Neuronal migration depends on blood flow in the adult brain

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Abstract

In animal tissues, several cell types migrate along blood vessels, raising the possibility that blood flow influences cell migration. Here, we show that blood flow promotes the migration of new olfactory-bulb neurons in the adult brain. Neuronal migration is facilitated by blood flow, leading to accumulation of new neurons near blood vessels with abundant blood flow. Blood flow inhibition attenuates blood vessel-guided neuronal migration, suggesting that blood contains factors beneficial to neuronal migration. We found that ghrelin, which is increased in blood by hunger, directly influences neuronal migration. Ghrelin signaling promotes somal translocation by activating actin cytoskeleton contraction at the rear of the cell soma. New neurons mature in the olfactory bulb and contribute to the olfactory function for sensing odorants from food. Finally, we show that neuronal migration is increased by calorie restriction, and that ghrelin signaling is involved in the process. This study suggests that blood flow promotes neuronal migration through blood-derived ghrelin signaling in the adult brain, which could be one of the mechanisms that improve the olfactory function for food-seeking behavior during starvation.

Introduction

Blood vessel-guided cell migration has been reported in various types of cells, including lymphatic endothelial cells in the embryonic zebrafish trunk (Bussmann et al., 2010), Schwann cells, oligodendrocyte precursor cells, glioma cells, and astrocyte progenitors in rodent tissues (Cattin et al., 2015; Farin et al., 2006; Tabata et al., 2022; Tsai et al., 2016), suggesting that it is a common mechanism for efficient cell migration within complex animal tissues. Previous reports have extensively studied the role of blood vessels as a physical scaffold for migration of neurons produced in the postnatal brain tissues (Bovetti et al., 2007; Fujioka et al., 2017; Grade et al., 2013; Ohab et al., 2006; Snapyan et al., 2009; Sun et al., 2015; Thored et al., 2007; Whitman et al., 2009; Yamashita et al., 2006). New neurons generated in the postnatal ventricular-subventricular zone (V-SVZ) migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS), forming elongated aggregates called chains (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Lois and Alvarez-Buylla, 1994). Within the OB, new neurons migrate individually and radially and terminate their migration in the granule cell layer (GCL) or glomerular layer (GL), where they are differentiated into GABAergic interneurons and incorporated into the OB neural circuit (Bressan and Saghatelyan, 2021; Kaneko et al., 2017). Previous studies have shown that both chain-forming and individually migrating neurons use blood vessels as physical scaffolds for their migration in the RMS and OB (Bovetti et al., 2007; Snapyan et al., 2009; Whitman et al., 2009). However, whether blood flow plays a role in blood vessel-guided cell migration has not been reported.

Ghrelin, a peripheral hormone produced in the stomach, is delivered to the brain through the bloodstream and accumulates in the brain parenchyma, especially at high levels in the OB (Rhea et al., 2018). The ghrelin concentration in blood has been reported to increase during fasting (Toshinai et al., 2001; Tschöp et al., 2000). In addition to its role in regulating metabolism (al Massadi et al., 2017; Chopin et al., 2012; Soleyman-Jahi et al., 2019; Stoyanova and Lutz, 2021), ghrelin influences neurogenesis in the V-SVZ and subgranular zone of the hippocampal dentate gyrus (Hornsby et al., 2016; Kent et al., 2015; Kim et al., 2015; Li et al., 2014, 2013; Moon et al., 2009). However, its role in blood vessel-guided neuronal migration has not been studied.

To elucidate the effects of blood flow on blood vessel-guided cell migration, we focused on neuronal migration along blood vessels in the adult RMS and OB. The results showed that new neurons migrate along blood vessels with high blood flow and that their

migration is affected by changes in blood flow. We also found that ghrelin promotes neuronal migration through activation of actin cytoskeleton contraction at the rear of the cell soma, indicating that blood flow influences neuronal migration via ghrelin signaling. Furthermore, we found that calorie restriction promotes the migration of OB neurons, suggesting that the blood flow-dependent mechanism of neuronal migration could be part of a system to improve sensory function in response to physiological change in the body.

Results

New neurons migrate along blood vessels with abundant blood flow

Previous studies have revealed that V-SVZ-derived new neurons migrate along blood vessels in the RMS and GCL (Bovetti et al., 2007; Snapyan et al., 2009; Whitman et al., 2009). However, the neuron-vessel interactions along the entire migration route remain unknown. Therefore, we first studied blood vessel-guided neuronal migration in the RMS and OB using three-dimensional imaging, which enables analysis of the *in vivo* spatial relationship between new neurons and blood vessels. We measured the distance from the soma of new neurons, labeled with green fluorescent protein (GFP)-encoding adenovirus, to the nearest blood vessel labeled with RITC-Dex-GMA. The results showed that GFP+ new neurons interact closely with blood vessels in the RMS, GCL, external plexiform layer, and GL (Figure. 1A–D, Supplemental movie S1, S2, S3). In particular, in the RMS and GL, the majority of cells were present within 5 μm of the inner surface of vessels (Figure. 1D), suggesting that new neurons in these regions frequently use blood vessels as migration scaffolds. Transmission electron microscopy revealed direct attachment of migratory neurons, identified as cells with an elongated cell body, a dark cytoplasm with many free ribosomes, and an electron-dense nucleus with multiple nucleoli (Doetsch et al., 1997), to thin astrocytic endfeet enwrapping blood vessels (Figure. 1E), as previously reported in the RMS and GCL (Bovetti et al., 2007; Whitman et al., 2009). These results indicate that new neurons migrate along blood vessels through their entire migration route, suggesting the possibility that their movement may be influenced by blood flow.

To examine the relationship between neuronal migration and blood flow, we recorded movement of new neurons and red blood cell (RBC) flow using two-photon laser scanning microscopy. Neuronal migration was recorded in the GL of *Dcx-EGFP* mice, in which new

neurons were labeled with enhanced GFP (EGFP) (Figure. 1F). RBC flow was also recorded in each vessel segment in the GL by line-scan measurements (Figure. 1F). We identified migrating new neurons as EGFP+ cells whose position changed during the imaging period in *Dcx-EGFP* mice, in which the majority of EGFP+ cells are stationary. Most EGFP+ migrating cells (90.2%) were located within 10 μm of the nearest vessel (Figure. 1G). To compare the blood vessel-guided migration of new neurons among blood vessels with different flows, we classified the vessels into two groups, high-flow and low-flow vessels, using the median RBC flow velocity as the criterion (Figure. 1H). New neurons were more abundant near high-flow vessels than near low-flow vessels (Figure. 1H). These cells migrated at a small angle to the longitudinal axis of blood vessels (Figure. 1I), indicating that new neurons use blood vessels as migration scaffolds. Next, we compared the migration speed of new neurons between the low- and high-flow perivascular region, the area within 10 μm from the nearest vessel. Migration speed, as measured by the migration distance of the cell soma, was significantly higher in the perivascular region of high-flow vessels than in that of low-flow vessels (Figure. 1J). A previous study showed that new neurons undergo discontinuous migration, including migratory and stationary periods in the GL (Liang et al., 2016). The proportion of the migratory period of new neurons in the total imaging period was larger in high-flow perivascular regions than in low-flow regions (Figure. 1K). Furthermore, the maximum migration speed, calculated from two consecutive imaging frames, was significantly higher for neurons migrating along high-flow vessels than for those migrating along low-flow vessels (Figure. 1L). These results suggest that neuronal migration is promoted in perivascular regions with abundant blood flow.

To compare the distribution of new neurons in perivascular regions with different blood flows in deep brain regions, we used endomucin, which has been reported to be downregulated by shear stress on vascular endothelial cells *in vitro* (Zahr et al., 2016). Endomucin-negative vessels showed higher RBC flow than endomucin-positive vessels (Figure. S1A–C), indicating that endomucin can be used as a marker for RBC flow velocity *in vivo*. The density of bromodeoxyuridine (BrdU)-labeled doublecortin (Dcx)+ new neurons was higher in the perivascular region of endomucin-negative vessels than in that of endomucin-positive vessels in the GL, GCL, and RMS (Figure. S1D–G), suggesting that new neurons migrate along vessels with faster blood flow throughout their migration route.

We next compared the distribution of new neurons near arteriole-side and venule-side vessels identified using SLC16A1, which is expressed in venules and venule-side

capillaries (Vanlandewijck et al., 2018). The density of perivascular Dcx+/BrdU+ new neurons was significantly higher in SLC16A1-negative perivascular areas than in SLC16A1-positive areas (Figure. S2A, B), indicating that they actively migrate in the vicinity of arteriole-side vessels (Figure. S2C). To further examine the localization of perivascular new neurons, we analyzed the cell distribution in *NG2-DsRed* mice (Zhu et al., 2008), in which we could identify arterioles, venules, and capillaries based on the morphological differences in NG2+ mural cells as performed previously (Hartmann et al., 2015; Hill et al., 2015). Arterioles, capillaries, and venules were characterized by band-like smooth muscle cells, pericytes, and fenestrated-shaped smooth muscle cells, respectively (Figure. 1M). A large proportion of Dcx+/BrdU+ new neurons was observed near capillaries in the GL (arterioles; 11.8 ± 2.39%, venules; 4.34 ± 0.212%, capillaries; 83.8 ± 2.25%) (Figure. 1N). The frequency of perivascular neuronal migration was compared in arteriole-side and venule-side capillaries (defined as capillaries one to three order branches away from the nearest arterioles and venules, respectively). The density of Dcx+/BrdU+ cells was higher near arteriole-side capillaries than that near venule-side capillaries (Figure. 1O). Taken together, these data suggest that most neuronal migration occurs near capillaries and that new neurons prefer capillaries on the arteriole side to those on the venule side (Figure. S2C).

We next investigated whether new neurons prefer arteriole-side vessels in the postnatal common marmoset brain, where V-SVZ-derived new neurons migrate in the neocortex and ventral striatum as individual cells or cell aggregates (Akter et al., 2021) (Supplemental movie S4, S5). In the ventral striatum of 3 to 4-month-old common marmosets, Dcx+ immature neurons localized more frequently near SLC16A1-negative vessels than near SLC16A1-positive vessels, regardless of migration modes (Figure. S2D, E). A similar tendency was also observed in the neocortex (Figure. S2F). These results suggest that immature neurons prefer to migrate along arteriole-side vessels in the common marmoset brain, and this phenomenon is common between rodents and primates.

Finally, we examined whether new neurons mature near high-flow vessels following their migration along these blood vessels. Neuronal maturation and blood flow were recorded in *VGAT-Venus* mice, in which GABAergic neurons are labeled with Venus. Maturation of new neurons was confirmed by observation of the same regions with an interval of 21 days (Figure. 1P). The density of newly added neurons was significantly higher near high-flow vessels than near low-flow vessels (Figure. 1Q). Consistently, BrdU-labeled mature neurons were frequently observed in the perivascular region of endomucin-negative vessels (Figure. S1H). These results

suggest that blood vessel-guided neuronal migration supplies new neurons in regions of high blood flow.

Decreases in blood flow affect neuronal migration

To investigate the effects of blood flow on neuronal migration, we performed bilateral carotid artery stenosis, which decreases blood flow in the anterior portion of the brain (Hattori et al., 2016; Shibata et al., 2004). Lentiviruses expressing Venus were injected into the V-SVZ to label new neurons migrating toward the OB (Figure. 2A). The proportion of Dcx+/Venus+ cells in the OB was significantly lower in the bilateral carotid artery stenosis group than that in the Sham-operated group at 5 dpi (Figure. 2B, C), suggesting that blood flow reduction inhibited tangential migration of new neurons in the RMS. To further examine the influence of blood flow changes on neuronal migration, we induced photothrombotic clot formation to reduce blood flow in individual vessels (Figure. 2D). Migration of EGFP+ cells along vessels in *Dcx-EGFP* mice was recorded using two-photon imaging (Figure. 2E). To prevent effects other than blood flow inhibition, clot formation was induced in upstream vessel fragments distant from vessels close to migrating neurons (Figure. 2D). Clot formation resulted in blood flow termination in downstream vessels (Figure. 2F), which was followed by a decrease in the migration speed of EGFP+ new neurons along downstream vessels (Figure. 2E, H). Laser irradiation without rose bengal did not affect the speed of neuronal migration (Figure. 2G), indicating that the inhibition of neuronal migration was not due to laser irradiation. These data suggest that blood flow facilitates neuronal migration in the RMS and OB and that blood contains factors influencing neuronal migration.

Ghrelin increases neuronal migration speed by promoting somal translocation

We focused on ghrelin, which can be delivered from the bloodstream to the brain parenchyma, including the OB tissue, by transcytosis across vascular walls (Rhea et al., 2018). A previous study showed that migration of V-SVZ-derived new neurons is attenuated in ghrelin knockout mice (Li et al., 2014), suggesting that ghrelin stimulates neuronal migration. At first, to examine transcytosis of ghrelin in the OB, we introduced fluorescently labeled ghrelin into the bloodstream. We found accumulation of fluorescent ghrelin in the RMS and OB as

reported previously(Rhea et al., 2018) (Figure 3A, B, Figure S3). Fluorescence signals were observed in vascular endothelial cells and parenchymal tissue in the RMS and OB (Figure 3B), indicating that blood-derived ghrelin crosses the vascular wall into the brain parenchyma and is delivered to new neurons. In addition, high fluorescent signals were found in vascular endothelial cells in endomucin-negative high-flow vessels (Figure 3C, D), suggesting that ghrelin transcytosis is active in the blood vessels with abundant flow. To examine the direct effect of ghrelin on migrating neurons, we added recombinant ghrelin protein to the culture medium of V-SVZ cells (Figure. 4A). The migration distance of new neurons was significantly increased in the ghrelin-containing medium (Figure. 4B). To further clarify the effects of ghrelin signaling, we performed super-resolution time-lapse imaging of cultured new neurons with or without knockdown (KD) of the growth hormone secretagogue receptor 1a (ghsr1a), a ghrelin receptor expressed in V-SVZ-derived new neurons (Li et al., 2014)(Figure. 4C). In lacZ-KD (control) cells, migration speed was increased when cells were cultured in ghrelin-containing medium (Figure. 4C, D). V-SVZ-derived new neurons exhibit saltatory migration consisting of a migratory phase and a resting phase (Ota et al., 2014). Ghrelin application increased the migratory phase proportion (Figure. 4E) but not the length of the migration cycle (Figure. 4F), suggesting that ghrelin signaling elongates the migratory phase of neuronal migration. Cultured new neurons alternate between leading process extension and somal translocation (Figure. 4C). Ghrelin application did not affect the length or speed of leading process extensions (Figure. 4G, H). In contrast, the somal translocation speed and somal stride length were significantly increased by ghrelin application (Figure. 4I, J). No such effects were observed in ghsr1a-KD cells (Figure. 4D, E, I, J), suggesting that ghrelin promotes neuronal migration through ghsr1a. Taken together, these results suggest that ghrelin signaling promotes somal translocation and thus increases the efficacy of neuronal migration.

Somal translocation of migrating cortical interneurons is driven by formation of the actin cup, an accumulation of F-actin at the rear of the cell soma (Martini and Valdeolmillos, 2010). Time-lapse imaging of cultured new neurons expressing EGFP fused to the calponin homology domain of utrophin (EGFP-UtrCH), a fluorescent reporter for F-actin (Burkel et al., 2007), revealed discontinuous formation of the actin cup at the rear of the cell soma (Figure. 4K). Ghrelin application extended the duration of actin cup formation (Figure. 4L) and increased the migration distance during actin cup formation (Figure. 4M, Supplemental movie S6). These effects were not observed in ghsr1a-KD cells (Figure. 4L, M, Supplemental movie S7),

suggesting that ghsr1a-mediated ghrelin signaling promotes somal translocation of new neurons by activation of actin cup formation at the rear of the cell soma.

To investigate the effects of ghrelin signaling on neuronal migration in the OB *in vivo*, ghsr1a-KD or control lentiviruses were injected into the OB core (Figure. 5A). Of the total labeled Dcx+ cells, the percentage of Dcx+ cells observed in the GL was smaller in the ghsr1a-KD group than that in the control group (Figure. 5B, C). When lentiviruses were injected into the V-SVZ (Figure. 5D), ghsr1a-KD (DsRed+) cells exhibited decreased distribution in the GL (Figure. 5E, F) and increased distribution in the RMS compared with control (EmGFP+) cells (Figure. 5G, H). These data indicate that ghrelin signaling facilitates both individual and chain migration in the migration route of new neurons. It is possible that blood-derived ghrelin promotes neuronal migration in the RMS and OB by activating actin cytoskeleton contraction in the cell soma. Finally, we examined whether calorie restriction, which has been reported to increase blood ghrelin levels (Toshinai et al., 2001; Tschöp et al., 2000) , affects neuronal migration. The proportion of labeled cells in the GL (Figure 5I) and NeuN+/Dcx- cells among BrdU+ cells in the OB (FigureS4) was larger in the calorie restriction group than in the ad libitum group. However, there was no significant difference in the proportion of ghsr1a-KD cells in the GL between the control and calorie restriction groups (Figure. 5J). Taken together, these data suggest that blood flow promotes the migration of OB neurons during starvation via ghrelin signaling and that promoted migration of new neurons increases the number of mature neurons in the OB.

Discussion

Previous studies have shown the role of blood vessels as physical scaffolds in the migratory routes of new neurons in various situations (Bovetti et al., 2007; Fujioka et al., 2017; Grade et al., 2013; Kojima et al., 2010; Ohab et al., 2006; Snapyan et al., 2009; Sun et al., 2015; Thored et al., 2007; Whitman et al., 2009; Yamashita et al., 2006). Proteins expressed by vascular cells have been reported to facilitate neuronal migration by binding to transmembrane receptors of new neurons (Fujioka et al., 2017; Grade et al., 2013; Ohab et al., 2006; Snapyan et al., 2009). However, whether neuronal migration is affected by blood flow remains unknown. Therefore, in this study, we focused on the specific feature of the vasculature as a pipeline for blood delivery. The effects of blood flow on neuronal migration are difficult to detect with previously used experimental procedures such as

immunohistochemistry of vascular endothelial cell markers in fixed tissue sections and time-lapse live imaging of brain slice cultures. Therefore, we classified blood vessels using molecular markers that reflect blood flow properties and performed *in vivo* live imaging to record blood flow. This approach enabled us to reveal the effects of blood flow on neuronal migration.

Previous studies reported that new neurons migrate along blood vessels in the RMS and GCL of the OB (Bovetti et al., 2007; Snapyan et al., 2009; Whitman et al., 2009). In the present study, three-dimensional imaging was performed over a wide area across the entire migration route in transparent brains, where the positional relationship between new neurons and blood vessels can be observed. The results suggest that new neurons use blood vessels as migration scaffolds throughout their migration route (Figure. 1A–D). The distance we found between new neurons and blood vessels was larger than that reported in a previous study (Snapyan et al., 2009). This might be due to our method of measuring the distance from the blood vessels to the cell soma of new neurons, rather than the distance to the entire new neurons. To investigate the effects of blood flow on blood vessel-guided neuronal migration, we used two-photon imaging to record neuronal migration and blood flow *in vivo* in the GL in the superficial area of the OB, which is a useful model for analyzing blood vessel-guided neuronal migration. The results suggest that new neurons migrate faster near blood vessels with high flow than in the areas of those with low flow. Neural stem cells have been reported to increase blood flow in the adult V-SVZ (Lacar et al., 2012), raising the possibility that new neurons may increase blood flow in the OB. However, our observation that inhibition of blood flow suppressed neuronal migration suggests that blood flow facilitates neuronal migration. New neurons terminated their migration and differentiated into mature interneurons in perivascular regions with abundant flow. Thus, the blood flow-dependent mechanism of neuronal migration may supply new neurons to areas appropriate for their function.

Based on the finding that neuronal migration was attenuated in our BCAS and photothrombosis experiments, we hypothesized that a blood-derived factor facilitates neuronal migration. In this study, we demonstrated that ghrelin signaling promotes neuronal migration through its receptor. Ghrelin signaling promoted somal translocation of new neurons by activating actin cytoskeletal dynamics in the rear of the cell soma. We also found that ghrelin signaling increases the migration distance of cell soma, which increases the migratory phase duration. These results suggest that ghrelin signaling promotes neuronal migration in the RMS and OB *in vivo*, which could further strengthen our finding that blood flow plays a role in neuronal migration. Previous studies have shown that a ghrelin receptor, ghsr1a, is expressed

in many brain regions (Zigman et al., 2006). New neuron-specific KD of ghsr1a revealed that ghrelin signaling acts cell-autonomously on neuronal migration. Furthermore, we found that blood-derived ghrelin crosses the vascular wall into the RMS and OB. Although we could not exclude the possibility that ghrelin is produced in the brain parenchyma (Howick et al., 2017), these results suggest that blood-derived ghrelin is provided to new neurons and promotes somal translocation by activating actin cup formation. Because blood ghrelin levels increase during fasting (Toshinai et al., 2001; Tschöp et al., 2000), we examined the possibility that neurogenesis in the OB is affected by feeding conditions. We found that calorie restriction promoted the migration of OB neurons, an effect which was cancelled by ghsr1a-KD, suggesting that calorie restriction facilitates the OB neurogenesis through ghrelin signaling. Since the supply of new neurons to the OB has been suggested to improve olfactory function in food-seeking behavior(Lazarini and Lledo, 2011), increased neurogenesis caused by long-term calorie restriction may contribute to improved olfactory function for food seeking during starvation. The increased speed of somal translocation and elongated duration of the migratory phase in cultured new neurons are in accord with the *in vivo* increase in migration speed and duration of the migratory period of new neurons around high-flow vessels, respectively. Effective somal translocation has been suggested to be advantageous for cell migration in densely packed tissues (Kengaku, 2018). It is possible that the promotion of somal translocation by ghrelin signaling and blood flow overcomes difficulties in smooth migration of new neurons in dense tissues of the adult brain.

Previous reports have shown that new neurons migrate along blood vessels to damaged areas after brain injury (Fujioka et al., 2017; Grade et al., 2013; Kojima et al., 2010; Ohab et al., 2006; Thored et al., 2007; Yamashita et al., 2006). Neuronal migration may be influenced by blood flow under pathological conditions as well as during blood vessel-guided migration under the physiological conditions shown in this study. It is possible that blood contains factors in addition to ghrelin that regulate neuronal migration. Blood flow may coordinate biological events between different organs by sending beneficial factors produced outside the brain to influence regionally restricted neuronal migration. Further studies could identify unknown factors involved in the mechanism of blood flow-dependent cell migration, which could contribute to the development of blood flow-based therapies for neurological diseases.

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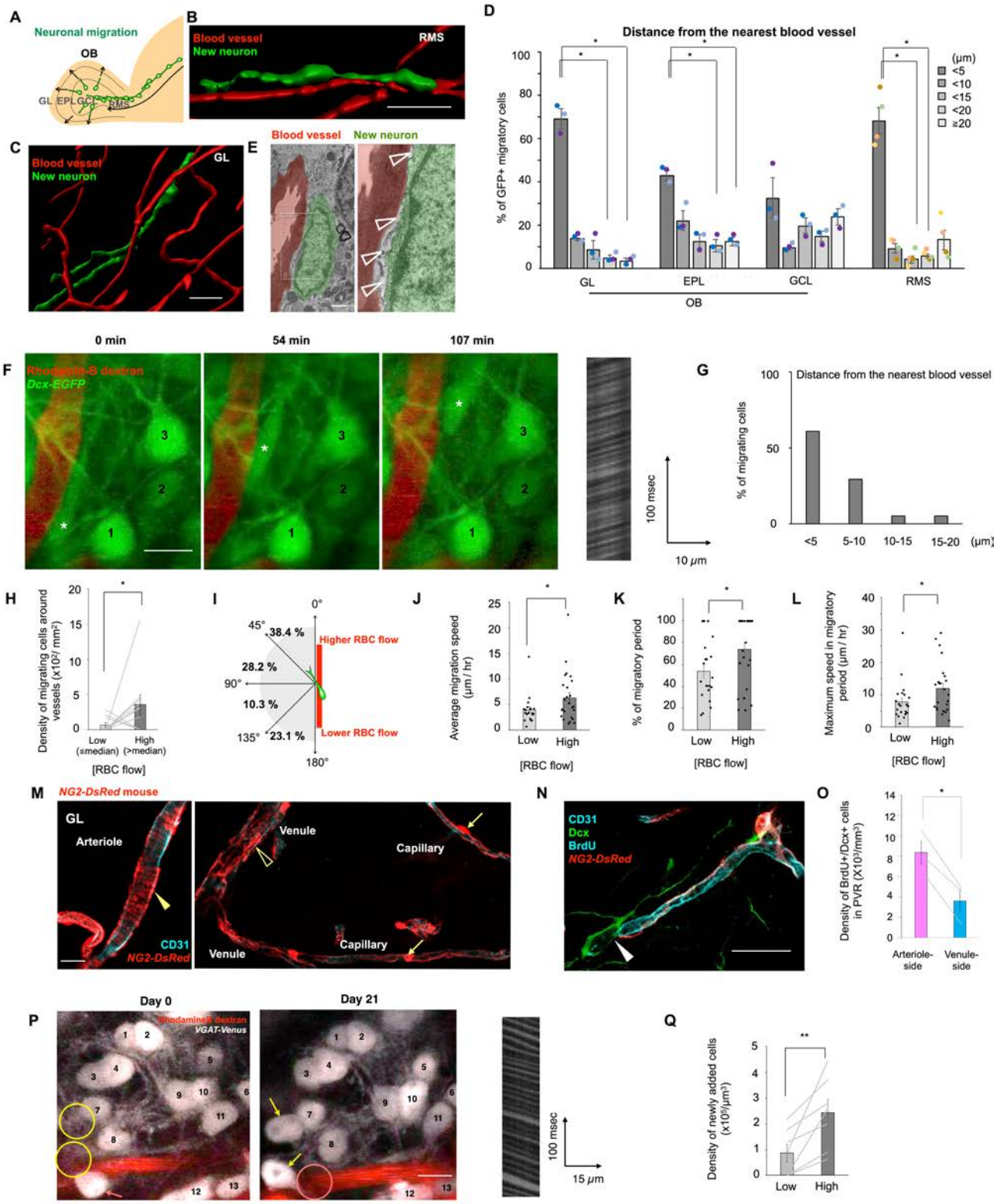

Figure. 1 New neurons migrate along blood vessels with abundant flow.

(A) Experimental scheme.

(B, C) Three-dimensional reconstructed images of a new neuron (green) and vasculature (red) in the rostral migratory stream (RMS) (B) and glomerular layer (GL) (C).

(D) Distance between new neurons and nearest vessels in the olfactory bulb and RMS (one-way repeated measures ANOVA followed by Bonferroni's test; 3 and 4 mice for the analysis in the OB and RMS, respectively).

(E) Transmission electron microscopy image of a new neuron (green) and a blood vessel (red) in the GL. Astrocytes (clear arrowheads).

(F) Time-lapse images of a migrating neuron (asterisks) in the *Dcx-EGFP* mouse GL. Red blood cell (RBC) flow is recorded as a two-photon line-scan image as shown in the right panel. Stationary cells are indicated by sequential numbers.

(G) Average distance between migrating cells and nearest blood vessels (41 cells from 38 mice).

(H) Density of perivascular migrating cells (Wilcoxon signed-rank test; 10 mice).

(I) Proportions of new neurons migrating in different directions along vessels (39 interactions from 26 mice).

(J) Average migration speed (Welch's *t*-test; low, 19 cells, high, 25 cells from 39 mice).

(K) Percentage of migratory period (Mann-Whitney U-test; low, 19 cells, high, 24 cells from 38 mice).

(L) Maximum migration speed (unpaired *t*-test; low, 22 cells, high, 24 cells from 39 mice).

(M) Fluorescent images in the *NG2-DsRed* mouse GL. Arterioles, capillaries, and venules were characterized by band-like smooth muscle cells (solid arrowhead), pericytes (arrows), and fenestrated smooth muscle cells (clear arrowhead), respectively. CD31 (blue), DsRed (red).

(N) Fluorescent image of a Dcx+/BrdU+ new neuron (solid arrowhead) attached to a capillary. Dcx (green), BrdU (blue), CD31 (blue, tube-like structures), DsRed (red).

(O) Density of BrdU+/Dcx+ cells in the perivascular region of arteriole-side and venule-side capillaries (paired *t*-test; 3 mice).

(P) Two-photon images of GABAergic neurons (white) and a blood vessel (red) in the *VGAT-Venus* mouse GL. Circles show positions of added (yellow) and lost (pink) Venus+ cells. Added and lost neurons are indicated by yellow and pink arrows, respectively. RBC flow on Day 21 is shown in the right panel.

(Q) Density of newly added neurons in the perivascular region (paired *t*-test; 7 mice).

Data are presented as the means ± standard error of the mean (SEM). Scale bars: B, 30 μm; C, 40 μm; E, 1 μm; F, 10 μm; M, 20 μm; N, 20 μm; P, 10 μm. See also Figure. S1 and S2.

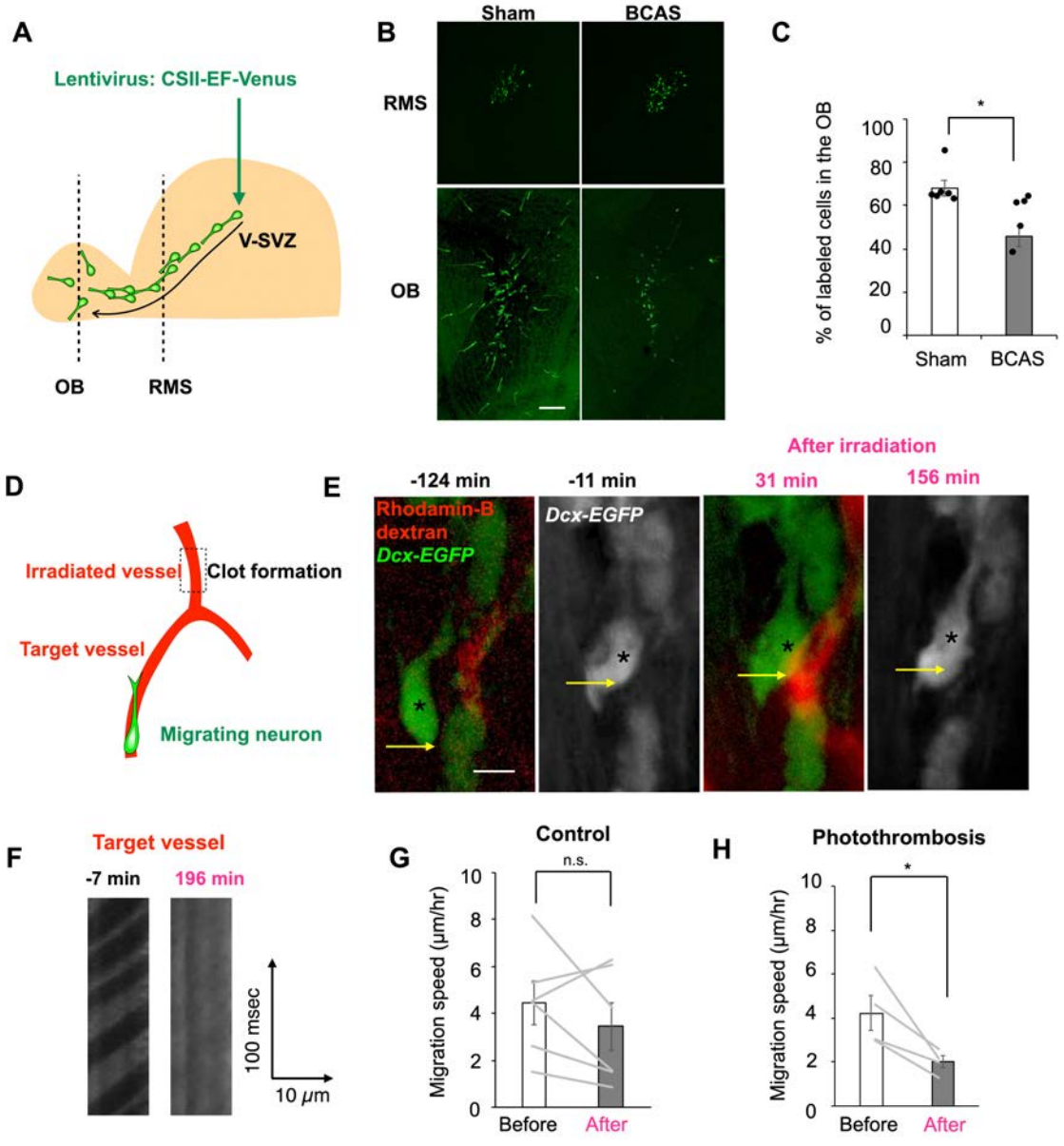

Figure. 2 Blood flow inhibition attenuates neuronal migration.

(A, D) Experimental schemes.

(B) Fluorescent images of Venus+ new neurons (green) in the rostral migratory stream and olfactory bulb (OB).

(C) Proportion of Venus+ cells in the OB in the Sham and bilateral carotid artery stenosis groups (unpaired *t*-test; Sham, 6 mice, BCAS, 5 mice).

(E) Two-photon images of neuronal migration (arrows) before and after photothrombotic clot formation in a *Dcx-EGFP* mouse. A new neuron (green), a blood vessel (red).

(F) Line-scan images from a blood vessel shown in (E).

(G, H) Comparison of migration speed before and after laser irradiation in the control (G) (paired *t*-test; 7 cells from 7 mice) and photothrombosis groups (H) (paired *t*-test; 4 cells from 4 mice). Data are presented as the means ±SEM. Scale bars: B, 100 μm; E, 10 μm.

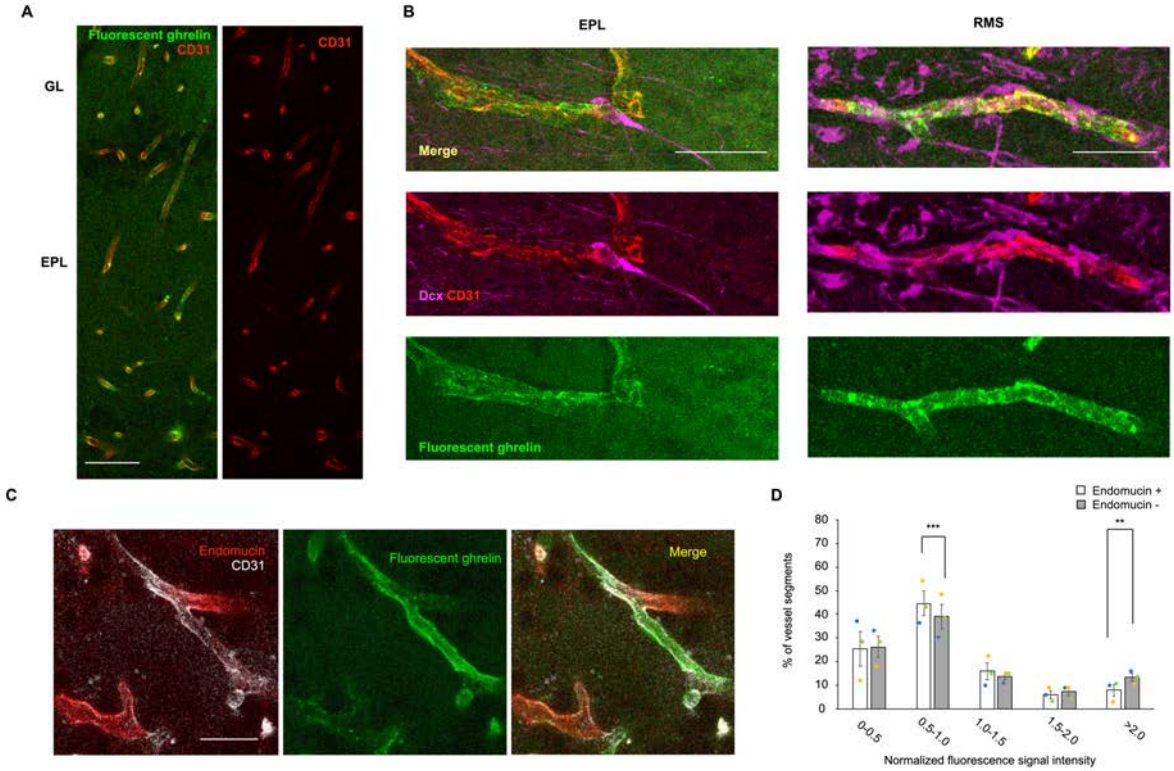

Figure. 3 Ghrelin is delivered from the bloodstream to the RMS and OB.

(A) Representative images of the OB and the cortex from fluorescent ghrelin-infused mice. CD31 (red), Dcx (magenta), fluorescent ghrelin (green).

(B) Fluorescent images of neuronal migration along blood vessels in the EPL and the RMS. CD31 (red), Dcx (magenta), fluorescent ghrelin (green).

(C) Fluorescent images of blood vessels in the GL (C). CD31 (white), endomucin (red), fluorescent ghrelin (green).

(D) Normalized fluorescence signal intensity in vascular endothelial cells (paired *t*-test; 3 mice). Data are presented as the means ±SEM. Scale bars: A, 50 μm; B, 20 μm (EPL), 10 μm (RMS); C, 20 μm. See also Figure. S3.

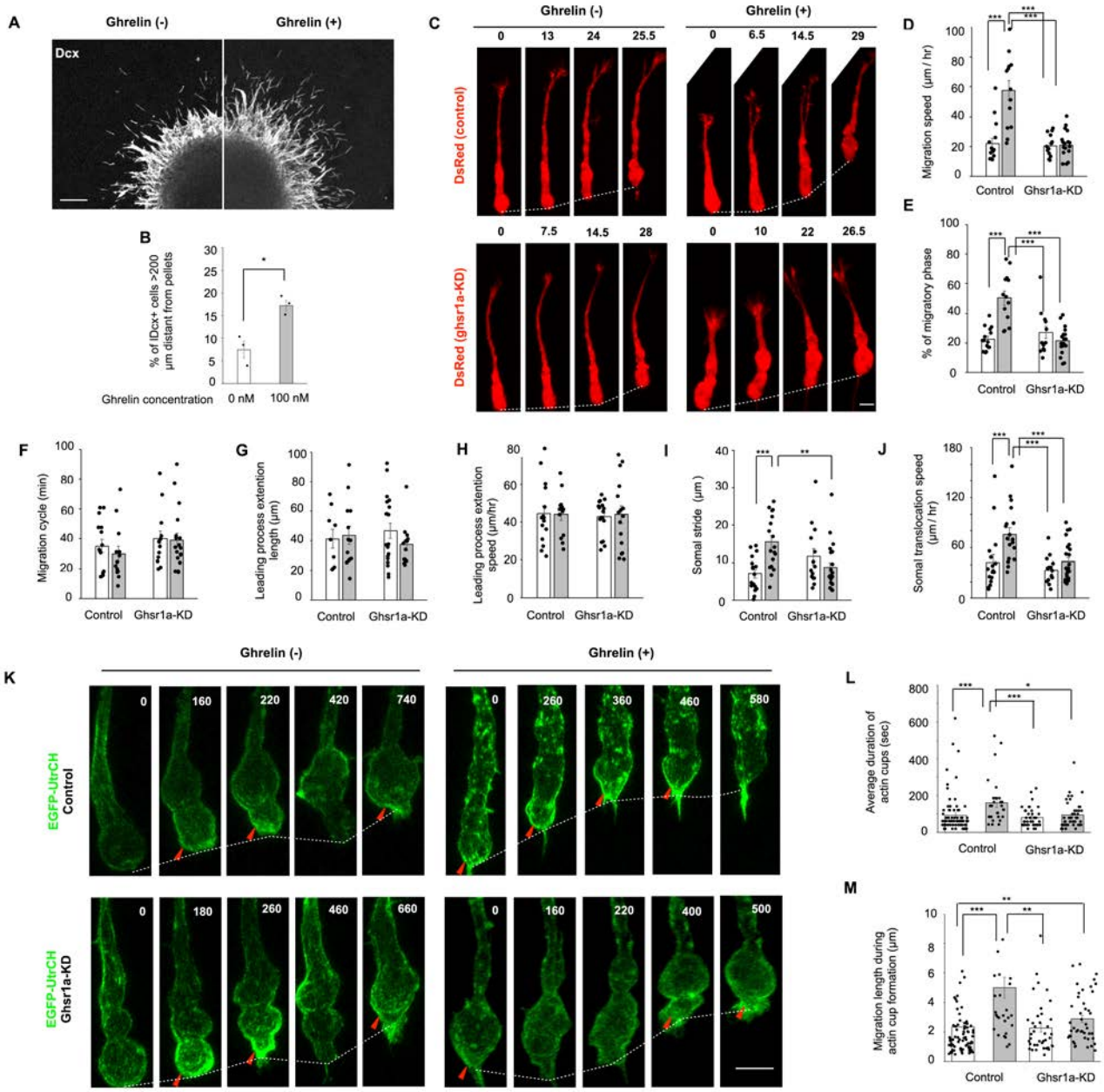

Figure. 4 Ghrelin promotes neuronal migration by activation of actin cup formation.

(A) Fluorescent images of Matrigel culture. Dcx (white).

(B) Percentage of Dcx+ cells > 200 µm distant from the edge of pellets (unpaired *t*-test; 3 independent cultures prepared on different days).

(C) Time-lapse images of cultured new neurons expressing DsRed (red). The number above each panel indicates minutes after initiation of migration.

(D–J) Migration speed (D), percentage of migratory phase (E), migration cycle (F), length/speed of leading process extension (G, H), and stride/speed of somal translocation (I, J) in neuronal

migration (one-way ANOVA followed by Turkey-Kramer test; D-F; control / Ghrelin (-), 15 cells, control / Ghrelin (+), 13 cells, KD / Ghrelin (-), 13 cells, KD / Ghrelin (+), 18 cells, I, J; control / Ghrelin (-), 17 events, control / Ghrelin (+), 18 events, KD / Ghrelin (-), 14 events, KD / Ghrelin (+), 23 events).

(K) Time-lapse images of actin cup formation (arrowheads) in the cell soma of new neurons. EGFP-UtrCH (green). Condensed dots of F-actin were scattered throughout the elongated cell soma in a control cell with ghrelin application.

(L, M) Average duration of actin cups (N) and migration distance during actin cup formation (O) in new neurons (Kruskal-Wallis test followed by the Steel-Dwass test; control / Ghrelin (-), 79 cells, control / Ghrelin (+), 31 cells, KD / Ghrelin (-), 39 cells, KD / Ghrelin (+), 44 cells).

Data are presented as the means ±SEM. Scale bars: A, 100 μm; C, 5 μm; K, 5 μm.

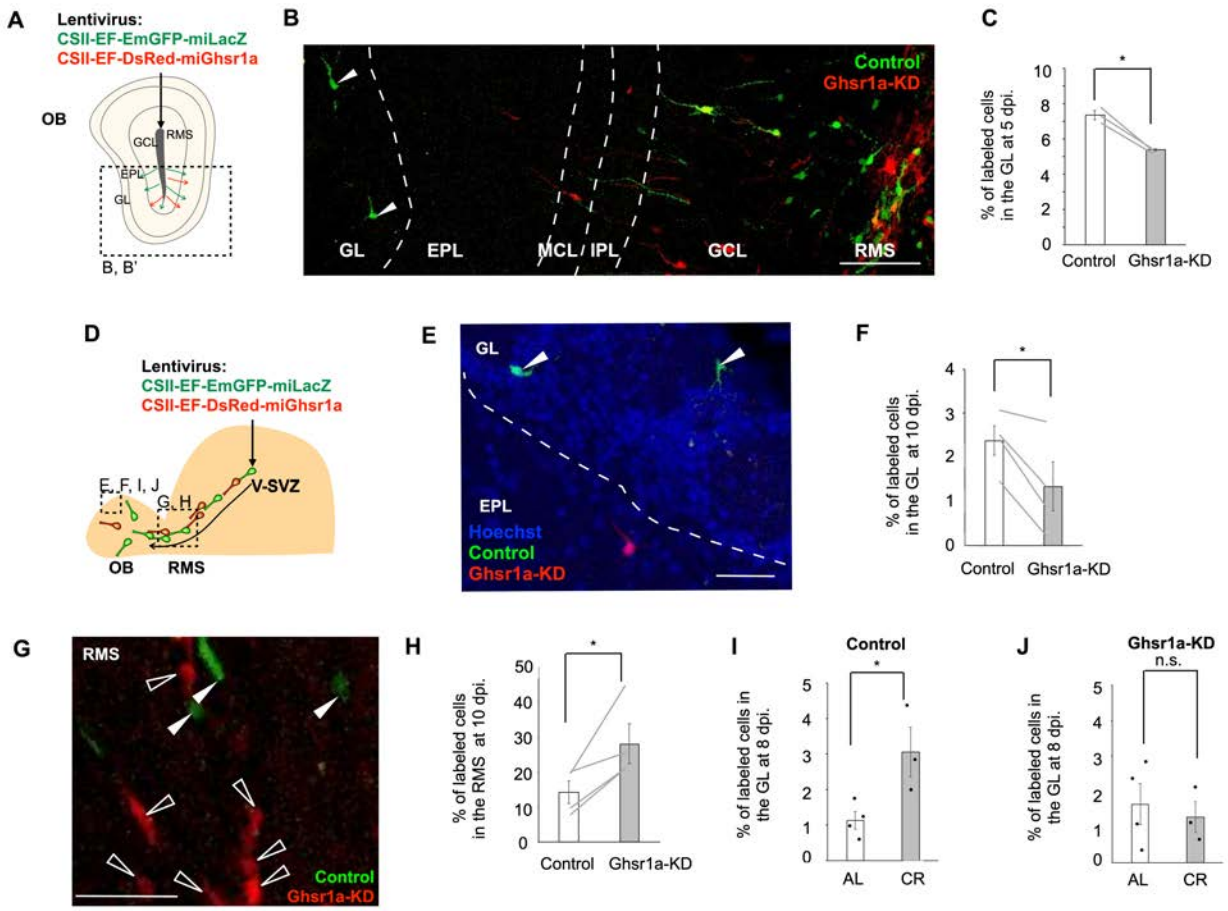

Figure. 5 Ghrelin signaling promotes neuronal migration in the adult brain.

(A, D) Experimental schemes.

(B) Fluorescent images of new neurons in the olfactory bulb in (A).

(C) Proportion of labeled cells in the GL at 5 dpi. in (A) (paired *t*-test; 3 mice).

(E, G) Fluorescent images of new neurons in the glomerular layer (GL) (E) and rostral migratory stream (RMS) (G) for the experiments shown in (D). Control cells (white arrowheads), ghsr1a-KD cells (clear arrowheads).

(F, H) Proportion of labeled cells in the GL (F) and the RMS (H) at 10 dpi. in (D) (paired *t*-test; 4 mice).

(I, J) Proportion of labeled cells in the GL at 8 dpi in the ad libitum (I) and calorie restriction (J) groups (Control, unpaired *t*-test; AL, 4 mice, DR, 3 mice) (KD, unpaired *t*-test; AL, 4 mice, CR, 3 mice).

Control (green), ghsr1a-KD (red). GL (glomerular layer), EPL (external plexiform layer), MCL (mitral cell layer), IPL (internal plexiform layer), GCL (granule cell layer), RMS (rostral migratory

1 stream). AL (ad libitum), CR (calorie restriction). Data are presented as the means ±SEM. Scale
2 bars: B, 100 μm; E, 40 μm; G, 40 μm.
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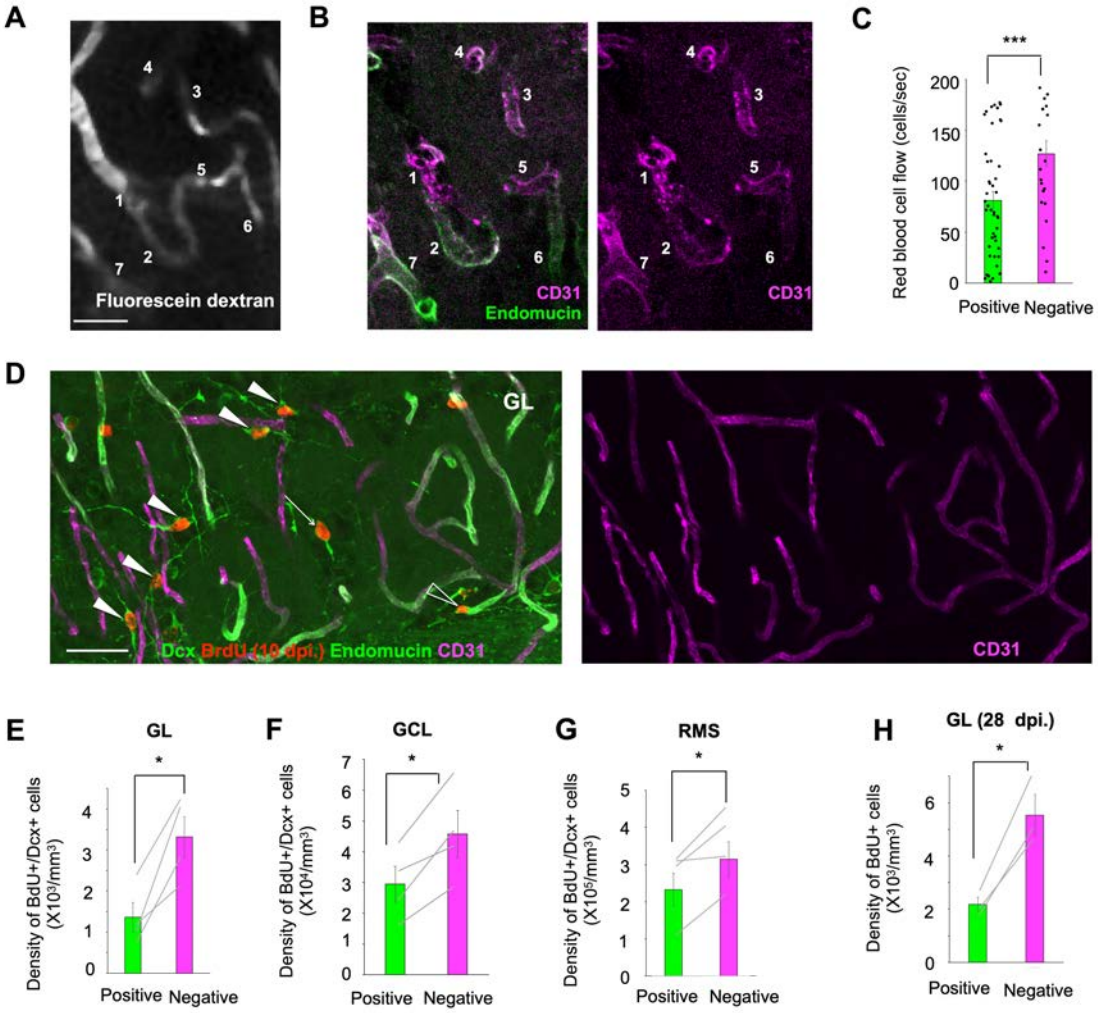

Figure. S1 New neurons migrate along endomucin-negative vessels.

(A, B) Representative images of vasculature in the glomerular layer (GL) of the olfactory bulb (OB). Red blood cell (RBC) flow was recorded in a live animal (A), followed by immunostaining of endomucin/CD31 in a fixed brain section (B). Identical vessels are indicated by different numbers (endomucin-positive; 2, 6, 7, endomucin-negative; 1, 3, 4, 5).

(C) Average RBC flow in endomucin-positive and endomucin-negative vessels (Mann-Whitney U-test; endomucin-negative, 24 vessels, endomucin-positive, 46 vessels).

(D) Fluorescent image of new neurons distributed in the vasculature in the GL. BrdU+/Dcx+ cells are shown in the perivascular region of endomucin-negative vessels (white arrowheads), endomucin-positive vessels (clear arrowhead), and distant from vessels (arrow). CD31 (magenta), endomucin (green, tube-like structures), Dcx (green), BrdU (red).

(E–G) Density of BrdU+/Dcx+ cells in the vicinity of endomucin-positive and endomucin-negative vessels in the GL (E), granule cell layer (F), and rostral migratory stream (OB core) (G) (paired *t*-test; 4 mice).

(H) Density of BrdU+ mature neurons at 28 dpi. in the vicinity of endomucin-positive and endomucin-negative vessels in the GL (paired *t*-test; 4 mice).

Data are presented as the means ±SEM. Scale bars: A, B, 20 μm; D, 20 μm.

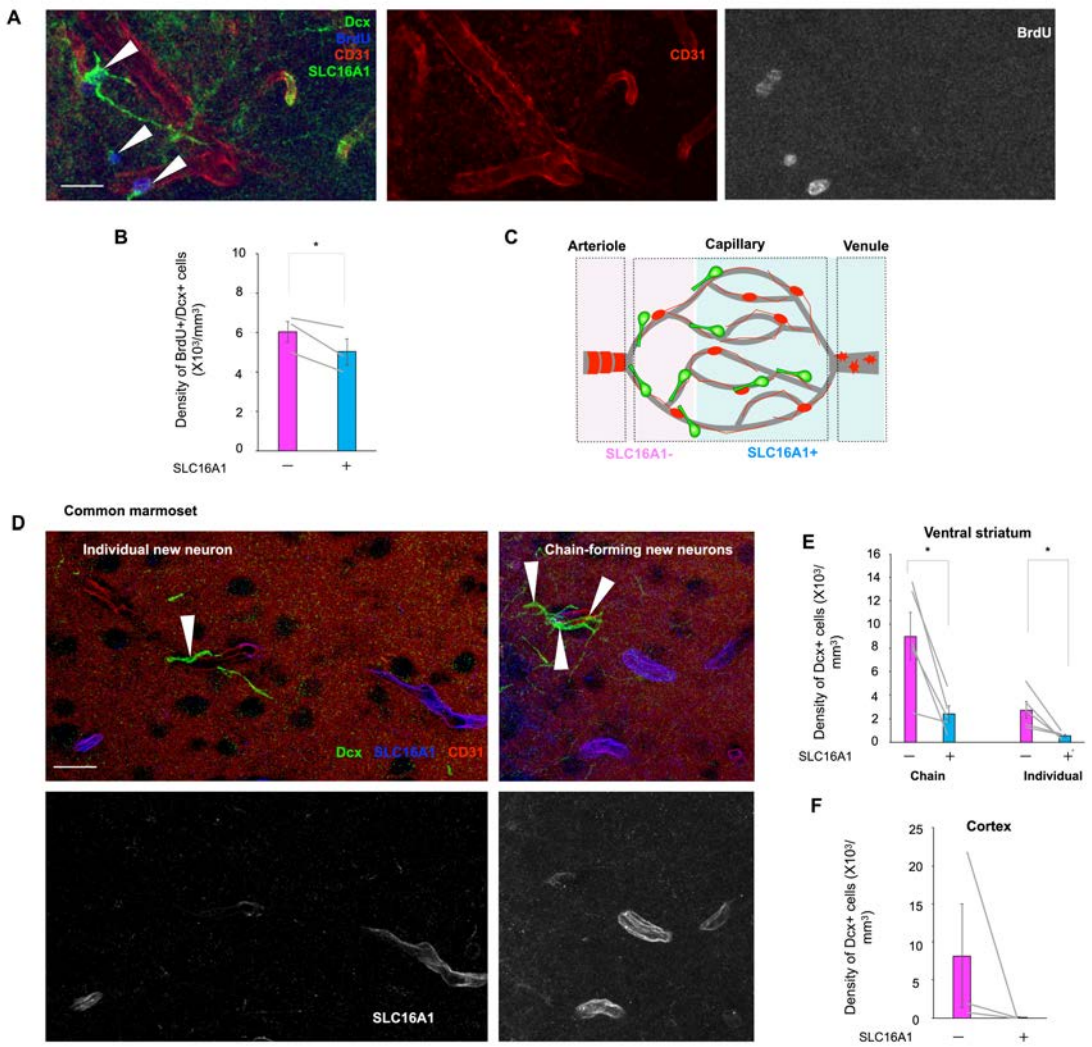

Figure. S2 New neurons exhibit a preference for arteriole-side vessels.

(A) Fluorescent image of immunostained tissue sections from the glomerular layer of the olfactory bulb. Dcx (green), BrdU (deep blue), CD31 (red), SLC16A1 (green, tube-like structures).

(B) Density of perivascular new neurons in the vicinity of SLC16A1-positive and SLC16A1-negative vessels (paired *t*-test; 4 mice).

(C) Schematic illustration of the distribution of new neurons and vessel identification.

(D) Fluorescent images of the ventral striatum from a 4-month-old common marmoset. Immature neurons are indicated by solid arrowheads. Dcx (green), SLC16A1 (deep blue), CD31 (red).

(E, F) Density of BrdU+/Dcx+ cells in the vicinity of SLC16A1-positive and SLC16A1-negative vessels in the ventral striatum (E) (paired *t*-test; 5 animals) and in the neocortex (F). Data are presented as the means ±SEM. Scale bars: A, 20 μm; D, 20 μm.

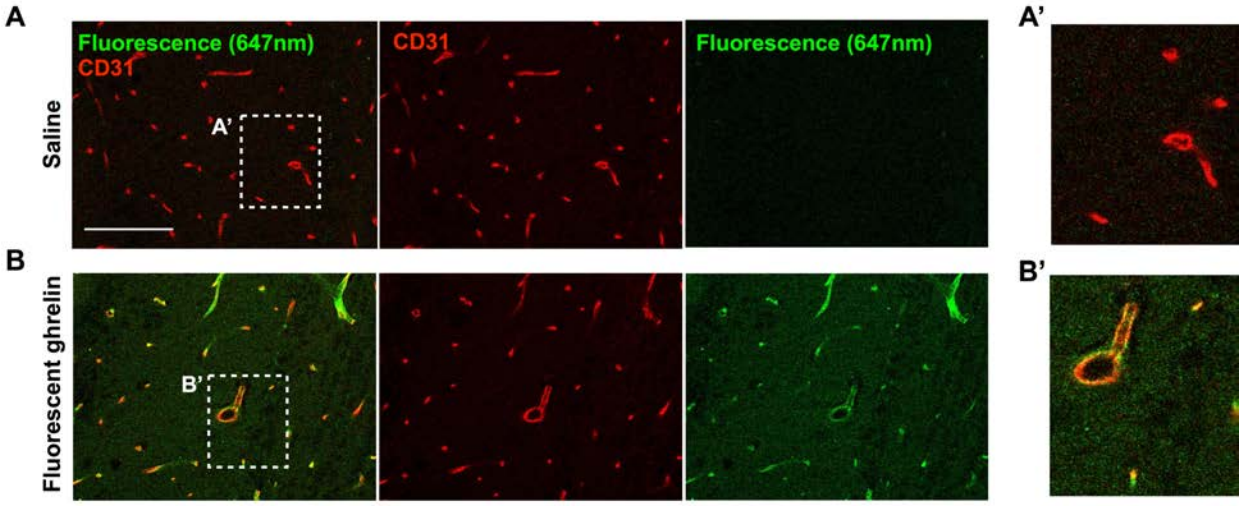

Figure. S3 Blood-derived ghrelin enters the RMS and OB.

(A, B) Representative images of the OB from mice with saline injection (A) and with fluorescent ghrelin injection (B). High-magnification images are shown in (A'), (B'). It was found that the experimental process did not affect the brightness of sections. CD31 (red), Dcx (magenta), fluorescence (647 nm) (green).

Scale bars: A,B, 100 μm.

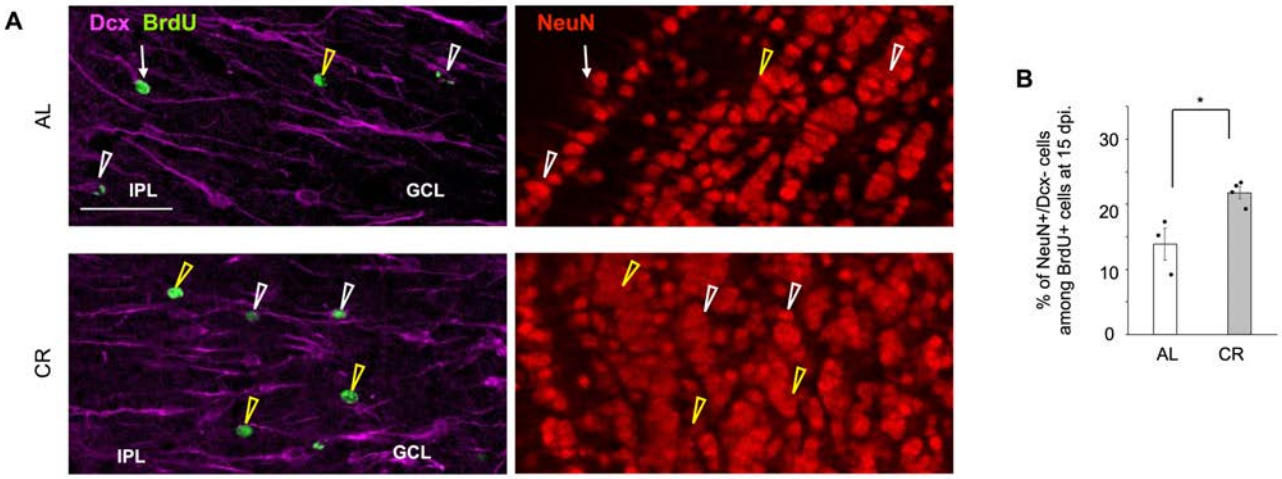

Figure. S4 Calorie restriction promotes neuronal maturation in the OB.

(A) Fluorescent images of new neurons in the OB. BrdU (green), NeuN (red), Dcx (magenta). A NeuN-/Dcx+ cell (white arrow), NeuN+/Dcx+ cells (white arrowheads), NeuN+/Dcx- cells (yellow arrowheads).

(B) Proportion of NeuN+/Dcx- cells among total BrdU+ cells in the OB (KD, unpaired *t*-test; AL, 3 mice, CR, 4 mice).

IPL (internal plexiform layer), GCL (granule cell layer). Data are presented as the means ±SEM. Scale bars: A, 50 μm.

Supplemental movie legends

Supplemental movie S1

A three-dimensional image from the rostral migratory stream of a *Dcx-EGFP* (green) mouse infused with RITC-Dex-GMA (red).

Supplemental movie S2

A three-dimensional image from the rostral migratory stream of a *Dcx-EGFP* / *Flt1-DsRed* mouse. EGFP (green), DsRed (red).

Supplemental movie S3

A three-dimensional, high-magnification image of chain-forming new neurons in the rostral migratory stream. *Dcx-EGFP* (green), *Flt1-DsRed* (red).

Supplemental movie S4

A three-dimensional image of immature neurons leaving the ventral ventricular-subventricular zone in a 1-month-old common marmoset infused with RITC-Dex-GMA (red). Dcx (green).

Supplemental movie S5

A three-dimensional image of an immature neuron from the ventral striatum in a 1-month-old common marmoset infused with RITC-Dex-GMA (red). Dcx (green).

Supplemental movie S6

Actin cup imaging in control cells. EGFP-UtrCH (green).

Supplemental movie S7

Actin cup imaging in ghsr1a-knockdown cells. EGFP-UtrCH (green).

Methods

Animals

All *in vivo* experiments were performed on 6 to 12-week-old C57BL/6J male mice. Wild-type mice were purchased from Japan SLC (Shizuoka, Japan). The following transgenic mice were used: *Dcx-EGFP* mice (Gong et al., 2003) provided by the Mutant Mouse Research Resource Center (MMRRC), *VGAT-Venus* line #39 (Wang et al., 2009), *Flt1-DsRed* (Matsumoto et al., 2012) and *NG2-DsRed* (Zhu et al., 2008). Cells were dissociated from postnatal day 0–1 (P0–P1) pups for *in vitro* experiments. Three to four-month-old postnatal common marmosets were obtained from three mating pairs in a domestic animal colony and used for immunohistochemistry. All animals were housed under a 12-hour light /dark cycle with ad libitum access to food and water. All experiments involving live animals were performed in accordance with the guidelines and regulations of Nagoya City University and the National Institute for Physiological Sciences.

BrdU administration

Bromodeoxyuridine (BrdU, MilliporeSigma), dissolved in sterile phosphate-buffered saline (PBS), was intraperitoneally administered to mice (50 mg/kg) twice with an interval of 2 hours. Mice were fixed at 10 days post injection (dpi) to observe immature neurons or 28 dpi to observe mature olfactory neurons.

Immunohistochemistry

Immunohistochemistry was performed as previously described for brain tissues of mice (Sawada et al., 2011)and common marmosets (Akter et al., 2021). Animals were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were removed from the skull and postfixed in the same fixative (24 hours for mice, 48 hours for common marmosets). Coronal sections were prepared using a vibratome (VT-1200S; Leica) (50 μm thick in mice, 60 μm thick in common marmosets). The sections were incubated with 10% normal donkey serum/0.2% Triton X-100 in PBS (blocking solution) for 30 min at room temperature (RT), the primary antibodies in blocking solution for 24 hours at 4°C, and AlexaFluor-/biotin-conjugated secondary antibodies (1:1000, Invitrogen) in the same solution for 2 hours at RT. For signal amplification, the sections were pretreated with 1% H_2O_2 in PBS for 40 min at RT before blocking. The signals were amplified with biotin-conjugated antibodies and a Vectastain Elite ABC Kit (Vector Laboratories) and

visualized using Tyramide Signal Amplification (Thermo Fisher Scientific). For BrdU staining, sections were treated with 1 M HCl at 37°C for 30 min after 1% H_2O_2 treatment. After staining, the sections were mounted with aqueous mounting medium (PermaFluor, Lab Vision Corporation). Z-stack images were obtained using an LSM700 confocal laser scanning microscope (Carl Zeiss) with a 20× objective (512 × 512 pixels, 1.25 μm per pixel, 1 μm z-step size). For cell density analysis, the perivascular region was defined as the area within 10 μm of the edge of CD31+ vessels. BrdU administration was performed to identify relatively immature cells in the Dcx+ cell population including newly generated cells with different differentiation states.

The following primary antibodies were used: rat anti-GFP (1:500, 04404-84, Nacalai Tesque, Inc.); rabbit anti-GFP (1:500, No. 598, Medical and Biological Laboratoies Co., LTD.); rabbit anti-DsRed (1:2000, 632496, Clontech); guinea pig anti-doublecortin (Dcx) (1:400, ab2253, MilliporeSigma); rabbit anti-Dcx (1:500, #4604, Cell Signaling Technology); rat anti-CD31 (1:100, 550274, BD Biosciences); mouse anti-human CD31 (1:100, Dako); rat anti-endomucin (1:500, sc-65495, Santa Cruz Biotechnology); rat anti-BrdU (1:100, ab6326, Abcam); rabbit anti-NeuN (1:1000, ab177487, abcam); and rabbit anti-SLC16A1 (1:500, TA321556, Origene). Nuclei were stained with Hoechst (1:5000, H1399, Thermo Fisher Scientific).

Three-dimensional imaging

For obtaining three-dimensional images from the rostral migratory stream (RMS), new neurons were visualized in *Dcx-EGFP* or *Dcx-EGFP/Flt1-DsRed* mice. Because the population of EGFP+ cells includes not only V-SVZ-derived migrating new neurons but also other types of cells in *Dcx-EGFP*, new neurons were labeled with adenoviruses encoding enhanced GFP (Ad-GFP, Vector Biolabs) for the analysis of new neuron-blood vessel interactions. Adenoviruses were stereotaxically injected into the V-SVZ (1.0 mm anterior, 1.1 mm lateral to bregma, and 1.6–2.0 mm deep) to label new neurons in the RMS and injected into the RMS (2.8 mm anterior, 0.82 mm lateral to bregma, and 2.8–3.0 mm deep) to label new neurons in the OB. The blood vessel lumen was visualized with RITC-Dex-GMA as previously reported with modifications (Miyawaki et al., 2020). At 5 dpi, mice were transcardially perfused with PBS and 4% PFA/0.1M PB followed by RITC-Dex-GMA. Mouse bodies were incubated in a 37°C water bath for 3 hours for polymerization. The brains were postfixed with SHIELD solutions (Lifecanvas Technologies) as previously reported (Park et al., 2018). Then, they were cleared using SmartClear II Pro (Lifecanvas Technologies). For visualization of new neurons in the common

marmoset brain, the brains were incubated in 10% normal donkey serum/ 0.5% Triton X-100 in PBS for 30 min, the anti-Dcx primary antibody in blocking solution for 5 days, and the AlexaFluor-conjugated secondary antibodies (1:1000, Invitrogen) in the same solution for 3 days at 37°C. Refractive index matching was performed using EasyIndex (Lifecanvas Technologies) before imaging. Z-stack images were acquired with a light-sheet fluorescent microscope (Carl Zeiss) with a 5× and 20× objective (1216 × 1216 pixels, 1.3 μm per pixel, 1.2–1.4 μm z-step size). Three-dimensional reconstruction was performed using Imaris software (Carl Zeiss). The three-dimensional distance was measured using ZEN software (Carl Zeiss) in light-sheet Z-stack images containing all of the GFP-positive cells in the OB hemisphere per mouse.

Bilateral carotid artery stenosis

Bilateral carotid artery stenosis was performed as previously described with modifications (Hattori et al., 2016; Shibata et al., 2004). After midline incision of the mouse cervical region, microcoils with an inner diameter of 0.18 mm (Sawane Spring Co., Ltd.) were wrapped around the common carotid arteries on both sides. Blood flow changes were confirmed by laser Doppler flowmetry in the anterior regions of brains (data not shown). A lentivirus encoding CSII-EF-Venus was stereotaxically injected into the V-SVZ to label new neurons. To analyze the cell distribution in the RMS and OB, mice were fixed at 5 dpi when glial activation is reported not to occur (Shibata et al., 2004).

Two-photon imaging

As described previously (Sawada et al., 2011), thinned-skull surgery was performed on wild-type mice for comparisons between RBC flow and endomucin expression in identical vessels and on *Dcx-EGFP* and *VGAT-Venus* mice for observation of neuronal migration and maturation, respectively. Blood vessels were visualized by intravenous injection of Rhodamine-B dextran (D1841, Invitrogen) or Fluorescein dextran (D1823, Invitrogen). Mice were anesthetized by inhalation of isoflurane. The heads were immobilized with ear bars on a stereotactic stage before surgery. The skull over the OB was carefully thinned with a high-speed drill (MINITER Co., Ltd.) and a surgical blade (Fine Science Tools). The thinned-skull window was observed under a two-photon laser scanning microscope (Nikon) and mode-locked system at 950 nm (Mai Tai HP, Spectra Physics) with a 25× water-immersion objective. Neuronal migration was recorded by identification of EGFP+ cells that changed their positions during the imaging period

in the whole visible imaging field. During imaging, mice were anesthetized by intraperitoneal administration of a mixture of medetomidine (Meiji Seika Pharma Co., Ltd.), midazolam (SANDOZ), and butorphanol (Meiji Seika Pharma Co., Ltd.) (0.75 mg/kg, 4 mg/kg, and 5 mg/kg, respectively) and kept on a heating pad for maintenance of the body temperature at 37°C. Image stacks (2048 × 2048 pixels, 0.25 μm per pixel, 2-μm z-step size) were acquired every 30–60 min during 3 hours. RBC flow was recorded by serial line-scans as previously reported (Kleinfeld et al., 1998). Line-shaped regions of interest were drawn along the longitudinal axis of each blood vessel. The RBC flow/s was calculated from repetitive scans obtained during 10 s at the beginning of the neuronal-migration recording. The median RBC flow velocity (38.4 RBCs/s) was used as a criterion for classification of vessels with different blood flows, whose distribution is not normal. Neuronal maturation was recorded as previously described with modifications (Sawada et al., 2011). After mice were anesthetized by isoflurane inhalation, the thinned-skull window was observed to record positions of Venus+ cells and RBC flow from each vessel in square-shaped regions (512 × 512 pixels, 0.5 μm per pixel, 2-μm z-step size). The same region of the GL was observed with an interval of 21 days. Stationary cells were defined as cells that were observed in the same position at both time points. Maturation and cell death in the GL were identified as addition and loss of Venus+ cells at the second time point. Data analysis was performed using NIS Element software (Nikon).

Photothrombotic clot formation

Photothrombotic clot formation was performed as previously reported with modifications (Schaffer et al., 2006). After identification of a blood vessel close to migrating neurons, an upstream vessel fragment was surrounded by a rectangular region of interest. Mice were intravenously injected with 20 mg/ml rose bengal (330000, Sigma-Aldrich) in PBS at concentration 0.05 mg/g. Immediately after injection, a selected fragment was irradiated using a two-photon laser at 950 nm. Irradiation by a 100-mW laser was performed for 5~10 seconds until the clot formed. The inner space of the vessel was equally irradiated by continuous movement of the imaging area. Ten minutes after introduction of rose bengal, the RBC flow of a target vessel was recorded to confirm blood flow inhibition. The behavior of migrating neurons was observed for 3 hours after clot formation and compared with that before photothrombosis. As a control experiment, vessels without rose Bengal injection were irradiated with a two-photon laser. Samples were excluded if bleeding occurred or clots were dissolved during observation.

Transmission electron microscopy

Transmission electron microscopy analysis was performed as previously described with modifications(Matsumoto et al., 2019; Sawada et al., 2018). Brain were fixed in 2.5% glutaraldehyde and 2% PFA in 0.1 M PB (pH 7.4) at 4°C, postfixed with 2% OsO_4 in the same buffer at 4°C, dehydrated in a graded ethanol series, placed in propyleneoxide, and embedded in Durcupan resin for 72 hours at 60°C to ensure polymerization. Ultra-thin sections (60–70 nm) were cut using an ULTRACUT-E (Reichert-Jung) with a diamond knife, stained with 2% uranyl acetate in distilled water for 15 min, and stained with modified Sato's lead solution for 5 min. Sections were analyzed with a transmission electron microscope (JEM-1011J; JEOL, Tokyo, Japan). Migratory neurons was identified as cells with an elongated cell body, a dark cytoplasm with many free ribosomes, and an electron-dense nucleus with multiple nucleoli (Doetsch et al., 1997; Matsumoto et al., 2019; Sawada et al., 2018).

Protein labeling

For observation of ghrelin transcytosis across the vascular wall, recombinant octanoylated ghrelin (Human, sc-364689, Santa Cruz Biotechnology) was fluorescently labeled with Atto 647N NHS ester (18373, Sigma-Aldrich) as previously described with modifications(Yang et al., 2020). Ghrelin was dissolved in 0.1 M bicarbonate buffer (pH 8.3) at 2 mg/ml and reacted with Atto 647N NHS ester for 60 min at RT. After reactions, free label was removed by gel permeation chromatography with PD MiniTrap™ G-25 columns (28918007, Cytiva). Mice were fixed as described above at 1 hour after intravenous injection of fluorescently labeled ghrelin (0.02 mg / 30g). Fluorescence signal intensity was measured by ZEN software. The average intensity among all vessels in each mouse was normalized to 1.0.

V-SVZ culture experiments

The V-SVZ was dissected from P0–P1 mice, cut into blocks, and embedded in 60% Matrigel (BD Biosciences) in L-15 medium (Gibco). Cell aggregates were cultured in Neurobasal medium containing 2% NeuroBrew-21 (Invitrogen), 2 mM L-glutamine (Gibco), and 50 U/ml penicillin-streptomycin (Gibco) at 37°C in a 5% incubation system (Tokai Hit). For migration distance analysis, octanoylated ghrelin (Human, Rat, 1-10, Peptides International) was added to the medium at a final concentration of 100 nM at 24 hours post-embedding (hpe). Then, cell aggregates were fixed in 4% PFA/0.1M PB at 48 hpe. For immunocytochemistry, aggregates were incubated in blocking solution for 30 min at RT, treated with the primary antibodies in

blocking solution for 24 hours at 4°C, and treated with AlexaFluor-secondary antibodies (1:1000) in the same solution for 2 hours at RT. The migration distance was analyzed in three independent cultures prepared on different days.

Viral vectors and plasmids

The pCSII lentiviral expression vectors were provided by Dr. Hiroyuki Miyoshi (RIKEN Tsukuba BioResource Center). The lacZ- and ghsr1a-KD plasmids were generated as previously described (Ota et al., 2014; Sawada et al., 2018). The target sequences of lacZ mRNA and mouse ghsr1a mRNA (Invitrogen) were inserted into modified Block-iT Poll II miR RNAi expression vectors containing EmGFP or DsRed-Express (Invitrogen). The Gateway System (Invitrogen) was used to generate pCSII-EF-Venus, pCSII-EF-Ghsr1a-IRES-Venus, pCSII-EF-EmGFP-express-miR-lacZ, pCSII-EF-DsRed-express-miR-ghsr1a, pCAGGS-DsRed-express-miR-lacZ, and pCAGGS-DsRed-express-miR-ghsr1a. For lentivirus production, the packaging vectors (pCAG-HIVgp, pCMV-VSV-G-RSV-Rev) and pCSII viral vectors were co-transfected into HEK-293T cells to generate lentivirus particles. Then, the culture supernatants were concentrated by centrifugation at 8,000 rpm for 16 hours at 4°C in an MX-307 refrigerated microcentrifuge (Tomy).

Ghsr1a KD experiments

The following sequence was inserted into siRNA expression vectors for targeting ghsr1a mRNA: TGCTGAAGATGAGCAGATCGGAGAAGGTTTTGGCCACTGACTGACCTTCTCCGCTGCTCA TCTTCAGG. For confirming efficacy of ghsr1a KD, pCSII-EF-Ghsr1a-IRES-Venus and/or pCSII-EF-DsRed-express-miR-ghsr1a were co-transfected in HEK-293T cells. Venus signal was not observed in DsRed+ ghsr1a-KD cells (data not shown).

For *in vitro* experiments, the dissected V-SVZ was dissociated with trypsin-EDTA (Invitrogen). The pCS2-EGFP-UtrCH was provided by Dr William Bement (University of Wisconsin-Madison) and Dr David J. Solecki (St. Jude Children's Research Hospital). The cells were washed in L-15 medium with 40 μg/ml DNaseI (Roche) and transfected with 2 μg plasmid DNA using the 4D-Nucleofector (Lonza). The cells were recovered in RPMI-1640 medium (Thermo Fisher Scientific) and embedded in 60% Matrigel in L-15. After cultivation in Neurobasal medium for 48 hours, time-lapse imaging was performed using an LSM880 confocal laser scanning microscope with a 40× objective (Carl Zeiss). Time-lapse images were captured at 30-s (Figure. 4C–J) and 20-s (Figure. 4K–M) intervals. The migration distance of cultured new neurons was

measured using ImageJ manual tracking tools. A migratory phase was defined as a phase in which the cell soma traveled ≥ 30 μm during 1 hour, and a resting phase was defined as a phase in which the cell soma migrated < 30 μm. ZEN software (Carl Zeiss) was used to analyze actin cup formation. Actin cups were defined as over 4-μm-long continuous EGFP-UrtCH signals that were 1.3 times brighter than those in other cell soma regions.

For *in vivo* experiments, the lentiviral solution was stereotaxically injected into the V-SVZ and OB core of adult mice (V-SVZ: 1.0 mm anterior, 1.1 mm lateral to bregma, and 1.6–2.0 mm deep) (OB core: 4.6 mm anterior, 0.9 mm lateral to bregma, and 0.6–0.9 mm deep). The proportions of EmGFP+/DsRed- and EmGFP-/DsRed+ cells among total labeled Dcx+ new neurons in the RMS and OB were analyzed using ZEN software (Carl Zeiss). Calorie restriction was performed as previously reported (Hornsby et al., 2016). In migration analysis, calorie-restricted animals were fed 70% of the total food consumed by animals fed ad libitum daily for the last 5 days prior to fixation at 8 dpi. In maturation analysis, calorie restriction was performed from Day 3 to Day 8 after BrdU administration at Day 0, following fixation at Day 15.

Statistics

Statistical analysis was performed using EZR software (Kanda, 2013). The normality of the data was analyzed using a Kolmogorov-Smirnov test or Shapiro-Wilk test. The equality of variance was analyzed using an *F* test. Comparisons of data between two groups were performed with unpaired/paired *t*-tests or Welch's *t*-test for normally distributed data and by Mann-Whitney *U*-tests/Wilcoxon signed-rank tests for abnormally distributed data. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA)/one-way repeated measures ANOVA/Kruskal-Wallis tests followed by a post hoc Tukey-Kramer test, Bonferroni test, or Steel-Dwass test. Numerical data are presented as the means ± standard error of the mean. A p value < 0.05 was considered statistically significant. Significance is indicated in graphs as follows: *p < 0.05, **p < 0.01, ***p < 0.005, n.s., not significant.

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Author contributions

TO, AS, MS, ST, JN, HK, HI, VHP, YM, ME, JMGV, JN, and KS performed experiments and analyzed the data. TO and KS wrote the manuscript.

Declaration of interests

The authors declare that they have no competing interests.

Data and materials availability

All data are available in the main text or in the supplementary materials.