

**Lercanidipine rescues hippocampal pyramidal neurons from mild ischemia-induced delayed neuronal death in SHRSP.**

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## Abstract

Stroke-prone spontaneously hypertensive rats (SHRSPs) are vulnerable to ischemia and delayed neuronal death (DND) of hippocampus pyramidal cells when bilateral carotid arteries are occluded for only 10 minutes. Since this occlusion induces just mild ischemia, the resulting DND may be an appropriate animal model for dementia in patient with essential hypertension exposed to small ischemic insults. The present study was designed to compare the effects of the antihypertensive drugs lercanidipine, nicardipine, lisinopril, valsartan, and hydralazine on occlusion-induced DND in SHRSPs. Drugs were administered for 2 weeks, from 15–17 weeks of age. 0.1% nicardipine and 0.01 or 0.03% lercanidipine were administered in the SP diet (about 61.3, 5.7, and 18.8 mg/kg/day, respectively), and the remaining drugs were administered at 10 mg/kg/day using the mini-osmotic pump. The animals were operated on at 16 weeks of age and DND was analyzed by histological examination 1 week later. Systolic blood pressure was measured at 15, 16, and 17 weeks of age. For chronic treatment, Ca<sup>2+</sup>-channel blockers were administered from 8–17 weeks of age. All antihypertensive drugs significantly lowered systolic blood pressure at 16 weeks of age. Hydralazine and lisinopril were associated with the greatest reduction; however, lercanidipine, nicardipine, and valsartan effectively reduced systolic blood pressure to within a medium range. DND was significantly inhibited only by 0.03% lercanidipine. Chronic treatment with 0.03% lercanidipine also protected pyramidal neurons. The results of this study demonstrate that the long-acting, lipophilic Ca<sup>2+</sup>-channel blocker lercanidipine inhibits occlusion-induced DND in

SHRSPs and that lercanidipine may effectively reduce dementia induced by small ischemic insults in patients with essential hypertension.

Key words: hypertension, ischemia, hippocampus, delayed neuronal death, lercanidipine

Abbreviations: SHRSP, stroke-prone spontaneously hypertensive rat; DND, delayed neuronal death;

CCB, Ca<sup>2+</sup> - channel blocker

## 1. Introduction

Hypertension is the main risk factor for stroke leading to cognitive impairment and dementia. Therefore, antihypertensive treatment is an essential therapy to prevent the cerebral complications of hypertension and dementia in the elderly (Staessen et al., 2000). Dihydropyridine  $\text{Ca}^{2+}$ -channel blockers (CCBs) are effective at reducing blood pressure and improving cerebral vasculature (Frishman, 2002; Kuriyama et al., 1993). Lercanidipine, which is a long-acting and lipophilic dihydropyridine CCB, has been associated with desirable vasodilatory activity due to its vascular selectivity compared to other dihydropyridine derivatives (Angelico et al., 1999; Makarounas-Kirchmann et al., 2009).

The stroke-prone spontaneously hypertensive rat (SHRSP) is an animal model widely used in the study of cerebrovascular diseases. SHRSPs that were given a high salt diet, the SP diet (0.8% NaCl), were found to demonstrate very severe hypertension from a young age and cerebral apoplexy after around 20 weeks of age (Niwa et al., 1994; Okamoto et al., 1974; Sakurai-Yamashita et al., 1997). SHRSPs were considerably vulnerable to ischemic insult and just 10-minute occlusion of the bilateral carotid arteries induced delayed neuronal death (DND) in the CA1 subfield of the hippocampus (Qiang et al., 1989; Sakurai-Yamashita et al., 2003; Yamashita et al., 1994). In contrast to Mongolian gerbils, bilateral occlusion of the carotid arteries, 2-vessel-occlusion is not enough to induce DND in most rat strains, because of the blood supply through the vertebral artery

(Kirino, 1982; Pulsinelli et al., 1982). The reduction in cerebral blood flow in SHRSPs during occlusion of the carotid arteries was found to be the same as that in normotensive WKY rats, with mean-blood flow after occlusion about 43% of the pre-occlusion value, although no DND was observed in WKY rats (Kinugawa et al., 2008). DND in WKY and other normotensive control rats was found to occur, when the vertebral arteries were cauterized before carotid artery-occlusion, leading to 4- vessel- occlusion, and their blood flow decreased to about 10% (Kinugawa et al., 2008; Pulsinelli et al., 1982). The susceptibility of SHRSPs to ischemic insults, including DND in the hippocampus induced by mild ischemia, is likely to be dependent on genetic factors, but independent of the severity of hypertension, because a congenic strain for the blood pressure quantitative trait locus on chromosome 1 also showed DND following 2- vessel- occlusion (Sakurai-Yamashita et al., 2010). Neuronal, glial, and vascular tolerability to ischemic insults are likely to differ between SHRSP and normotensive WKY rats (Cai et al., 1998; Jeffs et al., 1997; Tagami et al., 1998). The susceptibility of SHRSPs to DND suggests that patients with essential hypertension may also be susceptible to neuronal damage not only by severe ischemic attacks, but also mild ischemia, even when their blood pressure is lowered by antihypertensive drugs. Therefore, DND could be a suitable animal model for hypertensive patients with mild ischemic insults leading to dementia.

Several types of antihypertensive drugs are used clinically; however, the effectiveness of these agents in preventing the neuronal damage induced by mild ischemic insults in hypertensive

animals has not yet been elucidated. The present study was designed to identify drugs able to protect neurons from mild ischemia-induced DND. Nicardipine is a first-generation dihydropyridine CCB and lercanidipine is a highly lipophilic third-generation dihydropyridine CCB. Valsartan is an angiotensin II type 1 (AT1) receptor antagonist and lisinopril is a long-acting angiotensin converting enzyme (ACE) inhibitor. Among these antihypertensive drugs examined, only 0.03% lercanidipine protected hippocampal neurons while lowering blood pressure to within a medium range.

## **2. Material and methods**

### **2.1. Animals**

All SHRSPs used in the present study were SHRSP/*Izm* rats, which were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). All animals were fed the SP diet containing 0.8% NaCl (Funabashi Farm Co., Chiba, Japan) and water ad libitum. Groups of 3 or 4 rats were housed in a cage in an air-conditioned room with a temperature of  $24 \pm 1$  °C and a humidity of  $65\% \pm 5\%$  in a light:dark cycle of 12:12 h. All animals used for these procedures were treated in strict accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and as approved by the Nagasaki University Animal Care Committee.

### **2.2. Treatment with antihypertensive drugs and measurement of blood pressure**

Lercanidipine and nicardipine were included in the SP diet; 0.01% and 0.03% of lercanidipine was administered as a dose of  $5.7 \pm 0.2$  mg/kg and  $18.8 \pm 0.4$  mg/kg, respectively

calculated according to daily dietary intake; and 0.1% nicardipine was administered as  $61.3 \pm 1.3$  mg/kg. Control animals for lercanidipine and nicardipine were given the SP diet without drugs. Valsartan, lisinopril, and hydralazine were administered using the mini-osmotic pump (alzet, Cupertino, U.S.A.). The pump was implanted subcutaneously under pentobarbital (60 mg/kg, ip) anesthesia after blood pressure was measured at 15 weeks of age, and the drugs were continuously released for 14 days. Control animals for these groups were implanted with the pump filled with vehicle. Blood pressure was measured at 15, 16 and 17 weeks of age using the tail-cuff method. Valsartan was a gift from Novartis Pharm Company, and lisinopril dehydrate and hydralazine hydrochloride were purchased from Wako Pure Chemical Industries.

### **2.3. Surgical procedure and histological evaluation of DND**

SHRSPs at 16 weeks of age were anesthetized with 1.5% halothane in the air. The common carotid arteries were exposed bilaterally, and occluded for 10min with aneurismal clips. Body temperature was maintained at 37 °C with a heating pad. Seven days after transient occlusion, rats were deeply anesthetized and perfused with 4% paraformaldehyde. Coronal paraffin sections (6  $\mu$ m thick) at the level shown in plates 21–23 of the stereotaxic map of the rat brain (Paxinos and Watson, 1986) were stained with hematoxylin-eosin, and loss of pyramidal cells in the CA1 subfield of the hippocampus was evaluated. The degree of neuronal damage in the pyramidal layer of the CA1 subfield was graded as follows: 0, no

morphological change; 1, damaged cells less than 10%; 2, damaged cells 10–50%; 3, damaged cells 50–90%; 4, damaged cells more than 90%, as described previously (Fig 1, Sakurai-Yamashita et al., 2003).

#### **2.4. Statistical Analysis**

Comparison of the DND score was performed using analysis of variance (Kruskal-Wallis test) followed by a Mann-Whitney U-test for non-parametric multiple comparison. Blood pressure was analyzed by ANOVA and Scheffe's test, and is presented as mean (SD). A difference was considered significant when  $p < 0.05$ .

### **3. Results**

At 15 and 16 weeks of age, the systolic blood pressure of SHRSPs fed the SP diet was  $269 \pm 3.3$  and  $277 \pm 3.0$  mmHg, respectively. Fig 2A shows that ingestion of 0.03% and 0.01% lercanidipine or 0.1% nicardipine in the SP diet for 7 days decreased blood pressure significantly. At 16 weeks of age, the systolic blood pressure of SHRSPs treated with 0.03% lercanidipine was  $218 \pm 11.9$  mmHg, which was lower than that of 0.01% lercanidipine- or 0.1% nicardipine-treated animals. Treatment with hydralazine and lisinopril 10 mg/kg/day for 7 days dramatically decreased systolic blood pressure, to  $144 \pm 5.7$  and  $170 \pm 5.7$  mmHg (Fig 2B), respectively. Control SHRSPs implanted with the mini-osmotic pump releasing vehicle showed the same blood pressure as control animals without the pump. Valsartan also decreased systolic blood pressure to lower than 200 mmHg (Fig



2B). Each group of animals showed similar blood pressures at 16 and 17 weeks of age.

Fig 3 shows the median value (indicated by small square) and box-plot (25–75%) of the DND score in each group. Only treatment with 0.03% lercanidipine significantly decreased DND score compared to control (A). Lercanidipine 0.01% decreased the median value numerically, but not significantly. No protective effects were observed with lisinopril, hydralazine, and valsartan (B).

Table 1 shows the rate at which animals graded to each score in each group. About 70% of animals in the control groups showed DND. Treatment with 0.03% lercanidipine decreased the number of animals with a score of 4 (Fig 1 E, more than 90% of neurons degenerated) to 33% and increased the number of animals with a score of 0 (Fig 1A, no morphological change) to 53%. Lercanidipine 0.01% increased the number of animals with a score of 0 or 1 (Fig 1B, less than 10% of neurons were degenerated) with about half the animals almost completely protected from DND. However, 50% of animals treated with 0.01% lercanidipine showed a score of 4; therefore, 0.01% lercanidipine could not inhibit DND significantly. Lisinopril, hydralazine, and valsartan did not decrease the number of animals with a score of 3 or 4, suggesting that these agents did not protect the pyramidal neurons from the mild ischemia-induced DND.

As treatment with 0.01% lercanidipine during 15–17 weeks of age did not significantly decrease the median DND value, we assessed the effects of chronic treatment with CCBs; 0.01%-, and 0.03%- lercanidipine or 0.1% nicardipine was administered from 8 weeks of age and animals

were operated on at 16 weeks of age. Fig 4 demonstrates that the drugs lowered systolic blood pressure significantly compared to control treatment from 14 weeks of age, when control SHRSPs showed systolic blood pressures higher than about 250 mmHg. Chronic treatment of 0.03% lercanidipine inhibited DND significantly; however, 0.01% lercanidipine did not protect the animals from DND (Fig 5). Although chronic treatment with nicardipine 0.1% decreased the median value of the DND score, it did not reduce the score significantly.

#### **4. Discussion**

The long-acting and highly lipophilic CCB lercanidipine protected pyramidal neurons from DND induced by occlusion of the carotid arteries in SHRSPs when administered in the SP diet at 0.03% for only 2 weeks, 1 week before and 1 week after the operation. Other antihypertensive drugs examined in the present study did not inhibit DND in SHRSPs, although they decreased systolic blood pressure significantly. As the DND in SHRSPs may be a model of dementia in patients with essential hypertension, lercanidipine may be effective at inhibiting this dementia.

Ingestion of 0.03% lercanidipine in SHRSPs corresponded to treatment with a dose of 18.8 mg/kg/day, which lowered systolic blood pressure more than 0.01% lercanidipine or 0.1% nicardipine, and to the same extent as valsartan. Hydralazine and lisinopril decreased the systolic blood pressure dramatically at 16 weeks of age, after just 1 week of treatment. As hydralazine, lisinopril, and valsartan were administered with the mini-osmotic pump, the drugs were continuously

released subcutaneously. Therefore, it cannot be excluded that there may be differences in the pharmacodynamics between these drugs and those administered orally only during the dark hours (12h); however, lercanidipine is very long-acting, and nicardipine, which is administered twice a day in clinical practice, reaches steady state within 1 week. The similar reductions in systolic blood pressure between 1 and 2 weeks after the initiation of all treatments suggest that the effects of these drugs at 16 weeks of age, when the animals were operated on, was sufficient to exert maximum antihypertensive efficacy. Nevertheless, we believe that it is better to administer the drugs in unstressed conditions, because stress has been shown to affect DND in SHRSPs (Sakurai-Yamashita et al., 2003).

Hypertension is a risk factor for cognitive impairment and dementia, in addition to cerebrovascular morbidity and mortality (Frishman, 2002). There are several types of antihypertensive drugs, with varying mechanisms to lower blood pressure. Mackenize et al. (2009) reported that atenolol, a  $\beta$ -blocker, did not affect central pulse pressure despite a similar reduction in peripheral blood pressure to lercanidipine, perindopril, and bendrofluazide, suggesting that the choice of therapy may be important. Webb et al. (2010) also described that drug- class effects on interindividual variation in blood pressure can account for differences in the effects of antihypertensive drugs on the risk of stroke, independently of the effects on mean systolic blood pressure; CCBs and non-loop diuretic drugs reduce the risk of stroke, whereas ACE inhibitors, AT1

receptor blockers, and  $\beta$ -blockers increase the risk. These studies suggest that CCBs or combinations of CCBs with other classes of antihypertensive drugs may have therapeutic benefits to prevent stroke in hypertensive patients.

Voltage-dependent  $\text{Ca}^{2+}$ -channels have been divided into L-, P/Q-, N-, R-, and T-type channels (Tsien et al., 1995). Most CCBs for hypertensive therapy affect the L-type, and lercanidipine and nicardipine are also L-type CCBs. Cilnidipine, an L- and N- type CCB, has been reported to reduce the infarction volume induced by transient focal brain ischemia in rats (Takahara et al., 2004), suggesting that N-type CCBs may also be effective for the prevention of neuronal degeneration induced by ischemic insults in normotensive animals. Nicardipine is a first- generation CCB and has been widely used. Our previous studies demonstrated that nicardipine has antioxidant, antihypertensive, and stroke-preventive activity in SHRSPs (Carlos et al., 1998; Niwa et al., 1994). In the present study, 1- week- treatment with 0.1% nicardipine lowered systolic blood pressure significantly, but less so than 0.03% lercanidipine, and DND was not inhibited with the lower dose. Eight weeks- of treatment with nicardipine in SHRSPs from 8 weeks of age lowered systolic blood pressure less than both 0.03% and 0.01% lercanidipine, and decreased the median value of the DND score, although this reduction was not significant. These results suggest that a high dose of nicardipine or the addition of another antihypertensive drug may prevent DND, such as mild ischemic attack-induced neuronal death. Lercanidipine is a third- generation CCB, that is long-acting

and highly lipophilic. Lercanidipine has been reported to inhibit tissue inflammation in animal models of overexpression of human renin and angiotensinogen genes (Menne et al., 2006). Chronic treatment of 0.01% and 0.03% lercanidipine from 8 weeks of age prevented spontaneous stroke in SHRSPs until 30 weeks of age, although about 70% of control animals experienced stroke. Both nifedipine and lercanidipine prevented spontaneous stroke in SHRSPs treated from 8–30 weeks of age, but only 0.03% lercanidipine also prevented DND with semi-chronic or chronic treatment. The protective effects of lercanidipine may be related to the blockage of the  $\text{Ca}^{2+}$ -channel and the high lipophilicity of the agent, improvements in endothelial dysfunction, and the prevention of tissue inflammation (Menne et al., 2006). As lercanidipine has been shown to be associated with a low risk of peripheral edema or any adverse event (Makarounas-Kirchmann et al., 2009), the agent may be a safe and effective antihypertensive treatment option. A longitudinal study in hypertensive patients aged 40 years or older showed that lercanidipine, administered daily at a dose of 10 mg for 6 months, improved cognitive function in patients with good blood pressure control (Tisaire-Sánchez et al., 2006).

## **5. Conclusions**

In conclusion, as the DND induced by mild ischemia in SHRSPs may be related to genetic factors associated with the vulnerability to ischemia, lercanidipine could be a beneficial

antihypertensive drug for patients with essential hypertension to prevent dementia. Further studies assessing the efficacy of combination therapy with lercanidipine and other classes of antihypertensive drugs may result in the discovery of more effective therapeutics options.

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### Figure captions

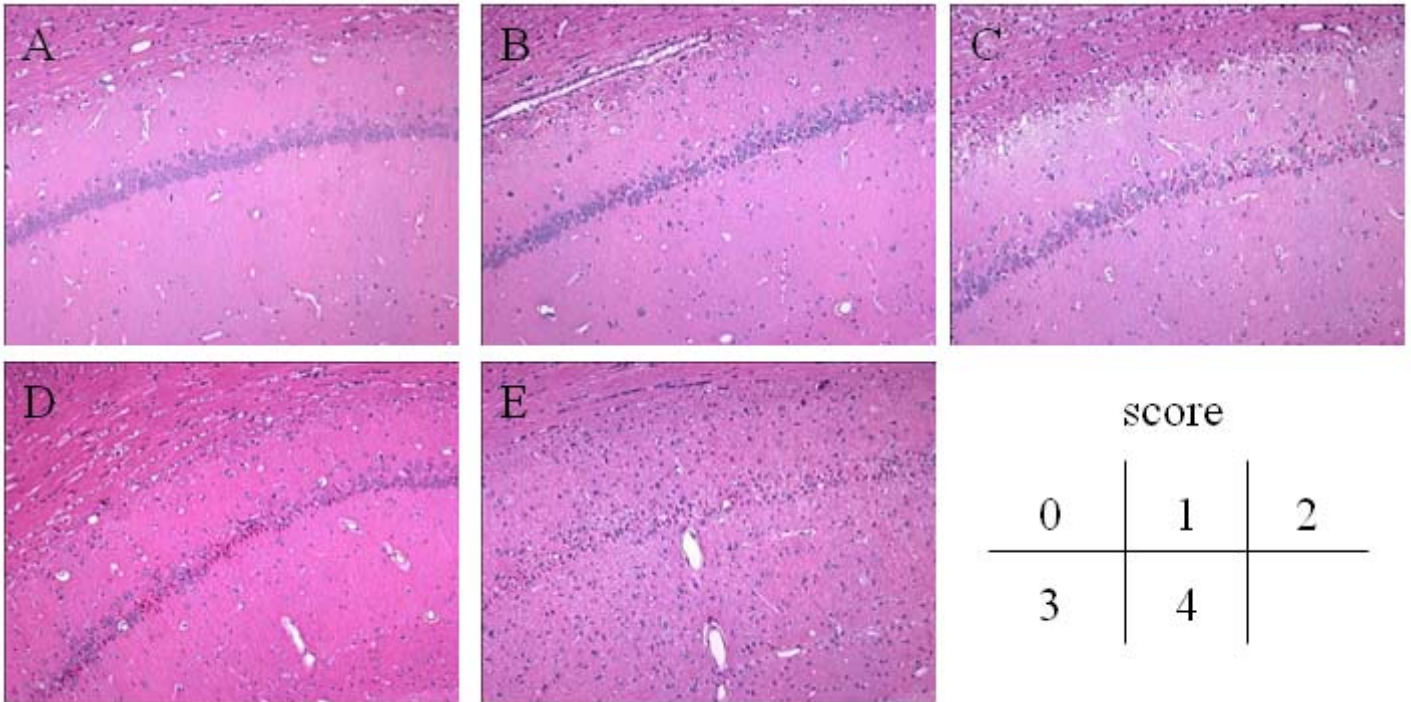


Fig. 1 Histological photographs of the hippocampal pyramidal neurons of SHRSP with delayed neuronal death corresponded to each score of neuronal damage. (A) Score 0, no morphological change, (B) Score 1, damaged cells less than 10%, (C) Score 2, damaged cells 10–50%, (D) Score 3, damaged cells 50–90%, and (E) Score 4, damaged cells more than 90%. Coronal sections (6  $\mu$ m thick) were stained with hematoxylin-eosin, and loss of pyramidal cells in the CA1 subfield of the hippocampus was evaluated along the score of neuronal damage, 0 to 4.

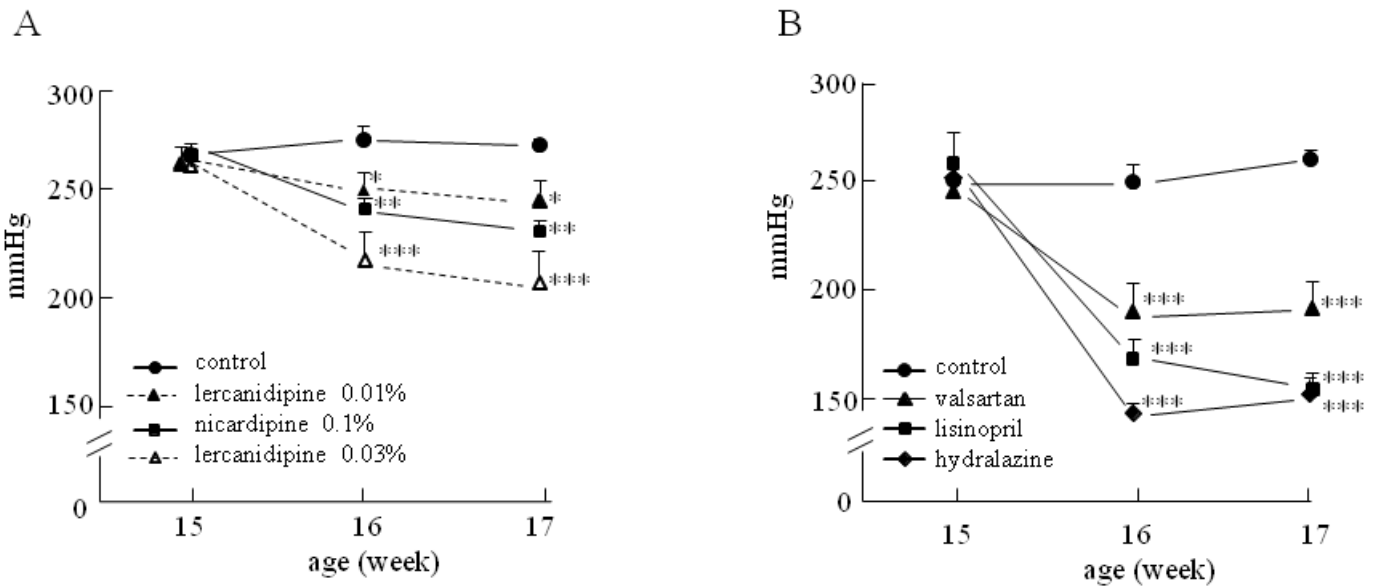


Fig. 2 Effects of antihypertensive drugs on the systolic blood pressure of stroke-prone spontaneously hypertensive rats (SHRSPs). (A) Effects of Ca<sup>2+</sup> - channel blockers on systolic blood pressure in SHRSPs; 0.1% nicardipine, 0.01% lercanidipine, and 0.03% lercanidipine were administered in the SP diet. (B) Effects of hydralazine, lisinopril, and valsartan on systolic blood pressure in SHRSPs. The drugs were administered using the mini-osmotic pump. \*, \*\*, and \*\*\* indicates  $p < 0.05$ , 0.01, and 0.001, respectively, by the Scheffe's-test. The vertical line shows SE.

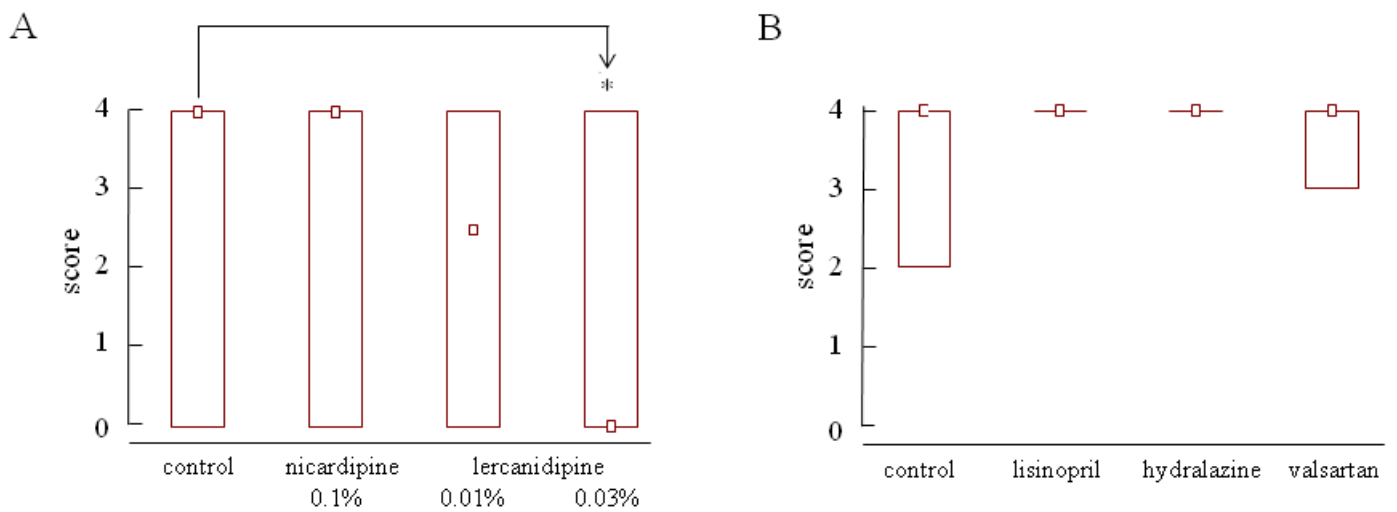


Fig. 3 Effects of antihypertensive drugs on delayed neuronal death (DND) induced by carotid artery occlusion in SHRSPs. (A) Effects of Ca<sup>2+</sup> - channel blockers on DND in SHRSPs; 0.1% nicardipine, 0.01% lercanidipine, and 0.03% lercanidipine were administered in the SP diet. (B) Effects of hydralazine, lisinopril and valsartan on DND in SHRSPs. The drugs were administered using the mini-osmotic pump. DND was graded for each animal as follows: 0, no morphological change; 1, damaged cells less than 10%; 2, damaged cells 10–50%; 3, damaged cells 50–90%; and 4, damaged cells more than 90%、 indicated in Fig 1. The square in the box is the median, with 25–75% of the score denoted by the box (maximum and minimum). \* indicates  $p < 0.05$  by the Mann-Whitney U-test.

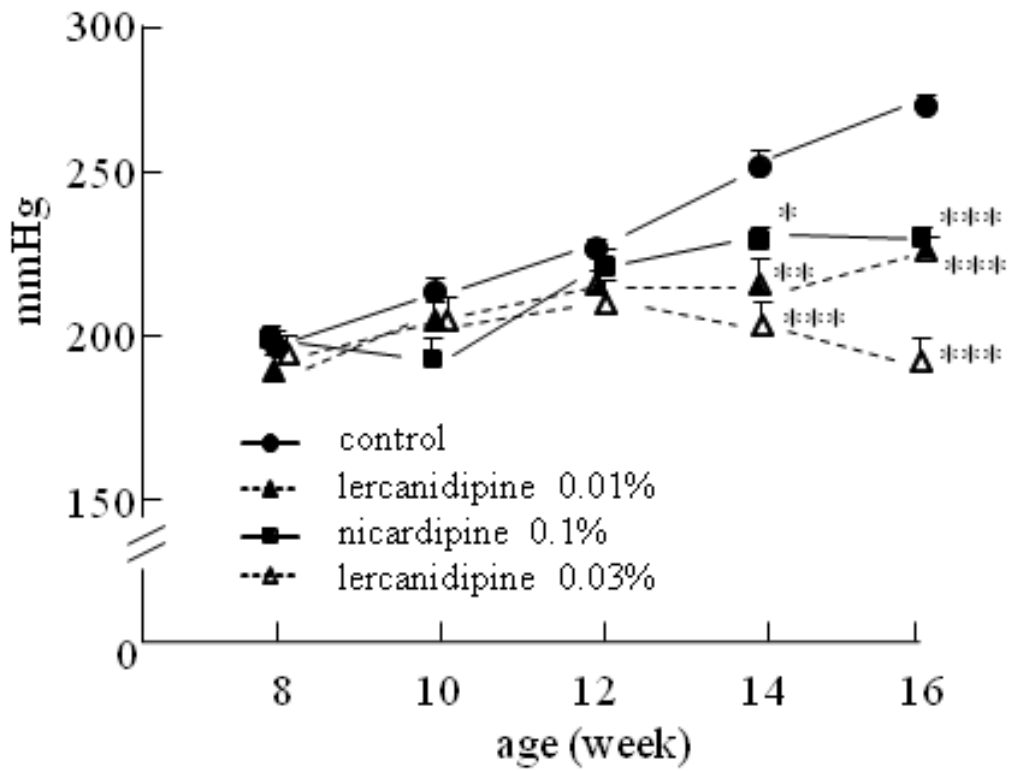


Fig. 4 Effects of chronic treatment with  $\text{Ca}^{2+}$  - channel blockers on systolic blood pressure. 0.1% nicardipine, 0.01% lercanidipine, and 0.03% lercanidipine were administered in the SP diet from 8 weeks of age. \*, \*\*, and \*\*\* indicates  $p < 0.05$ , 0.01, and 0.001, respectively by the Scheffe's-test.

The vertical line shows SE.

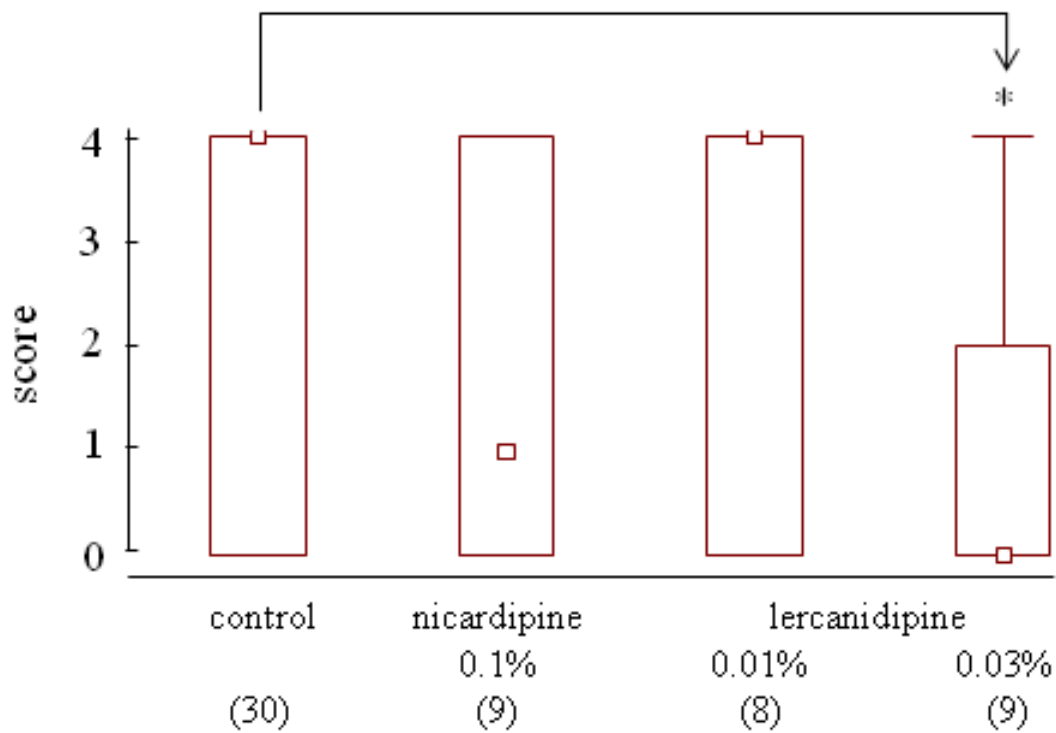


Fig. 5 Effects of chronic treatment with  $\text{Ca}^{2+}$  - channel blockers on DND in SHRSPs. DND was graded for each animal as follows: 0, no morphological change; 1, damaged cells less than 10%; 2, damaged cells 10–50%; 3, damaged cells 50–90%; and 4, damaged cells more than 90%, indicated in Fig 1. The square in the box is the median, with 25–75% of the score denoted by the box (maximum and minimum). \* indicates  $p < 0.05$  by the Mann-Whitney U-test.

**Table 1. Number of stroke-prone spontaneously hypertensive rats graded to each delayed neuronal death following treatment with antihypertensive drugs.**

Treatment	Number of animals for each score (%)					(Total number)
	0	1	2	3	4	
Control	27	3	0	0	70	(30)
Nicardipine	31	6	0	0	63	(16)
Lercanidipine (0.01%)	38	13	0	0	50	(16)
Lercanidipine (0.03%)	53	0	7	0	33	(15)
Control (Vehicle) *	0	0	29	14	57	(7)
Lisinopril	10	0	0	0	90	(10)
Hydralazine	11	0	11	0	78	(9)
Varsartan	0	20	0	20	60	(10)

\*This control group was injected with vehicle using the mini-osmotic pump.