J Physiol Sci (2017) 67:629–635
DOI 10.1007/s12576-017-0549-5

TECHNICAL NOTE

An in vitro experimental model for analysis of central control of sympathetic nerve activity

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Received: 15 February 2017 / Accepted: 5 June 2017 / Published online: 10 June 2017

Abstract Newborn rat brainstem-spinal cord preparations are useful for in vitro analysis of various brainstem functions including respiratory activity. When studying the central control of sympathetic nerve activity (SNA), it is important to record peripheral outputs of the SNA. We developed an in vitro preparation in which neuronal connections between the cardiovascular center in the medulla and SNA peripheral outputs are preserved. Zero- to 1-day-old rats were deeply anesthetized with isoflurane, and the brainstem and spinal cord were isolated with a partial right thoracic cage to record sympathetic nerve discharge from the right thoracic sympathetic nerve trunk (T9–T11). SNA in this preparation was strongly modulated by inspiratory activity. Single-shot electrical stimulation of the ipsilateral rostral ventrolateral medulla (RVLM) induced a transient increase of SNA. Bath application of angiotensin II induced an increase of SNA, and local ipsilateral microinjection of angiotensin II to the RVLM induced a transient increase of SNA. This preparation allows analysis of the central control of the SNA in vitro.

Keywords Sympathetic nerve activity · Respiratory activity · In vitro · Angiotensin II · Newborn rat

Introduction

The in vitro brainstem-spinal cord preparation isolated from newborn rat is a useful experimental model for studying respiratory neuronal network function [1]. Such en bloc preparations can also be used to analyze many other types of brain function such as the cardiovascular center, locomotor control, suckling, and nociceptive-related responses [2]. Regarding sympathetic nerve activity (SNA), in 1999 Su [3] reported for the first time a brainstem-spinal cord preparation in which peripheral sympathetic nerve discharge could be recorded from the splanchnic nerve. Although functional connections from the cardiovascular center in the medulla to the peripheral sympathetic nerves could be preserved in this preparation, literature on the study of the central control of SNA outputs using this type of preparation is rather limited. In several previous studies of the central control of SNA in en bloc preparation, the activity of the intermediolateral nucleus (IML) neurons in the spinal cord was used as an indication for outputs of neuronal activity involved in the sympathetic nervous system [4–7]. To further facilitate the study of the central control of SNA in vitro, we developed a simple en bloc preparation in which functional connections from the cardiovascular center in the medulla to the peripheral sympathetic nerve discharge were confirmed.

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Materials and methods

The experimental protocols were approved by the Animal Research Committee of Showa University, which operates in accordance with Law No. 105 of the Japanese Government for the care and use of laboratory animals.

Preparations and recordings

Experiments were performed with brainstem-spinal cord preparations from newborn (0- to 1-day-old) Wistar rats. The newborn rats were deeply anesthetized with isoflurane, and the brainstem and spinal cord were isolated by modifying previously reported methods [1, 8]. The following surgical procedures were performed in artificial cerebrospinal fluid (ACSF, see below for the composition) at room temperature. The vertebrae and left side of the thoracic cage were removed, and a part of the right side of the thoracic cage attached with the right side of the spinal nerve roots was retained to record the sympathetic nerve discharge from the right thoracic sympathetic nerve trunk (T9–T11) [9, 10] (Fig. 1). The rostral end of the preparations was cut transversely at a level just rostral to the anterior inferior cerebellar artery, corresponding to the level between the roots of the sixth cranial nerve and the lower border of the trapezoid body. The inspiratory activity corresponding to phrenic nerve activity was monitored from the fourth cervical ventral root (C4) at the right side. Glass suction electrodes were used to record the C4 nerve activity and sympathetic nerve discharge. The preparations were continuously superfused with ACSF [1] [composition (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 30 glucose, equilibrated with 95% O_2 and 5% CO_2; pH 7.4] at a rate of 2.5–3 ml/min in a 2-ml chamber and were maintained at a temperature of 25–26 °C. The muscle relaxant pancuronium bromide (1 μg/ml; Sigma-Aldrich, Japan) was added to the superfusate to immobilize the rib. Neuronal activities were recorded by an AC amplifier (MEG-5200, Nihon Kohden, Tokyo, Japan) through a 0.5-Hz low-cut filter and stored on hard-disc memory through a PowerLab system (ADInstruments, Castle Hill, Australia) with a 4-kHz sampling rate.

Drugs

Mecamylamine (a broad-spectrum nicotinic receptor antagonist, Sigma-Aldrich) was stocked as a 10-mM solution in purified water and was bath applied at a final concentration of 10 μM, which would be enough for ganglionic blockade [11] after dissolving with ACSF. Angiotensin II (ANG II) (Sigma-Aldrich) was stocked as a 1-mM solution in purified water. For bath application, ANG II (at a final concentration of 5 μM) [5, 12] was dissolved with ACSF. For microinjection, ANG II (at a final concentration of 100 μM) was dissolved with ACSF containing 0.5% fluorescent beads (FluoSpheres Carboxylate-Modified Microspheres, 0.2 μm, yellow-green fluorescent (505/515), 2% solids; Thermo Fisher Scientific Inc., Yokohama, Japan) and filled into a glass micropipette (50–100-μm tip diameter).

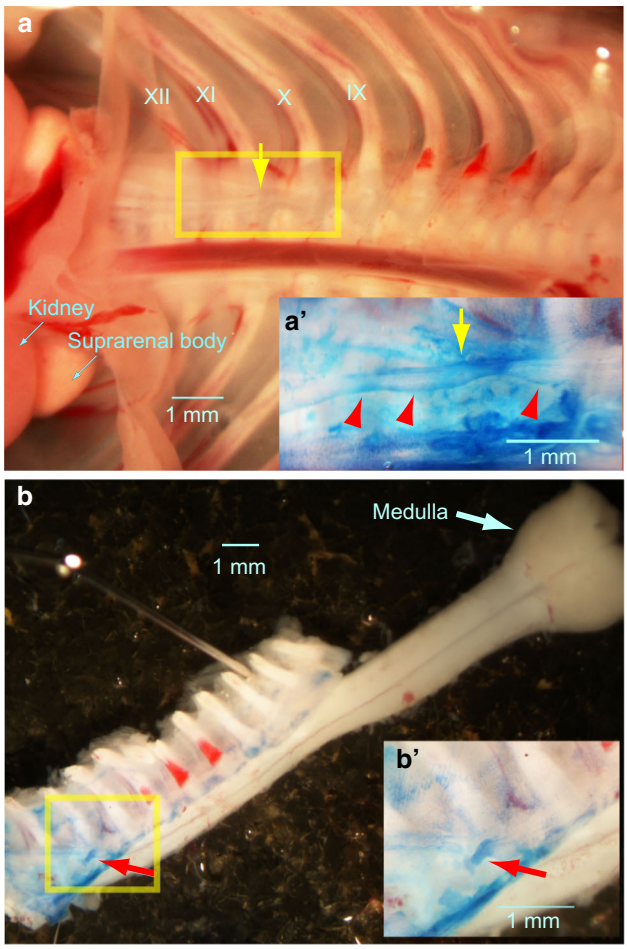

Fig. 1 Photographs of a preparation. **a** Photograph of a thoracic cage before isolation of the brainstem-spinal cord preparation with the sympathetic nerve trunk. IX–XII, rib bones. *a'* Higher magnification view of the highlighted *yellow square* in **a** after 0.5% methylene blue staining. *Red arrowheads* denote the sympathetic nerve trunk and *yellow arrows* the point at which the sympathetic nerve trunk was cut (between the Xth and XIth ribs). **b** Whole preparation used for the experiment. *Upper right* medulla; *lower left* sympathetic nerve trunk recorded at the level of the 10th thoracic spinal cord (*red arrow*). *b'* Higher magnification view of the highlighted *yellow square* in **b**. *Red arrow* denotes the stump of the sympathetic nerve trunk recorded

Microinjection and electrical stimulation

The glass micropipette was inserted into the rostral ventrolateral medulla (RVLM) (at the level of the IXth cranial

nerve roots and 1.0–1.2 mm lateral to the midline [13]) ipsilateral to the site of the SNA recording, and ANG II was ejected by a microinjection pump (Pneumatic PicoPump, PV 820; World Precision Instruments, Sarasota, FL, USA) with one shot of a 20-ms command pulse (approximately 10-nl injection volume). The preparation was fixed with 4% paraformaldehyde for confirmation of the injection site in 100-μm sections of the medulla. To examine the effects of electrical stimulation on the SNA, a stainless steel electrode (tip diameter 30 μm) was inserted into the RVLM ipsilateral to the site of SNA recording, and single-pulse stimulation (0.1 m s, 2–5 V) was performed. This electrical stimulation is the simplest test to confirm whether neuronal connections from the RVLM to the thoracic sympathetic nerve trunk are preserved in the preparation.

Data analysis

All data analyses were performed using the LabChart 7 Pro software program (ADInstruments). If necessary, the C4 activity and SNA were integrated with a 0.1- or 0.2-s time constant in off-line analysis. Cycle-triggered averaging was calculated from 30 respiratory cycles (using integrated C4 activity as the triggering signals). The power spectrum was calculated from approximately 10 min of continuous recordings. The C4 burst rate (bursts/min) was calculated from the mean rate for 3–5 min. To compare the SNAs before and after mecamylamine treatment, the integrated SNA (mean value during 1 min) was subtracted with background recording noise that was determined after addition of 0.1 M KCl to the bath solution to achieve a depolarization blockade of action potential generation [11]. The data are presented as the mean ± SD for all preparations. The significance of the values was analyzed by paired *t* test or one-way ANOVA followed by a Tukey-Kramer multiple comparisons test (GraphPad InStat; GraphPad Software Inc., La Jolla, CA, USA). *P* values of <0.05 were considered statistically significant.

Results

Figure 2 shows a typical example of C4 inspiratory activity and SNA in a brainstem-spinal cord preparation. The SNA indicated respiratory modulation as is clearly shown in the cycle-triggered averages (Fig. 2b). The peak of the SNA during the inspiratory phase was delayed 172 ± 86 ms (mean from 9 preparations) from the peak of the C4 burst. The single-shot electrical stimulation of the ipsilateral RVLM induced a transient increase of SNA and a C4 reflex response (Fig. 1c). The latencies to the induction of the reflex response from the stimulation were 89.4 ± 5.1 ms in SNA (mean from 5 preparations).

We performed a power spectral analysis of the SNA, which showed widely distributed frequency components (Fig. 3). The frequency component related to respiratory modulation of the SNA (Fig. 3b) was revealed by the power spectral analysis of the integrated SNA, in which the peak frequency was 0.080 ± 0.039 Hz and the peak frequency of C4 discharge (i.e., respiratory frequency) was 0.073 ± 0.027 Hz ($n = 9$). In the range higher than the respiratory rate, the peak frequency of the SNA showed a broad bell-shaped distribution with maximum values of 1–3 Hz (1.2 ± 0.62 Hz, $n = 9$). The peak frequency of the SNA in the range lower than the respiratory rate was detected in four preparations and was 0.022 ± 0.0063 Hz.

To evaluate the contribution of postganglionic neurons to the SNA recorded in the present study, we examined effects of a ganglion blocking agent, mecamylamine. After 15-min application of 10 μM mecamylamine, the integrated SNA decreased to 68.9 ± 18.7% of control ($n = 7$, $P < 0.05$), whereas the C4 inspiratory burst rate did not change significantly (5.4 ± 0.9 bursts/min in control, 5.6 ± 1.0 bursts/min in mecamylamine).

In the next step, we tested whether the SNA in this preparation could respond to bath application of physiologically active substances. We chose ANG II, which is known to modulate SNA [14–16]. Bath application of ANG II induced an increase of SNA followed by a decrease of the activity during a 10-min application (Fig. 4a). To compare the SNA in the control and during ANG II bath application, we calculated the integrated SNA. The maximum amplitude of the SNA during ANG II application was 124 ± 11.4% of control ($P < 0.05$, $n = 6$). The respiratory frequency (C4 burst rate) did not change significantly (4.7 ± 1.7 bursts/min in control, 5.5 ± 1.9 bursts/min with ANG II), whereas tonic activity was transiently induced in the C4 activity, possibly due to excitation of non-respiratory neurons in the spinal cord and/or medulla. Previous studies demonstrated that targets of ANG II could be the RVLM, IML and the peripheral sympathetic ganglions [5, 14–20]. Therefore, we examined the effects of ANG II injection to the RVLM. Local ipsilateral injection of ANG II to the RVLM induced a transient increase of SNA (138 ± 23.2% of control, $P < 0.01$) with a 252 ± 72-ms delay ($n = 5$) (Fig. 4b). The increased activity continued for approximately 1 s after the injection with no significant effect on C4 inspiratory activity (burst interval = 15.1 ± 6.6 s before injection vs. 15.6 ± 7.1 s after injection, $n = 5$).

Discussion

Properties of SNA in the in vitro preparation

Cycle-triggered averages and power spectral analysis revealed that the SNA in the in vitro preparation was

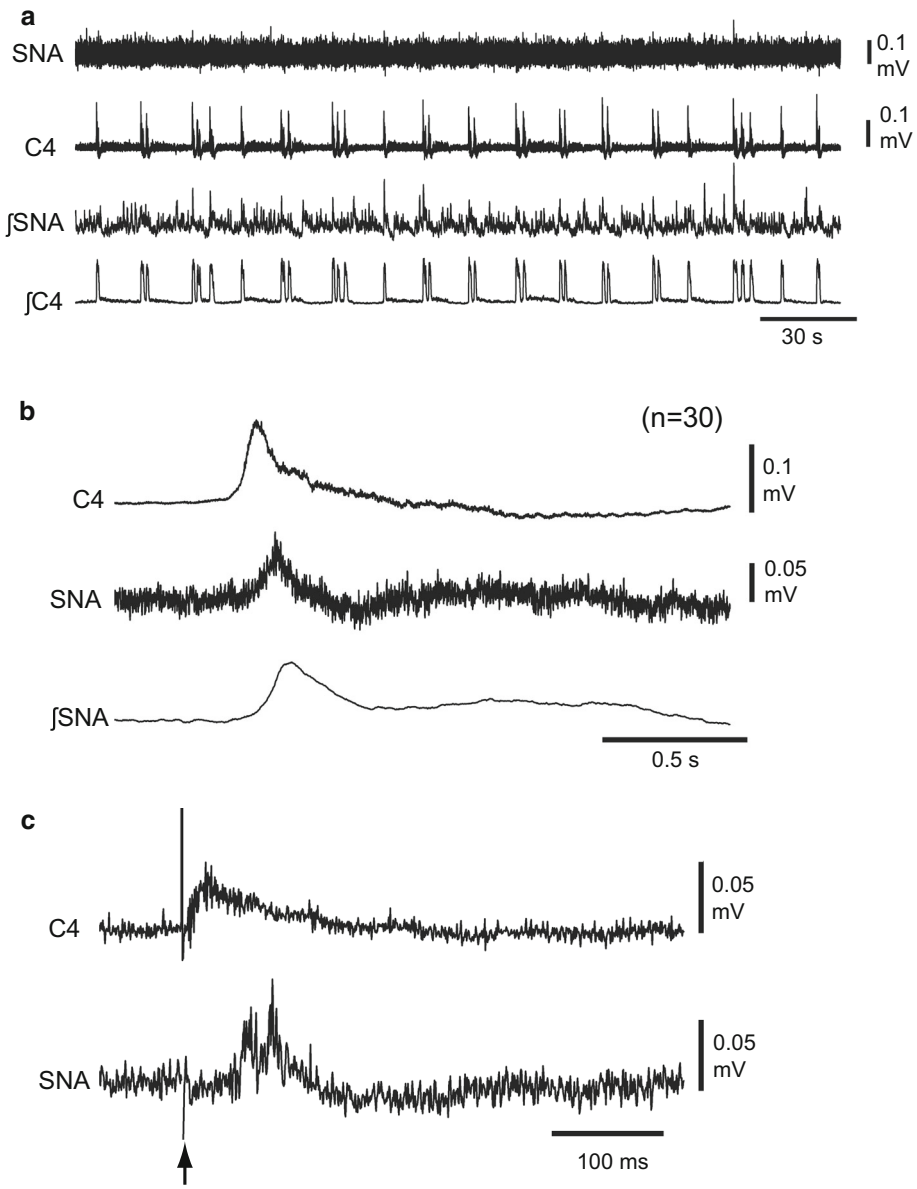

Fig. 2 A typical example of the SNA and C4 recordings. **a** Upper trace to lower trace: raw SNA, raw C4 activity, integrated SNA, and integrated C4 activity. **b** Cycle-triggered average of the SNA set by the rising phase of C4. The cycle number was set at 30. Note the inspiratory phase-related SNA. **c** Reflex potentials in C4 and SNA in response to electrical stimulation of the ipsilateral RVLM

strongly modulated by inspiratory activity as reported in a previous study [3]. This result is consistent with previous studies suggesting the presence of a central interaction between the respiratory system and the sympathetic nervous system [9, 10, 21, 22]. The latency from the RVLM electrical stimulation to induction of the SNA reflex response (approximately 90 m s) was shorter than that from the peak of the C4 inspiratory burst to the peak of the SNA in the cycle-triggered averages (172 m s), possibly because of the presence of multisynaptic connections between respiratory neurons and sympathetic-related neurons in the medulla and spinal cord. The SNA also showed peak frequencies of around 1 Hz in the range higher than the respiratory rate, consistent with that of a previous study [3]. The peak in the very low frequency range (0.02 Hz) was also detected in 44% of the preparations. Similar frequency components have been reported in renal SNA of in vivo adult rats [23]. A ganglion blocker, mecamylamine [11], induced about a 30% decrease in the SNA (but no significant change in the respiratory rate) compared to control, suggesting some contribution of post-ganglionic activity to the SNA in the present recordings.

Effects of ANG II

The application of ANG II into the RVLM is known to increase SNA and blood pressure in vivo [14, 16]. The angiotensin type 1 (AT1) receptor, a major receptor subtype, has been characterized pharmacologically and histologically in the RVLM, and the precise

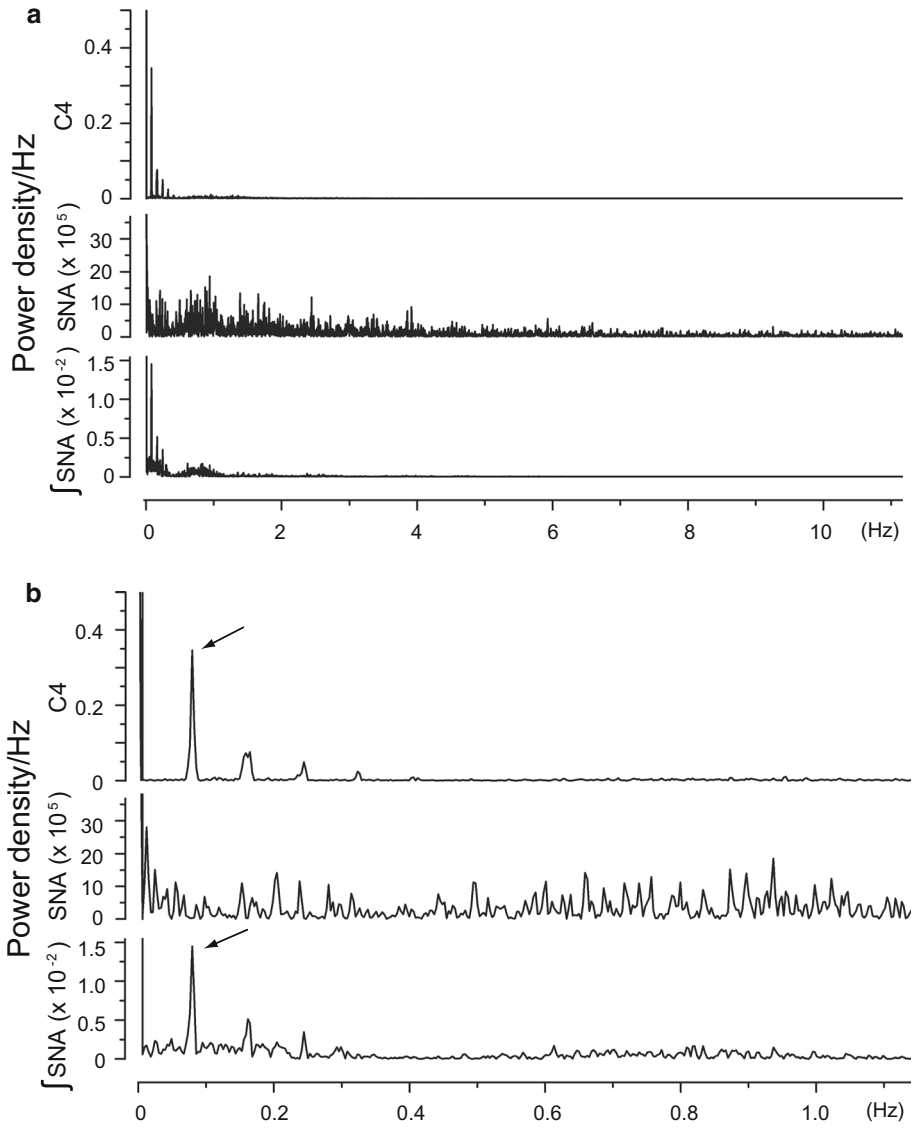

Fig. 3 An example of power density histograms of C4, SNA and integrated SNA. **a** Components from 0 to 10 Hz. **b** Components from 0 to 1 Hz. Bin size, 0.003 Hz. *Arrows* denote respiratory components in C4 and integrated SNA

electrophysiological responses of RVLM sympathetic neurons to ANG II have been elucidated in previous studies [14, 24]. IML neurons have also been confirmed to express AT1 receptors [17], and previous studies reported that IML neurons were excited in response to ANG II [5, 6, 15]. In addition, it was suggested that neurons in the sympathetic ganglions were directly excited by ANG II [19, 20]. Therefore, AT1 receptors in the RVLM, IML, and sympathetic ganglionic neurons could contribute to enhancement of the SNA by the bath application of ANG II. The local injection to the RVLM and the bath application of ANG II excited the SNA of the present in vitro preparation, whereas they were less effective on respiratory activity. The respiratory center (e.g., the Bötzinger complex and caudal parafacial respiratory group) and cardiovascular center (e.g., the C1 adrenergic area) are at least partially overlapped in the RVLM [13, 22]. The results suggested the more specific expression of AT1 receptors in cardiovascular neurons than in respiratory-related neurons in the RVLM [16]. In contrast, SNA response induced by electrical stimulation in the RVLM was a simple test to confirm that functional connections between the medulla and peripheral outputs of the SNA were preserved in the preparation, although it included non-specific excitation of the medullary centers and passing fibers.

Technical limitations

Regarding the physiological relevance of the present study, it is noteworthy that there were several points of difference between the present experimental conditions and those in the in vivo juvenile or adult preparation. Newborn rats of postnatal days 0–1 were used, and the temperature used in the experiments was 25–26 °C. The brainstem-spinal cord

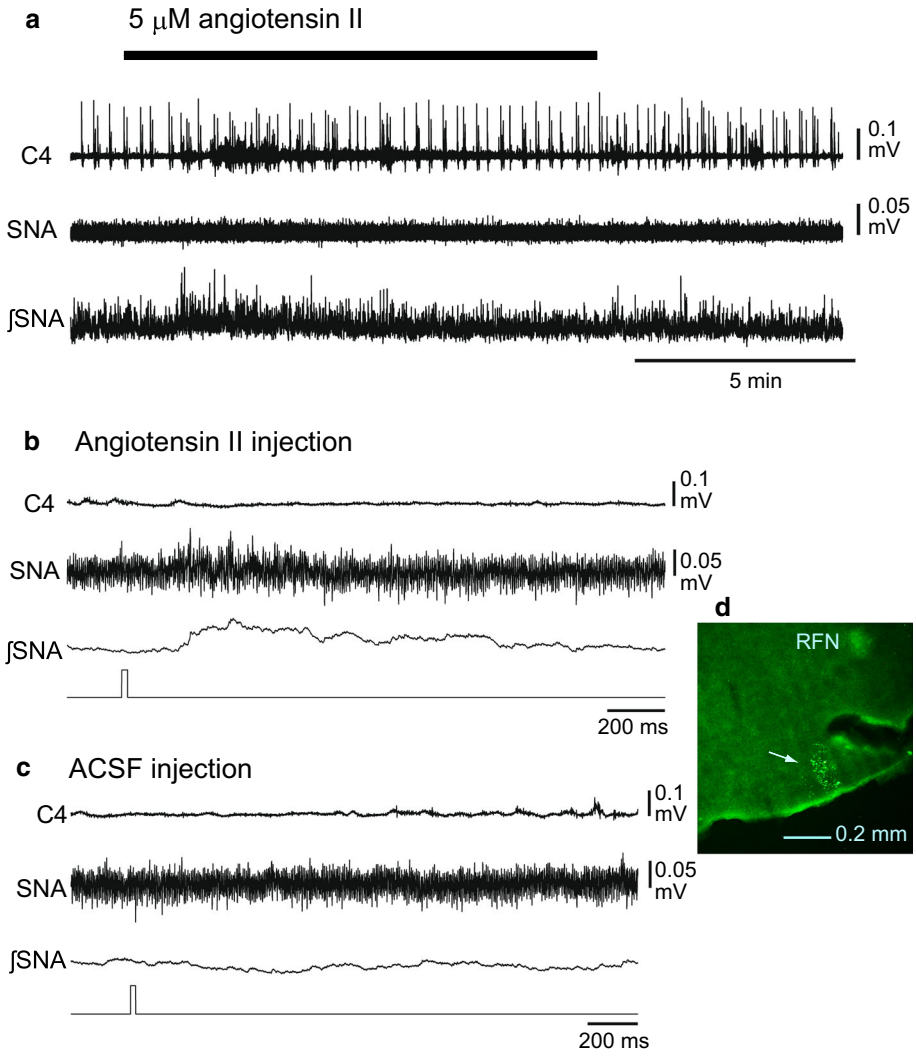

Fig. 4 Effects of ANG II on SNA. **a** Effects of bath application of 5 μM ANG II. Note the transient increase of the integrated SNA after application of ANG II. **b** Effects of microinjection of ANG II into the ipsilateral RVLM. The microinjection induced a transient increase of the SNA. **c** No effect of ACSF injection into the same site as in **b**. **d** Injection site marked with fluorescent beads (*arrow*). *RFN* retrofacial nucleus

preparation from newborn rats younger than 4 days old can be used for in vitro analysis of various brainstem functions including respiratory activity. In the present study, we retained an ipsilateral whole sympathetic nerve trunk together with a part of the thoracic cage. Therefore, oxygen supply to the tissues in the present preparation might be a more critical problem than that in the simple en bloc preparation without peripheral structures such as the thoracic cage. Although preparations from 2- to 3-day-old rats can be used for SNA recordings, it might be better to reduce the size of the thoracic cage and the sympathetic nerve trunk (unpublished observation by Oyama and Onimaru). The diffusion of the perfusate into tissues should be better in the present preparation than in that of the previous study [3] in which both thoracic cages were retained together with the vertebral bones covering the ventral part of the spinal cord. The addition of ascorbic acid to the ACSF might also be helpful to increase the viability of the preparations, as reported in the previous study [3], although we did not use it in the present study. The SNA in the in vitro sympathetic nerve trunk recordings was not related to a specific organ or tissue. However, preparations could be easily extended to record the SNA as outputs to more specific tissues.

Conclusion

We developed a simple in vitro preparation in which the output of both SNA and respiratory activity could be recorded. This preparation allows us to analyze neurophysiological and pharmacological mechanisms of the central control of the SNA in vitro, and it could be extended to various analyses of SNA and other functions. For instance, we previously reported a relation between ventromedial hypothalamus (VMH) oscillation and SNA in an arterially perfused preparation from juvenile rats [10]. We suggest that the VMH-attached en bloc preparation is

also useful for in vitro analysis of the relation among VMH oscillation, SNA and respiratory rhythm.

Author contributions YO, KI, and HO designed and performed the electrophysiological recordings, analyzed the data, and wrote the manuscript. YM, TO and MI helped to draft the manuscript. All authors approved the final version of the manuscript.

Complaince with ethical standards

Funding This work was supported by Grants-in Aid for Scientific Research (KAKENHI: 15K01835, 25461070).

Conflict of interest The authors declare that they have no conflict of interest.

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