Persistent brain exposure to high sodium induces stroke onset by upregulation of cerebral microbleeds and oxidative stress in hypertensive rats.

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Abstract

High salt intake induces hypertension and enhances stroke onset. However, whether an increase in brain sodium exposure itself is harmful and has poor prognosis remains unknown. Therefore, we employed hypertensive rats that underwent intracerebroventricular (ICV) infusion of sodium for 28 days and evaluated stroke onset and related cytotoxic brain injuries.

Forty-seven spontaneously hypertensive stroke-prone (SHRSP) and thirty-nine normotensive rats (Wistar Kyoto rats [WKY]) underwent persistent ICV infusion of the following four solutions: artificial cerebrospinal fluid, 0.9% 2.7% and 9% saline for 28 days. We evaluated stroke onset and all-cause mortality between SHRSP and WKY at each ICV sodium concentration as the primary endpoints. Our secondary objective was to explore histological brain injuries associated with SHRSP by ICV high sodium.. The results indicated that ICV infusion of 2.7% and 9% sodium showed significant increase in stroke onset and decrease in body weight in SHRSP compared to WKY. Increased blood pressure was not observed for ICV infusion of high sodium, while serum sodium concentration was increased in SHRSP and increase rate of brain water content were significantly higher in SHRSP with 2.7 and 9% saline than WKY. Histological evaluations revealed that ICV infusion of 2.7% and 9% sodium was associated with significantly increased superoxide and microbleeds in brain parenchyma and 9% sodium was associated with significantly increased activated microglia and neuronal cell loss.

We conclude that persistent exposure to high sodium in the brain is one of the risk factors for stroke onset upregulating cerebral microbleeds and oxidative stress in hypertensive rats.

Key words: sodium, stroke onset, blood pressure, oxidative stress, microbleeds

Introduction

High salt intake is strongly associated with hypertension, which is a risk factor for stroke onset. However, recent studies have reported that salt intake causes direct brain toxicity independent of hypertension [1-3]. Currently, salt intake itself affects the development of dementia without causing an increase in blood pressure. For example, salt intake produces interleukin-17 in the small intestine, which circulates throughout the body, causing cerebrovascular endothelial cell damage and dysfunction, leading to cognitive dysfunction [2,4]. However, whether brain sodium exposure induces brain damage, including stroke onset, remains unknown.

In addition to elevated blood pressure, high salt intake can also enhance sympathetic nerve activity. Although sodium cannot pass through the blood–brain barrier (BBB), the subfornical organ and organum vasculosum of the lamina terminalis, which have structurally fragile BBB, detect elevated blood sodium level and plasma osmolarity and transmit this information to the ventral lateral area of the rostral medulla via the paraventricular nucleus of the hypothalamus, thus activating sympathetic nerve activity and causing brain toxicity [5,6]. We previously demonstrated that renal denervation, which has been introduced to reduce blood pressure by way of sympathetic outflow [7,8], reduced high-salt diet-induced stroke onset in hypertensive rats, which was correlated with a reduction in brain oxidative stress and microglial activation [9]. Other studies have reported that intraventricular infusion of hypertonic saline induces hypertension, activates microglia, desensitizes baroreceptor reflexes, and affects the inflammatory response [10-12] . Although sympathetic nerve activation and brain oxidative stress may be key targets for salt-induced stroke onset, whether direct brain sodium exposure is correlated with stroke onset remains unclear.

In the present study, we aimed to test the hypothesis that persistent brain exposure with high sodium levels enhances stroke onset in hypertensive rats. To test this hypothesis, we employed hypertensive and normotensive rats, continuously administered multiple doses of sodium intracerebroventricularly, and evaluated stroke onset and the related brain injuries between the rats.

Materials and Methods

2.1 Animals and experimental protocol

All experiments were approved by the Institutional Animal Care and Use Committee of Kurume University and performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Eleven-week-old male spontaneously hypertensive stroke-prone rats/Izm (SHRSP, n=47) weighting 238–269 g and age-matched male Wistar Kyoto rats/Izm (WKY, n=39) weighting 279–348 g were assigned to the following groups: (1) intracerebroventricular (ICV) infusion with commercially available artificial cerebrospinal fluid (CSF) (ARTCEREB; Otsuka Pharmaceutical Factory, Tokushima, Japan) to SHRSP (SHRSP-CSF, n=10), (2) ICV infusion with 0.9% saline to SHRSP (SHRSP-0.9%, n=13), (3) ICV infusion with 2.7% saline to SHRSP (SHRSP-2.7%, n=10), (4) ICV infusion with 9% saline to SHRSP (SHRSP-9%, n=10), (5) ICV infusion with 0.9% saline to WKY (WKY-0.9%, n=13, control group), (6) ICV infusion with 2.7% saline to WKY (WKY-2.7%, n=11), and (7) ICV infusion with 9% saline to WKY (WKY-9%, n=11). In addition, precondition animals without ICV saline both SHRSP (n=4) and WKY (n=4) were included. Those animals were purchased from Japan SLC, Inc., Shizuoka, Japan keeping the strains officially sustained by the Disease Model Cooperative Research Association, Kyoto, Japan. All animals were fed a 0.3% sodium diet from 11 to 15 weeks of age. Experiment 1 was performed to compare the rate of stroke onset between CSF and 0.9% saline in SHRSP. The next experiments were performed to explore the dose-dependent effects of sodium on stroke onset in hypertensive rats compared with normotensive rats. We monitored the rate of stroke onset and all-cause mortality as the primary endpoints in all experiments, and our secondary objective was to explore histological brain injuries of SHRSP, including activated microglia, superoxide, neuronal cell loss, and microbleeds, compared to WKY with 0.9% saline as a control. Detailed protocols are shown in Supplemental Fig. 1.

2.2 Surgery

Implantation of an infusion cannula into the cerebral ventricles and an osmotic pump was performed according to our previous method [13,14]. Briefly, the rats were anesthetized with 2% isoflurane through a face mask, and stainless-steel cannulas (ALZET Brain Infusion Kit 1, Durect Co., Cuperito, CA, USA)

using osmotic minipumps (Model 2004, Durect Co.) were inserted into the right cerebral ventricle at 1.0 mm posterior and 2.0 mm lateral from the bregma through the subcutaneous pockets on their backs. The solutions were continuously administered at a rate of 6 μL/day for 4 weeks. After the surgery, the operative lesion was disinfected with iodine, and meloxicam (1 mg/kg) was administered subcutaneously for appropriate analgesia [15].

2.3 Monitoring of stroke-related neurological symptoms and death

Stroke-related neurological symptoms were assessed every day through 4 weeks from the start of ICV infusion and scored as follows: 6, normal; 5, slight decrease/increase in motor activity; 4, evident decrease/increase in motor activity; 3, paralysis of hind limbs or involuntary movement; 2, slight movement; 1, unable to stand; and 0, death [16] . The score was measured as a symptom test, and stroke onset was defined as a score ≤4. All mortalities were also assessed daily.

2.4 Measurement of body weight and blood pressure

The weight of the rats was monitored weekly, and their systolic blood pressure was measured before the start of ICV infusion and 2 weeks after infusion using a tail sphygmomanometer (MK-2000ST, Muromachi Kikai Co., Ltd., Tokyo, Japan) [17].

2.5 Measurement of rotarod test and beam walking test

To assess motor function, coordination, and activity, we performed rotarod and beam walking tests according to our previous method[15,16,18].

In the rotarod test, the rats were placed on a horizontal drum (MK-630B, Muromachi Kikai) and walked at a speed of four rotations per minute (RPM) for a maximum of 60 s as a training session. The animals were then subjected to a trial on the accelerating spindle (4–40 RPM) for 5 min, and the latency to fall off the cylinder was recorded. The mean times for the three test trials were assigned to each animal.

In the beam walking test, the rats were placed on a beam (100 cm in length, 2.5 cm in width, and 20 cm in height), and their performance was evaluated as follows: 0 or 1 point for rats that hung/stood off the beam without walking, 2 points for animals that walked but fell down from the beam within 1 min, and 3 or 4

points for animals that could walk less than or at least 20 cm on the beam for 1 min. The trial was performed thrice for each rat, and the mean scores were used for each animal.

2.6 Measurement of the brain water content

At the end of the experiment, blood samples from the left ventricle were taken from each animal, euthanized under deep anesthesia with an overdose of isoflurane, and their brains were quickly resected. The brains were cut at the point of the bregma, and the caudal side was kept in 4% paraformaldehyde solution, embedded in paraffin, and cut into 5-μm section (K.I. Stainer Inc., Kumamoto, Japan). The serum sodium concentration was measured at SRL, Inc. (Tokyo, Japan).

According to the evaluation of brain water content (BWC), the brains, including the left hemisphere of the rostral side, cerebellum, and brain stem, were separated and weighed (wet weight) and subsequently incubated in an oven at 105°C for 72 h and weighed again (dry weight) [15]. The following formula was used to calculate the percentage of BWC: ([wet weight – dry weight]/wet weight) × 100. In addition, we calculated increase rate as follows; (the value at the endpoint/the value at the baseline) × 100.

2.7. Histology

2.7.1 Ionized calcium binding adaptor molecule-1 staining

To assess the number of microglia, brain sections were immunostained with anti-ionized calcium binding adaptor molecule-1 (Iba-1; 1:2000; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), as previously described [19]. The number of positive cells was counted using images taken from three fields of the left somatosensory cortex at 200× magnification. The number of resting microglia (resting and ramified microglia) and activated microglia (reactive and phagocytic microglia) was counted separately based on their morphological appearance [20] and expressed as cells/mm^2. Additionally, we quantified microglial morphology using skeleton analysis, as previously described[21-23].

2.7.2 Dihydroethidium staining

To detect superoxide levels in the cortex, we employed dihydroethidium (DHE; Sigma-Aldrich St. Louis, MO, USA) because we previously confirmed that the fluorescence was derived from superoxide [24]. Brain sections were incubated with DHE for 30 min, as previously described with slight modification[19,25].

Superoxide levels were detected by density of DHE fluorescence dye using Lumina Vision version 2.2.0 analysis software (Mitani Corporation, Tokyo, Japan) and quantified using images taken from three fields of the left somatosensory cortex at 200× magnification. The mean values in the SHRSP groups were divided by those in the WKY-0.9% group in the same trial.

2.7.3 Nissl staining

Nissl staining was performed to evaluate the number of surviving neurons in the cortex. The number of positive cells was counted using images taken from three fields of the left somatosensory cortex at 200× magnification. The mean number of cells was expressed as cells/mm^2.

2.7.4 Prussian blue staining (iron staining)

To detect hemosiderin deposits, Prussian blue staining was performed according to the manufacturer's instructions (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Briefly, the sections were washed twice with distilled water and incubated with Prussian blue staining solution at room temperature for 30 min. The staining solution was prepared by blending 75 mL of a 2% potassium ferrocyanide solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and 1% hydrochloric acid in equal volumes. The sections were then rinsed twice in distilled water for 5 min and incubated in a cologne echolate solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 5 min. Microbleeds in the ipsilateral hemisphere of each animal were counted at 400× magnification, and the number was compared between the groups.

2.8 Statistical analyses

We performed all measurements in a blinded manner. Statistical analyses were performed using GraphPad Prism (version 9) for Windows (GraphPad Software Inc., San Diego, CA, USA) and Ekuseru-Tokei 2019 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan). All data are presented as the median ± interquartile. The incidence of stroke onset and mortality was analyzed using a standard Kaplan–Meier curve with a log-rank test and chi-squared analysis. Statistical significance was determined using the Mann-Whitney U-test between the two groups. Since the main purpose of the secondary endpoints was to compare the phenotype between the control (WKY-0.9% or SHRSP-0.9) and other SHRSP groups, statistical significance was tested using the Kruskal–Wallis test, followed by Shirley–

Williams' multiple comparison test. Differences were considered statistically significant at $p<0.05$ in all tests.

Results

To avoid confounding factors, such as direct traumatic brain injuries by stainless steel cannulas, we excluded rats that showed injury-related symptoms within 7 days after the start of both experiments (WKY-0.9%, 2; SHRSP-CSF, 0; SHRSP-0.9%, 2; SHRSP-2.7%, 0; SHRSP-9%, 0; WKY-2.7%, 0; WKY-9%, 0).

Experiment 1

No significant difference in the rate of stroke onset was observed between the SHRSP-CSF (20%, 2 of 10 rats) and SHRSP-0.9% (27.3%, 3 of 11 rats) groups (data not shown). Therefore, we evaluated experiment 2 using 0.9% saline as the solvent.

Experiment 2

Effect of mortality and incidence of stroke

The incidence rate of stroke onset in SHRSP-2.7% (5 of 10 rats, 50%) and SHRSP-9% (7 of 10 rats, 70%) was significantly higher than that in WKY-2.7 % (0 of 11 rats, 0%) and WKY-9% (1 of 11 rats, 9.1%) respectively, while there were no changes between WKY-0.9% (2 of 11 rats, 18.2%) and SHRSP-0.9% (3 of 11 rats, 27.3%) (Fig. 1a). The mortality rate did not differ among the six groups (1 of 11 rats, 0.9%-WKY group; 0 of 11 rats, 2.7%-WKY group; 1 of 11 rats, 9%-WKY group; 2 of 11 rats, SHRSP-0.9% group; 0 of 10 rats, SHRSP-2.7% group, 1 of 10 rats; SHRSP-9% group) (Fig. 1b). The score of the symptom test at 3 weeks in the SHRSP-2.7% (4.5 [2.8-6], n=10) and SHRSP-9% group (3.5 [2.5-6.0], n=10) was significantly lower than that in the WKY-2.7% group (6.0 [6.0-6.0], n=11) and WKY-9% group (6.0 [6.0-6.0], n=11) respectively, while there was no changes between WKY-0.9% (6.0 [6.0-6.0], n=11) and SHRSP-0.9% (6.0 [2.0-6.0], n=11). (Fig. 1c).

Effect of systolic blood pressure and weight

No significant increase of blood pressure was observed between WKY and SHRSP groups with any ICV sodium concentration (Fig. 2a; Supplemental Fig. 2a). SHRSP demonstrated lower body weight than WKY before the start of experiment 2 (Supplement Fig. 2b). The increase of body weight through 4 weeks in the SHRSP-2.7% (-5.7 [-48.6-39.9] g, n=10) and 9%-SHRSP (-23.0 [-38.1-22.1] g, n=9) groups was significantly lower compared with that in the WKY-2.7% (60.8 [40.7-66.8] g, n=11) and WKY-9% (59.4 [50.3-72.4] g, n=10) groups respectively, while there were no changes between WKY-0.9% (39.9 [33.3-50.9], n=11) and SHRSP-0.9% (13.2 [-19.5-41.7], n=11) (Fig. 2b).

Effect of neurological functions

There were no significant between-group differences in rotarod, while beam walking tests in SHRSP-2.7% (50.8 [0-68.8] sec) group was lower than those in WKY-2.7% (56.7 [49.7-58.7] sec) group (Figs. 3a and b).

Effect of serum sodium concentration

In comparison with the baseline sodium concentration of WKY (139.0 [139.0-139.0], n=4) mEq/L and SHRSP (139.5 [138.3-140], n=4) mEq/L, the values of sodium concentration were 140.0 [139.0-141.0] mEq/L in WKY-0.9% (n=9), 144.0 [143.5-145.5] mEq/L in SHRSP-0.9% (n=9), 138.0 [136.0-139.0] mEq/L in WKY-2.7% (n=11), 142.0 [141.0-145.0] mEq/L in SHRSP-2.7% (n=9), 136.5 [134.5-138.0] mEq/L in WKY-9% (n=11), and 143.0 [139.5-144.5] mEq/L in SHRSP-9% (n=9). The values in all groups were within normal range regardless of sodium concentration, but those in SHRSP were increased throughout 28 days and significantly higher than those in WKY in each ICV sodium concentration (Fig. 3c).

Effect of brain edema

As shown in Table 1, compared with the WKY groups, the BWCs of the SHRSP groups were significantly higher in at the both baseline and endpoint. However, increase rate of SHRSP-2.7% and SHRSP-9% were significantly higher than that of WKY in each ICV sodium concentration.

Histological examination

Because no significant changes were observed in primary endpoints among WKY groups, we further used only WKY-0.9% as a control in the secondary endpoint. The number of total and resting microglia was not significantly different among the group, whereas the number of activated microglia was significantly higher in the SHRSP-9% group (87.6 [50.7-375.8] cells, n=9) than in the WKY-0.9% group (35.7 [8.6-42.1] cells, n=10) (Fig. 4a). We then evaluated skeleton analysis by quantifying the number of microglial process endpoints and length per cell to confirm the significant microglial activation, for which characteristics should be reduction of the endpoints and length[20]. The number of microglia process endpoints and length were significantly lower in the SHRSP-9% group (13.6[11.4-15.9] and 9.7[7.7-11.9], respectively) than those in WKY-0.9% group (19.4[16.2-34.8] and 14.6[13.3-25.9], respectively) (Supplemental Fig. 3). Brain superoxide levels detected by DHE staining was significantly higher in the SHRSP-2.7% group (133.4 [104.7-184.8] %, n=10) and SHRSP-9% (157.7 [95.5-222.0] %, n=9) compared to the 0.9%-WKY group (92.8 [83.5-113.7] %, n=10) (Fig. 4b). The number of surviving neuronal cells detected by Nissl staining in SHRSP-9% (622.5 [283.6-819.7] cells, n=9) were less than WKY-0.9% group (854.3 [747.6-1056.6] cells, n=10) (Fig. 5a). Cerebral microbleeds (CMBs) detected by Prussian blue staining were significantly higher in the SHRSP-2.7% (4.5 [2.8-6.3], n=10) and SHRSP-9% (7.0 [5.5-8.5], n=9) groups than in the WKY-0.9% group (1.0 [0.8-2.3], n=10) (Fig. 5b). According to the dose-dependent effects of ICV infusion of sodium, higher sodium infusion significantly increased the number of reactive microglia, superoxide, neuronal cell loss, and microbleeds (4a, 4b, 5a, and 5b).

Discussion

Our previous studies revealed that oral feeding of a high-sodium diet for 28 days significantly induced stroke onset in hypertensive rats, which was hypothesized to be due to the elevation of blood pressure and sympathetic activity[9,13,16]. These results encouraged us to explore whether persistent brain exposure to high sodium levels enhanced stroke onset. Therefore, we used hypertensive and normotensive rats and evaluated the effects of persistent brain exposure to multiple doses of sodium on stroke onset and related brain injuries. In comparison with normotensive animals, persistent ICV infusion with high sodium in hypertensive rats induced stroke onset and worsened morbidity, representing symptom score and poor weight gain, independently increased blood pressure. Additionally, the hypertensive animals exhibited brain edema and increased activated microglia, superoxide, neuronal cell loss, and microbleeds. Based on

these findings, we suggest that continuous exposure of the brain to high sodium levels is a risk factor for stroke onset and related brain injuries in rats with a hypertensive background.

In experiment 2, our primary endpoint was significant, which represented a significant difference in the stroke onset and symptom test between SHRSP and WKY in 2.7 and 9% ICV sodium concentration, whereas there were no changes in the mortality rate in any group. As brain superoxide and microbleeds were upregulated in 2.7% and 9% SHRSP groups compared to the 0.9% WKY group, we suspected that those brain injuries were associated with the phenotype of primary endpoint. Although serum sodium concentration was higher in SHRSP than WKY, the concentration was similar and within normal range, regardless of sodium concentration in each hypertensive and normotensive rats, suggesting that primary and secondary endpoints of the animals represented the effects of exposure to higher sodium in the cerebral ventricle and the surrounding organs, rather than that in blood. Andersson et al. reported that the subfornical organ and organum vasculosum of the lamina terminalis in the anterior wall of the third ventricle were the sites of sodium sensing [26] and another study revealed that long-term ICV administration of hypertonic sodium solution induced sympathetic activation and blood pressure elevation through sodium channels in the organum vasculosum of the lamina terminalis [27]. Although we did not evaluate the parameters of sympathetic nerve activation nor showed significant increase of blood pressure by ICV infusion with high sodium, ICV infusion with high sodium might enhance deteriorative effects in SHRSP easily rather than WKY. Further research is needed to elucidate the mechanism of how ICV infusion with high sodium increases stroke onset in rats with hypertensive background by evaluating sympathetic nerve activation, etc.

According to our secondary endpoint, the study showed upregulating CMBs and superoxide in the hypertensive rats with 2.7% and 9% sodium. The CMBs are thought to be correlated with the rupture of small arteries, arterioles, and/or capillaries and occurred by disruption of basement membrane [28]. As high sodium intake increased vascular superoxide level[29], we speculate that circulating sodium in cerebrospinal fluid along with perivascular space upregulated cerebrovascular superoxide and disrupted basement membrane, resulting development of CMBs. On the other hand, microglia are resident innate immune cells. Resting microglia are activated by multiple pathological events, such as cerebral ischemia, transform into amoeba with large cell bodies, and play significant roles in oxidative stress and inflammatory responses [30]. Therefore, we suspect that ICV infusion with high sodium also activated microglia and

participated in production of oxidative stress, resulting in CMBs induction and neuronal cell loss. However, it cannot be excluded that the activated microglia and increased oxidative stress were secondary to CMBs induction and neuronal cell loss.

Hypertensive rats showed increased serum sodium concentration which may not reflect dehydrate status, as no body weight reduction was observed in SHRSP. As blood sodium concentration levels at baseline were similar between WKY and SHRSP, we believe that the responses to ICV infusion with sodium differed between WKY and SHRSP, although we did not clarify why serum sodium concentration was significantly increased in SHRSP only. On the other hand, brain water content in SHRSP was significantly increased in comparison with WKY at both baseline and endpoint, while increase rate of SHRSP-2.7% and SHRSP-9% were significantly higher than that of WKY in each ICV sodium concentration. The findings were consistent with the data seen in stroke onset, suggesting that ICV infusion with sodium induced significant brain edema which was associated with stroke onset. Although we did not clarify the mechanism further, we suspect that brain injuries by ICV infusion with sodium induced clinical and subclinical vasogenic edema by partially upregulating oxidative stress and microbleeds in hypertensive rats.

This study has limitations that must be acknowledged. First, we did not measure the sodium concentration in either urine or CSF. Second, stroke onset was observed in a few animals in the WKY-0.9% group, which we considered the control group. Basically, WKY feeding with normal sodium diet did not represent stroke onset throughout 28 days, speculating that the discrepancy might come from an unfavorable effect by long-term ICV infusion procedure.

Conclusions

In this study, we demonstrated that persistent brain exposure to high sodium levels increased stroke onset and morbidity, such as stroke-related symptoms and poor weight gain, in hypertensive rats. In addition, ICV infusion with high sodium upregulated cerebral microbleeds and oxidative stress in hypertensive rats.

Author Contributions

SK and YH contributed to study conception and design. SK, KF, ST, KK, and HU performed the experiments. YH performed the statistical analysis. MM helped with the interpretations. SK wrote the first draft of the manuscript. YH revised the manuscript. All authors reviewed and approved the manuscript.

Compliance with Ethical Standards

All procedures performed in studies involving animals were conducted in accordance with the ethical standards of the institution or practice at which the studies conducted.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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446 **Figure Legends**

447 **Fig. 1**: The incidence of stroke onset (a) and mortality (b) through the experiments, and symptom test (c) at
448 3 weeks after intracerebroventricular infusion of sodium in experiment 2.
449 Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats. Asterisk
450 indicates statistical significance ($p<0.05$). vs. WKY-0.9%
451
452 **Fig. 2**: The changes of blood pressure between the 14 days (a) and changes of body weight between the 28
453 days (d) in experiment 2.
454 Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats Asterisk
455 indicates statistical significance ($p<0.05$).
456
457 **Fig. 3**: Rotarod test (a) and beam walking test (b) at 21 days, and the values of serum sodium
458 concentration at 28 days (c) in experiment 2.
459 Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats. Asterisk
460 indicates statistical significance ($p<0.05$).
461
462
463 **Fig. 4**: The number of total, resting, and activated microglia (a) and superoxide detected by
464 dihydroethidium (b) in the left somatosensory cortex in experiment 2.
465 Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats. $^{*}p<0.05$
466 vs. WKY-0.9% and $^{\#}p<0.05$ vs. SHRSP-0.9%. Bar indicates 200 μm
467
468 **Fig. 5**: The number of surviving neuron detective by Nissl staining (a) in the left somatosensory cortex and
469 number of microbleeds in the left hemisphere (b) in experiment 2.
470 Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats. $^{*}p<0.05$
471 vs. WKY-0.9% and $^{\#}p<0.05$ vs. SHRSP-0.9%. Bar indicates 200 μm. Arrows indicate Prussian blue
472 positive microbleeds.
473
474

Table. 1: The brain water content in the left hemispheres (a), cerebellum (b), and brain stem (c) at 28 days after the intracerebroventricular infusion of sodium in experiment 2. Abbreviations: SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats. Asterisk indicates statistical significance between WKY and SHRSP in same sodium concentration groups ($p<0.05$).

	WKY-pre (n=4)	SHRSP-pre (n=4)	WKY-0.9 (n=10)	SHRSP-0.9 (n=9)	WKY-2.7 (n=11)	SHRSP-2.7 (n=10)	WKY-9 (n=10)	SHRSP-9 (n=9)
hemisphere (%)	79.0 [78.9-79.1]	79.4* [79.3-79.6]	80.0 [79.6-80.2]	80.4* [79.9-82.9]	79.0 [78.8-79.2]	80.2* [79.9-81.7]	79.2 [79.0-79.4]	81.0* [80.0-81.4]
increase rate (x100%)			101.3 [100.8-101.5]	101.2 [100.5-104.3]	100.1 [99.7-100.2]	101.0* [100.5-102.8]	100.3 [100.0-100.5]	101.9* [100.7-102.5]
cerebellum (%)	77.7 [76.6-78.0]	78.4* [78.3-78.5]	77.9 [77.8-78.1]	78.7* [78.6-78.7]	77.5 [77.4-77.6]	78.7* [78.4-78.7]	77.7 [77.4-77.9]	78.6* [78.4-78.8]
increase rate (x100%)			100.2 [100.1-100.4]	100.4 [100.2-100.4]	99.7 [99.5-99.8]	100.4* [100.1-100.4]	99.9 [99.4-100.1]	100.3* [100.1-100.6]
brainstem (%)	72.7 [72.6-73.0]	74.0* [73.8-74.2]	72.8 [72.6-73.1]	74.1* [73.9-74.4]	72.2 [72.1-72.2]	73.8* [73.5-74.9]	72.2 [71.8-72.4]	74.3* [73.8-74.9]
increase rate (x100%)			100.1 [99.8-100.4]	100.2 [100.0-100.5]	99.2 [99.0-99.3]	99.8* [99.4-101.3]	99.2 [98.7-99.5]	100.5* [99.8-101.3]

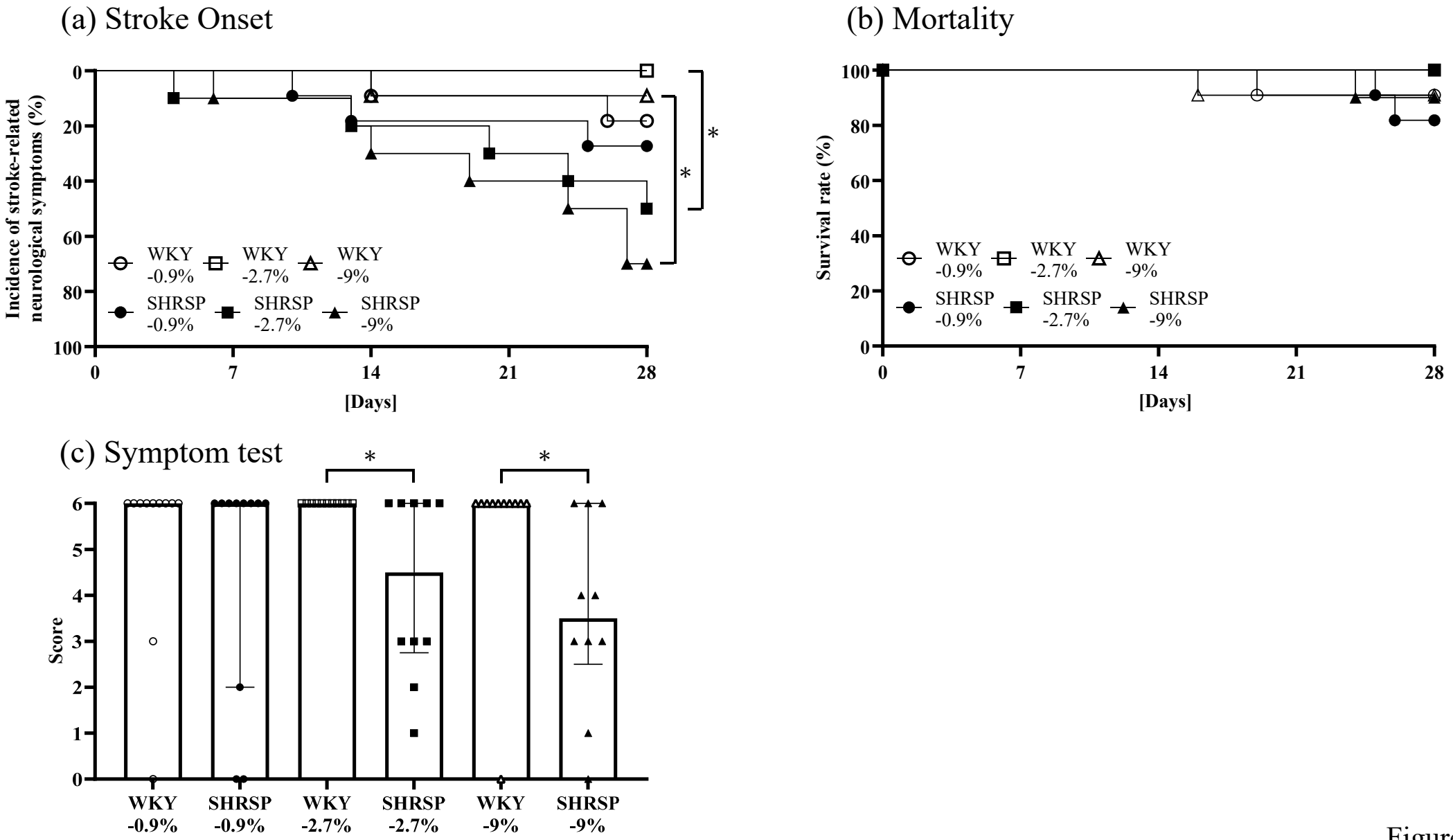
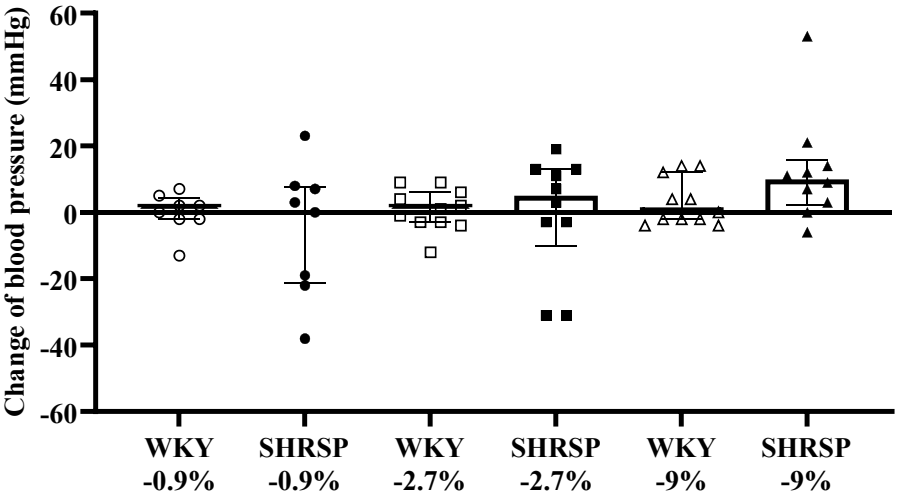

(a) Stroke Onset
Incidence of stroke-related neurological symptoms (%)
0
20
40
60
80
100
0
7
14
21
28
[Days]
WKY -0.9%
WKY -2.7%
WKY -9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%
*
*
(b) Mortality
Survival rate (%)
100
80
60
40
20
0
0
7
14
21
28
[Days]
WKY -0.9%
WKY -2.7%
WKY -9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%
(c) Symptom test
Score
6
5
4
3
2
1
0
*
*
WKY -0.9%
SHRSP -0.9%
WKY -2.7%
SHRSP -2.7%
WKY -9%
SHRSP -9%

Figure. 1

(a) Changes of blood pressure



(b) Changes of body weight

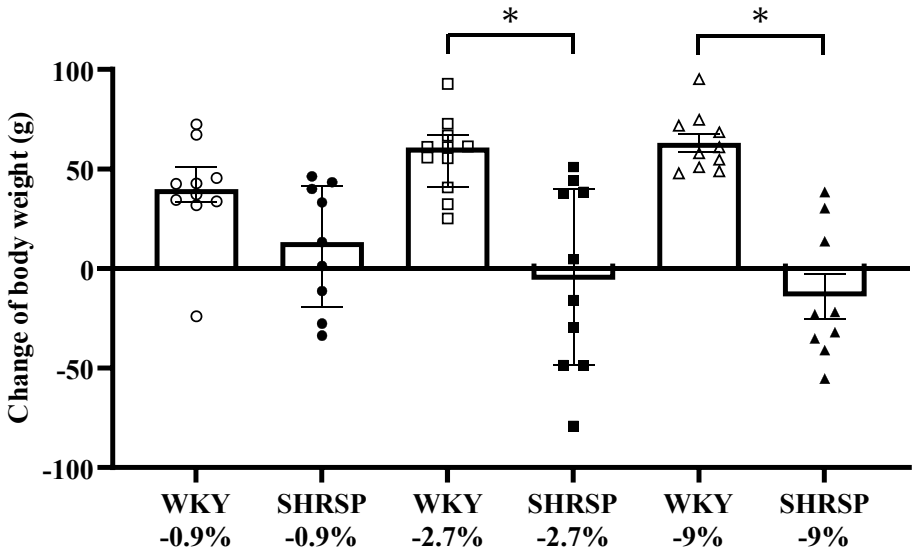


Figure. 2

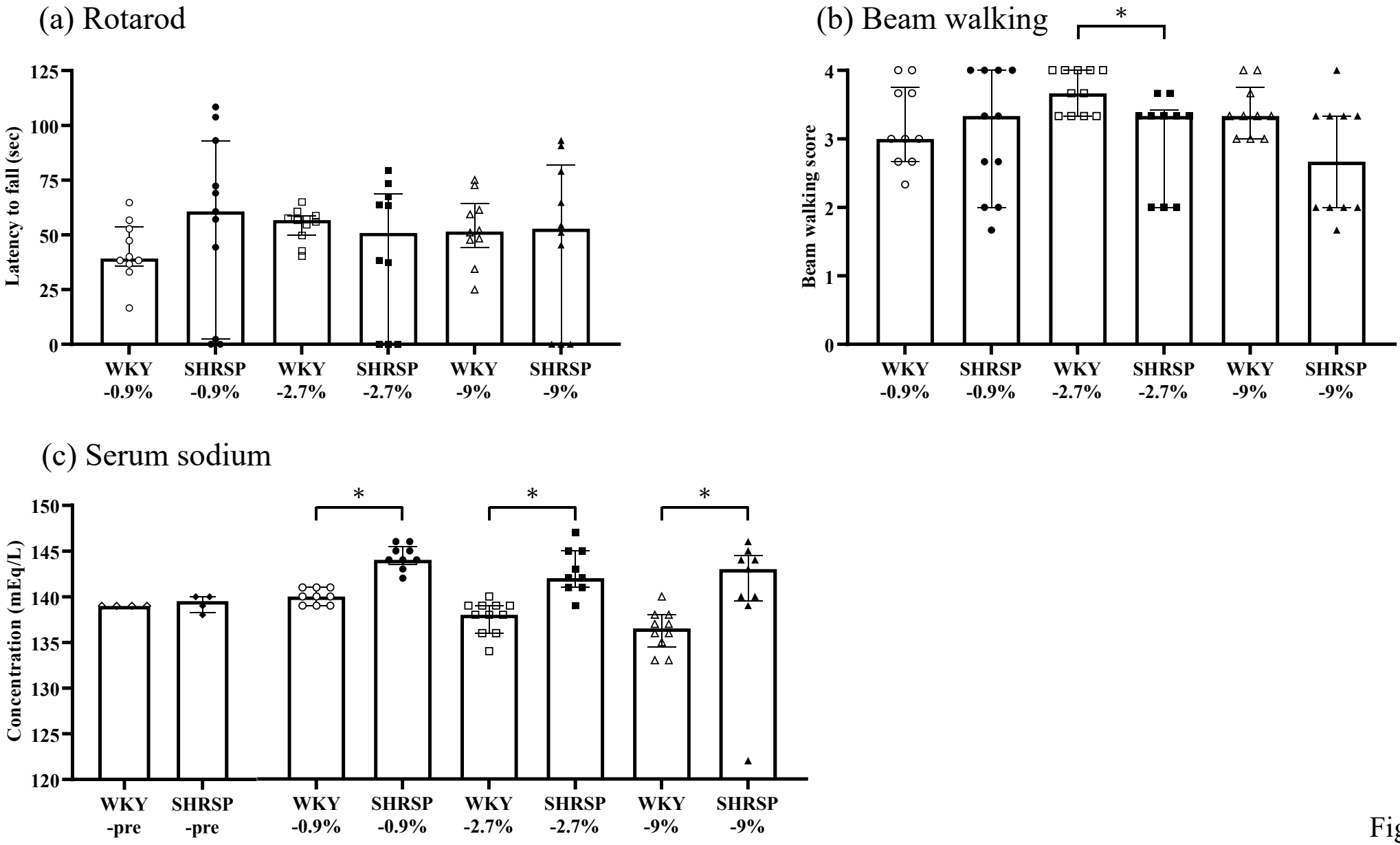
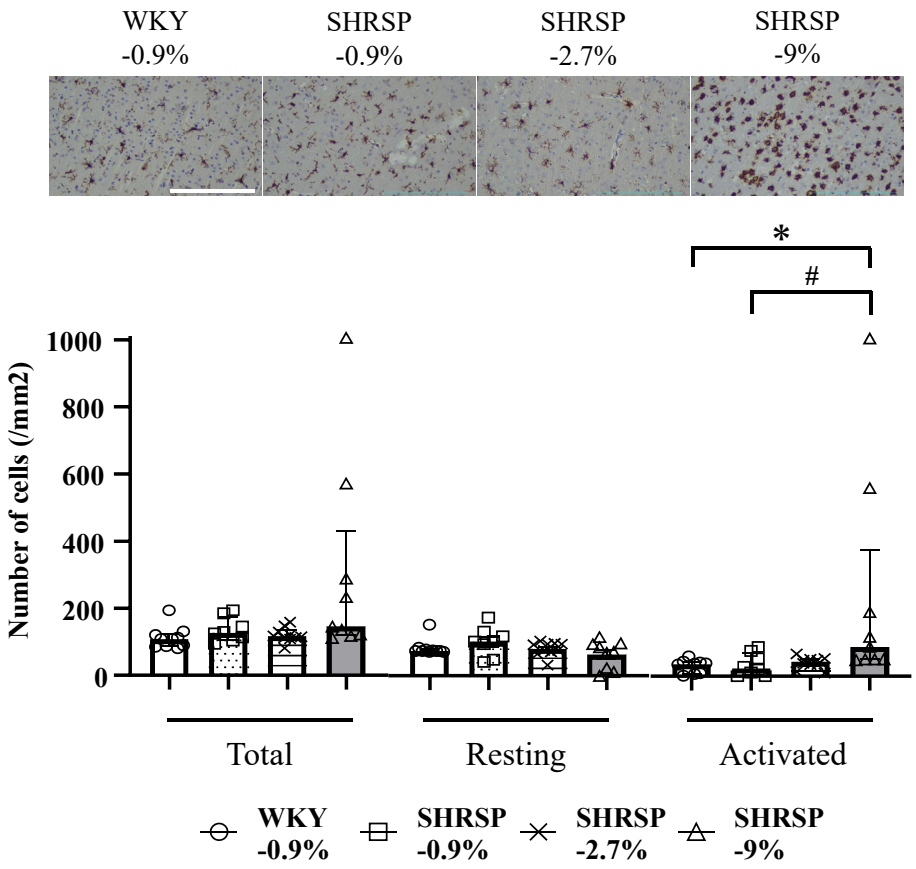

(a) Rotarod
Latency to fall (sec)
125
100
75
50
25
0
WKY -0.9%
SHRSP -0.9%
WKY -2.7%
SHRSP -2.7%
WKY -9%
SHRSP -9%
(b) Beam walking
*
Beam walking score
4
3
2
1
0
WKY -0.9%
SHRSP -0.9%
WKY -2.7%
SHRSP -2.7%
WKY -9%
SHRSP -9%
(c) Serum sodium
*
*
*
Concentration (mEq/L)
150
145
140
135
130
125
120
WKY -pre
SHRSP -pre
WKY -0.9%
SHRSP -0.9%
WKY -2.7%
SHRSP -2.7%
WKY -9%
SHRSP -9%

Figure. 3


(a) Microglia
WKY -0.9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%
*
#
Number of cells (/mm2)
1000
800
600
400
200
0
Total
Resting
Activated
WKY -0.9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%

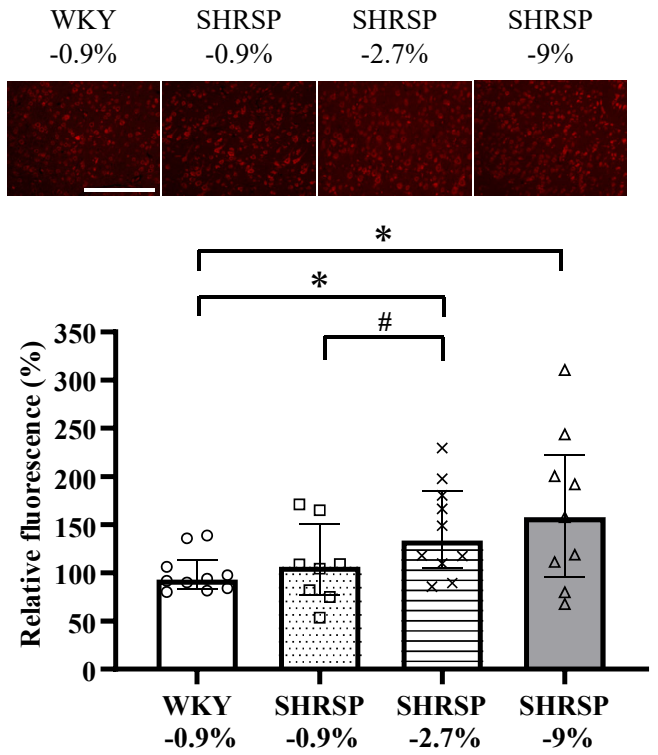
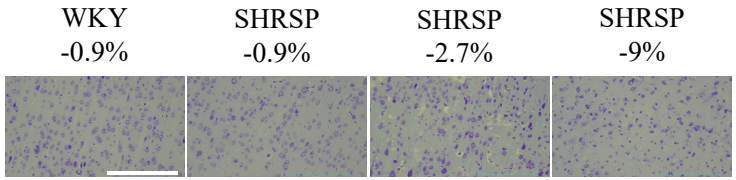
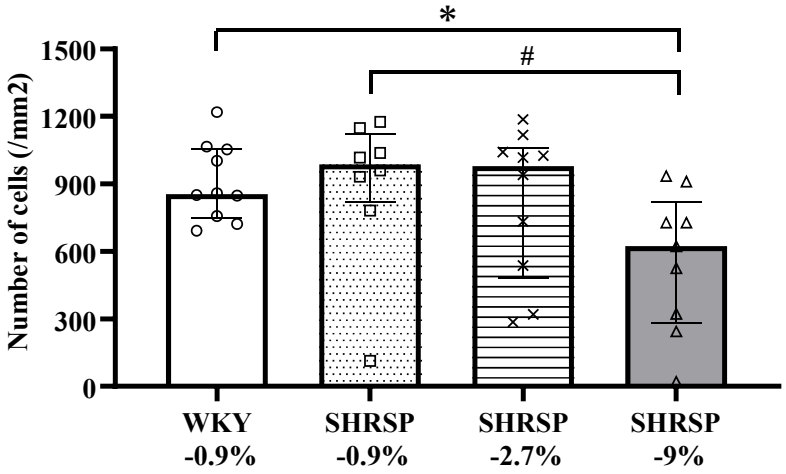

(b) DHE stain
WKY -0.9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%
*
*
#
Relative fluorescence (%)
350
300
250
200
150
100
50
0
WKY -0.9%
SHRSP -0.9%
SHRSP -2.7%
SHRSP -9%

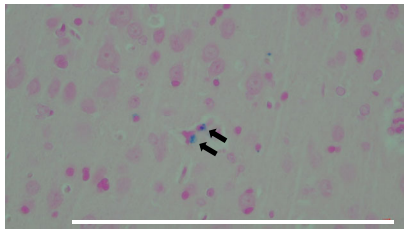
Figure. 4

(a) Neuron





(b) Microbleeds



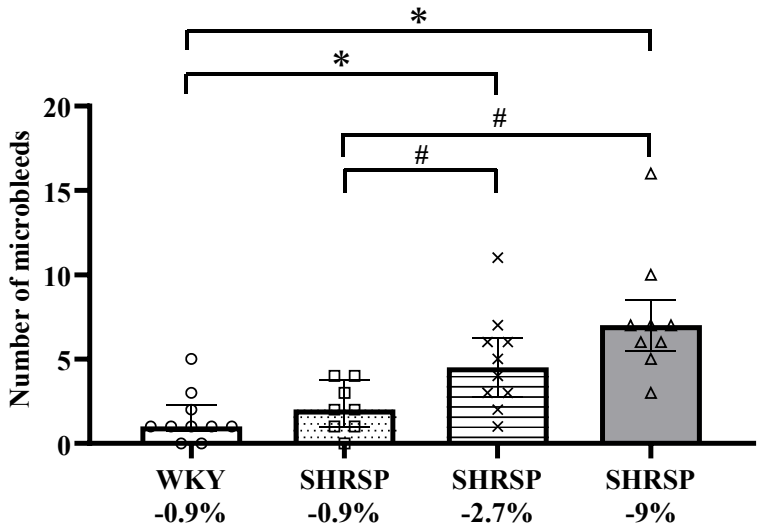
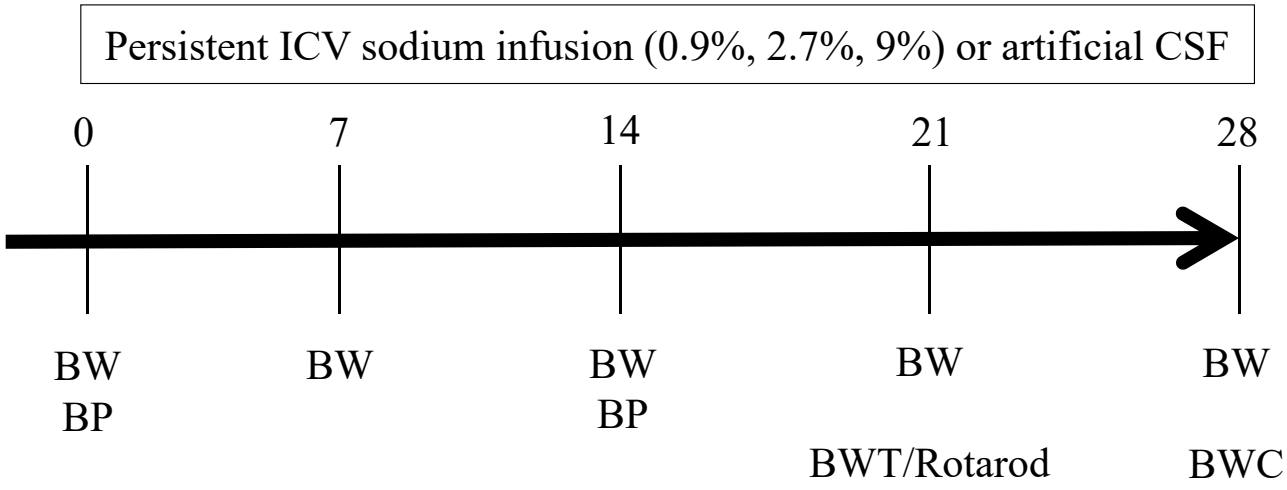
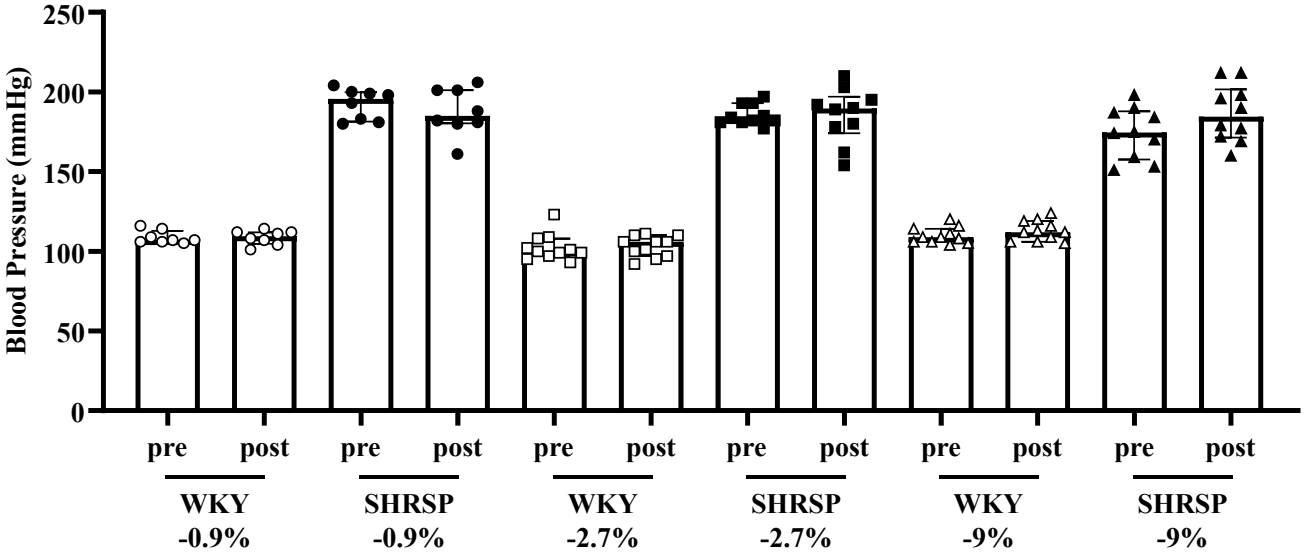


Figure. 5

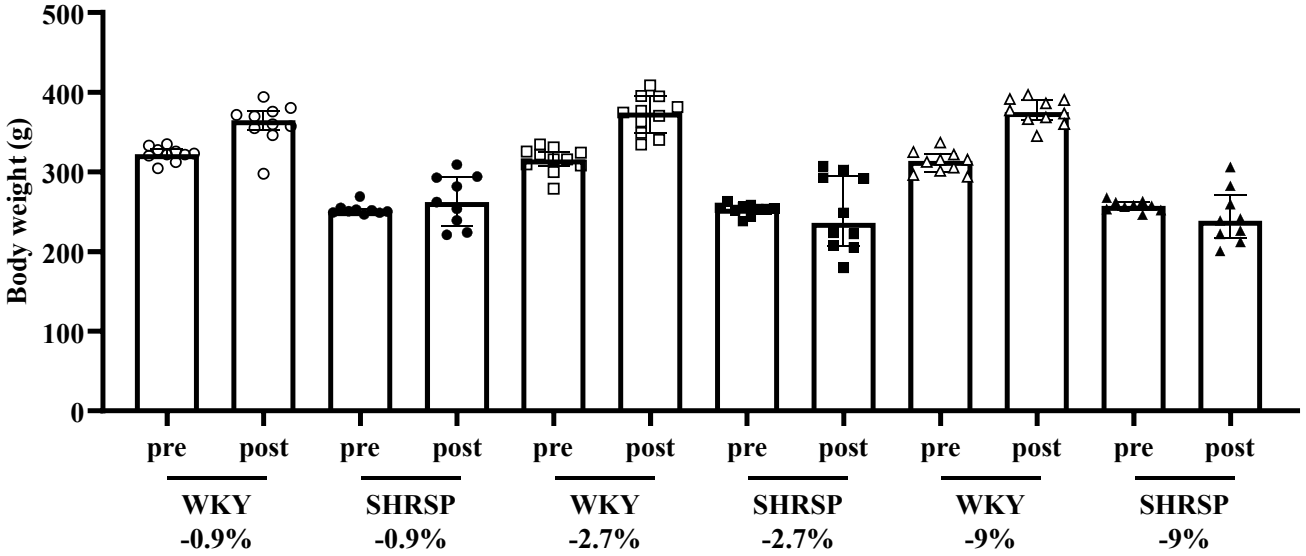


Suppl. Figure 1

(a) Systolic blood pressure

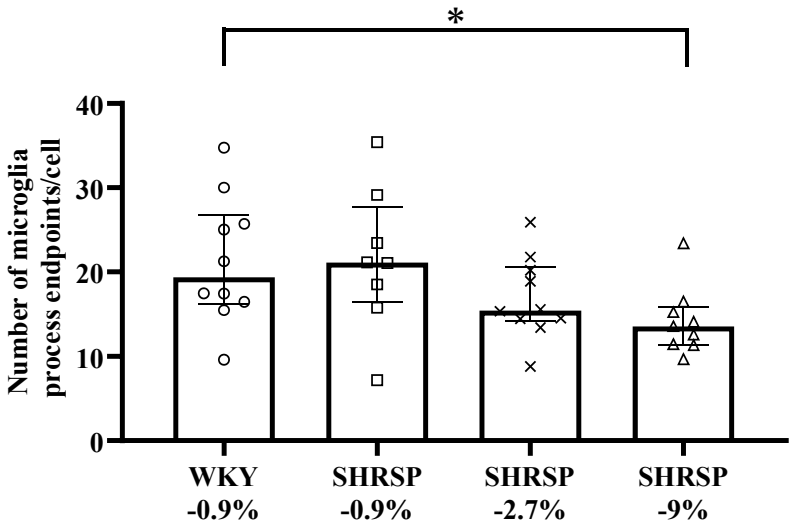


(b) Body weight

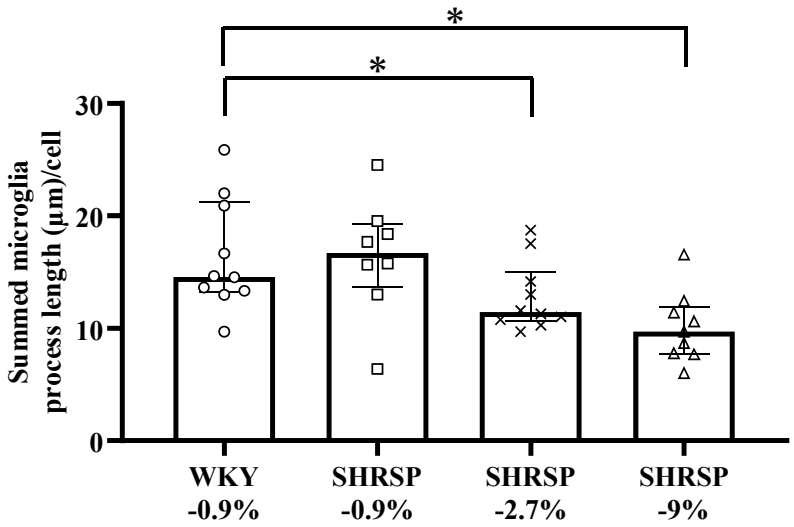


Suppl. Figure 2

(a) Process endpoints



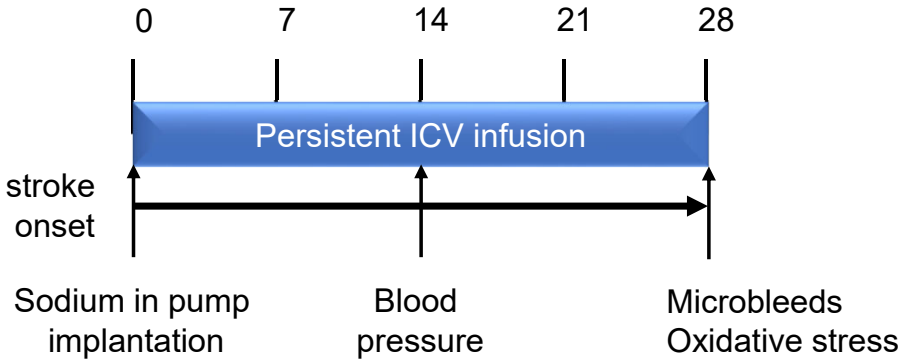
(b) Process length



Suppl. Figure 3

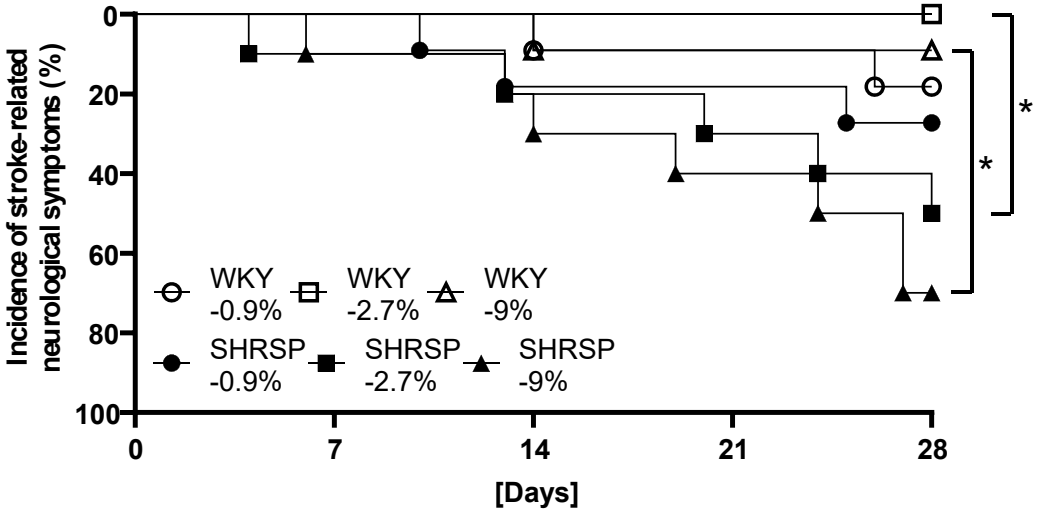
It is undetermined whether persistent brain exposure to high sodium induces stroke onset.

Protocol (SHRSP and WKY)

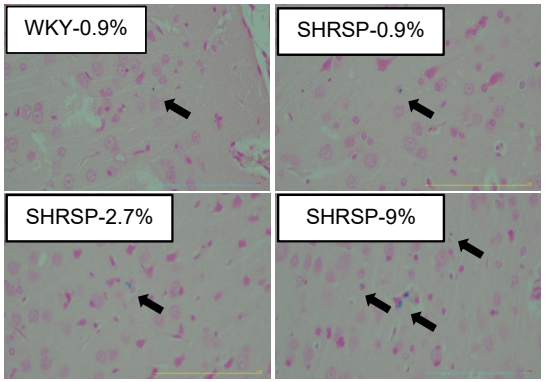


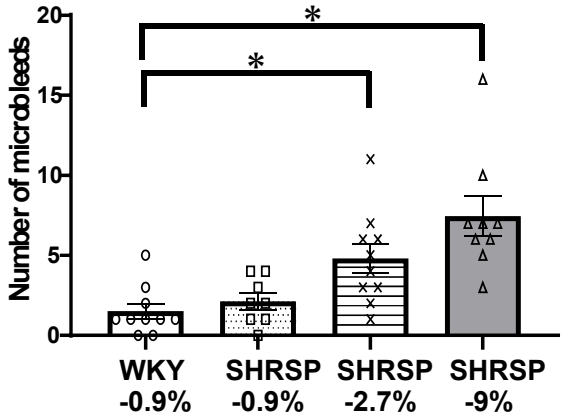
SHRSP, spontaneously hypertensive stroke-prone rats; WKY, Wistar Kyoto rats

Stroke Onset

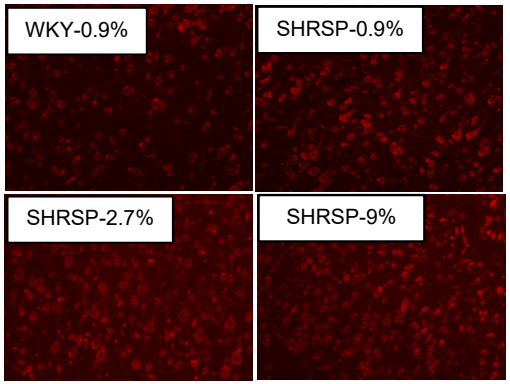


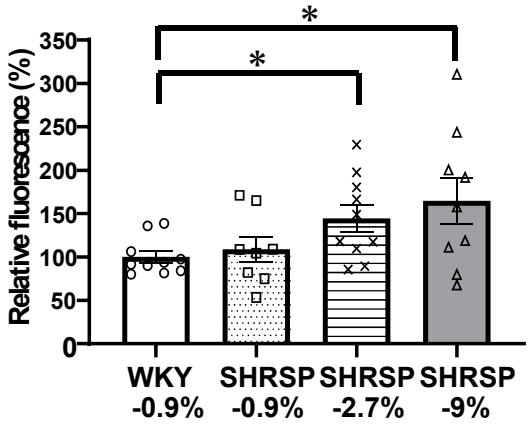
Microbleeds





Superoxide





Persistent brain exposure to high sodium induces stroke onset by upregulating cerebral microbleeds and oxidative stress in hypertensive rats.