Title: Dual cholinergic signals regulate daily migration of hematopoietic stem cells and leukocytes

Short title: cholinergic control of daily HSC&WBC traffic

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- Central parasympathetic signals repress sympathetic tone at night to orchestrate day/night HSPC traffic.
- Local sympathetic cholinergic signals modulate HSPC traffic by inhibiting diurnal BM vascular adhesion and β_3-adrenergic signaling at night.

Keywords: Hematopoietic stem cell niche, cholinergic, traffic, circadian rhythms, day/night, oscillations, autonomic nervous system, sympathetic, parasympathetic

Abstract

Hematopoietic stem and progenitor cells (HSPCs) and leukocytes circulate between the bone marrow (BM) and peripheral blood following circadian oscillations. Autonomic sympathetic noradrenergic signals have been shown to regulate HSPC and leukocyte trafficking, but the role of the cholinergic branch has remained unexplored. We have investigated the role of the cholinergic nervous system in the regulation of day/night traffic of HSPCs and leukocytes in mice. We show here that the autonomic cholinergic nervous system (including parasympathetic and sympathetic) dually regulates daily migration of HSPCs and leukocytes. At night, central parasympathetic cholinergic signals dampen sympathetic noradrenergic tone and decrease BM egress of HSPCs and leukocytes. However, at daytime, de-repressed sympathetic noradrenergic activity causes predominant BM egress of HSPCs and leukocytes via β_3-AR. This egress is locally supported by light-triggered sympathetic cholinergic activity, which inhibits BM vascular cell adhesion and homing. In summary, central (parasympathetic) and local (sympathetic) cholinergic signals regulate day/night oscillations of circulating HSPCs and leukocytes. This study shows how both branches of the autonomic nervous system cooperate to orchestrate daily traffic of HSPCs and leukocytes.

Introduction

The BM microenvironment for hematopoietic stem cells (HSCs) includes many different cell types that dynamically regulate HSC quiescence, maintenance, activation, proliferation, differentiation and migration.[1] We hypothesized that a master regulator of tissue homeostasis –the autonomic nervous system– might orchestrate different HSC responses to meet physiological demands. This system

comprises the enteric, sympathetic (SNS) and parasympathetic (PNS) nervous systems. Norepinephrine and acetylcholine are generally postganglionic neurotransmitters of (noradrenergic) SNS and (cholinergic) PNS. However, some sympathetic neurons switch from noradrenergic to cholinergic phenotype during postnatal development in periosteum and salivary glands,[2-4] but the role of sympathetic cholinergic fibers in bone has remained elusive.

Sympathetic noradrenergic fibers innervate BM[5] and regulate physiological traffic of HSCs and leukocytes, which follows day/night oscillations in mice[6,7] and humans.[8,9] BM noradrenergic fibers[10] and central cholinergic muscarinic signals[11] regulate HSPC mobilization enforced by granulocyte colony-stimulating factor (G-CSF). However, whether the cholinergic (sympathetic/ parasympathetic) nervous system regulates physiological HSPC traffic is unknown. Elucidating this regulation might explain rhythmic HSC and leukocyte traffic and, more importantly, suggest new approaches to therapeutically improve HSC homing/egress.

In mice, the SNS has been previously involved in 1) HSC and leukocyte release from BM into circulation at daytime[6] and 2) their BM homing during evening/night.[12] Therefore, it has remained enigmatic how similar noradrenergic signals can stimulate two opposite processes (BM egress and homing) at a different time. Therefore, we studied whether the cholinergic nervous system interacts with the noradrenergic nervous system to orchestrate day/night migration of HSCs and leukocytes.

Mice show two daily peaks of adrenergic receptor-mediated HSPC activity: one during darkness (when mice are more active) and another peak following light exposure (when the resting period starts).[13] This is consistent with the observed day/night fluctuations of their ligands norepinephrine (noradrenergic) and epinephrine (adrenergic) in murine/human plasma.[14,15] Whereas norepinephrine is the principal sympathetic neurotransmitter released in BM, blood epinephrine derives mainly from the adrenal glands. Furthermore, epinephrine and norepinephrine exhibit opposite, >30-fold higher affinities for β_2-AR and β_3-AR, respectively.[16] Thus, we hypothesized that norepinephrine and epinephrine might play differential roles in HSPC and leukocyte migration by activating different β-ARs.

To understand this complex regulatory pathway, we have analyzed HSPC and leukocyte traffic in rodents with impaired cholinergic neurotransmission during day/night cycles. We have uncovered a dual cholinergic regulation of the rhythmic migration of HSPCs and leukocytes. At night, central

parasympathetic signals antagonize sympathetic noradrenergic activity to restrict HSPC egress. Parasympathetic cholinergic regulation cooperates with endocrine epinephrine-β_2-AR signaling to trigger BM homing. Light exposure acutely induces sympathetic activity and suppresses parasympathetic tone.[17,18] Consequently, dawning triggers sympathetic release of norepinephrine (noradrenergic) and acetylcholine (cholinergic) in the BM, which activate β_3-AR signaling and inhibit vascular adhesion, respectively. This concerted action in the morning reduces BM homing and permits daily egress of HSPCs and leukocytes. Therefore, this study shows how central (parasympathetic) and peripheral (sympathetic) cholinergic neural signals regulate physiological migration of HSCs and leukocytes.

Methods

Age and sex-matched *Gfra2*$^{-/-}$ [19], *Nes-gfp* [20] (gift from G.E. Enikolopov), *Adrb2*$^{tm1Bkk/J}$ [21] (gift from G. Karsenty), *Adrb3*^{tm1Lowl}, *B6;129X1-Nrtn*^{tm1Jmi}*/J (Nrtn*$^{-/-}$*)* [22] and congenic CD45.1 and CD45.2 C57BL/6J mice (Jackson Laboratories) were used in this study. Mice were housed in specific pathogen free facilities. All experiments followed protocols approved by the Animal Welfare Ethical Committees in Centro Nacional de Investigaciones Cardiovaculares (CNIC) and University of Cambridge (PPL 70/8406) and were compliant with EU recommendations. Detailed methods for *in vivo* experiments, flow cytometry, cell culture, LT-CRU, homing assays, immunofluorescence, ELISA and Q-PCR are available in the supplemental Methods online.

Results

The parasympathetic nervous system inhibits sympathetic noradrenergic activity centrally to reduce circulating HSPCs at night

The GDNF family receptor alpha 2 (GFRα2) promotes development and survival of cholinergic neurons (either parasympathetic or sympathetic).[19,23] Therefore, we used *Gfra2*$^{-/-}$ mice as a model to study the cholinergic regulation of HSPC traffic. *Gfra2*$^{-/-}$ mice have deficient parasympathetic cholinergic innervation of lacrimal and salivary glands, small bowel,[19] endocrine pancreas[24] and reproductive organs.[25] Interestingly, parasympathetic activity follows daily oscillations in mice, with increased activity during day-night shift in stomach,[26] lungs[27] and heart.[28,29] Consistent with these studies, we found that

acetylcholinesterase (AChE) cholinergic activity appeared higher in wild type (WT) urine collected from nightshift to morning, compared with daytime harvest (Figure 1A). Urine AChE activity was halved in *Gfra2*$^{-/-}$ mice at night, suggesting widespread deficient parasympathetic activity in *Gfra2*$^{-/-}$ mice manifesting at night.

Murine HSPCs and leukocytes are preferentially released into circulation during day[6], whereas more white blood cells (WBCs) home to BM at night.[12] We quantified WBCs and circulating HSPCs, measured as colony forming units in culture (CFU-Cs), over 24h. Control mice exhibited normal oscillations of circulating HSPCs and WBCs.[6] These cells peaked 5h after light onset (at *Zeitgeber* time 5, ZT5; morning) and reached a nadir early at night (ZT13) similarly in WT mice and *Gfra2*$^{+/-}$ mice (Figure 1B-C and Figure S1A), which were used as control littermate mice in most experiments. In contrast, *Gfra2*$^{-/-}$ mice showed normal daytime values but at ZT13 exhibited 2-3-fold more circulating HSPCs and WBCs (Figure 1B-C), whereas other blood parameters remained unchanged (Figure S1B-E). To measure circulating HSCs, we performed competitive long-term repopulation assays using limiting dilutions of blood harvested at ZT13. *Gfra2*$^{-/-}$ mice showed 3.5 times more circulating HSCs early at night (Figure 1D). Despite this difference, BM nucleated cells and BM HSPCs remained unchanged (Figure S1F-G), as expected since their numbers are much higher in BM than in bloodstream. The frequency of leukocyte subsets (Figure S2A-H) and other cell types (not shown) appeared normal in *Gfra2*$^{-/-}$ blood and BM, excluding differentiation defects. These results show a time-specific accumulation of circulating HSPCs and WBCs in cholinergic-deficient mice at night.

Previous studies have suggested that sympathetic noradrenergic fibers contribute to BM HSC egress,[6,7] G-CSF-induced mobilization[10] and WBC BM homing.[12] We stained tyrosine hydroxylase (TH)$^{+}$ noradrenergic fibers and found them doubled in *Gfra2*$^{-/-}$ BM (Figure 2A-C). In addition, urine concentration of norepinephrine was doubled in *Gfra2*$^{-/-}$ mice at night (Figure 2D), suggesting that parasympathetic deficiency in *Gfra2*$^{-/-}$ mice de-represses noradrenergic activity at night, possibly triggering abnormal nocturnal release of HSPCs and leukocytes. To investigate whether cholinergic signals repress noradrenergic activity centrally or peripherally, we i.p. injected WT mice with blood-brain barrier (BBB)-permeable or BBB-non-permeable cholinergic (acetylcholine) antagonists (Figure 2E). Only BBB-permeable antagonists (mecamylamine and scopolamine) increased circulating HSPCs and WBCs at ZT13 (Figure 2F-G), suggesting a central inhibition of sympathetic tone by the PNS. This

result expands previous findings on the central cholinergic regulation of HSC mobilization enforced by G-CSF[11] to the regulation of physiological traffic of HSPCs and leukocytes. Therefore, central parasympathetic inhibition of sympathetic tone reduces BM release of HSPCs and leukocytes at night.

Cholinergic signaling regulates HSPC BM homing by modulating β_2-AR-dependent BM vascular adhesion

Next, we investigated whether nocturnal BM homing alteration in *Gfra2*$^{-/-}$ mice could further explain the accumulation of circulating HSPCs and WBCs. We transplanted donor cells at ZT10 (evening) using *Gfra2*$^{-/-}$ mice and control *Gfra2*$^{+/-}$ mice as donors/recipients. We analyzed recipient mice at ZT2 (early morning) to measure overall nocturnal BM homing (Figure 3A), which appeared normal in myeloablated mice (Figure 3B). However, because lethal irradiation enforces BM homing and might mask differences, we independently transplanted non-irradiated mice (Figure 3C). Surprisingly, nocturnal HSPC BM homing was not impaired (as it would be expected from HSPCs accumulating in circulation), but instead doubled in non-myeloablated *Gfra2*$^{-/-}$ mice (Figure 3D and Figure S3A-C). Homing of donor *Gfra2*$^{-/-}$ HSPCs in control recipient mice was normal (Figure 3D), indicating non-cell-autonomous cholinergic regulation of HSPC homing, which we next investigated at molecular level.

Firm adhesion to endothelium is the first step during BM homing[30] and requires endothelial selectins and vascular cell adhesion protein 1 (Vcam1).[31-33] Moreover, expression of *Vcam1* and *Sele* (E-selectin) mRNA peaks at night, when HSPCs and WBCs preferentially home to BM.[12] In control mice, we confirmed day/night oscillations of *Vcam1* and *Sele* mRNA peaking at ZT13. P-selectin (*Selp*) showed a similar trend (Figure 3E-G). Interestingly, *Gfra2*$^{-/-}$ BM showed higher mRNA expression of *Vcam1* (4.5-fold) and *Sele* (6.2-fold) at night (Figure 3E-F and 4A-B). *Selp* showed similar but non-significant trend (Figure 3G and 4C). Deregulated adhesion molecule expression specifically affected endothelial cells, which also exhibited enriched *Sele* and *Selp* mRNA expression, compared with non-endothelial stromal cells (Figure 4D-F). Flow cytometry confirmed increased Vcam1 protein in endothelial cells from *Gfra2*$^{-/-}$ mice at ZT13 (Figure S4A). To test whether increased adhesion explains enhanced BM homing in *Gfra2*$^{-/-}$ mice, we injected *Gfra2*$^{-/-}$ mice and control *Gfra2*$^{+/-}$ mice with control IgG or blocking antibodies against integrin α_4 (VLA-4, receptor for Vcam1), E-selectin and P-selectin before the peak of homing (ZT11; Figure 4G). Adhesion-blocking antibodies increased circulating HSPCs in both *Gfra2*$^{-/-}$ mice and control *Gfra2*$^{+/-}$ mice (as expected) but cancelled out the nocturnal differences between them

(Figure 4H). Therefore, enhanced nocturnal HSPC homing in *Gfra2*$^{-/-}$ mice is caused by increased BM vascular adhesion.

Nocturnal induction of these adhesion molecules by noradrenergic signals promotes WBC BM homing mainly through β_2-AR.[12] To dissect β-AR function in enhanced BM homing in cholinergic-neural-deficient mice, we intercrossed *Gfra2*$^{-/-}$ mice with mice lacking the β-ARs involved in HSPC/leukocyte traffic (β_2-AR and β_3-AR).[34] The 5-6-fold-increased mRNA expression of *Vcam1* and *Sele* found in *Gfra2*$^{-/-}$ mice normalized in *Gfra2*$^{-/-}$;*Adrb2*$^{-/-}$ mice (Figure 4A-B). To confirm the β_2-AR role, we measured HSPC homing in recipient mice previously injected with β_2-AR antagonist. This treatment normalized BM HSPC homing in *Gfra2*$^{-/-}$ mice (Figure 4I and Figure S4B), suggesting that increased sympathetic noradrenergic activity in cholinergic-neural-deficient mice promotes HSPC BM homing through β_2-AR.

Exacerbated sympathetic noradrenergic activity in cholinergic-neural-deficient mice causes abnormal BM egress of HSPCs and leukocytes via β_3-adrenergic receptors

Despite increased BM homing, cholinergic-neural-deficient mice accumulate circulating HSPCs and WBCs at night (Figure 1B-C). Therefore, we hypothesized that abnormal nocturnal BM egress might override increased BM homing in *Gfra2*$^{-/-}$ mice. Cxcl12-Cxcr4 axis regulates HSPC and leukocyte migration.[35,36] We have previously shown that light-triggered sympathetic signals decrease BM Cxcl12 expression and permit HSPC egress to circulation.[6] Therefore, we investigated whether exacerbated sympathetic noradrenergic activity in *Gfra2*$^{-/-}$ mice (in absence of inhibitory cholinergic signals) causes abnormal nocturnal BM release of HSPCs and leukocytes. To dissect hematopoietic-cell-autonomous and non-cell-autonomous effects, we generated chimeric mice through long-term reciprocal BM transplantations. Control recipients of *Gfra2*$^{-/-}$ hematopoietic cells showed normal WBCs. In contrast, *Gfra2*$^{-/-}$ recipient mice showed nocturnal accumulation of circulating HSPCs and WBCs (Figure 5A-B), suggesting non-cell-autonomous defects in HSPC and leukocyte traffic in *Gfra2*$^{-/-}$ mice.

Light-triggered sympathetic activity reduces BM Cxcl12 expression and permits HSPC egress.[6,37] We found that increased circulating HSPCs and WBCs correlated with reduced *Cxcl12* mRNA and protein expression in *Gfra2*$^{-/-}$ BM at the same time, whereas Cxcr4 expression was unchanged (Figure 5C-D and Figure S5A-B). Stem cell factor (*Kitl*) also showed reduced mRNA expression in *Gfra2*$^{-/-}$ BM during this period (Figure 5E). Both *Cxcl12* and *Kitl* are highly expressed by nestin^{+} BM mesenchymal

stem/progenitor cells (BMSCs), which regulate HSPC migration.[38] Therefore we intercrossed *Gfra2*$^{-/-}$ mice with *Nes-gfp* mice to measure nestin^{+} BMSCs. We dissected different Nes-GFP^{+} subpopulations[39] by mechanical separation of endosteal/non-endosteal cells.[40] Nes-GFP-high (Nes-GFP^{hi}) cells have been previously found associated with central arterioles and endosteal transition zone vessels connecting arterioles with sinusoids.[39,41,42] Nes-GFP-low (Nes-GFP^{lo}) cells coincide with Lepr^{+} cells in non-endosteal sinusoids,[43] through which HSPCs and leukocytes transmigrate.[41] Whereas Nes-GFP^{hi} cells were unchanged, Nes-GFP^{lo} cells were 40%-reduced in non-endosteal *Gfra2*$^{-/-}$ BM (Figure 5F). Therefore, decreased Cxcl12-dependent HSPC retention at ZT13 correlates with reduced non-endosteal Nes-GFP^{lo} cells in *Gfra2*$^{-/-}$ BM. Whereas this cholinergic regulation clearly involves nestin^{+} BMSCs and endothelial cells, it might also target other BM cells.

Since β-adrenergic signaling regulates *Cxcl12* expression in nestin^{+} cells,[6] we studied its possible role in enforced BM egress in cholinergic-neural-deficient mice. For that purpose, we measured circulating HSPCs and WBCs in *Gfra2*$^{-/-}$ mice and compound *Gfra2*$^{-/-}$;*Adrb2*$^{-/-}$ or *Gfra2*$^{-/-}$;*Adrb3*$^{-/-}$ mice. Importantly, nocturnal (ZT13) circulating HSPCs and WBCs increased similarly in *Gfra2*$^{-/-}$ mice and *Gfra2*$^{-/-}$;*Adrb2*$^{-/-}$ mice, but normalized in *Gfra2*$^{-/-}$;*Adrb3*$^{-/-}$ mice (Figure 5G-H). Thus, contrasting β_2-AR-regulated BM homing, abnormal nocturnal β_3-AR activation reduces *Cxcl12* expression and triggers BM egress of HSPCs and leukocytes in cholinergic-neural-deficient mice.

BM expression of β_2-AR and β_3-AR follows day/night oscillations, which are altered in cholinergic-neural-deficient mice

Since β_3-AR and β_2-AR respectively mediated egress and homing of BM HSPCs and leukocytes, we hypothesized that expression of β-ARs might follow day/night oscillations regulated by cholinergic signals. We measured BM mRNA expression of β_2-AR (*Adrb2*) and β_3-AR (*Adrb3*) at different time points during day/night and found oscillations in control mice. Whereas *Adrb2* mRNA expression was higher at night –when BM homing becomes predominant–, *Adrb3* mRNA expression was increased during the day, when β_3-AR triggers HSC egress (Figure 6A-B). In contrast, day/night oscillations of *Adrb2* were blunted and those of *Adrb3* mRNA were inverted in *Gfra2*$^{-/-}$ BM. Notably, BM *Adrb3* mRNA expression was 3-fold-increased in *Gfra2*$^{-/-}$ mice at night (Figure 6B), further indicating that exacerbated β_3-adrenergic signaling at night enforces BM release of HSPCs and leukocytes possibly explaining why BM egress overrides BM homing in cholinergic-neural-deficient mice.

Sympathetic cholinergic fibers modulate migratory oscillations by locally regulating the expression of vascular adhesion molecules and β-ARs

Altered BM expression of vascular adhesion molecules (Figure 3E-G and Figure 4A-F) and β-ARs (Figure 6A-B) in cholinergic-neural-deficient mice suggested their local regulation by cholinergic signals. Supporting this possibility, treatment with acetylcholine reduced *Adrb3* (not *Adrb2*) mRNA expression in MS-5 stromal cells (Figure 6C and Figure S6A). Mirroring the *in vitro* results, *in vivo* i.p. administration of acetylcholine antagonists increased BM *Adrb3* (not *Adrb2*) mRNA expression at night (Figure 6D and Figure S6B). These results suggest that local cholinergic signals reduce nocturnal β_3-AR signaling, which contributes to reduce BM egress of HSPCs and leukocytes. However, only very few studies (focused on bone turnover) have investigated this possible local cholinergic regulation. Cholinergic signals might have bone anabolic effects by antagonizing the SNS, but have not agreed between central and local effects.[44,45] A recent study showed that central cholinergic signals regulate the hypothalamus-hypophysis axis and G-CSF-induced HSPC migration.[11]

To study local cholinergic signals, we immunostained skull bones of WT and *Gfra2*$^{-/-}$ mice for vesicular acetylcholine transporter (VAChT)–a validated marker of periosteal sympathetic cholinergic fibers.[23] VAChT^{+} sympathetic cholinergic fibers were present in WT skull but were 3-4-fold reduced in *Gfra2*$^{-/-}$ skull (Figure 7A-C). Binding of the neurotrophic factor neurturin (Nrtn)[22] to its receptor GFRα2 [19,23] promotes development and survival of cholinergic neurons. Therefore, we used *Nrtn*$^{-/-}$ mice as an additional model to study the cholinergic regulation of HSPC traffic. For verification we stained WT/*Nrtn*$^{-/-}$ skull for Gfrα2 (another validated marker of periosteal sympathetic cholinergic fibers).[23] Contrasting *Gfra2*$^{-/-}$ mice, *Nrtn*$^{-/-}$ mice exhibited doubled periosteal cholinergic fibers (Figure 7D-F) and unchanged BM noradrenergic innervation (Figure S7A-C) and Cxcl12 content (Figure S7D). Thus, *Nrtn*$^{-/-}$ mice exhibit increased sympathetic cholinergic fibers in bone, but apparently normal central sympathetic/parasympathetic activity (Table S1). Therefore, *Gfra2*$^{-/-}$ mice and *Nrtn*$^{-/-}$ mice are respectively loss- and gain-of-function models of bone sympathetic cholinergic innervation. These opposite phenotypes are likely explained by compensatory 3-fold upregulation of *Gfra2* mRNA in *Nrtn*$^{-/-}$ BM (Figure 7G) and potential Gfrα2 activation by other neurotrophic factors, as previously reported.[46-48] Supporting this compensatory ligand/receptor upregulation, *Gfra2*$^{-/-}$ mice exhibited doubled BM *Nrtn*

mRNA expression (Figure 7H). Nocturnal WBCs were unchanged in *Nrtn*$^{-/-}$ mice (Figure 7I), consistently with normal noradrenergic innervation and Cxcl12 expression in these mice.

We used *Nrtn*$^{-/-}$ mice to study the influence of local cholinergic signals on vascular adhesion. Previous findings have shown that cholinergic signaling through nicotinic receptor α7 (Chrnα7) activation in endothelial cells reduces Vcam1, E-selectin and inflammation in the Schwartzman reaction model.[49] Interestingly and mirroring *Gfra2*$^{-/-}$ mice, *Nrtn*$^{-/-}$ mice exhibited 4-fold-reduced *Vcam1* and *Sele* mRNA expression (Figure 7J-K), suggesting that bone sympathetic cholinergic fibers inhibit vascular adhesion.

Expression of adhesion molecules follows day/night oscillations (Figure 3E-G) dropping at morning. Since bone noradrenergic and cholinergic fibers share sympathetic origin, we hypothesized that light-induced SNS activity might locally inhibit vascular adhesion and BM homing through cholinergic axons, to facilitate noradrenergic-mediated release of HSPCs and leukocytes. We measured diurnal HSPC BM homing in control and *Gfra2*$^{-/-}$ mice (which lack sympathetic cholinergic innervation) transplanted with labelled cells one hour before light onset (ZT23). Importantly, diurnal HSPC homing was 40%-increased in *Gfra2*$^{-/-}$ BM but 25% reduced in *Nrtn*$^{-/-}$ BM (Figure 7L). Therefore, whereas β_2-adrenergic signals promote nocturnal BM homing, sympathetic cholinergic signals inhibit BM homing at dawn.

These data suggested that sympathetic noradrenergic and cholinergic signals favor diurnal BM egress of HSPCs and leukocytes through different mechanisms: whereas noradrenergic signals decrease BM Cxcl12 expression, cholinergic signals might inhibit vascular adhesion. For confirmation we injected WT mice with antagonists of β_2-AR, β_3-AR or cholinergic nicotinic receptors one hour before light onset (ZT23). We measured 6h later (ZT5) circulating HSPCs and BM adhesion molecule expression. Contrasting β_2-AR's role in nocturnal BM homing, β_2-AR blockade did not alter dawning circulating HSPCs or adhesion molecules (Figure 7M-O). Therefore, BM β_2-AR does not seem responsive at dawn (likely due to well-known β_2-AR desensitization; this might follow sustained stimulation by high nocturnal plasma epinephrine, which preferentially binds β_2-AR). In contrast, β_3-AR blockade decreased dawning BM egress of HSPCs (Figure 7M) consistently with our previous study.[6] Nicotinic receptor blockade increased *Vcam1* and *Sele* mRNA BM expression 6h later (ZT5, morning) (Figure 7N-O), confirming that cholinergic nicotinic signals repress dawning BM homing by inhibiting vascular adhesion. Interestingly, β_3-AR blockade had a similar effect on adhesion molecule expression, suggesting the possible inhibition of vascular adhesion by β_3-adrenergic signaling at dawn.

Altogether, our results show that sympathetic cholinergic signals instruct differential responses by BM β-ARs. Sympathetic cholinergic signals cause reduced β_3-AR BM expression at night, when higher endocrine-derived epinephrine promotes β_2-AR-dependent vascular adhesion and BM homing. This regulation adds to central parasympathetic inhibition of sympathetic noradrenergic tone, to promote nocturnal BM homing. In contrast, light-triggered BM sympathetic activity facilitates HSPC and leukocyte egress to bloodstream through two neurotransmitters: norepinephrine reduces Cxcl12-dependent chemotaxis through β_3-AR (previously shown),[6] whereas acetylcholine inhibits vascular adhesion and BM homing (found here).

Discussion

Previous studies have demonstrated that sympathetic noradrenergic fibers regulate HSPC and leukocyte migration,[50] but the underlying mechanisms were not fully dissected. Consequently, similar noradrenergic signals were proposed to trigger opposite migratory behaviors at different circadian time in mice: BM egress during daytime[6] (resting period) and nocturnal BM homing (activity period).[12] Moreover, these studies assumed that all sympathetic fibers innervating bone are noradrenergic. However, some of these fibers acquire cholinergic properties postnatally,[51] but their function has remained unknown.[2-4] Whereas central cholinergic signals have been recently proposed to regulate G-CSF-induced HSC mobilization,[11] whether the cholinergic nervous system regulates physiological HSC traffic was unknown.

In this study we uncover a dual cholinergic regulation which cooperates with noradrenergic signals to control day/night traffic of HSPCs and leukocytes. These cholinergic signals involve central parasympathetic and peripheral sympathetic innervation, which coordinately act to orchestrate physiological migration of HSPCs and leukocytes.

Our results show that, central (parasympathetic) and local (sympathetic) cholinergic signals cooperate to restrict BM egress and render β_2-AR-mediated homing predominant at night. Central inhibition of sympathetic tone by parasympathetic signals explains reduced BM egress of HSPCs and leukocytes, which is regulated by β_3-AR.[6] Locally, sympathetic cholinergic signals modulate the adrenergic response of target cells by reducing their β_3-AR expression. In contrast, light triggers local sympathetic (noradrenergic and cholinergic) activity. Noradrenergic signaling (norepinephrine) triggers β_3-AR-

Cxcl12-dependent BM egress.[6] Our novel findings demonstrate that sympathetic cholinergic activity facilitates HSC and leukocyte release by reducing BM vascular adhesion and homing.

This data agrees with previous studies reporting increased parasympathetic activity in mice towards day-night interphase,[27] explaining reduced central sympathetic noradrenergic tone[45] and BM β_3-AR activation, and consequently decreased nocturnal egress of HSPCs and leukocytes. However, epinephrine is preferentially released by the adrenal glands at night in mice in response to the hypothalamus-pituitary-adrenal axis.[52,53] Moreover, norepinephrine oscillations are abolished by sympathectomy[54] or under constant light,[55] confirming that neuronal norepinephrine is modulated by photic cues. In contrast, epinephrine oscillations are not affected by these interventions, whereas epinephrine is secreted in response to endocrine signals, such as cortisol.[53] Importantly, β_2-AR and β_3-AR show opposite, >30-fold higher affinities for epinephrine and norepinephrine, respectively.[16] This evidence and our data suggest that nocturnal endocrine-(not SNS)-released epinephrine triggers β_2-AR-dependent BM homing, whereas light-triggered sympathetic activity induces BM release through norepinephrine (via β_3-AR) and inhibits vascular adhesion through acetylcholine (sympathetic cholinergic). Moreover, we show that β_2-AR and β_3-AR expression oscillates daily and peaks at the same time as those neurotransmitters with highest affinity (dawning β_3-AR-norepinephrine; nocturnal β_2-AR-epinephrine), suggesting that coordinated regulation of ligand and receptor ensures robust, timely responses.

This evidence is supported by two complementary murine models, *Gfra2*$^{-/-}$ mice and Neurturin-(one of GFRα2 ligands)-deficient mice (Table S1). The receptor GFRα2 is required for cholinergic neuron survival (either parasympathetic or sympathetic).[19,23] We found that *Gfra2*$^{-/-}$ mice[19,56] exhibit halved acetylcholinesterase activity and doubled norepinephrine in urine at night, consistent with extensive parasympathetic deficiency causing de-repression of nocturnal sympathetic tone. Exacerbated noradrenergic activity in *Gfra2*$^{-/-}$ mice increases bidirectional traffic of HSCs and leukocytes (BM egress and homing). Interestingly, whereas abnormal nocturnal egress is only normalized in β_3-AR (not β_2-AR) KO mice (Figure 5G), increased BM homing is rescued by β_2-AR blockade (Figure 4I), further revealing the different roles of both β-ARs. Diurnal homing to *Gfra2*$^{-/-}$ microenvironment is also exacerbated due to increased vascular adhesion caused by lack of sympathetic cholinergic innervation (Figure 7L). However, because circulating HSPCs and WBCs accumulate in *Gfra2*$^{-/-}$ mice at night, β_3-AR signaling

seems predominant and enforces BM egress, overriding increased BM homing. Supporting this possibility, *Gfra2*$^{-/-}$ mice exhibit increased β_3-AR mRNA expression at night (Figure 6B).

In contrast, *Nrtn*$^{-/-}$ mice have preserved central parasympathetic innervation and, consequently, normal noradrenergic activity.[22] However, *Nrtn*$^{-/-}$ mice exhibit doubled sympathetic cholinergic innervation in bone (Figure 7D-F). Therefore, whereas *Gfra2*$^{-/-}$ mice lack cholinergic innervation, *Nrtn*$^{-/-}$ mice represent a gain-of-function model for bone sympathetic cholinergic innervation. Indeed, despite some similarities between both mouse models, *Gfra2*$^{-/-}$ mice (but not *Nrtn*$^{-/-}$ mice) exhibit retarded growth. Given that GFRα2 and its co-receptor RET can signal in the same cell (cis) or between neighboring cells (trans) during development,[57] future studies will address differences between *Gfra2*$^{-/-}$ and *Nrtn*$^{-/-}$ mice. Mirroring *Gfra2*$^{-/-}$ mice, *Nrtn*$^{-/-}$ mice exhibit reduced expression of adhesion molecules (Figure 7J-K), further supporting the inhibition of vascular adhesion by sympathetic cholinergic fibers through nicotinic receptors, as previously proposed in other models.[49,58]

In summary, our results indicate that both branches of the autonomic nervous system (parasympathetic and sympathetic noradrenergic/cholinergic) cooperate to orchestrate rhythmic traffic of HSCs and leukocytes. Light triggers BM sympathetic activity, which through (1) norepinephrine reduces β_3-AR-Cxcl12-dependent BM chemotaxis and (2) acetylcholine inhibits vascular adhesion. Both effects of local sympathetic activity (not only noradrenergic, but also cholinergic) permit HSC and leukocyte release into circulation. Parasympathetic activation towards day-night interphase antagonizes the SNS. However, high plasma concentration of endocrine-derived epinephrine at night preferentially stimulates β_2-AR to increase vascular adhesion and BM homing.

Author contributions: A.G.-G performed most experiments and analyses, prepared figures and drafted the manuscript. C.K., M.G.-F, O.D, J.V., D.M.-P., J.I. and J.A.B.-G. performed *in vivo* experiments and tissue analysis. J.Z., J.A.P.-S., J.J.T.-A., T.M. and M.A provided mice and helped with data analysis. S.M.-F. designed the overall study, performed, supervised and analyzed experiments, prepared figures and wrote the manuscript. All authors edited the manuscript. The authors declare no competing financial interests.

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References

1. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature*. 2014;505(7483):327-334.

2. Francis NJ, Landis SC. Cellular and molecular determinants of sympathetic neuron development. *Annu Rev Neurosci*. 1999;22:541-566.

3. Apostolova G, Dechant G. Development of neurotransmitter phenotypes in sympathetic neurons. *Auton Neurosci*. 2009;151(1):30-38.

4. Cane KN, Anderson CR. Generating diversity: Mechanisms regulating the differentiation of autonomic neuron phenotypes. *Auton Neurosci*. 2009;151(1):17-29.

5. Yamazaki K, Allen TD. Ultrastructural morphometric study of efferent nerve terminals on murine bone marrow stromal cells, and the recognition of a novel anatomical unit: the "neuro-reticular complex". *Am J Anat*. 1990;187(3):261-276.

6. Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature*. 2008;452(7186):442-447.

7. Spiegel A, Shivtiel S, Kalinkovich A, et al. Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling. *Nat Immunol*. 2007;8(10):1123-1131.

8. Lucas D, Battista M, Shi PA, Isola L, Frenette PS. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. *Cell Stem Cell*. 2008;3(4):364-366.

9. Zhao Y, Liu M, Chan XY, et al. Uncovering the mystery of opposite circadian rhythms between mouse and human leukocytes in humanized mice. *Blood*. 2017;130(18):1995-2005.

10. Katayama Y, Battista M, Kao WM, et al. Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*. 2006;124(2):407-421.

11. Pierce H, Zhang D, Magnon C, et al. Cholinergic Signals from the CNS Regulate G-CSF-Mediated HSC Mobilization from Bone Marrow via a Glucocorticoid Signaling Relay. *Cell Stem Cell*. 2017;20(5):648-658 e644.

12. Scheiermann C, Kunisaki Y, Lucas D, et al. Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity*. 2012;37(2):290-301.

13. Golan K, Kumari A, Kollet O, et al. Daily Onset of Light and Darkness Differentially Controls Hematopoietic Stem Cell Differentiation and Maintenance. *Cell Stem Cell*. 2018.

14. De Boer SF, Van der Gugten J. Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. *Physiol Behav*. 1987;40(3):323-328.

15. Sauerbier I, von Mayersbach H. Circadian variation of catecholamines in human blood. *Horm Metab Res*. 1977;9(6):529-530.

16. Hoffmann C, Leitz MR, Oberdorf-Maass S, Lohse MJ, Klotz KN. Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*. 2004;369(2):151-159.

17. Mutoh T, Shibata S, Korf HW, Okamura H. Melatonin modulates the light-induced sympathoexcitation and vagal suppression with participation of the suprachiasmatic nucleus in mice. *J Physiol*. 2003;547(Pt 1):317-332.

18. Niijima A, Nagai K, Nagai N, Nakagawa H. Light enhances sympathetic and suppresses vagal outflows and lesions including the suprachiasmatic nucleus eliminate these changes in rats. *J Auton Nerv Syst*. 1992;40(2):155-160.

19. Rossi J, Luukko K, Poteryaev D, et al. Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor. *Neuron*. 1999;22(2):243-252.

20. Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J Comp Neurol*. 2004;469(3):311-324.

21. Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK. Targeted disruption of the beta2 adrenergic receptor gene. *J Biol Chem*. 1999;274(24):16694-16700.

22. Heuckeroth RO, Enomoto H, Grider JR, et al. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron*. 1999;22(2):253-263.

23. Hiltunen PH, Airaksinen MS. Sympathetic cholinergic target innervation requires GDNF family receptor GFR alpha 2. *Mol Cell Neurosci*. 2004;26(3):450-457.

24. Rossi J, Santamaki P, Airaksinen MS, Herzig KH. Parasympathetic innervation and function of endocrine pancreas requires the glial cell line-derived factor family receptor alpha2 (GFRalpha2). *Diabetes*. 2005;54(5):1324-1330.

25. Wanigasekara Y, Airaksinen MS, Heuckeroth RO, Milbrandt J, Keast JR. Neurturin signalling via GFRalpha2 is essential for innervation of glandular but not muscle targets of sacral parasympathetic ganglion neurons. *Mol Cell Neurosci*. 2004;25(2):288-300.

26. Kentish SJ, Vincent AD, Kennaway DJ, Wittert GA, Page AJ. High-Fat Diet-Induced Obesity Ablates Gastric Vagal Afferent Circadian Rhythms. *J Neurosci*. 2016;36(11):3199-3207.

27. Bando H, Nishio T, van der Horst GT, Masubuchi S, Hisa Y, Okamura H. Vagal regulation of respiratory clocks in mice. *J Neurosci*. 2007;27(16):4359-4365.

28. Gavioli M, Lara A, Almeida PW, et al. Cholinergic signaling exerts protective effects in models of sympathetic hyperactivity-induced cardiac dysfunction. *PLoS One*. 2014;9(7):e100179.

29. Hasan W, Smith PG. Decreased adrenoceptor stimulation in heart failure rats reduces NGF expression by cardiac parasympathetic neurons. *Auton Neurosci*. 2014;181:13-20.

30. Sackstein R. Lymphocyte migration following bone marrow transplantation. *Ann N Y Acad Sci*. 1995;770:177-188.

31. Papayannopoulou T, Priestley GV, Nakamoto B, Zafiropoulos V, Scott LM. Molecular pathways in bone marrow homing: dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. *Blood*. 2001;98(8):2403-2411.

32. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci U S A*. 1998;95(24):14423-14428.

33. Katayama Y, Hidalgo A, Furie BC, Vestweber D, Furie B, Frenette PS. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. *Blood*. 2003;102(6):2060-2067.

34. Mendez-Ferrer S, Battista M, Frenette PS. Cooperation of beta(2)- and beta(3)-adrenergic receptors in hematopoietic progenitor cell mobilization. *Ann N Y Acad Sci*. 2010;1192:139-144.

35. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996;382(6592):635-638.

36. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*. 2006;25(6):977-988.

37. Schajnovitz A, Itkin T, D'Uva G, et al. CXCL12 secretion by bone marrow stromal cells is dependent on cell contact and mediated by connexin-43 and connexin-45 gap junctions. *Nat Immunol*. 2011;12(5):391-398.

38. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*. 2010;466(7308):829-834.

39. Kunisaki Y, Bruns I, Scheiermann C, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*. 2013;502(7473):637-643.

40. Grassinger J, Nilsson SK. Methods to analyze the homing efficiency and spatial distribution of hematopoietic stem and progenitor cells and their relationship to the bone marrow endosteum and vascular endothelium. *Methods Mol Biol*. 2011;750:197-214.

41. Itkin T, Gur-Cohen S, Spencer JA, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature*. 2016;532(7599):323-328.

42. Kusumbe AP, Ramasamy SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature*. 2014;507(7492):323-328.

43. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*. 2012;481(7382):457-462.

44. Bajayo A, Bar A, Denes A, et al. Skeletal parasympathetic innervation communicates central IL-1 signals regulating bone mass accrual. *Proc Natl Acad Sci U S A*. 2012;109(38):15455-15460.

45. Shi Y, Oury F, Yadav VK, et al. Signaling through the M(3) muscarinic receptor favors bone mass accrual by decreasing sympathetic activity. *Cell Metab*. 2010;11(3):231-238.

46. Trupp M, Raynoschek C, Belluardo N, Ibanez CF. Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. *Mol Cell Neurosci*. 1998;11(1-2):47-63.

47. Jing S, Yu Y, Fang M, et al. GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. *J Biol Chem*. 1997;272(52):33111-33117.

48. Sanicola M, Hession C, Worley D, et al. Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. *Proc Natl Acad Sci U S A*. 1997;94(12):6238-6243.

49. Saeed RW, Varma S, Peng-Nemeroff T, et al. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med*. 2005;201(7):1113-1123.

50. del Toro R, Mendez-Ferrer S. Autonomic regulation of hematopoiesis and cancer. *Haematologica*. 2013;98(11):1663-1666.

51. Asmus SE, Parsons S, Landis SC. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. *J Neurosci*. 2000;20(4):1495-1504.

52. Scheer FA, Hu K, Evoniuk H, et al. Impact of the human circadian system, exercise, and their interaction on cardiovascular function. *Proc Natl Acad Sci U S A*. 2010;107(47):20541-20546.

53. Dimitrov S, Benedict C, Heutling D, Westermann J, Born J, Lange T. Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. *Blood*. 2009;113(21):5134-5143.

54. Maestroni GJ, Cosentino M, Marino F, et al. Neural and endogenous catecholamines in the bone marrow. Circadian association of norepinephrine with hematopoiesis? *Exp Hematol*. 1998;26(12):1172-1177.

55. Akerstedt T, Levi L. Circadian rhythms in the secretion of cortisol, adrenaline and noradrenaline. *Eur J Clin Invest*. 1978;8(2):57-58.

56. Rossi J, Herzig KH, Voikar V, Hiltunen PH, Segerstrale M, Airaksinen MS. Alimentary tract innervation deficits and dysfunction in mice lacking GDNF family receptor alpha2. *J Clin Invest*. 2003;112(5):707-716.

57. Fleming MS, Vysochan A, Paixao S, et al. Cis and trans RET signaling control the survival and central projection growth of rapidly adapting mechanoreceptors. *Elife*. 2015;4:e06828.

58. Pavlov VA, Chavan SS, Tracey KJ. Molecular and Functional Neuroscience in Immunity. *Annu Rev Immunol*. 2018;36:783-812.

59. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods*. 2009;347(1-2):70-78.

FIGURE LEGENDS

Figure 1. Cholinergic signals regulate circadian traffic of HSCs and leukocytes in mice.

(A) Acetylcholinesterase (AChE) activity in urine samples from *Gfra2*$^{-/-}$ and WT mice collected in the nocturnal (black) and diurnal (yellow) periods.

(B-C) HSPCs (B) measured as colony-forming units (CFU-Cs), and (C) white blood cells (WBC) in peripheral blood of *Gfra2*$^{-/-}$ mice (green) and control *Gfra2*$^{+/-}$ mice (purple) harvested at the specified *Zeitgeber* time (ZT, hours after light onset). ZT1 has been duplicated to facilitate viewing.

(D) HSCs, measured by long-term competitive repopulation assay, in peripheral blood harvested at ZT13 from *Gfra2*$^{-/-}$ mice (red) and control *Gfra2*$^{+/-}$ mice (black). The log fraction of mice which failed reconstitution is plotted against the transplanted blood volume using ELDA software.[59] Likelihood ratio test of single-hit model, $p = 0.006$; Chi square test. Blood HSC concentrations are indicated (n = 5).

(A-D) Data are means ± SEM; n (inside bars/symbols) and p values (multivariate analysis for >2 groups) are indicated. (A) One-way ANOVA and Bonferroni comparisons. (B-C) Multiple two-tailed test. (D) Chi square test. * $p < 0.05$; *** $p < 0.001$.

Figure 2. Cholinergic signals regulate HSPC and leukocyte traffic by modulating sympathetic noradrenergic tone centrally.

(A-B) Representative immunofluorescence of CD31^{+} endothelial cells (blue), tyrosine hydroxylase (TH)$^{+}$ sympathetic nerve fibers (red) and Nestin-GFP^{+} cells (green) in the skull BM of *Nes-gfp*;*Gfra2*$^{+/-}$ and *Nes-gfp*;*Gfra2*$^{-/-}$ compound mice. Scale, 100 μm.

(C) Quantification of the skull BM area covered by TH^{+} sympathetic noradrenergic nerve fibers from *Gfra2*$^{+/-}$ mice and *Gfra2*$^{-/-}$ mice (a.u, arbitrary units).

(D) Nocturnal (black) and diurnal (yellow) norepinephrine concentration in the urine of *Gfra2*$^{+/-}$ and *Gfra2*$^{-/-}$ compound mice.

(E) Scheme illustrating the different types of cholinergic antagonists used and their capacity to cross the blood-brain barrier (BBB). Mecamylamine and scopolamine are BBB-permeable antagonists, while hexamethonium and methylatropine are BBB-non-permeable antagonists.

(F-G) Blood circulating HSPCs, measured as CFU-Cs (F), and white blood cells (WBCs) (G) at ZT13 in WT mice treated with acetylcholine antagonists (i.p) at ZT5.

(C-D, F-G) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. (C) Unpaired two-tailed *t* test. (D, F-G) One-way ANOVA and Bonferroni comparisons. * $p < 0.05$; ** $p < 0.01$.

Figure 3. The parasympathetic nervous system regulates nocturnal HSC BM adhesion and homing.

(A) Scheme showing the protocol used for HSPC BM homing assay with irradiation (12 Gy).

(B) Frequencies of HSPCs (homing efficiency) at ZT2 that homed during the night to the BM after i.v. transplantation in lethally-irradiated mice (12 Gy) (to promote homing) at ZT10. *Gfra2*$^{-/-}$ mice and control *Gfra2*$^{+/-}$ mice were used as donor (lower genotypes) or recipients (upper genotypes) in all combinations. Homing efficiency is determined as the percentage of CFU-Cs obtained from BM harvested from irradiated mice in comparison to CFU-Cs obtained from a non-irradiated mouse.

(C) Scheme showing the protocol used for HSPC BM homing assay without irradiation.

(D) Frequencies of donor-derived *Gfra2*$^{+/+}$ or *Gfra2*$^{-/-}$ lin^{-} sca-1^{+} c-kit^{+} HSPCs (identified by flow cytometry) at ZT2 that homed during the night to the BM after i.v. transplantation in non-irradiated congenic mice at ZT10.

(E-G) *Vcam1, Sele* and *Selp* mRNA expression in the unfractionated BM of *Gfra2*$^{-/-}$ and *Gfra2*$^{+/-}$ control mice at the specified ZT. ZT21 has been duplicated to facilitate viewing.

(B, D-G) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. (B, D) One-way ANOVA and Bonferroni comparisons. (E-G) Multiple two-tailed test. * $p < 0.05$.

Figure 4. Parasympathetic deficiency increases nocturnal HSPC BM adhesion and homing through β_2-adrenergic signaling in the microenvironment.

(A-C) *Vcam1*, *Sele* and *Selp* mRNA expression at ZT13 in the unfractionated BM of control *Gfra2*$^{+/-}$ mice, *Gfra2*$^{-/-}$ mice, single β_2-AR (*Adrb2*) or β_3-AR (*Adrb3*)-deficient mice, or compound *Gfra2*$^{-/-}$*Adrb2*$^{-/-}$ and *Gfra2*$^{-/-}$*Adrb3*$^{-/-}$ mice.

(D-F) *Vcam1*, *Sele* and *Selp* mRNA expression at ZT13 in CD45^{-}Ter119^{-} endothelial (CD31^{+}) or non-endothelial (CD31^{-}) cells from *Gfra2*$^{-/-}$ and control *Gfra2*$^{+/-}$ mice.

(G) Scheme showing the protocol used for blockade of *in vivo* HSPC adhesion to blood vessels using antibodies against α_4-integrin, P- and E-selectins (i.v. injection at ZT11 and analysis at ZT13).

(H) HSPCs circulating at ZT13, 2h after injection of blocking antibodies (Abs) or control IgG. Please note that CFU-C fold change goes from 3.2-fold increase in control IgG-treated mice to 1.3-fold in blocking antibodies-treated mice.

(I) Frequencies of donor-derived WT CD45.1^{+} lin^{-} sca1^{+} ckit^{+} HSPCs at ZT2 that homed during the night to the BM after i.v. transplantation (at ZT10) in non-irradiated *Gfra2*$^{-/-}$ mice or control *Gfra2*$^{+/-}$ mice pre-conditioned with saline or β_2 adrenergic antagonist (ICI118,551) 4h before transplantation.

(A-F, H-I) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. One-way ANOVA and Bonferroni comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. The parasympathetic nervous system inhibits β_3-adrenergic-dependent BM egress of HSPCs at night.

(A-B) (A) HSPCs, measured as colony-forming units (CFU-Cs), and (B) WBCs circulating at ZT13, 16 weeks after BM transplantation in lethally-irradiated mice. *Gfra2*$^{-/-}$ mice and control *Gfra2*$^{+/-}$ mice were used as donor (lower genotypes) or recipients (upper genotypes) in all combinations.

(C) *Cxcl12* mRNA expression in the BM of *Gfra2*$^{-/-}$ and *Gfra2*$^{+/-}$ control mice at the specified ZT. ZT21 has been duplicated to facilitate viewing.

(D) Cxcl12 concentration in BM extracellular fluid (BMECF) at ZT13.

(E) *Kitl* mRNA expression in the BM of *Gfra2*$^{-/-}$ and *Gfra2*$^{+/-}$ control mice at ZT13.

(F) Number of stromal Nes-GFP$^{hi/lo}$ cells in endosteal and non-endosteal BM (upper panel). Representative flow cytometry showing CD31 and Nes-GFP expression in CD45^{-}Ter119^{-} cells isolated from endosteal BM of *Gfra2*$^{-/-}$ and control *Gfra2*$^{+/+}$ mice (lower panel).

(G) CFU-C fold change at ZT13 in control *Gfra2*$^{+/-}$ mice, *Gfra2*$^{-/-}$ mice, single β_2- or β_3-AR (*Adrb2*, *Adrb3*)-deficient mice, or compound *Gfra2*$^{-/-}$*Adrb2*$^{-/-}$ and *Gfra2*$^{-/-}$*Adrb3*$^{-/-}$ mice.

(H) WBCs circulating at ZT13 in control *Gfra2*$^{+/-}$ mice, *Gfra2*$^{-/-}$ mice, single β_2- or β_3-AR (*Adrb2*, *Adrb3*)-deficient mice, or compound *Gfra2*$^{-/-}$*Adrb2*$^{-/-}$ and *Gfra2*$^{-/-}$*Adrb3*$^{-/-}$ mice.

(A-H) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. (A-B, F-H) One-way ANOVA and Bonferroni comparisons. (C) Multiple two-tailed test. (D-E) Unpaired two-tailed *t* test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6. Local cholinergic signals regulate oscillatory expression of β_3-AR in BM.

(A-B) *Adrb2* and *Adrb3* mRNA expression in the BM of *Gfra2*$^{-/-}$ and *Gfra2*$^{+/-}$ control mice at the specified ZT. ZT21 has been duplicated to facilitate viewing.

(C) *Adrb3* mRNA expression in MS-5 cell line cultures treated with vehicle or acetylcholine (10 μM) for 6h.

(D) *Adrb3* mRNA expression in the BM of WT mice treated with acetylcholine antagonists (i.p) at ZT5 and analyzed at ZT13.

(A-D) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. (A-B) Multiple two-tailed test. (C) Unpaired two-tailed *t* test. (D) One-way ANOVA and Bonferroni comparisons. * $p < 0.05$; *** $p < 0.001$.

Figure 7. Sympathetic cholinergic signals locally repress adhesion to BM vessels during daytime.

(A-B) Z-stack projection showing immunofluorescence of CD31^{+} endothelial cells (blue) and vesicular acetylcholine transporter (VAChT)$^{+}$ nerve fibers (red) in the skull of *Gfra2*$^{-/-}$ and WT mice. Scale bar, 100 μm.

(C) Quantification of VAChT^{+} fibers in the skull periosteum of *Gfra2*$^{-/-}$ and WT mice.

(D-E) Immunofluorescence of CD31^{+} endothelial cells (blue) and GDNF family receptor alpha 2 (Gfrα2)$^{+}$ nerve fibers (red) in the skull of *Nrtn*$^{-/-}$ and WT mice. Scale bar, 100 μm.

(F) Quantification of Gfrα2^{+} fibers in the skull periosteum of *Nrtn*$^{-/-}$ and WT mice.

(G-H) *Gfra2* and *Nrtn* mRNA expression at ZT13 in the BM of *Gfra2*$^{-/-}$, *Nrtn*$^{-/-}$ and WT mice.

(I) Normalized white blood cell (WBC) counts in peripheral blood of *Gfra2*$^{-/-}$, *Nrtn*$^{-/-}$ and WT mice at ZT13.

(J-K) *Vcam1* and *Sele* mRNA expression at ZT13 in the unfractionated BM of control *Nrtn*$^{+/+}$ and compound *Nrtn*$^{-/-}$ mice.

(L) Frequencies of donor-derived WT lin^- $sca1^+$ $ckit^+$ HSPCs that homed to the BM at ZT5, 6h after i.v. transplantation in non-irradiated *Gfra2*$^{-/-}$, *Nrtn*$^{-/-}$ and WT mice (at ZT23).

(M) Blood CFU-C fold change at ZT5 in WT mice treated with saline, β-AR antagonists or cholinergic nicotinic (Chrn) antagonist at ZT23.

(N-O) *Vcam1* and *Sele* mRNA expression at ZT5 in the BM of WT mice treated with saline, β-AR antagonists or cholinergic nicotinic antagonist at ZT23.

(C, F-O) Data are means ± SEM; n (inside bars) and p values (multivariate analysis for >2 groups) are indicated. (C, F, J-K) Unpaired two-tailed *t* test. (G-I, L-O) One-way ANOVA and Bonferroni comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.