolini *et al.*, 2020). The amygdala belongs to the limbic system that arranges emotional expression (Templin *et al.*, 2019), and the projection may reach to the NTS (Takeuchi *et al.*, 1983; Watanabe *et al.*, 2015). The NTS receives inhibitory input from the amygdala, and then the input from the caudal ventrolateral medullary depressor area (CVLM) to the ventrolateral medullary pressor area (RVLM) is suppressed; thus, RVLM neurons are activated, which lead to increased blood pressure (Chopra *et al.*, 2011; Watanabe *et al.*, 2015). The amygdala may affect the activity of the autonomic nervous system with its own signal in response to stress via the NTS (Takeuchi *et al.*, 1983; Templin *et al.*, 2019), and the risk of cardiovascular disease is elevated with increased amygdala activity (Tawakol *et al.*, 2017). These findings indicate that the cochlear nucleus and amygdala may partly have contribution in mGluR2/3-treated rats during the BP regulation through the dorsal medulla oblongata.

The expression level of mGluR2/3 in the NTS is quite volatile in early postnatal development, and has a higher level at the juvenile age (Khozhai, 2018). The abnormal increase of Gi protein levels in the heart of SHRs starts from 2 weeks of age, which causes impaired BP regulation (Anand-Srivastava, 1996). Taking the result of LY341495 as an example, the augmented expression level of mGluR2 in the medulla oblongata could have reduced the amount of glutamate released, followed by the increased release of dopamine (Gołembiowska *et al.*, 2002; Khozhai, 2018). Then, the sympathetic outflow is enhanced by the greater dopamine release (Rea *et al.*, 1990). On the other hand, the increased expression level of mGluR2 may affect NTS indirectly, which adjusts the activation of mGluR2/3 (Takeuchi *et al.*, 1983; Watanabe *et al.*, 2015; Khozhai, 2018). Changing the activation of

mGluR2/3 decreases glutamate release, and brings less glutamatergic inputs to the CVLM (Sved *et al.*, 2000; Viard and Sapru, 2002). When the CVLM is not excited, the inhibitory projection of gamma-aminobutyric acid (GABA) to the RVLM is blocked (Viard and Sapru, 2002; Mandel *et al.*, 2008). The activated RVLM leads to the dominance of sympathetic vasomotor tone, and then increases the levels of catecholamine and vasopressin in plasma (Ross *et al.*, 1984; Mandel *et al.*, 2008), which is consistent with our findings in HRV analysis and catecholamine level in the blood sample. Although no significant change was observed in the levels of gene expression and serum catecholamine with LY379268, the inference from LY341495 may be useful to explain the association between agonist-altered BP and dominance of parasympathetic nerve activity.

The activation of mGluR2/3 with LY379268 decreases the release of glutamate (Imre, 2007). However, the period of stimulation should be deliberated in this study, because difference between short-term and long-term is obvious and effective. In consideration of long-term stimulation is the chosen method, it includes the activation of N-methyl-d-aspartate (NMDA) receptors. The binding of glutamate activates NMDA receptor (Lüscher and Malenka, 2012). The amount of activated NMDA receptors decreases during the competition with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors on binding glutamate. At the same time, the threshold of long-term stimulation will increase, keeping the whole network and neurons nearly steady and stable (Lüscher and Malenka, 2012; Diering and Huganir, 2018).

In the medulla oblongata, the cardiovascular network includes the NTS, CVLM, and RVLM for controlling the baroreflex function (Colombari *et al.*, 2001). The NTS, the first cardiovascular site in the dorsal medulla oblongata, has higher firing rate of SHR neurons in

the caudal area than WKY neurons, which is related to higher blood pressure (Abdel-Rahman and Tao, 1996; Talman, 1997; Colombari *et al.*, 2001). The CVLM receives glutamatergic projections from the NTS, has weaker input in the caudal area of SHRs compared with WKYs, and may affect the sympathetic outflow (Sved *et al.*, 2000; Viard and Sapru, 2002; Mandel *et al.*, 2008). The firing rate of the RVLM was faster in SHRs than in WKYs since the neonatal stage (Matsuura *et al.*, 2002). End-organ damage may appear from impaired baroreflex function, especially in hypertension (Shan *et al.*, 1999). In our study, LY379268 improved the reflex bradycardia in baroreflex function probably through the release of more glutamate, which inhibits GABA release in NTS; thus, signal transmission of baroreceptor is also remodeled accordingly (Chen and Bonham, 2005; Potts, 2006). SHRs have a quantitative trait locus on chromosome 8 that is related to the regulation of heart rate, which causes tachycardia and hypertension (Silva *et al.*, 2007). Therefore, LY379268 seems unable to modify this genetic region. Apparently, the response of reflex tachycardia was still on the same level in the sham control group and LY341495-treated SHRs. Due to the same reason, the cardiac and renal ultrasound examination detected no difference between sham control group and experimental groups in SHR.

The respiratory-related sympathetic nerve activity of SHRs was higher than that of WKYs since 9 days of age, which affected vascular tone; then, the SHR's vascular resistance was adjusted from 5 weeks of age (Simms *et al.*, 2009). mGluR2/3 treatment in the dorsal medulla oblongata may change the BP with the balance of the autonomic nervous system by CVLM, the vasomotor center, via modulating sympathetic vasomotor tone (Mandel *et al.*, 2008; Joyner and Limberg, 2014). In the present study, LY379268 enhanced the parasympathetic activity, while LY341495 augmented the sympathetic activity. Two reasons

can explain the partial attenuation of BP and improved baroreflex function only after giving LY379268 chronic treatment on SHR. Vascular hypertrophy and minimal left ventricular hypertrophy existed in SHRs since 4 weeks of age, although the BP was still similar to that of age-matched WKYs (Adams *et al.*, 1989). Moreover, the predysfunctional state from neonatal stage brought upward resetting of central sympathetic center in SHR (Judy and Farrell, 1979; Takahashi *et al.*, 2011). Although LY379268 brought dominance of the sympathetic nervous system in the dorsal medulla oblongata of SHRs in the whole duration of hypertension development, the sympathetic nerves were still activated during this parasympathetic dominance, and may compensate the dysfunctional structures that ascending standard level of sympathetic center. This inference can also explain why LY341495-treated SHRs had higher BP and sympathetic dominance than sham control ones. In severe progression of hypertension, the hyperactivity of the sympathetic nervous system was observed in the renal region since 2 weeks of age and consequently resulted in significant end-organ damage, including fibrohyalinosis in arterial walls and myocardial hypertrophy with thickened fibers, at the adult age (Grisk and Rettig, 2004; Shan *et al.*, 1999). Given that the cardiac and renal ultrasound examination was performed around 18 weeks of age (juvenile age), the end-organ damage was not shown in this study. The end-organ damage in the renal area is anticipated in LY341495-treated SHRs because the catecholamine level in the blood was increased.

6.2 Conclusion

SHRs are considered to have hypofunctional glutamatergic system in earlier studies

(Glazewski *et al.*, 1995), but the specific reason still remains unclear. In this study, the 6-week chronic stimulation/inhibition of mGluR2/3 in the dorsal part of the medulla oblongata either attenuates or exacerbates the development of hypertension by changing the activity of the autonomic nervous system (Figure 6-1). The crucial mechanism behind the alteration of hypertension development might be the regulation of mGluR2 for neurons in the medulla oblongata. These findings can provide novel information for understanding the role of mGluR2/3 in the dorsal medulla oblongata in blood pressure regulation and potential therapeutic strategy.

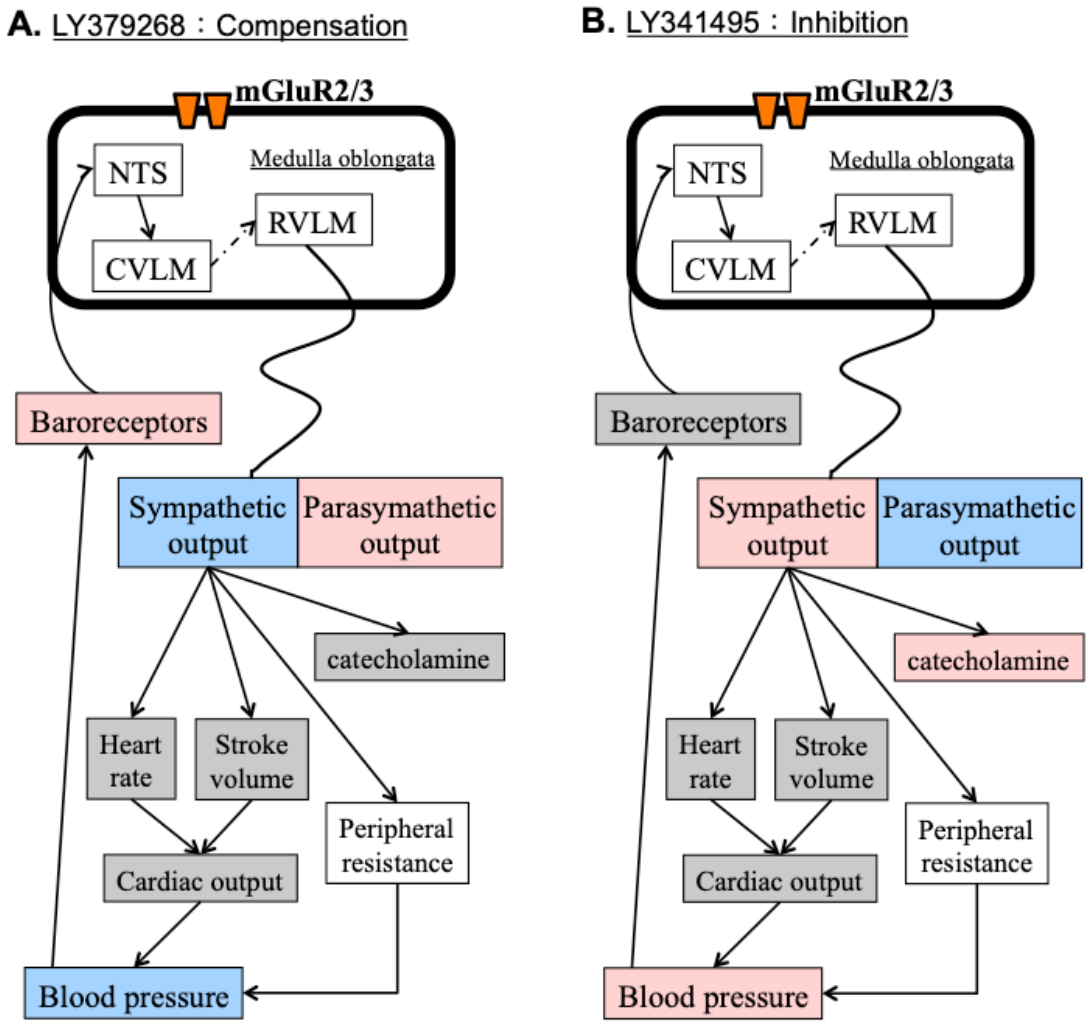

Figure 6-1. Schemes for blood pressure regulation via dorsal hindbrain mGluR2/3 treatment of LY379268 (A) and LY341495 (B). The grey boxes indicate no alteration, the red boxes indicate increase, the blue box indicates decrease, and the white boxes indicate obscureness. Compensating mGluR2/3 function with LY379268 increased the parasympathetic activity, which attenuated hypertension development and improved baroreflex function. On the other hand, inhibiting mGluR2/3 function with LY341495 led to sympathetic dominance, which accelerated the progression of hypertension and released more catecholamine. Therefore, mGluR2/3-altered BP is associated with the activity of the autonomic nervous system, and might also be related to peripheral resistance given that the heart rate and stroke volume were not affected. mGluR2/3, group II metabotropic glutamate receptor; NTS, nucleus tractus solitarius; CVLM, caudal ventrolateral medulla; RVLM, rostral ventrolateral medulla.

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