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Regular Article

Title: Functional connectivity in the central nucleus of the amygdala, paraventricular hypothalamus, and the nucleus of the tractus solitarius circuits during high-intensity treadmill exercise in rats

Running Title: CeA-PVH-NTS circuits in high-intensity exercise

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Abstract (250 words)

High-intensity exercise (HIE) induces negative emotions alongside fatigue, suffering, and changes in cardiovascular responses, and these regulations could be important for athletic performance. Previous studies have reported that limbic and brain stem regions, including the central nucleus of the amygdala (CeA), the paraventricular nucleus of the hypothalamus (PVH), and the nucleus of the tractus solitarius (NTS), play important roles in emotional response and autonomic cardiovascular regulation. However, how these brain regions interact during HIE remains unclear. In this study, Wistar rats were subjected to 90-min treadmill running sessions at different exercise intensities (sedentary, low-intensity, and high-intensity: 0, 20, and 34 m/min, respectively; n = 9 per group). After exercise, brain tissues were extracted and examined for c-Fos immediate early gene expression in brain regions such as the CeA, PVH, and NTS at each exercise intensity. The c-Fos-positive cells were counted, and then a partial correlation analysis was performed to examine the functional connectivity during exercise. As a result, the numbers of c-Fos-positive cells in the CeA, PVH, and NTS increased in an exercise intensity-dependent manner. Furthermore, partial correlation analyses of c-Fos-positive cells between CeA and NTS (CeA-NTS), PVH and NTS (PVH-NTS), and CeA and PVH (CeA-PVH) exhibited significant correlation coefficients during HIE but not during sedentary and low-intensity exercises. Thus, these results suggest that functional connectivity between CeA-PVH, PVH-NTS, and CeA-NTS may be enhanced during HIE. This enhanced functional connectivity may also be involved in emotional and cardiovascular regulation during exercise.

Keywords: high-intensity exercise, functional connectivity, amygdala, paraventricular nucleus, nucleus of the solitary tract, rat

Title: 高強度運動時のラットにおける扁桃体、視床下部室傍核、延髄孤束核の機能的接続について

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Abstract:

高強度運動は、疲労、苦痛、心血管応答の変化とともに負の情動を引き起こすが、これらの制御は運動パフォーマンスの発揮に重要である。先行研究において、扁桃体中心核（CeA）、視床下部室傍核（PVH）、孤束核（NTS）を含む辺縁系および脳幹領域が、情動反応や自律神経系の調節に重要な役割を果たすことが報告されている。しかしながら、高強度運動時にこれらの脳領域がどのように相互作用して機能しているかは、未だ不明である。本研究では、Wistar ラットに、異なる運動強度（強制運動なし群、低強度運動群、高強度運動群：それぞれ 0、20、34m/分、各群 n＝9）で 90 分間のトレッドミル走行をさせた。その後、脳組織を摘出し、各運動強度における CeA、PVH、NTS の脳領域での c-Fos 陽性細胞数を定量化し、偏相関解析を行い、運動中の機能的接続を検討した。その結果、CeA、PVH、NTS の c-Fos 陽性細胞数は、運動強度依存的に増加した。さらに、CeA と NTS（CeA-NTS）、PVH と NTS（PVH-NTS）、CeA と PVH（CeA-PVH）間の c-Fos 陽性細胞の偏相関解析では、高強度運動群においては有意な相関を示したが、強制運動なし群および低強度運動群においては有意な相関を示さなかった。したがって、これらの結果は、高強度運動時に CeA-PVH-NTS 回路の機能的接続が強くなる可能性を示唆するものである。この機能的接続は、運動中の情動や心血管系の調節にも関与している可能性がある。

Introduction

High-intensity exercises (HIEs) such as those performed by athletes cause fatigue and distress and also induce changes in negative emotions and cardiovascular responses. It has been reported that negative emotions affect cardiovascular regulation through the autonomic nervous system[1]. Proper regulation of cardiovascular responses during exercise is important for maintaining athletic performance, including the provision of adequate energy to active skeletal muscles and washing metabolites that lead to fatigue. Cardiovascular regulation is performed by the autonomic (sympathetic and parasympathetic) nervous system and the endocrine system. The sympathetic nervous system increases heart rate and blood pressure (a phenomenon referred to as the "fight-or-flight response") and prepares the body for exercise. The parasympathetic nervous system, on the other hand, decreases blood pressure and heart rate (a phenomenon known as the "rest and digest" response) and relaxes the body.

The brain region involved in the autonomic regulations that occur during exercise is the nucleus tractus solitarius (NTS) in the medulla oblongata, which integrates peripheral inputs (feedback control) and central command inputs (feedforward control). The NTS has a projection to the rostral ventrolateral medulla with sympathetic premotor neurons via the caudal ventrolateral medulla, as well as to the nucleus ambiguus with parasympathetic preganglionic neurons, contributing to the maintenance and increments in arterial pressure and heart rate that occur during exercise[2]. The paraventricular nucleus of the hypothalamus (PVH) plays an important role in the regulation of the autonomic nervous and endocrine systems[3]. The PVH also has sympathetic premotor neurons and can activate sympathetic nerves via projections to the rostral ventrolateral medulla[4]. It has been reported that neurons in both NTS and PVH are activated in an exercise intensity-dependent manner[5,6].

It is known that HIE often produces negative emotions such as suffering. The

amygdala, one of the most well-known brain regions that process emotions, is involved in the autonomic responses[7]. Our previous studies have demonstrated that electrical and/or chemical stimulation of the central nucleus of the amygdala (CeA) increases blood pressure and causes vascular resistance[8,9]. We have also shown that HIE causes CeA activation[10] and that CeA lesions are associated with prolonged maximal treadmill running time and cardiovascular regulation during HIE[9]. Thus, the CeA may affect cardiovascular regulation and limit athletic performance by disrupting circulatory dynamics (e.g., accumulation of metabolites due to increased muscle vascular resistance) during HIE. The CeA has anatomical connections with the PVH and NTS, suggesting that these three brain regions may cooperate to regulate cardiovascular responses during HIE[9]. However, it remains unclear whether and how the CeA-PVH-NTS regions interact in a coordinated manner during HIE.

To capture functional connectivity and interactions between different brain regions, there are known methods of describing statistical relationships based on biological signal information such as neuron firing activity signals of immediate early gene c-Fos, blood-oxygen-level-dependent signals in functional magnetic resonance imaging (fMRI), and electroencephalogram (EEG)[11-15].

Given that peripheral metabolic demands are gradually increased when stronger negative emotions are elicited during HIE, it is possible that interactions between the CeA, PVH, and NTS regions may become stronger during HIE and that the functional connectivity of the network may change in an exercise intensity-dependent manner. Therefore, in this study, we aim to examine the functional connectivity between CeA, PVH, and NTS at different exercise intensities, including HIE.

Materials and Methods

Animals

Male Wistar rats (n = 27, age: 8–9 weeks and weight: 190.3 ± 2.4 g) were purchased from Japan SLC Inc., Japan. These rats were housed in a temperature-controlled room (21℃−22℃, 50%−60%) under a fixed 12/12-h dark/light cycle (6:00–18:00/18:00–6:00) schedule. Food and water were available in the home cage *ad libitum*. All study procedures were approved by the Ethics Committee for Animal Experiments of Juntendo University (Registered/Approved number: S38/2022-31), and they adhere to the guidelines of the Japan Physiological Society.

Procedure

To determine whether the functional connectivity between CeA, PVH, and NTS regions changes in an exercise intensity-dependent manner, the rats were subjected to forced running exercises of different intensities on a treadmill (Fig. 1A; 55 cm × 10 cm, width measured from the shock grid; MK-680, Muromachi Kikai, Tokyo, Japan). First, as in the previous study[9], the rats were familiarized with the treadmill using a habitual protocol lasting 60 min per day for three days. They were made to start running at an initial speed of 10 m/min, and the speed was increased by 2 m/min every 10 min up to a maximum of 20 m/min (Fig. 1B).

The protocol of treadmill running was determined based on previous studies[9,10]. After habituation, the rats were classified into three groups: sedentary (SED, n = 9), low-intensity exercise (LIE, n = 9), and HIE (HIE, n = 9) (Fig. 1C). On the test day, following an initial 60-min rest, the treadmill exercise intensity was started at a speed of 10 m/min and increased by 2 m/min every 3 min up to a maximum of 20 m/min (LIE group) or 34 m/min (HIE group) for 90 min. Rats in the SED group were simply placed on the treadmill with the belt kept stationary.

<Fig. 1>Thereafter, the animals were returned to their home cages where they waited for over 90 min, after which they were deeply anesthetized using isoflurane and perfused

transcardially with heparinized saline followed by 4% paraformaldehyde. Then, their brains were extracted and post-fixed with 4% paraformaldehyde for at least 48 h and transferred into phosphate-buffered saline (PBS) containing 30% sucrose before sectioning (thickness: 50 μm) with a freezing microtome (REM-710, Yamato Kohki Industrial Co., Saitama, Japan).

Brain sections containing CeA, PVH, and NTS were immunohistochemically labeled with c-Fos. The sections were washed in PBS, placed in 10% serum with 0.3% Triton X-100 for 20 min at room temperature, washed once again, and then incubated overnight at 4℃ with an anti-c-Fos antibody (1:200 dilution; RPCA-c-Fos, Encor Biotechnology Inc., FL, USA) using an immunostaining enhancer (IMMUNO SHOT Fine, IS-F-20, Cosmo Bio, Tokyo, Japan). The following day, the sections were washed in PBS and incubated with biotinylated secondary antibody (1:500 dilution; biotinylated goat anti-rabbit c-Fos IgG, BA-1000, Vector Laboratories, CA, USA) using an immunostaining enhancer for 1 h. Following another round of washing, the sections were incubated with streptavidin-conjugated Alexa 594 (1:500 dilution; streptavidin-conjugated Alexa Fluor 594, S32356, Thermo Fisher Scientific, USA) for 1 h. Finally, the sections were washed with PBS, mounted on VECTASHIELD (H-1000-10, Vector Laboratories, Burlingame, CA, USA), and imaged using fluorescence microscopy with a 10× objective lens (EVOS FL Auto 2 Cell Imaging System, Thermo Fisher Scientific, USA). Using Image J (Version 1.53) software, the number of c-Fos-positive cells per area (mm^2) in 2–4 sections of each brain region was counted and averaged.

Statistical analysis

The number of c-Fos-positive cells was represented as the mean ± standard error of the mean. Group comparisons of the number of c-Fos-positive cells in each brain region of the SED, LIE, and HIE groups were analyzed using the one-way analysis of variance

(ANOVA) with Tukey's HSD post-hoc test. Previous studies have reported that functional connectivity investigated through correlation analysis of activity between brain regions measured using immediate early genes[16,17]and functional magnetic resonance imaging[18,19]. In the current study, to examine the functional connectivity between CeA-PVH-NTS at each exercise intensity, partial correlation analyses were performed on the number of c-Fos-positive cells between CeA and PVH (independent of that of NTS) and CeA and NTS (independent of that of PVH) and PVH and NTS (independent of that of CeA).The threshold for statistical significance was set at $p < 0.05$.

Results

Exercise intensity-dependent increments in the number of c-Fos-positive cells

First, the numbers of c-Fos-positive cells in the CeA, PVH, and NTS regions were compared between SED, LIE, and HIE. The numbers of c-Fos-positive cells in the CeA, PVH, and NTS were significantly increased in an exercise intensity-dependent manner (Figs. 2A and 2B, CeA, $F(2, 24) = 37.6$, SED vs. LIE: $p < 0.05$, LIE vs. HIE: $p < 0.001$, SED vs. HIE: $p < 0.001$; Figs. 2C and 2D, PVH, $F(2, 23) = 18.5$, SED vs. LIE: $p < 0.05$, LIE vs. HIE: $p < 0.01$, SED vs. HIE: $p < 0.001$; Figs. 2E and 2F, NTS, $F(2, 24) = 37.1$, SED vs. LIE: $p < 0.01$, LIE vs. HIE: $p < 0.001$, SED vs. HIE: $p < 0.001$; the one-way ANOVA with Tukey's HSD post-hoc test).

<Fig. 2>

Functional connectivity between CeA-PVH-NTS network in an exercise intensity-dependent manner

Functional connectivity in the CeA-PVH-NTS network was investigated using partial correlation analyses. The number of c-Fos-positive cells in the CeA did not show any significant correlation to that in the PVH, independent of that of NTS, in the SED (Figs.

3A and 3D left panel, CeA-PVH: partial $r = 0.10$, $p = 0.84$) and LIE (Figs. 3A and 3D middle panel, CeA-PVH: partial $r = 0.49$, $p = 0.22$). Similarly, the CeA-NTS and PVH-NTS pathways did not show significant partial correlations in the SED (Figs. 3B and 3D left panel, CeA-NTS: partial $r = 0.02$, $p = 0.96$; Fig. 3 C and D left panel, PVH-NTS: partial $r = 0.20$, $p = 0.68$) and LIE groups (Figs. 3B and 3D middle panel, CeA-NTS: partial $r = 0.37$, $p = 0.36$; Figs. 3C and 3D middle panel, PVH-NTS: partial $r = -0.02$, $p = 0.96$). However, it is noteworthy that in the HIE group, the number of c-Fos-positive cells between CeA-NTS and PVH-NTS showed a significant positive partial correlation (Figs. 3B and 3D right panel, CeA-NTS: partial $r = 0.73$, $p < 0.05$; Figs. 3C and 3D right panel, PVH-NTS: partial $r = 0.75$, $p < 0.05$), while that between CeA-PVH showed a significant negative partial correlation (Figs. 3A and 3D right panel, CeA-PVH: partial $r = -0.75$, $p < 0.05$).

<Fig. 3>

Discussion

In this study, to test whether the CeA, PVH, and NTS regions are functionally connected during HIE, we analyzed the correlation of the expression of the immediate early gene c-Fos, a marker of neuronal activation, between brain regions of rats performing treadmill running exercises. We found significant correlations between the CeA-PVH, PVH-NTS, and CeA-NTS regions during HIE rats but not during SED and LIE in rats. These results suggest that the functional connectivity of CeA-PVH-NTS circuit is enhanced during HIE but not during SED and LIE.

Exercise intensity-dependent activation in CeA, PVH, and NTS regions

The number of c-Fos-positive cells in the CeA, PVH, and NTS increased in an exercise intensity-dependent manner, which is consistent with the findings of previous

studies[5,6]. In general, the PVH is known to contain corticotropin-releasing hormone (CRH) and vasopressin (VP) neurons, which release adrenocorticotropic hormone (ACTH) from the pituitary gland and corticosterone from the adrenal glands (in a pathway called the hypothalamic-pituitary-adrenal (HPA) axis) and is important for metabolic responses to mental and physical stress[20]. Exercise excites CRH and VP neurons in the PVH and increases blood ACTH levels, both of which are involved in energy metabolism[21]. Since the metabolic demands of active skeletal muscles increase with exercise intensity, PVH may play a role in compensating for those demands of peripheral tissues via the endocrine and autonomic nervous systems.

The NTS, which receives and integrates signals both from baroreceptors via the carotid sinus and aortic nerves and from the higher brain regions, is known to play a key role in cardiovascular regulation during exercise[2]. The result of the exercise intensity-dependent increase in c-Fos in the NTS could be due to the increase in central and peripheral inputs to the NTS with HIE.

Previously, it has been reported that CeA activation in response to acute exercise occurs specifically in the HIE (34 m/min) and not in the SED or LIE (20 m/min) group; however, the present study showed significant neuronal activation in the CeA, even in the LIE group. The difference in results could be because the exercise duration was 45 min in the previous study, whereas it was 90 min in the present study[10]. Given that the c-Fos expression of CeA has been reported to be upregulated by skeletal muscle fatigue with metabolic disturbances[22], it is possible that differences in skeletal muscle fatigue with increasing exercise duration may have led to our results being different from those of the previous study. Exercise intensity-dependent CeA activation, which increases sympathetic outflow, blood pressure, and heart rate via output to lower brain regions (central command)[23], may play a role in responding to the increased metabolic demands of peripheral tissues during exercise.

258

259 ***Enhanced CeA-PVH-NTS functional connectivity during high-intensity exercise***

260 CeA-PVH, PVH-NTS, and CeA-NTS showed significant correlations during HIE but
261 not during SED and LIE, suggesting these circuits have specific functional connectivity
262 during HIE. CeA lesions attenuate the response of the HPA axis to PVH stimulation[24].
263 It is considered that the CeA and PVH act to modulate the stress response to high-
264 intensity endurance exercise through the HPA axis. In addition, simultaneous electrical
265 stimulation of the CeA and PVH increases blood pressure and muscle vascular
266 resistance[9], suggesting that enhanced CeA-PVH functional connectivity during HIE
267 may be involved in the regulation of cardiovascular responses.

268 PVH has anatomical projections to the NTS[25]. VP neurons of the PVH, which are
269 involved in autonomic cardiovascular regulation during exercise via the NTS, cause
270 resetting of the baroreceptor reflex set point[26], suggesting that PVH-NTS functional
271 connectivity during HIE may be utilized in maintaining the blood pressure and heart rate
272 high during HIE.

273 CeA has anatomical projections to the NTS[27], and the number of NTS-projecting CeA
274 neurons is increased during HIE compared to during no exercise[9]. Based on these results,
275 our finding that CeA-NTS functional connectivity is enhanced suggests that CeA may
276 regulate the NTS during HIE.

277

278 ***Limitations***

279 In summary, enhanced functional connectivity of the CeA-PVH-NTS circuit may
280 contribute to increasing or maintaining blood pressure and heart rate during high-
281 intensity endurance exercise. However, how functional connectivity contributes during
282 exercise is uncertain. In this study, positive or negative correlation coefficients were
283 obtained for functional connectivity; however, it was difficult to determine whether

these connections had excitatory or inhibitory effects on the targeted brain regions because of neuronal type diversity (including inhibitory interneurons) except for excitatory projection neurons[28,29]. To address this issue, it is necessary to test how functional connections affect cardiovascular regulation by selectivity manipulating neuronal pathways using optogenetics or chemogenetics while recording the hemodynamic parameters of animals performing exercises.

While electrical foot shocks were used to motivate treadmill running in this study, electrical stimulation is stressful for animals, and the possibility that it affects c-Fos expression could not be ruled out[30,31].

Finally, although this present study defined high-intensity endurance exercise using the 34 m/min for 90 min value, it remains unclear how functional connectivity is altered at even higher exercise intensities; in other words, at an exercise intensity at the limit of performance. It is speculated that when the exercise intensity is further increased (such as in a progressive-exercise test), sympathetic hyperactivity causes vasoconstriction in active skeletal muscles, resulting in impaired blood flow to peripheral tissues and, consequently, the inability to continue exercises.

Conclusion

Our findings demonstrate that CeA, PVH, and NTS exhibit exercise intensity-dependent activation and that the CeA-PVH-NTS circuit enhances functional connectivity during high-intensity endurance exercise.

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Conflict of Interests

The authors declare no competing financial interests.

Author Contributions

SE, KY, and HW designed the study. SE and HI performed the experiments and analyzed the data. SE drafted the manuscript. KY and HW edited and revised the manuscript. SE, KY, HI, and HW interpreted the results and discussed the results. SE, KY, HI, and HW approved the final version of the manuscript for submission.

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Figure Legends

Figure 1. Treadmill exercise test.

(A) A photograph of rats performing the treadmill exercise task. To encourage the rats to run, electrical foot shocks were applied using the electrical stimulation unit located behind the running belt.

(B) Experimental schedule. A test session was performed on the 4^{th} day after three days of habituation for acclimatization to the treadmill. Rats were perfused 90 min after the end of the exercise during the test session.

(C) Changes in running speed during the test session. The running speed started at 10 m/min and increased by 2 m/min every 3 min until it reached 34 m/min for the high-intensity exercise (HIE) group and 20 m/min for the low-intensity exercise (LIE) group, for 90 min. Rats in the sedentary (SED) group, which was the control group, were simply put on the stationary treadmill (0 m/min) for 90 min.

Figure 2. c-Fos is expressed in an exercise intensity-dependent manner in the CeA, PVH, and NTS.

(A–F) Distribution and number (per mm^2) of c-Fos-positive cells in the sedentary (SED; left panel), low-intensity exercise (LIE; middle panel), and high-intensity (HIE; right panel) groups at anterior-posterior levels of −2.6 mm (A), −1.8 mm (C), −13.8 mm (E), respectively, from the bregma. Scale bar = 500 μm. Box plots (n = 9 per group) of the number (per mm^2) of c-Fos cells in the CeA (B), PVH (D), and NTS (F). Data are shown as group averages and measurements in individual rats. For these data, the one-way analysis of variance (ANOVA) with Tukey HSD post-hoc test was used to identify statistically significant mean differences, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

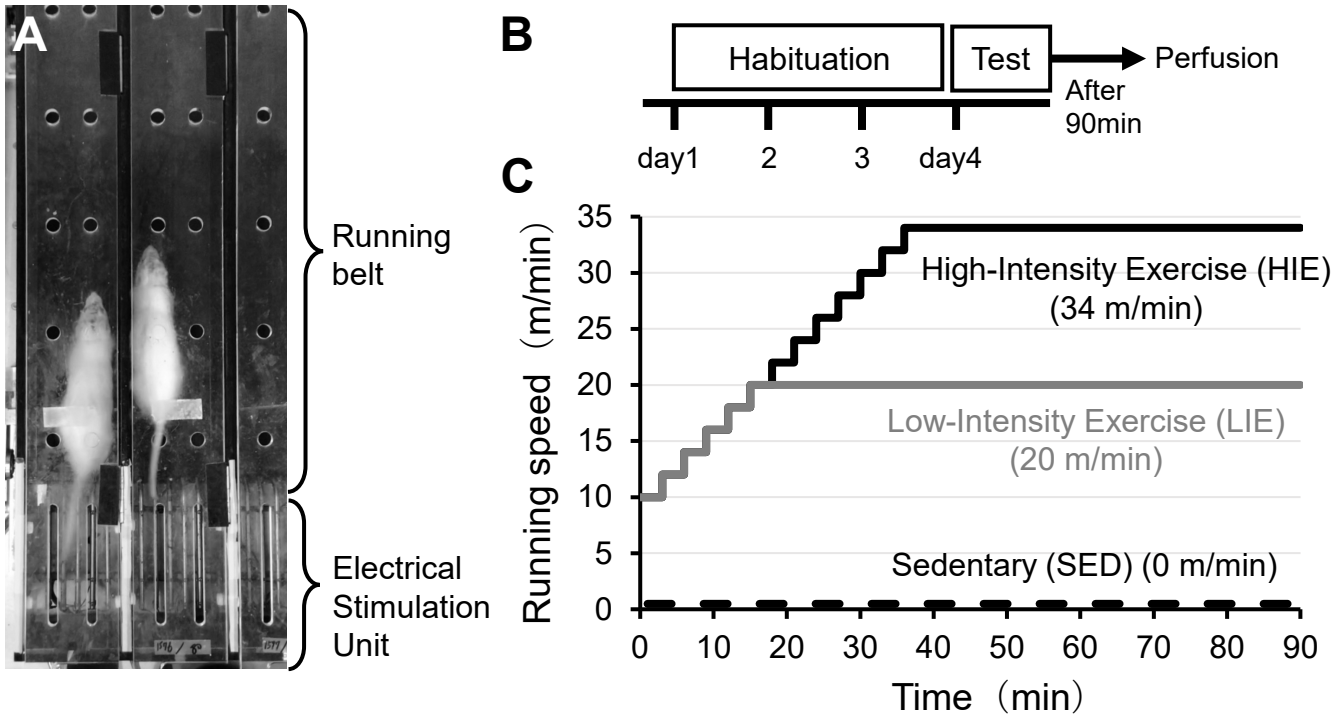
CeA, central nucleus of the amygdala; BLA, basolateral amygdala; PVH,

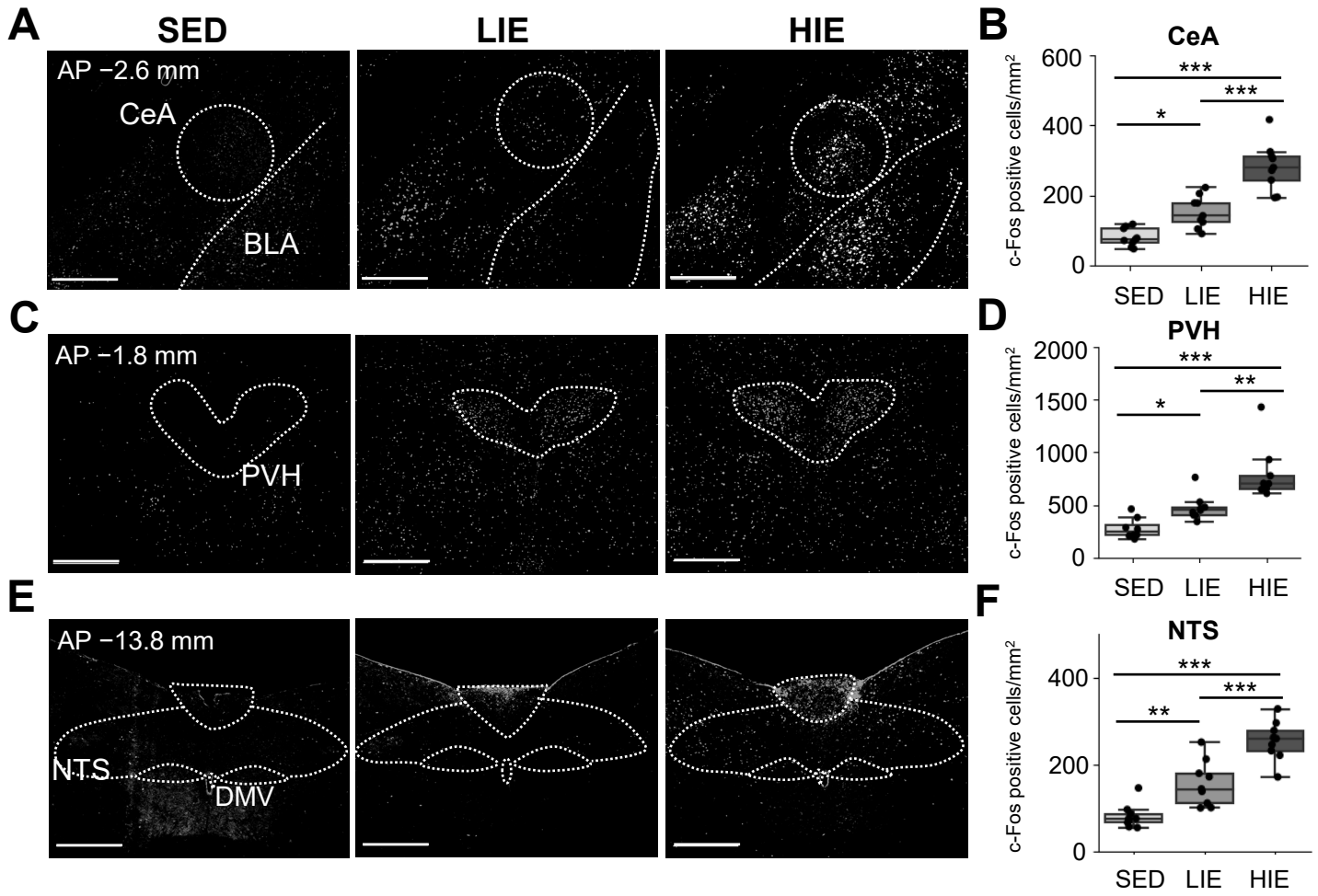
paraventricular nucleus of the hypothalamus; NTS, nucleus tractus solitarius; DMV, dorsal motor vagal nucleus.

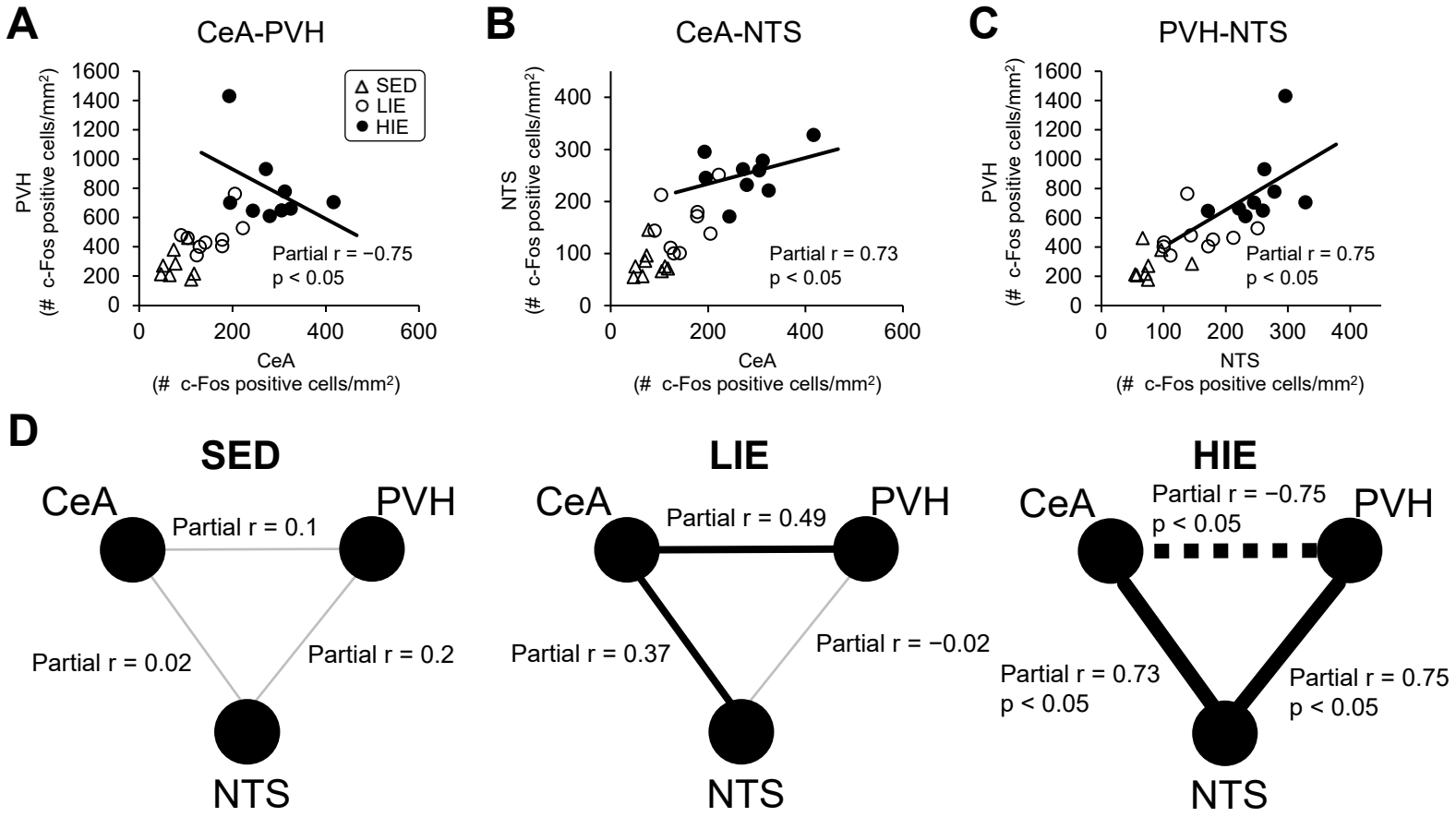
Figure 3. Enhanced CeA-PVH-NTS functional connectivity during high-intensity endurance treadmill exercise.

(A-C) Correlation of the number (per mm^2) of c-Fos-positive cells between CeA-PVH (A), CeA-NTS (B), and PVH-NTS (C). Each symbol represents the number of c-Fos-positive cells in each rat at different exercise intensities: triangle (△), sedentary (SED) group; white circle (○), low-intensity exercise (LIE) group; black circle (●), high-intensity exercise (HIE) group.

(D) Correlation maps of CeA-PVH-NTS networks in the SED (left panel), LIE (middle panel), and HIE (right panel) groups. The thicknesses of lines between brain regions indicate the magnitude of the partial correlation coefficient. Solid and dotted lines represent positive and negative correlation coefficients, respectively.


A
Running belt
Electrical Stimulation Unit
B
Habituation
Test
Perfusion
After 90min
day1
2
3
day4
C
Running speed（m/min）
35
30
25
20
15
10
5
0
High-Intensity Exercise (HIE) (34 m/min)
Low-Intensity Exercise (LIE) (20 m/min)
Sedentary (SED) (0 m/min)
0
10
20
30
40
50
60
70
80
90
Time（min）


A
SED
LIE
HIE
AP −2.6 mm
CeA
BLA
B
CeA
c-Fos positive cells/mm²
600
400
200
0
***
*
***
SED
LIE
HIE
C
AP −1.8 mm
PVH
D
PVH
c-Fos positive cells/mm²
2000
1500
1000
500
0
***
**
*
SED
LIE
HIE
E
AP −13.8 mm
NTS
DMV
F
NTS
c-Fos positive cells/mm²
400
200
0
***
***
**
SED
LIE
HIE


A
CeA-PVH
SED
LIE
HIE
PVH
(# c-Fos positive cells/mm²)
CeA
(# c-Fos positive cells/mm²)
Partial r = −0.75
p < 0.05
B
CeA-NTS
NTS
(# c-Fos positive cells/mm²)
CeA
(# c-Fos positive cells/mm²)
Partial r = 0.73
p < 0.05
C
PVH-NTS
PVH
(# c-Fos positive cells/mm²)
NTS
(# c-Fos positive cells/mm²)
Partial r = 0.75
p < 0.05
D
SED
CeA
PVH
Partial r = 0.1
Partial r = 0.02
Partial r = 0.2
NTS
LIE
CeA
PVH
Partial r = 0.49
Partial r = 0.37
Partial r = −0.02
NTS
HIE
CeA
Partial r = −0.75
p < 0.05
PVH
Partial r = 0.73
p < 0.05
Partial r = 0.75
p < 0.05
NTS