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Q:1 Negative energy balance hinders prosocial helping behavior

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Q:11 The internal state of an animal, including homeostatic requirements, modulates its behavior. Negative energy balance stimulates hunger, thus promoting a range of actions aimed at obtaining food. While these survival actions are well established, the influence of the energy status on prosocial behavior remains unexplored. We developed a paradigm to assess helping behavior in which a free mouse was faced with a conspecific trapped in a restrainer. We measured the willingness of the free mouse to liberate the confined mouse under diverse metabolic conditions. Around 42% of ad libitum–fed mice exhibited a help-Q:12 ing behavior, as evidenced by the reduction in the latencies to release the trapped cagemate. This behavior was independent of subsequent social contact reward and was associated with changes in corticosterone indicative of emotional contagion. This decision-making process was coupled with reduced blood glucose excursions and higher ATP:ADP ratios in the forebrain of helper mice, suggesting that it was a highly energy-demanding process. Interestingly, chronic (food restriction and type 2 diabetes) and acute (chemogenetic activation of hunger-promoting AgRP neurons) situations mimicking organismal negative energy balance and enhanced appetite attenuated helping behavior toward a distressed conspecific. To investigate similar effects in humans, we estimated the influence of glycated hemoglobin (a surrogate of long-term glycemic control) on prosocial behavior (namely charity donation) using the Understanding Society dataset. Our results evidenced that organismal energy status markedly influences helping behavior and that hypothalamic AgRP neurons are at the interface of metabolism and prosocial behavior.

helping behavior | energy status | AgRP neurons | hunger | hypothalamus

The internal state of an animal (including arousal, motivation, emotion, and varying homeostatic needs) can profoundly influence its behavioral decisions (1). Indeed, the integration of external and internal cues orchestrates appropriate behavioral and physiological responses that are crucial for survival (1). For example, limited food resources entail a situation of negative energy balance that stimulates hunger. Hunger is a universally recognized signal that triggers a repertoire of behaviors aimed at fulfilling organismal energy requirements (2). In this context, it is well established that hunger modulates sensory perception and promotes a range of orchestrated and prioritized behaviors that are intuitively connected with food acquisition (locomotion, exploratory drive, foraging, etc.) (2). However, less is known about the impact of hunger on emotions and, in particular, on prosocial behaviors.

Prosocial behaviors are voluntary actions intended to benefit others, such as sharing, comforting, caring, and helping in the absence of reward (3). In the context of the present research, the word "intended" refers to a goal-directed learned action in order to be more suitable for interpreting mouse behavior (4). It is believed that the basis of targeted helping is empathy, an advanced mental capacity that has been traditionally restricted to humans (5). However, growing experimental findings evidence the existence of empathy-like behaviors in diverse animal species (3) including rodents (6). Indeed, rats and mice are able to perceive negative experiences of conspecifics via emotional contagion (7–9) and even rescue conspecifics in distress under threatening situations (10–13).

In the current study, we aimed at investigating whether perturbations in the organismal metabolic status influence prosocial helping behavior in mice. We found that diverse interventions mimicking a state of negative energy balance compromised helping performance, as measured by the liberation of distressed conspecifics under restrained conditions. This process, which was guided by emotional contagion, was highly demanding in terms of brain energy costs. Our data also provide evidence that pathological conditions associated with negative energy balance interfere with helping behavior in both mice and humans.

Materials and Methods

Q:13 **Animals and Husbandry.** Mice were maintained in a 12-h light–dark cycle with free access to water and standard chow diet unless stated. C57BL/6 and *AgRP^cre/+* mice (14) were bred in-house. All experimental protocols

Significance

In the current study, we investigate the influence of the metabolic internal state of an animal on prosocial helping behavior. We found that situations that entail hunger or limited nutrient availability correlated with a reduced helping behavior toward a conspecific in distress. These Q:10 results represent a significant advance in the field of prosocial science as they provide insights into complex animal behaviors. Furthermore, our work also evidence that specific hypothalamic neurons are at the interface of metabolic control and helping behavior, thus integrating homeostatic and social cues.

were approved by the University of Barcelona Ethics Committee complying with the current Spanish and European legislation.

Behavioral Procedures. General behavioral procedures are detailed in *SI Appendix, Materials and Methods.*

Helping Behavior Test (HBT).Apparatus. The HBT for mice described herein was adapted from a previously established protocol for rats (12). Briefly, a rodent restrainer (5 cm diameter and 18 cm long) was divided into two equal compartments (5 cm diameter and 4 cm long) that were large enough to permit the trapped mouse to move and turn around. The restrainer, which was laid on a methacrylate platform (21 × 18 cm), had two rabbets housing sliding doors on both sides. These doors could be opened by either pushing or pulling. This action required perseverance before accomplishing effective opening and could not be executed randomly or by chance. A trapped dummy mouse was used as a control to ensure that door opening was motivated by helping behavior rather than arbitrary behaviors.

Subjects. Animals were weaned in groups of four mice per cage and at 6 to 8 wk of age were housed in dyads. Trapped and free mice were randomly designated, and no selection criteria was used prior to the actual study. Free mice were labeled with an ear perforation. Tests were conducted at 10 to 12 wk of age.

Protocol. It comprises the habituation phase, the helping testing phase, and, in some cases, the crossover phase. Habituation consisted of daily sessions during 4 consecutive days, where trapped and free mice were allowed to freely explore the arena and the empty restrainer for 15 min. The helping testing phase consisted of daily sessions during 10 consecutive days. The free mice were exposed to the restrainer empty or with a dummy and trapped mouse (in a counterbalanced position) for 30 min. After this time, if the free mouse was unable to liberate the conspecific, the experimenter manually half-opened the door and allowed the trapped and free mice to remain in the arena for 10 additional minutes. The crossover phase consisted of extending the helping testing phase for 10 additional daily sessions or until achieving 5 consecutive opening days but exchanging treatments between groups. Each dyad performed only one trial per day during the entire protocol.

Nonhelper and helper mice. Free mice performed the task for 10 consecutive days (always with the same paired trapped mouse). Mice were considered nonhelpers when failed to liberate the trapped conspecific after the 10-d protocol. A free mouse that liberated its trapped cagemate for at least five consecutive sessions was considered a helper mouse. Thus, the exposure time to the HBT was 10 d for nonhelper mice and 10.4 to 12.1 d for helper animals [as they began to release their cagemates around the sixth day of testing; mean (95% CI) = 6.3 (5.4 to 7.2) d].

Latency to door opening. Helper mice that started opening after the fifth session were tested until they achieved five consecutive door openings. However, door-opening latencies were plotted only until day 10 of testing. In the crossover phase, the latency to door opening was plotted from the first day of crossover treatment until helper mice achieved 5 consecutive days of door opening.

Separated helping test. To investigate whether door opening was motivated by subsequent social contact rather than a genuine helping behavior, we modified the HBT in a way that the released and helper mice remained physically separated. Details are provided in *SI Appendix, Materials and Methods*.

Food restriction. Twelve-week-old C57BL/6 mice were submitted to food restriction using an automated feeder system (ClockLab Feeder Control, Actimetrics) that provided scheduled dustless precision pellets (BioServe). Control ad libitum–fed mice were provided with the same diet. Food restriction consisted of ad libitum access to food only during the dark cycle and at the necessary amount to maintain 85 to 90% of the initial body weight. This protocol started the night of the first habituation session and was maintained across the sessions of each phase of the protocol.

Sucrose consumption test. After the regular HBT, mice were submitted to an extra session with locked doors. A bottle containing 1% sucrose was offered in the arena. Sucrose consumption was measured at the end of the test.

High palatable food accessibility. To test if the presence of a high palatable food influenced helping behavior, we replaced the dummy mouse during the HBT with a pellet containing 45% of Kcal derived from fat (Research Diets). This diet was presented in the home cage in small quantities (0.6 g/d) for 2 d before the test. Experimental setup, testing, and measurements were performed as described above.

Streptozotocin (STZ)-induced diabetes. Six-week-old C57BL/6 mice were intraperitoneally injected with STZ (50 mg/kg, Sigma) or vehicle (sodium citrate dihydrate, pH 5.4) for 5 consecutive days after 5-h fasting. One week after the final injection, blood glucose was measured. Animals were considered diabetic when random-fed blood glucose levels were ≥200 mg/dL. Mice were submitted to the HBT at 12 wk of age.

Chemogenetic activation of AgRP neurons. $AgRP^{Cre/+}$ mice were injected with an adeno-associated virus (AAV) encoding excitatory Designer Receptors Exclusively Activated by Designer Drugs [DREADDs; AAV8-hSYN-DIO-hM3D(Gq)-mCherry, Addgene] into the ARC. Detailed information is described in *SI Appendix, Materials and Methods*.

Quantification of Social Interaction Test. Assessment of social interaction between helper/nonhelper mice and trapped counterparts was conducted following previously published protocols (15). Detailed information is described in *SI Appendix, Materials and Methods.*

Elevated Plus Maze Test. Twelve-week-old naive free mice, after four sessions of habituation to the arena and the empty restrainer, were randomly exposed to a dummy or trapped mouse during 20 min under the same setup as in the HBT. The elevated plus maze test was based on previously published protocols (16). Detailed information is described in *SI Appendix, Materials and Methods.*

Physiological Tests. Body weight, blood glucose, and blood sampling for corticosterone quantification were performed during the morning (1 to 6 h after lights on) of day 5 of the HBT. Blood samples were taken 30 min before and immediately after the HBT. Each session included four parallel arenas with nonhelper and helper dyads in a counterbalanced manner. In this particular experimental setting, the researcher did not open the doors at the end of the test, thus avoiding potential interferences of social contact with the glucose or corticosterone levels. All animals received the same cues and the same time exposure to the behavioral paradigm to minimize confounding factors. Blood glucose was measured using a glucometer (Arkray). Blood samples were collected via the tail vein using a capillary collection system with EDTA (Sarstedt) and centrifuged at 3,600 rpm for 20 min at 4 °C to obtain plasma. Corticosterone was measured via the ELISA Kit (Immunodiagnostic Systems).

Fluorescent mRNA In Situ Hybridization (RNAscope) and Quantification. Fluorescent in situ hybridization for the simultaneous detection of oxytocin (*Oxt*) and *Fos* mRNA was performed using RNAScope. Detailed information is described in *SI Appendix, Materials and Methods*.

FOS Brain Immunostaining and Quantification. Brain slices from helper and nonhelper mice were stained with rabbit anti-FOS primary antibody (1:200; Santa Cruz Biotechnology) following standard protocols. Detailed information is described in *SI Appendix, Materials and Methods*.

Hexokinase (HK) Brain Immunostaining and Analysis. Brain slices were incubated with rabbit anti-HK antibody (1:200; Merck Millipore) following standard protocols. A custom-made macro was programmed with instructions for the automated image analysis pipeline. HK-positive cells were segmented as previously described (17). Detailed information is described in *SI Appendix, Materials and Methods*.

Metabolite Analysis by NMR. After an extra session of HBT with locked doors, mice were killed, and brain regions were rapidly dissected in a cold matrix/plate. Brain metabolite extraction was performed according to the methanol:chloroform protocol as previously described (18). Metabolite quantification was performed by comparing the area of the peaks of interest to that of TPS using Chenomx (19). Detailed information is described in *SI Appendix, Materials and Methods*.

Prosociality in a Sample of the British Public. We used data from the Understanding Society, the main UK Household Longitudinal Survey (20), which contains a sample of biomarkers. Detailed information is described in *SI Appendix, Materials and Methods.*

Results

Mice Exhibit Prosocial Helping Behavior toward a Trapped Conspecific. To investigate the influence of metabolic state on prosocial helping behavior in mice, we adapted a previously validated paradigm described by Bartal and collaborators (12). The paradigm, hereafter called the HBT, consisted of placing a free mouse in an arena with a two-compartment restrainer (containing a trapped cagemate or a dummy mouse) closed by sliding doors (Fig. 1A and *SI Appendix*, Fig. S1A). The dummy mouse served as the control condition to rule out any motivation for door opening other than goal-directed helping. Under this setting, the liberation of the trapped cagemate required a free decision-making task (Fig. 1*A* and *SI Appendix*, Fig. S1A and Movie S1).

Testing sessions were conducted for 10 consecutive days and lasted 40 min. During the first 30 min, the free mouse was able to undisturbedly explore the arena and the restrainer containing the trapped and dummy mouse. If the free mouse failed to liberate the confined conspecific, the experimenter manually half-opened the sliding door (to prevent learned helplessness) and allowed both mice to remain together in the arena for 10 min. A mouse that liberated its cagemate for at least five consecutive sessions was considered a "helper." On average, helper mice began to release their cagemates around the sixth day of testing [mean (95% CI) = 6.3 (5.4 to 7.2) d], and the latencies of liberation rapidly decreased in subsequent sessions showing a directed and effective execution of the door-opening task [Fig. 1*B*; median (95% CI) for empty = 30.0 (30.0 to 30.0) min, trapped = 22.9 (6.8 to 30.0) min, dummy = 24.7 (11.7 to 30.0) min]. Helper mice also opened the door where the dummy mouse was located but invariably after liberating their cagemate (Fig. 1*B*). In contrast, helper mice exposed to an empty restrainer did not pull the sliding doors throughout a whole week of testing sessions (Fig. 1*B*). The proportion of helper mice versus the total tested was 42%, while free mice opening the dummy mouse compartment only accounted for 25% (P = 0.02, Fisher exact test; helpers = 14 of 34 male dyads, three independent experiments; *SI Appendix*, Fig. S1B). Altogether, these results suggested that door opening is a learned action requiring motivation (higher interest for trapped vs. dummy mouse) that is not merely driven by the innate exploratory behavior of mice (empty condition). Accordingly, helper mice exhibited similar exploratory behavior to nonhelpers, indicated by an equivalent number of entries into the zones where trapped or dummy mice were located (Fig. 1*C*). However, helper mice spent more time in the trapped mouse quadrant than in the dummy mouse area (Fig. 1*D*), indicating increased interest of helper mice toward liberating the confined conspecific. Consistently, interaction time with the trapped mouse was higher in helper mice (Fig. 1*E*).

Release of Trapped Mice Is Independent of Social Contact–Seeking Reward. To test whether door opening was motivated by subsequent social contact–seeking reward or by a genuine helping behavior, we modified our experimental paradigm in a way that the released mouse was physically separated from the helper cagemate, thus permitting sensory but not physical interactions (*SI Appendix*, Fig. S1C). Under this setting, helper mice were consecutively exposed to either a cagemate or a dummy mouse in a counterbalanced order to avoid exposure bias. When a conspecific was locked in the restrainer, latencies of door opening decreased throughout sessions as expected (Fig. 1*F*). However, when a dummy mouse was presented, door-opening latencies gradually increased consistent with a decline in motivation (Fig. 1*F*). Notably, under social interaction avoidance conditions, 70% of helper mice continued releasing the trapped mice, while only 50% of them persisted in opening the door for the dummy mouse (P = 0.006, Fisher exact test; n = 14 male dyads, two independent experiments; *SI Appendix*, Fig. S1D). Collectively, and coherent with other studies in rodents (12, 21), our results suggested that the underlying foundation for helping behavior in mice is based on affective motivation that is independent of social and physical contact reward.

Release of Trapped Mice Is Promoted by Emotional Contagion. Emotional contagion is considered a primitive form of empathy, which is a key motivation factor for prosocial helping behavior (5). To investigate if the directed response of helper mice was driven by the perception of stress in restrained conspecifics, naive free mice were submitted to the elevated plus maze test immediately after being exposed either to a dummy or trapped mouse in the first session. Exposure to a trapped mouse dramatically increased the time spent in closed arms, indicating enhanced anxiety-like behavior (Fig. 1*G*). Additionally, we also measured the increase in plasma corticosterone (difference between the final and initial values) as a proxy of emotional contagion (7). As expected, trapped mice from either nonhelper or helper dyads showed a similar increase in plasma corticosterone (nonhelper: 180.5 ± 27.2 ng/mL vs. helper: 150.6 ± 22.7 ng/mL, unpaired *t* test, P = 0.49). However, helper mice displayed a lesser increase in corticosterone compared with nonhelper mice (Fig. 1*H*). This is in agreement with previous findings indicating that helping behavior requires a mild increase in the stress response since intense stress or anxiolytic treatment impairs helping (13). Furthermore, the increase in plasma corticosterone positively correlated between helper dyads (although marginally) but not between nonhelper ones (Fig. 1*I*). This combination of physiological and behavioral state matching observed in helper mice suggests the engagement of emotional contagion.

Helping Behavior Is a Highly Energy-Demanding Process for the Brain. To initially explore the link between metabolic state and helping behavior, we measured blood glucose concentration in free mice immediately before and after performing the HBT. Interestingly, we observed a smaller increase in blood glucose levels in helper mice suggesting that psychological stress and decision-making processes were associated with high glucose consumption by the brain (Fig. 2*A*). To assess brain energy metabolism during the HBT, we measured a range of metabolites via NMR spectroscopy right after the paradigm. Among the numerous metabolites determined (Fig. 2*B* and *SI Appendix*, Fig. S2 *A* and *B*), we observed a significant increase in the ATP:ADP ratio [an indicator of cellular energy status (22)] specifically in the forebrain of helper mice (Fig. 2*C*). These results were consistent with a higher brain energy state, likely reflecting the energy-demanding requirements of the task. To corroborate these findings, we analyzed HK expression as a correlate of cellular glucose uptake and consumption (23). The arcuate nucleus of the hypothalamus
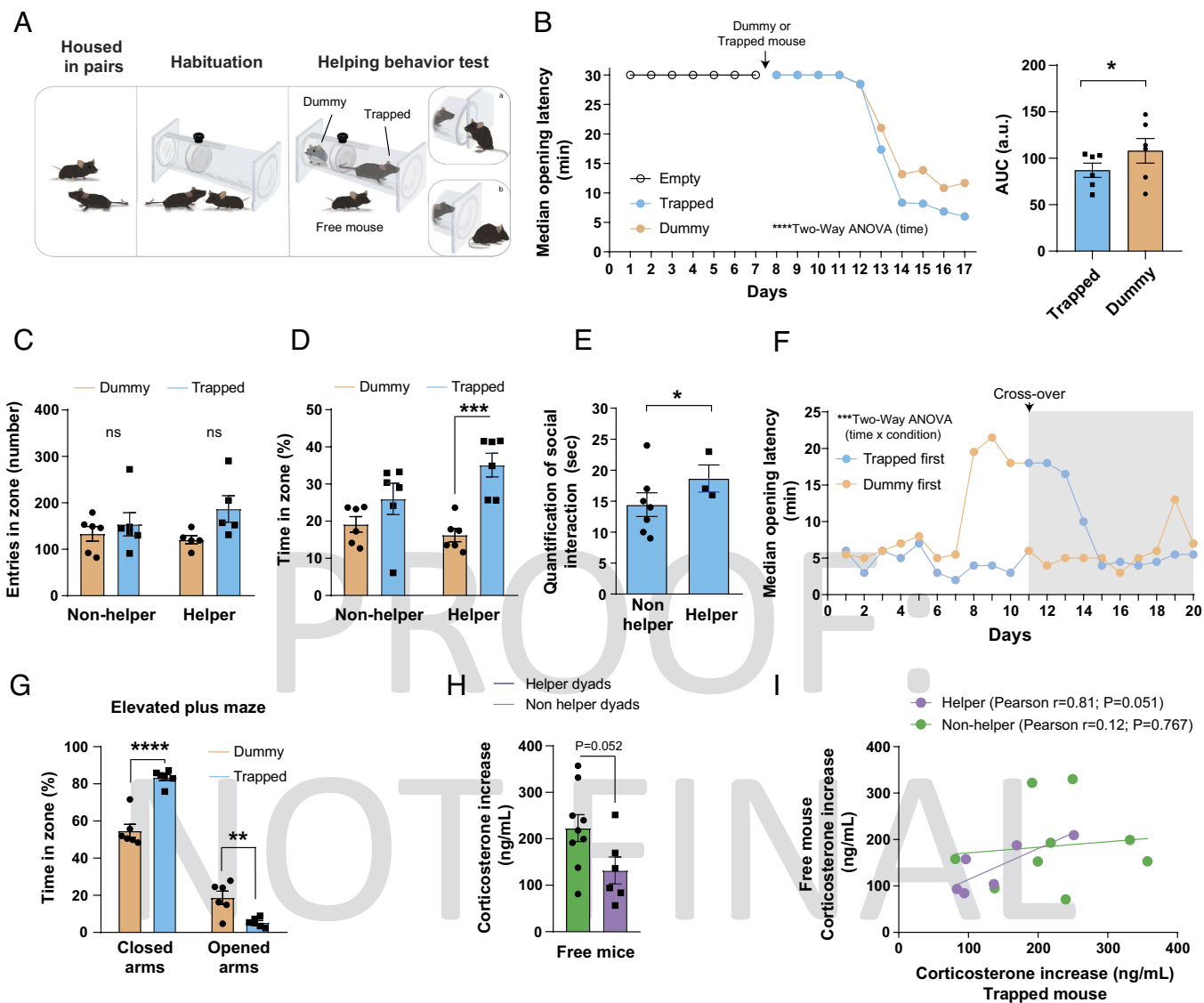
Fig. 1. Mice exhibit prosocial helping behavior toward a restrained conspecific. (*A*) Schematic view of the HBT consisting of three phases: housing in pairs (for 4 to 6 wk), habituation (4 consecutive days), and test. The image depicts the restraining apparatus, which is divided into two equivalent spaces where a dummy mouse and a cagemate were placed. Free mice were allowed to freely explore the arena, and these can become (*a*) helpers if they release the conspecific for 5 consecutive days or (*b*) nonhelpers if they do not. (*B*) Latency time to door opening when free mice were exposed to an empty restrainer (white circles) or simultaneously exposed to a trapped (blue circles) and dummy mouse (orange circles) (n = 6). Door-opening latencies are shown using the median as this variable was not normally distributed. Area under the curve (AUC) is shown as *Inset*. (*C*) Exploratory activity of nonhelper and helper mice during the HBT as measured by the number of entries into the dummy or trapped quadrants. Note that one mouse from the helper group was excluded from the study as it was identified as an outlier (using the Rout method). (*D*) Place preference of nonhelper and helper mice during the HBT as measured by the percentage of time spent in the dummy or trapped quadrants. (*E*) Social interaction time between nonhelper and helper mice with trapped mice during the HBT. Data from a second independent experiment are shown (n = 10; seven nonhelper and three helper mice). (*F*) Latency to door opening of helper mice when exposed to a trapped (blue circles) or dummy mouse (orange circles) in the modified HBT that prevented social contact upon the release of the conspecific (n = 5 to 8). Note the crossover experimental design. Door-opening latencies are shown using the median as this variable was not normally distributed. (*G*) Assessment of the anxiety-like state of helper mice by the elevated plus maze test. Time spent (%) in closed and open arms was measured after exposure to a dummy or trapped mouse. These are the same mice shown in *B*. (*H*) Plasma corticosterone increase (difference between before and after the HBT on day 5 of test) of free mice belonging to nonhelper and helper dyads. Data from a third independent experiment are shown (n = 5 to 9). (*I*) Correlation of plasma corticosterone increase between free and trapped mice. Data expressed as mean ± SEM or otherwise stated. Dots represent individual sample data. Statistical analysis was performed by two-way ANOVA with the Bonferroni multiple comparisons test for (*B–D*, *F*, and *G*), Wilcoxon test for (*B Inset*), unpaired *t* test for (*E* and *H*), and Pearson correlation test for (*I*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.
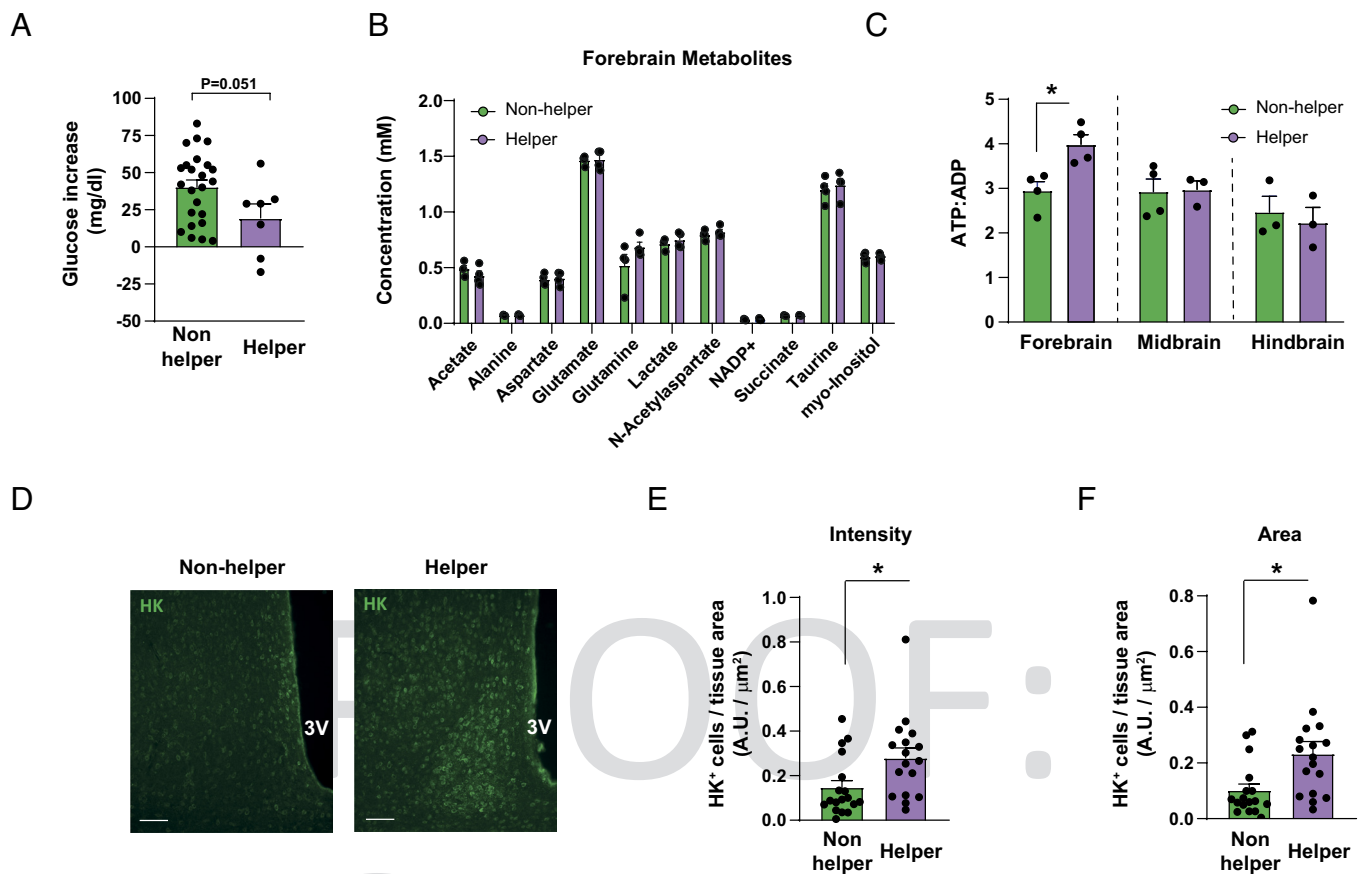
(ARC) is a major forebrain area implicated in glucose sensing and the systemic integration of energy state (24). Therefore, we studied the ARC as a prototypical brain region involved in energy status monitoring. Immunofluorescence analysis of HK in the ARC revealed stronger immunolabeling in helper than in nonhelper mice after performing the HBT (Fig. 2 *D–F*). Collectively, our data support the notion that emotional contagion and helping behavior are costly energy processes that are fueled by peripheral glucose.

Food Restriction Hinders Helping Behavior. Based on these results, we hypothesized that being in a negative energy balance would adversely impact helping behavior in mice. To this aim, we submitted food-restricted (FR) and control ad libitum–fed mice to the HBT (Fig. 3*A*). Similar to previous experiments (Fig. 1*B*), fed mice started liberating the trapped conspecific around the sixth day of the test (Fig. 3*B*). In contrast, FR mice did not open the restrainer door in any of the 10 test sessions (Fig. 3*B*). To ensure that FR mice were capable of performing the task to

Fig. 2. Helping behavior is highly energy demanding. (*A*) Blood glucose increase (difference between before and after the last session of the HBT) of nonhelper and helper mice exposed to a trapped mouse. A pool of three independent experiments is shown. (*B*) Concentration of diverse metabolites in the forebrain of nonhelper and helper mice after the HBT. (*C*) ATP:ADP ratio in the forebrain, midbrain, and hindbrain of nonhelper and helper mice after the HBT. (*D*) Representative immunofluorescence images showing HK staining in the mediobasal hypothalamus of nonhelper and helper mice after the HBT. 3V: third ventricle. (*E*) Intensity quantification of HK staining in the mediobasal hypothalamus of nonhelper and helper mice after the HBT. Dots represent brain sections from 3 to 4 mice/group. (*F*) Area quantification of HK staining in the mediobasal hypothalamus of nonhelper and helper mice after the HBT. Data expressed as mean ± SEM. Dots represent individual sample data. Statistical analysis was performed by an unpaired *t* test. *$P < 0.05$.

the same extent as controls, a crossover experimental design was implemented. Remarkably, former nonhelper FR mice became helpers once fed ad libitum as evidenced by decreased door-opening latencies across sessions [Fig. 3*B*, shaded area; median (95% CI) for fed = 12.8 (9.7 to 27.2) min; FR = 24.1 (18.3 to 30.0) min]. The ratio of helper vs. nonhelper mice occurred to a similar extent as in fed mice [38% helpers under fed conditions vs. 41% helpers under FR; *P* = 0.77, Fisher exact test; n = 21 (fed) and 26 (FR) male dyads, three independent experiments; *SI Appendix*, Fig. S3*A*], suggesting that prior exposure to food restriction did not compromise subsequent prosocial behavior. Similarly, food restriction of previously ad libitum–fed mice partially inverted the preceding door-opening trend (Fig. 3*B*, shaded area). Together, these results indicated that the systemic energy status robustly affects helping behavior in mice ($F_{(17, 248)}$ = 3.396; *P* < 0.0001).

Food restriction can affect locomotion, motivation, and exploratory behaviors. Therefore, we undertook studies to assess the potential influence of alterations in these parameters on helping behavior. FR mice did not show differences in locomotor activity (*SI Appendix*, Fig. S3*B*), speed (*SI Appendix*, Fig. S3*C*), and number of entries or time spent in trapped- or dummy-defined areas when compared to fed mice (*SI Appendix*, Fig. S3 *D* and *E*). Together, these results indicated that the energy balance status strongly influences empathically based helping behavior without altering attentiveness toward a trapped cagemate.
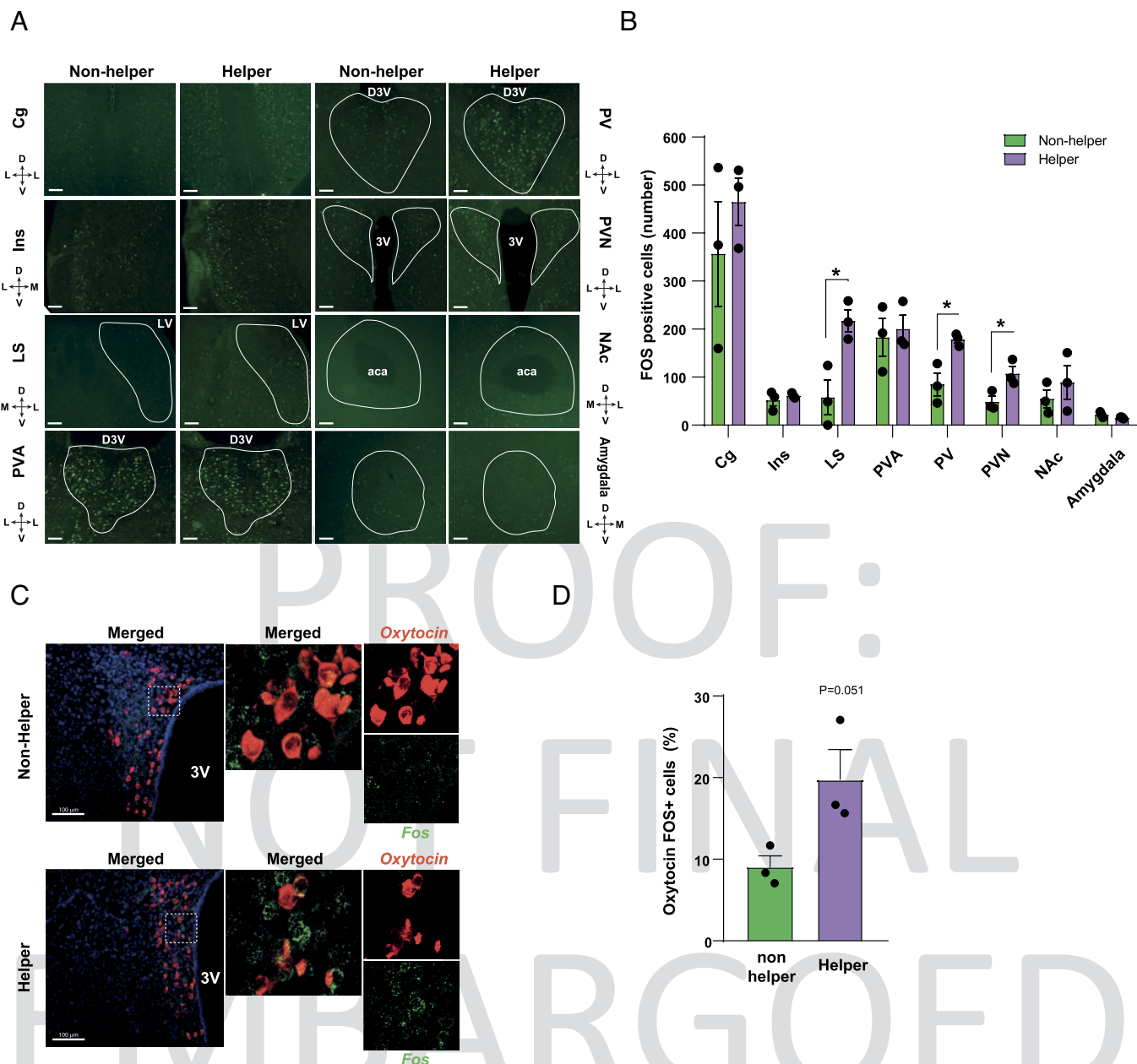
Next, we modified the HBT by the addition of a bottle containing 1% sucrose (Fig. 3*C*). We reasoned that helper mice would increase sucrose intake to compensate for the task's high energy demand (Fig. 2 *A–F*). Unexpectedly, helper mice showed a trend for less sucrose consumption during the test (Fig. 3*D*), despite manifesting an equivalent rewarding response to sucrose (Fig. 3*E*). Under this setting, nonhelper mice exhibited a positive correlation between time spent in the trapped mouse quadrant and sucrose intake (Fig. 3*F*). In contrast, helper mice did not display this trend (Fig. 3*F*).

In another set of experiments, the value of high palatable food availability in fed and FR mice was assessed in the context of helping behavior. Here, the HBT was modified by replacing the dummy mouse with a high palatable food pellet (Fig. 3*G*). Under this setting, free mice under both dietary conditions opened the door for the first time around the third session [mean (95% CI) = 3.2 (2.4 to 4.0) d] (Fig. 3 *H* and *I*), suggesting that the presence of high palatable food exerted a greater motivation for executing the door-opening action. FR mice showed a faster incentive not only for pellet acquisition than fed mice [Fig. 3*H*; median (95% CI) for fed = 7.0 (2.5 to 20.4) min; FR = 5.5 (3.2 to 9.6) min] but also for the liberation of trapped conspecifics [Fig. 3*I*; median (95% CI) for fed = 16.4 (5.3 to 23.9) min; FR = 5.7 (2.0 to 19.8) min]. While 40% of FR mice first liberated their cagemate and afterward obtained the food pellet, only 25% of fed mice liberated the trapped mouse as the first option (*SI Appendix, Fig. S3F*). Overall, the availability of palatable food revealed its greater reward in promoting motivation for door
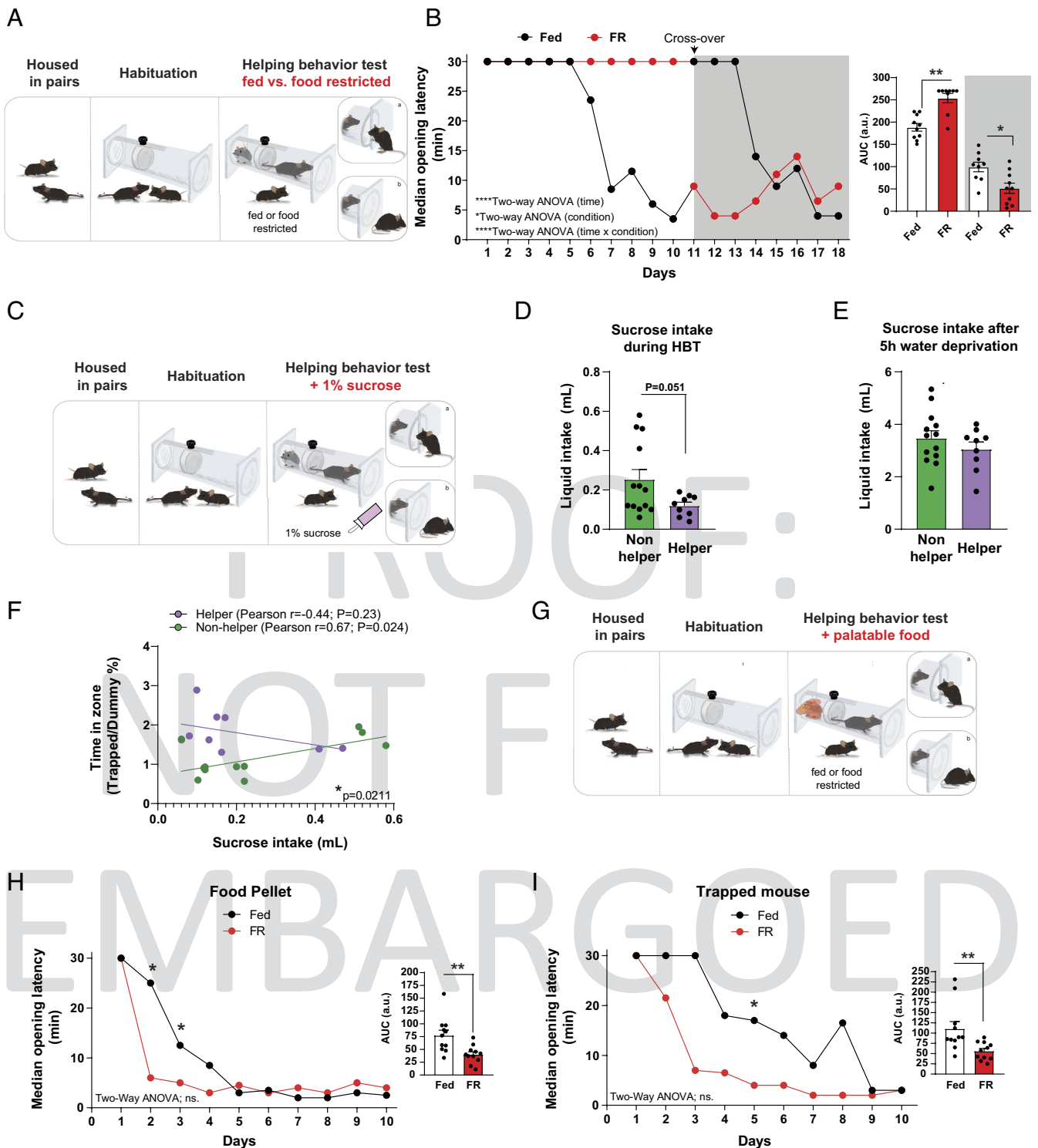
Fig. 3. Food restriction prevents helping behavior. (*A*) Schematic view of the HBT on fed and food-restricted (FR) mice. (*B*) Latency to door opening of free mice ad libitum fed (black circles) or FR (red circles) (n = 9 to 10/group). Door-opening latencies are shown using the median as this variable was not normally distributed. Note the crossover experimental design. Area under the curve (AUC) is shown as *Inset*. (*C*) Schematic view of the modified version of the HBT in which 1% sucrose was available. (*D*) Sucrose intake in nonhelper and helper mice during the HBT. (*E*) Sucrose intake after 5-h water deprivation in nonhelper and helper mice. (*F*) Correlation between time in quadrant and sucrose intake in nonhelper and helper mice during the HBT. Pearson correlation indexes and *P* values are shown for each experimental group. Linear regression slopes from both groups were significantly different (*P* = 0.021). (*G*) Schematic view of the modified version of the HBT in which the dummy mouse was replaced by a high palatable food pellet. (*H* and *I*) Latency time to door opening of the (*H*) food pellet or (*I*) trapped mouse for ad libitum–fed (black circles) and FR (red circles) mice (n = 11/group). Door-opening latencies are shown using the median as this variable was not normally distributed. *Insets* represent the area under the curve (AUC). Data expressed as mean ± SEM or otherwise stated. Dots represent individual sample data. Statistical analysis was performed by two-way ANOVA with the Bonferroni multiple comparisons test for (*B*, *H*, and *I*), unpaired *t* test for (*D* and *E*), Mann–Whitney *U* test for (*B Inset*, *H Inset*, and *I Inset*), and Pearson correlation test and linear regression for (*F*). ns: not significant. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

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Fig. 4. Helping behavior is associated with the activation of oxytocin neurons in the PVN. (*A* and *B*) Representative immunofluorescence images of FOS staining in diverse brain regions from nonhelper and helper mice (*A*) and quantification (*B*). Cingulate cortex (Cg), insular cortex (Ins), LS, paraventricular thalamus anterior nuclei (PVA), paraventricular thalamus (PV), paraventricular nucleus of the hypothalamus (PVN), and nucleus accumbens (NAc). Orientation planes are shown (D: dorsal; V: ventral; L: lateral; M: medial). (Scale bar, 50 μm.) (*C* and *D*) Representative fluorescent in situ hybridization images of oxytocin and Fos in the PVN from nonhelper and helper mice (*C*) and quantification (*D*). 3V: third ventricle; D3V: dorsal third ventricle; LV: lateral ventricle; aca: anterior cerebral artery. Data expressed as mean ± SEM. Dots represent individual sample data. Statistical analysis was performed by an unpaired *t* test. *$P < 0.05$.

opening. However, once the task was learned, the presence of food favored a prosocial helping behavior in FR mice when compared with ad libitum–fed mice (*SI Appendix*, Fig. S3F).

Helping Behavior Associates with Oxytocin Neuron Activation in the Paraventricular Nucleus. To map the pattern of activation involved in helping behavior, we quantified the immediate early gene marker FOS as an index of neural activity in selected brain areas. To this aim, an extra test session was conducted in which restrainer doors were locked to avoid different time exposures to the trapped mice. Brain regions, previously implicated in prosocial behavior (25), from nonhelper and helper mice, were assessed for FOS immunolabeling. This study revealed enhanced FOS positivity in the paraventricular thalamus (PV), lateral septum (LS), and

paraventricular nucleus of the hypothalamus (PVN) of helper mice (Fig. 4 *A* and *B*). No changes were observed in the cingulate cortex (Cg), insular cortex (Ins), paraventricular thalamus anterior nuclei (PVA), nucleus accumbens (NAc), and amygdala (Fig. 4 *A* and *B*).

Oxytocin neurons of the PVN have been associated with empathy, emotion recognition, and social engagement (26). Hence, we examined the activation status of oxytocin neurons after the HBT. Using fluorescent in situ hybridization, we determined that helper mice exhibited higher oxytocin neural activity than nonhelper mice at the end of the HBT (Fig. 4 *C* and *D*).

Activation of Hunger AgRP Neurons Prevents Helping Behavior. Oxytocin neurons of the PVN receive direct inhibitory inputs from ARC AgRP neurons (27). AgRP neurons are an integral

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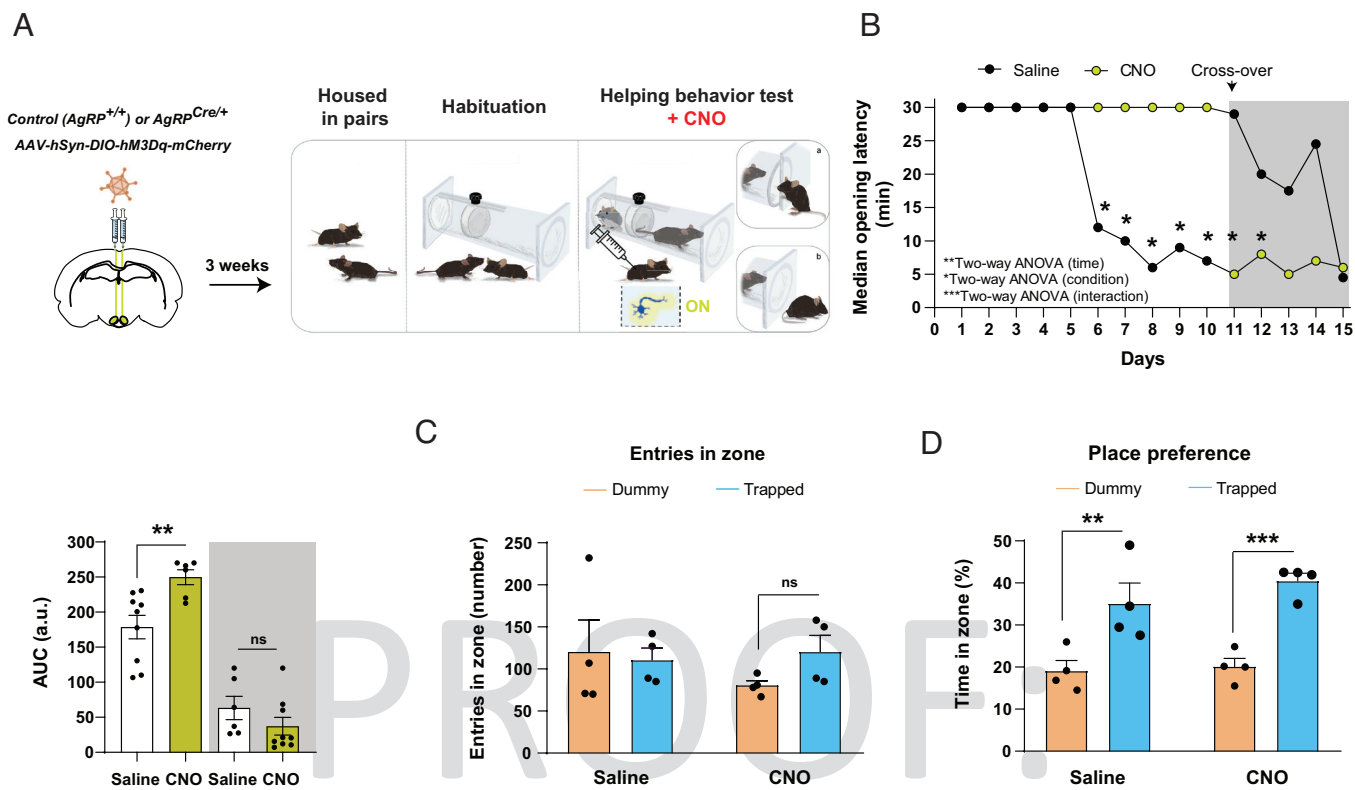
Fig. 5. Chemogenetic activation of AgRP neurons hinders helping behavior. (*A*) Schematic view of the experimental design. (*B*) Latency to door opening of free *AgRP*^{hM3Dq} mice injected with either saline (n = 9) or chemogenetic ligand CNO (n = 6) (n = 15/group considering the crossover experimental design). Door-opening latencies are shown using the median as this variable was not normally distributed. Area under the curve (AUC) is shown as *Inset*. (*C*) Exploratory activity of *AgRP*^{hM3Dq} mice injected with either saline or chemogenetic ligand CNO during the HBT, as measured by the number of entries in the dummy or trapped quadrants. Data show a random subset of mice shown in (*B*). (*D*) Place preference of *AgRP*^{hM3Dq} mice injected with either saline or chemogenetic ligand CNO during the HBT, as measured by the percentage of time spent in the dummy or trapped quadrants. Data show the same subset of mice shown in (*C*). Data expressed as mean ± SEM or otherwise stated. Dots represent individual sample data. Statistical analysis was performed by two-way ANOVA with the Bonferroni multiple comparisons test for (*B*–*D*) or Mann–Whitney *U* test (*B Inset*). ns: not significant. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

element of the neurocircuits that crucially control systemic energy balance and metabolism and are strongly activated by energy deficits (28). Furthermore, AgRP neurons have been proposed to also participate in complex behaviors (29–31). Thus, we hypothesized that this population of neurons connects organismal energy status with prosocial helping behavior. To investigate this, we chemogenetically activated AgRP neurons via the viral expression of DREADDs in ad libitum–fed *AgRP*$^{cre/+}$ free mice and submitted them to the HBT (Fig. 5*A*). Control animals displayed the expected decrease in latency times in liberating the trapped cagemate (Fig. 5*B*). However, activation of AgRP neurons by the chemogenetic ligand CNO mirrored the behavior observed in FR mice, preventing the helping behavior of free mice (Fig. 5*B*). Crossover treatment reversed this behavior as, when AgRP neurons were no longer activated, free mice liberated the trapped cagemate [Fig. 5*B*, shaded area; median (95% CI) for saline = 15.1 (12.6 to 24.5) min; CNO = 27.0 (21.3 to 28.7) min]. However, in contrast to FR mice, former helper mice when subjected to AgRP activation continued liberating the trapped mice. This indicated that stimulation of AgRP neurons in fed mice is unable to reverse the previously acquired prosocial behavior (Fig. 5*B*) and that this does not completely recapitulate the FR condition. The efficiency of the DREADD system was confirmed by the correct assessment of viral expression in the ARC (*SI Appendix*, Fig. S4*A*) and increased food intake (*SI Appendix*, Fig. S4*B*) at the end of the test.

AgRP activation has been shown to induce foraging behavior in the absence of food, acting as a competing state for other behavioral tasks (30). To rule out potential interferences with helping
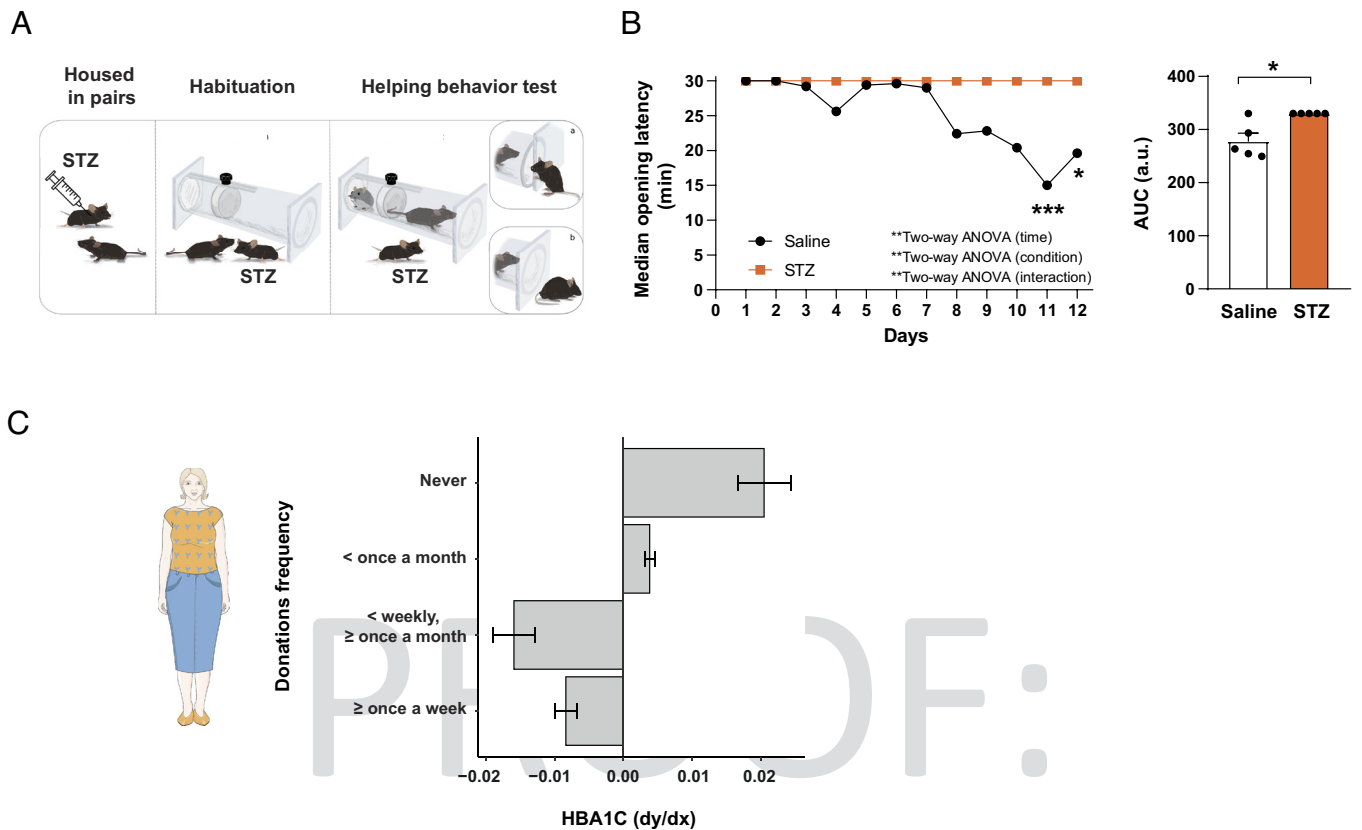
behavior, we assessed exploratory drive (*SI Appendix*, Fig. S4 *C* and *D*) and attentiveness to dummy/trapped mice after AgRP neuron activation (Fig. 5 *C* and *D*). None of these parameters were affected by CNO treatment, suggesting a similar motivation to the distress of a conspecific in both groups. The ratio of helper mice occurred to a similar extent in both groups after crossover treatments (control: 43%; CNO: 32%; *P* = 0.10, Fisher exact test; control n = 19 male dyads; CNO n = 21; three independent experiments; *SI Appendix*, Fig. S4*E*).

Pathological States Affecting Energy Balance Influence Helping Behavior. Next, we explored the influence of metabolic pathological states on prosocial helping behavior (Fig. 6*A*). To this aim, we generated STZ-induced diabetes as a disease model of negative energy balance in which mice were ad libitum fed, but glucose utilization was limited due to the lack of insulin. As expected, STZ mice were hyperglycemic compared with saline-treated counterparts (*SI Appendix*, Fig. S5*A*). During the HBT, free control mice showed the expected decreasing door-opening latencies, but diabetic mice displayed nonhelping behavior [Fig. 6*B*; median (95% CI) for saline = 27.3 (20.4 to 29.6) min; STZ = 30.0 (30.0 to 30.0) min]. Saline-injected mice tended to spend more time in the area where trapped mice were located, a behavior that was not observed in STZ mice (*SI Appendix*, Fig. S5*B*). Exploratory drive was equivalent between control and STZ mice (*SI Appendix*, Fig. S5 *C–E*). However, while the proportion of helpers was around 50% in control mice, this parameter dramatically decreased to 12% in STZ-treated mice

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Fig. 6. Declined helping behavior in pathological conditions associated with negative energy balance. (*A*) Schematic view of the HBT in STZ-induced diabetic mice. (*B*) Latency time to door opening of control (saline; n = 5) and STZ-diabetic mice (n = 5). Door-opening latencies are shown using the median as this variable was not normally distributed. *Inset* represents the area under the curve (AUC). (*C*) Derivative (dy/dx) of HbA1c concentrations in relation to the frequency of donations in the Understanding Society dataset. Data expressed as mean ± SD. Data expressed as mean ± SEM or otherwise stated. Dots represent individual sample data. Statistical analysis was performed by two-way ANOVA with the Bonferroni multiple comparisons test for (*B*) and Mann–Whitney *U* test for (*B Inset*). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

(*P* < 0.0001; Fisher exact test; n = 5 male dyads; *SI Appendix,* *Fig. S5F*).

To investigate a comparable effect among humans, we estimated the effect of glycated hemoglobin (HbA1c, a surrogate marker of long-term glycemic control) on prosocial behavior (namely charity donation). We used a Likert scale that measured the frequency of donations available in the Understanding Society database, which follows approximately 40,000 households in the United Kingdom (www.understandingsociety.ac.uk) (20). *SI Appendix,* *Table S1* summarizes the descriptive statistics of the variables included in the analysis. Our results show a positive association between HbA1c levels and less frequent charity donations, that is, lower HbA1c concentration increases the likelihood of prosocial behaviors (Table 1 and Fig. 6*C*). Fig. 6*C* reports the marginal effect of variations in HbA1c for each category of frequency of charity donations (rather than the monetary amount which would depend on individual income). A 1% change in HbA1c increased the probability of nondonating to charity in approximately 2 percentual points (pp) and reduced the likelihood of donating monthly or weekly in around 1.5 and 1 pp, respectively (Table 1 and Fig. 6*C*). These estimates were retrieved after controlling for age, gender, and its quadratic effects and interactions. We did not control for additional covariates to avoid the potential inclusion of inadequate controls influenced by HbA1c.

Discussion

Internal states permit the integration of external and internal cues, resulting in appropriate behavioral and physiological responses (1).

How this complex interplay between constantly changing environmental conditions and physiological cues shapes prosocial behaviors remains poorly understood. In the present study, we introduced a reward-independent robust paradigm to investigate helping behavior in mice. We found that acute (AgRP neuron activation) or chronic (food restriction and diabetes) strategies mimicking organismal negative energy balance and hunger prevented helping behavior toward a distressed conspecific.

Prosocial behavior has been observed across the animal kingdom. It is speculated that it emerged from primitive affective circuits supporting maternal care that evolved into other social contexts (5). From the evolutive perspective, prosocial behavior facilitates the biological success of the community by ensuring

Table 1.   Ordered logit estimates of the association of HbA1c on charity donations

Variable	Coefficient	SD	P value
HbA1c	0.06320****	0.01191	P < 0.0001
Age	−0.04180***	0.00508	P < 0.0001
Age²	0.00028***	0.00005	P < 0.0001
Male	−0.52629**	0.19378	P = 0.007
Male × Age	0.02543**	0.00758	P = 0.001
Male × Age²	0.00022**	0.00007	P = 0.002
No. of observations	10,587	–	–
Wald chi-squared test (6)	349.50****	–	P < 0.0001

the endurance of the kin genome (32). However, helping others requires a decision-making process that must be dynamically evaluated considering past experiences and environmental and interoceptive trade-offs. Under our experimental conditions, a primary feature of helper mice was that they exhibited an attenuated rise in circulating glucose levels during the HBT. It is unlikely that this effect was caused by increased stress as stress is associated with the development of hyperglycemia. Instead, this observation suggested that the helping process was coupled with higher glucose consumption. In line with this, we also found that helper individuals presented an augmented forebrain ATP:ADP ratio (likely reflecting an enhanced cellular energy potential) and increased HK expression (a gateway enzyme of glucose metabolism). These findings support the idea that the psychological stress of helper mice witnessing a conspecific in distress, and the subsequent decision-making processes, is associated with high-energy costs for the brain. Consistently, helping behavior is less likely to occur in situations of negative energy balance.

In rodents, it is believed that the basis of helping behavior is emotional contagion (33). The transfer of emotional suffering among individuals is complex and multifactorial, including diverse external (visual, auditive, and olfactory) and interoceptive factors (33, 34). Measures of stress (i.e., circulating corticosterone) in focal animals have been used as a proxy for this phenomenon (7, 35). In this context, we observed that mice displaying higher corticosterone transitions upon exposure to trapped cagemates were more reluctant to help than mice with modest responses. Blood sampling was conducted in a group counterbalanced manner following identical protocols and time frames. Hence, the observed differences in corticosterone levels are not due to methodological differences. Our data suggest that helping behavior was associated with the induction of mild stress (moderate increase in corticosterone levels). This concept is in line with other studies proposing that both insufficient and excessive stress limit the motivation to act for the benefit of conspecifics (13).

The brain regions and neural identities mediating helping behavior are beginning to be elucidated (25), and it is likely that diverse brain structures and cell types contribute to this complex behavior. Our HBT engaged the neural activity of the PV, LS, and PVN of helper mice. Striatal areas, such as the LS, have also been reported to be significantly more active in rats that help in-group but not out-group conspecifics (11). These regions have been classically implicated in emotional and motivational processing, including social reward (36–38). Nevertheless, it is important to note that helping behavior can progress independently of subsequent social contact as indicated by our data and other reports (10, 12, 21). Therefore, these results confirm that the LS may be relevant and predictive target regions of helping behavior in rodents as opposed to the Cg, Ins, and amygdala which exhibit similar activation patterns (between nonhelper and helper mice) that could be related to vicarious stress (39).

The PVN is another distinct brain region that was activated in helper versus nonhelper individuals. In this area, oxytocin neurons represent a prominent neural population that plays crucial roles in social cognition and emotional processing (40). Consistently, we found that helper mice exhibited an increased number of activated oxytocin neurons, suggesting that this neural subset may also be implicated in helping behavior. It is relevant to note that oxytocin neurons of the PVN receive inputs from ARC AgRP neurons (27). AgRP circuits are not only key for appetite control but also influence other motivated processes. For example, the promotion of a hunger-like state via activation of AgRP neurons hinders a variety of behaviors including sleep, territorial aggressiveness, and reproduction (30, 41–43). Our findings are congruent with these

observations as chemogenetic stimulation of AgRP neurons suppressed helping behavior. This outcome may be the result of cues promoting food foraging, energy preservation, or the prioritization of behaviors based on their energy requirements. Together, these results reinforce the idea that energetic needs compete with other motivations, thus guiding social and prosocial behaviors.

In a series of experiments, mice were submitted to diverse variations of the HBT under food restriction conditions (i.e., 1% sucrose or food availability). Interestingly, helping behavior was associated with reduced sucrose intake and lack of correlation between time spent in the trapped mouse quadrant and sucrose consumption. These results suggested that perseverance in trying to liberate their cagemate was stronger than the caloric reward. The presence of palatable food during the HBT also provided intriguing results. Food availability greatly sharpened learning as denoted by the rapid reduction in latency times to open at the early stages of the paradigm. Interestingly, energy-restricted mice in the presence of palatable food exhibited a marked helping behavior when compared with fed mice. It is likely that under these circumstances, self-distress caused by food restriction could lead to helping behavior since it has been shown that self-referential anticipation of a reward can facilitate self-other differentiation of stress (44).

A common characteristic of the experimental energy-deficit conditions assessed in this study that prevented helping behavior (food restriction, STZ-induced diabetes, and chemogenetics) is that they were associated with hypercorticosteronemia (45) and AgRP neuron activation (46). This population of neurons expresses glucocorticoid receptors (47, 48), and *Agrp* gene expression is up-regulated by increased circulating corticosterone concentration (49). In line with this, it has also been reported that corticosterone modulates synaptic input organization and firing of AgRP neurons (50, 51). Collectively, it is reasonable to speculate that conditions of energy deficit promote hypercorticosteronemia, which in turn activates ARC AgRP neurons and subsequently inhibits oxytocin neurons of the PVN (27). This provides a plausible nexus that posits AgRP neurons at the crux between energy status and prosocial helping behavior.

To understand to what extent our findings in mice could resemble human biology, we examined biomarker evidence available in the UK Understanding Society dataset. Specifically, we estimated the effect of HbA1c (a biomarker of long-term glycemic control) on the frequency of charity donations (as a measure of prosocial behavior). Consistent with our observations in mice, we documented a positive association between higher HbA1c concentrations and less frequent charity donations. These results indicated that poorly controlled diabetes may influence prosocial behavior.

In conclusion, in this study, we developed and tested a paradigm Q:22 to assess helping behavior in mice, thus paving the way toward a molecular understanding of this biological process via mouse genetics and modern system neuroscience. We found that chronic and acute variations in internal energy status markedly affect helping predisposition and that hypothalamic AgRP neurons are at the interface of energy balance and helping behavior.

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Q23	All data shared in this article that do not appear within the main text or SI Appendix, including your own data that have been deposited to an external source, must be cited in text with an entry in the reference list. For each new reference, please provide the following information: 1) author names, 2) data/page title, 3) database name, 4) a direct URL to the data, 5) the date on which the data were accessed or deposited (not the release date), and 6) where the new reference citation should be added in the main text and/or data availability statement. Please provide accession number for the Figshare database.
Q24	Abbreviations must be used at least twice in the main text; as such, ERC, ISCIII, and FEDER have been deleted in the acknowledgments section.
Q25	If you have any changes to your Supporting Information (SI) file(s), please provide revised, ready-to-publish replacement files without annotations.