

Anaphylactic hypotension causes renal and adrenal sympathoexcitation and induces c-fos in the hypothalamus and medulla oblongata

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Abstract

We previously reported that sympathetic nerve activity (SNA) to the kidney and the hind-limb increases during anaphylactic hypotension in anaesthetized rats. Based on this evidence, we examined effects of anaphylactic hypotension on SNA to the brown adipose tissue (BAT), and the adrenal gland and kidney in anaesthetized rats. We demonstrated that adrenal and renal SNA, but not BAT-SNA, were stimulated. In addition, the effects of anaphylaxis on neural activities of the hypothalamic and medullary nuclei, which are candidates for relaying efferent SNA to the peripheral organs, were investigated via immunohistochemical staining of c-fos. Anaphylaxis increased c-fos expression in the neurons of the paraventricular nucleus (PVN) of the hypothalamus and in those of the nucleus tractus solitarius (NTS) and rostral ventrolateral medulla (RVLM) of the medulla oblongata; c-fos was expressed in γ -aminobutyric acid (GABA)-ergic neurons of the NTS and in the catecholaminergic neurons of the RVLM. In addition, c-fos expression in the rostral NTS and mid NTS during anaphylaxis was reduced by sino-aortic baroreceptor denervation; however, increased c-fos expression in the caudal NTS and RVLM or adrenal sympathoexcitation were not affected by sinoaortic baroreceptor denervation. These results indicated that anaphylactic hypotension activates the hypothalamic PVN and the medullary NTS and RVLM independently of the baroreflex pathway. Further, it stimulated efferent SNA to the adrenal gland and kidney to restore blood pressure.

KEYWORDS

Adrenal gland, Anaphylactic shock, Arterial baroreflex, Autonomic nervous system, C1 neurons

1 | INTRODUCTION

Anaphylactic shock-accompanied hypotension is potentially life threatening. In our previous experiments, blood pressure in rats sensitized by subcutaneous injections of an emulsion made by mixing equal volumes of complete Freund's adjuvant with ovalbumin dissolved in physiological saline decreased after intravenous injection of antigen (Wang, Tanida, Shibamoto, & Kurata, 2013; Sun et al., 2014; Wang et al., 2014; Song et al., 2016).

The sympathetic nervous system serves as a defence system against anaphylactic shock (Koyama et al., 1990; Song et al., 2016; Sun et al., 2014; Wang et al., 2013). Although efferent sympathetic nerve activity (SNA) to the kidney (Sun et al., 2014) and hind-limb (Song et al., 2016) increased during anaphylactic hypotension in anaesthetized rats, the SNA in the liver did not change (Wang et al., 2013). These results indicated regional differences in the SNA response to anaphylactic hypotension. It is well known that SNA to the adrenal gland regulates its catecholamine release, which modulates

New Findings

- **What is the central question of this study?**

Whether anaphylaxis affects sympathetic outflows to the brown adipose tissue (BAT) and adrenal gland and whether anaphylaxis affects some brain areas in association with sympathetic regulation.

- **What is the main finding and its importance?**

Sympathoexcitatory responses to anaphylaxis occurred regionally in the kidney and adrenal gland, but not in the thermogenesis-related BAT. Further, anaphylactic hypotension also caused increase in c-fos immunoreactivity in the hypothalamic and medullary areas. Moreover, catecholaminergic neurons of the brainstem cause adrenal sympathoexcitation in a baroreceptor-independent manner.

the cardiovascular system (Guyenet, 2006). Moreover, adrenal SNA responds to hypotension and hypertension via baroreflex (Scislo, Robert, & Donal, 1998). Conversely, although the sympathetic branch to brown adipose tissue (BAT) contributes to the regulation of energy metabolism and thermogenesis (Morrison, Madden, & Tupone, 2014; Tanida et al., 2007; Tupone, Cano, & Morrison, 2017), it does not have barosensitivity to blood pressure changes (Morrison, 2001). Further, body temperature decreases (Manzano-Szalai et al., 2016) and blood catecholamine levels increase during anaphylactic hypotension (Wang et al., 2014; Zhang, Shibamoto, Kuda, Ohmukai, & Kurata, 2011). However, the responses of BAT-SNA and adrenal SNA to anaphylactic hypotension are currently not known. Thus, the primary aim of the present study was to determine how the SNA to BAT and adrenal gland changes in anaesthetized rats during anaphylactic hypotension.

Some brain areas regulate the SNA to peripheral organs to maintain cardiovascular and metabolic functions. These brain areas include the paraventricular nucleus (PVN) of the hypothalamus and the nucleus tractus solitarius (NTS)–rostral ventrolateral medulla (RVLM) pathways, which are involved in cardiovascular control (Kline, King, Austgen, Heesch, & Hasser, 2010; Malpas, 2010; Sly, Colvill, McKinley, & Oldfield, 1999; Tanida et al., 2015), and the ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and arcuate nucleus (ARC)–PVN pathways of the hypothalamus, which regulate BAT-SNA and thermogenesis (Labbé et al., 2015; Morrison et al., 2014). Thus, neural pathways controlling the cardiovascular organs and metabolic organs are localized differently in the brain (Morrison, 2001). Immunohistochemical studies revealed an increase in c-fos-positive cells (indicative of activated neurons) in the PVN and ARC of the hypothalamus and in the NTS and RVLM of the medulla oblongata following acute hypotension in rats (Graham, Hoffman, & Sved, 1995) and rabbits (Polson, Mrljak, Potts, & Dampney, 2002). Furthermore, c-fos expression in the hypothalamic PVN and medullary NTS increased in response to systemic anaphylaxis in rats (Castex,

Fioramonti, Fargeas, & Bueno, 1995; Földes, Némethy, Szalay, & Kovács, 2000). However, it is currently unknown whether systemic anaphylaxis affects other brain sites, such as the VMH, DMH, ARC and RVLM, in association with sympathetic regulation. Thus, the second aim of the current study was to determine whether c-fos was induced in the hypothalamic and medullary nuclei during anaphylactic hypotension.

2 | METHODS

2.1 | Ethical approval

All experiments were approved by the animal research committee of Kanazawa Medical University (2014-39) and carefully performed in accordance with relevant guidelines and regulations (Guidelines for Proper Conduct of Animal Experiments, 1 June 2006, Science Council of Japan) and the principles and regulations described by Grundy (2015).

2.2 | Animals

Male Sprague–Dawley rats (330–360 g, $n = 69$) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and housed in a room maintained on a light–dark cycle with 12 h light (08.00–20.00 h) at $24 \pm 1^\circ\text{C}$ with food and water provided *ad libitum*. To sensitize rats, 2 weeks before experiments, an emulsion made by mixing equal volumes of complete Freund's adjuvant (0.5 ml) with 1 mg ovalbumin (grade V, Sigma-Aldrich, St Louis, MO, USA) dissolved in physiological saline (0.5 ml) was injected subcutaneously (Song et al., 2016; Sun et al., 2014; Wang et al., 2013). Non-sensitized rats were used as the control animals.

2.3 | Electrophysiology

General preparations were performed as previously described (Song et al., 2016; Sun et al., 2014; Tanida et al., 2007; Tanida et al., 2015). Briefly, under pentobarbital sodium anaesthesia (50 mg kg^{-1} , i.p.), rats with the right femoral vein, right femoral artery and trachea cannulated with plastic catheters were fixed in a stereotaxic apparatus. In the experiment, depth of anaesthesia was checked by cardiovascular responses to paw pinch (Tanida et al., 2005), and if any one of these responses was observed, additional pentobarbital sodium (5 mg kg^{-1}) was injected subcutaneously. Their body temperature was maintained at $36.0\text{--}36.5^\circ\text{C}$ using a heating pad, unless otherwise stated, and was monitored using a thermometer inserted into the rectum. For measurement of renal SNA or adrenal SNA, a nerve branch of the left kidney or left adrenal gland, respectively, was exposed retroperitoneally through a left flank incision. To record the BAT-SNA, the left sympathetic nerve innervating interscapular BAT was exposed through a left dorsal incision, and the distal side of the nerve bundle was incised to prevent interfusion of the electrocardiographic signal. The nerve was attached to a pair of stainless steel wire electrodes for

SNA recordings. The recording electrodes were fixed with a silicone gel (liquid A & liquid B, Kagawa kikai Co., Miki, Japan) to prevent dehydration and for electrical insulation.

The electrical change in the SNA was amplified 20,000–50,000 times using a bandpass of 100–1000 kHz, which was monitored using an oscilloscope. The raw data were converted to standard pulses using a window discriminator. Both the discharge rate and the neurogram were sampled using a PowerLab analog-to-digital converter (4sp, ADInstruments, Bella Vista, NSW, Australia) for recording and data analysis on a computer. Background noise, which was determined 30–60 min after the animal was killed, was subtracted. The SNA was rectified, integrated and normalized to the baseline nerve activity. The systemic arterial pressure measured from the left femoral artery and heart rate (HR) were calculated from systemic arterial pressure signals that were also sampled using PowerLab and stored on a hard disk for off-line analysis.

Following surgery, each rat was allowed to stabilize for 10–20 min and injected intravenously with a bolus of sodium nitroprusside (SNP; 30 mg kg⁻¹) to induce baroreceptor-mediated sympathoexcitation. This treatment did not affect renal sympathetic and cardiovascular responses to anaphylaxis (Figure 3). Next, the baseline was measured for 5 min prior to the intravenous injection of the ovalbumin antigen (0.6 mg) dissolved in saline. The parameters were recorded for 60 min after the antigen injection. At the end of the experiment, hexamethonium chloride (10 mg kg⁻¹) was intravenously administered to ensure the post-ganglionic efferent SNA. After all experiment procedure, the rats were killed by intraperitoneal injection of an overdose of pentobarbital sodium (100 mg kg⁻¹).

2.4 | Sino aortic denervation

To perform sinoaortic denervation (SAD) (Song et al., 2016; Sun et al., 2014) in rats in which adrenal SNA was measured, after the neurovascular trunk in the neck was exposed, the carotid sinus, aortic depressor and recurrent laryngeal nerves were sectioned bilaterally. Successful SAD was confirmed by the absence of reflex increases in HR and adrenal SNA in response to the hypotension induced by SNP (Figure 9d, e).

2.5 | Immunohistochemistry

The expression of c-fos proteins in the hypothalamus and brainstem was investigated in rats anaesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg kg⁻¹), and renal SNA, BP and HR were recorded before and after the antigen injection. In all rats, renal sympathoexcitation and tachycardia by SNP-induced hypotension were observed before antigen injection. General histological preparations were made as previously described (Tanida et al., 2008). An injection of hexamethonium caused hypotension and increase in the number of c-fos-positive cells that were colocalized with tyrosine hydroxylase (TH) in the RVLM (Figure 10f). Thus, 60 min after the antigen injection, without hexamethonium injection, rats were perfused intracardially with ice-cold saline followed by 4% paraformaldehyde. Brains were dissected out and sliced into 40 µm-thick sections

with a cryomicrotome (CM1900, Leica, Wetzlar, Germany). Coronal brain sections were incubated with an anti-c-fos polyclonal rabbit antibody (lot no. 2878126, ABE457, Merck Millipore, Billerica, MA, USA), followed by a biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA). Sections were then treated with ABC kits (Vector Laboratories) according to the manufacturer's instructions. To quantify the expression of c-fos in the nuclei of the brain (PVN, ARC, VMH, DMH, NTS, RVLM), images of the slices were acquired using a photon microscope (TDI camera, Nano zoomer, Hamamatsu Photonics K.K., Hamamatsu, Japan) and examined with reference to the rat brain atlas (Paxinos & Watson, 2007). The numbers of c-fos-positive cells in the bilateral areas were counted using the ImageJ software program. Serial sections were used to count c-fos-positive cells, in all the areas (i.e. the PVN, ARC, VMH, DMH and RVLM), except for the NTS. To analyse the NTS, it was divided into the three regions, i.e. the rostral, mid and caudal regions, and each region was further subdivided into two or three areas as follows: rostral NTS, two areas –13.1 and –13.3 mm anteroposterior; mid NTS, three areas –13.5, –13.7 and –13.9 mm anteroposterior; caudal NTS, two areas –14.1 and –14.3 mm anteroposterior, according to a previous study (King, Heesch, Clark, Kline, & Hasser, 2012).

Double staining of slices containing the NTS and RVLM was performed using fluorescence immunohistochemistry. GABAergic neurons and catecholaminergic neurons were identified using antibodies against glutamic acid decarboxylase isoform 67 (GAD67), a GABA-synthesizing enzyme, and TH, respectively. Brain sections were incubated with a primary antibody using the following dilutions: rabbit anti-c-fos, 1:1000; mouse anti-GAD67, 1:500 (lot no. 2676521, MAB5406, Merck Millipore); and mouse anti-TH, 1:600 (lot no. 2742733, MAB5296, Merck Millipore). To perform double staining of c-fos and GAD67, following the overnight incubation with the anti-GAD67 antibody, the slices were treated with biotinylated anti-mouse IgG (Vector Laboratories). Thereafter, it was incubated with the ABC kits and Tyramide Signal Amplification (TSA plus fluorescein, Perkin Elmer Japan co. Ltd., Yokohama, Japan). Thereafter, the stained slices were washed and incubated with anti-c-fos antibody overnight, followed by Alexa Fluor 543-labelled secondary antibody and 4',6-diamidino-2-phenylindole (DAPI).

To perform double staining using c-fos and TH, the slices were treated with anti-c-fos and anti-TH antibodies overnight. Thereafter, the slices were incubated with Alexa Fluor 488-labelled and Alexa Fluor 543-labelled secondary antibody. The stained slices were observed using an all-in-one fluorescence microscope system (BZ-9000, Keyence, Osaka, Japan).

2.6 | Statistical methods

All results are expressed as the mean ± SD. Comparison of individual values within a group was performed using one-way ANOVA followed by the Bonferroni *post hoc* test. Comparison of sympathetic and haemodynamic responses to the antigen between the two groups was performed using a two-way repeated measures ANOVA with the Bonferroni *post hoc* test. Student's *t* test or one-way ANOVA followed by the Bonferroni *post hoc* test was used to compare c-fos response in

two groups or between three groups, respectively. Differences were considered statistically significant at $P < 0.05$.

3 | RESULTS

3.1 | Sympathetic responses to anaphylactic hypotension in anaesthetized rats

In the anaphylaxis group, renal SNA and adrenal SNA began to increase significantly at 2 min after antigen injection, and thereafter progressively increased to $174 \pm 21\%$ and $263 \pm 22\%$ of the baseline, respectively, at the end of the experimental period of 60 min ($P < 0.05$, Figure 1a and b). In contrast, BAT-SNA did not change significantly during anaphylaxis, regardless of whether the body temperature was maintained at $36.0\text{--}36.5^\circ\text{C}$ (Figures 1c and 2a). In the anaphylaxis group, the mean systemic arterial pressure (MAP) decreased, while the HR increased after antigen injection ($P < 0.05$, Figure 1d and e). Representative recordings during anaphylaxis are shown in Figure 2a. The baroreceptor reflex was confirmed by increases in renal SNA and adrenal SNA, but not in BAT-SNA, in response to SNP-induced hypotension (Figure 2b). In addition, in both groups with or without pretreatment of SNP injection, renal sympathoexcitation and hypotension were observed (Figure 3a and b). None of the parameters changed significantly in the control groups, throughout the experimental period (Figure 1).

To mimic the hypotensive response to the antigen, a subcutaneous injection of SNP was performed in a non-sensitized rat. Representative data of a sensitized rat with antigen injection and a non-sensitized rat with SNP injection are shown in Figure 4. In both rats, hypotensive response was induced, but the renal sympathoexcitation in a SNP-treated rat was smaller than that in a rat with evoked anaphylaxis (Figure 4b and c).

3.2 | Effects of anaphylactic hypotension on c-fos expression in the neurons of the hypothalamus and medulla oblongata

Furthermore, we examined whether anaphylactic hypotension had an effect on the neurons in the PVN, ARC, VMH and DMH in the hypothalamus. Anaphylaxis increased c-fos expression in the neurons of the PVN, but not the ARC, VMH or DMH ($P < 0.05$, Figure 5). In addition, anaphylaxis significantly increased c-fos-positive neurons in all three NTS areas (i.e. the rostral NTS, mid NTS and caudal NTS; $P < 0.05$, Figure 6a and b) and the RVLM ($P < 0.05$, Figure 7a and b).

Following anaphylactic hypotension, the majority of c-fos in the NTS was expressed by GAD67-immunoreactive cells (Figure 6c), while in the RVLM it was found in TH-positive neurons (Figure 7c).

To mimic the hypotensive response to the antigen, a subcutaneous injection of SNP was performed in a non-sensitized rat. Immunohistochemical data of a sensitized rat with antigen injection and a non-sensitized rat with SNP injection are shown in Figure 4. In both rats, c-fos-positive neurons in the PVN (Figure 4d) and the NTS (Figure 4e) increased ($P < 0.05$). However, c-fos-positive cells in the TH neurons in

the RVLM in a SNP-treated rat were fewer than in a rat with evoked anaphylaxis (Figure 4f).

3.3 | Effects of sinoaortic denervation on increased c-fos expression in the neurons of the NTS and RVLM and adrenal sympathoexcitation during anaphylactic hypotension

The c-fos expression in the caudal NTS and RVLM increased after anaphylaxis in both intact rats and SAD rats (Figure 8a and b), and c-fos was localized to TH-positive neurons and was induced in the RVLM of both groups (Figure 8c). However, c-fos expression in the rostral NTS and mid NTS was significantly lower in SAD rats, compared to intact rats ($P < 0.05$, Figure 8a and b). Notably, increased adrenal SNA was observed in the SAD rats after anaphylactic hypotension (Figure 9a). We examined adrenal SNA and HR responses to the SNP injection to evaluate the baroreceptor reflex, and demonstrated that the stimulatory responses of adrenal SNA and HR to the SNP-induced hypotension in the SAD rats were significantly less than in the intact rats ($P < 0.05$, Figure 4d and e). As a control experiment, saline injection did not induce renal sympathetic response or c-fos expression of TH neurons in the RVLM or adrenal sympathoexcitation in either intact rats or SAD rats (Figure 10a, b, d and e).

4 | DISCUSSION

In the present study, we demonstrated, for the first time, that anaphylactic hypotension accelerated not only renal SNA but also adrenal SNA, with no effect on BAT-SNA. Further, it also increased c-fos expression in the PVN of the hypothalamus and in the rostral, mid and caudal areas of the NTS and RVLM of the brainstem, but not in the DMH, VMH or ARC of the hypothalamus. In addition, c-fos was expressed in GABAergic and catecholaminergic neurons of the NTS and RVLM, respectively. These lines of evidence suggest that anaphylactic hypotension tissue-specifically stimulates SNA to the kidney and adrenal gland to drive the cardiovascular system, via the neural pathway between the hypothalamic PVN and brainstem. To our knowledge, this is the first study to report increases in adrenal SNA, but not BAT-SNA, and c-fos immunoreactivity in the hypothalamus and medulla oblongata during anaphylactic hypotension.

During anaphylactic hypotension, increased renal SNA and adrenal SNA may contribute to the blood pressure recovery through noradrenergic vasomotion and catecholamine secretion, respectively. Plasma catecholamine levels were also elevated during anaphylaxis (Wang et al., 2013; Zhang et al., 2011). Further, α -adrenoceptor blockers and chemical sympathectomy attenuated blood pressure recovery during anaphylaxis (Wang et al., 2013). A novel finding of the current study was that BAT-SNA did not significantly change during anaphylactic hypotension. The sympathetic branch to BAT plays an important role in thermoregulation and energy metabolism; increased BAT-SNA caused thermogenesis and metabolic activation

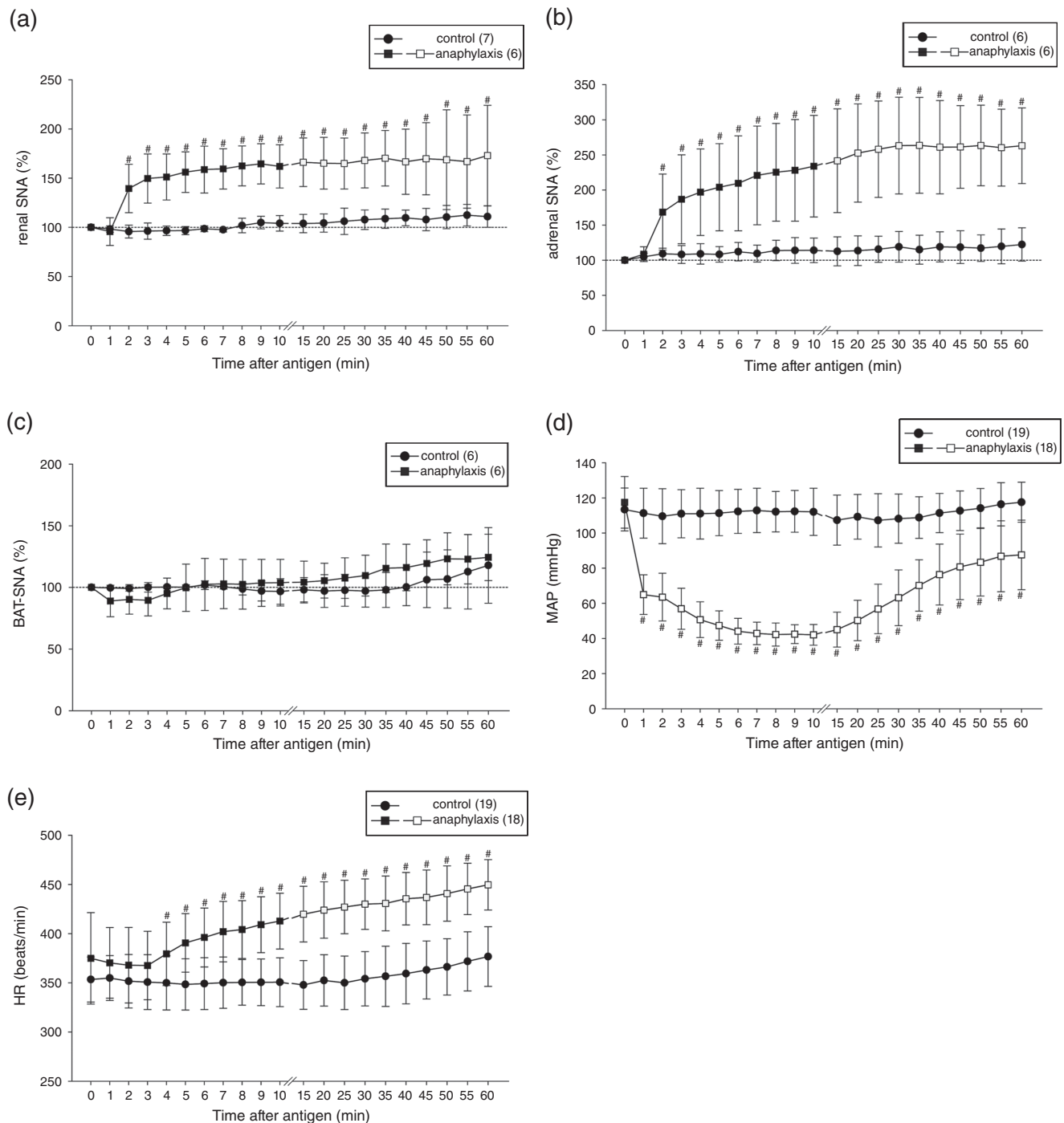


FIGURE 1 Summary of the sympathetic and cardiovascular responses to anaphylaxis. Time course data of the renal SNA (a), adrenal SNA (b), BAT-SNA (c), MAP (d) and HR (e) after an intravenous injection of the antigen in non-sensitized rats (control, ●) and sensitized rats (anaphylaxis, ■, □). Numbers of animals used are shown in parentheses. Values are expressed as means $\pm$ SD. # P < 0.05 vs. the control group; open symbols, P < 0.05 vs. the baseline

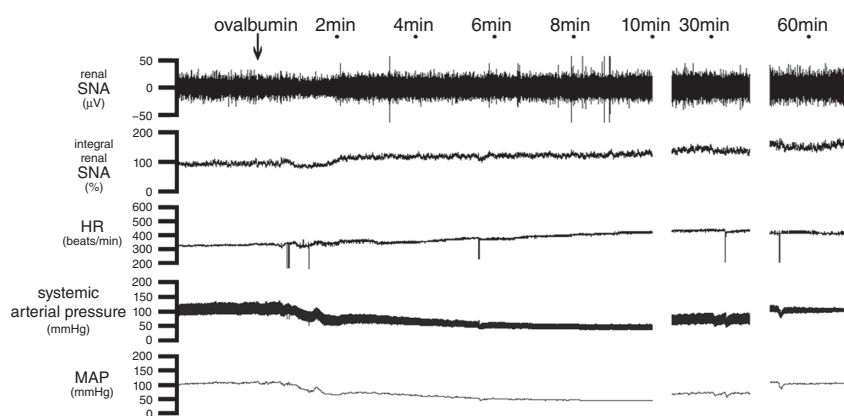
(Morrison et al., 2014; Scislo et al., 1998; Tanida et al., 2007), while decreased BAT-SNA evoked hypothermia and metabolic inhibition (Kannan, Nijima, & Yamashita, 1988). Interestingly, since anaphylaxis caused hypothermia in conscious mice (Manzano-Szalai et al., 2016), BAT-SNA was expected to be inhibited during anaphylactic hypotension. However, this did not occur in the current study. The sympathetic response to anaphylaxis was attenuated in anaesthetized rats, compared to conscious rats (Sun et al., 2014). Thus, anaesthesia

might have influenced the BAT-SNA response to anaphylactic hypotension. However, further studies are required to test this assumption.

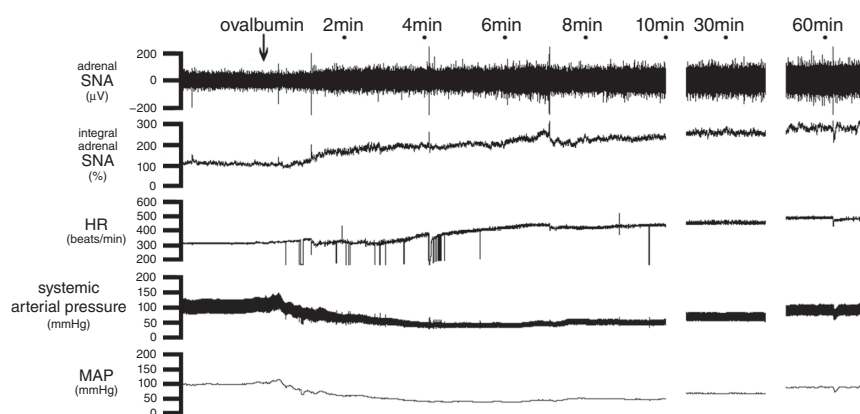
In the hypothalamus, following anaphylactic hypotension, the number of c-fos-immunoreactive cells increased in the PVN, but not in the other areas (i.e. the ARC, VMH or DMH). This finding is consistent with previous observations (Földes et al., 2000). The hypothalamic PVN efferent sympathetic branch projects to the kidney and is a crucial area for regulating blood pressure (Kannan et al., 1988; Sly et al., 1999;

(a) Responses to anaphylaxis

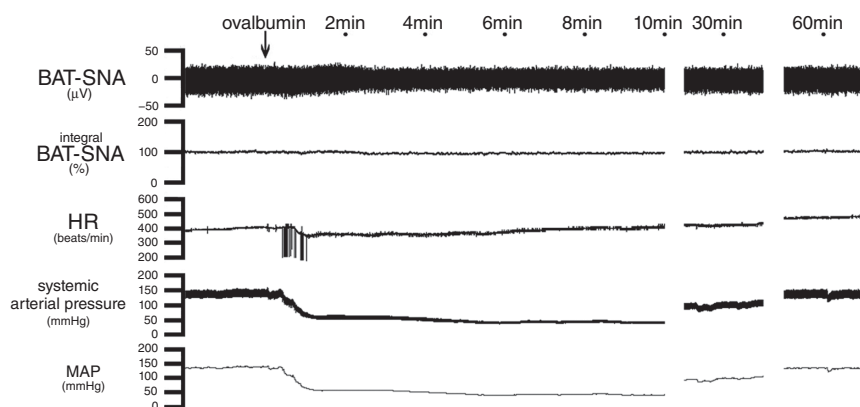
renal SNA



adrenal SNA

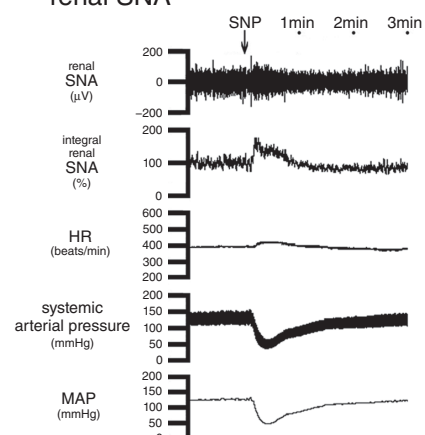


BAT-SNA

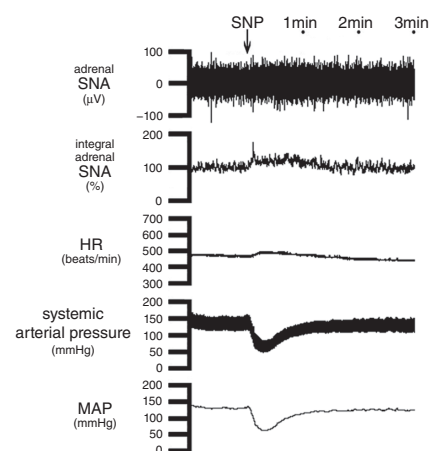


(b) Responses to SNP

renal SNA



adrenal SNA



BAT-SNA

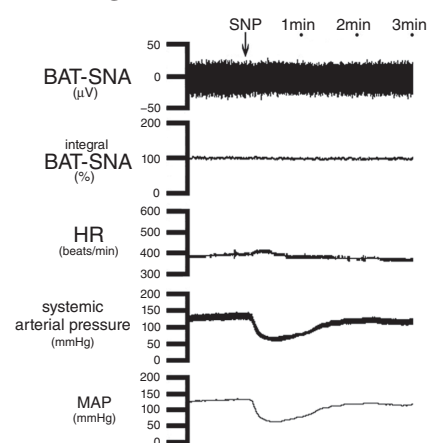


FIGURE 2 Sympathetic nerve activity to the kidney, adrenal gland and BAT and haemodynamics after antigen injection. (a) Representative data of renal SNA, adrenal SNA and BAT-SNA with systemic arterial pressure, MAP and HR before and after an intravenous injection of the antigen in a sensitized rat. (b) Typical data of renal SNA, adrenal SNA and BAT-SNA with systemic arterial pressure, MAP and HR during SNP-induced hypotension

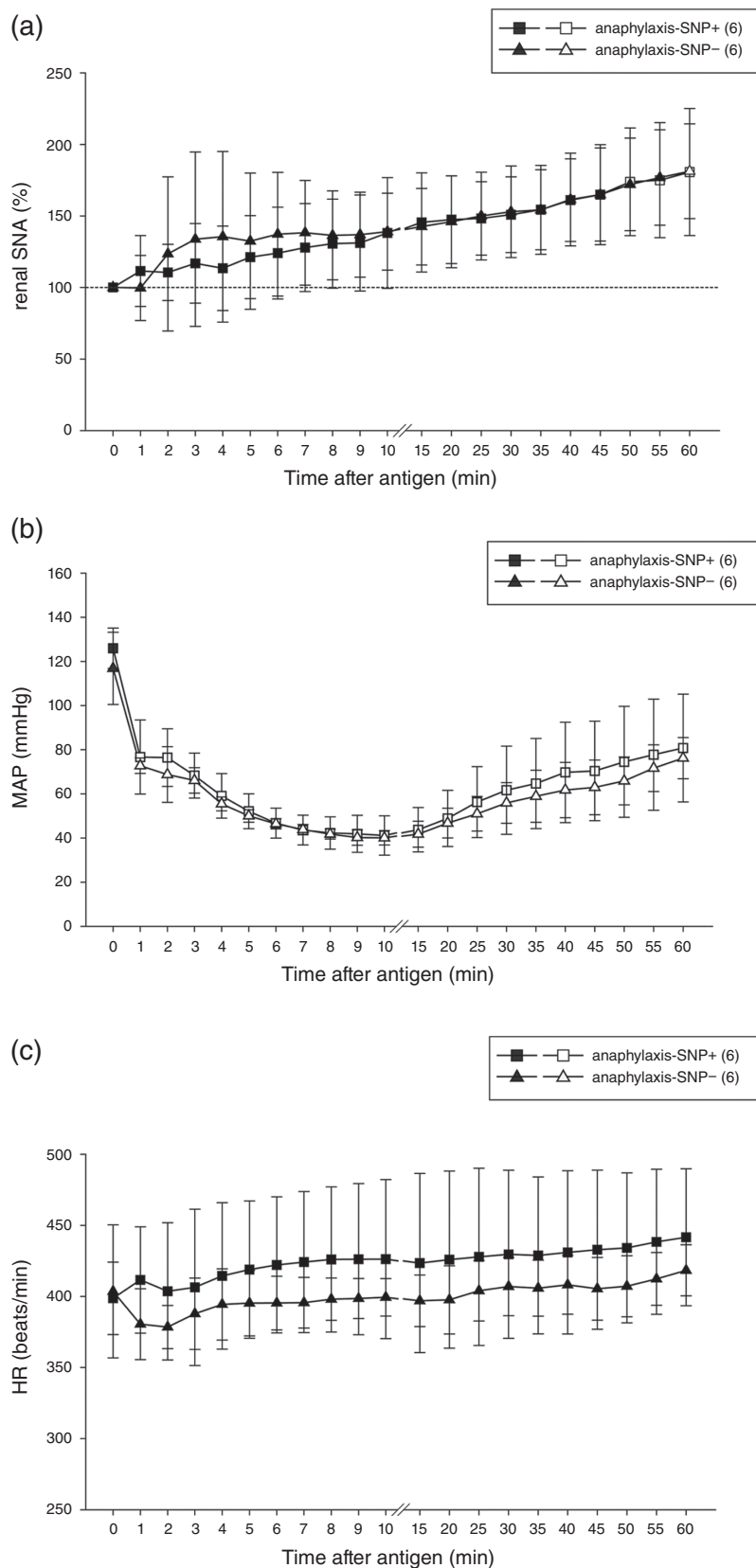


FIGURE 3 Effects of SNP injection on the antigen-induced renal sympathoexcitation and hypotension. Time course data of the changes in renal SNA (a), MAP (b) and HR (c) after an intravenous injection of the antigen in sensitized rats with preinjection of SNP (anaphylaxis-SNP+, ■, □) and sensitized rats without preinjection of SNP (anaphylaxis-SNP-, ▲, △). Numbers of animals used are shown in parentheses. The values are expressed as means $\pm$ SD. Open symbols, $P < 0.05$ vs. baseline

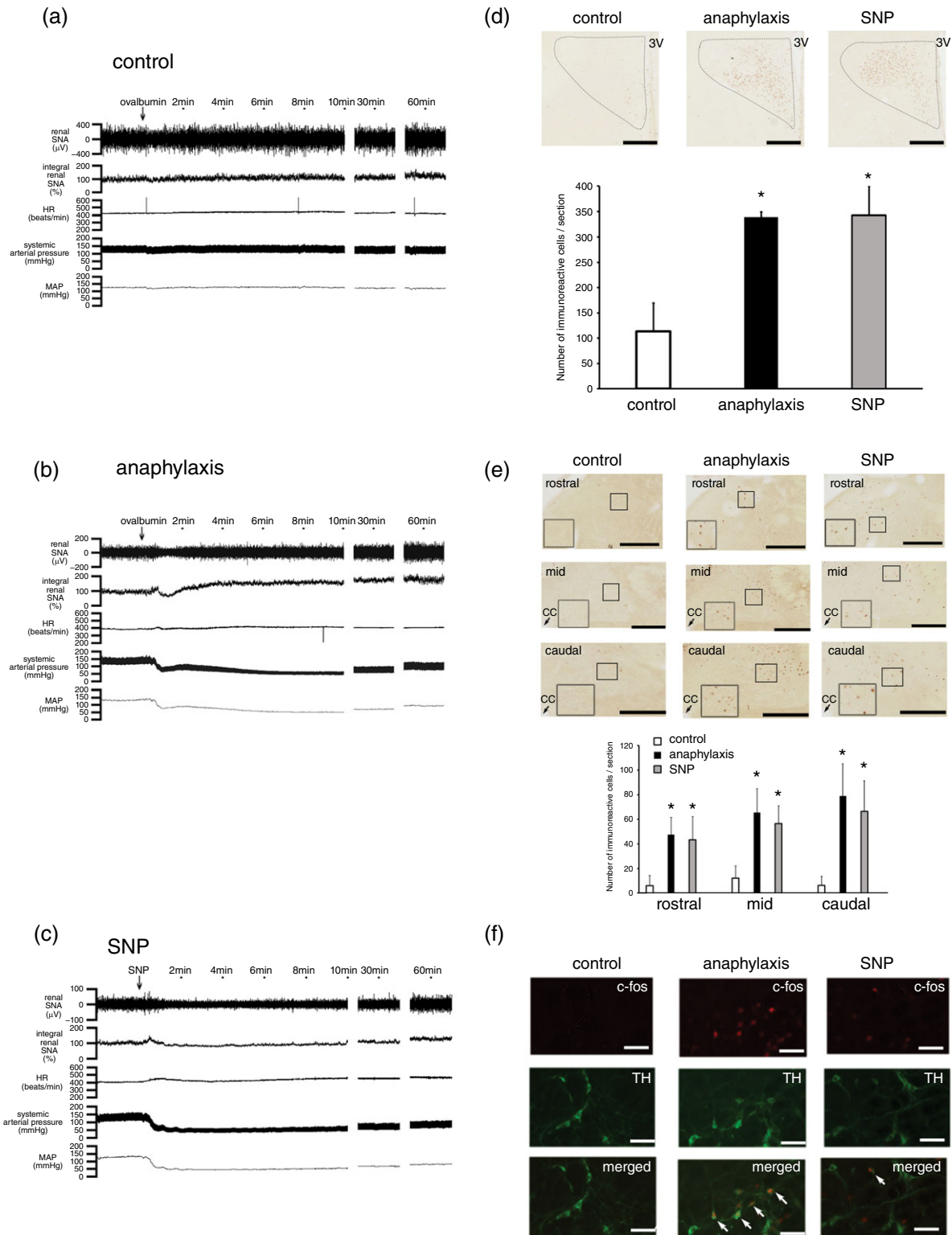


FIGURE 4 Effects of SNP-induced hypotension on renal SNA and c-fos-immunoreactive cells in the NTS and the RVLM. (a–c) Representative data of renal SNA with systemic arterial pressure, MAP and HR before and after an intravenous injection of ovalbumin in a non-sensitized rat (a, control) and in a sensitized rat (b, anaphylaxis), and those in response to subcutaneous injection of SNP (3 mg kg⁻¹) in a non-sensitized rat (c, SNP). (d, e) Representative photomicrographs of coronal sections and bar graph show the c-fos-immunoreactive cells in the PVN (d) and the rostral, mid, and caudal areas of the NTS (e) in a non-sensitized rat (control, $n = 3$), in a sensitized rat (anaphylaxis, $n = 3$) and in non-sensitized rat with SNP injection (SNP, $n = 3$). The values are expressed as means $\pm$ SD. * $P < 0.05$ vs. the control group. 3V, the third ventricle; CC, central canal. Scale bars: 250 μ m. (e) Higher magnification of smaller boxed areas in the NTS is shown at the larger areas at the lower left in each sections. (f) Double staining images from a non-sensitized rat (control), a sensitized rat (anaphylaxis) and a non-sensitized rat with SNP injection (SNP) showing c-fos-positive cells (red) and TH-immunoreactive cells (green) in the RVLM separately. The cells with colocalization are indicated by the white arrows in the merged photo. Scale bars: 50 μ m

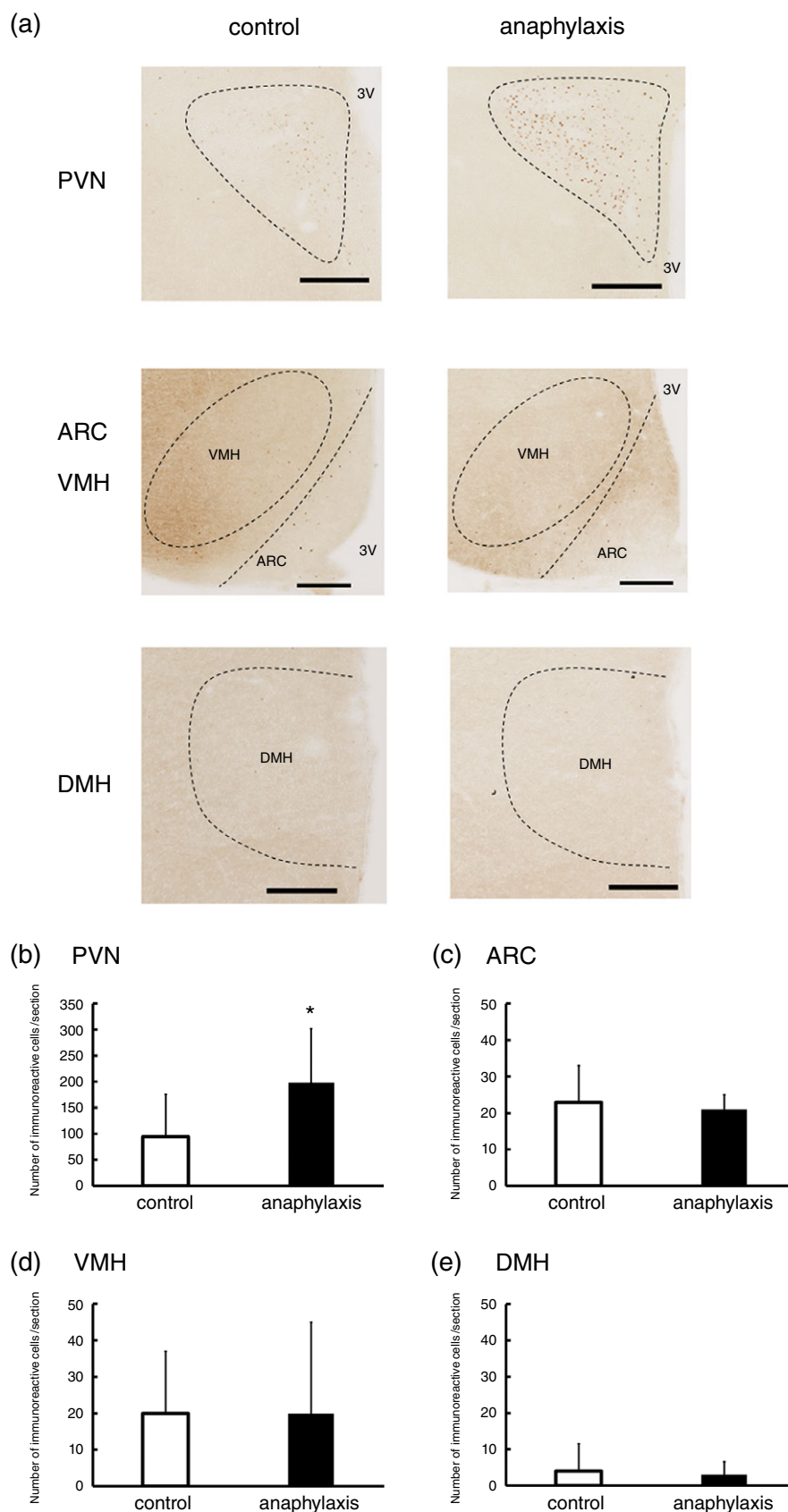


FIGURE 5 Effects of anaphylaxis on c-fos-immunoreactivity in the hypothalamic nuclei. (a) Representative photomicrographs of coronal sections showing the c-fos-immunoreactive cells in the PVN, ARC, VMH and DMH in a non-sensitized rat (control) and in a sensitized rat (anaphylaxis). 3V, the third ventricle. Scale bars = 250 μ m. (b–e) Bar graphs showing the number of c-fos-immunoreactive cells counted in the PVN (b), ARC (c), VMH (d) and DMH (e) in non-sensitized rats (control, $n = 6$) and in sensitized rats (anaphylaxis, $n = 6$). The values are expressed as means $\pm$ SD. * $P < 0.05$ vs. the control group

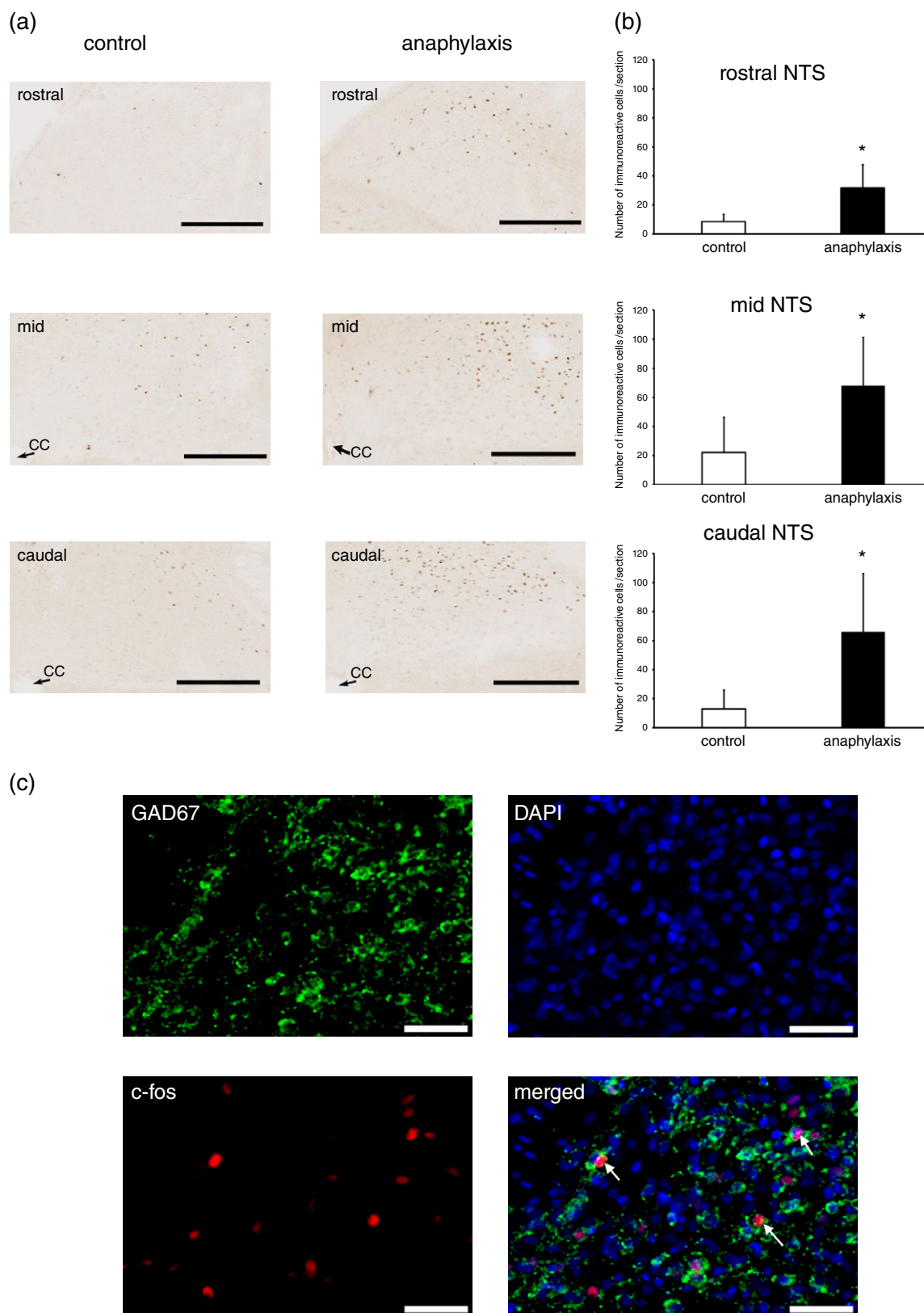


FIGURE 6 Effects of anaphylaxis on c-fos-immunoreactivity in the NTS. (a) Representative photomicrographs of coronal sections showing the c-fos-positive cells in the rostral, mid and caudal areas of the NTS in a non-sensitized rat (control) and in a sensitized rat (anaphylaxis). Scale bars = 250 μ m. CC, central canal. (b) Bar graphs showing the number of c-fos-immunoreactive cells counted in the rostral, mid and caudal areas of the NTS in non-sensitized rats (control, $n = 6$) and in sensitized rats (anaphylaxis, $n = 6$). The values are expressed as means $\pm$ SD. * $P < 0.05$ vs. the control group. (c) Triple-immunofluorescence images obtained from a rat suffering from anaphylaxis show c-fos-positive cells (red), GAD67-immunoreactive cells (green) and DAPI-immunoreactive cells (blue) separately; the cells with colocalization of c-fos and GAD67 are induced by the white arrows. Scale bars = 50 μ m

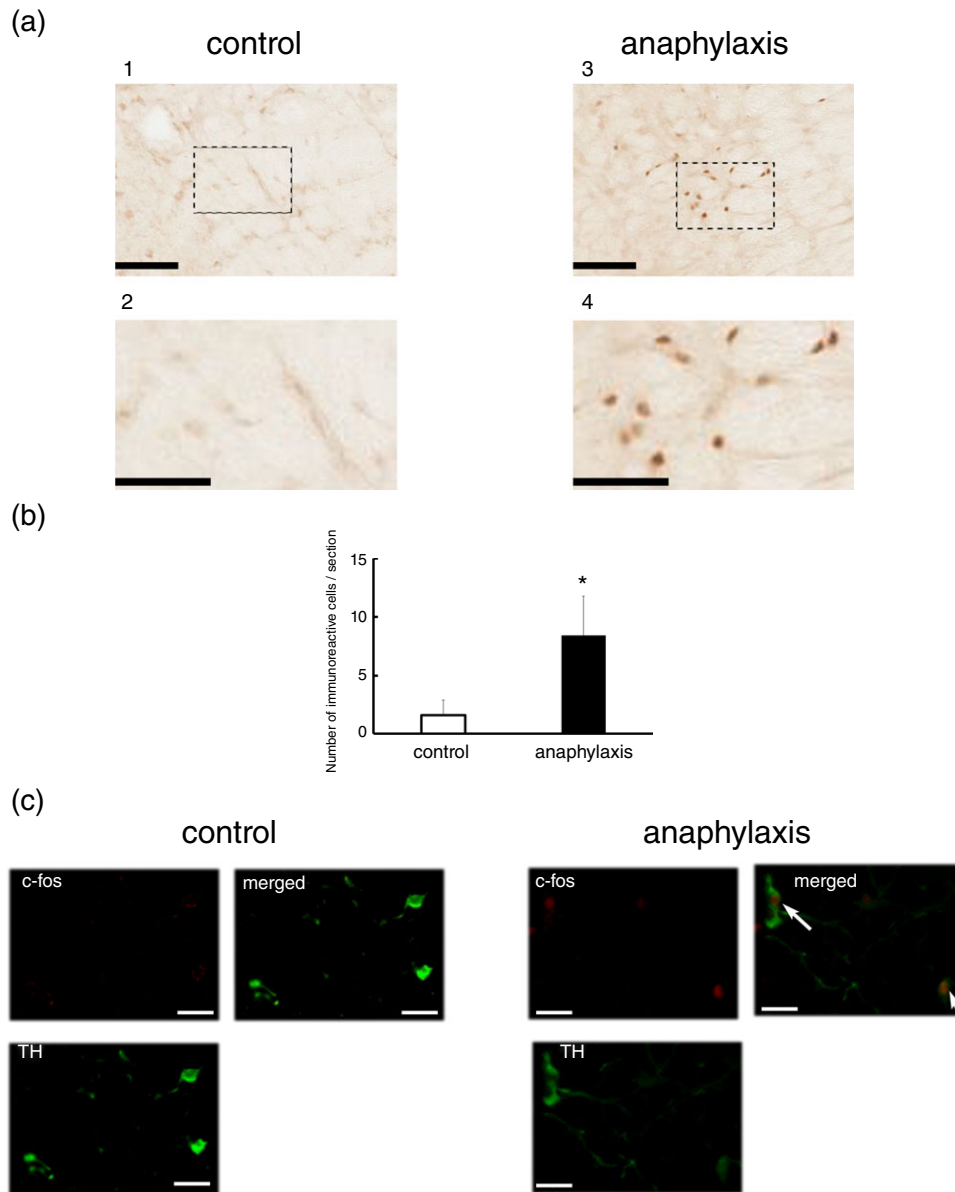


FIGURE 7 Effects of anaphylaxis on c-fos-immunoreactivity in the RVLM. (a) Representative photomicrographs of coronal sections showing the c-fos-positive cells in the RVLM in a non-sensitized rat (control) and in a sensitized rat (anaphylaxis). High magnification photos of the boxed area in the upper photos (1, 3) are shown at the bottom (2, 4). Scale bars (1, 3) = 100 μ m, (2, 4) = 50 μ m. (b) Bar graph showing c-fos-immunoreactive cells counted in the RVLM in non-sensitized rats (control, $n = 6$) and in sensitized rats (anaphylaxis, $n = 6$). The values are expressed as means $\pm$ SD. * $P < 0.05$ vs. the control group. (c) Double-immunofluorescence images from a control rat and a rat suffering from anaphylaxis showing c-fos-positive cells (red) and TH-immunoreactive cells (green) separately. The cells with colocalization are indicated by the white arrows in the merged photo. Scale bars = 50 μ m

Tanida et al., 2015). In fact, increased c-fos expression in the PVN was accompanied by renal sympathoexcitation, when anaphylaxis was present. In the PVN, c-fos was co-expressed by vasopressin-positive neurons during anaphylaxis (Földes et al., 2000). Vasopressin contributed to the recovery of anaphylactic hypotension in our previous study (Wang et al., 2014). Thus, these lines of evidence suggested that anaphylactic hypotension activated the PVN and caused the cardiovascular compensation humorally, via vasopressin secretion, and neurally, via renal sympathoexcitation. In contrast, c-fos immunoreactivity in the ARC, VMH and DMH, all of which send sympathetic efferent signals to BAT, was not increased during anaphylaxis. This

finding is consistent with the absence of change in the BAT-SNA. Thus, anaphylactic hypotension did not affect metabolic and thermogenesis systems, via the hypothalamus–BAT sympathetic pathway.

The medulla oblongata containing the NTS and RVLM is a critical area that controls cardiovascular function by receiving afferent signals from peripheral organs and sending efferent signals to other sites in the brain and back to peripheral organs. It was previously reported that anaphylaxis increased c-fos expression in the NTS (Castex et al., 1995). We validated this finding in the current study and newly demonstrated that c-fos increased in GAD67-immunoreactive neurons in the NTS following antigen injection. This finding suggested that anaphylactic

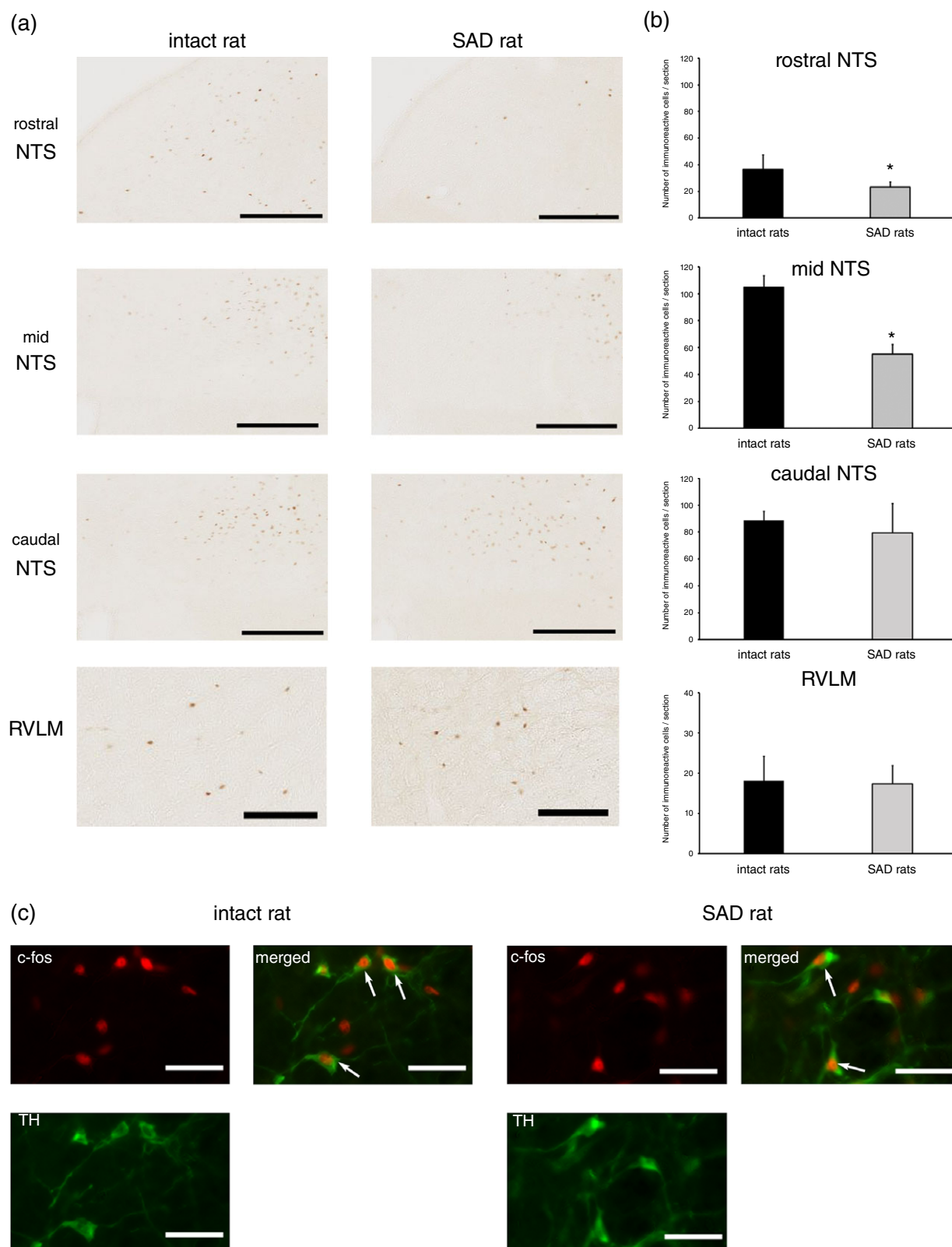


FIGURE 8 Effects of SAD on the antigen-induced increase in c-fos-immunoreactive cells in the NTS and the RVLM. (a) Representative photomicrographs of coronal sections showing the c-fos-positive cells in the rostral, mid and caudal areas of the NTS and the RVLM after anaphylaxis in an intact rat and in a SAD rat. Scale bars: 250 μ m in the NTS and 50 μ m in the RVLM. (b) Bar graphs show the numbers of c-fos-immunoreactive cells counted in the rostral, mid and caudal areas of the NTS in sensitized intact rats (black bar, $n = 6$) and in sensitized SAD rats (grey bar, $n = 6$). The values are expressed as means $\pm$ SD. (c) Double-immunofluorescence images from an intact rat and a SAD rat suffering from anaphylaxis, showing c-fos-positive cells (red) and TH-immunoreactive cells (green) separately. The cells with colocalization are indicated by the white arrows in the merged photos. Scale bars: 50 μ m

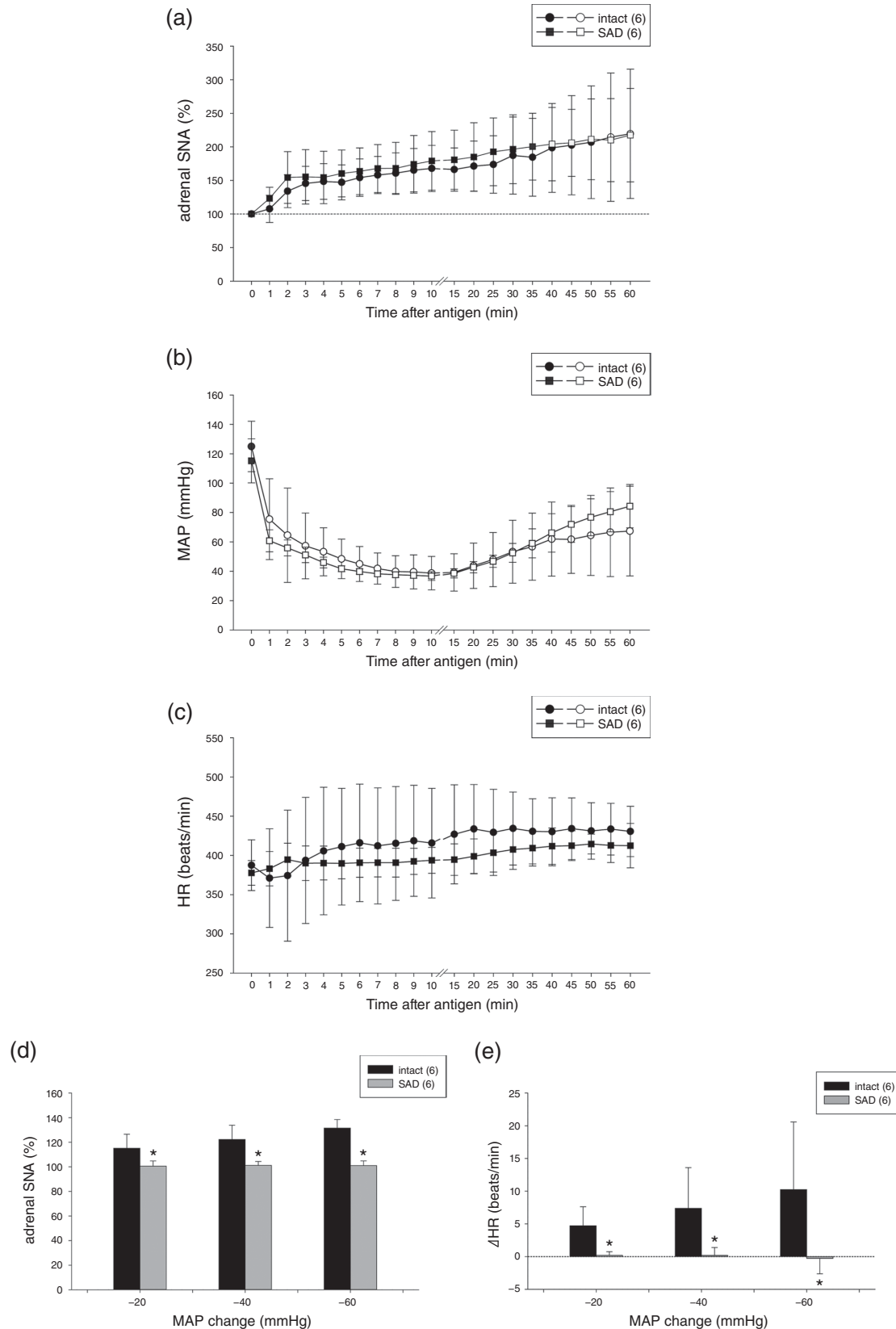


FIGURE 9 Effects of SAD on the antigen-induced adrenal sympathoexcitation. (a–c) Time course data of the changes in adrenal SNA (a), MAP (b) and HR (c) after an intravenous injection of the antigen in sensitized intact rats (●, ○) and sensitized SAD rats (■, □). Numbers of animals used are shown in parentheses. The values are expressed as means $\pm$ SD. Open symbols, $P < 0.05$ vs. baseline. (d, e) Adrenal SNA (d) and HR (e) responses to decreases in blood pressure (–20, –40 and –60 mmHg) induced by the SNP injection in the intact rats (black bar) and SAD rats (grey bar). Numbers of animals used are shown in parentheses. Values are expressed as the mean $\pm$ SEM. * $P < 0.05$ vs. the intact rats

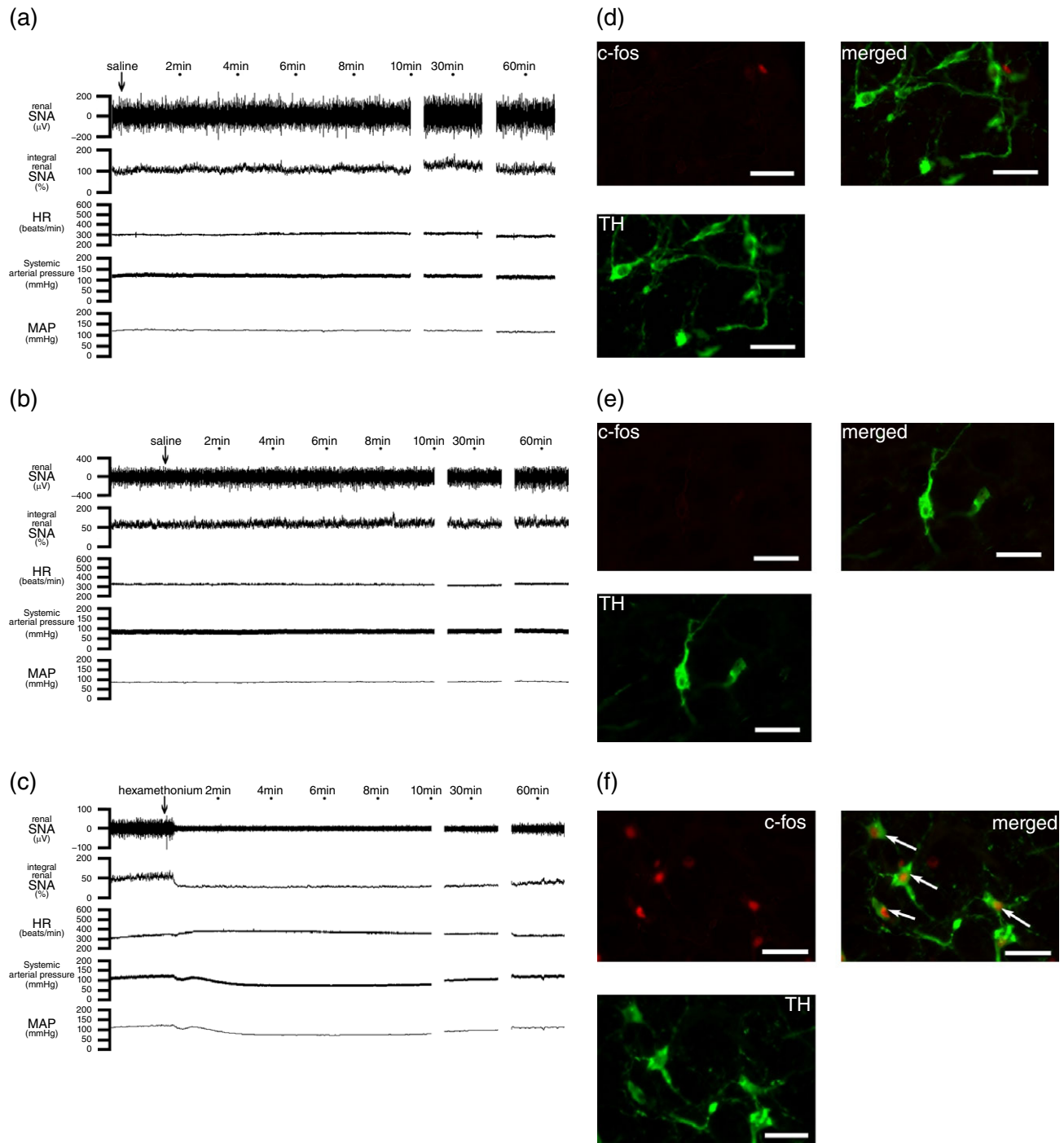


FIGURE 10 Effects of injections of saline or hexamethonium on renal SNA, haemodynamics and RVLM neurons in non-sensitized rats. (a–c) Representative data of renal SNA with systemic arterial pressure, MAP and HR before and after an intravenous injection of saline in a non-sensitized intact rat (a) and a non-sensitized SAD rat (b), and in response to an intravenous injection of hexamethonium in a non-sensitized rat (c). (d–f) Double-immunofluorescence images from a non-sensitized intact rat (d) and a non-sensitized SAD rat (e) with saline injection, and from a non-sensitized rat with hexamethonium injection (f) showing c-fos-positive cells (red) and TH-immunoreactive cells (green) separately. The cells with colocalization are indicated by the white arrows in the merged photo. Scale bars = 50 μm

hypotension activated GABA neurons in the NTS. The c-fos expression in the three regions of the NTS (i.e. rostral, mid and caudal areas) was also analysed. The results showed that SAD reduced c-fos expression in the rostral and mid regions, but not in the caudal NTS (Figure 8). The activation of the caudal NTS neurons, which was independent of the baroreflex during anaphylaxis, may partially

account for why SAD did not affect the anaphylaxis-induced renal and adrenal sympathoexcitation. Afferent information from the caudal NTS, rather than that from the rostral or mid NTS, may be important in relaying afferent information to efferent SNA during anaphylaxis. The caudal NTS plays a crucial role in the cardiovascular reflex (Finley & Katz, 1992). The mechanism for anaphylaxis-induced activation of

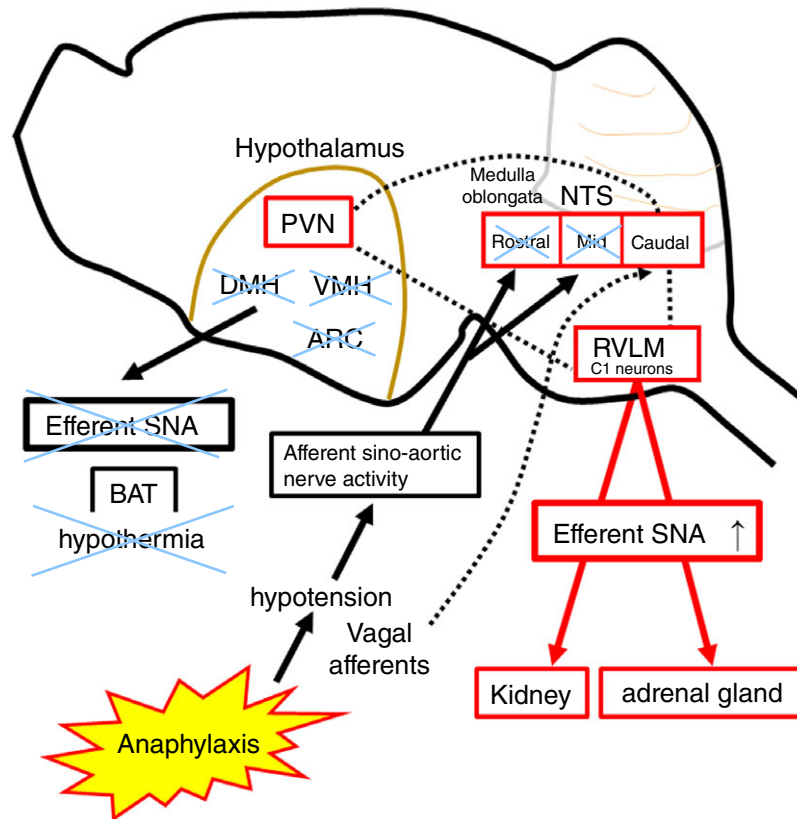


FIGURE 11 Summary diagram of the neural pathway of efferent sympathetic control during anaphylactic hypotension in anaesthetized rats. Anaphylaxis causes hypotension and region-specific sympathoexcitation to the kidney and the adrenal gland, but not to the BAT, and stimulates three nuclei, hypothalamic PVN, and NTS and RVLM in the medulla oblongata. Renal and adrenal sympathoexcitation might be mediated by a medullary neural circuit containing neurons in the caudal NTS and C1 neurons in the RVLM without involvement of the baroreceptor reflex. Dotted lines indicate the connections remaining unclear. X indicates non-commitment of the rostral and mid areas in the NTS via afferent sinoaortic nerve, no change in hypothalamic DMH, ARC, VMH and BAT-SNA, and no induction of hypothermia. In this diagram, the unknown effects of the regional anaphylaxis which might occur in the brain or humoral mediators on the central regions are not shown.

NTS, other than the baroreflex, is not known, but may be related to inputs from visceral afferents (Bailey, Hermes, Andresen, & Aicher, 2006). Afferent signals of the gastric vagal nerve also increased during anaphylactic hypotension in anaesthetized rats (unpublished data).

We also demonstrated that anaphylaxis induced *c-fos* expression in the RVLM, which is connected to the NTS neurons and is a critical area for the central control of sympathetic neurotransmission and cardiovascular function. Furthermore, the immunohistochemical study of the RVLM revealed that *c-fos* was expressed in TH-positive neurons, which are known as catecholamine neurons or C1 neurons. These findings are consistent with previous studies reporting that C1 neurons in the RVLM and GABA neurons in the NTS were influenced by hypotension (King et al., 2012; Nedoboy et al., 2016) or hypertension (Weston, Wang, Stornetta, Sevigny, & Guyenet, 2003). Thus, anaphylactic hypotension may affect the medullary neural pathway containing GABA neurons in the NTS and C1 neurons in the RVLM to enhance renal and adrenal SNA. Notably, *c-fos* immunoreactivity was increased in the RVLM and caudal NTS by anaphylactic hypotension in SAD rats, with renal (Sun et al., 2014) and adrenal sympathoexcitation. Thus, these lines of evidence suggested that anaphylaxis in the anaesthetized rats activated the medullary areas, including the caudal NTS and RVLM, independently of baroreceptors, which resulted in

sympathoexcitation. The mechanism for this anaphylaxis-associated baroreflex-independent sympathoexcitation is currently unknown. One possible mechanism is the chemoreflex in the medulla oblongata, where anaphylactic hypotension may cause decreases in P_{aO_2} and pH (Davidson et al., 2012), activating C1 neurons in the RVLM in anaesthetized rats (King et al., 2012; Sun & Reis, 1994). Indeed, RVLM neurons are connected to adrenal gland through the sympathetic nerves (Kerman, Enquist, Watson, & Yates, 2003). Another possible mechanism might be associated with direct actions of anaphylactic mediators released in the brain or in other tissues on the brain regions responsible for autonomic integration. However, further studies are required to confirm this hypothesis.

In general, systemic hypotension causes sympathetic changes, via the central neural circuit. Further, *c-fos* expression was induced in some nuclei of the brain following systemic hypotension evoked by haemorrhage and endotoxin (Chan & Sawchenko, 1994; Göktaay & Millington, 2016; Kung, Glasgow, Ruszaj, Gray, & Scrogin, 2010; Lin, Wan, Kang, Wu, & Tseng, 1999; Zhang, Lu, Elmquist, & Saper, 2000). Similar to the present findings regarding anaphylactic hypotension, haemorrhagic and endotoxin (lipopolysaccharide; LPS) shock also increased *c-fos* expression in the hypothalamic PVN, NTS and RVLM (Chan & Sawchenko, 1994; Göktaay & Millington, 2016; Lin

et al., 1999; Zhang et al., 2000). However, in haemorrhagic hypotension, serotonin neurons of the caudal raphe nuclei play a critical role (Kung et al., 2010), while in LPS-induced shock, neurons which release nitric oxide and glutamate in the NTS (Lin et al., 1999) or corticotropin-releasing hormone neurons in the PVN (Singru, Sánchez, Acharya, Fekete, & Lechan, 2008) are activated. These findings indicated that central areas and types of activated neurons may differ depending on the cause of hypotension, whether haemorrhage, LPS or anaphylaxis. Notably, C1 neurons in the RVLM, which were activated by anaphylaxis in the present study, were also stimulated during decreases in blood pressure (King et al., 2012; Nedoboy et al., 2016), and during haemorrhagic and LPS-induced hypotension (Chan & Sawchenko, 1994; Göktalay & Millington, 2016). Recently, C1 neurons have also been reported to contribute to efferent sympathetic regulation (Wenker et al., 2017). Thus, these lines of data suggested that in the brain circuit when hypotension is induced by anaphylaxis, haemorrhage, or LPS injection, C1 neurons in the RVLM are finally activated, leading to the efferent sympathetic activation to reset the decrease in blood pressure.

In conclusion, the present study demonstrated that sympathoexcitatory responses to anaphylactic hypotension regionally occurred in the kidney and adrenal gland, but not in the thermogenesis-related BAT. Further, there was also an increase in c-fos immunoreactivity in the hypothalamic PVN and medullary NTS and RVLM, but not in the DMH, VMH or ARC of the hypothalamus. In addition, renal and adrenal sympathoexcitation during anaphylactic hypotension might be mediated by caudal NTS and catecholamine neurons in the RVLM. Figure 11 shows a summary of the efferent sympathetic control, via the central nervous system during anaphylaxis, presented in the current study. Overall, anaphylactic hypotension in anaesthetized rats activates PVN neurons in the hypothalamus. Moreover, neurons in the caudal NTS and catecholaminergic neurons in the RVLM of the brainstem cause renal and adrenal sympathoexcitation in a baroreceptor-independent manner.

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COMPETING INTERESTS

The authors declare no relevant conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

M.T. and T.Z. contributed equally to this work and performed the experiments. M.T. and T.S. conceived and designed the experiments. M.T., Y.K. and T.S. wrote most parts of the paper. L.S., J.S., W.Y. and Y.K. performed part of the experiments, analysed the data and wrote the Methods section of the paper. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy

or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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