Original Article

MOLECULAR METABOLISM

Regulation of muscle and metabolic physiology by hypothalamic erythropoietin independently of its peripheral action

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ABSTRACT

Objective: The glycoprotein hormone erythropoietin (EPO) is required for erythropoiesis, and the kidney is the primary site of adult EPO synthesis. Limited evidence has suggested that EPO could be detectable in the brain under certain conditions, but it remains unknown if the brain might have its own EPO system for biological functions that are independent of peripheral EPO production and action. We performed this study to address this question using mice under normal conditions versus pathophysiological conditions including aging and dietary obesity.

Methods: EPO expression was measured in different brain regions as well as in the cerebrospinal fluid. Hypothalamic ventricular EPO was administered to physiologically examine possible therapeutic effects on the conditions of aging and dietary obesity. Body weight, body composition, insulin tolerance, and glucose tolerance were measured to assess the central effects of EPO on metabolic physiology, and muscle strength and histology were analyzed to assess the central effects of EPO on muscle function. In addition, β2-adrenergic receptor knockout bone marrow transplant was employed to determine the potential role of bone marrow in linking the brain to some of these peripheral functions.

Results: This study revealed that EPO is expressed in the ventromedial hypothalamus in addition to a few other brain regions and is present in the cerebrospinal fluid. Unlike blood EPO concentration, which increased with aging and dietary obesity, hypothalamic EPO decreased in these disease conditions. Therapeutically, aged mice were chronically treated with EPO in the hypothalamic ventricle, showing an increase in lean mass, while body weight and fat mass decreased as a result of a moderate reduction of food intake. Both muscle and metabolic functions were improved by this central treatment, and mechanistically, adrenergic signals to the bone marrow played a role in conveying hypothalamic EPO to these peripheral actions. Dietary obesity was also studied, showing that hypothalamic EPO treatment caused a reduction in food intake and obesity, leading to improved metabolic functions related to decreased fat as well as increased lean mass.

Conclusions: Hypothalamic EPO plays a role in the central regulation of muscle and metabolic physiology, while its decline contributes to aging and obesity physiology in a manner that is independent of peripheral EPO.

Keywords Hypothalamus; Brain; Erythropoietin; Aging; Obesity; Muscle; Metabolism; Glucose tolerance

1. INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that is essential for erythropoiesis, including survival, proliferation, and differentiation of erythrocytic progenitors. On the other hand, EPO has been shown to also influence nonhematopoietic tissues and functions and even possibly body weight and glucose control [1], but it is still less clear if these effects could be mediated indirectly by erythropoiesis or if there might exist unidentified mechanisms. Systemically, EPO is primarily produced by the liver in the fetus and by the kidney in adults [2]. Apart from its peripheral production and actions, limited evidence exists showing that EPO or its receptor (EpoR) could be detected in cultured neural cells and even in the brain under particular conditions [3–10]. However, little is known about whether brain EPO could be physiologically important and especially whether this system in the brain could be critical for certain disease mechanisms.

EPO synthesis is transcriptionally regulated, and EPO mRNA can be detectable in a number of peripheral tissues other than the kidney, despite the fact that EPO in these tissues cannot substitute for kidney-derived EPO in chronic kidney disease [2]. Kidney EPO production is known to be hypoxia inducible via a hypoxia response element that is bound by hypoxia inducible factor-1 (HIF-1). In terms of signaling, EPO binds to EpoR and signals through Janus kinase 2 to various intracellular enzymes and transcription factors that promote erythroid progenitor survival, proliferation, and differentiation [2]. EPO has a molecular weight of about 30 kDa, which should limit its passage across the blood–brain barrier (BBB) of most brain regions when at its physiologic concentrations [11]. Thus, if EPO is important for the brain and given that the brain contains its receptor and EPO can be detected in cerebrospinal fluid (CSF) [11], the prediction could be made that some site of the central nervous system (CNS) is important for EPO production, even in normal physiology. In the literature, the analysis of

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EPO concentration in the CSF after aneurysmal subarachnoid hemorrhage in human patients or intraventricular hemorrhage in neonates found that it indeed did not correlate with serum EPO [12,13]. Regarding the origin of EPO in the brain, it was shown to be hypoxia inducible [6,14] and produced by astrocytes [4,6,8,10] and neurons [10]. Similarly, EPO mRNA was detectable in various brain regions in humans, including the hippocampus, amygdala, and temporal cortex [6]. In experiments, peripheral EPO injection has been reported to improve several aspects of cognitive functions including learning and memory [15–17], hinting at the possibility that EPO at pharmacological doses might limitedly access selective brain regions where the BBB is permeable, such as the mediobasal hypothalamus. In neurobiology, EPO has been much less studied, although there is limited information suggesting that it is neurotrophic and neuroprotective for cerebral diseases including Alzheimer's [18], depression [19], epilepsy [20], and ischemia [21].

In terms of EPO in aging, clinical studies reported that peripheral red blood cell counts decline with aging, and further analysis of elderly anemic patients revealed that the underlying abnormality is due to diminished erythropoiesis [22]. Analysis of EPO levels in elderly non-anemic subjects has not led to a defined pattern, as studies have reported either an increase or no change [22]. However, a longitudinal analysis of a patient cohort over 8–30 years did report a steady increase in EPO over time [23]. Animal models have also demonstrated that aging increases basal serum EPO levels and EPO mRNA in the kidney [24–26]. Based on these findings from human studies and animal models, it can be speculated that there might be defects of erythroid progenitor cells in responding to EPO, which secondarily leads to increased production and release of EPO from the kidney. One interesting area of research on EPO signaling is related to its potential role in metabolic physiology. When EpoR was ablated in the body, except for the erythroid compartment, animals were found to have increased fat mass and insulin resistance as well as altered proopiomelanocortin (POMC) production from the hypothalamus [27]. Systemically, EPO administration was seen as protective against obesity [27,28] and glucose intolerance [29–31], but it was unclear if EPO at pharmacological doses could have access to the hypothalamus for these metabolic effects. In this study, we observed that the hypothalamus is a key site for the production and action of neural EPO, and because hypothalamic EPO declined under aging or obesity, we studied whether hypothalamic ventricle EPO administration could help certain physiological functions under these conditions.

2. EXPERIMENTAL PROCEDURES

2.1. Animal models

C57BL/6 mice were obtained from Jackson Laboratories and the National Institute of Aging and were housed under standard conditions in a temperature- and humidity-controlled facility with 12-h light:12-h dark cycles. For the dietary obesity model, mice were maintained on a high-fat diet (HFD; 60 kcal% fat, 20 kcal% protein, and 20 kcal% carbohydrate), and control mice were fed with normal chow (10 kcal% fat, 20 kcal% protein, and 70 kcal% carbohydrate) for 3 months. Mice were maintained on an HFD for 3 months and then continued to be maintained on HFD feeding when receiving EPO treatment. Food intake and body weight were measured on a regular basis during the treatment. Food intake was measured with attention paid to any spilled food in cages. Magnetic resonance imaging was used to determine body composition of mice. Male mice were employed throughout this study. All procedures were approved by the Institutional Care and Use Committee of Albert Einstein College of Medicine.

2.2. Hypothalamic ventricle cannulation and injection

As established in our previous studies [32–36], an ultra-precise small animal stereotactic apparatus (David Kopf Instrument) was used to implant a guide cannula into the hypothalamic third ventricle of anesthetized mice at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the bregma of the animals. For surgical recovery, mice were given at least 1 week for postcannulation recovery. Individual mice were restrained in a mouse restrainer and infused with EPO using a 26- gauge guide cannula and 33-gauge injector (Plastics One) connected to a Hamilton Syringe and infusion pump (Harvard Apparatus). A dose of 30 pg EPO (Sigma) versus the vehicle (artificial CSF) at a volume of 1.5 μL was administered over approximately 5 min through the cannula 3 times per week for the indicated experimental durations.

2.3. Glucose tolerance test and insulin tolerance test

A glucose tolerance test (GTT) was performed using overnight-fasted mice that received an intraperitoneal (i.p.) injection of glucose (Sigma–Aldrich, Munich, Germany) at a dose of 2 g/kg body weight. Blood was collected from the tail vein at time points before and after injection to measure blood glucose levels with an Accu-Chek glucose meter (Roche, Manheim, Germany). For the insulin tolerance test (ITT), mice were fasted for about 6 h prior to receiving an i.p. insulin injection (Lilly, Bad Homburg, Germany; 0.75 U/kg body weight), and blood glucose levels were measured at various time points.

2.4. Bone marrow transplantation

Adrb2^{tm1Bkk} mice were a kind gift from P. Frenette's lab. These mice were homozygous null for the β2-adrenergic receptor (*Adrb2*) and were viable, fertile, and normal sized, and did not have any gross physical or behavioral abnormalities or any reported changes in hematopoiesis. Mice were euthanized, and the whole BM was extracted by established methods [37–39]. β2AR knockout chimera mice were generated by reconstituting whole BM cells from *Adrb2*^{tm1Bkk} mice into near lethally irradiated (1200 cGY) male C57BL/6 mice by a single retro-orbital injection at a ratio of 1:4 (donor-recipient). Control chimera mice were generated by reconstitution of C57BL/6J whole BM cells into age- and sex-matched C57BL/6 mice by comparable irradiation and reconstitution protocols [39]. Successful BM ablation and reconstitution was confirmed by real-time polymerase chain reaction (PCR) of BM using primers for the *Adrb2* receptor. More than 90% reconstitution was considered successful, in accordance with previous protocols [39] and as measured by significantly lower expression of *Adrb2* receptors in the blood of knockout chimera mice, which corresponded to the levels seen in the BM of naive *Adrb2*^{tm1Bkk} mice. All reconstituted mice were allowed to recover for about 3 months prior to EPO treatment and experiments.

2.5. Quantitative PCR

Total RNA extraction was performed using TRIzol (Life Technologies), followed by treatment with DNase to eliminate genomic DNA with RNase-free DNase Set (Qiagen, Hilden, Germany) and RNA clean-up with the RNeasy mini kit (Qiagen). RNA concentration was measured using Nanodrop (Life Technologies, Thermo Fisher Scientific). Reverse transcription was performed using the Reverse Transcription System (Promega, Madison, WI, USA), following the manufacturer's recommendations. Levels of mRNA expression were measured by quantitative real-time reverse transcriptase PCR (RT-PCR) using the SYBR green method. Specific primer pairs were mixed with SYBR Green PCR Master Mix (Applied Biosystems), and reactions were run in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Analysis was performed using SDS software (Applied Biosystems). All values were normalized to GAPDH as an internal control. The sequence of primers used for quantitative RT-PCR are shown as:
MuRF: 5′-GCTGGTGGAAAACATCATTGACAT-3′, and 5′-CATCGGGTGG CTGCCTTT-3′;
MAFbx: 5′-CTTTCAACAGACTGGACTTCTCGA-3′, and 5′-CAGCTCCAA-CAGCCTTACTACGT-3′;
FOXO1: 5′-TTCCTTCATTCTGCACACGA-3′, and 5′-GTCCTACGCC-GACCTCATC-3′;
ADRB2: 5′-AAGAATAAGCCCGAGTGGT-3′, and 5′-GTAGGCCTGG TTCGTGAAGA-3′;
GAPDH: 5′-AACAGCAACTCCCACTCTTC-3′, and 5′-CCTGTTGCTGTA GCCGTATT-3′.

2.6. Immunofluorescence staining

Animals under anesthesia were perfused with 4% paraformaldehyde, after which brains were harvested, post-fixed, and infiltrated in 20%—30% sucrose. Brain sections were made at 20-μm thickness using a cryostat at −20 °C. Fixed tissues were blocked with serum of appropriate species, penetrated with 0.3% Triton-X 100, and treated with primary antibodies including anti-Epo 1:50 (Santa Cruz), anti-NeuN 1:200 (Novus Biologicals), anti-Pax-7 1:200 (Abcam), anti-SF1 1:100 (Invitrogen), and anti-p-STAT3 1:100 (Cell Signaling Technology). Sections were then incubated with appropriate Alexa Fluor 488 or 555 secondary antibody (Invitrogen). DAPI nuclear staining was used to reveal all the cells in tissue sections. Images were captured on a Leica SP5 confocal microscope.

2.7. Skeletal muscle assessments

Mice were euthanized immediately prior to tissue collection. Muscles were removed and weighed. Quadriceps, gastrocnemius, and soleus muscle were collected, and hematoxylin and eosin staining was performed on 9-μm frozen sections. Image J software was used to analyze the muscle fiber cross-section area based on muscle images that were captured under a microscope (Axiovert 100; Zeiss, Göettingen, Germany).

2.8. Biochemical and molecular analyses

The concentrations of EPO protein in the serum and CSF were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (LSBio, Mouse EPO/Erythropoietin ELISA Kit). Briefly, serum and CSF were added to a 96-well plate with enzyme-labeled anti-mouse EPO antibodies. The plates were incubated for 2 h at room temperature. Then, the substrate was added and the reaction was stopped with sulfuric acid. The plates were read at 450 nm with a microplate reader, and the concentration of EPO was calculated on the basis of the standard curve of lyophilized synthetic EPO protein. Western blot analyses were performed using samples collected from mice. The hypothalamus was cut along the anterior border of the optic chiasm, the posterior border of the mammillary body, the upper border of the anterior commissure, and lateral border halfway from the lateral sulcus in the ventral side of the brain. Tissues were homogenized, proteins dissolved in lysis buffer, and Western blots were performed. Protein extracts were separated by sodium dodecyl sulfate—polyacrylamide gel electrophoresis and detected by immunoblotting using antibodies against EPO 1:200 (Santa Cruz) and β-actin 1:1000 (Cell Signaling) and then reacted with horseradish peroxidase—conjugated anti-rabbit or anti-mouse secondary antibodies (Pierce). Quantification of specific blots was performed using Image Lab 3.0 software (Bio-Rad).

2.9. Behavioral tests

A weight-lift test was performed as described previously [40]. Mice were held by the middle/base of their tails and lowered to allow them to grab the weight (20 g) and timed until the link was cleared off the bench. A hold time of 3 s was set as the criterion. If a mouse dropped the weight in less than 3 s, the time the weight was held was recorded. If a mouse failed to hold the weight three times, the trial was terminated, and the mouse was assigned the maximum time/weight achieved. Heavier weights were used if a mouse could hold the initial weight for 3 s. If it could lift the heavier weight for 3 s, it was tested with the next heavier weight once all cage mates had been tested with the initial weight. Again, mice were given three chances to hold the weight for 3 s. A final total score was calculated as the product of the number of links in the heaviest chain held for the full 3 s, multiplied by the time (seconds) it was held. If the heaviest weight was dropped before 3 s, an appropriate intermediate value was calculated. Grip strength test was performed as described previously [34,35]. Each mouse was lifted by its tail and placed on a homemade square grid (1-cm mesh size). The grid was then inverted 30 cm over a soft pad, and the mouse was allowed to hang by its paws for 2—6 min dependent on its age. The time that the mouse was able to hang was recorded during a 2-minute test period. Three repeat tests were performed for each mouse, with at least a 10-minute rest between each trial. Rotarod test was performed as similarly described in our prior studies [34,35]. Mice were trained on a rotarod (Columbus instruments) that was moving at a constant speed of 6 rpm for 60 s. After a 10-minute rest, each mouse was given three trials, during which the rotarod started at 6 rpm and accelerated by 2 rpm per minute until 10 min, with a 30-minute rest period between each trial.

2.10. Statistical analyses

All measured data are presented as mean ± SE. Samples and mice were assigned to different experimental groups in randomized manners, and sample sizes with adequate power were estimated based on our previous experience and studies. Analysis of variance and Turkey' post hoc analyses were used for data comparisons when experiments were designed to have more than two groups. Student's *t*-test was used when experiments involved only two groups for comparison. Software Excel and GraphPad Prism were used for statistical analyses. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. EPO is locally expressed and provides signaling in the hypothalamic subregions

Since previous research has suggested that EPO and its receptor are expressed in the brain, this prompted us to examine in which subregions and neural cell types brain EPO is expressed [3—10]. Using Western blotting, we first generally compared EPO protein levels among various brain regions including the hypothalamus, hippocampus, total cortex, cerebellum, and brainstem of normal chow-fed young mice. As shown in Figure 1A, EPO protein levels were high in the hypothalamus, cortex, and hippocampus but low or undetectable in other regions. Since we observed strong EPO expression in the hypothalamus, we decided to further dissect whether EPO was localized to specific hypothalamic subregions. We found that EPO was more highly expressed in the ventromedial hypothalamus (VMH) but weakly in the arcuate nucleus (ARC) and barely in the periventricular nucleus or dorsomedial hypothalamus (Figure 1B). We also examined various cortical subregions to determine if EPO was expressed in the cortex distinctly or diffusely. As shown in Figure 1C, our results revealed that

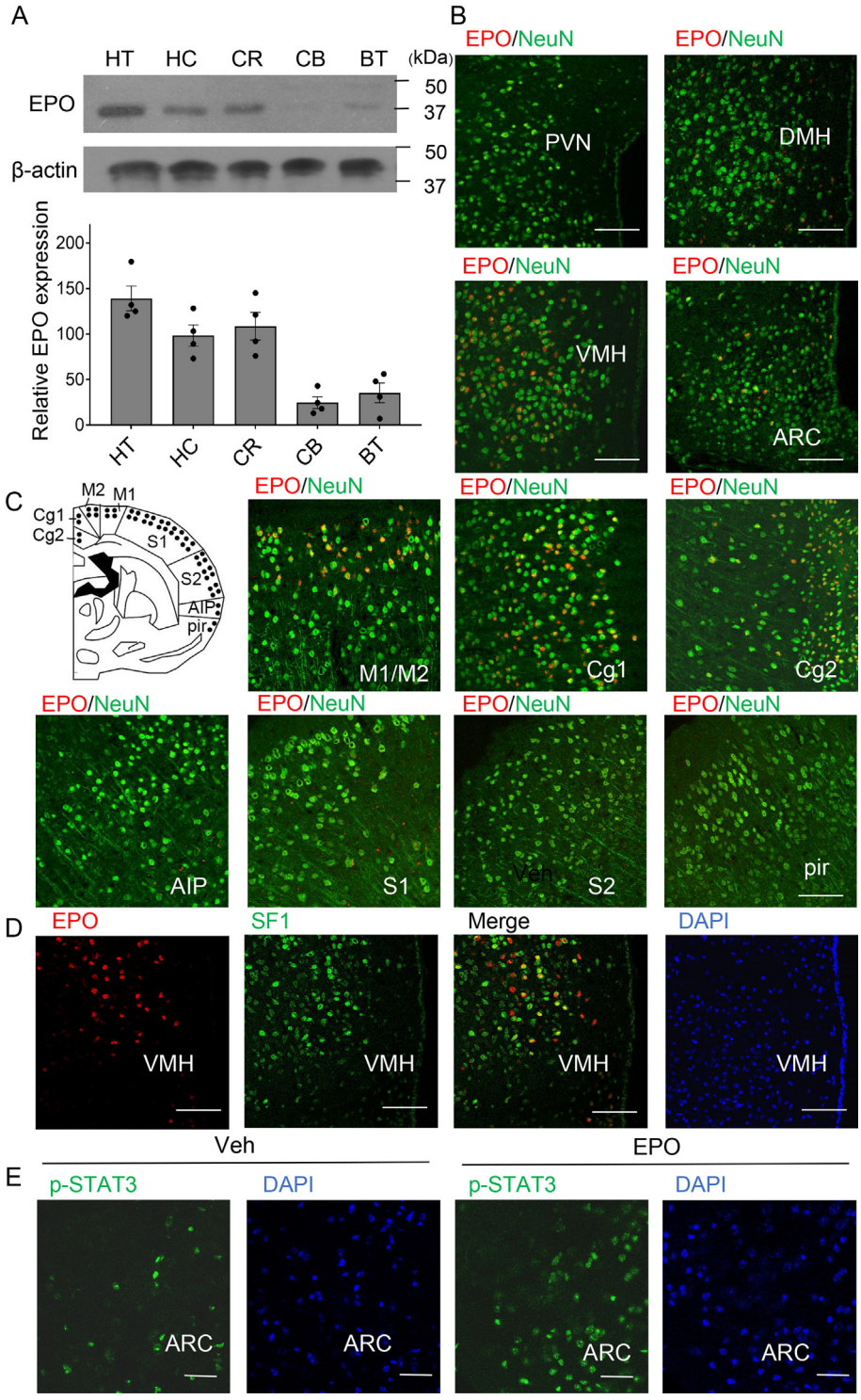

Figure 1: Brain EPO expression in the brain and hypothalamus. *A,* Western blot analysis and quantification (n = 4 mice per group, biological replicates) for EPO in different brain regions including the hypothalamus (HT), hippocampus (HC), cerebral cortex (CR), cerebellum (CB), and brainstem (BT) of chow-fed young mice. EPO was normalized by β-actin. Bars represent mean ± SE. *B,* Immunostaining for EPO (red) and NeuN (green) in different subregions of the hypothalamus including the periventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC) of chow-fed young mice. Scale bar = 100 μm. *C,* Immunostaining for EPO (red) and NeuN (green) in different subregions of the cortex including the motor cortex (M1), cingulate cortex (Cg1 and Cg2), anterior intraparietal area (AIP), primary somatosensory cortex (S1), secondary somatosensory cortex (S2), and piriform cortex (pir). A diagram is included to elucidate these cortex subregions. Scale bar = 100 μm. *D,* Immunostaining for EPO (red) and SF1 (green) in the VMH. DAPI staining (blue) was induced to indicate cells in the sections. Scale bar = 100 μm. *E,* Immunostaining for p-STAT3 (green) in the arcuate nucleus (ARC) sections of mice 30 min after receiving an injection of EPO (30 pg) versus vehicle (Veh) via the hypothalamic third ventricle. DAPI staining (blue) was induced to indicate cells in the sections. Scale bar = 50 μm.

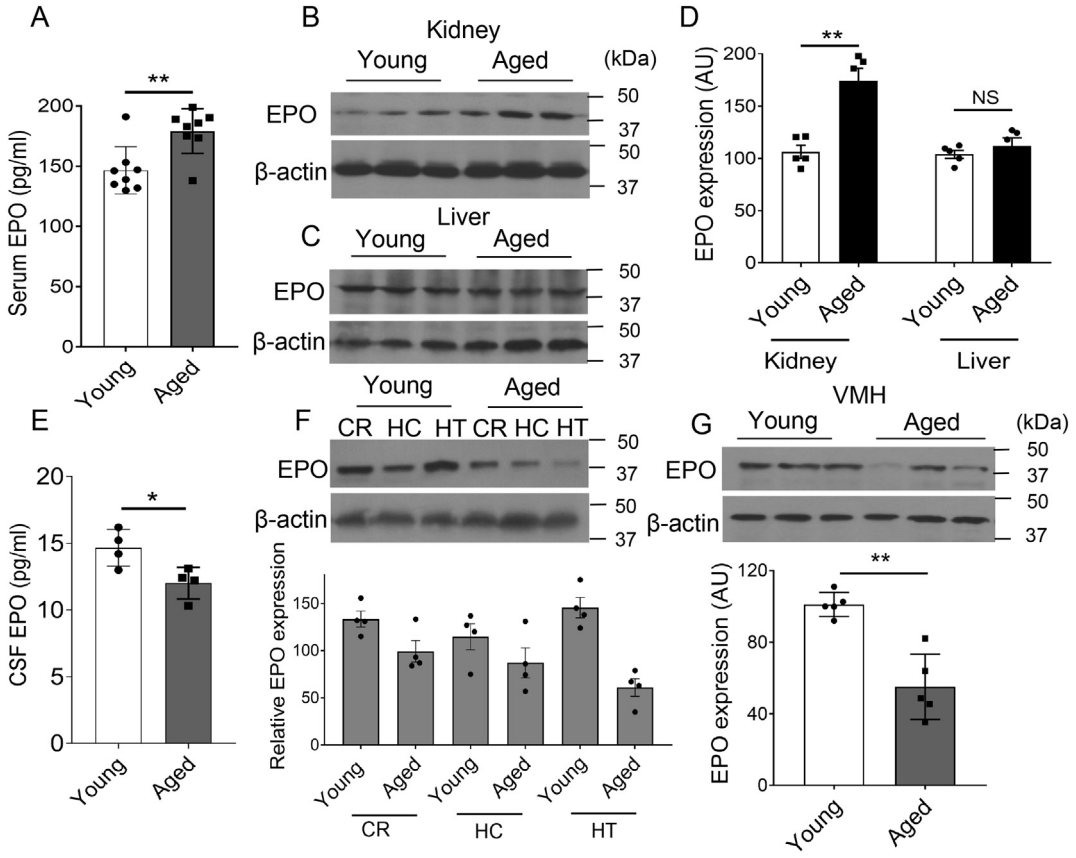

Figure 2: Changes of serum and brain EPO in middle aging. *A*, ELISA for EPO in serum of young (~3 months) and middle-aged (~16 months) mice (n = 8 mice per group, biological replicates). *B*, Western blot for EPO in kidney tissue of young and middle-aged mice (n = 5 mice per group, biological replicates). *C*, Western blot for EPO in liver tissue of young and middle-aged mice (n = 5 mice per group, biological replicates). *D*, Quantification of kidney and liver EPO expression. *E*, ELISA for EPO in the CSF of young and middle-aged mice (n = 4 mice per group, biological replicates). *F*, Western blot and quantification of EPO in different brain regions including the hypothalamus (HT), hippocampus (HC), and cerebral cortex (CR) of young and middle-aged mice (n = 4 mice per group, biological replicates). *G*, Western blot and quantification of EPO in the VMH of young and middle-aged mice (n = 5 mice per group, biological replicates). $^{**}p < 0.01$, $^{*}p < 0.05$; *NS*, not significant. Bars represent mean ± SE. *AU*, arbitrary unit.

EPO is expressed selectively in the motor cortex (M1, M2) and cingulate cortex (Cg1, Cg2). In contrast to this, there was weak or no expression in other parts of the cortex, including the anterior intraparietal area, somatosensory cortex (S1, S2), and piriform cortex. Using co-immunostaining, we labeled neurons using a neuronal marker (NeuN) and found EPO expression co-localized with this marker in about 50% of EPO-positive cells; hence, these neurons could be EPOergic with the functional capacity to express and produce EPO. Further, because EPO was highly expressed within the VMH, we co-stained for EPO with steroidogenic factor 1 (SF1), a biomarker for VMH neuronal subtypes that are important for metabolic regulation. The results showed that some SF1 neurons were positive for EPO expression (Figure 1D), further indicating that EPO in the VMH might have relevance for metabolic regulation. Furthermore, we checked EPO signaling in the hypothalamus by examining the level of signal transducer and activator of transcription 3 (STAT3) phosphorylation upon EPO injection to the hypothalamic third ventricle (Figure 1E). This finding suggested that there exists an interaction between the VMH and the ARC through local EPO production and signaling. Altogether, based on these observations, the hypothalamus should represent an important brain region for EPO production and signaling.

3.2. Aging is associated with increased peripheral EPO but decreased hypothalamic EPO

Serum EPO has been shown to increase with aging, including human subjects [23–26]. In light of this, we sought to examine how brain EPO and serum EPO might change with aging, bearing in mind that the BBB is weakly permeable to EPO transport into the brain [11]. Based on ELISA, we found that circulating EPO in the blood slightly increased in middle-aged (16 months old) mice compared with young (3 months old) mice (Figure 2A). This result was corroborated by Western blots, revealing higher levels of EPO protein in the kidney (Figure 2B,D) but not the liver (Figure 2C,D) of middle-aged mice compared with young mice. This result agrees with the appreciation in the literature that adult circulating EPO is primarily released by the renal cortex [2]. However, analysis of brain EPO level by ELISA revealed that brain EPO did not follow the same trend as serum EPO with aging; instead, EPO concentration in the CSF decreased with aging (Figure 2E). This indicated that serum EPO level is not representative of brain EPO level, and EPO diffusion across the BBB should have minimal effects on EPO in the CSF. Since we observed that EPO protein expression was high in the hypothalamus, hippocampus, and cortex (Figure 1A), we compared EPO levels in these tissues between middle-aged and

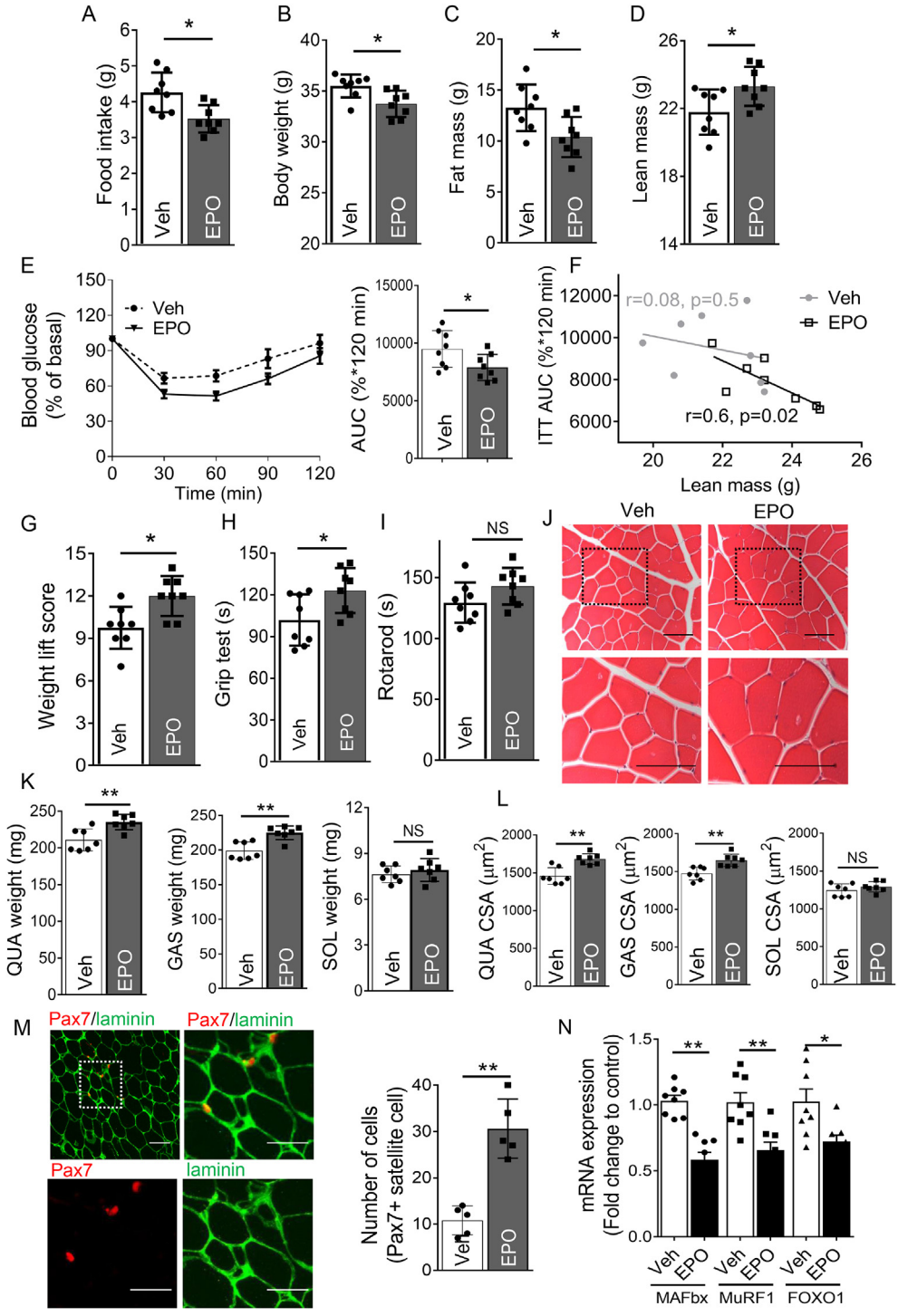

Figure 3: The physiologic effects of hypothalamic EPO injection in middle-aged mice. Middle-aged mice were injected with EPO or vehicle (Veh) in the hypothalamic third ventricle 3 times per week for 12 weeks. *A, B*, Representative daily food intake during the treatment (*A*) and body weight at the end of treatment (*B*) (n = 8 mice per group, biological replicates). C, *D*, Fat mass (*C*) and lean mass (*D*) measurement after the treatment (n = 8 mice per group, biological replicates). *E*, ITT measurement at 12 weeks after EPO injection (n = 8 mice per group, biological replicates). Data are presented according to the area under curve (AUC) values of ITT (the baseline blood glucose was designated as 100%). *F*, Correction analysis between ITT AUC and lean mass of EPO- and Veh-injected mice. *G-I*, Weight-lift score (*G*), grip test performance (*H*), and rotarod performance (*I*) (n = 8 mice per group, biological replicates). *J*, Hematoxylin and eosin staining for gastrocnemius (GAS) in middle-aged mice. Bottom images are high magnification of outlined regions in the top panels. Scale bar = 50 μm. *K*, Muscle weight of GAS and other limb muscle types including quadriceps (QUA) and soleus (SOL) (n = 7 mice per group, biological replicates). *L*, Muscle cross-sectional area (CSA) quantification for QUA, GAS, and SOL. *M*, Immunostaining (Pax7 [red] with laminin [green]) and quantification of Pax7+ cells in the GAS muscle of middle-aged mice (n = 5 mice per group, biological replicates). The outlined region in the low-magnification image (top left) was cropped to show high-magnification details of Pax7 and laminin staining (other panels). Scale bar = 50 μm. *N*, mRNA expression for muscle atrophy genes (MAFbx, MuRF1, and FOXO1) between hypothalamic third-ventricle EPO-injected and vehicle-injected middle-aged mice (n = 8 mice per group, biological replicates). $^{**}p < 0.01$, $^{*}p < 0.05$; *NS*, not significant. Bars represent mean ± SE.

young conditions. As shown in Figure 2F, EPO expression in these brain regions decreased in middle-aged mice compared with young mice, and the decrease in the hypothalamus was the most remarkable. Given that the VMH is an important hypothalamic subregion for EPO expression, we further dissected out the VMH in middle-aged versus young mice and then subjected these samples to Western blot for EPO. The results verified a significant loss of EPO expression in the VMH of the hypothalamus under the aging condition (Figure 2G). Taken together, while brain EPO in general decreases with aging, the hypothalamus is extremely sensitive to this effect of aging in suppressing neural EPO.

3.3. Hypothalamic EPO protects against aging-related metabolic and muscular dysfunctions

In animal models, i.p. or subcutaneous administration of EPO has been shown to have protective effects against obesity and glucose disorder [27,28,30,41], but it is unclear if these effects might involve the hypothalamus, considering that EPO at pharmacological doses could limitedly access the ARC, which has an incomplete BBB. Previous research did not explore hypothalamic EPO and how it could influence systemic physiology. In this work, we studied the physiological effects of EPO delivery to the hypothalamic third ventricle (henceforth *hypothalamic EPO injection*). We chose an injection dose of 30 pg; this dose is too low to affect blood EPO levels, and in fact, blood EPO concentrations did not significantly change even when this dose was entirely injected intravenously. In the context of our finding that middle aging was associated with hypothalamic EPO decline, our initial focus was on therapeutic interest through treating middle-aged mice via hypothalamic EPO injection (3 times per week for 12 weeks). We confirmed that central EPO treatment did not cause changes in blood EPO levels (EPO-injected vs. vehicle-injected mice: 175.3 ± 5.1 vs. 179.2 ± 6.6 pg/mL, $p = 0.48$). We observed that hypothalamic EPO treatment led to a moderate reduction in food intake, and thus, these EPO-treated mice became leaner than the control mice (Figure 3A–C). Through body composition examination, we found that while fat mass decreased, lean mass increased in mice following the treatment (Figure 3C,D). To profile the metabolic changes, we performed ITT and found that EPO-treated mice were more sensitive than vehicle-treated mice for the effect of insulin in lowering blood glucose (Figure 3E), and this metabolic sensitivity correlated with increased lean mass (Figure 3F). Considering that skeletal muscle is an important component of lean mass, we behaviorally assessed the muscle function of these mice through two commonly used tests for muscle strength in rodents, the weight-lift test and grip-strength test. As shown in Figure 3G,H, EPO treatment significantly improved muscle strength of middle-aged mice. Muscle coordination was also examined using a rotarod test, but this function was not affected by EPO treatment (Figure 3I). To summarize, hypothalamic EPO can provide an effect in counteracting against aging-associated metabolic and muscular dysfunctions.

3.4. Hypothalamic EPO injection leads to increased myogenesis but decreased muscle wasting

Because hypothalamic EPO injection increased muscle strength and mass, we analyzed muscle size at the end of the treatment. We weighed lower-limb muscles including quadriceps, gastrocnemius, and soleus and performed muscle tissue histology. As shown in Figure 3J–L, hypothalamic EPO injection led to increased muscle weight and muscle fiber size of the quadriceps and gastrocnemius. Compared with the effects on the quadriceps and gastrocnemius (which contain lots of fast-twitch fibers), the soleus (which is composed of slow-twitch fibers) was not affected by this EPO treatment (Figure 3J–L). In parallel, we sought to examine if the muscle phenotype in EPO-treated mice was related to any changes in muscle building versus decay. To assess muscle building, we stained muscle sections for Paired Box 7 (Pax7), a transcription factor that labels precursor muscle cells (also termed *satellite cells*). We found that EPO treatment led to an increase in the number of Pax7-positive muscle cells (Figure 3M), indicating that central EPO can work to promote the entry of muscle satellite cells into muscle generation. In parallel, we attempted to analyze muscle decay, since it is progressively associated with aging. To reflect the level of muscle decay, we examined the expression levels of several muscle degradation biomarkers, including muscle atrophy F-box (MAFbx), muscle RING finger 1 (MuRF1), and Forkhead Box O1 (FOXO1). Our results revealed that mRNA levels of these genes in muscles of middle-aged mice were suppressed by hypothalamic EPO injection (Figure 3N). Hence, while aging is associated with decreased myogensis and increased muscle decay, hypothalamic EPO treatment can provide a protective effect against these aging-associated muscular problems.

3.5. Hypothalamic EPO controls muscle through an adrenergic signaling axis to the BM

EPO was recently reported to stimulate sympathetic neurons of the rostral medulla [42], and BM was found to be responsive to adrenergic stimulation [43,44]. We were interested in testing if BM could be involved in relating the hypothalamus to the peripheral organs. Our interest was also heightened because BM is an important endocrine organ and is known to release many myogenic factors that promote muscle size. To address this question, using the design elucidated in Figure 4A, we transplanted donor β2-adrenergic receptor (β2AR) knockout BM into middle-aged BM-depleted wild-type (WT) mice, henceforth β2AR knockout chimera mice, to test whether inhibition of sympathetic stimulation to the BM could abrogate the therapeutic effects of EPO. We verified the success of this model by confirming that β2AR expression was barely detectable in the BM of the chimera mice (Figure 4B). These animals and WT controls then received hypothalamic EPO injection, as described in Figure 3. We noted that the loss of adrenergic signaling in the BM did not comprise the hypophagic effect of hypothalamic EPO injection (Figure 4C) but abrogated the therapeutic effects of EPO treatment in improving muscle functions (Figure 4D,E). Rotarod test was included a behavioral control, showing no difference between β2AR knockout and WT chimera with EPO or vehicle injection (Figure 4F), indicating that the muscular effects of BM β2AR knockout were rather specifically related to the central EPO treatment. Metabolically, we profiled these mice via insulin and GTTs. While WT chimera showed metabolic improvements in both tests, these benefits were also abrogated in β2AR knockout chimeras (Figure 4I,J), suggesting that the BM-muscle axis is important for the therapeutic effects of central EPO on glucose metabolism. At the end of the experiment, we examined muscle histology, showing that while hypothalamic EPO administration increased the weight and fiber size of the quadriceps and gastrocnemius in WT chimeras, these effects were lost in β2AR knockout chimeras (Figure 4G,H). The soleus was used as a tissue control, since this is the type of muscle that is not affected by central EPO, and no difference occurred among all groups (Figure 4G,H). Hence, through this transplantation model, we further confirmed that the effects of central EPO administration were independent of peripheral EPO and identified a downstream endocrine organ that relates the hypothalamus to the control of muscle size and functions.

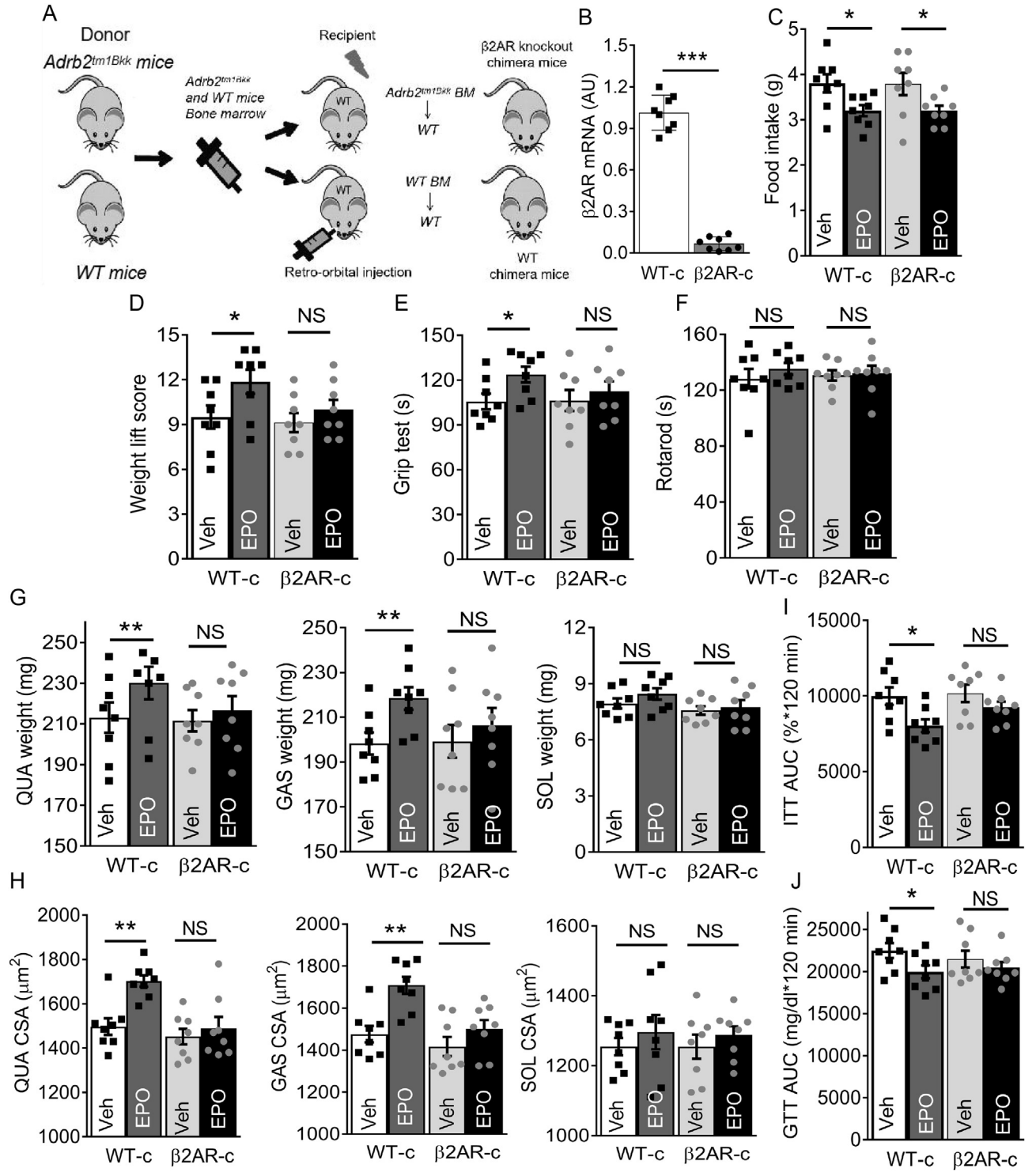

Figure 4: Bone marrow via adrenergic signaling links hypothalamic EPO to muscle. *A*, Experimental model for inhibition of bone marrow adrenergic signaling. BM was harvested from donor wild-type or β2AR knockout mice and transplanted into recipient, irradiated, middle-age, wild-type mice, which were referred to as β2AR knockout chimera (β2AR-c) and WT chimera (WT-c), respectively. *B*, mRNA expression for β2AR after bone marrow transplant in WT-c and β2AR knockout chimera mice (n = 8 mice per group, biological replicates). *C*, Representative food intake after hypothalamic third-ventricle injection of EPO versus vehicle in WT or β2AR knockout chimeras (n = 8 mice per group, biological replicates). *D-F*, Weight-lift score (*D*), grip test performance (*E*), and rotarod performance (*F*) with hypothalamic third-ventricle injection of EPO versus vehicle in WT or β2AR knockout chimeras (n = 8 mice per group, biological replicates). *G*, Muscle weight of quadriceps (QUA), gastrocnemius (GAS), and soleus (SOL) (n = 7 mice per group, biological replicates). *H,* Cross-sectional area (CSA) quantification for QUA, GAS, and SOL (n = 8 mice per group, biological replicates). *I-J*, ITT (*I*) and GTT (*J*) AUC measurement at 12 weeks after EPO injection (n = 8 mice per group, biological replicates). $^{**}p < 0.01$, $^{*}p < 0.05$; *NS*, not significant. Bars represent mean ± SE.

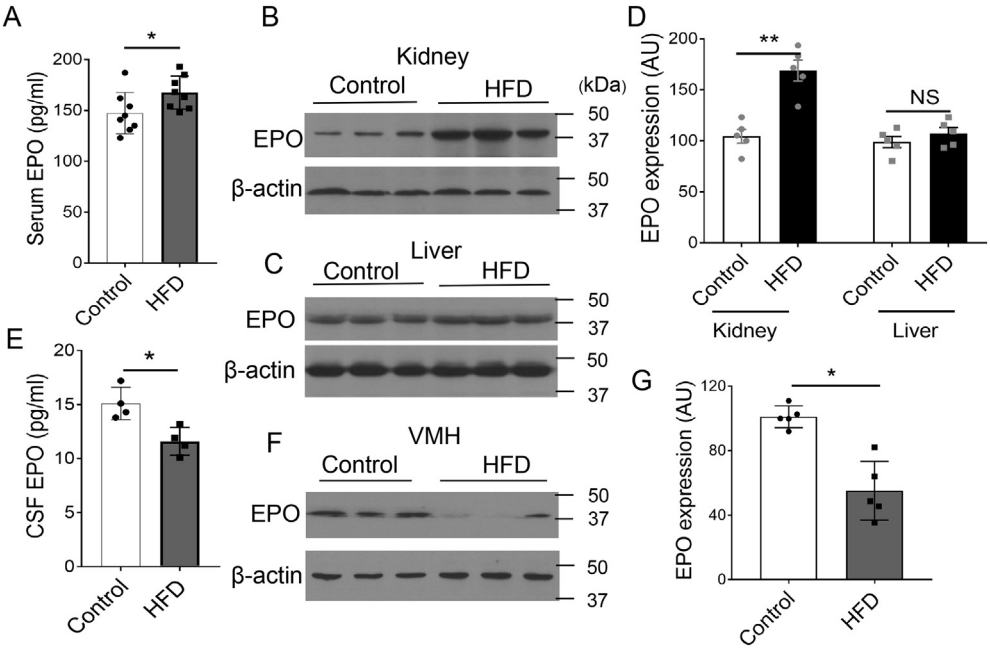

Figure 5: Serum and brain EPO in HFD-induced obesity. *A*, ELISA for EPO in serum of control (chow feeding) and DIO (3-months HFD) mice (n = 8 mice per group, biological replicates). *B*, Western blot for EPO in kidney tissue of control and DIO mice (n = 5 mice per group, biological replicates). *C*, Western blot for EPO in liver tissue of control and DIO mice (n = 5 mice per group, biological replicates). *D*, Quantification of kidney and liver EPO expression. *E*, ELISA for EPO in CSF of control and DIO mice (n = 4 mice per group, biological replicates). *F*, Western blot for EPO in the VMH of control and DIO mice (n = 5 mice per group, biological replicates). *G*, Quantification for VMH EPO expression. *$p < 0.05$; *NS*, not significant. Bars represent mean ± SE. *AU*, arbitrary unit.

3.6. Dietary obesity mimics aging in decreasing brain EPO despite increasing peripheral EPO

In parallel with our studies based on middle aging, we examined the condition of dietary obesity, a basic etiological condition that is important not only for metabolic disease but also for aging. Despite a wealth of research examining how exogenous EPO could affect physiology under certain metabolic conditions [27,28,30,41], it was of surprise that measurement of serum EPO in models of diet-induced obesity (DIO) through HFD feeding is still lacking, despite the fact that a clinical study has reported increased serum EPO in obese or aging subjects [45]. Thus, we compared 2 groups of mice that were maintained on an HFD versus normal chow for 3 months. Similar to the observations in middle aging, we found that chronic HFD feeding also led to a slight increase of EPO in the blood (Figure 5A) and kidney (Figure 5B,D), while other peripheral organs such as the liver (Figure 5C,D) were not affected. Also, EPO levels in both the CSF (Figure 5E) and the VMH (Figure 5F,G) were elevated in HFD-fed mice compared with the levels in chow-fed mice, resembling the brain pattern of EPO under middle aging. Thus, declining brain and hypothalamic EPO in the chronic condition of HFD-induced obesity is reminiscent of the middle-aging condition, which again suggests an etiologically close relationship between obesity and aging from this new biochemical perspective.

3.7. Hypothalamic EPO injection is protective against metabolic dysfunction with HFD feeding

We then investigated whether hypothalamic EPO treatment could affect metabolic disorders in the mouse model of HFD-induced obesity. Experimentally, we first studied chow-fed normal mice to obtain baseline information from hypothalamic EPO injection. A group of chow-fed young mice received an EPO injection via cannula in the hypothalamic ventricle for 8 weeks (3 times per week), and we confirmed that this central EPO treatment did not cause changes in blood EPO levels (EPO-injected vs. vehicle-injected mice: 143.9 ± 7.1 vs. 146.7 ± 6.9 pg/mL, $p = 0.93$). We observed that this EPO treatment decreased food intake, body weight, and fat mass in these chow-fed mice (Figure 6A–C), which was pretty similar to the observations in middle-aged chow-fed mice (Figure 3A–C). However, this EPO treatment did not lead to major changes in lean mass or glucose metabolism of these young healthy mice (Figure 6D–F). Compared with young healthy conditions, we appreciated that these effects of EPO treatment were more evident in middle-aged mice, suggesting that central EPO could be highly significant therapeutically. To study the model of dietary obesity, a group of mice were established with HFD-induced obesity and glucose intolerance and then subjected to the hypothalamic injection of EPO for 12 weeks (3 times per week). This central EPO treatment did not cause changes in blood EPO levels (EPO-injected vs. vehicle-injected mice: 167.3 ± 6.3 vs. 169.8 ± 5.3 pg/mL, $p = 0.67$). This hypothalamic EPO treatment led to a reduction in food intake in association with a protective effect against HFD-induced weight gain (Figure 6G), together with a reduction in fat mass (Figure 6H). Of note, EPO-treated mice had increased lean mass as compared with control mice (Figure 6I), suggesting that chronic HFD feeding recapitulated the middle-aging condition in revealing the effect on lean mass. As shown in Figure 6J,K, as both glucose and insulin tolerance were impaired in control HFD-fed mice, both of these impairments in HFD-fed mice were significantly reversed by the central EPO treatment. Correction for lean mass with insulin sensitivity showed that EPO treatment in this HFD feeding model increased insulin sensitivity with increasing lean mass (Figure 6L,M). Therefore, in addition to the benefit for muscle physiology in aging, hypothalamic EPO provides metabolic benefits in counteracting obesity and associated glucose disorders.

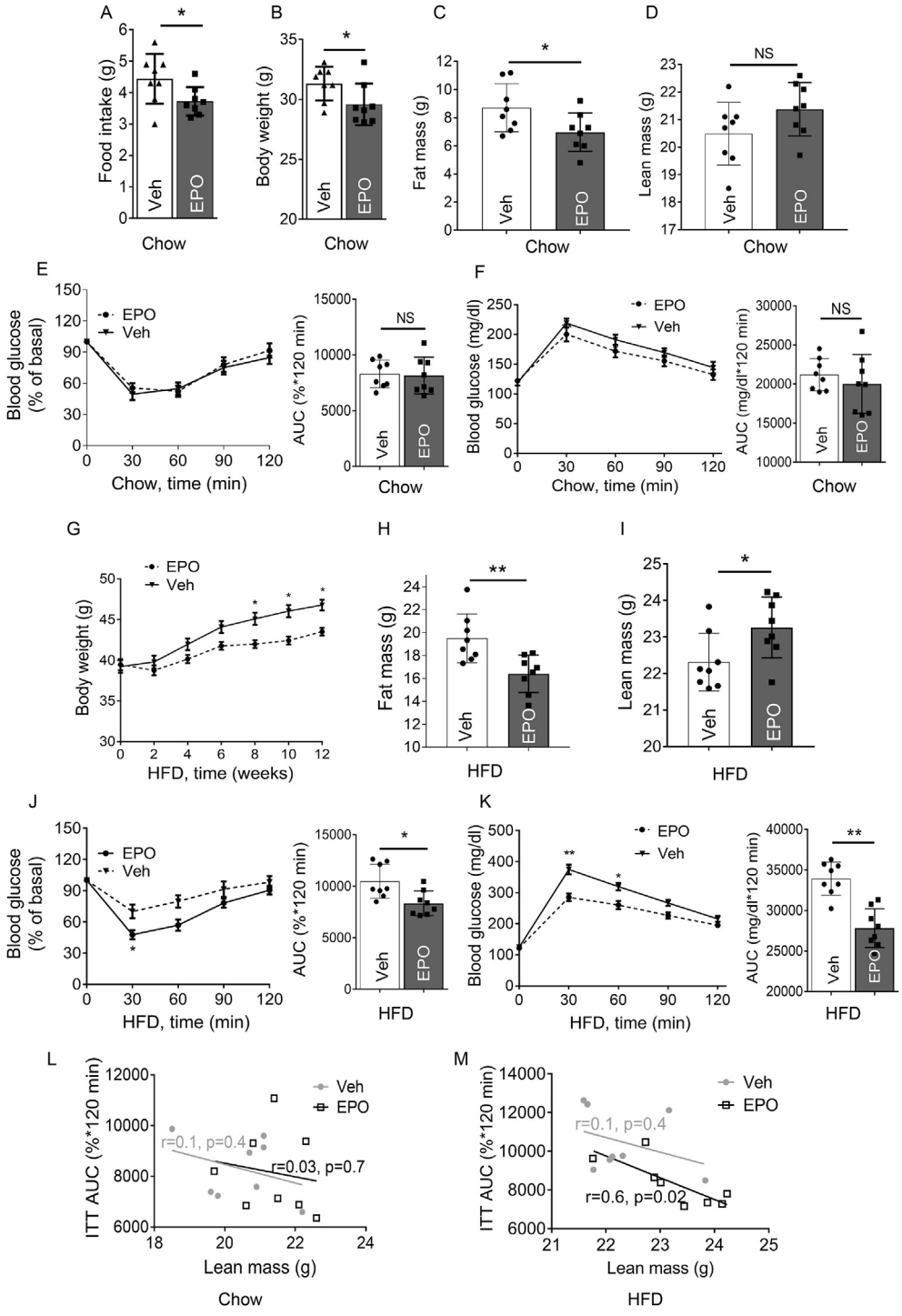

Figure 6: The metabolic effects of hypothalamic EPO injection in DIO. *A-F* Normal chow-fed young mice (2- to 3-month-old C57BL/6) were injected in the hypothalamic third ventricle with of EPO versus vehicle (Veh), 3 times per week, for 8 weeks. *A, B*, Representative daily food intake during the treatment (*A*) and body weight at the end of the treatment (*B*) (n = 8 mice per group, biological replicates). *C, D*, Fat mass (*C*) and lean mass (*D*) measurement after 8 weeks of EPO injections in normal chow-fed mice (n = 8 mice per group, biological replicates). *E, F*, ITT (*E*) and GTT (*F*) measurement at 7–8 weeks of EPO injections (n = 8 mice per group, biological replicates). *G-K* Standard mice (2-month-old C57BL/6) were fed with an HFD for 3 months and then divided into two groups to receive a hypothalamic third ventricle injection of EPO versus vehicle, 3 times per week, for 12 weeks, during which these mice continued to be maintained on HFD feeding. *G*, Body weight measurement every 2 weeks for 12 weeks with hypothalamic third-ventricle injection of EPO versus vehicle in HFD-fed mice (n = 8 mice per group, biological replicates). *H–I*, Fat mass (*H*) and lean mass (*I*) measurement after 12 weeks of EPO injection in HFD-fed mice (n = 8 mice per group, biological replicates). *J-K*, ITT (*J*) and GTT (*K*) measurement at 11 t 12 weeks of EPO injection (n = 8 mice per group, biological replicates). Baseline blood glucose levels were designated as 100% for ITT. *M, N*, Correction analysis between ITT AUC values and lean mass in chow-fed (*L*) or HFD (*M*) mice (n = 8 mice per group, biological replicates). $^{**}p < 0.01$, $^{*}p < 0.05$; *NS*, not significant. Bars represent mean ± SE.

4. DISCUSSION

Our findings showed that the hypothalamus, hippocampus, and cerebral cortex are the main sites of EPO expression. In the cortex, EPO expression was high in the M1/M2, Cg1, and Cg2 subregions, which seems to be in line with the physiological role of brain EPO in improving muscle strength given that the M1/M2 cortex regulates motor function. Our finding that the hippocampus and Cg1/Cg2 cortex subregions have high EPO expression is consistent with prior work showing that EPO improves cognition and memory [15–17]. In the hypothalamus, we observed high EPO expression in the VMH, a hypothalamic subregion that has been appreciated for its pivotal role in satiety and inhibitory control over feeding [46]. A previous study observed that POMC neurons in the ARC express EpoR [27]. Thus, this study supported our findings that hypothalamic EPO injection can reduce food intake and body weight. In addition, the strong EPO expression we observed in the VMH and co-staining of EPO with SF1 suggests that there exists a crosstalk between the VMH and ARC; for instance, EPO is produced and secreted from the VMH and travels to the nearby ARC to act on ARC neurons. This prediction was further supported by our finding that EPO leads to STAT3 activation broadly in the ARC of the hypothalamus.

Several studies have documented that systemic EPO increases with aging [23–26]. Since the primary function of systemic EPO is to stimulate erythropoiesis, as suggested in the literature [22], it is possible that an increase in systemic EPO with aging could be a result of an erythroid progenitor defect that makes these cells insensitive to EPO, thus leading to a compensative increase in renal production and release of EPO. In contrast to this pattern in the circulation, we found that brain EPO decreases with aging. This would support evidence that the BBB is relatively impermeable to EPO [11], and brain EPO has its own production and regulation that is independent of peripheral EPO. It is also important to note that EPO concentration in the CSF is an order of magnitude less than plasma EPO concentration but still decreases with aging or HFD feeding, further supporting an independent EPO system in the brain rather than through EPO transport across the BBB from the periphery. But it should also be pointed out that circumventricular organs in the brain such as the hypothalamus do not have a complete BBB and can have limited access to peripheral hormones; thus, in addition to its central production, peripheral EPO might have access to selective brain subregions and contribute to certain central signaling and functions. Another differential aspect is, compared with peripheral EPO production, which is strongly responsive to hypoxia, the brain normally does not have hypoxia, indicating that it is likely to be regulated by other mechanisms not explored in this study. Further research is needed to determine how EPO synthesis and secretion in the brain are regulated and to identify the stimulatory cues.

Hypothalamic EPO treatment in middle aging offers benefits in improving muscle size and function, and this protection is coupled with an enhanced metabolic capacity of handling blood glucose, which can be better demonstrated in the model of dietary obesity. To understand how brain EPO influences peripheral tissue function, we generated mice with inhibited adrenergic signaling in the BM. The rostral ventrolateral medulla is a central component of sympathetic activation in the brainstem, and it was previously shown to be stimulated by EPO [42]. We observed that β2AR expression in the BM is necessary for hypothalamic EPO treatment to improve muscle mass, strength, and glucose metabolism in middle aging. This suggests a signaling axis by which hypothalamic EPO increases adrenergic nerve activation of the BM, which potentially leads to the release of myogenic factors that are important for muscle growth and repair. The increased muscle mass could be responsible for the improvements in insulin sensitivity. However, the effects of hypothalamic EPO in the regulation of feeding are independent of this hypothalamus-BM axis, indicating that central EPO employs multiple pathways and processes in affecting the physiology of peripheral tissues. Peripheral EPO administration has also been shown to be beneficial against obesity and glucose disorder [27,28,30,41], and in the context of our findings within this work, we suggest that peripheral EPO travels to the hypothalamus (although limitedly) and acts upon certain hypothalamic neurons (such as POMC neurons), leading to changes in metabolic regulation. This suggestion should be studied experimentally in the future.

5. CONCLUSION

Brain EPO is present in the hypothalamus, cerebral cortex, and hippocampus. Brain and hypothalamic EPO decrease with aging and dietary obesity. Hypothalamic EPO treatment in aging or obesity offers benefits in muscle protection, which involves a role of BM, as well as in metabolic protection against energy and glucose imbalance. Thus, this study revealed how the brain and in particular hypothalamic EPO contribute to the pleiotropic functionality of EPO and how brain and systemic EPO are distinctly compartmentalized with differential significance in physiology and pathophysiology.

AUTHORS' CONTRIBUTION

D.C. conceived, designed, and supervised the project. Z.W. carried out the experiments and performed data collection and analysis. All authors were involved in data interpretation and discussion. S.K. and Z.W. drafted the manuscript, and D.C. edited and completed the writing of the paper.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with regard to this article.

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