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Abstract

Bromocriptine, a dopamine D2 receptor agonist originally used for the treatment of hyperprolactinemia, is largely successful in reducing hyperglycemia and improving glucose tolerance in type 2 diabetics. However, the mechanism behind bromocriptine's effect on glucose intolerance is unclear. Here, we tested three hypotheses, that bromocriptine may exert its effects on glucose metabolism by *1*) decreasing prolactin secretion, *2*) indirectly increasing activity of key melanocortin receptors in the central nervous system, or *3*) improving/restoring circadian rhythms. Using a diet-induced obese (DIO) mouse model, we established that a 2-wk treatment of bromocriptine is robustly effective at improving glucose tolerance. We then demonstrated that bromocriptine is effective at improving the glucose tolerance of both DIO prolactin-deficient and melanocortin-4 receptor (MC4R)-deficient mice, pointing to bromocriptine's ability to affect glucose tolerance independently of prolactin or MC4R signaling. Finally, we tested bromocriptine's dependence on the circadian system by testing its effectiveness in environmental (e.g., repeated shifts to the light-dark cycle) and genetic (e.g., the *Clock* mutant mouse) models of circadian disruption. In both models of circadian disruption, bromocriptine was effective at improving glucose tolerance, indicating that a functional or well-aligned endogenous clock is not necessary for bromocriptine's effects on glucose metabolism. Taken together, these results do not support the role of prolactin, MC4R, or the circadian clock as integral to bromocriptine's underlying mechanism. Instead, we find that bromocriptine is a robust diabetic treatment and resilient to genetically induced obesity, diabetes, and circadian disruption.

INTRODUCTION

Bromocriptine, a dopamine D2 receptor agonist, serotonin antagonist, and norepinephrine antagonist, increases insulin sensitivity and reduces hyperglycemia. In a controlled clinical trial, bromocriptine improved Hb A_{1c} levels in diabetic humans by 0.55–1.2% and also reduced fasting glucose (28). Additionally, some studies have indicated that bromocriptine may lead to weight loss. Two clinical trials observing the effects of bromocriptine over a 3-mo period found that bromocriptine treatment led to significant reductions (7), whereas other studies found no significant changes in body mass (1). Unfortunately, little work has tested the specific mechanism responsible for these therapeutic effects.

Accumulating evidence supports a centrally regulated mechanism chiefly targeting the hypothalamus. The strongest evidence for this comes from work in Syrian hamsters, where bromocriptine was equally effective at improving glucose tolerance when injected intraperitoneally versus intracerebroventricularly (22). Beyond a general consensus for a central mechanism of action, some hypothesize that bromocriptine affects glucose homeostasis via prolactin, the melanocortin system, and/or circadian rhythms.

Because bromocriptine is robustly effective at reducing prolactin levels (24), it is possible that its therapeutic effects on glucose regulation are secondary to its effects on prolactin. Moreover, prolactin is known to exhibit a circadian pattern of expression that can be disrupted by obesity, opening the

possibility that bromocriptine may affect glucose regulation by modulating prolactin rhythms (18). Whereas bromocriptine has unmistakable effects on prolactin, the involvement of prolactin in bromocriptine's effects on glucose tolerance has not been tested.

Another possibility is that bromocriptine acts on existing metabolic pathways in the brain to alter glucose regulation. Bromocriptine is known to stimulate hypothalamic neurons within the arcuate nucleus and lead to an increase in proopiomelanocortin (POMC) production (29). POMC is a precursor peptide to multiple hormones, including melanocyte-stimulating hormone (α-MSH), which has dramatic effects on body weight and glucose homeostasis via action on the melanocortin-4 receptor (MC4R) (5). Rodents deficient in MC4R are obese and glucose intolerant (16, 23). Although it is possible that bromocriptine's effects on glucose homeostasis are dependent on the MC4R, this hypothesis has not been tested specifically.

Historically, many of the founding studies that pioneered the use of bromocriptine for the treatment of diabetes have focused on circadian and circannual rhythms as possible mediators of bromocriptine's action. Indeed, bromocriptine's therapeutic effects on hyperglycemia were initially discovered to prevent the onset of seasonal insulin resistance in Syrian hamsters (6). This work eventually led to bromocriptine's FDA approval for diabetes in 2009. One can presume that this circadian hypothesis influenced the dosage instructions for Cycloset, the quick-release form of bromocriptine, in which individuals are advised to take bromocriptine within the first 2 h of wakening (28). However, few studies have rigorously tested the involvement of the circadian system in bromocriptine's effectiveness. Moreover, the importance of taking bromocriptine within the first 2 h of wakening becomes unclear for individuals with disrupted day/night rhythms (such as nightshift or 3rd-shift workers). Indeed, to our knowledge, none have tested how bromocriptine may function in a model of circadian disruption or the required dosage timing in such a model.

Here, we test these three hypotheses using preclinical mouse models. We determine bromocriptine's dependence on prolactin and MCR4 by administrating bromocriptine to mice deficient in prolactin or MC4R, respectively. To determine bromocriptine's dependence on the circadian clock, we conduct a series of experiments in diabetic, circadian disrupted mice as well as chronotherapy experiments to determine how the timing of bromocriptine administration affects glucose tolerance.

MATERIALS AND METHODS

Animals and housing.

Wild type (WT) C57Bl/6J mice were ordered from Jackson Laboratories (Bar Harbor, ME). Homozygous Clock$^{\Delta 19}$ knockout mice (*Clock* mutant), homozygous prolactin-knockout mice (PRL-KO), and homozygous melanocortin-4 receptor-knockout mice (MC4R-KO) were bred in-house at the University of Cincinnati and Marquette University. All mice were genotyped to verify gene expression (as previously described; see Ref. 4). Four to eight weeks before bromocriptine treatment, all mice were placed on a 60% kcal/fat high-fat diet (HFD; Teklad TD 06414) and maintained on the HFD ad libitum throughout the experiment. For mice in Fig. 1 only, lean controls were placed on a 10% kcal/fat low-fat diet (LFD; Teklad TD 08806) and maintained on the LFD ad libitum throughout the experiment. Initial experiments to test the effectiveness of bromocriptine were conducted in both male and female mice. Additional experiments used male mice only. For all experiments, mice were age- and weight-matched

between treatments conditions. All studies were reviewed, approved by, and performed according to the guidelines of the Institutional Animal Care and Use Committee of the sponsoring institutions, which included the University of Cincinnati (Cincinnati, OH), the University of Michigan (Ann Arbor, MI), and Marquette University (Milwaukee, WI).

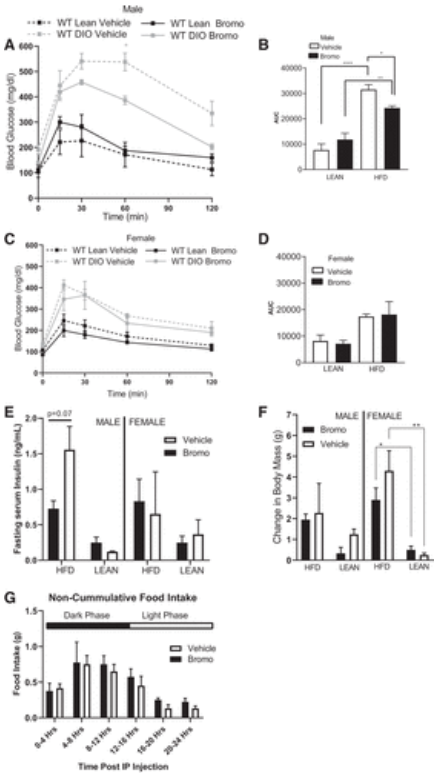

Fig. 1. Bromocriptine (Bromo) improves glucose tolerance in diet-induced obese (DIO) male mice. *A*: Bromo treatment (solid line) in DIO male mice improves glucose clearance compared with vehicle (dashed line) in response to a glucose tolerance test (2 mg/kg). Repeated-measures ANOVA with a Tukey's multiple-comparison test. Gray asterisk corresponds to the significance between the male DIO wild-type (WT) vehicle and Bromo treatment. *B*: corresponding area under the curve (AUC) in vehicle-treated (open bars) and Bromo-treated (black bars) DIO and lean male mice. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001; 1-way ANOVA with a Tukey's multiple-comparison test. *C*: Bromo treatment (solid line) in female mice has no effect on glucose clearance compared with vehicle (dashed line) in response to a glucose tolerance test (2 mg/kg) in both high-fat diet (HFD) and lean groups. Repeated-measures ANOVA with a Tukey's multiple-comparison test. *D*: corresponding AUC in vehicle-treated (open bars) and Bromo-treated (black bars) in both DIO and lean female mice; 1-way ANOVA with a Tukey's multiple-comparison test. *E*: fasting serum insulin for both lean and DIO male and female mice. Bromo (black bars) and vehicle treatment (open bars); 2-way ANOVA with a Sidak's multiple-comparison test. *F*: Bromo (black bars) did not lead to weight loss in DIO, lean, male, or female mice. **P* < 0.05, ***P* < 0.01; 2-way ANOVA with a Tukey's multiple-comparison test. (Male mice: DIO treated with Bromo, *n* = 6; DIO treated with vehicle, *n* = 4; lean treated with Bromo, *n* = 3; lean treated with vehicle, *n* = 2). (female mice: DIO treated with Bromo, *n* = 6; DIO treated with vehicle, *n* = 3; lean treated with Bromo, *n* = 4; lean treated with vehicle, *n* = 4). *G*: DIO male mice treated with Bromo did not differ in their daily food intake from vehicle-treated mice. Repeated-measures ANOVA (*n* = 8 DIO male mice treated with Bromo; *n* = 6 DIO male mice treated with vehicle).

Bromocriptine treatment.

At 9–17 wk old, mice began bromocriptine or vehicle treatment for 2 wk. For injections, bromocriptine (B2134; Sigma) was mixed with 30% EtOH immediately before use to create an 8 mg/kg dose and given

intraperitoneally. Injections were administered 1 h before the dark phase unless otherwise noted. For the continuous delivery of bromocriptine, mini-osmotic pumps (no. 1002; Alzet) were implanted subcutaneously and delivered at 8 $mg \cdot kg^{-1} \cdot day^{-1}$ for a period of 2 wk.

Environmental circadian disruption.

To induce circadian disruption by way of altering light-dark cycles, "shift work" mice were exposed to a 6-h advance of the light-dark cycle by extension of the light cycle every Monday through Friday and returned to the previous light-dark cycle on the weekends.

Food intake, body weight, and body composition.

Food was provided ad libitum and weighed weekly to determine food intake. Noncumulative food intake was measured after the 2nd day of bromocriptine treatment. Food was weighed every 4 h for the following 24 h for both vehicle- and bromocriptine-treated mice. Body weights were measured daily. Body composition was measured by EchoMRI (Houston, TX) to determine total fat and lean mass in awake animals, as previously described (3).

Glucose tolerance test.

All animals were fasted within 1 h of lights on for 4–6 h before the glucose bolus, which was given between 11 AM and 12 PM. Twenty-five percent dextrose at either 1.5 or 2 mg/kg was administered to each mouse via an intraperitoneal injection, as noted in each experiment. The glucose dosage for each experiment was designed to sufficiently challenge all groups. Blood was collected from the tail for the assessment of blood glucose via glucometer before glucose injection (*time 0*) and then 15, 30, 60, and 120 min postinjection. Glucose tolerance tests were given the day following the last bromocriptine injection, unless otherwise noted.

Insulin measurements.

Blood plasma collected during the glucose tolerance test was tested via an ELISA mouse insulin kit (Crystal Chem). The homeostasis model assessment (HOMA) index was calculated as fasting serum insulin (μU/ml) × fasting blood glucose (mmol/l)/22.5, as previously described (11).

Statistical analysis.

Statistical analysis was preformed using Graph Pad Prism 8. All values are reported as means ± SE.

RESULTS

Bromocriptine improves the glucose tolerance of diet-induced obese male mice.

In alignment with previous reports (22), we found that 2 wk of daily bromocriptine treatment given 1 h before the animals' active phase was sufficient to improve fasting glucose and overall glucose tolerance in diet-induced obese (DIO) male mice [main effect of treatment in HFD males: $F_{(1,8)} = 11.11$, $P = 0.010$; 1-way ANOVA: $F_{(3,11)} = 34.37$, $P < 0.0001$, and Tukey's multiple-comparison test, $P = 0.025$ respectively; Fig. 1, *A* and *B*]. Whereas bromocriptine does not significantly affect fasting insulin (Sidak's multiple-comparisons test for males on HFD bromocriptine vs. vehicle $P = 0.07$; Fig. 1*E*), bromocriptine lowers the HOMA index of DIO male mice (Supplemental Fig. S2; https://doi.org/10.6084/m9.figshare.10286966.v1). In contrast to male DIO mice, we found no effect of bromocriptine on fasting glucose, fasting insulin, or overall glucose tolerance in DIO female

mice (Fig. 1, *C*–*F*). Moreover, bromocriptine had no glucose regulatory effects in lean mice regardless of sex (Fig. 1, *A*–*E*).

A 2-wk treatment of bromocriptine had no effect on weight loss in either male or female mice [main effect of treatment: $F_{(1,24)} = 1.44$, $P = 0.243$; Fig. 1*G*]. In a separate experiment using only DIO male mice, we found that bromocriptine had no effect on overall food intake or 24-h feeding patterns (main effect of treatment: $F_{(1,12)} = 0.446$, $P = 0.517$; Fig. 1*G*].

Prolactin is not necessary for the glucose-regulatory effect of bromocriptine.

Because bromocriptine leads to a potent decrease in prolactin and the circadian expression of prolactin release has been implicated in obesity (18), we next investigated whether prolactin was necessary for bromocriptine's effect on glucose regulation. Prolactin-deficient mice (PRL-KO) were maintained on a high-fat diet to induce obesity and glucose intolerance. Despite being maintained on a high-fat diet, PRL-KO mice had improved glucose tolerance as compared with the wild-type (WT) littermates maintained on the same diet [main effect of genotype: $F_{(1,23)} = 16.83$, $P = 0.0004$; and main effect of genotype: $F_{(1,23)} = 10.12$, $P = 0.004$, respectively; Fig. 2, *A* and *B*]. Nevertheless, 2 wk of daily bromocriptine treatment led to a significant improvement in glucose tolerance in both WT and PRL-KO mice [main effect of treatment: $F_{(1,23)} = 25.2$, $P < 0.001$, Tukey's multiple comparison between PRL-KO mice, $P = 0.007$; Fig. 2*B*]. However, bromocriptine treatment did not affect the fasting serum insulin (Fig. 2*D*) of either PRL-KO or WT mice. Bromocriptine treatment resulted in weight loss within the PRL-KO mice [main effect of treatment: $F_{(1,24)} = 6.46$, $P = 0.018$, Tukey's multiple comparison between PRL-KO mice, $P = 0.015$; Fig. 2*C*].

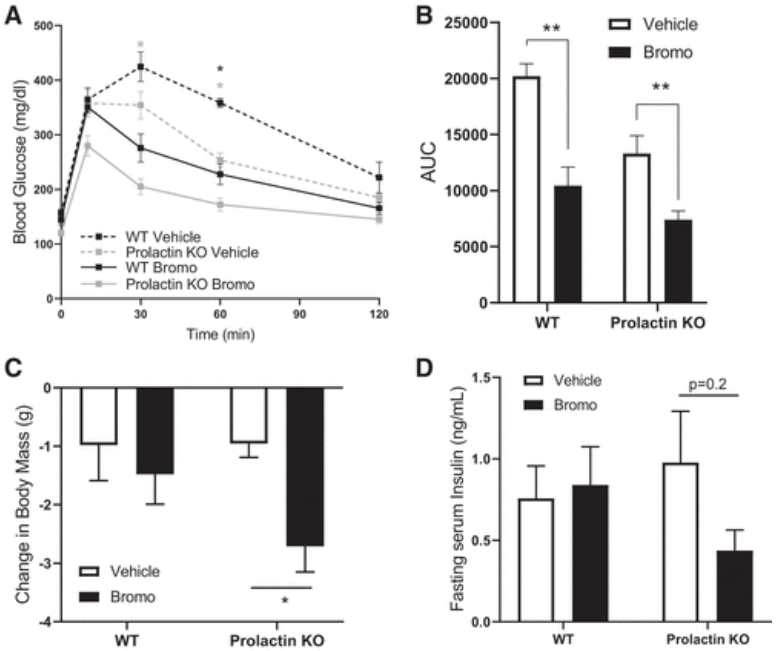

Fig. 2. Bromocriptine (Bromo) improves glucose tolerance and induces weight loss in prolactin-knockout (KO) mice. *A*: Bromo treatment [solid black line for wild type (WT) and solid gray line for prolactin-KO] improves glucose clearance compared with vehicle (dotted black line for WT and dotted gray line for prolactin-KO) in response to a glucose tolerance test (2 mg/kg). *$P < 0.05$ (gray asterisks represent differences between prolactin-KO mice and black asterisk represents differences between WT mice). Repeated-measures ANOVA with a Tukey's multiple-comparison test. *B*: corresponding area under the curve (AUC) in vehicle-treated (open bars) and Bromo-treated (black bars) mice. **$P < 0.01$, 2-way ANOVA with a Sidak's multiple-comparison test. *C*:

Bromo (black bars) leads to an increased loss of body mass compared with vehicle (open bars). *$P < 0.05$, 2-way ANOVA with a Tukey's multiple-comparison test. *D*: fasting serum insulin for WT and prolactin-KO mice, Bromo (black bars) and vehicle treatment (open bars); 2-way ANOVA with Sidak's multiple-comparison test, 2-way ANOVA with Sidak's multiple-comparison test (prolactin-KO treated with Bromo, $n = 8$; prolactin-KO treated with vehicle, $n = 10$; WT treated with Bromo, $n = 5$; WT treated with vehicle, $n = 4$).

The MC4R is not necessary for the glucose-regulatory effect of bromocriptine.

Previous research demonstrates an increase in hypothalamic POMC following bromocriptine treatment (29). Because the MC4R is a key downstream receptor of POMC signaling, we determined whether the MC4R was necessary for bromocriptine's effect on glucose tolerance. As previously reported (16), MC4R-knockout (MC4R-KO) mice exhibit glucose intolerance and obesity compared with WT littermate controls (Fig. 3). After a 2-wk treatment with bromocriptine, MC4R-KO and WT mice exhibited an improvement in glucose tolerance [main effect of treatment: $F_{(1,29)} = 9.02$, $P = 0.005$; repeated-measures ANOVA for MC4R vehicle vs. MC4R bromocriptine treated: $F_{(1,19)} = 4.89$, $P = 0.040$; Fig. 3*A*]. Overall, there was a trend for bromocriptine to improve the glucose tolerance area under the curve (AUC) in MC4R-KO-treated mice [main effect of treatment, $F_{(3,29)} = 2.55$, $P = 0.075$; Sidak's multiple-comparison test for MC4R mice, $P = 0.053$; Fig. 3*B*]. Bromocriptine treatment also led to a decreased fasting serum insulin [main effect of treatment: $F_{(3,29)} = 21.34$, $P < 0.0001$; Fig. 3*E*] and a decreased HOMA index (Supplemental Fig. S3; https://doi.org/10.6084/m9.figshare.10286996.v1) in MC4R-KO mice. Food intake was significantly increased in MC4R-KO mice but not affected by bromocriptine treatment [main effect of genotype: $F_{(1,29)} = 94.01$, $P < 0.0001$; Fig. 3*C*]. Moreover, bromocriptine treatment did not lead to weight loss in WT or MC4R-KO mice [main effect of treatment: $F_{(1,29)} = 2.603$, $P = 0.12$; Fig. 3*D*].

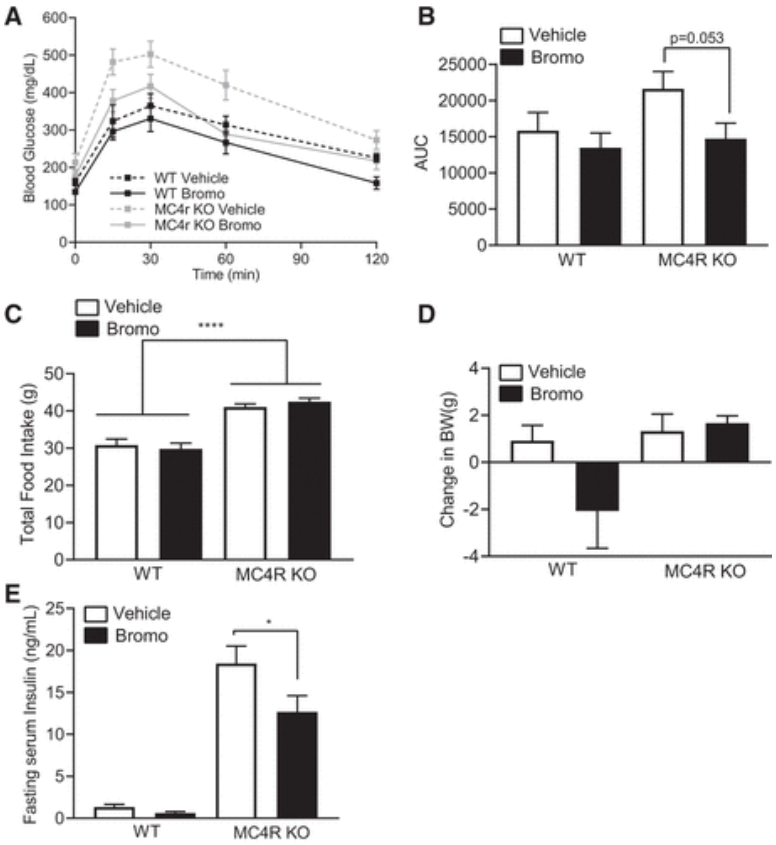

Fig. 3. Bromocriptine (Bromo) improves glucose tolerance independent of weight loss in melanocortin-4 receptor-knockout (MC4R-KO) mice. *A*: Bromo treatment [solid black line for wild-type (WT) and solid gray line for MC4R-KO] improves glucose clearance compared with vehicle (dashed black line for WT and dashd gray line for MC4R-KO) in response to a glucose tolerance test (1.5 mg/kg). Repeated-measures ANOVA with a Tukey's multiple-comparison test. *B*: corresponding area under the curve (AUC) in vehicle-treated (open bars) and Bromo-treated (black bars) WT and MC4R mice; 1-way ANOVA with a Sidak's multiple-comparison test. *C*: total food intake in vehicle-treated (open bars) and Bromo-treated (black bars) WT and MC4R mice. ****$P < 0.0001$, 1-way ANOVA with Tukey's multiple-comparison test. *D*: Bromo (black bars) and vehicle treatment (open bars) do not lead to significant weight loss in WT or MC4R-KO mice; 1-way ANOVA with Tukey's multiple-comparison test. *E*: Bromo treatment (black bars) led to an improved fasting serum insulin for MC4R-KO mice compared with vehicle-treated (open bars) MC4R-KO mice. *$P < 0.05$, 1-way ANOVA with Sidak's multiple-comparison test; 2-way ANOVA with Tukey's multiple-comparison test (MC4R-KO treated with Bromo, $n = 11$; MC4R-KO treated with vehicle, $n = 10$; WT treated with bromocriptine, $n = 6$; WT treated with vehicle $n = 6$).

Bromocriptine is effective in genetic and environmental models of circadian disruption.

One hypothesis postulates that bromocriptine works through the circadian system to produce its glucose-regulatory effects. To determine whether a functional or well-aligned circadian clock is necessary for bromocriptine's effects on glucose tolerance, we administered bromocriptine to DIO circadian-disrupted mice. Genetic circadian disruption was induced by a mutation to a core circadian gene, CLOCK, whereas environmental circadian disruption was induced by repeated shifts to the light-dark cycle.

Mutation of the CLOCK gene in $Clock^{\Delta 19}$ (hereafter *Clock* mutant) mice led to the expected obese phenotype (30); however, in this data set *Clock* mutant mice were not more glucose intolerant than WT littermates [main effect of genotype: $F_{(1,15)} = 2.294$, $P = 0.1507$; and main effect of

genotype: $F_{(1,15)} = 1.272$, $P = 0.2771$, respectively; Fig. 4, *A* and *B*]. Nevertheless, 2 wk of daily bromocriptine treatment led to a significant improvement in glucose tolerance independent of a functioning CLOCK gene [main effect of treatment: $F_{(1,15)} = 20.68$, $P = 0.004$; genotype × treatment interaction $F_{(1,15)} = 1.235$, $P = 0.2840$; repeated-measures ANOVA for Clock vehicle vs. Clock bromocriptine-treated mice: $F_{(1,6)} = 13.72$, $P = 0.010$; Fig. 4*A*]. In this data set, bromocriptine treatment led to weight loss in the WT littermates [main effect of treatment, $F_{(1,18)} = 13.75$, $P = 0.0016$, Sidak's multiple-comparison test for WT vehicle vs. WT bromocriptine mice, $P = 0.0014$; Fig. 4*C*], which was primarily due to loss of fat mass [main effect of treatment: $F_{(1,18)} = 23.69$, $P = 0.0001$; Sidak's multiple-comparison test for WT vehicle vs. WT bromocriptine mice, $P = 0.0002$; Fig. 4*D*]. However, bromocriptine treatment did not lead to weight loss in *Clock* mutant mice [main effect of treatment: $F_{(1,18)} = 13.75$, $P = 0.0016$, Sidak's multiple-comparison test for *Clock* vehicle vs. *Clock* bromocriptine mice, $P = 0.3843$; Fig. 4*C*]. This loss in weight was not due to changes in total caloric intake, as all mice ate a similar amount of food [main effect of treatment: $F_{(1,18)} = 0.07$, $P = 0.79$; main effect of genotype: $F_{(1,18)} = 0.004$, $P = 0.95$; Fig. 4*E*].

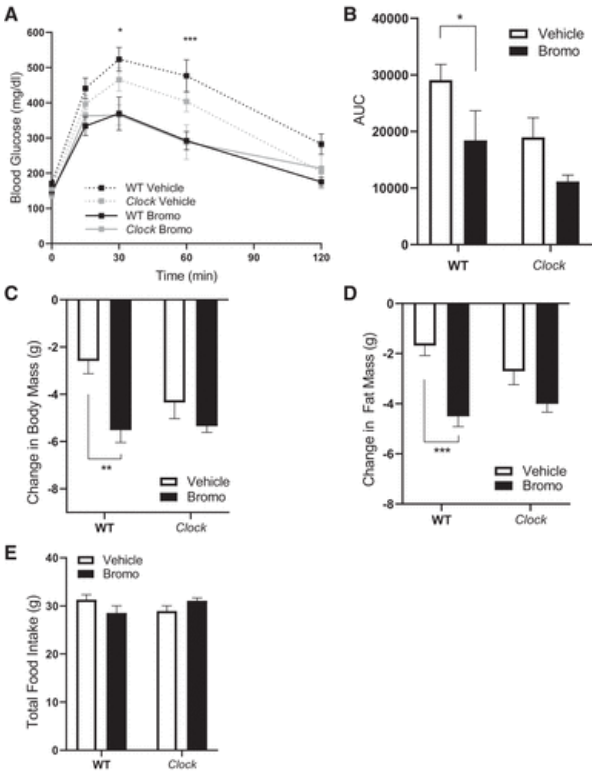

Fig. 4. Bromocriptine (Bromo) improves glucose tolerance in *Clock*-knockout (KO) mice without weight loss. *A*: Bromo treatment (solid line) improves glucose clearance compared with vehicle (dotted lines) in response to a glucose tolerance test (2 mg/kg) in wild-type (WT; black solid and dotted lines) and *Clock* mutant (gray solid and dotted lines) mice. Repeated-measures ANOVA with a Bonferroni's multiple-comparison test. *$P < 0.05$ and ***$P < 0.001$, differences between treatment groups of the same genotype. *B*: corresponding area under the curve (AUC) in vehicle-treated (open bars) and Bromo-treated (black bars) mice. *$P < 0.05$. *C*: Bromo (black bars) leads to an increased loss of body mass compared with vehicle (open bars) in WT mice. **$P < 0.01$. *D*: weight loss is chiefly from loss of fat mass with Bromo treatment (black bars), leading to increased fat mass loss compared with vehicle (open bars). ***$P < 0.001$. *E*: total food intake did not differ between Bromo- (black bars) and vehicle-treated (open bars) mice. *B*–*E*: analyzed using 2-way ANOVA with a Sidak's multiple-comparison test. (*Clock* mutant treated with Bromo, $n = 2$; *Clock* mutant treated with vehicle, $n = 5$; WT treated with bromocriptine, $n = 6$; WT treated with vehicle, $n = 7$).

We next induced environmental disruption of circadian rhythms by repeatedly shifting the light-dark cycle of DIO WT mice and then tested the effectiveness of bromocriptine administered either in 24-h intervals (i.e., “every 24 h”) or 1 h before the shifting light-dark cycle (i.e., “relative to light”). We found that bromocriptine was successful at improving the glucose tolerance of DIO environmentally disrupted mice [main effect of treatment: $F_{(1,16)} = 31.23$, $P < 0.0001$; and main effect of treatment: $F_{(1,16)} = 32.30$, $P < 0.0001$, respectively; Fig. 5, *A* and *B*]. Moreover, both treatment strategies (i.e., every 24 h or relative to light) were equally successful at improving glucose tolerance [main effect of treatment: $F_{(1,16)} = 32.3$, $P = <0.0001$; Fig. 5*B*]. Whereas there was no significant difference in overall weight loss [t (8) = 1.722, $P = 0.12$; Fig. 5*C*], mice treated with bromocriptine every 24 h experienced greater fat mass loss than those treated relative to the shifting light-dark period [t (8) = 2.96, $P = 0.018$; Fig. 5*D*].

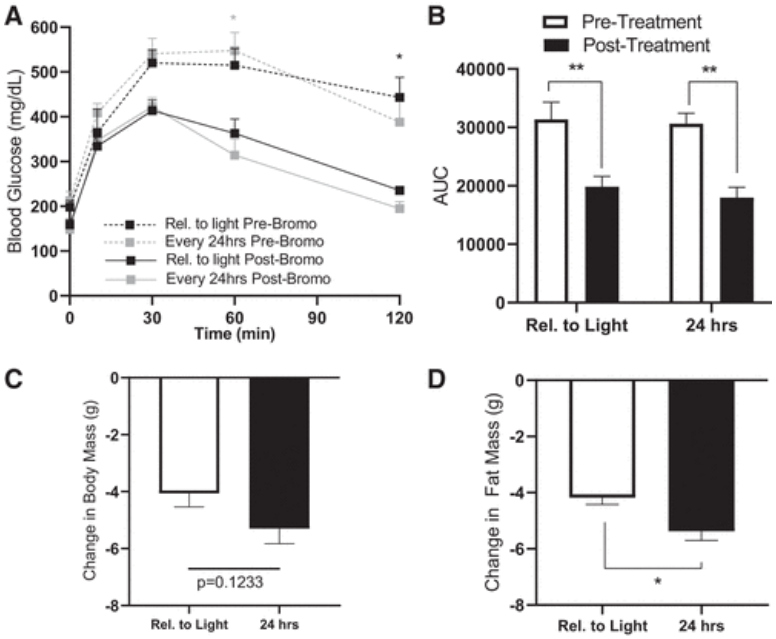

Fig. 5. Bromocriptine [Bromo; provided relative to shifting light-dark cycle (relative to light)] improves glucose tolerance in mouse models of environmental circadian rhythm disruption. *A*: Bromo treatment (black solid line for relative to light and gray solid line for every 24 h) improves glucose clearance in response to a glucose tolerance test (2 mg/kg) compared with pretreatment (black dashed line for relative to light and gray dashed line for every 24 h). Gray asterisk corresponds to significant difference between pre- and post-Bromo given every 24 h. *$P < 0.05$, significant difference between pre- and post-Bromo given relative to light. Repeated-measures ANOVA with a Tukey’s multiple-comparison test ($n = 5$ in each group). *B*: corresponding area under the curve (AUC) in pretreatment (open bars) and Bromo-treated (black bars) mice. **$P < 0.01$, 2-way ANOVA with a Sidak’s multiple-comparison test. *C*: Bromo treatment leads to weight loss under both treatment strategies. Unpaired 2-tailed *t*-test. *D*: Bromo given every 24 h leads to a greater fat mass loss. *$P < 0.05$, unpaired 2-tailed *t*-test.

Timing of bromocriptine treatment has marginal effects on glucose tolerance and weight loss.

Previous reports, as well as clinical guidelines, suggest that the timing of bromocriptine treatment is important for maximal effectiveness (10). Indeed, we found that DIO mice treated with a once daily

injection of bromocriptine have better glucose regulatory outcomes than DIO mice treated with the same dose of bromocriptine continuously delivered over a 24-h period via pump (Supplemental Fig. S1; https://doi.org/10.6084/m9.figshare.9548405.v1). To determine whether the specific timing of bromocriptine affects glucose tolerance, we administered bromocriptine to DIO mice at either Zeitgeber time (ZT) 11, ZT 17, or ZT 23 (ZT marks the onset of the light period). In replication of Fig. 1, we find that 2 wk of daily bromocriptine administered 1 h before lights off (at ZT 11) results in a significant improvement in glucose tolerance in DIO mice [main effect of treatment: $F_{(1,14)} = 7.37$, $P = 0.017$; Fig. 6*A*]. Daily bromocriptine treatment administered during the middle of the dark period (ZT17) shows a trend to improve glucose tolerance [$F_{(1,14)} = 3.93$, $P = 0.067$; Fig. 6*B*], whereas bromocriptine administered at the end of the dark period (ZT 23) resulted in a significant improvement in glucose tolerance in DIO mice [$F_{(1,14)} = 5.83$, $P = 0.030$; Fig. 6*C*]. Bromocriptine administered at ZT 11 significantly lowered the glucose tolerance AUC and tended to lower the AUC when administered at ZT 23 [main effect of treatment: $F_{(1, 42)} = 17.19$, $P = 0.0002$; Fig. 6*D*] but had no effect on the AUC when administered at ZT 17. Although bromocriptine did not lead to a change in total body mass (Fig. 6*E*), bromocriptine did lead to a significant fat mass loss in mice receiving drug at ZT 11 and ZT 17 as compared with the vehicle [main effect of treatment: $F_{(1,42)} = 18.98$, $P < 0.0001$, Sidak's multiple-comparisons test for vehicle vs bromocriptine at ZT 11, $P = 0.0082$ and ZT 17, $P = 0.048$; Fig. 6*F*]. In this data set, bromocriptine treatment did not significantly affect fasting serum insulin (Fig. 6*G*) at any time.

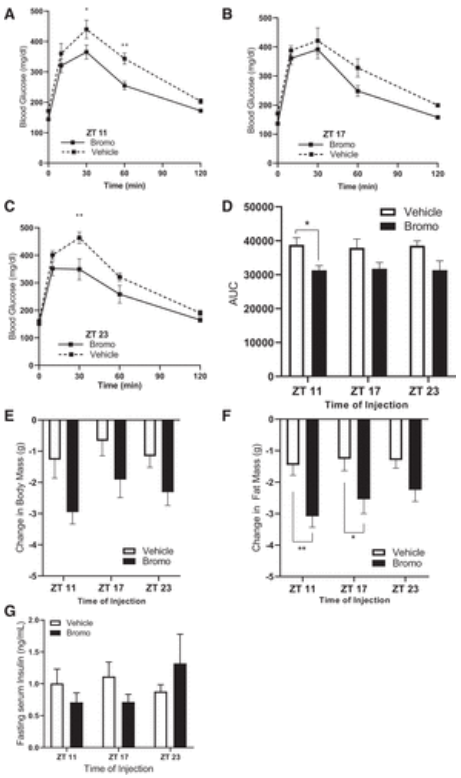

Fig. 6. Bromocriptine (Bromo) injections in diet-induced obese (DIO) Zeitgeber time (ZT) mice at ZT 11 and ZT 23 improves glucose tolerance. *A*: Bromo treatment (solid line) at ZT 11 improves glucose clearance compared with vehicle (dashed line) in response to a glucose tolerance test (2 mg/kg). *B*: Bromo treatment (solid line) at ZT 17 does not improve glucose clearance compared with vehicle (dashed line) in response to a glucose tolerance test (2 mg/kg). *C*: Bromo treatment (solid line) at ZT 23 improves glucose clearance compared with vehicle (dashed line) in response to a glucose tolerance test (2 mg/kg). *A*–*C*: **P* < 0.05, ***P* < 0.01. Repeated-measures ANOVA

with a Sidak's multiple-comparison test. *D*: corresponding area under the curve (AUC) in vehicle-treated (open bars) and Bromo-treated (black bars) mice for all injection timepoints. *E*: Bromo did not result in significant changes in body mass. *F*: Bromo treatments at ZT 11 and ZT 17 resulted in a significant loss of fat mass. *G*: fasting serum insulin for ZT11, ZT17, and ZT23 was unaffected by bromocriptine (black bars) and vehicle treatment (open bars). *D*–*G*: **P* < 0.05, ***P* < 0.01. 2-way ANOVA with a Sidak's multiple-comparison test (*n* = 8 for each group).

DISCUSSION

As demonstrated in clinical trials (1), bromocriptine is an effective treatment for type 2 diabetes. Bromocriptine is hypothesized to act predominantly through a central mechanism to modulate glucose homeostasis; however, the precise mechanism is unknown. Here, we utilize a preclinical, obese mouse model to demonstrate that bromocriptine's effect on glucose tolerance is not dependent on prolactin, the MC4R, or circadian rhythms.

As previously reported (21), we find that a 2-wk treatment of bromocriptine is effective at improving the glucose tolerance of obese male mice. However, bromocriptine was ineffective in female mice. Previous research indicates that female mice are more resistant to the diabetic effects of a HFD diet (26). Our results support this conclusion, as we find that female mice maintained on the HFD have overall lower glucose tolerance curves and fasting insulin compared with males (Fig. 1). Because lean mice are also unaffected by bromocriptine treatment, we speculate that female DIO mice are unaffected by bromocriptine due to their inability to become sufficiently diabetic on the high-fat diet. In the clinic, bromocriptine has previously been reported as effective at improving glucose tolerance of women (7). In agreement with clinical studies (1), we find that bromocriptine treatment in C57Bl6/J (WT) mice does not lead to weight loss (Fig. 1*F*). These data suggest that bromocriptine improves glucose tolerance in a weight-independent manner. However, we did observe weight loss in the WT littermates of the *Clock* mutant mice following bromocriptine treatment (Fig. 4). This apparent inconsistency in bromocriptine's effect on body weight may be attributed to deviations in the genetic makeup of the WT mouse of *Clock* littermates, which may differentially influence bromocriptine's effect on body weight regulation. Clinically, bromocriptine's effect on body weight is mixed, with some studies reporting weight loss (7) and others indicating no effect (1).

Using obese prolactin-knockout (PRL-KO) male mice, we tested bromocriptine's dependence on prolactin in mediating the effect on glucose metabolism. Bromocriptine has robust effects on prolactin (24) and is commonly used in the treatment of hyperprolactinemia. Prolactin exhibits a rhythmic expression pattern that rises with sleep (15). In obese humans, prolactin exhibits a secondary peak in the early morning that is not present in lean individuals (9). It is hypothesized that bromocriptine may be decreasing this early morning prolactin rise to improve glucose tolerance (18). In contrast with the previous literature (20), we observed that diet-induced obese PRL-KO mice had improved glucose tolerance compared with similarly-fed WT mice. Nevertheless, we found that a 2-wk administration of bromocriptine was equally effective at reducing the glucose tolerance of PRL-KO mice as it was in WT littermates (Fig. 2*B*), indicating that prolactin is not necessary for bromocriptine's effect on glucose tolerance. Although it is possible that bromocriptine is improving the glucose tolerance of the PRL-KO mice totally or in part through body weight loss, it must be noted that that PRL WT littermates do not lose weight and still exhibit an improvement in glucose tolerance when treated with bromocriptine.

Therefore, it is unlikely that bromocriptine is solely affecting the glucose tolerance by way of weight loss.

Multiple studies have demonstrated bromocriptine's central effects on the hypothalamus as critical to its therapeutic effects on glucose regulation (22, 29) and notably lead to an increase in POMC in the arcuate nucleus (29). Because the MC4R is a key downstream receptor of POMC responsible for the control of body weight and glucose regulation, we determined whether melanocortin signaling through the MC4R receptor was necessary for bromocriptine's effect on glucose tolerance by administrating bromocriptine to MC4R-knockout (MC4R-KO) mice. Like our studies in PRL-KO mice, we found that bromocriptine was effective at improving the glucose tolerance of MC4R-KO mice (Fig. 3*B*). Bromocriptine's effectiveness in MC4R-KO mice suggests that bromocriptine would be an effective diabetic treatment for individuals with genetic mutations of the melanocortin system. Indeed, individuals with mutations in the MC4R and melanocyte-stimulating hormones represent the most commonly known monogenetic form of obesity (13) and are at increased risk of developing type 2 diabetes.

Given that bromocriptine's therapeutic effect on glucose regulation was originally discovered in the seasonal, insulin-resistant hamster (6), we determined whether bromocriptine would be an effective treatment in animal models of circadian disruption, including genetic circadian disruption, as in the *Clock* mutant mouse (30), and environmental circadian disruption induced by repeatedly altering the light-dark cycle of WT mice (2). We found that bromocriptine improves the glucose tolerance of both genetic (Fig. 4*B*) and environmental models of circadian disruption (Fig. 5*B*). Interestingly, bromocriptine leads to a significant improvement in the glucose tolerance of environmentally circadian-disrupted mice regardless of whether bromocriptine is administered every 24 h or relative to the animals' active phase (Fig. 5*B*). These data have intriguing implications for human shift workers, suggesting that shift workers may experience a benefit from bromocriptine even if bromocriptine is taken when an individuals' active period is not aligned with daylight hours.

Whereas previous research has posited that bromocriptine must be taken in the morning to increase hypothalamic dopamine levels (10) and/or to "reset" the circadian clock (19) to improve glucose tolerance, our data do not support this conclusion. We tested this hypothesis by administering bromocriptine in male DIO WT mice at three different times (ZT 11, ZT 17, and ZT 23) and found that the timing of bromocriptine administration had little effect on glucose tolerance (Fig. 6*D*). However, the timing of bromocriptine administration did have a significant effect on weight loss, with the most amount of weight loss achieved when bromocriptine was administered at ZT 11 (Fig. 6*E*). Clinically, bromocriptine's effect on weight loss has yielded mixed results (1, 7). In light of the present data, it is possible that slight deviations in patient consumption of bromocriptine may be a confounding variable in clinical weight loss outcomes.

Additionally, there is clinical evidence that bromocriptine lowers plasma triglyceride levels (17). However, in our studies, bromocriptine had no effect on fasting blood triglyceride levels (data not shown). This difference between our mouse model and the clinical literature may be due in part to our relatively short-term treatment with bromocriptine (i.e., 2 wk). It is possible that chronic treatment with bromocriptine may significantly affect fasting blood triglycerides in a manner more consistent with clinical observations.

The present studies were conducted in mouse models that have been heavily utilized for identifying clinically meaningful treatment options for individuals with type 2 diabetes. Nevertheless, it is possible that bromocriptine's mechanism of action in mice differs from humans. Of note is that mice and humans exhibit opposing active periods, with humans being diurnal and mice nocturnal. To help control for such differences, bromocriptine was administered to mice just before the onset of the dark period, which is the active phase for mice and similar to the time when humans are advised to take bromocriptine.

We find that bromocriptine decreases fasting insulin and/or the HOMA index in very glucose-intolerant mice (Figs. 1 and 3 and Supplemental Figs. S2 and S3). It is noteworthy to mention that although the HOMA index has been used in mice (11), it is not a validated method for rodent use. In less glucose-intolerant animals, bromocriptine did not affect fasting insulin (Fig. 2, 3, and 6), and yet overall glucose tolerance was still improved, consistent with previous studies (25). We conclude that although bromocriptine can lead to an increase in insulin sensitivity, these effects are not critical for bromocriptine's overall effect on glucose tolerance (8, 27).

Overall, these experiments indicate that bromocriptine is effective at improving glucose tolerance in prolactin-deficient, MC4R-deficient, and circadian-disrupted mice. These results demonstrate that bromocriptine's effect on glucose tolerance is independent of prolactin, MC4R, or circadian rhythms. Although the precise mechanism of bromocriptine remains undefined, there is a general agreement that bromocriptine has a central mechanism of action (10, 14, 22). Within the brain, it is postulated that bromocriptine may lead to effects on glucose tolerance by way of decreasing sympathetic activity (10). Bromocriptine's reduction in sympathetic activity is believed to occur via a direct increase in brain dopamine levels, most likely within the ventromedial hypothalamus (10), which in turn leads to beneficial effects on glucose hemostasis (14). In agreement, previous research indicates that bromocriptine improves glucose tolerance via suppression of hepatic glucose production (8, 10, 12). The present data cannot rule out this hypothesis. Further preclinical and clinical research is necessary to determine bromocriptine's mechanistic action on glucose metabolism.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

S.N.F.-D. and D.M.A. conceived and designed research; S.N.F.-D., E.B., S.Y., H.P., and D.M.A. performed experiments; S.N.F.-D., E.B., and D.M.A. analyzed data; S.N.F.-D. and D.M.A. interpreted results of experiments; S.N.F.-D., E.B., and H.P. prepared figures; S.N.F.-D. and D.M.A. drafted manuscript; S.N.F.-

D., D.A.S., R.J.S., and D.M.A. edited and revised manuscript; S.N.F.-D., S.Y., H.P., D.A.S., R.J.S., and D.M.A. approved final version of manuscript.

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AUTHOR NOTES

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