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Hypothalamic Orexinergic Neurons Projecting to the Mesencephalic Locomotor Region Are Activated by Voluntary Wheel Running Exercise in Rats

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ABSTRACT

Background Cardiovascular changes during exercise are regulated by a motor volitional signal, called central command, which originates in the rostral portions of the brain and simultaneously regulates somatomotor and autonomic nervous systems. Whereas we recently elucidated mesencephalic locomotor region (MLR) neurons projecting to the rostral ventrolateral medulla as a crucial component of the central circuit responsible for transmitting central command signals, upstream circuits that regulate the MLR neurons remain unknown. Orexinergic neurons, which primarily originate from the perifornical area (PeFA) of the hypothalamus and reportedly play roles in eliciting locomotion and elevating sympathetic activity, send axonal projection to the MLR. The knowledge led us to investigate whether central command signals are relayed through orexinergic neurons projecting to the MLR.

Methods We performed anterograde transsynaptic tagging with AAV1 encoding Cre to confirm the presence of MLR neurons postsynaptic to the PeFA in rats. We also conducted retrograde neural tracing with retrograde AAV, combined with immunohistochemical staining, to examine the excitability of MLR-projecting orexinergic neurons in rats that were allowed to freely run on the wheel for 90 min.

Results A significant number of MLR neurons were labeled with Cre, indicating that PeFA neurons make synaptic contacts with MLR neurons. Moreover, immunoreactivities of Fos, a marker of neuronal excitation, were found in many MLR-projecting orexinergic neurons by voluntary wheel running exercise, compared to non-exercising control rats, especially in the intermediate-posterior, rather than anterior, and medial, rather than lateral, portions within the orexinergic neuron-distributing domain.

Conclusion The findings suggest that specifically located orexinergic neurons transmit central command signals onto the MLR for running exercise. Elucidating the role of these MLR-projecting orexinergic neurons in somatomotor control and autonomic cardiovascular control deserves further study to unveil central circuit mechanisms responsible for central command function.

Key words adeno-associated virous vector; exercise; Fos; mesencephalic locomotor region; orexinergic neuron

Exercise including locomotor activities requires cardiovascular adjustments to meet the increased oxygen demand in contracting skeletal muscles. Autonomic cardiovascular changes during exercise are regulated by a feedforward neural drive originating in rostral portions of the brain, that is associated with motor volition.[1, 2] This mechanism is called central command, involving activation of subcortical circuits that regulate somatomotor and autonomic nervous systems.[3, 4] Our recent study identified a brainstem monosynaptic pathway as a crucial component of the brain circuit mechanism responsible for transmitting central command signals.[5] In this previous study, we found that mesencephalic locomotor region (MLR) neurons projecting to the rostral ventrolateral medulla (RVLM), an important region for regulating vasomotor tone, are activated by voluntary locomotor exercise.[6] Furthermore, optogenetic excitation of MLR→RVLM neurons not only evoked activation of both α motor neurons and sympathetic postganglionic neurons but also elicited locomotion and cardiovascular response, whereas inhibition of their activity during voluntary running exercise suppressed locomotion and blood pressure elevation. However, the upstream circuits that regulate the activity of MLR neurons during running exercise remain to be determined.

Orexinergic neurons, primarily located in the dorsal hypothalamus (DH) and particularly abundant in the hypothalamic perifornical area (PeFA), play roles in eliciting locomotion and elevating sympathetic activity.[7–11] Remarkably, the MLR receives axonal

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Abbreviations: AAV, adeno-associated virus; AAVrg; retrograde AAV; ChAT, choline acetyltransferase; CnF, cuneiform nucleus; DH, dorsal hypothalamus, DMH, dorsomedial hypothalamus; DLH, dorsolateral hypothalamus; MLR, mesencephalic locomotor region; PeFA, perifornical area; PPTg, pedunculopontine tegmental nucleus

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projections from hypothalamic orexinergic neurons in rats.[12, 13] Given their functional roles and connectomes, orexinergic neurons projecting to the MLR may hypothetically transmit central command signals, thereby regulating motor and autonomic nervous systems for locomotor exercise. However, the excitability of MLR-projecting orexinergic neurons in response to exercise has been undetermined.

In this study, we conducted immunohistochemical examinations to assess the expression of Fos, a marker of neural activation, in orexinergic neurons projecting to the MLR following spontaneous wheel running exercise in rats.[14] We genetically labeled neural circuits by utilizing two adeno-associated virus (AAV) vectors: AAV1 encoding Cre for anterograde transsynaptic tagging and retrograde AAV encoding Cre for retrograde neural tracing.[15, 16]

MATERIALS AND METHODS

Ethical approval

The experiments of this study were approved by the Animal Care Committee (ref #: 20-Y-39) and the Gene Recombination Experiment Safety Committee (ref #: 31-067) of Tottori University. All procedures conformed to the guidelines of animal care by the Tottori University and the ARRIVE guidelines 2.0. AAV vectors were obtained under material transfer agreements with Addgene.

Animals

Male (n = 4) and female (n = 14) Sprague-Dawley (SD) rats (Japan SLC, Inc) were used in this study. Rats were housed in cages with ad libitum access to water and food in environmentally controlled conditions at 25 °C with a 12-h light/12-h dark cycle. To ensure appropriate anesthesia during surgical procedures described below, the absence of a withdrawal reflex response to nociceptive hind paw stimulation was confirmed. Throughout the postsurgical recovery period, daily checks were conducted on the rats.

Anterograde transsynaptic tagging to label MLR neurons postsynaptic to hypothalamic neurons

Male SD rats (n = 4, 9–12 weeks, 347–486g) were anesthetized via inhalation of 5% isoflurane in oxygen followed by subcutaneous administration of a combined anesthetic (0.15 mg/kg of medetomidine hydrochloride, 2.0 mg/kg of midazolam, 2.5 mg/kg of butorphanol) and were then placed in a stereotaxic frame (SR-6R, Narishige, Tokyo, Japan). With a calibrated pressure microinjection system (Nanoject II, Drummond Scientific Co., Broomall, PA), AAV1-hsyn-Cre-WPRE-hGH (184 nL) (donated by Wilson JM: Addgene Cat # 105553-AAV1) was injected unilaterally into the PeFA (3.0–3.1 mm caudal, 1.6 mm lateral, and 8.8–9.1 mm ventral to the bregma). AAV1 encoding Cre exhibits anterograde transsynaptic spread properties, allowing us to label postsynaptic neurons of PeFA neurons with Cre.[15] More than 29 days after the injection, rats were paraformaldehyde-fixed, and their brains were histologically analyzed as described later.

Fos screening in MLR-projecting orexinergic neurons in voluntarily exercising rats

Female SD rats (> 4 weeks old, n = 14) were used to examine the expression of Fos in the DH neurons following running exercise. After weaning, they were housed in the cage in which a vertically rotating wheel (SWY-30, Melquest, Toyama, Japan) or a flying saucer wheel (36 cm diameter, Exotic Nutrition, Newport News, VA) was equipped. Consequently, they became willing to run on the wheel. At 8–9 weeks old (186–226g), under anesthesia, retrograde AAV (AAVrg)-pkg-Cre (184 nL) (donated by Aebischer P: Addgene Cat # 24593-AAVrg) containing fluorescent microspheres (FluoSpheres™ Carboxylate-Modified Microspheres, F8784, Thermo Fisher Science, Waltham, MA) was unilaterally injected into the MLR (8.0 mm caudal, 1.7 mm lateral, and 6.7–6.8 mm ventral to the bregma). Following this surgery, the rats spent more than 21 days in the cage with a wheel before the experimental day.

For 24–48 h immediately prior to the experiment, the wheel was removed from the cage. A pair of the rats were randomly assigned to either "Exercise" or "Control" groups. During the experimental period for 90 min in the dark phase, the "Exercise" rat was allowed free access to the vertical wheel re-installed in the cages (7 females, 253 ± 5 g), while no wheel was installed for the "Control" rat (7 females, 265 ± 5 g). Immediately after this period, they were paraformaldehyde-fixed, and their brains were removed as described later.

Fixation, immunohistochemistry, and imaging

Rats were deeply anesthetized via inhalation of 5% isoflurane in oxygen and subcutaneous administration of a combined anesthetic. They were then transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in 0.1-M phosphate-buffered saline. Subsequently, the brains were removed and post-fixed in the fixative at 4 °C for 2–12 h. After immersion in a 30% sucrose solution at 4 °C for 24–48 h, they were frozen at –80 °C and subsequently cut into 30 µm-thick coronal sections with a cryostat (CM1900, Leica, Wetzlar, Germany). The anteroposterior levels of

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coronal sections obtained were 2.5, 2.75, 3.0, 3.25, and 3.5 mm, and 8.0 mm caudal to the bregma.

Immunofluorescence staining procedures followed our previous studies.[5, 17] The primary antibodies were mouse anti-Cre Recombinase antibody (1:1000, MAB3120, Merck Millipore, Burlington, MA), goat anti-choline acetyltransferase (ChAT) antibody (1:100, AB144P, Merck Millipore), rabbit anti-c-Fos antibody (1:500, ab190289, Abcam, Cambridge, UK), and goat anti-orexin antibody (1:125, sc-8070, Santa Cruz Biotechnology, Dallas, TX). The secondary antibodies were Alexa Fluor 405-conjugated donkey anti-goat IgG (1:125, ab175665, Abcam), Alexa Fluor 488-conjugated donkey anti-goat IgG (1:500, ab150129, Abcam), Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:500, ab150065, Abcam), and Alexa Fluor 555-conjugated donkey anti-mouse IgG (1:500, ab150110, Abcam). The sections were mounted on slides and coverslipped with liquid mountant (P36930, Thermo Fisher Scientific. The slides were scanned under a digital microscope (BZ-X700, Keyence, Osaka, Japan).

Mapping and quantitative analyses of immunoreactive neurons

To investigate the distribution of immunoreactive neurons, we mapped them onto digital images of the sections and transcribed onto illustrations of the corresponding sections provided in a standard brain atlas by Paxinos and Watson.[18] According to the atlas, the cuneiform nucleus (CnF), the dorsal part of the MLR at 8.0 mm caudal to the bregma (bregma –8.0 mm), was defined as the area delineated by the ventral edge of the central nucleus inferior colliculus, the medial edge of the lateral lemniscus, the lateral edge of periaqueductal gray, and the dorsal edge of the isthmic reticular formation. The pedunculopontine tegmental nucleus (PPTg), which is ventrally located to the CnF, was defined as the area where ChAT-immunoreactive neurons were densely distributed. The DH at each bregma level (–2.5 to –3.5 mm) was defined as a rectangle area delimited by a horizontal line along the ventral edge of the fornix, another horizontal line along the dorsal edge of the third ventricle, and two vertical lines placed on the medial edges of both sides of internal capsules. Moreover, the DH at bregma –3.0 mm was divided into three regions; the dorsomedial hypothalamus (DMH), the perifornical area (PeFA), and the dorsolateral hypothalamus (DLH). In both sides of each DH region, cell numbers were counted. Counting the immunoreactive neurons was performed with the assistance of BZ-X image analysis application (Keyence).

Statistics

All statistical analyses were performed with SigmaPlot 14.0 (Systat Software Inc., San Jose, CA). Repeated measure two-way analysis of variance (ANOVA) was used to assess differences in the number or percentage of immunoreactive neurons within the DH between "Control" and "Exercise" rats among bregma levels or medio-lateral regions. If appropriate, the ANOVA was followed by Tukey's post hoc tests. Student's *t* test was used for independent group comparisons. All tests were two-sided. Statistical significance was defined as $P < 0.05$. Data are presented as means ± SEM.

RESULTS

PeFA neurons make synaptic contacts with MLR neurons

While Arima et al. previously demonstrated the existence of MLR-projecting, orexinergic lateral hypothalamus (LH) neurons using retrograde neural tracing technique in rats, the synaptic contacts between LH and MLR neurons remained unclear.[13] To address this issue, we performed anterograde transsynaptic tagging by injecting AAV1 encoding Cre into the PeFA (Fig. 1A). Cre-labeled cells were distributed predominantly around the PeFA, indicating that AAV was adequately injected to the PeFA (Fig. 1B). It is noted, nevertheless, that Cre-labeled hypothalamic neurons should include not only directly infected cells by AAV1 but also those infected transsynaptically. By investigating the distribution of Cre-immunoreactive neurons on mesencephalic coronal sections including the MLR at bregma –8.0 mm, we found that Cre-labeled, postsynaptic neurons of PeFA neurons were abundantly distributed in the midbrain, including the CnF and PPTg, with a dominant presence in the ipsilateral midbrain to the PeFA into which AAV was injected (Figs. 1C and D). These observations indicate that many MLR neurons make synaptic contacts with axons derived from the PeFA.

Orexinergic neurons projecting to the MLR are excited by voluntary running exercise

We then examined whether orexinergic neurons projecting to the MLR are excited by running exercise. Immunohistochemical staining of Fos was performed in this population, which was labeled by Cre expressed in nuclei of MLR-projecting neurons following the injection of AAVrg into the MLR, in "Exercise" and "Control" rats ($n = 7$ for each group) (Figs. 2A and B). Throughout the 90-min experimental period, "Exercise" rats were intermittently engaged in wheel running (900 to 3,632 wheel rotations).

Immunofluorescence staining carried out on

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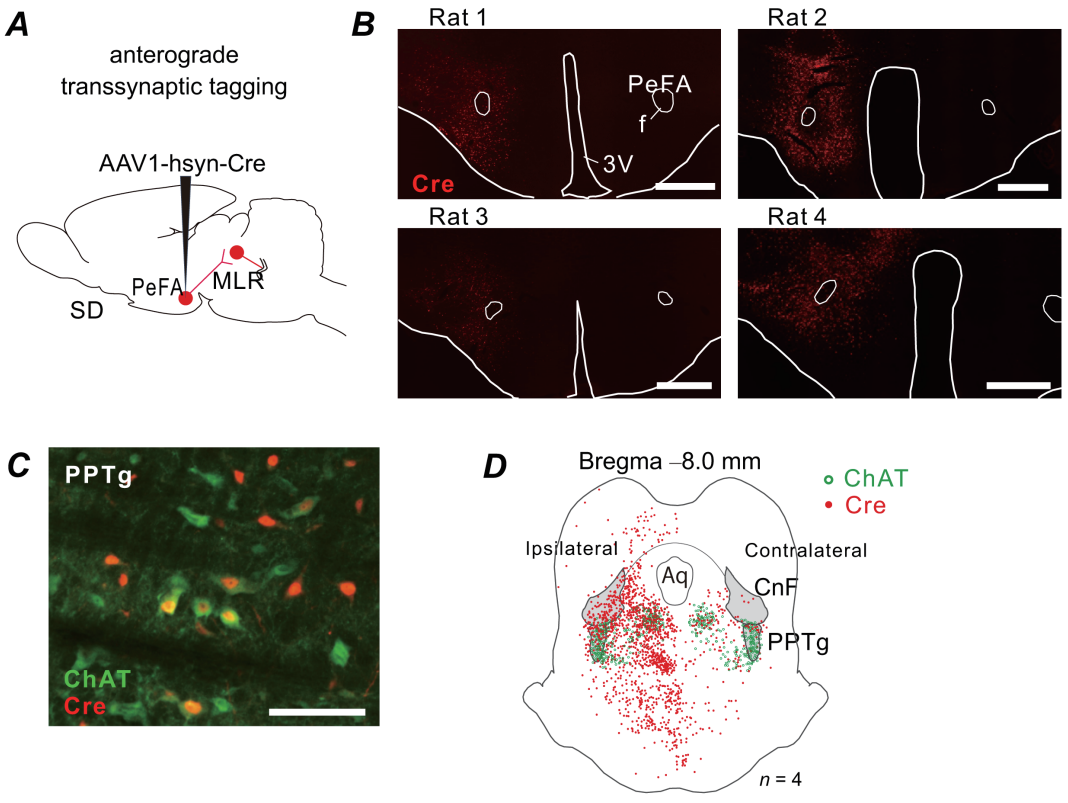

Fig. 1. PeFA neurons make synaptic contacts with MLR neurons. **A**, anterograde transsynaptic tagging. AAV, adeno-associated virus; MLR, mesencephalic locomotor region; PeFA, the perifornical area. SD rat, Sprague-Dawley rat. **B**, images showing distribution of Cre-labeled hypothalamic neurons at 3.0 mm caudal to the bregma (bregma –3.0 mm) of four rats. 3V, third ventricle; f, fornix. Scale bars, 1 mm. **C**, an image showing distribution of Cre-immunoreactive neurons in the pedunculopontine tegmental nucleus (PPTg) of coronal section at bregma –8.0 mm where choline acetyltransferase (ChAT)-immunoreactive neurons are abundantly distributed. Scale bar, 100 μm. **D**, a drawing of distribution of ChAT- and Cre-immunoreactive neurons of 4 rats at bregma –8.0 mm. The brain section illustrations used were adapted from the Paxinos and Watson atlas.[18] Aq, cerebral aqueduct; CnF, cuneiform nucleus.

hypothalamic coronal sections (Figs. 2C and D) revealed that both orexinergic neurons and Cre-labelled, MLR-projecting orexinergic neurons were distributed in the DH across the anteroposterior levels from bregma –2.5 to –3.5 mm, with the greatest number observed at bregma –3.0 mm (Figs. 3A and B). Across these levels, approximately 10% of orexinergic neurons were labeled with Cre, owing to unilateral injection of AAVrg within a restrictive part of the MLR (Fig. 3C). This suggests that, irrespective of anteroposterior levels, at least one-tenth of orexinergic neurons send axonal projections to the unilateral MLR.

Because of the most abundant distribution of MLR-projecting orexinergic neurons at bregma –3.0 mm, the results presented below focus on the data at this level. Within the DH at bregma –3.0 mm, the axons of these neurons exhibited ipsilaterally dominant projections because Cre-labeled orexinergic neurons were more abundantly distributed in the DH ipsilateral to the MLR which received AAVrg by injection, compared to those in the contralateral DH, although their difference in percentage was less than 24% on average (Fig. 3D). Moreover, orexinergic neurons and Cre-labeled orexinergic neurons in both sides of the DH were predominantly distributed in the PeFA compared to the DMH and DLH (Figs. 2D, 3E and F).

Additionally, in the DH, Cre-labelled MLR-projecting, non-orexinergic neurons were most distributed in the DLH compared to the other areas (Figs. 2D and 3G). The effect of the rat group on the densities of Cre-immunoreactive and/or orexinergic DH neurons was not observed (Figs. 3A–G).

Across the anteroposterior levels from bregma –2.5 to –3.5 mm, DH neurons of "Exercise" rats exhibited higher Fos expression compared to "Control" rats, irrespective of co-immunoreactivities with orexin and/or Cre, with the highest numbers observed at bregma –3.0 mm (Figs. 4A–C). In "Exercise" rats, 59% of orexinergic neurons expressed Fos at bregma –3.0 mm, the greatest among the anteroposterior levels investigated

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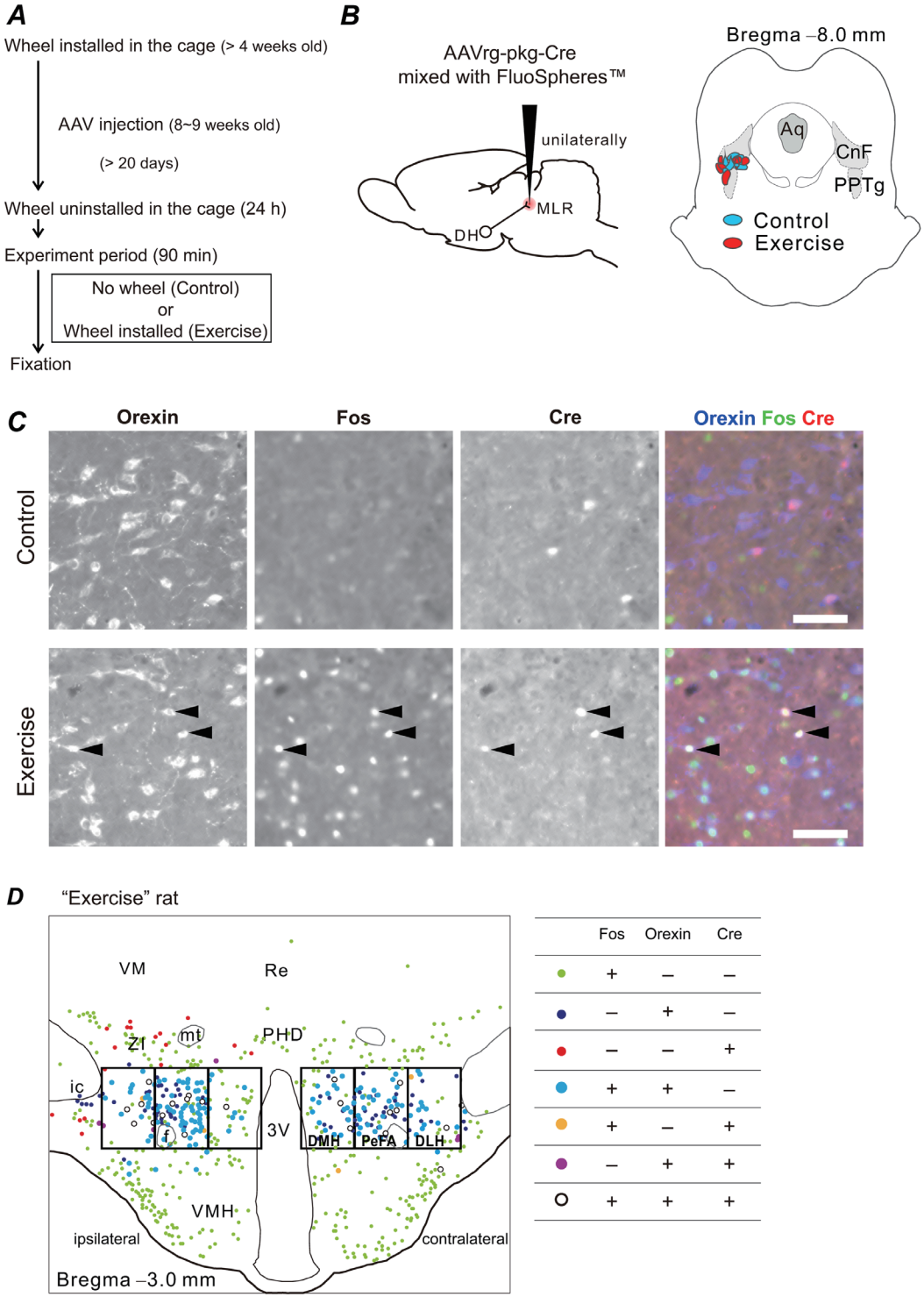

Fig. 2. Immunofluorescence staining of Fos in MLR-projecting orexinergic neurons in "Control" and "Exercise" rats. **A**, a timeline diagram of the experiment. **B**, retrograde neural tracing (left) and injectate spread labeled by fluorescent microspheres diluted in the solution (right). AAVrg, retrograde AAV; DH, the dorsal hypothalamus; MLR, the mesencephalic locomotor region. **C**, representative images showing orexin- (orexin$^+$), Fos- (Fos$^+$), and Cre-immunoreactive (Cre$^+$) neurons in the PeFA of "Control" (top) and "Exercise" (bottom) rats. Arrowheads indicate orexin$^+$, Fos$^+$, and Cre$^+$ neurons. Scale bars, 100 µm. **D**, a drawing example showing representative distribution of orexin$^+$, Fos$^+$, and Cre$^+$ neurons in the hypothalamus of an "Exercise" rat. Outer rectangles represent the boundaries of the DH while inner small ones indicate the dorsomedial hypothalamus (DMH), PeFA, and dorsolateral hypothalamus (DLH) (See Methods). Ic, internal capsule; mt, mammillothalamic tract; PHD, dorsal part of posterior hypothalamic area; Re, reuniens thalamic nucleus; VM, ventromedial thalamic nucleus; VMH, ventromedial nucleus of the hypothalamus; ZI, Zona incerta.

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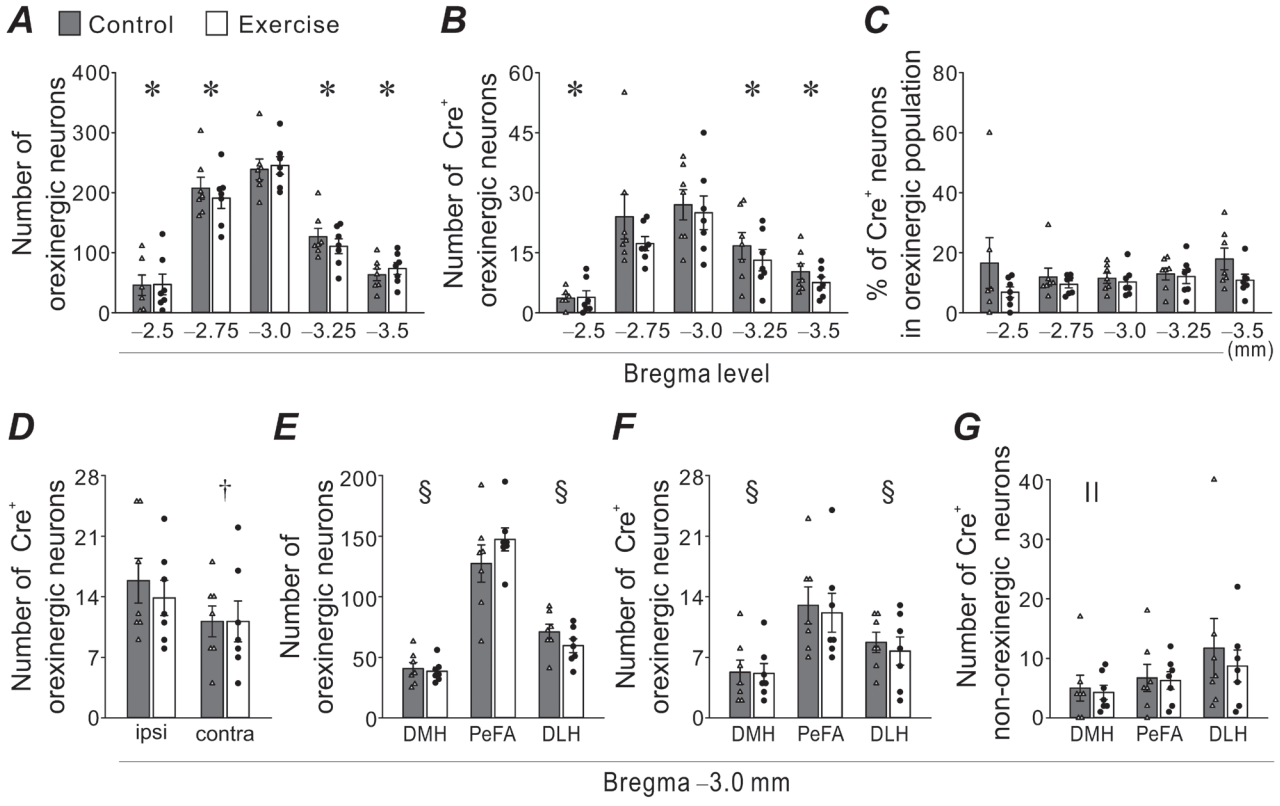

Fig. 3. Distribution of MLR-projecting orexinergic neurons. **A**–**C**, comparisons between "Control" and "Exercise" rats at bregma –2.5, –2.75, –3.0, –3.25, and –3.5 mm, of the numbers of orexinergic neurons **(A)**, of the number of Cre^+ orexinergic neurons **(B)**, and of the percentage of Cre^+ neurons in orexinergic population **(C)** in the DH (n = 7 for each group). **D**, comparisons between the ipsilateral and contralateral DHs (bregma –3.0 mm) to the MLR which received the AAVrg injection, of the numbers of Cre^+ orexinergic neurons. **E**–**G**, comparisons among the DMH, PeFA, and DLH at bregma –3.0 mm, of orexinergic neurons **(E)**, of the numbers of Cre^+ orexinergic neurons **(F)**, and of Cre^+ non-orexinergic neurons **(G)**. Data were analyzed by repeated measure two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. *$P < 0.05$ vs. bregma –3.0 mm. †$P < 0.05$ vs. ipsilateral DH. §$P < 0.05$ vs. PeFA. ‖$P < 0.05$ vs. DLH, irrespective of rat group. Data shown are mean ± SEM.

(Fig. 4D). Further, more than 60% of Cre-labeled orexinergic neurons expressed Fos in the intermediate to posterior, but not anterior, portion of the DH (bregma –2.75 to –3.5 mm) (Fig. 4E). At bregma –3.0 mm in "Exercise" rats, the PeFA exhibited the greatest number of Fos^+ orexinergic neurons compared to the DMH and DLH, irrespective of co-immunoreactivity with Cre (Figs. 5A and B). The densities of Fos^+ neurons, regardless of co-immunoreactivity with Cre, per orexinergic neurons were greater in the DMH and PeFA compared to DLH (Figs. 5C and D). These results indicate that orexinergic neurons, including those projecting to the MLR, are activated by voluntary running exercise. Moreover, running-excited, MLR-projecting orexinergic neurons are densely located in the intermediate-posterior, rather than anterior, and medial, rather than lateral, portion of the DH.

In addition, Cre-labeled, non-orexinergic DH neurons of "Exercise" rats expressed greater Fos compared to "Control" rats ($P = 0.029$), although the number of this population was smaller than that of Cre-labeled, Fos^+ orexinergic neurons (Figs. 5B and E). Likewise, in "Exercise" rats, the densities of Fos^+ neurons per Cre-labeled non-orexinergic neurons, although greater than those in "Control" rats, were less than the densities observed per Cre-labeled orexinergic neurons (Figs. 5D and F). These observations indicate that orexinergic neurons, rather than non-orexinergic neurons, are the major population in the monosynaptic DH→MLR pathway activated by exercise.

Fos expression in MLR neurons was also increased in "Exercise" rats compared to "Control" rats (Fig. 5F), indicating that MLR neurons were activated by voluntary running exercise.

DISCUSSION

Using anterograde transsynaptic tagging with AAV1, we found a significant number of MLR neurons receiving

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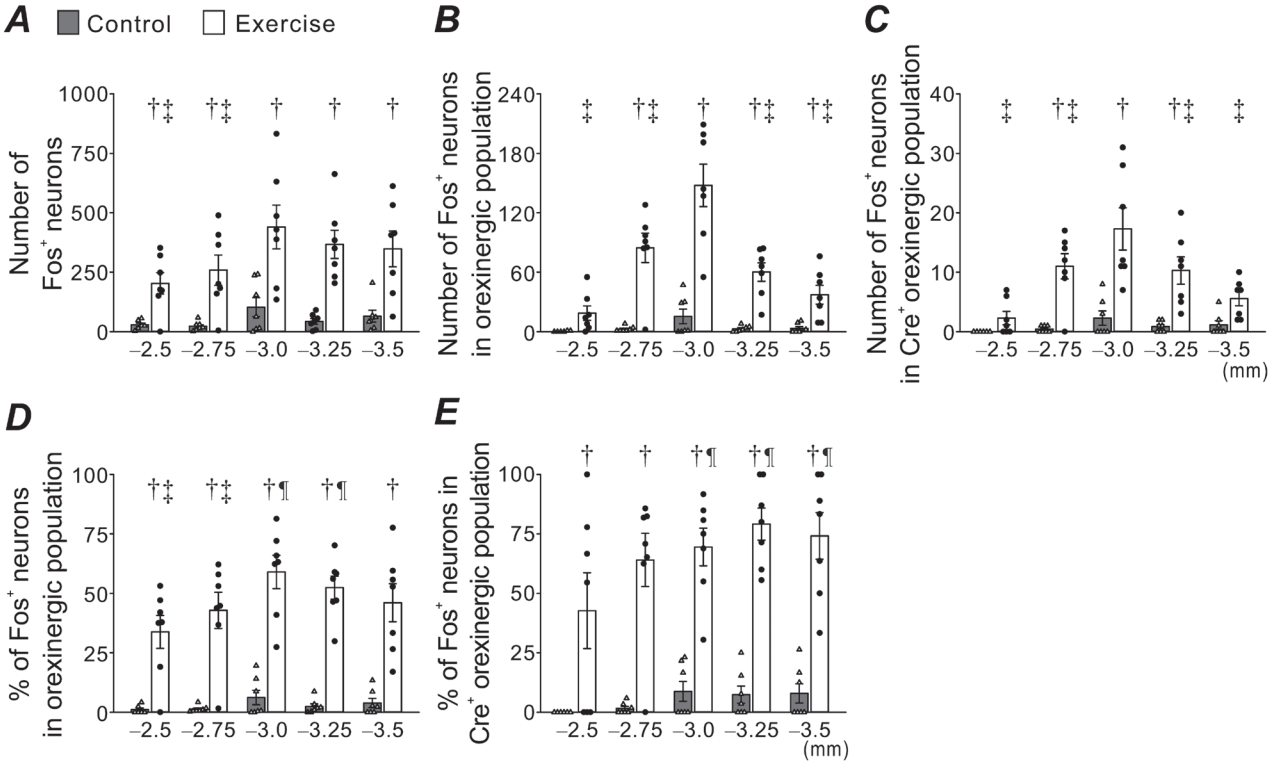

Fig. 4. The excitability of MLR-projecting orexinergic neurons by running at different bregma levels. **A**–**C**. comparisons between "Control" and "Exercise" rats among bregma –2.5, –2.75, –3.0, –3.25, –3.5 mm of the number of Fos^+ neurons in non-specific population **(A)**, in orexinergic population **(B)**, and in Cre^+ orexinergic population **(C)** in the DH. **D** and **E**, the comparisons between "Control" and "Exercise" rats among bregma –2.5, –2.75, –3.0, –3.25, and –3.5 mm of the percentage of Fos^+ neurons in orexinergic population **(D)** and in Cre^+ orexinergic population **(E)**. Data were analyzed by repeated measure two-way ANOVA followed by Tukey's post hoc test. †$P < 0.05$ vs. "Control". ‡$P < 0.05$ vs. bregma –3.0 mm within each group. ¶$P < 0.05$ vs. bregma –2.5 mm within each group **(D, E)**. Data shown are mean ± SEM.

synaptic inputs from PeFA neurons. Employing immunohistochemical staining combined with retrograde neural tracing via AAVrg, we discovered that MLR projecting orexinergic neurons were excited by voluntary wheel running exercise.[15, 16] Remarkably, running-excited, MLR-projecting orexinergic neurons are densely located in the intermediate-posterior, rather than anterior, and medial, rather than lateral, portion of the DH. These findings suggest that orexinergic neurons projecting to the MLR transmit central command signals triggered by motor volition for running exercise, thereby activating the MLR.

The orexinergic nervous system regulates various organismic functions, including wakefulness, metabolism, and motivation for behaviors.[19–23] Additionally, orexinergic neurons play a role in triggering locomotion and elevating sympathetic activity.[7–11] These roles lead us to hypothesize that orexinergic neurons may play a role in transmitting central command signals, although functional or anatomical linkage between the orexinergic nervous system and motor or autonomic nervous system engaged during exercise has remained unknown. In this regard, the present study has advanced our understandings of central circuit mechanisms responsible for central command function.[24, 25] The findings determined the orexinergic neurons projecting to the MLR, a crucial brain region simultaneously regulating somatomotor limb control and autonomic cardiovascular control for running exercise, as a neural pathway to relay central command signals.[5] Nevertheless, further research is demanded to identify the specific role of MLR-projecting orexinergic neurons in regulating somatomotor and autonomic functions during exercise.

Orexinergic neurons play different roles in regulating organismic functions based on their locations. Laterally distributed orexinergic neurons promote reward processing, whereas the medially located population mediates arousal and exhibits heightened excitability in responses to pain.[26, 27] Our observation

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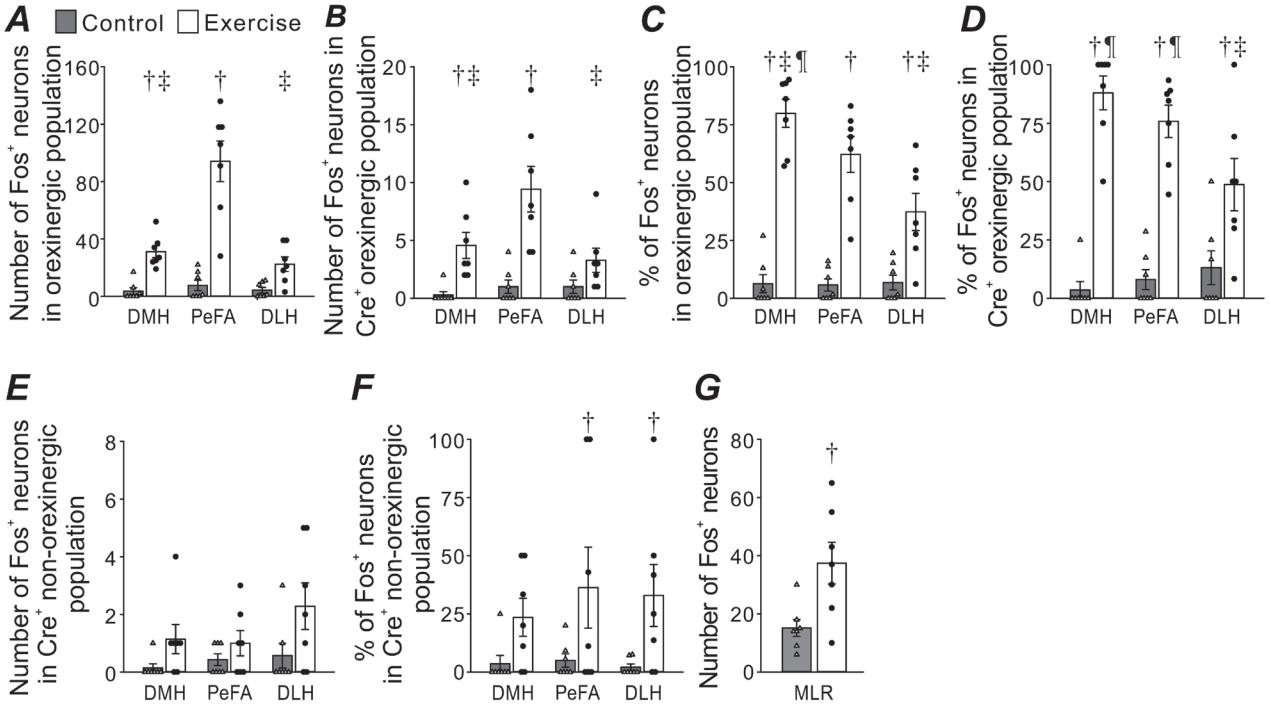

Fig. 5. The excitability of MLR-projecting orexinergic neurons by running, in the DMH, PeFA, and DLH at bregma –3.0 mm. **A** and **B**, comparisons between "Control" and "Exercise" rats among the DMH, the PeFA, and DLH at bregma –3.0 mm, of the numbers of Fos^+ neurons in orexinergic population **(A)** and in Cre^+ orexinergic population **(B)**. **C** and **D**, comparisons of the percentage of Fos^+ neurons in orexinergic population **(C)** and in Cre^+ orexinergic population **(D)**. **E** and **F**, comparisons of the number (**E**) and percentage (**F**) of Fos^+ neurons in Cre^+ non-orexinergic population. **G**, comparisons between "Control" and "Exercise" rats of the number of Fos^+ MLR neurons. Data were analyzed by repeated measure two-way ANOVA followed by Tukey's post hoc test (**A**–**F**) or by Student's *t* test (**G**). †$P < 0.05$ vs. "Control". ‡$P < 0.05$ vs. bregma –3.0 mm within each group. ¶$P < 0.05$ vs. DLH within each group **(C, D)**. Data shown are mean ± SEM.

that MLR-projecting orexinergic neurons primarily distributed in the intermediate-posterior and medial portion of the DH exhibit running sensitive characteristics (Figs. 2D, 4E and 5D) suggests a central role of these spatially distinct orexinergic neurons in transmitting central command signals for running exercise. The previous and present studies emphasize the importance of discerning the locations of orexinergic neuronal distribution across both the anteroposterior and mediolateral axes in future studies. Such distinction is indispensable not only for determining the precise role of orexinergic neurons in central command functions during running exercise but also for understanding the functional neural circuits involving a spatially distinct population of orexinergic neurons.

Whereas a significant number of MLR-projecting orexinergic neurons likely transmit central command signals for running exercise, it is noteworthy that the DH also contained many MLR-projecting, non-orexinergic neurons, a small portion of which exhibited running sensitive characteristics. This result suggests that MLR-projecting, non-orexinergic neurons play a minor role in relaying central command signals onto the MLR. The major roles of this non-orexinergic pathway to the MLR remain undetermined and warrant future study.

In conclusion, the present study demonstrates that orexinergic neurons projecting to the MLR, primarily located in the intermediate-posterior, rather than anterior, and medial, rather than lateral, portion of the DH, are excited by voluntary wheel running. These results suggests that a spatially distinct population of orexinergic neurons transmits central command signals onto the MLR via their monosynaptic projections. Whether this monosynaptic pathway contributes to simultaneous coordination of somatomotor and autonomic cardiovascular controls for running exercise deserves further study to unveil central circuit mechanisms responsible for central command function, a research issue for well over a century.[1, 2]

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The authors declare no conflict of interest.

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