Suppression of prolactin secretion partially explains the anti-diabetic effect of bromocriptine in *ob/ob* mice

Isadora C. Furigo, Miriam F. Suzuki, João E. Oliveira, Angela M. Ramos-Lobo, Pryscila D.S. Teixeira, João A. Pedroso, Amanda de Alencar, Thais T. Zampieri, Daniella C. Buonfiglio, Paula G.F. Quaresma, Patricia O. Prada, Paolo Bartolini, Carlos R.J. Soares, and Jose Donato Jr

Endocrinology
Endocrine Society

Submitted: June 29, 2018
Accepted: November 15, 2018
First Online: November 20, 2018

Advance Articles are PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. The manuscripts are published online as soon as possible after acceptance and before the copyedited, typeset articles are published. They are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes. No corrections/changes to the PDF manuscripts are accepted. Accordingly, there likely will be differences between the Advance Article manuscripts and the final, typeset articles. The manuscripts remain listed on the Advance Article page until the final, typeset articles are posted. At that point, the manuscripts are removed from the Advance Article page.

DISCLAIMER: These manuscripts are provided "as is" without warranty of any kind, either express or particular purpose, or non-infringement. Changes will be made to these manuscripts before publication. Review and/or use or reliance on these materials is at the discretion and risk of the reader/user. In no event shall the Endocrine Society be liable for damages of any kind arising references to, products or publications do not imply endorsement of that product or publication.

Downloaded from https://academic.oup.com/endo/advance-article-abstract/doi/10.1210/en.2018-00629/5185194 by FMRP user on 10 December 2018

Prolactin inhibition improves glucose homeostasis.

Suppression of prolactin secretion partially explains the anti-diabetic effect of bromocriptine in *ob/ob* mice

Isadora C. Furigo[1], Miriam F. Suzuki[2], João E. Oliveira[2], Angela M. Ramos-Lobo[1], Pryscila D.S. Teixeira[1], João A. Pedroso[1], Amanda de Alencar[1], Thais T. Zampieri[1], Daniella C. Buonfiglio[1], Paula G.F. Quaresma[1,3], Patricia O. Prada[4], Paolo Bartolini[2], Carlos R.J. Soares[2], and Jose Donato Jr[1,]*

[1]*Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil.*

[2] *Biotechnology Center, Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN/SP, São Paulo, SP, Brazil.*

[3] *Department of Internal Medicine, State University of Campinas (UNICAMP), Campinas, SP, Brazil.*

[4] *School of Applied Sciences, UNICAMP, Limeira, SP, Brazil.*

ORCiD numbers:

0000-0002-4166-7608

Donato

Jose

Received 29 June 2018. Accepted 15 November 2018.

Previous studies have shown that bromocriptine mesylate (Bromo) lowers blood glucose levels in adults with type 2 diabetes mellitus. However, the mechanism of action of the anti-diabetic effects of Bromo is unclear. As a dopamine receptor agonist, Bromo can alter brain dopamine activity affecting glucose control. However, Bromo also suppresses prolactin (Prl) secretion, and Prl levels modulate glucose homeostasis. Thus, the objective of the present study was to investigate whether Bromo improves insulin sensitivity via inhibition of Prl secretion. Male and female *ob/ob* animals (a mouse model of obesity and insulin resistance) were treated with Bromo and/or Prl. Bromo-treated *ob/ob* mice exhibited lower serum Prl concentration, improved glucose and insulin tolerance, as well as increased insulin sensitivity in the liver and skeletal muscle, compared to vehicle-treated mice. Prl replacement in Bromo-treated mice normalized serum Prl concentration without inducing hyperprolactinemia. Importantly, Prl replacement partially reversed the improvements in glucose homeostasis caused by Bromo treatment. The effects of the Prl receptor antagonist G129R-hPrl on glucose homeostasis were also investigated. We found that central G129R-hPrl infusion increased insulin tolerance of male *ob/ob* mice. In summary, our findings indicate that part of Bromo effects on glucose homeostasis are associated with decrease in serum Prl levels. Since G129R-hPrl treatment also improved the insulin sensitivity of *ob/ob* mice, pharmacological compounds that inhibit Prl signaling may represent a promising and novel therapeutic approach to control blood glucose levels in individuals with insulin resistance.

Introduction

Type 2 diabetes mellitus (T2DM) is a syndrome characterized by dysfunctions in the metabolism of glucose, amino acids and free fat acids (1). The present scenario of modern life contributes to the exacerbated growth of metabolic disorders. The number of individuals with T2DM has doubled over the past three decades, which turned this disease into a health global challenge (2). Importantly, changes in lifestyle and diet have not been enough to solve the burden of these diseases. Thus, ongoing efforts of health organizations, research groups and society are necessary to curb the frightening trends in morbidity and mortality attributable to this disease. Therefore, intensive investigations of more efficient therapeutic approaches to prevent and treat T2DM are urgently needed.

Currently, drugs used to treat T2DM act stimulating insulin secretion by the pancreas (sulfonylureas), reducing hepatic glucose production (biguanides), delaying digestion and intestinal absorption of carbohydrates (alpha-glucosidase inhibitors) or increasing insulin sensitivity (thiazolidinediones and biguanides) (3,4). Additionally, there are relatively new classes of anti-diabetic drugs, including incretin receptor agonists that stimulate insulin secretion and suppress glucagon secretion (5) or sodium-glucose co-transporter-2 (SGLT2) inhibitors, which decrease renal glucose reabsorption (6).

However, a growing interest in studying the central nervous system (CNS) as a potential target of anti-diabetic drugs is emerging since insulin sensitivity, hepatic glucose production and insulin/glucagon secretion are regulated by the autonomic nervous system (7). Moreover, insulin receptor is widely distributed in various brain nuclei that control glycemia and energy balance (8,9). Notably, central insulin signaling regulates peripheral glucose and fat metabolism (10). In addition to insulin, leptin has an important effect on glucose homeostasis via the CNS. Selective leptin receptor deletion in neurons leads to T2DM (11) and leptin action on proopiomelanocortin neurons is sufficient to normalize glucose and glucagon levels in mice otherwise knockout for the leptin receptor (12–14).

In the context of drugs acting on CNS, the USA Food and Drug Administration has approved a quick release formulation of bromocriptine mesylate (Bromo), a dopamine D2 receptor agonist, as a coadjutant treatment for T2DM (15). Human and animal tests have shown anti-diabetic effects of Bromo. Bromo lowers fasting blood glucose, improves glucose tolerance and reduces insulin levels in T2DM patients (16). Two weeks of treatment with Bromo plus a dopamine D1 receptor agonist reduced hyperglycemia, hyperinsulinemia and hyperlipidemia of leptin deficient (*ob/ob*) mice (17). However, the mechanism of action of the anti-diabetic effects of Bromo remains unclear. It has been suggested that Bromo acts via the CNS to improve glucose homeostasis (18). In this context, Bromo administration in the early morning could hypothetically reset the circadian rhythm of dopamine in the hypothalamus, leading to improvements in insulin sensitivity and other metabolic aspects (19), although no clear evidence about this effect has been reported.

As a dopamine receptor agonist, Bromo also inhibits prolactin (Prl) secretion. This occurs because tuberoinfundibular dopamine (TIDA) neurons are major regulators of pituitary Prl secretion via a negative feedback loop. Prl activates TIDA neurons, which in turn release dopamine into the hypophyseal portal system. Activation of dopamine receptors in pituitary lactotrophs inhibits Prl secretion (20,21). Importantly, Prl modulates glucose homeostasis (22) . In this sense, Prl level within the normal range is a protective factor against T2DM in healthy women (23). Additionally, Prl signaling plays an important physiological role favoring β-cell proliferation and expansion during pregnancy, thus contributing to the glucose homeostasis and preventing gestational diabetes mellitus (24,25). On the other hand, there is evidence that Prl may cause insulin resistance and produce diabetogenic effects. For example, hyperprolactinemia either induced by pituitary tumors or by antipsychotic drugs is an important risk factor for glucose intolerance and T2DM (22). Therefore, it is plausible to hypothesize that the anti-diabetic effects of Bromo are associated with reductions in Prl secretion. However, no study until now tested this hypothesis. In addition, Prl signaling seems to be an interesting target for the modulation of glucose metabolism. Of note, several Prl receptor antagonists have been developed, mainly to investigate their potential to treat mammary cancer (26,27). However, whether Prl antagonists could exhibit anti-diabetic effects has never been evaluated.

Therefore, the objective of the present study was to determine whether Bromo improves insulin sensitivity via inhibition of Prl secretion and to investigate if a specific Prl receptor antagonist is able to improve glucose homeostasis. For this purpose, we studied *ob/ob* mice since these animals are a natural model of obesity and insulin resistance (28) as well as being widely used in studies investigating the anti-diabetic effects of Bromo (17,29–31).

Material and Methods

Animals

All experiments were performed on 5-month-old *ob/ob* mice, weighing an average of 56 grams. As previously shown, *ob/ob* mice are infertile and females acyclic, maintaining a persistent estrous-like vaginal cytology (32–34). Male and female mice were produced in our local animal facility and the initial breeders were obtained from The Jackson Laboratory (stock#: 000632). Mice had *ad libitum* access to a regular rodent chow diet (2.99 kcal/g; 9.4% calories from fat) and were maintained under standard conditions of light (12-h light/dark cycle) and temperature (23 ± 1 °C). The animal procedures were approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences at the University of São Paulo and were performed according to the ethical guidelines adopted by the Brazilian College of Animal Experimentation.

Study of the mechanism of action of the anti-diabetic effects of bromocriptine

All mice were anesthetized with isoflurane and received subcutaneous implant of an osmotic mini-pump (Alzet; model #1002). Surgical incision was sutured with clips and asepsis was performed with iodine. Mice were separated into 4 experimental groups, according to the treatment (Fig 1A): Control group received mini-pump containing PBS and daily intraperitoneal (i.p.) injections of vehicle (50% DMSO + 50% saline) in the first hour of light cycle (9:00 am); Prl group received mini-pump containing Prl from sheep pituitary (infusion of 18 µg/day; Sigma) and daily i.p. injections of vehicle; Bromo group received mini-pump containing PBS and daily i.p. injections of bromocriptine mesylate (12 µg/g b.w.; Santa Cruz Biotechnology); Bromo+Prl group received mini-pump containing Prl and daily i.p. injections of bromocriptine mesylate. The treatments were performed for 16 days. Prl was administered via mini-pumps to maintain basal serum Prl levels due to its constant infusion. Bromo was provided via daily i.p. injections based on studies in humans and mice showing that a single daily administration of Bromo is sufficient to improve glucose homeostasis in insulin-resistant individuals (17,18,29,35).

Food intake and body weight were measured daily. Glucose tolerance test (GTT) and insulin tolerance test (ITT) were conducted after 4 hours of fasting at the 12th and 14th day of treatment, respectively (Fig 1A). In these tests, mice received i.p. injections of either 1 g glucose/kg b.w. or 3 IU insulin/kg b.w., respectively. Glycemia was measured via blood samples collected from the tail before the injection and 20, 40, 60, and 90 minutes afterwards. For euthanasia, mice were anesthetized with isoflurane. Serum Prl (Mouse Prolactin ELISA Kit, Sigma) and insulin (Crystal Chem, Inc.) concentrations were determined by ELISA in male *ob/ob* mice. The subcutaneous, perigonadal (males), periuterine (females) and retroperitoneal fat pads were collected and weighted as an indicator of body adiposity.

Tissue-specific insulin sensitivity

A subset of the animals previously subjected to GTT and ITT were used to determine the sensitivity to insulin in the liver and skeletal muscle. Thus, male mice received an i.p. injection of 6 IU insulin/kg and were euthanized 15 min later at the 16th day of treatment. The liver and gastrocnemius/soleus muscle were processed for Western blotting as previously described (36). We measured total AKT (1:1,000; Santa Cruz; sc-8312; RRID: AB_671714) and the phosphorylation of AKT ($pAKT^{Ser473}$; 1:1,000; Cell Signaling; #3787 RRID: AB_331170). The values were normalized to GAPDH expression (1:1,000; Santa Cruz; sc-25778; RRID: AB_10167668). Proteins were analyzed using the Li-COR Odyssey system (Li-COR).

Effects of the Prl receptor antagonist (G129R-hPrl) on glucose homeostasis of ob/ob mice

In order to confirm that the G129R-hPrl is capable of blocking Prl signaling *in vivo*, we implanted intracerebroventricular (icv) cannulas (Plastics One) to assess the capacity of the G129R-hPrl to block Prl-induced signal transducer and activator of transcription 5 phosphorylation (pSTAT5) in the hypothalamus. After 4 days of recovery from surgery, mice received an acute icv injection of G129R-hPrl (4 µg in 2 µl) and 5 minutes later an i.p. injection of human recombinant Prl (2.5 µg/g b.w.). After 40 minutes, mice were euthanized and their hypothalami collected to determine STAT5 phosphorylation via Western blotting ($pSTAT5^{Tyr694}$; 1:1000; Cell Signaling; #9351; RRID: AB_2315225). To study the effects of peripheral administration of G129R-hPrl on glucose metabolism, 5-month-old male *ob/ob* mice received subcutaneous implant of osmotic mini-pumps (Alzet; model #1002) filled either with PBS or G129R-hPrl (delivery of 14.4 µg/day for 14 days). To evaluate the effects of icv administration of G129R-hPrl, another group of 5-month-old male and female *ob/ob* mice were implanted with an icv cannula (Alzet; brain infusion kit 3) attached to an osmotic mini-pump (Alzet; model #1004) filled either with PBS or G129R-hPrl (delivery of 5.28 µg/day for 28 days). In this surgical procedure, mice were anesthetized with a cocktail of 8% ketamine + 6% xylazine. The following stereotaxic coordinates in relation to the Bregma were used to implant the cannula in the lateral ventricle: anteroposterior: 0.5 mm, mid-1ateral: 3 mm and dorsoventral: 2.2 mm. Glucose and insulin tolerance were evaluated in the same manner of the aforementioned experiment.

Synthesis, purification and characterization of G129R-hPrl

G129R-hPrl synthesis in the periplasm of *E. coli* followed the same procedures already used for other proteins, using the lambda PL promoter (37–39) . The DsbA signal sequence was utilized, replacing the “GGC” codon corresponding to amino acid 129 glycine with the “CGC” codon corresponding to arginine. Since hPRL tightly degrades at 42°C when it is present in the cytoplasm (38), G129R-hPRL expression was carried out at lower temperatures: 35°C and 37°C (38,39). Osmotic shock was then utilized to extract all proteins from the bacterial periplasm, as previously described (37).

The periplasmic fluid obtained by osmotic shock was purified using metal (Ni) affinity chromatography. As a first purification step, a Hisprep Fast Flow 16/10 was utilized with 0.05 M sodium phosphate pH 7.2, 0.8 M NaCl buffer, under two steps (10 mM and 20 mM Imidazole) and an Imidazole gradient from 20 mM to 100 mM, 5 column volume each, with a flow rate of 300 ml/h. G129R-hPrl was eluted from 50 to 90 mM Imidazole (peak at 72 mM) in the middle of gradient. The second purification step was a size exclusion chromatography (S100 Sephacryl resin in a 26 x 100 mm column), run under isocratic conditions and eluting with ammonium bicarbonate buffer 0.05 M, pH 7.9, at a flow rate of 60 ml/h. After purification and quantification via established methodologies, G129R-hPrl was lyophilized in ammonium bicarbonate buffer in aliquots of 1 mg/flask.

Physico-chemical and immunological characterization was carried out via SDS-PAGE, Western blotting, reversed-phase high-performance liquid chromatography (RP-HPLC) and high-performance size-exclusion chromatography (HPSEC), as previously described (40,41). Electrospray ionization mass spectrometry (ESI-MS) was also utilized in order to accurately confirm the exact molecular mass of G129R-hPRL, as carried out in previous work analyzing a different receptor antagonist (40).

Statistical analysis

The results are expressed as mean ± SEM. Data were analyzed using two-way ANOVA and Fisher's LSD post hoc test. Repeated measures two-way ANOVA was used to analyze the data during the GTT and ITT. The effects of G129R-hPrl were analyzed by unpaired two-tailed t-student test. GraphPad Prism software was used for the statistical analyses and only *p* values < 0.05 were considered to be statistically significant.

Results

Exogenous Prl infusion prevented the suppression of serum Prl concentration induced by Bromo treatment

As earlier mentioned, Bromo suppresses Prl secretion by activating dopamine receptors in the pituitary gland (15,42). Before investigating the metabolic effects of Bromo and/or Prl, we initially analyzed the consequences of our different treatments on serum Prl levels in male *ob/ob* mice (Fig 1B). Prl-treated mice (Prl group) showed similar serum Prl concentrations compared to control mice (Fig 1B). As expected, Bromo-treated mice exhibited a significant reduction in serum Prl levels, which was prevented by exogenous Prl infusion (Bromo+Prl group). Importantly, Prl replacement normalized serum Prl concentration without inducing hyperprolactinemia (Fig 1B).

Effects of Bromo and/or Prl on energy homeostasis of ob/ob mice

Evidence in literature indicates that either Prl or Bromo can produce significant effects on energy homeostasis (16,43,44). However, body weight, food intake and body adiposity remained unaffected in male *ob/ob* mice treated with Bromo and/or Prl for 16 days (Fig. 2). Since the metabolic effects of Prl are sexually dimorphic (43,45), we also studied female *ob/ob* mice. As seen in males, Bromo and/or Prl treatment caused no significant changes in body weight, food intake or body adiposity of female *ob/ob* mice (Fig. 3).

The anti-diabetic effects of Bromo in ob/ob mice is attenuated by Prl replacement

To study the effects of Bromo and/or Prl on glucose homeostasis, we evaluated the glucose and insulin tolerance of *ob/ob* mice. In males, we observed that Bromo treatment, independently of Prl replacement, led to improved glucose tolerance (Fig. 4A-B). In addition, Bromo increased insulin sensitivity of male *ob/ob* mice, but this improvement was prevented by Prl replacement (Fig. 4C-D). Bromo treatment also produced marked anti-diabetic effects in female *ob/ob* mice (Fig. 5). Importantly, the increased glucose and insulin tolerance caused by Bromo was blunted in Bromo+Prl group (Fig. 5), suggesting that Bromo effects on glucose homeostasis are partially associated with serum Prl levels.

Bromo lowers serum insulin concentration and increases insulin sensitivity in the liver and skeletal muscle of male ob/ob mice, whereas Prl replacement prevents these effects

Serum insulin concentration is an important indicator of insulin resistance. We found that Bromo reduced in 35% the serum insulin concentration of male *ob/ob* mice, suggesting increased insulin sensitivity (Fig 6A). However, Prl replacement prevented this improvement (Fig 6A). To further assess glucose homeostasis, we evaluated the capacity of insulin to induce the phosphorylation of AKT (pAKT), a well-established intracellular protein recruited by insulin receptor activation, in key insulin-responsive tissues. An acute insulin stimulus was unable to increase pAKT in the liver and skeletal muscle of control and Prl groups (Fig. 6B-C). These results are in accordance with the marked insulin resistance observed in *ob/ob* mice (46). Notably, Bromo treatment increased the response to insulin in the liver and skeletal muscle, whereas Prl replacement rendered the mice unresponsive to insulin in both tissues (Fig. 6B-C). Neither chronic treatments (Bromo and/or Prl) nor the acute insulin injection caused significant changes in total AKT levels in the liver and skeletal muscle (Fig. 6B-C).

Synthesis and purification of the Prl receptor antagonist – G129R-hPrl

The G129R-hPrl obtained in the periplasmic fluid of W3110 strain of *E. coli* containing the constitutive lambda P_L-DsbA-G129R-hPrl expression vector was purified from 3 L of 2x LB medium (final optical density = 5.05 ± 0.24 A_{600}). Specific productivity was higher when the expression was conducted at 35 °C (0.49 µg/ml/A_{600}) than at 37 °C (0.27 µg/ml/A_{600}) (47). The periplasmic fluid containing ~5 mg of G129R-hPrl in 40 ml obtained by osmotic shock was dialyzed against 0.05 M sodium phosphate pH 7.2. This sample was applied to a Nickel affinity column (Hisprep FF 16/10) and eluted with an Imidazole gradient from 20 mM to 100 mM (47). A pool of fractions (#53 through #60) was applied to a size exclusion chromatography column (Sephacryl S100) eluting with 0.05 M ammonium bicarbonate, pH 7.9. Analysis of the pool from purification steps was performed by Western blotting (47) and RP-HPLC. HPSEC analysis of the final product obtained or of the lyophilized protein after 1 month of storage at 4 °C showed less than 5% of dimeric and aggregated forms (47). The theoretical molecular mass of G129R-hPrl is 22997 Da. Electrospray ionization mass spectrometry (ESI-MS) provided a value of 22999 Da, which is 0.009% higher than the expected mass. Therefore, we were able to produce a highly purified G129R-hPrl, which was then used in the following *in vivo* experiments.

Anti-diabetic effects of the G129R-hPrl

Our hypothesis was that Bromo's effects on glucose homeostasis depend, at least partially, on the suppression of Prl secretion. To further test our hypothesis, we studied the anti-diabetic potential of the recombinant G129R-hPrl. Initially, we determined whether the G129R-hPrl is able to block Prl signaling *in vivo*. Thus, we assessed if a prior G129R-hPrl administration could prevent the capacity of Prl to induce pSTAT5 in the hypothalamus, a key brain area that contains a great number of Prl responsive neurons (48,49). We observed that systemic Prl administration induced a robust phosphorylation of STAT5 in the hypothalamus (Fig. 7A-B). Importantly, this increase was completely blocked by a prior icv injection of G129R-hPrl (Fig. 7A-B). These data confirm that G129R-hPrl is able to block Prl signaling *in vivo*. Next, we chronically infused G129R-hPrl either systemically or centrally in male *ob/ob* mice. We observed that subcutaneous administration of G129R-hPrl caused a non-significant improvement in glucose and insulin tolerance in male *ob/ob* mice (Fig. 7C-F). Icv G129R-hPrl infusion also did not affect glucose tolerance significantly (Fig. 7G-H). However, insulin responsiveness was increased by chronic icv G129R-hPrl infusion (Fig. 7I-J), indicating a modest but biologically significant impact of G129R-hPrl on glucose homeostasis. A group of female *ob/ob* mice also received icv cannulas to infuse either G129R-hPrl or PBS. However, we did not observe significant effects of the G129R-hPrl on glucose homeostasis of female *ob/ob* mice (Fig. 7K-N).

Discussion

In the present study, we investigated the hypothesis that at least part of the anti-diabetic effects of Bromo depend on the inhibition of Prl secretion. Of note, hyperprolactinemia commonly causes insulin resistance, hyperinsulinemia and glucose intolerance (22,50–53) and the treatment of hyperprolactinemic patients with dopamine agonists have beneficial effects in the glycemic control (54). However, it is worth mentioning that *ob/ob* mice are not hyperprolactinemic (55). Therefore, we propose that reduction of circulating Prl to levels below the normal range improves glucose homeostasis in previously insulin resistant animals. Our findings are in accordance with our hypothesis because Prl replacement partially reversed the improvements in glucose homeostasis induced by Bromo treatment.

Several methodological considerations are relevant for the present study. Our low-dose Prl infusion was able to normalize serum Prl concentration in Bromo-treated male mice, without inducing hyperprolactemia. Since Prl secretion is regulated by negative feedback

(20,21), adjustments in Prl secretion probably avoided high Prl levels in the Prl group. Thus, Prl treatment may have increased the central dopaminergic tone in the Prl group. However, this effect was likely specific for TIDA neurons which express Prl receptors (48,49), but not for other dopaminergic circuits, such as the mesolimbic dopamine system. Importantly, the Prl group did not differ from the control group in any of the analyzed parameters, indicating that if Prl administration affected the dopaminergic circuit, these effects did not change the main parameters evaluated. In addition, we cannot extrapolate our findings to other models, such as diet-induced obese animals. In fact, we showed in a previous study that while *ob/ob* mice are completely responsive to Prl, diet-induced obese mice exhibit both peripheral and central Prl resistance (56). Prl resistance in diet-induced obese mice is explained by their elevated serum leptin levels that can interact with Prl responsive cells in the brain and periphery (56,57). Therefore, additional studies are needed to investigate whether Bromo improves insulin sensitivity via inhibition of Prl secretion in other animal models of T2DM. Moreover, the storage capacity of the mini-pumps led us to study the animals for only two weeks. Thus, a longer treatment would have allowed additional experiments to evaluate energy and glucose homeostasis, as well as it could have amplified the effects we observed. Finally, the constant infusion by the mini-pump cannot reproduce the physiological pattern of Prl secretion, which is typically pulsatile and influenced by circadian and environmental factors (58).

Previous studies have shown that energy homeostasis can be affected by Bromo treatment (17,29,59). However, in the present study we observed that Bromo treatment did not change food intake nor body weight of *ob/ob* mice. In this sense, our results are in accordance with Pijl et al. (15) who demonstrated an improvement of glucose tolerance in T2DM obese subjects treated with Bromo, without alterations in their body weight or body composition. Thus, it is interesting to highlight that Bromo-induced improvements in glucose homeostasis of *ob/ob* mice were not secondary to changes in body composition or adiposity.

Bromo treatment improved glucose and insulin sensitivity of *ob/ob* mice and increased the capacity of insulin to activate its intracellular pathway in the skeletal muscle and liver. Previous studies suggested that Bromo has a direct action in pancreatic β-cells, regulating insulin secretion (60). However, other studies demonstrated that the anti-diabetic effects of Bromo depend on the CNS since icv Bromo infusion improves glucose homeostasis (18). Moreover, some authors propose that Bromo administration in the early morning modulates the dopaminergic system and this effect, particularly in the hypothalamus, could lead to increased postprandial insulin sensitivity in patients with T2DM (19,61). Although our study does not rule out that Bromo may have the aforementioned effects, we provided evidence that Prl replacement in Bromo-treated *ob/ob* mice can prevent major improvements in glucose and insulin tolerance. Thus, at least part of the anti-diabetic effects of Bromo depends on the changes induced in serum Prl levels. Accordingly, there is some evidence that other dopamine receptor agonists are also capable of improving glucose homeostasis in diabetic individuals (62). For example, cabergoline treatment reduced fasting and postprandial plasma glucose levels and HbA1c in type 2 diabetic patients (63). In addition, a combination of Bromo and SKF38393 (a dopamine D1 agonist) produces better effects on the glucose homeostasis of *ob/ob* mice than Bromo treatment only (17). Therefore, although Bromo may improve glucose homeostasis via several and perhaps unique mechanisms, the suppression of Prl secretion seems to play an important role in these effects and probably explains the anti-diabetic properties of different dopamine receptor agonists.

We observed some sexually dimorphic responses, particularly in the capacity of Prl replacement to affect glucose tolerance. In males, Prl replacement did not prevent the improvements in glucose tolerance caused by Bromo, whereas a significant effect of Prl treatment was observed in female *ob/ob* mice. Therefore, the anti-diabetic effects of Bromo seem to be more dependent on Prl in females, whereas in males these effects depend on both Prl and other mechanisms. A stronger effect of Prl on the metabolism of females is not surprising. For example, Prl receptor knockout mice are leaner than control littermates, although this effect is greater in females (43). In addition, hyperprolactinemia induced by selective disruption of dopamine D2 receptors in pituitary lactotrophs increases body weight and adiposity of female mice, while males show no significant metabolic abnormalities (45). Of note, hyperprolactinemia causes no metabolic disturbances in global D2 receptor knockout mice (64), suggesting that an intact central dopaminergic system is required for the metabolic effects of Prl.

We also tested our hypothesis by blocking Prl signaling using a Prl receptor antagonist. Prl receptor antagonists have been developed as potential drugs to treat breast cancer, prostate cancer and prolactinomas resistant to dopamine treatment (26). Therefore, this is the first study that aimed to investigate their anti-diabetic potential. The structure of these drugs comprises the Prl molecule itself, but with specific mutations that make them inverse agonists of the endogenous receptor. Different Prl receptor antagonists have been produced, including the G129R-hPrl which presents a substitution of glycine in position 129 for arginine, the S179D-hPrl, produced by introducing an aspartate in place of the normally phosphorylated serine 179, and the Del1-9-G129R-hPrl obtained by deleting the nine N-terminal residues of G129R-hPrl (26,65,66). The physical-chemical and biological properties of some Prl receptor antagonists were previously characterized by our group (67). In the present study we used the G129R-hPrl because it is primarily an antagonist of the Prl receptor with low residual action on other receptors (67–70). However, Prl receptor antagonists may have residual agonist activity in a dose-dependent manner, which is a difficult effect to measure *in vivo* (27,67). Using the capacity of Prl to phosphorylate STAT5 as a marker of Prl responsiveness, we observed that a prior icv injection of G129R-hPrl completely blocked the ability of a systemic Prl administration to induce pSTAT5 in the hypothalamus, providing evidence that G129R-hPrl is able to block Prl signaling *in vivo*. In addition, the injection of G129R-hPrl alone did not induce pSTAT5, discarding a possible agonistic effect of this drug, at least in this experimental design. Remarkably, we observed increased insulin responsiveness in *ob/ob* mice chronically treated with G129R-hPrl. Nonetheless, this effect was only observed when G129R-hPrl was administered centrally and in male mice. Although systemic Prl is fully capable of entering the CNS (48,71,72), we have no information about the central transportation of G129R-hPrl. Furthermore, systemic injections of Prl require higher doses to produce equivalent effects in the CNS, compared to central administration (49,73). Since the dose used in the peripheral infusion of the G129R-hPrl was only 3 times higher than the one administered centrally, we may have achieved greater inhibition of Prl signaling with the central infusion. Nevertheless, our results highlight the key role of the CNS in the regulation of glucose homeostasis (7).

In summary, our findings indicated that Prl replacement partially reversed the improvements in glucose homeostasis caused by Bromo treatment. Despite the clear anti-diabetic effects exhibited by Bromo treatment, dopamine agonists modulate several brain functions, including locomotion, cognition, eating behavior, energy homeostasis, motivation, reward, memory, mood, learning, and hormonal secretion (74). Thus, the identification of drugs with less potential side effects is of interest. Our study identified for the first time anti-diabetic properties of Prl receptor antagonists. Taken together, our findings indicate that Prl intracellular signaling cascade represents a potentially promising target of novel therapeutic approaches to improve insulin sensitivity.

Grants: This study was supported by the São Paulo Research Foundation (FAPESP, Brazil) via grants (12/24345-4 and 15/10992-6) and fellowships (12/15517-6, 13/21722-4, 13/25032-2, 14/11752-6 and 17/25281-3).

Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 12/24345-4, Carlos RJ Soares; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 15/10992-6, Jose Donato; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 12/15517-6, Thais T Zampieri; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 13/21722-4, Isadora C Furigo; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 13/25032-2, João A Pedroso; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 14/11752-6, Angela M Ramos-Lobo; Fundação de Amparo à Pesquisa do Estado de São Paulo
http://dx.doi.org/10.13039/501100001807, 17/25281-3, Paula GF Quaresma

* Corresponding author: All reprints should be addressed to Jose Donato Jr., PhD, Address: Av. Prof. Lineu Prestes, 1524, São Paulo, SP, Brazil 05508-000; Email: jdonato@icb.usp.br; Phone: +55-11-3091-0929; Fax: +55-11-3091-7285

Disclosure summary:
The authors have nothing to disclose.

References

1. Davidson MB. *Diabetes Mellitus. Diagnosis and Treatment.* 4th edition. Philadelphia: WB Saunders Company; 1998.
2. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract*. 2010;**87**(1):4-14.
3. Krentz AJ, Bailey CJ. Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs*. 2005;**65**(3):385-411.
4. Corathers SD, Peavie S, Salehi M. Complications of diabetes therapy. *Endocrinol Metab Clin North Am*. 2013;**42**(4):947-970.
5. Neumiller JJ. Clinical pharmacology of incretin therapies for type 2 diabetes mellitus: implications for treatment. *Clin Ther*. 2011;**33**(5):528-576.
6. Ghosh RK, Ghosh SM, Chawla S, Jasdanwala SA. SGLT2 inhibitors: a new emerging therapeutic class in the treatment of type 2 diabetes mellitus. *J Clin Pharmacol*. 2012;**52**(4):457-463.
7. Donato J. The central nervous system as a promising target to treat diabetes mellitus. *Curr Top Med Chem*. 2012;**12**(19):2070-2081.
8. Havrankova J, Roth J, Brownstein M. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature*. 1978;**272**(5656):827-829.
9. Marks JL, Porte D, Stahl WL, Baskin DG. Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology*. 1990;**127**(6):3234-3236.
10. Koch L, Wunderlich FT, Seibler J, Könner AC, Hampel B, Irlenbusch S, Brabant G, Kahn CR, Schwenk F, Brüning JC. Central insulin action regulates peripheral glucose and fat metabolism in mice. *J Clin Invest*. 2008;**118**(6):2132-2147.
11. Cohen P, Zhao C, Cai X, Montez JM, Rohani SC, Feinstein P, Mombaerts P, Friedman JM. Selective deletion of leptin receptor in neurons leads to obesity. *J Clin Invest*. 2001;**108**(8):1113-1121.
12. Hill JW, Elias CF, Fukuda M, Williams KW, Berglund ED, Holland WL, Cho Y-R, Chuang J-C, Xu Y, Choi M, Lauzon D, Lee CE, Coppari R, Richardson JA, Zigman JM, Chua S, Scherer PE, Lowell BB, Brüning JC, Elmquist JK. Direct insulin and leptin action on

pro-opiomelanocortin neurons is required for normal glucose homeostasis and fertility. *Cell Metab*. 2010;**11**(4):286-297.
13. Huo L, Gamber K, Greeley S, Silva J, Huntoon N, Leng X-H, Bjørbaek C. Leptin-dependent control of glucose balance and locomotor activity by POMC neurons. *Cell Metab*. 2009;**9**(6):537-547.
14. Berglund ED, Vianna CR, Donato J, Kim MH, Chuang J-C, Lee CE, Lauzon DA, Lin P, Brule LJ, Scott MM, Coppari R, Elmquist JK. Direct leptin action on POMC neurons regulates glucose homeostasis and hepatic insulin sensitivity in mice. *J Clin Invest*. 2012;**122**(3):1000-1009.
15. Pijl H, Ohashi S, Matsuda M, Miyazaki Y, Mahankali A, Kumar V, Pipek R, Iozzo P, Lancaster JL, Cincotta AH, DeFronzo RA. Bromocriptine: a novel approach to the treatment of type 2 diabetes. *Diabetes Care*. 2000;**23**(8):1154-1161.
16. Barnett AH, Chapman C, Gailer K, Hayter CJ. Effect of bromocriptine on maturity onset diabetes. *Postgrad Med J*. 1980;**56**(651):11-14.
17. Cincotta AH, Tozzo E, Scislowski PW. Bromocriptine/SKF38393 treatment ameliorates obesity and associated metabolic dysfunctions in obese (ob/ob) mice. *Life Sci*. 1997;**61**(10):951-956.
18. Luo S, Liang Y, Cincotta AH. Intracerebroventricular administration of bromocriptine ameliorates the insulin-resistant/glucose-intolerant state in hamsters. *Neuroendocrinology*. 1999;**69**(3):160-166.
19. Holt RIG, Barnett AH, Bailey CJ. Bromocriptine: old drug, new formulation and new indication. *Diabetes Obes Metab*. 2010;**12**(12):1048-1057.
20. Ben-Jonathan N. Dopamine: a prolactin-inhibiting hormone. *Endocr Rev*. 1985;**6**(4):564-589.
21. Grattan DR. 60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-prolactin axis. *J Endocrinol*. 2015;**226**(2):T101-122.
22. Landgraf R, Landraf-Leurs MM, Weissmann A, Hörl R, von Werder K, Scriba PC. Prolactin: a diabetogenic hormone. *Diabetologia*. 1977;**13**(2):99-104.
23. Li J, Rice MS, Huang T, Hankinson SE, Clevenger CV, Hu FB, Tworoger SS. Circulating prolactin concentrations and risk of type 2 diabetes in US women. *Diabetologia*. 2018;**61**(12):2549-2560.
24. Banerjee RR, Cyphert HA, Walker EM, Chakravarthy H, Peiris H, Gu X, Liu Y, Conrad E, Goodrich L, Stein RW, Kim SK. Gestational Diabetes Mellitus From Inactivation of Prolactin Receptor and MafB in Islet β-Cells. *Diabetes*. 2016;**65**(8):2331-2341.
25. Amaral MEC, Cunha DA, Anhê GF, Ueno M, Carneiro EM, Velloso LA, Bordin S, Boschero AC. Participation of prolactin receptors and phosphatidylinositol 3-kinase and MAP kinase pathways in the increase in pancreatic islet mass and sensitivity to glucose during pregnancy. *J Endocrinol*. 2004;**183**(3):469-476.
26. Jomain J-B, Tallet E, Broutin I, Hoos S, van Agthoven J, Ducruix A, Kelly PA, Kragelund BB, England P, Goffin V. Structural and thermodynamic bases for the design of pure prolactin receptor antagonists: X-ray structure of Del1-9-G129R-hPRL. *J Biol Chem*. 2007;**282**(45):33118-33131.
27. Bernichtein S, Kayser C, Dillner K, Moulin S, Kopchick JJ, Martial JA, Norstedt G, Isaksson O, Kelly PA, Goffin V. Development of pure prolactin receptor antagonists. *J Biol Chem*. 2003;**278**(38):35988-35999.
28. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;**372**(6505):425-432.

29. Zhang Y, Scislowski PW, Prevelige R, Phaneuf S, Cincotta AH. Bromocriptine/SKF38393 treatment ameliorates dyslipidemia in ob/ob mice. *Metab Clin Exp*. 1999;**48**(8):1033-1040.
30. Scislowski PW, Tozzo E, Zhang Y, Phaneuf S, Prevelige R, Cincotta AH. Biochemical mechanisms responsible for the attenuation of diabetic and obese conditions in ob/ob mice treated with dopaminergic agonists. *Int J Obes Relat Metab Disord*. 1999;**23**(4):425-431.
31. Liang Y, Lubkin M, Sheng H, Scislowski PW, Cincotta AH. Dopamine agonist treatment ameliorates hyperglycemia, hyperlipidemia, and the elevated basal insulin release from islets of ob/ob mice. *Biochim Biophys Acta*. 1998;**1405**(1-3):1-13.
32. Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet*. 1996;**12**(3):318-320.
33. Chakraborty S, Sachdev A, Salton SRJ, Chakraborty TR. Stereological analysis of estrogen receptor expression in the hypothalamic arcuate nucleus of ob/ob and agouti mice. *Brain Res*. 2008;**1217**:86-95.
34. Ng KY, Yong J, Chakraborty TR. Estrous cycle in ob/ob and ovariectomized female mice and its relation with estrogen and leptin. *Physiol Behav*. 2010;**99**(1):125-130.
35. Kamath V, Jones CN, Yip JC, Varasteh BB, Cincotta AH, Reaven GM, Chen YD. Effects of a quick-release form of bromocriptine (Ergoset) on fasting and postprandial plasma glucose, insulin, lipid, and lipoprotein concentrations in obese nondiabetic hyperinsulinemic women. *Diabetes Care*. 1997;**20**(11):1697-1701.
36. Zampieri TT, Pedroso JAB, Furigo IC, Tirapegui J, Donato J. Oral leucine supplementation is sensed by the brain but neither reduces food intake nor induces an anorectic pattern of gene expression in the hypothalamus. *PLoS ONE*. 2013;**8**(12):e84094.
37. Soares CRJ, Gomide FIC, Ueda EKM, Bartolini P. Periplasmic expression of human growth hormone via plasmid vectors containing the lambdaPL promoter: use of HPLC for product quantification. *Protein Eng*. 2003;**16**(12):1131-1138.
38. Soares CRJ, Ueda EKM, Oliveira TL, Gomide FIC, Heller SR, Bartolini P. Distinct human prolactin (hPRL) and growth hormone (hGH) behavior under bacteriophage lambda PL promoter control: temperature plays a major role in protein yields. *J Biotechnol*. 2008;**133**(1):27-35.
39. Suzuki MF, Arthuso FS, Oliveira JE, Oliveira NAJ, Goulart HR, Capone MVN, Ribela MTCP, Bartolini P, Soares CRJ. Expression, purification, and characterization of authentic mouse prolactin obtained in Escherichia coli periplasmic space. *Biotechnol Appl Biochem*. 2012;**59**(3):178-185.
40. Menezes ACSC, Suzuki MF, Oliveira JE, Ribela MTCP, Furigo IC, Donato J, Bartolini P, Soares CRJ. Expression, purification and characterization of the authentic form of human growth hormone receptor antagonist G120R-hGH obtained in Escherichia coli periplasmic space. *Protein Expr Purif*. 2017;**131**:91-100.
41. Soares CRJ, Camargo IMC, Morganti L, Gimbo E, Ezequiel de Oliveira J, Legoux R, Ferrara P, Bartolini P. Reversed-phase high-performance liquid chromatography method for the determination of prolactin in bacterial extracts and in its purified form. *J Chromatogr A*. 2002;**955**(2):229-236.
42. Doknic M, Pekic S, Zarkovic M, Medic-Stojanoska M, Dieguez C, Casanueva F, Popovic V. Dopaminergic tone and obesity: an insight from prolactinomas treated with bromocriptine. *Eur J Endocrinol*. 2002;**147**(1):77-84.
43. Freemark M, Fleenor D, Driscoll P, Binart N, Kelly P. Body weight and fat deposition in prolactin receptor-deficient mice. *Endocrinology*. 2001;**142**(2):532-537.

44. Cincotta AH, Meier AH, Cincotta M Jr. Bromocriptine improves glycaemic control and serum lipid profile in obese Type 2 diabetic subjects: a new approach in the treatment of diabetes. *Expert Opin Investig Drugs*. 1999;**8**(10):1683-1707.
45. Perez Millan MI, Luque GM, Ramirez MC, Noain D, Ornstein AM, Rubinstein M, Becu-Villalobos D. Selective disruption of dopamine D2 receptors in pituitary lactotropes increases body weight and adiposity in female mice. *Endocrinology*. 2014;**155**(3):829-839.
46. Dubuc PU. The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice. *Metab Clin Exp*. 1976;**25**(12):1567-1574.
47. Furigo IC, Suzuki MF, Oliveira JE, Ramos-Lobo AM, Teixeira PDS, Pedroso JA, Alencar A, Zampieri TT, Buonfiglio DC, Quaresma PGF, Prada PO, Bartolini P, Soares CRJ, Donato J Jr. Data from: Suppression of prolactin secretion partially explains the anti-diabetic effect of bromocriptine in ob/ob mice. Figshare Digital Repository. Deposited 14 November 2018. http://doi.org/10.6084/m9.figshare.7338947.
48. Furigo IC, Metzger M, Teixeira PDS, Soares CRJ, Donato J. Distribution of growth hormone-responsive cells in the mouse brain. *Brain Struct Funct*. 2017;**222**(1):341-363.
49. Brown RSE, Kokay IC, Herbison AE, Grattan DR. Distribution of prolactin-responsive neurons in the mouse forebrain. *J Comp Neurol*. 2010;**518**(1):92-102.
50. Johnston DG, Alberti KG, Nattrass M, Burrin JM, Blesa-Malpica G, Hall K, Hall R. Hyperinsulinaemia in hyperprolactinaemic women. *Clin Endocrinol (Oxf)*. 1980;**13**(4):361-368.
51. Schernthaner G, Prager R, Punzengruber C, Luger A. Severe hyperprolactinaemia is associated with decreased insulin binding in vitro and insulin resistance in vivo. *Diabetologia*. 1985;**28**(3):138-142.
52. Tuzcu A, Yalaki S, Arikan S, Gokalp D, Bahcec M, Tuzcu S. Evaluation of insulin sensitivity in hyperprolactinemic subjects by euglycemic hyperinsulinemic clamp technique. *Pituitary*. 2009;**12**(4):330-334.
53. Ben-Jonathan N, Hugo ER, Brandebourg TD, LaPensee CR. Focus on prolactin as a metabolic hormone. *Trends Endocrinol Metab*. 2006;**17**(3):110-116.
54. Berinder K, Nyström T, Höybye C, Hall K, Hulting A-L. Insulin sensitivity and lipid profile in prolactinoma patients before and after normalization of prolactin by dopamine agonist therapy. *Pituitary*. 2011;**14**(3):199-207.
55. Larson BA, Sinha YN, Vanderlaan WP. Serum growth hormone and prolactin during and after the development of the obese-hyperglycemic syndrome in mice. *Endocrinology*. 1976;**98**(1):139-145.
56. Buonfiglio DC, Ramos-Lobo AM, Freitas VM, Zampieri TT, Nagaishi VS, Magalhães M, Cipolla-Neto J, Cella N, Donato J. Obesity impairs lactation performance in mice by inducing prolactin resistance. *Sci Rep*. 2016;**6**:22421.
57. Nagaishi VS, Cardinali LI, Zampieri TT, Furigo IC, Metzger M, Donato J. Possible crosstalk between leptin and prolactin during pregnancy. *Neuroscience*. 2014;**259**:71-83.
58. Egli M, Leeners B, Kruger THC. Prolactin secretion patterns: basic mechanisms and clinical implications for reproduction. *Reproduction*. 2010;**140**(5):643-654.
59. Davis LM, Michaelides M, Cheskin LJ, Moran TH, Aja S, Watkins PA, Pei Z, Contoreggi C, McCullough K, Hope B, Wang GJ, Volkow ND, Thanos PK. Bromocriptine administration reduces hyperphagia and adiposity and differentially affects dopamine D2 receptor and transporter binding in leptin-receptor-deficient Zucker rats and rats with diet-induced obesity. *Neuroendocrinology*. 2009;**89**(2):152-162.
60. Rubí B, Ljubicic S, Pournourmohammadi S, Carobbio S, Armanet M, Bartley C, Maechler P. Dopamine D2-like receptors are expressed in pancreatic beta cells and mediate inhibition of insulin secretion. *J Biol Chem*. 2005;**280**(44):36824-36832.

61. Schwartz SS, Zangeneh F. Evidence-based practice use of quick-release bromocriptine across the natural history of type 2 diabetes mellitus. *Postgrad Med*. 2016;**128**(8):828-838.

62. Lamos EM, Levitt DL, Munir KM. A review of dopamine agonist therapy in type 2 diabetes and effects on cardio-metabolic parameters. *Prim Care Diabetes*. 2016;**10**(1):60-65.

63. Taghavi SM, Fatemi SS, Rokni H. Cabergoline effect on blood sugar in type 2 diabetic patients with oral agent failure. *Med J Malaysia*. 2012;**67**(4):390-392.

64. Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, Ben-Jonathan N, Grandy DK, Low MJ. Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron*. 1997;**19**(1):103-113.

65. Tallet E, Rouet V, Jomain J-B, Kelly PA, Bernichtein S, Goffin V. Rational design of competitive prolactin/growth hormone receptor antagonists. *J Mammary Gland Biol Neoplasia*. 2008;**13**(1):105-117.

66. Bridges R, Rigero B, Byrnes E, Yang L, Walker A. Central infusions of the recombinant human prolactin receptor antagonist, S179D-PRL, delay the onset of maternal behavior in steroid-primed, nulliparous female rats. *Endocrinology*. 2001;**142**(2):730-739.

67. Soares CRJ, Glezer A, Okazaki K, Ueda EKM, Heller SR, Walker AM, Goffin V, Bartolini P. Physico-chemical and biological characterizations of two human prolactin analogs exhibiting controversial bioactivity, synthesized in Chinese hamster ovary (CHO) cells. *Protein Expr Purif*. 2006;**48**(2):182-194.

68. Chen TJ, Kuo CB, Tsai KF, Liu JW, Chen DY, Walker AM. Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. *Endocrinology*. 1998;**139**(2):609-616.

69. Goffin V, Bernichtein S, Kayser C, Kelly PA. Development of new prolactin analogs acting as pure prolactin receptor antagonists. *Pituitary*. 2003;**6**(2):89-95.

70. DePalatis L, Almgren CM, Patmastan J, Troyer M, Woodrich T, Brooks CL. Structure and function of a new class of human prolactin antagonists. *Protein Expr Purif*. 2009;**66**(2):121-130.

71. Brown RSE, Wyatt AK, Herbison RE, Knowles PJ, Ladyman SR, Binart N, Banks WA, Grattan DR. Prolactin transport into mouse brain is independent of prolactin receptor. *FASEB J*. 2016;**30**(2):1002-1010.

72. Furigo IC, Kim KW, Nagaishi VS, Ramos-Lobo AM, de Alencar A, Pedroso JAB, Metzger M, Donato J. Prolactin-sensitive neurons express estrogen receptor-α and depend on sex hormones for normal responsiveness to prolactin. *Brain Res*. 2014;**1566**:47-59.

73. Sjoeholm A, Bridges RS, Grattan DR, Anderson GM. Region-, neuron-, and signaling pathway-specific increases in prolactin responsiveness in reproductively experienced female rats. *Endocrinology*. 2011;**152**(5):1979-1988.

74. Lopez Vicchi F, Luque GM, Brie B, Nogueira JP, Garcia Tornadu I, Becu-Villalobos D. Dopaminergic drugs in type 2 diabetes and glucose homeostasis. *Pharmacological Research*. 2016;**109**:74-80.

Figure 1. Experimental design and serum prolactin levels. A. *Ob/ob* mice received subcutaneous osmotic mini-pumps containing either ovine prolactin (infusion of 18 µg/day) or PBS, followed by daily intraperitoneal (i.p.) injections of bromocriptine (12 µg/g b.w.) or vehicle. Glucose and insulin tolerance tests were performed at 12th and 14th day of treatment, respectively, and mice were euthanized at 16th day. During this period, food intake and body weight were daily monitored. **B.** Serum prolactin concentration (n = 5-7/group) at the 16th day of treatment (main effect of Bromo [$F_{(1, 20)} = 7.155$, $P = 0.0146$], main effect of Prl [$F_{(1, 20)} = 5.470$, $P = 0.0298$] and interaction [$F_{(1, 20)} = 6.847$, $P = 0.0165$]). **$P<0.01$ vs Bromo group.

Figure 2. Energy balance of male *ob/ob* mice during treatments. A-B. Body weight (A) (main effect of Bromo [$F_{(1, 97)} = 3.145$, $P = 0.0793$], main effect of Prl [$F_{(1, 97)} = 0.7515$, $P = 0.3882$] and interaction [$F_{(1, 97)} = 0.4262$, $P = 0.5154$]) and daily food intake (B) (main effect of Bromo [$F_{(1, 85)} = 3.220$, $P = 0.0763$], main effect of Prl [$F_{(1, 85)} = 0.0054$, $P = 0.9418$] and interaction [$F_{(1, 85)} = 0.3116$, $P = 0.5782$]) of control, Prl, Bromo and Bromo+Prl groups. **C-E.** Body adiposity indicated by the weight of subcutaneous (C) (main effect of Bromo [$F_{(1, 83)} = 0.0682$, $P = 0.7946$], main effect of Prl [$F_{(1, 83)} = 0.0788$, $P = 0.7795$] and interaction [$F_{(1, 83)} = 0.0156$, $P = 0.9006$]), perigonadal (D) (main effect of Bromo [$F_{(1, 83)} = 0.0314$, $P = 0.8597$], main effect of Prl [$F_{(1, 83)} = 0.3336$, $P = 0.5651$] and interaction [$F_{(1, 83)} = 0.1108$, $P = 0.7401$]) and retroperitoneal (E) (main effect of Bromo [$F_{(1, 83)} = 0.02349$, $P = 0.8786$], main effect of Prl [$F_{(1, 83)} = 0.0078$, $P = 0.9295$] and interaction [$F_{(1, 83)} = 0.3261$, $P = 0.5695$]) fat pads.

Figure 3. Energy balance of female *ob/ob* mice during treatments. **A-B.** Body weight (A) (main effect of Bromo [$F_{(1, 56)} = 0.0511$, $P = 0.8219$], main effect of Prl [$F_{(1, 56)} = 0.1700$, $P = 0.6817$] and interaction [$F_{(1, 56)} = 0.1317$, $P = 0.7180$]) and daily food intake (B) (main effect of Bromo [$F_{(1, 36)} = 2.687$, $P = 0.1099$], main effect of Prl [$F_{(1, 36)} = 0.2591$, $P = 0.6139$] and interaction [$F_{(1, 36)} = 0.0152$, $P = 0.9025$]) of control, Prl, Bromo and Bromo+Prl groups. **C-E.** Body adiposity indicated by the weight of subcutaneous (C) (main effect of Bromo [$F_{(1, 56)} = 0.0545$, $P = 0.8162$], main effect of Prl [$F_{(1, 56)} = 0.0087$, $P = 0.9259$] and interaction [$F_{(1, 56)} = 0.0805$, $P = 0.7776$]), perigonadal (D) (main effect of Bromo [$F_{(1, 56)} = 1.248$, $P = 0.2687$], main effect of Prl [$F_{(1, 56)} = 0.0072$, $P = 0.9323$] and interaction [$F_{(1, 56)} = 0.0830$, $P = 0.7742$]) and retroperitoneal (E) (main effect of Bromo [$F_{(1, 56)} = 0.2522$, $P = 0.6175$], effect of Prl [$F_{(1, 56)} = 0.1034$, $P = 0.7490$] and interaction [$F_{(1, 56)} = 0.06714$, $P = 0.7965$]) fat pads.

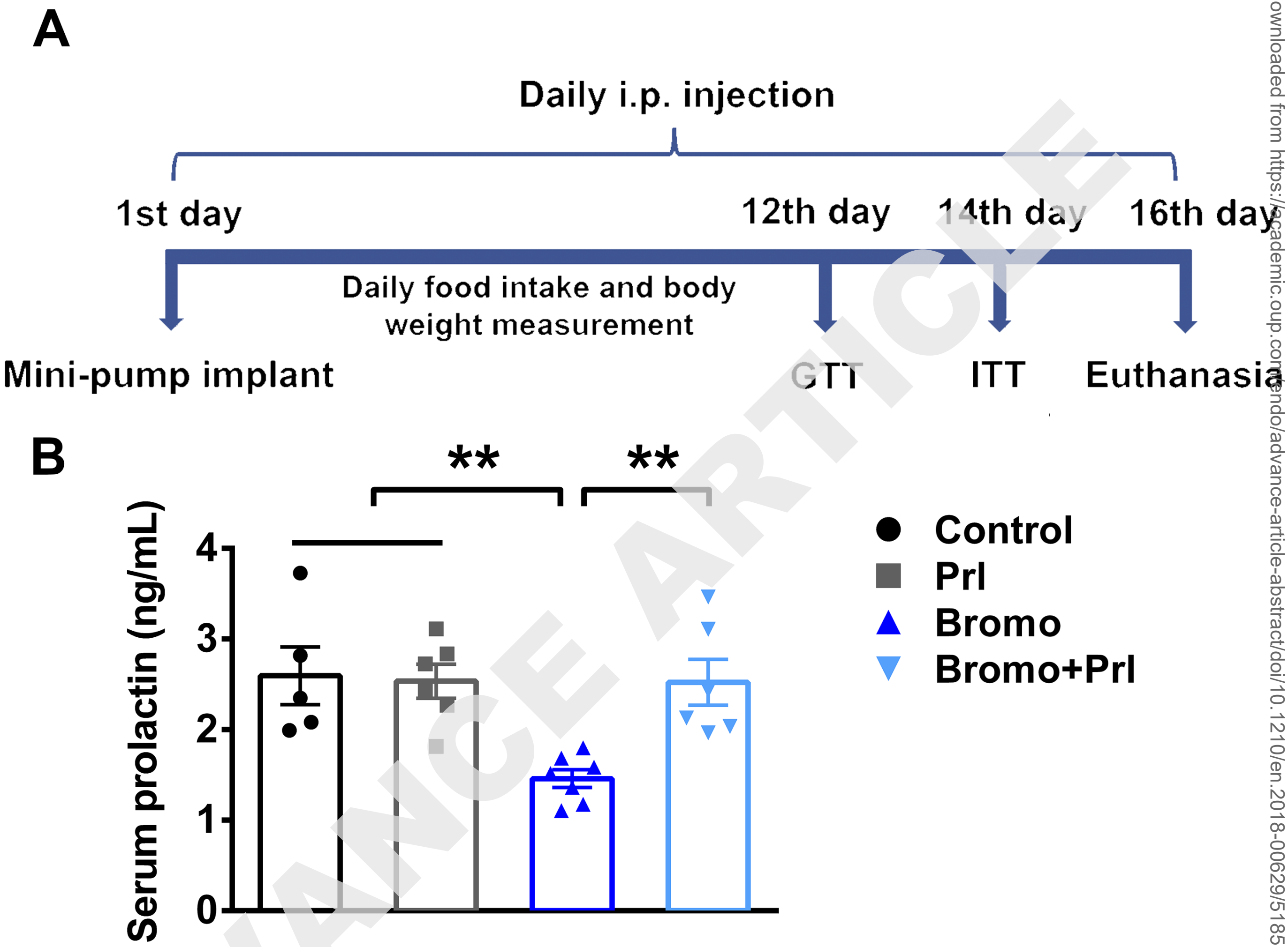
Figure 4. Glucose and insulin tolerance in male *ob/ob* mice during treatments. A. Area under curve of the glucose tolerance test (GTT; main effect of Bromo [$F_{(1, 57)} = 27.53$, $P < 0.0001$], main effect of Prl [$F_{(1, 57)} = 0.1461$, $P = 0.7037$] and interaction [$F_{(1, 57)} = 0.7926$, $P = 0.3770$]) ***$P < 0.001$ Bromo vs Control and Prl groups; ##$P < 0.01$ Bromo+Prl vs Control and Prl groups. **B.** Blood glucose changes during the GTT. *$P < 0.05$ Bromo vs Control and Prl groups; #$P < 0.05$ Bromo+Prl vs Control and Prl groups. **C.** Area under curve of the insulin tolerance test (ITT; main effect of Bromo [$F_{(1, 56)} = 2.991$, $P = 0.0893$], main effect of Prl [$F_{(1, 56)} = 5.746$, $P = 0.0199$] and interaction [$F_{(1, 56)} = 2.840$, $P = 0.0975$]) *$P < 0.05$ vs Bromo group; **$P < 0.01$ vs Bromo group. **D.** Blood glucose changes during the ITT. &$P < 0.05$ Bromo vs Prl group; #$P < 0.05$ Bromo+Prl vs Prl group; *$P < 0.05$ Bromo vs Bromo+Prl group.

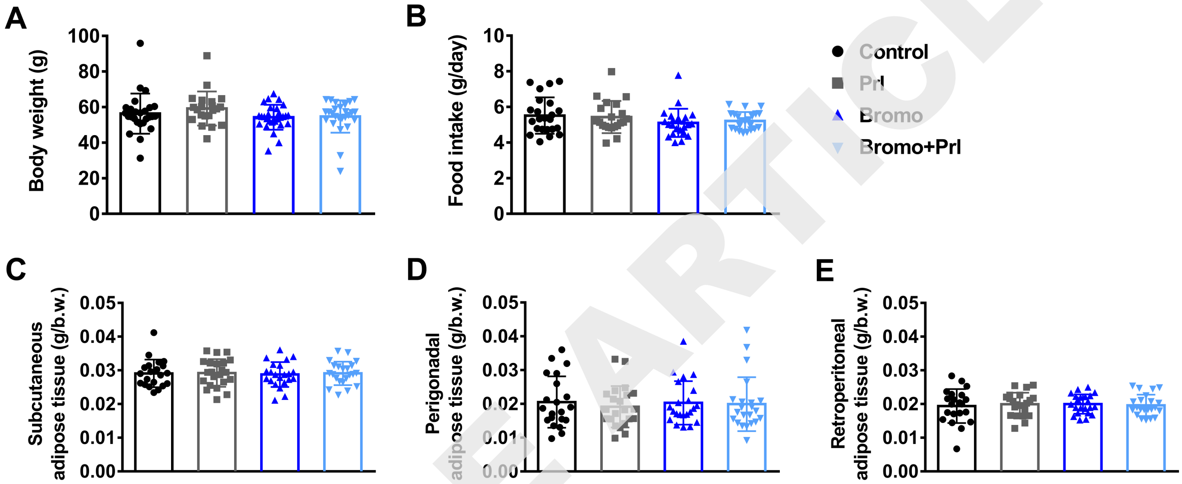
Figure 5. Glucose and insulin tolerance in female *ob/ob* mice during treatments. A. Area under curve of the glucose tolerance test (GTT; main effect of Bromo [$F_{(1, 50)} = 15.57$, $P = 0.0002$], main effect of Prl [$F_{(1, 50)} = 7.434$, $P = 0.0088$] and interaction [$F_{(1, 50)} = 0.5234$, $P = 0.4728$]. **B.** Blood glucose changes during the GTT. &$P < 0.05$ Bromo vs Prl group; *$P < 0.05$ Bromo vs all groups; @$P < 0.05$ Bromo+Prl vs all groups; #$P < 0.05$ Prl vs Bromo+Prl group; +$P < 0.05$ Control and Prl vs Bromo group. **C.** Area under curve of the insulin tolerance test (ITT; main effect of Bromo [$F_{(1, 31)} = 6.055$, $P = 0.0196$], main effect of Prl [$F_{(1, 31)} = 1.987$, $P = 0.1686$] and interaction [$F_{(1, 31)} = 2.588$, $P = 0.1178$]. *$P < 0.05$ vs Bromo group. **$P < 0.01$ vs Bromo group. **D.** Blood glucose changes during the ITT. +$P < 0.05$ Bromo vs Control and Prl groups; ˆ$P < 0.05$ Control vs Bromo+Prl group; * $P < 0.05$ Bromo vs all groups.

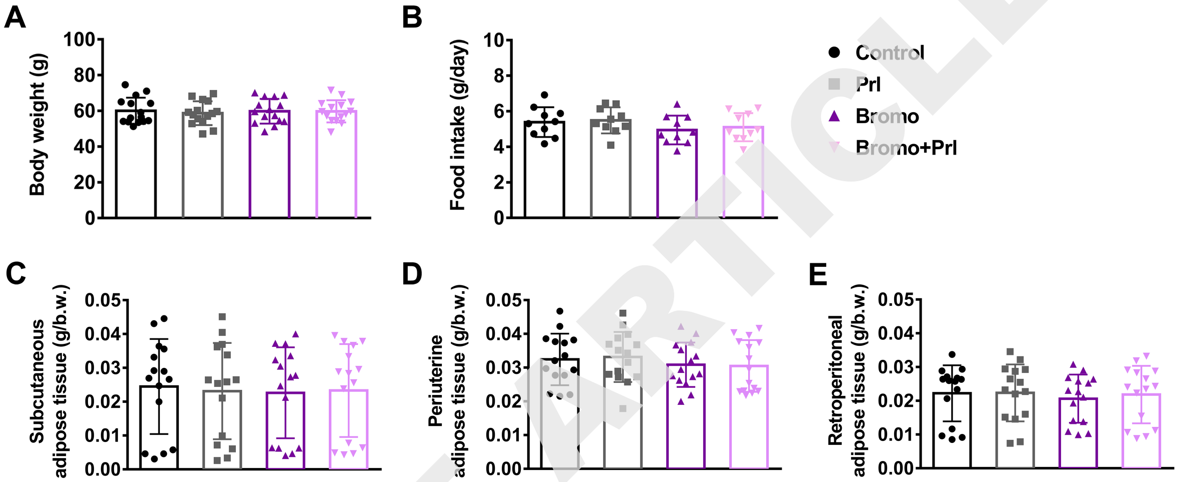
Figure 6. Bromocriptine treatment in male *ob/ob* mice decreases serum insulin concentration and ameliorates insulin sensitivity while Prl replacement reverts these effects. A. Serum insulin concentration at 16th day of treatment (main effect of Bromo [$F_{(1, 28)}$

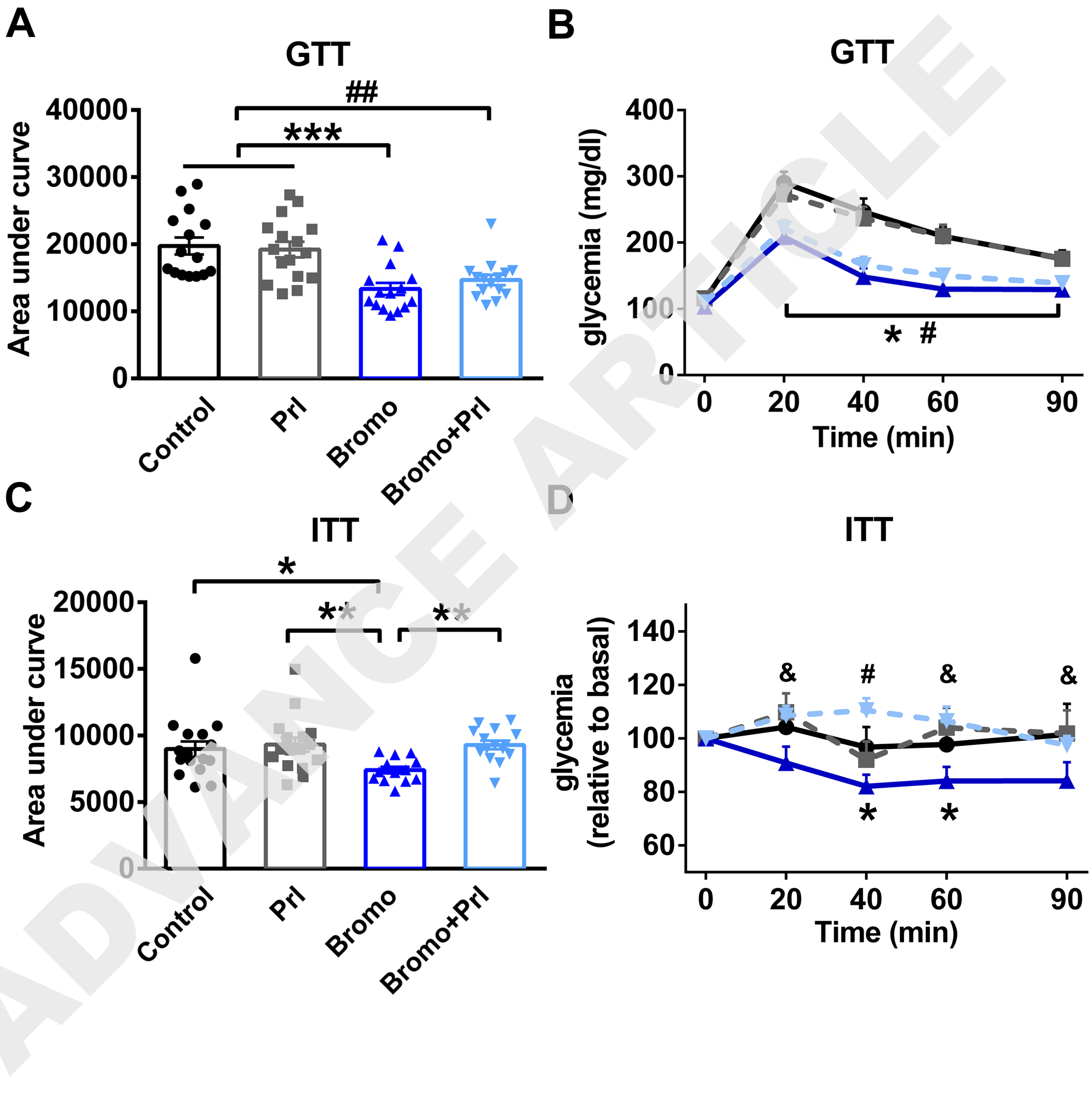
= 3.202, $P = 0.0844$], main effect of Prl [$F_{(1, 28)} = 1.167$, $P = 0.2892$] and interaction [$F_{(1, 28)} = 1.302$, $P = 0.2635$]; $n = 8$) **B-C.** Phospho-AKT-Ser^{473} quantification in the liver (main effect of Bromo [$F_{(3, 27)} = 0.9028$, $P = 0.4526$], main effect of Prl [$F_{(1, 27)} = 2.328$, $P = 0.1387$] and interaction [$F_{(3, 27)} = 1.332$, $P = 0.2847$]; $n = 3\text{-}5$) and in the skeletal muscle (main effect of Bromo [$F_{(3, 27)} = 2.558$, $P = 0.1085$], main effect of Prl [$F_{(1, 11)} = 0.03717$, $P = 0.8506$] and interaction [$F_{(3, 11)} = 0.9253$, $P = 0.4607$]; $n = 3$). Mice were infused with 6 IU insulin/kg (i.p.) or saline and euthanized 15 min later. * $P < 0.05$ vs Bromo group.

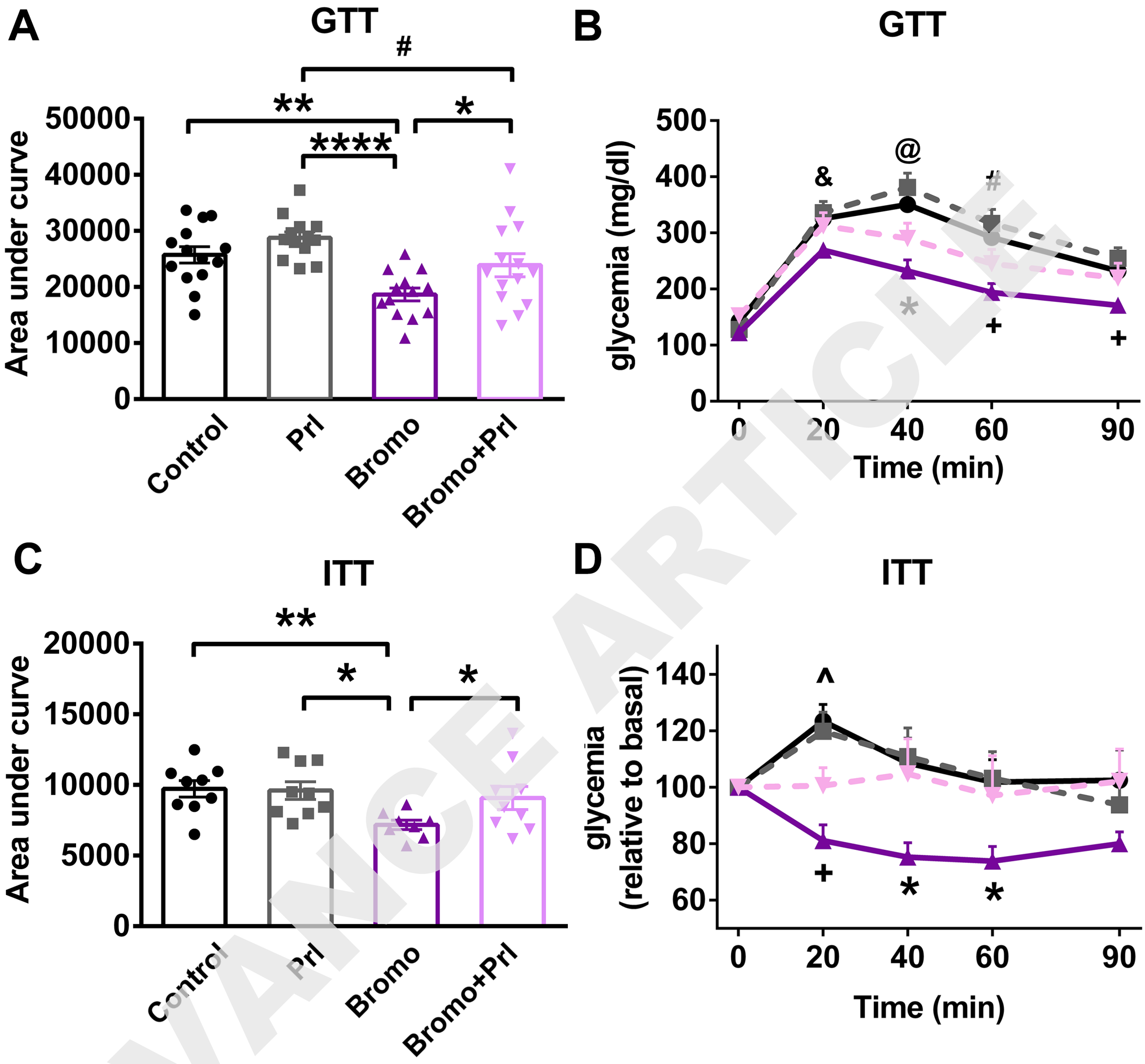
Figure 7. Glucose and insulin tolerance in male *ob/ob* mice that received systemic or central administration of prolactin receptor antagonist (G129R-hPrl). A-B. Phosphorylation of STAT5 (pSTAT5) in the hypothalamus of mice that received intracerebroventricular injection of G129R-hPrl (4 µg in 2 µl) or saline, and 5 minutes later i.p. injection of Prl (2.5 µg/g b.w.) or PBS (main effect of G129R-hPrl [$F_{(1, 8)} = 27.01$, $P = 0.0008$], main effect of Prl [$F_{(1, 8)} = 16.72$, $P = 0.0035$] and interaction [$F_{(1, 8)} = 15.58$, $P = 0.0043$]; $n = 3$/group). Hypothalami were collected 40 minutes after the i.p. injection. **C-F.** Area under the curve of the GTT (C), blood glucose changes during the GTT (D), area under the curve of the ITT (E) and blood glucose changes during the ITT (F) in male *ob/ob* mice that received subcutaneous infusion of G129R-hPrl ($n = 11$) or PBS ($n = 13$). **G-J.** Area under the curve of the GTT (G), blood glucose changes during the GTT (H), area under the curve of the ITT (I) and blood glucose changes during the ITT (J) in male *ob/ob* mice that received intracerebroventricular infusion of G129R-hPrl or PBS ($n = 4$/group). **K-N.** Area under the curve of the GTT (K), blood glucose changes during the GTT (L), area under the curve of the ITT (M) and blood glucose changes during the ITT (N) in female *ob/ob* mice that received intracerebroventricular infusion of G129R-hPrl or PBS ($n = 4$/group). * $P < 0.05$ (unpaired two-tailed t-student test).

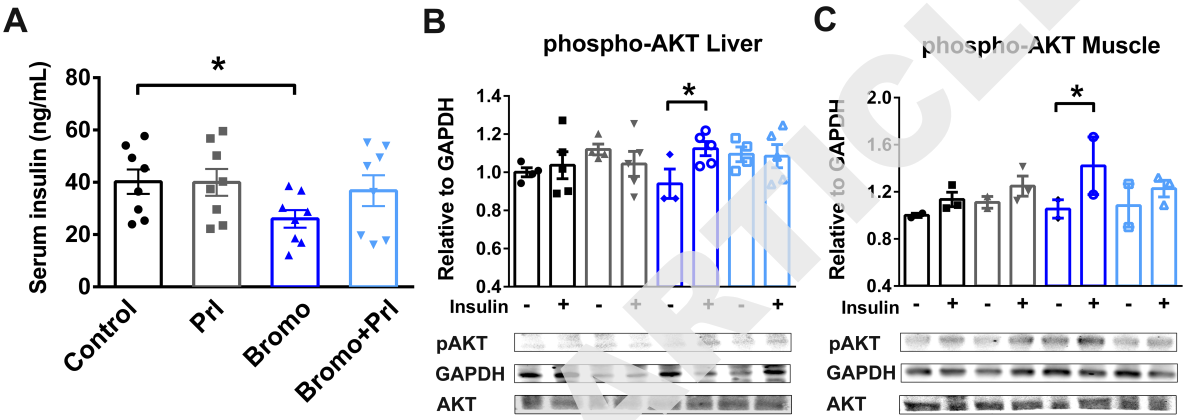

A
Daily i.p. injection
1st day
12th day
14th day
16th day
Daily food intake and body weight measurement
Mini-pump implant
GTT
ITT
Euthanasia
B
Serum prolactin (ng/mL)
0
1
2
3
4
**
**
● Control
■ Prl
▲ Bromo
▼ Bromo+Prl

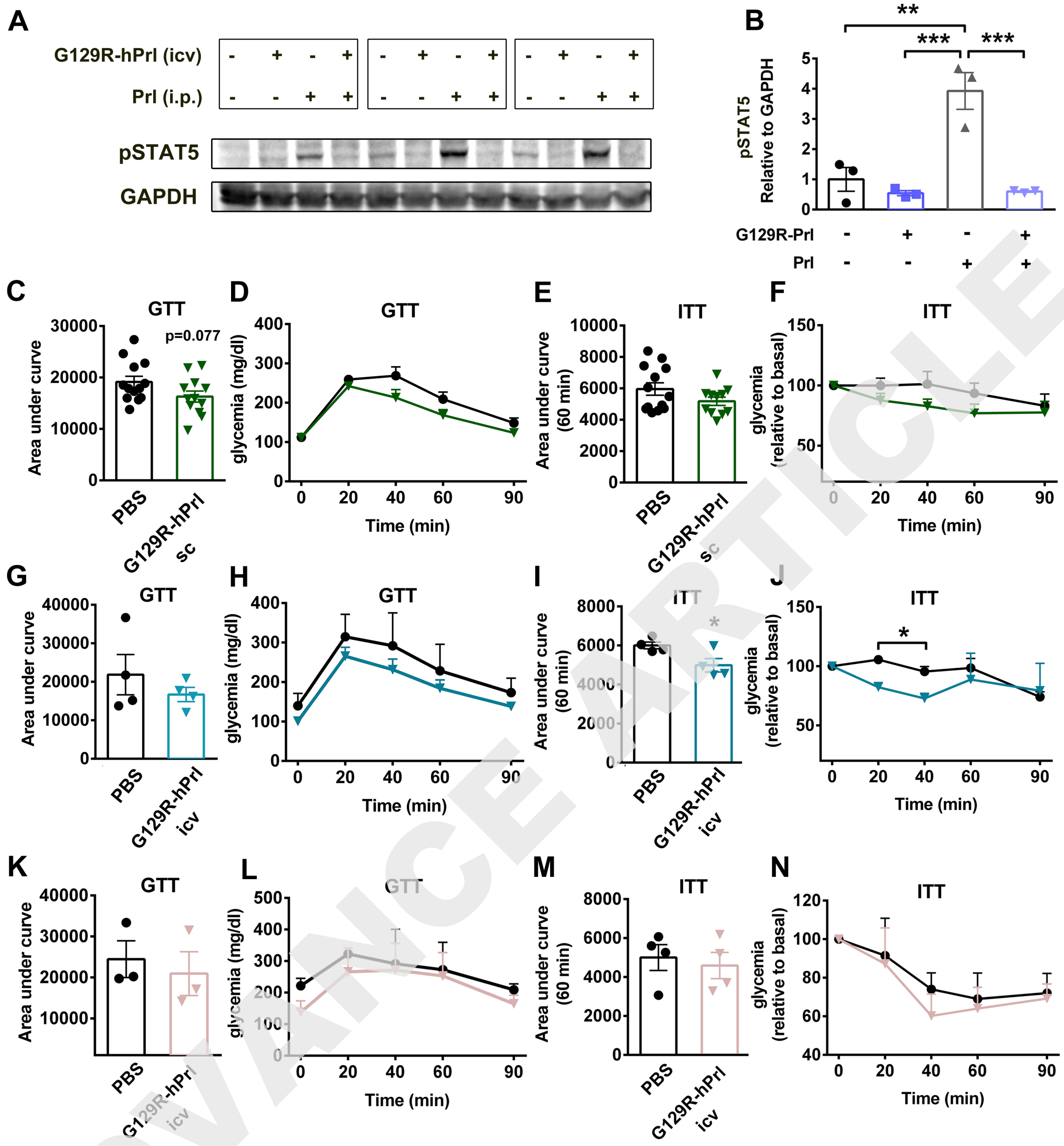

A
Body weight (g)
100
80
60
40
20
0
B
Food intake (g/day)
10
8
6
4
2
0
● Control
■ Prl
▲ Bromo
▼ Bromo+Prl
C
Subcutaneous adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00
D
Perigonadal adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00
E
Retroperitoneal adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00


A
Body weight (g)
100
80
60
40
20
0
B
Food intake (g/day)
10
8
6
4
2
0
● Control
■ Prl
▲ Bromo
▼ Bromo+Prl
C
Subcutaneous adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00
D
Periuterine adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00
E
Retroperitoneal adipose tissue (g/b.w.)
0.05
0.04
0.03
0.02
0.01
0.00


A
GTT
Area under curve
40000
30000
20000
10000
0
##
***
Control
Prl
Bromo
Bromo+Prl
B
GTT
glycemia (mg/dl)
400
300
200
100
0
* #
0
20
40
60
90
Time (min)
C
ITT
Area under curve
20000
15000
10000
5000
0
*
**
**
Control
Prl
Bromo
Bromo+Prl
D
ITT
glycemia
(relative to basal)
140
120
100
80
60
&
#
&
&
*
*
0
20
40
60
90
Time (min)


A
GTT
#
**
*
****
Area under curve
50000
40000
30000
20000
10000
0
Control
Prl
Bromo
Bromo+Prl
B
GTT
glycemia (mg/dl)
500
400
300
200
100
0
&
@
#
*
+
+
0
20
40
60
90
Time (min)
C
ITT
**
*
*
Area under curve
20000
15000
10000
5000
0
Control
Prl
Bromo
Bromo+Prl
D
ITT
glycemia
(relative to basal)
140
120
100
80
60
^
+
*
*
0
20
40
60
90
Time (min)


A
Serum insulin (ng/mL)
80
60
40
20
0
*
Control
Prl
Bromo
Bromo+Prl
B
phospho-AKT Liver
Relative to GAPDH
1.4
1.2
1.0
0.8
0.6
0.4
*
Insulin - + - + - + - +
pAKT
GAPDH
AKT
C
phospho-AKT Muscle
Relative to GAPDH
2.0
1.6
1.2
0.8
0.4
*
Insulin - + - + - + - +
pAKT
GAPDH
AKT


A
G129R-hPrl (icv)
- + - + - + - + - + - +
Prl (i.p.)
- - + + - - + + - - + +
pSTAT5
GAPDH
B
pSTAT5 Relative to GAPDH
0 1 2 3 4 5
**
***
***
G129R-Prl - + - +
Prl - - + +
C
GTT
Area under curve
0 10000 20000 30000
p=0.077
PBS
G129R-hPrl sc
D
GTT
glycemia (mg/dl)
0 100 200 300 400
0 20 40 60 90
Time (min)
E
ITT
Area under curve (60 min)
0 2000 4000 6000 8000 10000
PBS
G129R-hPrl sc
F
ITT
glycemia (relative to basal)
50 100 150
0 20 40 60 90
Time (min)
G
GTT
Area under curve
0 10000 20000 30000 40000
PBS
G129R-hPrl icv
H
GTT
glycemia (mg/dl)
0 100 200 300 400
0 20 40 60 90
Time (min)
I
ITT
Area under curve (60 min)
0 2000 4000 6000 8000
*
PBS
G129R-hPrl icv
J
ITT
glycemia (relative to basal)
50 100 150
*
0 20 40 60 90
Time (min)
K
GTT
Area under curve
10000 20000 30000 40000
PBS
G129R-hPrl icv
L
GTT
glycemia (mg/dl)
0 100 200 300 400 500
0 20 40 60 90
Time (min)
M
ITT
Area under curve (60 min)
0 2000 4000 6000 8000
PBS
G129R-hPrl icv
N
ITT
glycemia (relative to basal)
40 60 80 100 120
0 20 40 60 90
Time (min)