Glucose-responsive neurons of the paraventricular thalamus control sucrose-seeking behavior

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Feeding behavior is governed by homeostatic needs and motivational drive to obtain palatable foods. Here, we identify a population of glutamatergic neurons in the paraventricular thalamus of mice that express the glucose transporter Glut2 (encoded by Slc2a2) and project to the nucleus accumbens. These neurons are activated by hypoglycemia and, in freely moving mice, their activation by optogenetics or Slc2a2 inactivation increases motivated sucrose-seeking but not saccharin-seeking behavior. These neurons may control sugar overconsumption in obesity and diabetes.

The brain depends almost exclusively on glucose as a source of metabolic energy. Sufficient provision of glucose to the brain is ensured by homeostatic mechanisms, which maintain glycemia at a minimum level of ~5 mM (ref. 1), and by hedonic mechanisms, which attribute a high reward value to glucose-containing foods to increase the motivation to consume them2–4. In this hedonic response the nucleus accumbens (NAc) plays a central regulatory role by integrating feeding-related inputs originating from several brain areas4–11.

In previous studies, using mice with genetic deletion of Slc2a2 in the nervous system (Slc2a2ΔloxP;NesCre+, NG2KO mice), we showed that Glut2 is required for the control by glucose of sympathetic and parasympathetic nerve activities12. Here we tested whether neuronal Glut2-dependent glucose sensing was also involved in motivated feeding behavior. NG2KO mice and control littermates were monitored during operant conditioning tasks, which associate nose-poking behavior with a light stimulus (conditioned stimulus, CS+) and the delivery of a sweet reward (10% sucrose solution). In a first set of experiments, mice were trained for 30 min daily for 10 d on a fixed ratio 1 schedule of reinforcement (FR1) where they learned to associate the CS+ with sweet reward. Appetitive learning was observed in both genotypes: rewards obtained and active nosepokes performed increased over time (Fig. 1a,b), whereas inactive nosepokes remained similarly low in NG2KO mice and control mice (Supplementary Fig. 1a), indicating similar learning capacity. However, NG2KO mice performed better in this task (Fig. 1a,b). Next we subjected mice to a progressive-ratio (PR) schedule consisting of a systematic within-session increase in the number of nosepokes required to earn each successive reward. NG2KO mice again performed better, with higher numbers of active nosepokes and rewards obtained, and a higher maximal operant response (breakpoint) indicating higher motivation (Fig. 1c–e). Similar behavioral tasks were then performed with the non-caloric artificial sweetener saccharin. NG2KO mice and control mice performed identically in FR1 (Fig. 1f,g) and PR (Fig. 1h–j) experiments, indicating that neuronal Glut2-dependent glucose sensing responds to sucrose and is insensitive to artificial sweeteners.

Operant conditioning is associated with activity of the NAc13,14. We therefore investigated whether Glut2-positive neurons or axons could be observed in the NAc using Slc2a2-cre;Rosa26tdTomato mice15. We observed numerous red fibers within the medial part of the NAc, mainly close to the anterior commissure and around the ventral end of the lateral ventricle (Supplementary Fig. 2a). To determine the origin of those fibers, we performed retrograde tracing experiments in which a cholera toxin B subunit–Alexa Fluor 488 conjugate (CTxB) was injected bilaterally in the NAc of Slc2a2-cre;Rosa26tdTomato mice (Fig. 2a and Supplementary Fig. 2b). Three weeks later, we screened the entire brain for tdTomato cell bodies labeled with CTxB. We found no tdTomato–CTxB co-labeling in the rare Glut2 neurons present in the ventral tegmental area, the prefrontal cortex, the hippocampus or the various hypothalamic nuclei. Colabeled cells were found only in the paraventricular thalamic nucleus (PVT; Fig. 2b) where 91 ± 1.7% of the PVT Glut2 neurons were also labeled with CTxB.

Next, we assessed whether the firing activity of tdTomato-positive neurons was responsive to changes in extracellular glucose concentrations (Fig. 2c). In the presence of 5 mM glucose, these neurons showed a low basal activity, which was markedly enhanced in the presence of 0.5 mM glucose. Returning the glucose to 5 mM restored the initial firing activity (Fig. 2d–f). The same glucose-dependent increase in firing activity was seen in the presence of a cocktail of synaptic inhibitors, indicating that glucose sensing is cell-autonomous (Supplementary Fig. 3a). When glycolysis was inhibited by 10 mM 2-deoxy-d-glucose, the firing activity dropped dramatically (Fig. 2g–i). Fructose at 5 mM failed to suppress firing induced by 0.5 mM glucose (Supplementary Fig. 3b). Finally, patch-clamp analysis of Glut2 neurons from mice previously injected with 2-deoxy-d-glucose showed that their firing frequency at 5 mM glucose was identical to that seen during exposure to 0.5 mM glucose of Glut2-neurons from naive mice and did not change when glucose was lowered to 0.5 mM (Fig. 2j–l). tdTomato-negative cells were also recorded in the presence of 5 or 0.5 mM glucose; they were found to be unresponsive to hypoglycemia (Supplementary Fig. 3c). Thus, PVT Glut2 neurons are activated by low glucose concentrations or by glycolysis inhibition; their firing cannot be suppressed by fructose, and they are responsive to in vivo induced neuroglucopenia.

To investigate the nature of Glut2 projections to the NAc, Slc2a2-cre mice were injected in the PVT with a viral construct allowing Cre-dependent expression of ChR2-eYFP (AAV-DIO-ChR2-eYFP; Fig. 3a)16. Brain slices obtained 3 weeks later displayed numerous eYFP-positive neurons in the PVT and fibers in the NAc (Fig. 3b,c). Electrophysiological recordings

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Figure 1 Inactivation of Slc2a2 enhances motivated sucrose-seeking behavior. In operant conditioning experiments, under an FR1 schedule, NG2KO mice (n = 18) showed higher motivation to obtain a sucrose reward compared to control mice (n = 22). (a) Rewards obtained (30-min daily sessions). (b) Active nosepokes (reward; genotype effect, F$_{1,341}$ = 47.29, P < 0.0001; interaction, F$_{3,341}$ = 0.65, P = 0.7574; active nosepokes; genotype effect, F$_{1,341}$ = 29.07, P = 0.0001; interaction, F$_{3,341}$ = 0.38, P = 0.9449; two-way ANOVA followed by Bonferroni’s test). Under a PR schedule, NG2KO mice showed higher motivation to obtain sucrose as shown by (c) the total number of rewards (P = 0.0314), (d) the number of active nosepokes (P = 0.0320), and (e) the breakpoint (P = 0.03; t-test). With saccharin as the reward (0.2% solution), NG2KO (n = 17) and control (n = 19) littermates behaved identically in FR1 schedules (f,g) (reward: genotype effect, F$_{1,335}$ = 0.47, P = 0.4915; interaction, F$_{3,335}$ = 0.67, P = 0.7403; active nosepokes: genotype effect, F$_{1,335}$ = 0.14, P = 0.7111; interaction, F$_{3,335}$ = 0.42, P = 0.9235; two-way ANOVA followed by Bonferroni’s test), or PR schedules (h–j) (P > 0.05, t-test). Graphs show means ± s.e.m.; box plots show median, lower and upper quartiles (boxes), and minima and maxima (whiskers). *** P < 0.001; * P < 0.05; n.s., non-significant.

from medium spiny neurons (MSNs) of the NAc together with local light-stimulation of the ChR2-expressing terminals (Fig. 3c) revealed postsynaptic inward currents (Fig. 3d), which could be totally abolished by tetrodotoxin, a blocker of action potential–dependent neurotransmitter release (Fig. 3d,e). The typical fast kinetics of those inward currents suggested they were AMPA receptor (AMPA) excitatory postsynaptic currents (EPSCs). This was confirmed by the application of the AMPAR antagonist DNQX, which entirely suppressed light-induced EPSCs in all the cells tested (Fig. 3f,g). Thus, light stimulation of NAc-projecting PVT Glut2 neurons triggers glutamate release onto NAc MSNs.

Figure 2 PVT Glut2 neurons project to NAc and respond to hypoglycemia. (a) Experimental approach. CTXB was bilaterally injected in the NAc of Slc2a2-cre;Rosa26tdTomato mice. (b) CTXB (green) was found to co-localize with tdTomato-positive Glut2 neurons (red) only in the NAc region. (c) Slc2a2-cre;Rosa26tdTomato mice were used to prepare acute brain slices containing the PVT Glut2 neurons for patch-clamp analysis. Inset shows microscopic view of a patched Glut2 neuron (red); white dashed lines delimit the recording pipette. (d) Neuronal activity recorded in juxtacortical configuration from a PVT Glut2 neuron in the presence of the indicated extracellular glucose concentrations. (e) Mean firing frequencies monitored under various extracellular glucose (Glc) concentrations (n = 16 neurons in 7 mice). Hypoglycemia induced a significant increase of the firing rate (F$_{2,14}$ = 14.49, P < 0.0001; one-way ANOVA followed by Bonferroni’s test). (f) Individual plots of firing frequencies for the neurons described in e. (g,h) Glucosamine (GLN) induced an increase in Glut2 neuronal activity despite the presence of 5 mM extracellular glucose (n = 8 neurons in 4 mice; F$_{2,7}$ = 7.9, P = 0.0051; one-way ANOVA followed by Bonferroni’s test). (i) Individual plots for neurons described in h. (j) Neuroglucopenia induced by intraperitoneal injection of 2-deoxy-D-glucose 45 min before mice were killed increased neuronal activity of PVT Glut2 neurons. (k) Mean firing frequencies monitored under the indicated extracellular glucose concentrations (n = 15 neurons in 8 mice). Injection of 2-deoxy-D-glucose increased the firing activity in the presence of 5 mM glucose and blunted the response to hypoglycemia (compare with e; F$_{2,14}$ = 0.11, P = 0.8976; one-way ANOVA followed by Bonferroni’s test). (l) Individual plots for neurons described in k. Box plots show median, lower and upper quartiles (boxes), and minima and maxima (whiskers). 3V, third ventricle; *** P < 0.001; ** P < 0.01; * P < 0.05; n.s., non-significant.
To test the functional role of the PVT Glut2 excitatory inputs onto the NAc, we used an operant conditioning protocol associated with in vivo optogenetic activation of these neurons. Slc2a2-cre mice and control mice were injected in the PVT with an AAV-DIO-Chr2-eYFP and equipped with a fiber-optic cannula. Following recovery, mice were tested under operant conditioning to obtain sucrose. At the beginning of each behavioral session, mice were connected to a light source via an optical fiber and stimulated during the entire duration of the session. During the training and learning period (FR1 schedule), the Chr2-expressing mice consumed significantly more reward (Fig. 3h) and performed more active nosepokes (Fig. 3i) than the control mice. Moreover, during the PR schedule, mice expressing Chr2 showed increased motivated sucrose-seeking behavior by obtaining more rewards and showing increased breakpoint (Fig. 3j,k).

To confirm that increased motivated behavior was light-dependent, we tested the same mice in a PR schedule 1 month later without light stimulation. No difference in motivated behavior was seen between genotypes (Supplementary Fig. 4b,c). A similar experiment was repeated but with light stimulation through fiber-optic cannulas placed bilaterally at the NAc level. Light stimulation increased motivated sucrose-seeking behavior as assessed in FR1 and PR schedules (Supplementary Fig. 5). When tested 3 d later without light stimulation, the breakpoint value of control mice remained the same and Chr2-expressing mice exhibited motivation levels similar to those seen in controls. Learning during the FR1 protocol was slower than in the previous experiment (Fig. 3h,i), probably because these mice were implanted with two optical fibers instead of one, which may have hampered their access to the nosepoke holes.

Finally, we inactivated Slc2a2 selectively in the PVT of Slc2a2loxP/loxP mice by stereotactic injection of an AAV-Cre-GFP (Fig. 3l). PCR analysis of genomic DNA extracted from the PVT and fluorescence microscopy analysis confirmed proper injection of the virus and recombination of Slc2a2loxPloxP allele in the AAV-Cre-GFP injected mice (Supplementary Fig. 6). These mice displayed increased motivation to get sucrose compared to control mice as reflected by the higher number of rewards they obtained and increased breakpoint they displayed (Fig. 3m,n).

Collectively, our data show that Glut2 neurons from the PVT and projecting to the NAc form a population of hypoglycemia-activated glutamatergic neurons that induce EPSCs in MSN neurons. Their firing rate can be increased by glycolysis inhibition but cannot be suppressed by fructose, indicating that they are controlled by...
glucose metabolism. When activated by hypoglycemia, optogenetics or Slc2a2 inactivation, they increase motivated sugar-feeding behavior, indicating a dominant role in motivated sugar seeking. The possible relevance of these results to human physiology is suggested by the observations that mild hypoglycemia induces marked increases in synaptic activity in PVT and that variants in SLC2A2 are associated with increased intake of sugar-containing foods and conversion from impaired glucose tolerance to type 2 diabetes. This neuronal pathway may therefore represent a target for the prevention of metabolic disease aiming at restoring the normal balance between the homeostatic and hedonic control of food intake.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.T., G.L. and B.B. designed the experimental strategy and analyzed the data. D.T. performed histological analysis and supervised mouse breeding. All other experiments were performed by G.L. B.T. and G.L. wrote the manuscript. All authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All procedures were approved by the veterinary office of Canton de Vaud (Switzerland) under veterinary license number V2D153. Slc2a2

isoP/NeuN (NG2KO), Slc2a2-cre and Slc2a2-cre;Rosa26tdTomato mice were as described previously.11,15,16 NG2KO and Slc2a2-cpe;Rosa26tdTomato mice were on a mixed C57BL/6J congenic background. Slc2a2-cpe and Slc2a2-cpe;Rosa26tdTomato mice were on a C57BL/6J background. All studies used littermates as controls. For all experiments, mice were age-matched and randomly assigned to experimental groups to ensure an unbiased distribution of animals. No blinding was used. Unless otherwise stated, animals were collectively housed (maximum 5 individuals per cage) on a 12-h light/dark cycle (lights on at 7 a.m.) and fed with a standard chow (Diet 3436, Provimi Kliba AG).

Viral vectors. All viral constructs were produced by the Vector Core of the Gene Therapy Center at the University of North Carolina (UNC, North Carolina, USA).

Operant conditioning. Adult NG2KO males and control littermates (10 to 15 weeks old) were housed individually under a 12-h reversed light/dark cycle (lights on at 8:30 p.m.) at a constant temperature (22 ± 1 °C) and had ad libitum access to water. Starting from the first day of experiment, mice were slightly food restricted, receiving −3 g of laboratory chow per day. No more than 15% of body weight reduction over the entire duration of the experiment was allowed. Mice were trained in operant conditioning chambers (Med Associates) under a fixed ratio 1 schedule of reinforcement (FR1) during 30-min daily sessions. Animals had a choice between two nosepoke ports: an active nosepoke hole associated with a 3-s light cue and a concomitant delivery of a liquid reward through a central spout equipped with infrared head entry detector and an inactive nosepoke hole that remained inoperative. Each active nosepoke triggered the delivery of 10 µl of a 10% sucrose or 0.2% saccharin solution. Liquid rewards remained available for 3 s once access to the central spout was detected. Supplementary entries in the active nosepoke in the absence of head entry detection above the liquid dipper and entries in the inactive nosepoke were recorded but had no further consequence. After a training period of 7 to 10 d, the animals underwent three consecutive sessions (one 90-min session per day) under a progressive-ratio schedule of reinforcement (PR). Under this schedule the mice were required to progressively increase the number of active nosepokes between two successive rewards based on the progression sequence given by ref. 20: response ratio (rounded to nearest integer) = (5 × e (reward × 0.2)) − 5. Hence, the progressive-ratio schedule followed the progression 1, 2, 4, 8, 16, 32, 64, 128, 320, 640, 3200, 6400, 4500, 1500, 500, 100, 50, 10, 5, 2, 1. The maximal number of active nosepokes performed to reach the final ratio was defined as the breakpoint, a value reflecting the animals motivation to get the reward. Active/inactive nosepokes and rewards obtained were monitored online using an analog/digital interface coupling the operant chambers to a computer running the MED-PC behavioral software suite (Med Associates).

Neuronal retrograde tracing. Four adult Slc2a2-cpe;Rosa26tdTomato males (10 weeks old) were anesthetized with isoflurane (5% in/o for induction followed by 2.5% for maintenance (Attane, Piramal Healthcare)) and placed in a stereotaxic frame (David Kopf Instruments). NAC was infused bilaterally with CTxB conjugated with Alexa Fluor 488 (Molecular Probes, Life Technologies). The following stereotaxic coordinates were used: AP +1.5 / ML ± 1.4 / DV −4.5 mm (10° angle). A volume of 0.4 µl of 0.25% CTxB solution was injected in each hemisphere through a 33-gauge stainless steel injector (Hamilton) coupled to a precision pump (Harvard Apparatus) with an injection rate of 0.1 µl min−1. Two weeks later, animals were anesthetized (isoflurane) and perfused transcardially with a 0.1 M phosphate buffer (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. The perfused brains were dissected and postfixed with 4% paraformaldehyde in PBS at 4 °C for 1 h and then placed overnight at 4 °C in a solution containing 30% sucrose in PBS. Brains were frozen in isopentane and maintained at −20 °C until further analysis. Cryosections (25 µm) were prepared (Leica) and mounted onto glass slides with Mowiol anti-fade medium (Sigma-Aldrich). Images were taken using an Axio Imager D1 microscope interfaced with a Axiovision software or an LSM 510 Meta inverted confocal laser scanning microscope with LSM software 3.5 (Zeiss). For neuronal quantification, four sections containing PVT were screened in each mouse (n = 4) and Glut2 neurons were counted (n = 891) to determine percentage of those neurons were colabeled with CTxB. Injection sites were verified for each mouse at the end of the experiment.

Electrophysiology. Four- to 8-week-old males used for electrophysiology were deeply anesthetized with isoflurane before decapitation. The brains were removed immediately submerged in an ice-cold slushy ACSF solution saturated with 95% O2 / 5% CO2 containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1 MgCl2, 2 CaCl2, 26 NaHCO3, 12.5 sucrose and 5 mM glucose (300 ± 5 mOsm). Acute coronal sections (250 µm) containing PVT or NAc were obtained using a vibratome (VT1000S; Leica) and maintained at 32 °C for at least 1 h before recording. Experiments were performed using an upright epifluorescence microscope (BX51WI; Olympus) mounted on a motorized stage and coupled to micromanipulators (Sutter Instruments). Brain slices were placed in a recording chamber and continuously superfused at a rate of 2 ml min−1 with oxygenated ACSF maintained at 32–34 °C. Borosilicate pipettes (resistance: 3 to 5 MΩ; Harvard Apparatus) were shaped with a horizontal micropipette puller (Sutter Instruments). The firing rates of PVT neurons at different extracellular glucose concentrations (5 or 0.5 mM) were monitored after a 10–15 min baseline through juxtcacellular recordings in current-clamp mode using a MultiClamp 700B amplifier (Molecular Devices). The intrapipette solution contained (in mM) 130 potassium gluconate, 10 HEPES, 0.2 EGTA, 5 NaCl, 1 MgCl2, 10 sodium phosphocreatine, 4 Mg-ATP and 0.5 Na2-GTP (pH 7.2–7.3; 275 ± 5 mOsm). Where mentioned in the text, a cocktail of synaptic inhibitors was used, composed of picrotoxin (100 µM; GABA_A receptor antagonist), DNQX (10 µM; AMPA and kainate receptor antagonist), DL-AP5 (100 µM; NMDA receptor antagonist) and strychnine (1 µM; glycine receptor antagonist) (Tocris Bioscience). For ex vivo optogenetic experiments in medium spiny neurons of the NAc, neurons were voltage clamped at −70 mV in whole-cell configuration and light-induced currents were recorded with an intrapipette solution containing (in mM) 117 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 Mg-ATP and 0.5 Na2-GTP (pH 7.2–7.3; 275 ± 5 mOsm). Blue light (473-nm) pulses were delivered on the brain slice through the microscope objective. TTX 1 µM and DNXQ 10 µM were bath applied in the perfusion chamber. Currents traces were constructed by averaging 15 consecutive photocurrents. Signals were filtered at 2 kHz, digitized at 10 kHz and collected online using a pClamp 10 data acquisition system (Molecular Devices).

Optogenetics. Ten- to 12-week-old adult Slc2a2-cpe or Slc2a2-cspe males were used for optogenetics experiments. Under isoflurane anesthesia, mice were placed in a stereotaxic frame (David Kopf Instruments) and PVT was infused with the viral vector rAAV2-E1a-DIO-hChR2(H134R)-eYFP (titer, 3.08 × 1012 particles per ml) to allow the expression of channelrhodopsin-2 in a Cre-dependent manner (0.25 µl delivered at 0.1 µl min−1). To ensure a good expression of the ChR2 in the entire PVT, the virus was injected at two coordinates: AP −0.4 / ML +0.8 / DV −3.5 mm and AP −1.6 / ML +0.7 / DV −3 mm with a 10° angle to avoid the damage to the superior sagittal sinus. Three weeks after viral injection, mice were either used for ex vivo electrophysiological experiments or involved in in vivo operant conditioning behavioral tasks as described above. For ex vivo experiments, 4-ms light pulses were delivered by a high-power collimated blue LED system (M470L2-C1 + LED11B; Thorlabs) through the optical path of the microscope. For in vivo experiments, optical fiber cannulas (Precision Fiber Products) were lowered either unilaterally in PVT (AP −0.4 / ML +0.8 / DV −3.4 mm, 10° angle) or bilaterally in NAc (AP +1.5 / ML ± 1.35 / DV −4.2 mm, 10° angle) and fixed to the skull with surgical screws (PlasticsOne), tissue adhesive (VetBond; 3M) and dental cement (Paladur; Heraeus-Kulzer). A DSPP laser (LRS-0473-GFM-00100-05; Laserlow Technologies) was used to deliver light pulses (10-ms light pulses at 20 Hz; 1 s on/1 s off; 10–15 mW) through an optical fiber (0.22 NA: 200-µm core diameter; Doric Lenses) that was attached to the optic cannula at the beginning of each experimental session (FR1 or FR2). Light stimulation was provided during the entire duration of the experimental session. To prevent the optical fiber from twisting and to allow mice to move freely, a rotary joint (FRI 1 × 1; Doric Lenses) was placed outside the operant chamber right on top of the animal. Injection sites and optical fiber cannula placement were verified for each mouse at the end of the experiment.

PVT Glut2 deletion. Ten- to fifteen-week-old adult Slc2a2-cpe;Rosa26tdTomato males were injected with the viral vectors rAAV5-Cre-GFP (titer, 5 × 1012 particles per ml) or
rAAV5-TRUFR-GFP (titer, \(6.9 \times 10^{12}\) particles per ml) at the following injections sites: AP −0.4 / ML +0.8 / DV −3.5 mm (10° angle) and AP −1.6 / ML +0.7 / DV −3.0 mm (10° angle). Each injection consisted of an infusion of 0.25 µl virus at a rate of 0.1 µl min\(^{-1}\). At the end of the operant conditioning experiments, mice were anesthetized (isoflurane) and killed, and deletion of Glut2 was determined via PCR amplification of genomic DNA from dissected brain tissue containing PVT as previously described\(^{21}\). PCR amplification gels were made in duplicates. Injection sites were verified for each mouse at the end of the experiment.

**Statistical analysis.** Statistical significance was calculated using GraphPad Prism 5.0c (GraphPad Software). The sample size for each experiment was determined based on published studies using similar experimental designs together with pilot experiments from our laboratory. This allowed us to determine the sample size required for each experiment to ensure a statistical power of 0.8 and an alpha level of 0.05. Two-sided Student’s t-tests were used to analyze statistical significance between pairs of experimental groups. To ensure variance similarity between compared groups, Fisher’s F-tests were performed before each t-test. When multiple comparisons were required, a one-way or two-way ANOVA was performed followed by Bonferroni’s post hoc tests. The data distribution was assumed to be normal. Data are represented as means ± s.e.m. Levels of statistical significance are indicated as follows: ***\(P < 0.001\); **\(P < 0.01\); *\(P < 0.05\).

A **Supplementary Methods Checklist** is available.

**Data availability.** Data are available upon request from the corresponding author.

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