

Blood pressure-independent factors determine the susceptibility to delayed neuronal death in the stroke-prone spontaneously hypertensive rats

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Running title: Delayed neuronal death in SHRSP

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## Summary

The stroke-prone spontaneously hypertensive rat (SHRSP) is vulnerable to delayed neuronal death (DND) in the CA1 subfield of the hippocampus after the transient forebrain ischemia by the occlusion of the bilateral carotid arteries. The present study was designed to show that the genetic factors independent of high blood pressure contributed to the high incidence of DND in SHRSP. Male rats of the 4 strains, SHRSP/Izm, SHRSP/Ngsk, SHR/Izm and a congenic strain for the blood pressure quantitative trait locus on chromosome 1 [SHRSP.WKY-(*D1Wox29-D1Arb21*)/Izm] were used in the experiments. At 13 weeks of age, the bilateral carotid arteries of rats were occluded for 10 min under anesthesia with their body temperature kept at 37°C. Seven days after the transient ischemia, the loss of the pyramidal cells in the CA1 was evaluated histologically. In some experiments, the blood flow was monitored with a laser Doppler flowmeter during the transient ischemia. The blood pressure in SHRSP/Izm was significantly greater than that in the other 3 strains. The incidence of DND, however, was not significantly different among SHRSP/Izm, SHRSP/Ngsk and the congenic strain (82, 74 and 65%, respectively), while SHR/Izm

showed a significantly lower incidence (20%). Neither a significant correlation between the incidence of DND and the blood flow reduction during the occlusion, nor a significant inter-strain difference in the blood flow reduction was observed. The genetic factors independent of high blood pressure may contribute to the greater susceptibility to DND in SHRSP.

**Key words:** cerebral ischemia; SHRSP; hypertension; two-vessel occlusion model

## Introduction

The stroke-prone spontaneously hypertensive rat (SHRSP) is a genetic model rat widely used in the study of cerebrovascular diseases (CVD). Although severe hypertension is essential in the pathogenesis of CVD in SHRSP, a substantial amount of evidence has indicated that genetic factors independent of high blood pressure play important roles in the pathogenesis of CVD in this strain [Nabika et al., 2004]. The vulnerability of the SHRSP neurons to ischemia is one of the potential candidates for such factors [Cai et al., 1998; Jeffs et al., 1997; Tagami et al., 1998].

SHRSP was considerably vulnerable to the transient ischemia induced by the occlusion of the bilateral carotid arteries [Yamashita et al., 1994; Sakurai-Yamashita et al., 2003; Qiang et al., 1989]; almost all pyramidal cells in the CA1 subfield of the hippocampus disappeared 7 days after transient forebrain ischemia [known as “delayed neuronal death (DND)”]. In contrast to mongolian gerbils, the bilateral occlusion of the carotid arteries [2-vessel occlusion (2-VO)] is not enough to induce DND in most rat strains [Kirino, 1982; Kawamura et al., 2004; Liu et al., 2005]. To our knowledge, SHRSP is the only exception in rats suffering from DND after the transient 2-VO.

The vulnerability to DND observed in SHRSP is possibly due to the intrinsic weakness of neuronal and/or glial cells to ischemic insult. However, as SHRSP has severe hypertension, high blood pressure *per se* may be a unique factor responsible for the high incidence of DND. In this study, our goal was therefore to obtain compelling evidence indicating that there were factors independent of hypertension contributing to the susceptibility to DND in SHRSP. To accomplish this, we employed 3 additional rat strains, SHRSP/Ngsk, SHR/Izm and SHRSP.WKY-(*D1Wox29-D1Arb21*)/Izm (called SHRSPwch1.0/Izm hereafter), that were closely related to SHRSP/Izm; SHR/Izm was separated from SHRSP/Izm as a ‘stroke-resistant’ substrain more than 3 decades ago [Okamoto et al., 1974; Nabika et al., 1991], while SHRSP/Ngsk was separated from the early stage of SHRSP colony about 2 decades ago [Niwa M, personal communication]. SHRSPwch1.0 is a congenic strain for the blood pressure quantitative trait locus (QTL) on chromosome 1, in which the chromosomal fragment of Wistar-Kyoto (WKY) rat was introgressed into SHRSP/Izm to cover the QTL [Kato et al., 2003ab; Mashimo et al., 1999].

In this communication, we showed that the incidence of DND in the congenic strain and

SHRSP/Ngsk was not different from that in SHRSP/Izm despite of the lower blood pressure in the former 2 strains. In a complementary manner, the incidence of DND was significantly lower in SHR/Izm than in SHRSP/Ngsk and SHRSPwch1.0, though the blood pressure was comparable among the three strains. This observation clearly indicated that SHRSP substrains shared genetic factors, making them vulnerable to DND independently of its severe hypertension.

## Materials and Methods

### Animals

SHRSP/Izm were provided from the Disease Model Cooperative Research Association (Kyoto, Japan). SHRSP/Ngsk were separated from the original SHRSP colony in 1980 and have been maintained as an inbred strain in Nagasaki University School of Medicine. A congenic strain SHRSPwch1.0/Izm for the blood pressure QTL on chromosome 1 was constructed using the ‘speed congenic’ strategy as described previously [Kato et al., 2003b]. The congenic region between D1Wox29 and D1Arb21 covered the 100:1 confidence interval for the QTL [14]. Blood pressure was measured at 12 weeks of age

using the tail-cuff method. Rats were used in the experiments at 13 weeks old. All animal procedures were approved by the Nagasaki University Animal Care Committee.

### Surgical procedure

The 2-VO was performed as described in the previous reports [Sakurai-Yamashita et al., 2003]. In brief, rats were anesthetized with 1.5% halothane in the air. The common carotid arteries were exposed bilaterally, and occluded for 10min with aneurismal clips. Their body temperature was maintained at 37°C with a heating pad during the occlusion period.

### Histological evaluation of DND

Seven days after the transient occlusion, rats were deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde. Coronal paraffin sections (6 µm thick) at the level shown in the plates 21-23 of the stereotaxic map of the rat brain [Paxinos and Watson, 1986] were stained with hematoxylin-eosin and the loss of the pyramidal cells in the CA1 subfield of the hippocampus was evaluated. The histological appearance of the DND was shown in Fig. 1. In all of the cases with DND, 'moderate' (10-50%) to 'severe'

(>50%) loss of the pyramidal cells was clearly observed in the CA1, while cases without DND showed no apparent histological changes. We therefore used a dichotomous criterion for the DND, i.e. DND (+) and (-), in this study.

#### Measurement of blood flow in CA1 of the hippocampus

Three rats from each strain were used in the experiments. Rats anesthetized with pentobarbital (60 mg/kg) were placed in a stereotaxic frame. A flowmeter probe was implanted into the pyramidal layer of the CA1. Seven days after the operation, the 2-VO was performed under halothane anesthesia as described above. Blood flow was measured continuously with a laser Doppler flowmeter (Advance laser flowmetry, ALF21) throughout the experimental procedure. Blood flow during the occlusion was averaged and represented as a % over the 10-min average of the blood flow immediately before the occlusion started (Fig2A). Rats were kept for 7 days after the measurement and then sacrificed to examine the DND in the CA1, as well as the location of the probe (Fig.2B).

#### Statistics

The incidence of DND was compared among the strains using the Steel-Dwass test for non-parametric multiple comparison. The blood pressure and % blood flow were



represented as the mean  $\pm$  SD, and analyzed by ANOVA or Student's t-test. The difference was considered significant when  $p < 0.05$ .

## Results

Fig. 3 summarizes the incidence of DND and averaged systolic blood pressure of the 4 strains. The DND incidence of SHRSP/Ngsk and SHRSPwch1.0/Izm was 74 and 65%, respectively, which was significantly greater than that of SHR/Izm (20%). The difference of the incidence among SHRSP/Izm (82%), SHRSP/Ngsk and SHRSPwch1.0/Izm was not significant. In contrast, the blood pressure in SHRSP/Ngsk ( $211 \pm 15$  mmHg) and in SHRSPwch1.0/Izm ( $198 \pm 16$  mmHg) was not greater than that in SHR/Izm ( $212 \pm 18$  mmHg), while they were significantly lower than that in SHRSP/Izm ( $248 \pm 16$  mmHg).

These observations clearly indicated that the incidence of DND was not dependent on blood pressure in these 4 strains. In rats, it is known that the 2-VO can not shut down the blood flow in the hippocampus because of the collateral circulation from the vertebral arteries [Kirino, 1982]. The blood flow in the CA1 was therefore measured during the

transient occlusion to test whether the level of blood flow reduction influenced the incidence of DND.

As shown in Fig.2C, there is no significant difference in blood flow reduction between the rats with and without DND ( $43\pm 15$  and  $38\pm 11\%$ , respectively). Furthermore, the inter-strain difference in blood flow was not significant either ( $F=0.34$ ,  $p=0.8$  by ANOVA).

## Discussion

The major findings of the present study are 1) that the incidence of DND was significantly different between SHR/Izm and SHRSP/Izm, 2) the incidence in SHRSP/Ngsk and SHRSPwch1.0/Izm was as high as that in SHRSP/Izm despite of the blood pressure being significantly lower than that in SHRSP/Izm, and 3) the blood flow reduction during the occlusion was comparable between rats with and without DND.

SHRSP is a unique rat strain that suffers from DND after the 2-VO [Yamashita et al., 1994; Sakurai-Yamashita et al., 2003; Qiang et al., 1989]. As SHRSP/Izm is a model for hypertension, this unique character of SHRSP is possibly related to its severe hypertension. The present observation of the large difference in the DND incidence

between SHR/Izm and SHRSP/Izm, however, suggested that SHRSP/Izm harbored additional genetic factors making this strain more susceptible to DND. One of the possible factors was significantly higher blood pressure in SHRSP/Izm than in SHR/Izm; hypertension in SHR/Izm might not be severe enough to exceed a certain ‘threshold’ for DND. To assess this possibility, we examined two additional hypertensive strains, SHRSP/Ngsk and SHRSPwch1.0/Izm. These strains were expected to have a common genetic background with SHRSP/Izm; SHRSPwch1.0 shared 98% of the genome with SHRSP/Izm except a 40cM fragment on chromosome 1, and SHRSP/Ngsk had the same allele of SHRSP/Izm at 88% of 357 simple-sequence-repeat markers throughout the whole genome (National Bioresource Project for the Rat at <http://www.anim.med.kyoto-u.ac.jp/nbr/>). The two strains showed as a high incidence of DND as SHRSP/Izm, although their blood pressure was comparable with that of SHR/Izm (Fig.3). This observation strongly suggested that the greater susceptibility in SHRSP was independent of its severe hypertension. This was further supported by a separate study using an antihypertensive drug; when hydralazine (10mg/Kg body weight/day) was administrated intravenously to SHRSP/Izm throughout the experimental

procedure, the incidence of DND was not different between the hydralazine and the control group (100% and 80%, respectively, n=5), whereas blood pressure was significantly reduced from  $266\pm 4$  to  $150\pm 8$  mmHg (n=5).

Whereas the severity of ischemia during the occlusion was likely to be a major factor for the incidence of DND, the results of the blood flow measurement indicated no significant difference in the blood flow reduction between rats with and without DND (Fig.2C).

Further, the blood flow reduction did not seem markedly different among the strains employed in the experiments. These observations implied that, in SHRSP/Izm and the other two susceptible strains, DND was induced even under a moderate reduction of the cerebral blood flow. This suggests an attractive but as yet unproven hypothesis that the CA1 pyramidal cells in SHRSP/Izm, SHRSP/Ngsk and SHRSPwch1.0 are vulnerable to the oxidative stress induced by the reperfusion rather than to the ischemia *per se*.

In this context, it is of interest that Tagami et al. showed that cultured neuronal cells of SHRSP/Izm were more vulnerable to hypoxia/re-oxygenation than those of WKY/Izm [Tagami et al., 1998]. They also showed that the cell damage by hypoxia/re-oxygenation was prevented with antioxidant agents, such as vitamin E and the superoxide dismutase.

This hypothesis is worthy of being evaluated in future studies.

Through the genetic analysis of a F2 cohort obtained between SHRSP/HD and SHR/HD, Rubattu et al. showed that a QTL for 'stroke latency' after salt-loading was on chromosome 1 [Rubattu et al., 1996]. It should be noted that this QTL is in the same region introgressed in our SHRSPwch1.0. Although the present result on the congenic rat did not support the effects of the QTL on DND (because of the high incident of DND in SHRSPwch1.0 that had the WKY allele for the QTL), careful evaluation is necessary with respect to this issue because the two phenotypes examined in these studies, i.e., the cerebral stroke after salt-loading and the DND after the artificial transient ischemia, were not identical.

In conclusion, the present study suggested that the stroke-prone strains of SHR had genetic factors making them susceptible to DND. These factors were independent of the severe hypertension observed in SHRSP. The putative genetic factors should be dissected further in a QTL analysis using a F2 population between SHRSP and SHR.

Acknowledgement

We would express cordial thanks to Yoshiko Masuda for her skillful assistance.

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## Figure legends

Fig.1 Histological appearance of DND in the CA1 of the hippocampus.

‘Moderate’ and ‘severe’ forms of DND were defined as 10-50% and >50% loss of the pyramidal cells in the CA1, respectively. The scale bar in the figure indicates 100 $\mu$ m.

Fig. 2 Blood flow in the CA1 during the transient occlusion of the bilateral carotid arteries.

(A) A typical recording of the flowmetry. (B) Location of the flowmetry probe. The arrow shows a gap due to the probe insertion, indicating the tip of the probe on the CA1.

(C) The reduction of the blood flow during the transient occlusion. There was no significant difference between the DND(+) and the DND(-) [43 $\pm$ 15 and 38 $\pm$ 9%, respectively;  $p=0.4$  according to the Student’s t test]. The difference among the strains was not significant either ( $F=0.37$ ,  $p=0.8$  by ANOVA).

Fig.3 The incidence of DND (the upper panel) and the systolic blood pressure (the lower panel) in the 4 strains studied.

\*: The incidence in SHR/Izm is significantly different from that in the other three strains according to the Steel-Dwass test for a non-parametric multiple comparison ( $p<0.05$ ).

†: The blood pressure in SHRSP/Izm is significantly different from that in the other three strains according to the Tukey's multiple comparison test ( $p < 0.001$ ).

§: The blood pressure in SHRSPwch1.0 is significantly different from that in SHR/Izm according to the Tukey's multiple comparison test ( $p < 0.05$ ).

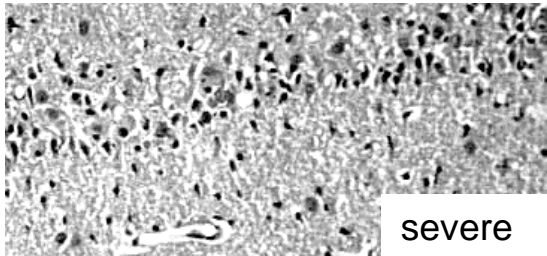
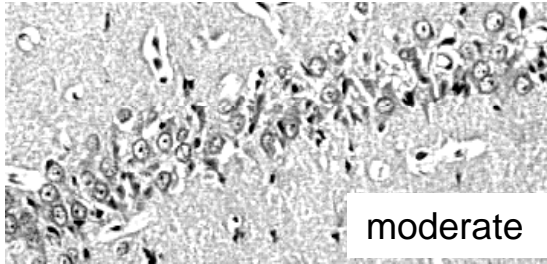
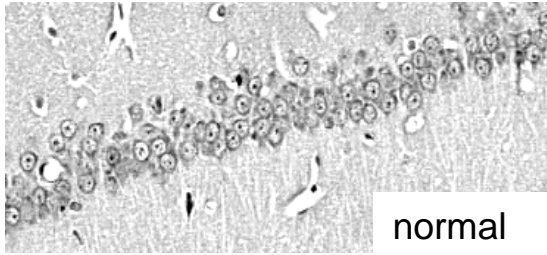


Fig.1

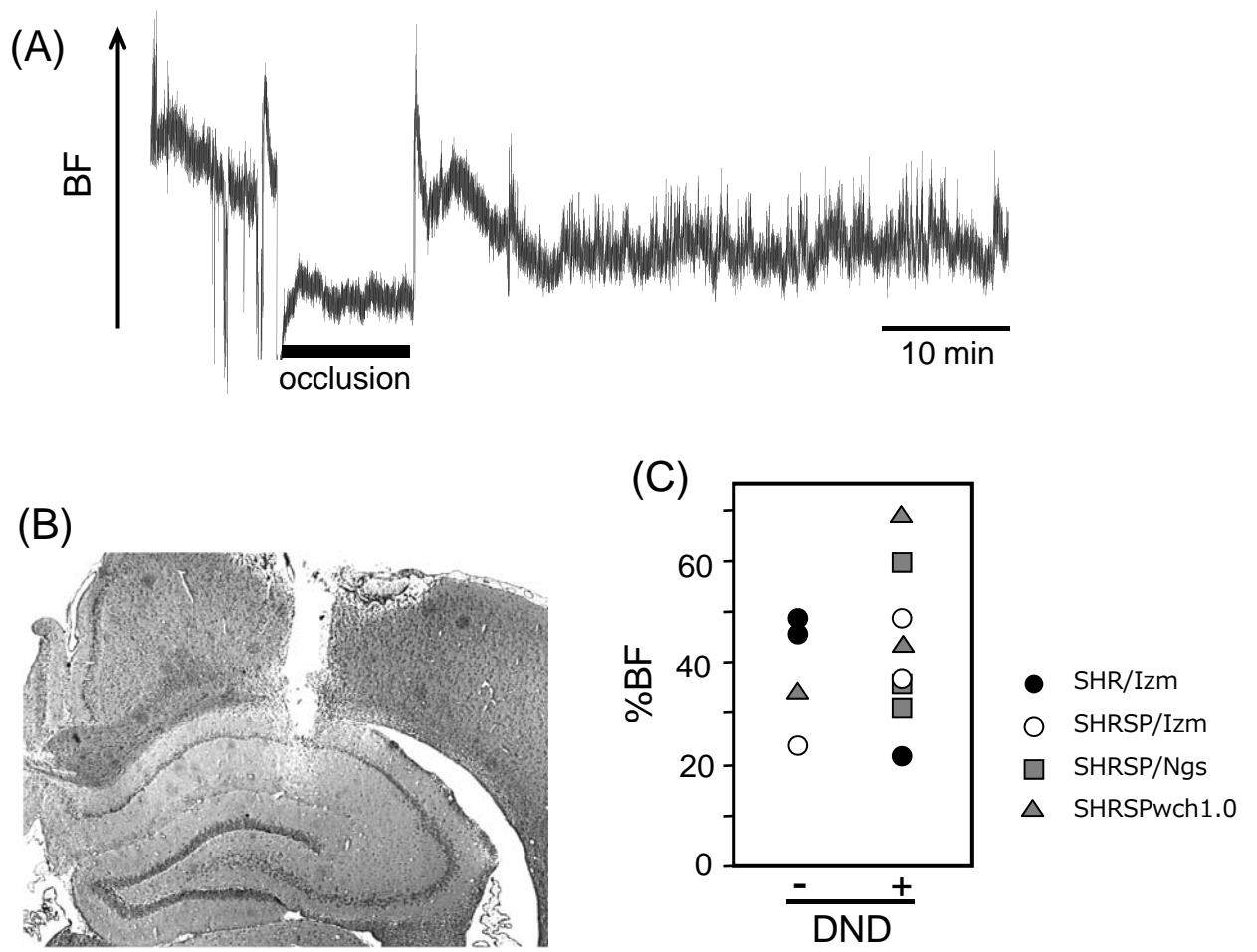
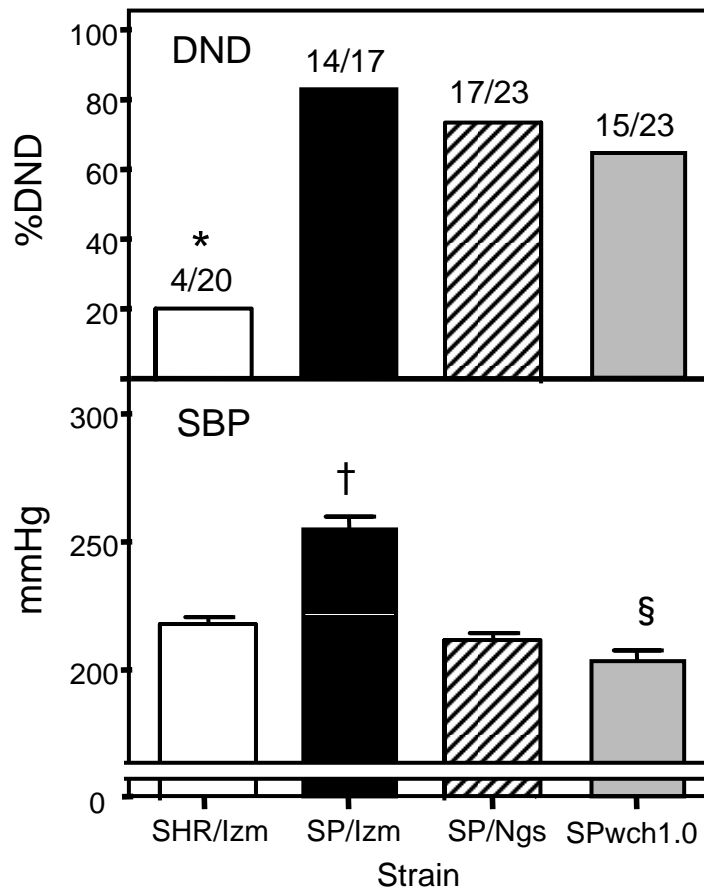


Fig.2

Fig.3



\*: SHR/lzm is significantly different from the other three strains by the Steel-Dwass test for a non-parametric multiple comparison.

†: SP/lzm is significantly different from the other three strains by the Tukey's multiple comparison test ( $p < 0.001$ ).

§ : SPwch1.0 is significantly different from SHR/lzm by the Tukey's multiple comparison test ( $p < 0.05$ ).