Ghrelin effects on neuropeptides in the rat hypothalamus depend on fatty acid metabolism actions on BSX but not on gender

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ABSTRACT

Purpose(s): The orexigenic effect of ghrelin is mediated by neuropeptide Y (NPY) and agouti-related protein (AgRP) in the hypothalamic arcuate nucleus (ARC). Recent evidence also indicates that ghrelin promotes feeding through a mechanism involving activation of hypothalamic AMP-activated protein kinase (AMPK) and inactivation of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). This results in decreased hypothalamic levels of malonyl-CoA, increased carnitine palmitoyltransferase 1 (CPT1) activity and mitochondrial production of reactive oxygen species (ROS). We evaluated whether these molecular events are part of a unique signaling cascade or represent alternative pathways mediating the orexigenic effect of ghrelin. Moreover, we examined the gender-dependency of these mechanisms, since recent evidence has proposed that ghrelin orexigenic effect is reduced in female rats. ***Procedures:*** We studied in both genders the effect of ghrelin on the expression of AgRP and NPY, as well as their transcription factors: cAMP response-element binding protein (CREB and its phosphorylated form, pCREB), forkhead box O1 (FoxO1 and its phosphorylated form, pFoxO1) and brain-specific-homeobox transcription factor (BSX). In addition, to establish a mechanistic link between ghrelin, fatty acid metabolism and neuropeptides, we evaluated the effect of ghrelin after blockage of hypothalamic fatty acid beta oxidation, by using the CPT1 inhibitor etomoxir. ***Findings:*** Ghrelin-induced changes in the AMPK-CPT1 pathway are associated with increased levels of AgRP and NPY mRNA expression through modulation of BSX, pCREB and FoxO1, as well as decreased expression of endoplasmic reticulum (ER) stress markers in a gender-independent manner. In addition, blockage of hypothalamic fatty acid beta oxidation prevents the ghrelin-promoting action on AgRP and NPY mRNA expression, also in a gender-independent manner. Importantly, this effect is associated with decreased BSX

expression and reduced food intake. ***Conclusions:*** Overall, our data suggest that BSX integrates changes in neuronal metabolic status with ARC-derived neuropeptides in a gender-independent manner.

INTRODUCTION

Ghrelin, a hormone produced in the stomach with orexigenic properties (1-8), has been proposed as a potential anti-obesity therapeutic target (9, 10). This is supported by evidence gathered during the last decade showing that ghrelin is an important signal in both rodents and humans, to prepare for meal initiation (11-13). Furthermore, ghrelin modulates peripheral metabolism, weight gain and adiposity (2, 14-18).

The orexigenic effects of ghrelin are exerted through the growth hormone secretagogue receptor 1a (GHS-R1a) (19), which is expressed in agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons in the arcuate nucleus of the hypothalamus (ARC) (20, 21). In keeping with this orexigenic role, male rats either fed or fasted, showed increased AgRP and NPY expression in the ARC when treated centrally with ghrelin (3, 4, 7, 8). The physiological relevance of both neuropeptides as mediators of ghrelin effects was firmly established by assessing the response to ghrelin in AgRP and NPY KO mice. These experiments revealed that while AgRP KO and NPY KO male mice exhibited the expected hyperphagic response to ghrelin, the double AgRP/NPY KO failed to show any response, indicating the existence of redundancy between these two neuropeptides as mediators of ghrelin orexigenic action (22). The molecular mechanisms linking ghrelin with the induction of *agrp* and *npy* gene expression have recently been identified. It was reported that the hypothalamic homeobox domain transcription factor BSX regulates ghrelin's stimulatory effect on *agrp* and *npy* gene expression in male rats (23, 24). Moreover, it has been recently reported that BSX interacts with two other transcription factors: the forkhead box O1 (FoxO1) and the phosphorylated cAMP response-element binding protein (pCREB) and to modulate the expression of *agrp* and

npy genes (25, 26), respectively. It is unclear whether ghrelin action on those neuropeptides is mediated by alterations in the level of pCREB and FoxO1.

Besides ARC-derived neuropeptides, we and others have recently demonstrated that, in male rats, the orexigenic effect of ghrelin requires the modulation of AMP-activated protein kinase (AMPK), a key upstream regulator of lipid metabolism (5-8). The physiological orexigenic effect of ghrelin requires AMPK-induced inhibition of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in the ventromedial nucleus of the hypothalamus (VMH). The net result of this effect is the decrease in the hypothalamic levels of malonyl-CoA and subsequent increase in carnitine palmitoyltransferase 1 (CPT1) activity, which leads to robust changes in hypothalamic mitochondrial respiration and production of reactive oxygen species (ROS)(7, 8).

Despite these results, the link between ghrelin-induced modulation of neuronal lipid metabolism and the orexigenic changes in neuropeptide expression remains uncertain. In this study, we demonstrate that ghrelin-elicited changes in the AMPK-CPT1 pathway are associated with increased levels of AgRP and NPY mRNA expression in the ARC and that this effect is dependent on the transcription factors BSX, FoxO1 and pCREB. Importantly, we also show that this effect is present in both male and female rats, indicating that this is a robust gender-independent physiological mechanism controlling feeding. These results are particularly relevant since the vast majority of data regarding ghrelin orexigenic effects are limited to males, and not many studies have examined the effect of ghrelin in females. In fact, the current assumption is that ghrelin orexigenic effect is reduced in female rats as a consequence of the action of ovarian estrogens (27). Furthermore, the gender issue is important given that: *a)* circulating ghrelin levels and

the expression of the GHS-R are influenced by gender (28); *b)* there are sex differences in the expression and regulation of pCREB in specific neuronal populations of the central nervous system (29, 30); *c)* the synthesis and release of AgRP and NPY, as well as some of its biological effects, are influenced by gender and gonadal status (31-33); and *d)* finally, differences in lipid metabolism in peripheral tissues are greatly dependent on the gender and gonadal status (34).

MATERIALS AND METHODS

Animals

We used adult male and female Sprague-Dawley (200-250g) rats. Rats were housed in a temperature controlled room, with a 12-h light, 12-h dark cycle (lights from 08:00 to 20:00 h). All experiments and procedures involved in this study were reviewed and approved by the Ethics Committee of the USC, in accordance with European Union normative for the use of experimental animals.

Implantation of intracerebroventricular cannulae

Chronic intracerebroventricular (ICV) cannulae were implanted under ketamine/xylazine anesthesia as previously described (7, 18, 24, 35). The correct location of the cannulae in the lateral ventricle was confirmed by methylene blue staining. Animals were individually caged and allowed to recover for 1 week before experiment. During the postoperative recovery period, the rats were handled regularly under non-stressful conditions.

Intracerebroventricular treatments

For the ghrelin acute experiments, male and female rats received either an ICV administration of vehicle (5 μl of saline) or ghrelin (5 μg = 1.5 nmol) in a total volume of 5 μl (*Bachem,* Bubendorf, Switzerland) (7, 18, 24). For the experiments with etomoxir, rats received an ICV injection of vehicle (5 μl of saline) or etomoxir (10 μg in a total volume of 5 μl; *Sigma*; St Louis, MO, USA) prior ghrelin administration. We used 8 rats per group and the experiments were repeated at least twice; animals were

treated at 09:00 AM (one hour later the light cycle had started), when they were satiated. Rats were killed by cervical dislocation. The hypothalamus (for western blotting and enzymatic activity analysis) or the whole brain (for studies of *in situ* hybridization) were dissected and stored at -80ºC until further processing.

Western blotting

Hypothalamic total protein lysates were subjected to SDS-PAGE, electrotransferred on a PVDF membrane and probed with the following antibodies: ACC, pACCα-Ser^{79}, AMPKα1 and AMPKα2 (*Upstate*; Temecula, CA, USA), C/EP homologous binding protein (CHOP-10), CREB, FoxO1, pCREB-Ser^{129}, CPT1m and CPT1l, phosphorylated eukaryotic translation initiation factor 2 α (peIF2α-Ser^{52}) and pFoxO1-Ser^{256} (*Santa Cruz*; Santa Cruz, CA, USA); CPT1c (*Proteintech;* Chicago, IL, USA); pAMPKα-Thr^{172}, (*Cell Signaling*; Danvers, MA, USA); β-Actin (*Sigma;* St Louis, MO, USA), as previously described (7, 18, 35). We used 8 rats per experimental group. Values are expressed in relation to β-Actin levels.

In situ hybridization

Coronal brain sections (16 μm) were probed with specific antisense oligonucleotides against AgRP, NPY and BSX mRNAs (Table 1). *In situ* hybridizations were performed as previously published (7, 18, 24, 35, 36). We used 8 rats per experimental group. We used between 16 and 20 sections for each animal (4-5 slides with four sections per slide). The mean of these 16-20 values was used as the final densitometry value for each animal.

CPT1 activity assay

The CPT1 activity was measured in the supernatant, using methods described by Bieber *et al.* and Zammit & Newsholm (37, 38), as previously published (7, 18). We used 8 rats per experimental group.

Statistical analysis

Data are expressed as mean±SEM. Statistic significance was determined by t-Student or ANOVA and *post-hoc* two-tailed Bonferroni test. $P<0.05$ was considered significant.

RESULTS

Central administration of ghrelin increases food intake, AgRP and NPY mRNA expression in male and female rats

It is well established that ghrelin administration induces a powerful acute orexigenic effect in male rats (1-8). However, the orexigenic effects of ghrelin in female rats are controversial (27). To determine whether ghrelin administration increases food intake in female rats, we centrally administered the same single dose of ghrelin to fed satiated male and female rats. Our data showed that ICV administration of ghrelin induced a marked increase in feeding in a gender-independent manner (Figures 1A and 1B). Next, we evaluated whether ghrelin orexigenic action was associated with changes in ARC-derived neuropeptides. Our data show that ICV administration of ghrelin increased the mRNA expression of AgRP (Figures 1C and 1E) and NPY (Figures 1D and 1F) in male and female rats.

Central administration of ghrelin increases BSX mRNA expression, FoxO1, pFoxO1 and pCREB protein expression in male and female rats

It was recently reported that the transcription factor BSX interacts with pCREB and FoxO1 and plays an essential role in controlling the expression of *npy* and *agrp* genes, respectively, in the ARC of male rats (23, 24). Our data show that acute ghrelin administration increased the mRNA expression of BSX in the ARC in a similar extent in male and female rats (Figures 2A and 2B). Furthermore, our results showed that acute ghrelin administration increased pCREB, FoxO1 and pFoxO1 hypothalamic protein levels (Figures 2C and 2D) in male and female rats, in parallel to the increased mRNA levels of AgRP, NPY and BSX. Current evidence has shown that in pancreatic β-cells, ghrelin treatment increases both FoxO1 and its phosphorylated (and inactive)

form pFoxO1, which elicits a protective effect against lipotoxicity, as shown by inhibition of endoplasmic reticulum stress (ER-stress) and reduced levels of C/EP homologous binding protein (CHOP-10)(39, 40). In keeping with these observations, our data show that ghrelin decreased the hypothalamic levels of CHOP-10 and the phosphorylated form of its upstream regulator the eukaryotic translation initiation factor 2 α (peIF2α) (Figures 2C and 2D).

Central administration of ghrelin inactivates fatty acid synthesis *de novo* and stimulates CPT1 activity in the hypothalamus of male and female rats

Current data from several groups, including ours, have demonstrated that fatty acid metabolism is a target of ghrelin actions in the hypothalamus, mediating its orexigenic actions in male rats (5-8). However, no data have been reported about the effect of ghrelin on fatty acid metabolism in the hypothalamus of female rats. Our data show that ghrelin administration induced a similar increase in AMPKα (pAMPKα) and ACCα (pACCα) phosphorylation levels, with no changes in the non-phosphorylated isoforms (AMPKα1, AMPKα2 and ACCα, respectively) in the hypothalamus of male and female rats (Figures 3A and 3B). As a result of the inactivation of fatty acid synthesis *de novo* and the subsequent decrease in the levels of malonyl-CoA (data not shown)(7), CPT1 activity was increased in the hypothalamus of both male and female rats (Figure 3C). No changes were detected in the hypothalamic protein levels of CPT1a, CPT1b and CPT1c (Figures 3D and 3E), suggesting that the stimulatory action of ghrelin on CPT1 activity is not related to increased CPT1 expression.

The orexigenic effect of ghrelin is mediated by stimulation of hypothalamic CPT1 activity in male and female rats

Recent data from our group and others have demonstrated that the orexigenic effect of ghrelin is associated with CPT1 activation in the hypothalamus of male rats (7, 8). To determine whether the effect of ghrelin on food intake involved the activation of CPT1 in female rats, we investigated the effect of etomoxir, an inhibitor of CPT1 (7, 8), on ghrelin orexigenic effect. The selected dose of etomoxir (10 μg) induced no anorectic effect *per se* (Figure 4A) but significantly inhibited hypothalamic CPT1 activity (Figure 4B). Our data showed that ICV injection of etomoxir decreased the orexigenic effect of ghrelin in male (Figure 4C) and female (Figure 4D) rats. Etomoxir action blunting ghrelin-induced feeding was longer lasting in female rats (up until 6 hours) than in male rats (up until 4 hours).

Inhibition of hypothalamic CPT1 activity prevents the ghrelin-induced increase in AgRP, NPY and BSX mRNA expression in the hypothalamus of male and female rats

Having shown that inhibition of CPT1 activity by using etomoxir decreased ghrelin's orexigenic effect in both genders, we investigated the effect of this treatment on the mRNA expression levels of AgRP, NPY and BSX in the ARC of male and female rats. Our results showed that administration of etomoxir prevented the ghrelin-induced increase in AgRP (Figures 5A and 5B, 6A and 6B), NPY (Figures 5C and 5D, 6C and 6D) and BSX (Figures 5E and 5F, 6E and 6F) in male (Figure 5) and female (Figure 6) rats. These data demonstrate that ghrelin's effects on AgRP, NPY and their upstream transcription factor BSX are mediated by a CPT1-dependent mechanism. Overall, these

results suggest that BSX integrates ghrelin-induced changes in fatty acid metabolism pathway with neuropeptide expression in a gender-independent manner.

DISCUSSION

In this study we first demonstrate that ghrelin-induced changes in the AMPK-CPT1 pathway elicit increased levels of AgRP and NPY mRNA expression in the ARC, through modulation of the transcription factors BSX, FoxO1 and pCREB in male and female rats. These data first suggest that BSX integrates changes in neuronal metabolic status with neuropeptide networks in the ARC and very importantly that this link is a gender-independent physiological mechanism controlling feeding.

Besides the well-established stimulatory action of ghrelin on AgRP/NPY neurons in the ARC (3, 4, 7, 8, 22), current evidence has demonstrated that ghrelin orexigenic effect is mediated by the selective modulation of hypothalamic fatty acid metabolism (5-8). Although it is clear that the above described pathways are both *bona fide* components of ghrelin signaling, the mechanistic link between them is still unclear. It has been recently reported that ghrelin-induced activation of hypothalamic fatty acid oxidation leads to robust changes in hypothalamic mitochondrial respiration and production of ROS, which are buffered by uncoupling protein 2 (UCP2) (8). This mechanism is critical for ghrelin-induced electric activation of AgRP/NPY neurons, as well for ghrelin-dependent gene transcription events in those cells. Thus, ghrelin-induced upregulation of *agrp, npy, ucp2, cpt1* and nuclear respiratory factor 1 (*nrf1*) gene expression is blunted in UCP2 KO mice (8). Although these results provide an interesting mechanism, linking ghrelin-induced changes in fatty acid metabolism and neuropeptide expression, how alterations in mitochondrial function lead to nuclear transcriptional events remains unresolved. In this study, we show compelling evidence demonstrating that BSX, a recently discovered transcription factor that regulates of AgRP and NPY expression (23, 24), connects ghrelin-promoted activation of hypothalamic fatty acid

beta oxidation with AgRP and NPY expression in the ARC. More importantly, we demonstrate that this association is a physiological gender-independent mechanism modulating feeding. In fact, pharmacological blockage of hypothalamic fatty acid beta oxidation, by using the CPT1 inhibitor etomoxir, blocks the ghrelin-induced effect on BSX and subsequently on AgRP and NPY mRNA expression in the ARC of both male and female rats. Additional work, using loss of function experiment, such as BSX KO mice (23), is necessary to confirm the existence of alternative pathways to BSX linking ghrelin fatty acid metabolism and neuropeptide expression. In fact, considering that BSX KO mice do not totally lack of fasting-induced response in AgRP and NPY mRNA levels (23), the involvement of alternative additional transcription factors cannot be excluded, albeit they role is likely to be of lesser importance.

We also first show that FoxO1 and pCREB, two well-established transcription factors modulating *agrp* and *npy* expression, respectively, (23, 25, 26, 41), are stimulated after central ghrelin administration, also in a gender independent manner. Recent evidence has demonstrated that peripheral signals, such as leptin and insulin, regulate *agrp* and *pomc* (proopiomelanocortin) gene expression through modulation of the balance between FoxO1 (active form) and phosphorylated FoxO1 (pFoxO1, inactive form). Thus, leptin and insulin promote pFoxO1 and prevent its translocation to the nucleus (41), which results in increased expression of *pomc* (42-44) and decreased expression of *agrp* (26). Contrary to leptin and insulin, we show that central ghrelin administration increased both hypothalamic FoxO1 and pFoxO1 in a similar extent. In keeping with previous literature, increased levels of FoxO1 would be the mechanism leading to increased expression of the *agrp* gene by ghrelin (26); however, the physiological relevance of increased pFoxO1 is intriguing. Current evidence has shown that in

pancreatic β-cells, ghrelin treatment increases both FoxO1 and pFoxO1. Increased levels of pFoxO1 after ghrelin treatment elicit a protective effect against lipotoxicity, as shown by inhibition of ER-stress and reduced levels of CHOP-10 (39, 40). Similar results showing ghrelin's protective effects against lipotoxicity-induced apoptosis and ER-stress has been also reported in an ischemic heart model (45). Consistent with these observations, our data show that ghrelin reduces the hypothalamic levels of CHOP-10 and the phosphorylated form of its upstream regulator peIF2α. Considering that *1)* hypothalamic ER-stress has recently been proposed as a pathophysiological mechanism under leptin resistance and obesity (46-49) and that *2)* central ghrelin administration increases hypothalamic ROS (8), which are well-recognized ER-stress inducers (49-53), it is tempting to speculate that ghrelin-induced increase in hypothalamic pFoxO1 might be a compensatory mechanism leading to protection against ER-stress, a hypothesis that will require further investigation.

Another particular question which remains unresolved is whether BSX, pCREB and FoxO1 mediate just ghrelin's effect or its role is extensible to other peripheral hormonal signals modulating hypothalamic fatty acid metabolism, such as leptin, insulin, glucagon-like peptide (GLP-1), adiponectin (ADPN), resistin (RSTN), glucocorticoids, thyroid hormones and endocannabinoids (54-56). In this sense, recent evidence from our group has demonstrated that BSX integrates leptin signaling in the ARC and may play a major role in the pathogenesis of leptin resistance (24). Considering that, similarly to ghrelin, leptin effects on feeding are mediated by the modulation of hypothalamic lipid metabolism (5, 57, 58), it is tempting to speculate that BSX may also be a mediator of those effects. In this sense, it has been currently published that acute central administration of palmitate induces leptin resistance in the central nervous

system (59). Further work will be required to address the possible implication of BSX in fatty-acid induced leptin resistance.

Ovarian steroids play a major role in the regulation of feeding and energy balance by modulating neuropeptide expression, synaptic plasticity and leptin effect on hypothalamic networks (27, 32, 33, 60-64). However, one particular black box about the effects of ghrelin on energy balance is that the vast majority of data have been generated by using male rats and mice, but not many studies have examined the effect of ghrelin in females (27). In fact, the current assumption is that ghrelin orexigenic effect is reduced in female rats as a consequence of the action of ovarian estrogens. In this sense, *Clegg and colleagues* have elegantly examined the effect of ghrelin in female rats and mice (27). They reported that ovariectomized (OVX) female rats (and also male rats) were significantly more sensitive than intact female rats to the orexigenic effects of both centrally and systemically administered ghrelin and that this effect is attenuated by estradiol administration (27). Our findings indicate that the whole molecular network under ghrelin orexigenic actions: inhibition of fatty acid synthesis, activation of fatty acid oxidation and increased pCREB, FoxO1, BSX, AgRP and NPY expression, is common in both males and females. These discrepancies are likely due to the different rat strain (Long Evans *vs*. Sprague-Dawley, as well as their physical parameters), experimental models: OVX (total lack of circulating estrogens) *vs.* intact female rats and time of the injection (1 hour after lights on *vs.* 6 hours before lights off). Finally and very importantly, the eating-stimulatory effect of ghrelin varies across different phases of the ovarian cycle in intact rats. *Cleeg's* and colleagues reported that central ghrelin had no reliable overall effect when cycle day was not taken into account; however, when cycle day was considered, central ghrelin increased eating during diestrus 1 and

diestus 2 but not during proestrus or estrus (27). In any case, the lack of differences that we report agrees with data obtained from ghrelin and GHS-R KO mice where a similar resistance to high fat diet (HFD)-induced obesity was found in both genders (15, 16). Moreover, these data indicate that the physiological response of hypothalamic fatty acid metabolism is more conserved than in peripheral tissues, where gender and gonadal status markedly modulates lipid metabolism (34). Altogether, this evidence demonstrates that ghrelin elicit feeding in a gender-independent manner, through a physiological mechanism involving the modulation of hypothalamic fatty acid metabolism, FoxO1, pCREB, BSX and neuropeptide expression in the ARC.

In summary, here we show that the orexigenic action of ghrelin is associated with the specific FoxO1, pCREB and BSX-mediated integration of hypothalamic fatty acid metabolism with AgRP and NPY expression in the ARC. Our data also confirm FoxO1, pCREB and BSX as gender-independent mediators of food intake of relevance for the understanding and treatment of obesity. Finally, our preliminary data showing that ghrelin influences the expression of two hypothalamic ER-stress markers, such as CHOP-10 and peIF2α merits further investigation in order to assess its biological relevance.

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REFERENCES

1. Kojima, M., Hosoda, H., Date, Y., Nakazato, M., Matsuo, H., and Kangawa, K. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**, 656-660
2. Tschop, M., Smiley, D. L., and Heiman, M. L. (2000) Ghrelin induces adiposity in rodents. *Nature* **407**, 908-913
3. Nakazato, M., Murakami, N., Date, Y., Kojima, M., Matsuo, H., Kangawa, K., and Matsukura, S. (2001) A role for ghrelin in the central regulation of feeding. *Nature* **409**, 194-198
4. Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Sugihara, H., and Wakabayashi, I. (2001) Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes* **50**, 2438-2443
5. Andersson, U., Filipsson, K., Abbott, C. R., Woods, A., Smith, K., Bloom, S. R., Carling, D., and Small, C. J. (2004) AMP-activated protein kinase plays a role in the control of food intake. *J Biol. Chem.* **279**, 12005-12008
6. Kola, B., Farkas, I., Christ-Crain, M., Wittmann, G., Lolli, F., Amin, F., Harvey-White, J., Liposits, Z., Kunos, G., Grossman, A. B., Fekete, C., and Korbonits, M. (2008) The orexigenic effect of ghrelin is mediated through central activation of the endogenous cannabinoid system. *PLoS. ONE.* **3**, e1797
7. López, M., Lage, R., Saha, A. K., Pérez-Tilve, D., Vàzquez, M. J., Varela, L., Sangiao-Alvarellos, S., Tovar, S., Raghay, K., Rodríguez-Cuenca, S., Deoliveira, R. M., Castañeda, T., Datta, R., Dong, J. Z., Culler, M., Sleeman, M. W., Álvarez, C. V., Gallego, R., Lelliott, C. J., Carling, D., Tschop, M. H., Diéguez, C., and Vidal-Puig, A. (2008) Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin. *Cell Metab* **7**, 389-399
8. Andrews, Z. B., Liu, Z. W., Walllingford, N., Erion, D. M., Borok, E., Friedman, J. M., Tschop, M. H., Shanabrough, M., Cline, G., Shulman, G. I., Coppola, A., Gao, X. B., Horvath, T. L., and Diano, S. (2008) UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals. *Nature* **454**, 846-851
9. Foster-Schubert, K. E., and Cummings, D. E. (2006) Emerging therapeutic strategies for obesity. *Endocr. Rev.* **27**, 779-793
10. Zorrilla, E. P., Iwasaki, S., Moss, J. A., Chang, J., Otsuji, J., Inoue, K., Meijler, M. M., and Janda, K. D. (2006) Vaccination against weight gain. *Proc. Natl. Acad. Sci. U. S. A* **103**, 13226-13231
11. Wren, A. M., Seal, L. J., Cohen, M. A., Brynes, A. E., Frost, G. S., Murphy, K. G., Dhillo, W. S., Ghatei, M. A., and Bloom, S. R. (2001) Ghrelin Enhances Appetite and Increases Food Intake in Humans. *J. Clin. Endocrinol. Metab* **86**, 5992
12. Cummings, D. E., Purnell, J. Q., Frayo, R. S., Schmidova, K., Wisse, B. E., and Weigle, D. S. (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50**, 1714-1719
13. Kirchner, H., Gutierrez, J. A., Solenberg, P. J., Pfluger, P. T., Czyzyk, T. A., Willency, J. A., Schurmann, A., Joost, H. G., Jandacek, R. J., Hale, J. E., Heiman, M. L., and Tschop, M. H. (2009) GOAT links dietary lipids with the endocrine control of energy balance. *Nat. Med.* **15**, 741-745
14. Tschop, M., Weyer, C., Tataranni, P. A., Devanarayan, V., Ravussin, E., and Heiman, M. L. (2001) Circulating ghrelin levels are decreased in human obesity. *Diabetes* **50**, 707-709
15. Wortley, K. E., Del Rincon, J. P., Murray, J. D., Garcia, K., Iida, K., Thorner, M. O., and Sleeman, M. W. (2005) Absence of ghrelin protects against early-onset obesity. *J. Clin. Invest* **115**, 3573-3578
16. Zigman, J. M., Nakano, Y., Coppari, R., Balthasar, N., Marcus, J. N., Lee, C. E., Jones, J. E., Deysher, A. E., Waxman, A. R., White, R. D., Williams, T. D., Lachey, J. L., Seeley, R. J., Lowell, B. B., and Elmquist, J. K. (2005) Mice lacking ghrelin receptors resist the development of diet-induced obesity. *J. Clin. Invest* **115**, 3564-3572
17. Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P., Nogueiras, R., Perez-Tilve, D., Pfluger, P., Castaneda, T. R., Muzzin, P., Schurmann, A., Szanto, I., Tschop, M. H., and Rohner-Jeanrenaud, F. (2006) Ghrelin action in the brain controls adipocyte metabolism. *J. Clin. Invest* **116**, 1983-1993
18. Sangiao-Alvarellos, S., Vazquez, M. J., Varela, L., Nogueiras, R., Saha, A. K., Cordido, F., Lopez, M., and Dieguez, C. (2009) Central ghrelin regulates peripheral lipid metabolism in a growth hormone-independent fashion. *Endocrinology* **150**, 4562-4574
19. Sun, Y., Wang, P., Zheng, H., and Smith, R. G. (2004) Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc. Natl. Acad. Sci. U. S. A* **101**, 4679-4684

20. Tannenbaum, G. S., Lapointe, M., Beaudet, A., and Howard, A. D. (1998) Expression of growth hormone secretagogue-receptors by growth hormone- releasing hormone neurons in the mediobasal hypothalamus. *Endocrinology* **139**, 4420-4423
21. Zigman, J. M., Jones, J. E., Lee, C. E., Saper, C. B., and Elmquist, J. K. (2006) Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J. Comp Neurol.* **494**, 528-548
22. Chen, H. Y., Trumbauer, M. E., Chen, A. S., Weingarth, D. T., Adams, J. R., Frazier, E. G., Shen, Z., Marsh, D. J., Feighner, S. D., Guan, X. M., Ye, Z., Nargund, R. P., Smith, R. G., Van Der Ploeg, L. H., Howard, A. D., MacNeil, D. J., and Qian, S. (2004) Orexigenic Action of Peripheral Ghrelin is Mediated by Neuropeptide Y (NPY) and Agouti-Related Protein (AgRP). *Endocrinology* **145**, 2607-2612
23. Sakkou, M., Wiedmer, P., Anlag, K., Hamm, A., Seuntjens, E., Ettwiller, L., Tschop, M. H., and Treier, M. (2007) A role for brain-specific homeobox factor bsx in the control of hyperphagia and locomotory behavior. *Cell Metab* **5**, 450-463
24. Nogueiras, R., López, M., Lage, R., Pérez-Tilve, D., Pfluger, P., Mendieta-Zeron, H., Sakkou, M., Wiedmer, P., Benoit, S., Datta, R., Dong, J. Z., Culler, M., Sleeman, M., Vidal-Puig, A., Horvath, T., Treier, M., Diéguez, C., and Tschop, M. H. (2008) Bsx, a Novel Hypothalamic Factor linking Feeding with Locomotor Activity, is Regulated by Energy Availability. *Endocrinology* **149**, 3009-3015
25. Shimizu-Albergine, M., Ippolito, D. L., and Beavo, J. A. (2001) Downregulation of fasting-induced cAMP response element-mediated gene induction by leptin in neuropeptide Y neurons of the arcuate nucleus. *J. Neurosci.* **21**, 1238-1246
26. Kitamura, T., Feng, Y., Ido, K. Y., Chua, S. C., Xu, A. W., Barsh, G. S., Rossetti, L., and Accili, D. (2006) Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. *Nat. Med.* **12**, 534-540
27. Clegg, D. J., Brown, L. M., Zigman, J. M., Kemp, C. J., Strader, A. D., Benoit, S. C., Woods, S. C., Mangiaracina, M., and Geary, N. (2007) Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. *Diabetes* **56**, 1051-1058
28. Kamegai, J., Wakabayashi, I., Kineman, R. D., and Frohman, L. A. (1999) Growth hormone-releasing hormone receptor (GHRH-R) and growth hormone secretagogue receptor (GHS-R) mRNA levels during postnatal development in male and female rats. *J. Neuroendocrinol.* **11**, 299-306
29. Mogi, K., Funabashi, T., Mitsushima, D., Hagiwara, H., and Kimura, F. (2005) Sex difference in the response of melanin-concentrating hormone neurons in the lateral hypothalamic area to glucose, as revealed by the expression of phosphorylated cyclic adenosine 3',5'-monophosphate response element-binding protein. *Endocrinology* **146**, 3325-3333
30. Nazarian, A., Sun, W. L., Zhou, L., Kemen, L. M., Jenab, S., and Quinones-Jenab, V. (2009) Sex differences in basal and cocaine-induced alterations in PKA and CREB proteins in the nucleus accumbens. *Psychopharmacology (Berl)* **203**, 641-650
31. Archer, Z. A., Findlay, P. A., McMillen, S. R., Rhind, S. M., and Adam, C. L. (2004) Effects of nutritional status and gonadal steroids on expression of appetite-regulatory genes in the hypothalamic arcuate nucleus of sheep. *J Endocrinol.* **182**, 409-419
32. Santollo, J., and Eckel, L. A. (2008) Estradiol decreases the orexigenic effect of neuropeptide Y, but not agouti-related protein, in ovariectomized rats. *Behav. Brain Res.* **191**, 173-177
33. Olofsson, L. E., Pierce, A. A., and Xu, A. W. (2009) Functional requirement of AgRP and NPY neurons in ovarian cycle-dependent regulation of food intake. *Proc. Natl. Acad. Sci. U. S. A* **106**, 15932-15937
34. Blaak, E. (2001) Gender differences in fat metabolism. *Curr. Opin. Clin. Nutr. Metab Care* **4**, 499-502
35. López, M., Lelliott, C. J., Tovar, S., Kimber, W., Gallego, R., Virtue, S., Blount, M., Vázquez, M. J., Finer, N., Powles, T., O'Rahilly, S., Saha, A. K., Diéguez, C., and Vidal-Puig, A. J. (2006) Tamoxifen-induced anorexia is associated with fatty acid synthase inhibition in the ventromedial nucleus of the hypothalamus and accumulation of malonyl-CoA. *Diabetes* **55**, 1327-1336
36. Chakravarthy, M. V., Zhu, Y., López, M., Yin, L., Wozniak, D. W., Coleman, T., Hu, Z., Wolfgang, M., Vidal-Puig, A., Lane, M. D., and Semenkovich, C. F. (2007) Brain fatty acid synthase activates PPAR-alpha to maintain energy homeostasis. *J. Clin. Invest* **117**, 2539-2552
37. Bieber, L. L., Abraham, T., and Helmrath, T. (1972) A rapid spectrophotometric assay for carnitine palmitoyltransferase. *Anal. Biochem.* **50**, 509-518
38. Zammit, V. A., and Newsholme, E. A. (1979) Activities of enzymes of fat and ketone-body metabolism and effects of starvation on blood concentrations of glucose and fat fuels in teleost and elasmobranch fish. *Biochem. J.* **184**, 313-322

39. Wang, W., Liu, Y., Chen, Y., Cao, C., Xiang, Y., Zhang, D., Han, L., Zhao, H., and Liu, G. (2009) Inhibition of Foxo1 mediates protective effects of ghrelin against lipotoxicity in MIN6 pancreatic beta-cells. *Peptides* (in press) doi:10.1016/j.peptides.2009.11.011
40. Wang, W., Zhang, D., Zhao, H., Chen, Y., Liu, Y., Cao, C., Han, L., and Liu, G. (2010) Ghrelin inhibits cell apoptosis induced by lipotoxicity in pancreatic beta-cell line. *Regul. Pept.* (in press) doi:10.1016/j.regpep.2009.12.017
41. Kim, M. S., Pak, Y. K., Jang, P. G., Namkoong, C., Choi, Y. S., Won, J. C., Kim, K. S., Kim, S. W., Kim, H. S., Park, J. Y., Kim, Y. B., and Lee, K. U. (2006) Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. *Nat. Neurosci.* **9**, 901-906
42. Plum, L., Lin, H. V., Dutia, R., Tanaka, J., Aizawa, K. S., Matsumoto, M., Kim, A. J., Cawley, N. X., Paik, J. H., Loh, Y. P., DePinho, R. A., Wardlaw, S. L., and Accili, D. (2009) The obesity susceptibility gene Cpe links FoxO1 signaling in hypothalamic pro-opiomelanocortin neurons with regulation of food intake. *Nat. Med.* **15**, 1195-1201
43. Belgardt, B. F., Husch, A., Rother, E., Ernst, M. B., Wunderlich, F. T., Hampel, B., Klockener, T., Alessi, D., Kloppenburg, P., and Bruning, J. C. (2008) PDK1 deficiency in POMC-expressing cells reveals FOXO1-dependent and -independent pathways in control of energy homeostasis and stress response. *Cell Metab* **7**, 291-301
44. Ernst, M. B., Wunderlich, C. M., Hess, S., Paehler, M., Mesaros, A., Koralov, S. B., Kleinridders, A., Husch, A., Munzberg, H., Hampel, B., Alber, J., Kloppenburg, P., Bruning, J. C., and Wunderlich, F. T. (2009) Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity. *J. Neurosci.* **29**, 11582-11593
45. Zhang, G. G., Teng, X., Liu, Y., Cai, Y., Zhou, Y. B., Duan, X. H., Song, J. Q., Shi, Y., Tang, C. S., Yin, X. H., and Qi, Y. F. (2009) Inhibition of endoplasm reticulum stress by ghrelin protects against ischemia/reperfusion injury in rat heart. *Peptides* **30**, 1109-1116
46. Zhang, X., Zhang, G., Zhang, H., Karin, M., Bai, H., and Cai, D. (2008) Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity. *Cell* **135**, 61-73
47. Hosoi, T., Sasaki, M., Miyahara, T., Hashimoto, C., Matsuo, S., Yoshii, M., and Ozawa, K. (2008) Endoplasmic reticulum stress induces leptin resistance. *Mol. Pharmacol.* **74**, 1610-1619
48. Ozcan, L., Ergin, A. S., Lu, A., Chung, J., Sarkar, S., Nie, D., Myers, M. G., Jr., and Ozcan, U. (2009) Endoplasmic reticulum stress plays a central role in development of leptin resistance. *Cell Metab* **9**, 35-51
49. Martínez de Morentin, P. B., Varela, L., Ferno, J., Nogueiras, R., Diéguez, C., and López, M. (2009) Hypothalamic lipotoxicity and the metabolic syndrome. *Biochim. Biophys. Acta* (in press) doi:10.1016/j.bbalip.2009.09.016
50. Tagawa, Y., Hiramatsu, N., Kasai, A., Hayakawa, K., Okamura, M., Yao, J., and Kitamura, M. (2008) Induction of apoptosis by cigarette smoke via ROS-dependent endoplasmic reticulum stress and CCAAT/enhancer-binding protein-homologous protein (CHOP). *Free Radic. Biol. Med.* **45**, 50-59
51. Santos, C. X., Tanaka, L. Y., Wosniak, J., and Laurindo, F. R. (2009) Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid. Redox. Signal.* **11**, 2409-2427
52. Lee, G. H., Kim, H. K., Chae, S. W., Kim, D. S., Ha, K. C., Cuddy, M., Kress, C., Reed, J. C., Kim, H. R., and Chae, H. J. (2007) Bax inhibitor-1 regulates endoplasmic reticulum stress-associated reactive oxygen species and heme oxygenase-1 expression. *J. Biol. Chem.* **282**, 21618-21628
53. Medina-Gomez, G., Yetukuri, L., Velagapudi, V., Campbell, M., Blount, M., Jimenez-Linan, M., Ros, M., Oresic, M., and Vidal-Puig, A. (2009) Adaptation and failure of pancreatic beta cells in murine models with different degrees of metabolic syndrome. *Dis. Model. Mech.* **2**, 582-592
54. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* **1**, 15-25
55. Kola, B., Boscaro, M., Rutter, G. A., Grossman, A. B., and Korbonits, M. (2006) Expanding role of AMPK in endocrinology. *Trends Endocrinol. Metab* **17**, 205-215
56. Lage, R., Diéguez, C., Vidal-Puig, A., and López, M. (2008) AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends Mol. Med.* **14**, 539-549
57. Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y. B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M. J., Stuck, B. J., and Kahn, B. B. (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428**, 569-574

58. Gao, S., Kinzig, K. P., Aja, S., Scott, K. A., Keung, W., Kelly, S., Strynadka, K., Chohnan, S., Smith, W. W., Tamashiro, K. L., Ladenheim, E. E., Ronnett, G. V., Tu, Y., Birnbaum, M. J., Lopaschuk, G. D., and Moran, T. H. (2007) Leptin activates hypothalamic acetyl-CoA carboxylase to inhibit food intake. *Proc. Natl. Acad. Sci. U. S. A* **104**, 17358-17363
59. Kleinridders, A., Schenten, D., Konner, A. C., Belgardt, B. F., Mauer, J., Okamura, T., Wunderlich, F. T., Medzhitov, R., and Bruning, J. C. (2009) MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity. *Cell Metab* **10**, 249-259
60. Shimizu, H., Ohtani, K., Kato, Y., Tanaka, Y., and Mori, M. (1996) Withdrawal of [corrected] estrogen increases hypothalamic neuropeptide Y (NPY) mRNA expression in ovariectomized obese rat. *Neurosci. Lett.* **204**, 81-84
61. Mystkowski, P., Seeley, R. J., Hahn, T. M., Baskin, D. G., Havel, P. J., Matsumoto, A. M., Wilkinson, C. W., Peacock-Kinzig, K., Blake, K. A., and Schwartz, M. W. (2000) Hypothalamic melanin-concentrating hormone and estrogen-induced weight loss. *J. Neurosci.* **20**, 8637-8642
62. Fodor, M., and Delemarre-van de Waal HA (2001) Are POMC neurons targets for sex steroids in the arcuate nucleus of the rat? *Neuroreport* **12**, 3989-3991
63. Gao, Q., Mezei, G., Nie, Y., Rao, Y., Choi, C. S., Bechmann, I., Leranth, C., Toran-Allerand, D., Priest, C. A., Roberts, J. L., Gao, X. B., Mobbs, C., Shulman, G. I., Diano, S., and Horvath, T. L. (2007) Anorectic estrogen mimics leptin's effect on the rewiring of melanocortin cells and Stat3 signaling in obese animals. *Nat. Med.* **13**, 89-94
64. Gao, Q., and Horvath, T. L. (2008) Cross-talk between estrogen and leptin signaling in the hypothalamus. *Am. J. Physiol Endocrinol. Metab* **294**, E817-E826

FIGURE LEGENDS

FIGURE 1

Central administration of ghrelin increases food intake, AgRP and NPY mRNA expression in male and female rats. Cumulative food intake (A and B; white rhombuses: vehicle; black circles = ghrelin), AgRP (C and E) and NPY (D and F) mRNA in the arcuate nucleus of the hypothalamus (ARC) of male and female rats ICV-treated with vehicle (white bars) or ghrelin (black bars). ***: P<0.001 *vs*. vehicle. 3V: third ventricle.

FIGURE 2

Central administration of ghrelin increases BSX mRNA expression, FoxO1, pFoxO1 and pCREB protein expression in male and female rats. BSX mRNA levels in the arcuate nucleus of the hypothalamus (ARC) (A and B) and pCREB, CREB, pFoxO1, FoxO1, CHOP-10 and peIF2α hypothalamic protein levels (C and D) of male and female rats ICV-treated with vehicle (white bars) or ghrelin (black bars). *: P<0.05 *vs*. vehicle; **: P<0.01 *vs*. vehicle; ***: P<0.001 *vs*. vehicle. 3V: third ventricle.

FIGURE 3

Central administration of ghrelin inactivates fatty acid synthesis *de novo* and stimulates CPT1 activity in the hypothalamus of male and female rats. pAMPKα, AMPKα1, AMPKα2, pACCα and ACCα (A and B), hypothalamic CPT1 activity (C) and hypothalamic CPT1a, CPT1b and CPT1c protein levels (D and E) of male and female rats ICV-treated with vehicle (white bars) or ghrelin (black bars). *: P<0.05 *vs*. vehicle; **: P<0.01 *vs*. vehicle; ***: P<0.001 *vs*. vehicle.

FIGURE 4

The orexigenic effect of ghrelin is mediated by stimulation of hypothalamic CPT1 activity in male and female rats. Cumulative food intake (A) and hypothalamic CPT1 activity (B) after ICV treatment with vehicle (white bars) or the CPT1 inhibitor etomoxir (grey and black bars). Cumulative food intake after ICV administration of vehicle or etomoxir prior ICV administration of vehicle or ghrelin in male (C) and female (D) rats; white rhombuses = vehicle/vehicle; black squares = vehicle/etomoxir; black circles = ghrelin/vehicle; white triangles = ghrelin/etomoxir. *: P<0.05 *vs.* vehicle or vehicle/vehicle; **: P<0.01 *vs*. vehicle/vehicle; ***: P<0.001 *vs*. vehicle/vehicle; #: P<0.05 ghrelin/vehicle *vs*. ghrelin/etomoxir; ##: P<0.01 ghrelin/vehicle *vs.* ghrelin/etomoxir; ###: P<0.001 ghrelin/vehicle *vs.* ghrelin/etomoxir.

FIGURE 5

Inhibition of hypothalamic CPT1 activity prevents the ghrelin-induced increase in AgRP, NPY and BSX mRNA expression in the hypothalamus of male rats. AgRP (A and B), NPY (C and D) and BSX (E and F) mRNA levels in the arcuate nucleus of the hypothalamus (ARC) of male rats after ICV administration of vehicle (white bars) or the CPT1 inhibitor etomoxir (black bars) prior ICV administration of vehicle or ghrelin. **: P<0.01 *vs.* vehicle/vehicle; ***: P<0.001 *vs.* vehicle/vehicle; #: P<0.05 ghrelin/vehicle *vs.* ghrelin/etomoxir; ##: P<0.01 ghrelin/vehicle *vs.* ghrelin/etomoxir; ###: P<0.001 ghrelin/vehicle *vs*. ghrelin/etomoxir.

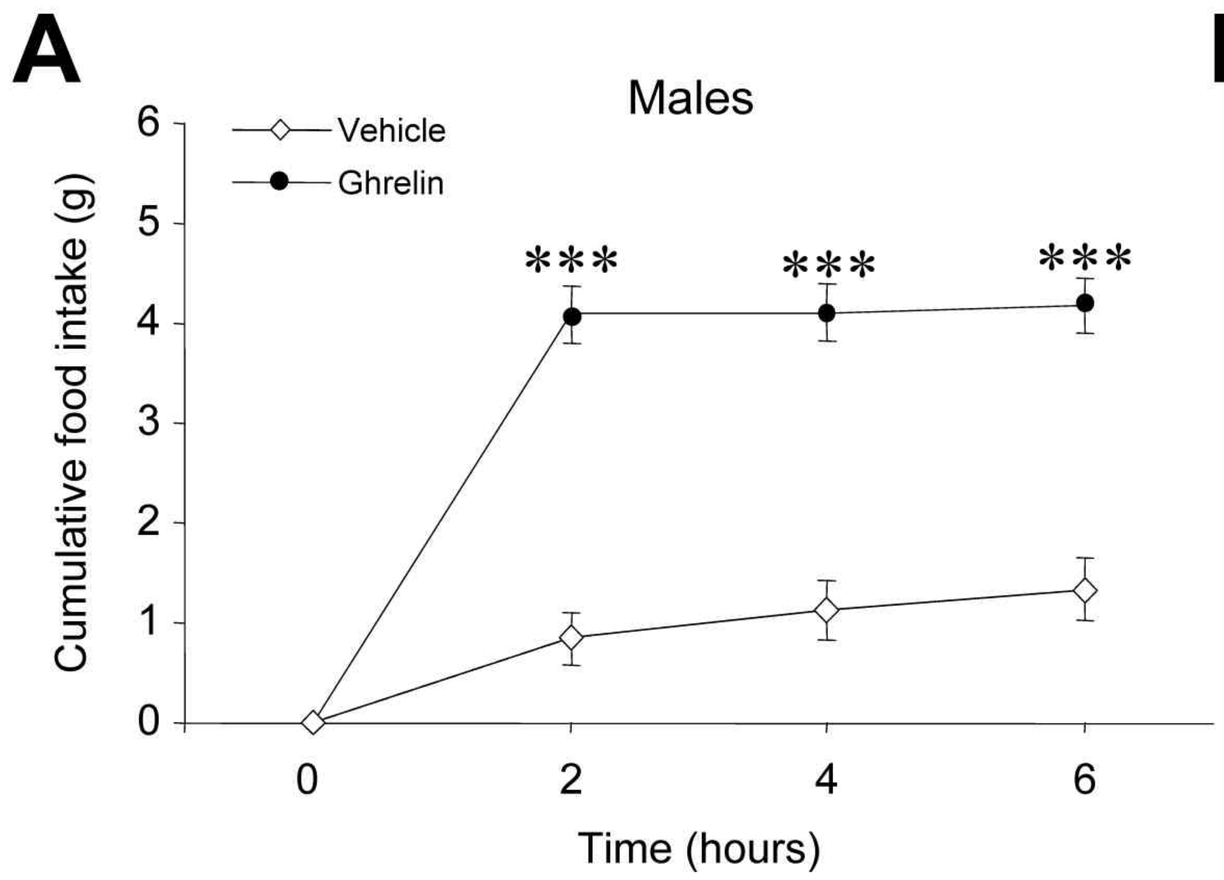
FIGURE 6

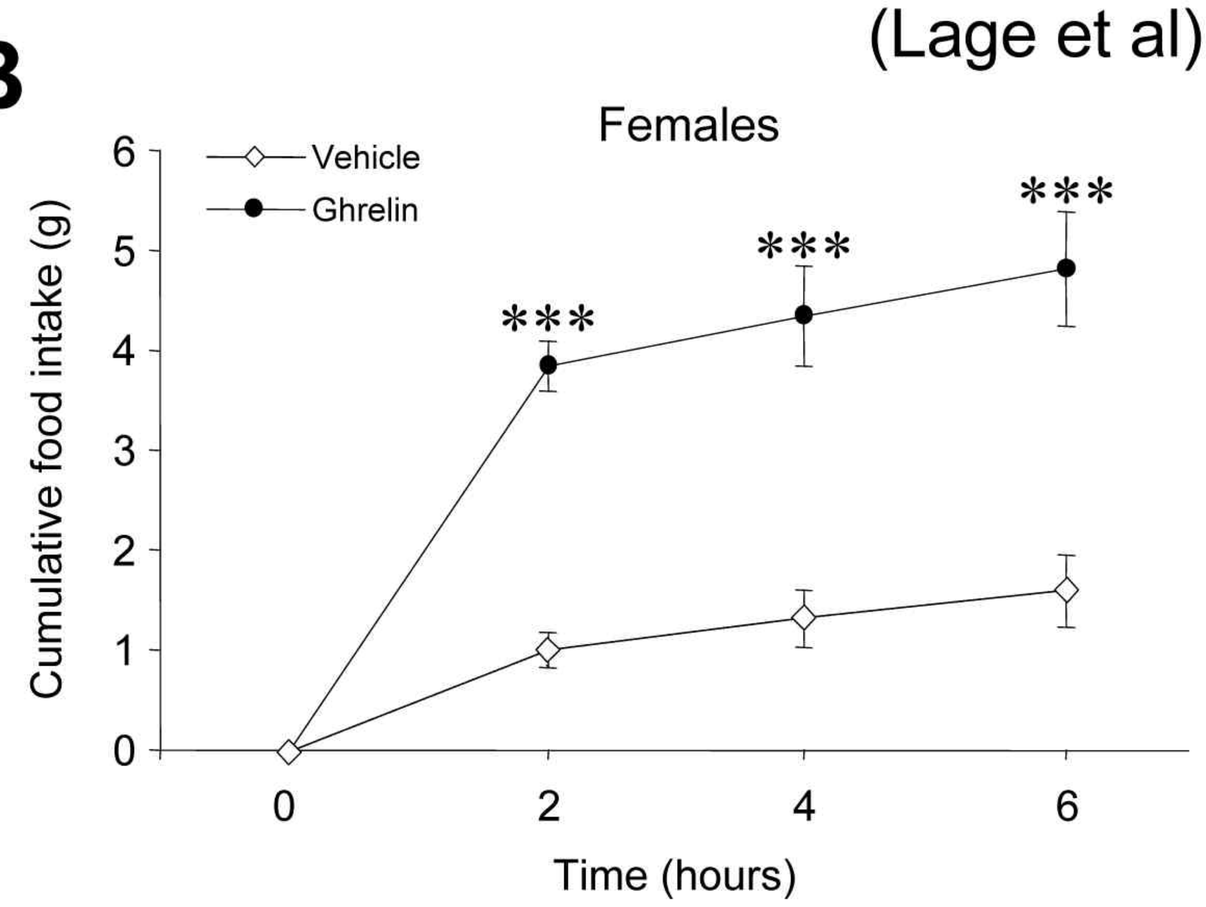
Inhibition of hypothalamic CPT1 activity prevents the ghrelin-induced increase in AgRP, NPY and BSX mRNA expression in the hypothalamus of female rats. AgRP (A and B), NPY (C and D) and BSX (E and F) mRNA levels in the arcuate nucleus of the hypothalamus (ARC) of female rats after ICV administration of vehicle (white bars) or the CPT1 inhibitor etomoxir (black bars) prior ICV administration of vehicle or ghrelin. **: P<0.01 *vs.* vehicle/vehicle; ***: P<0.001 *vs.* vehicle/vehicle; #: P<0.05 ghrelin/vehicle *vs.* ghrelin/etomoxir; ##: P<0.01 ghrelin/vehicle *vs.* ghrelin/etomoxir.

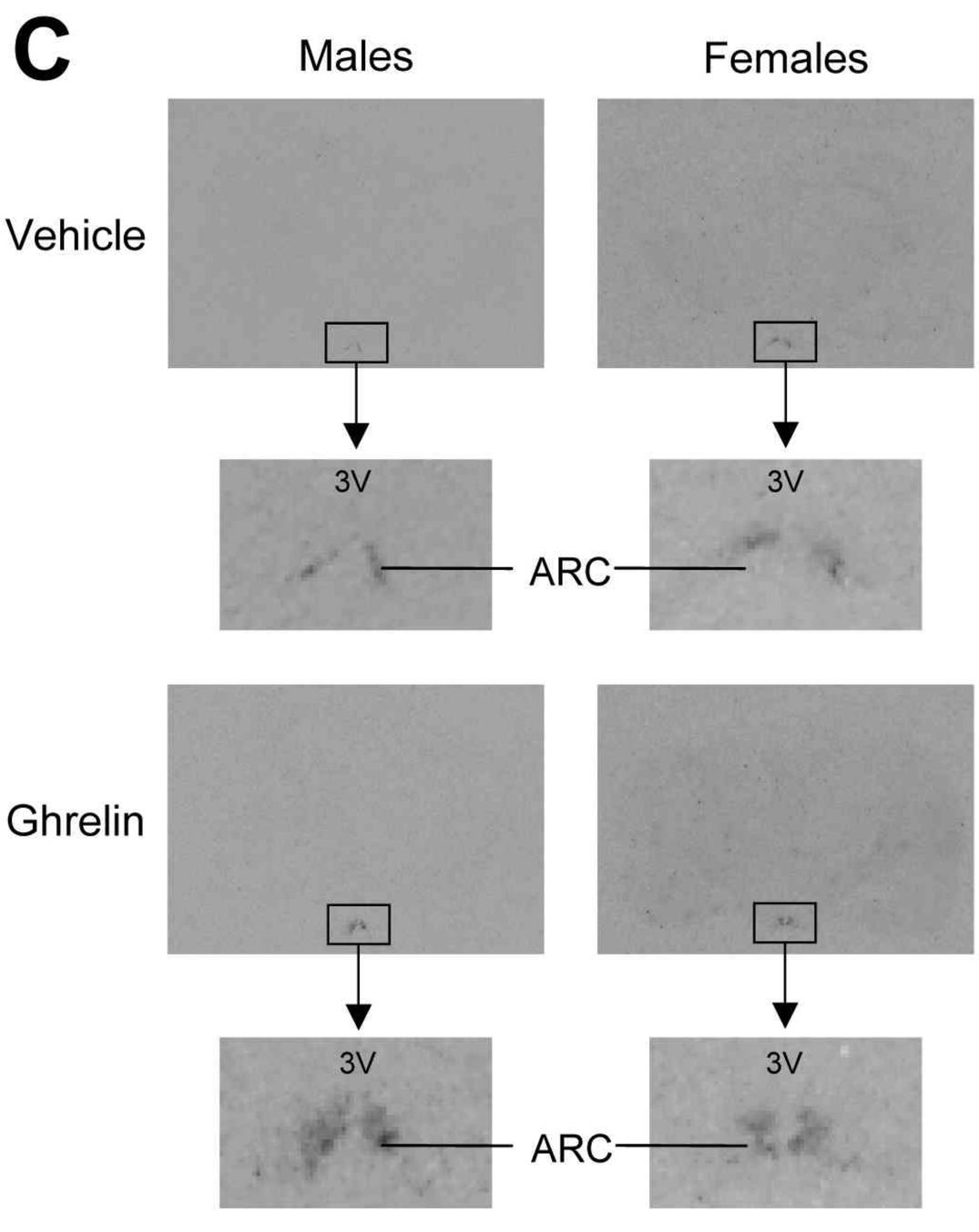
Table 1: Antisense oligonucleotides for in situ hybridization analysis

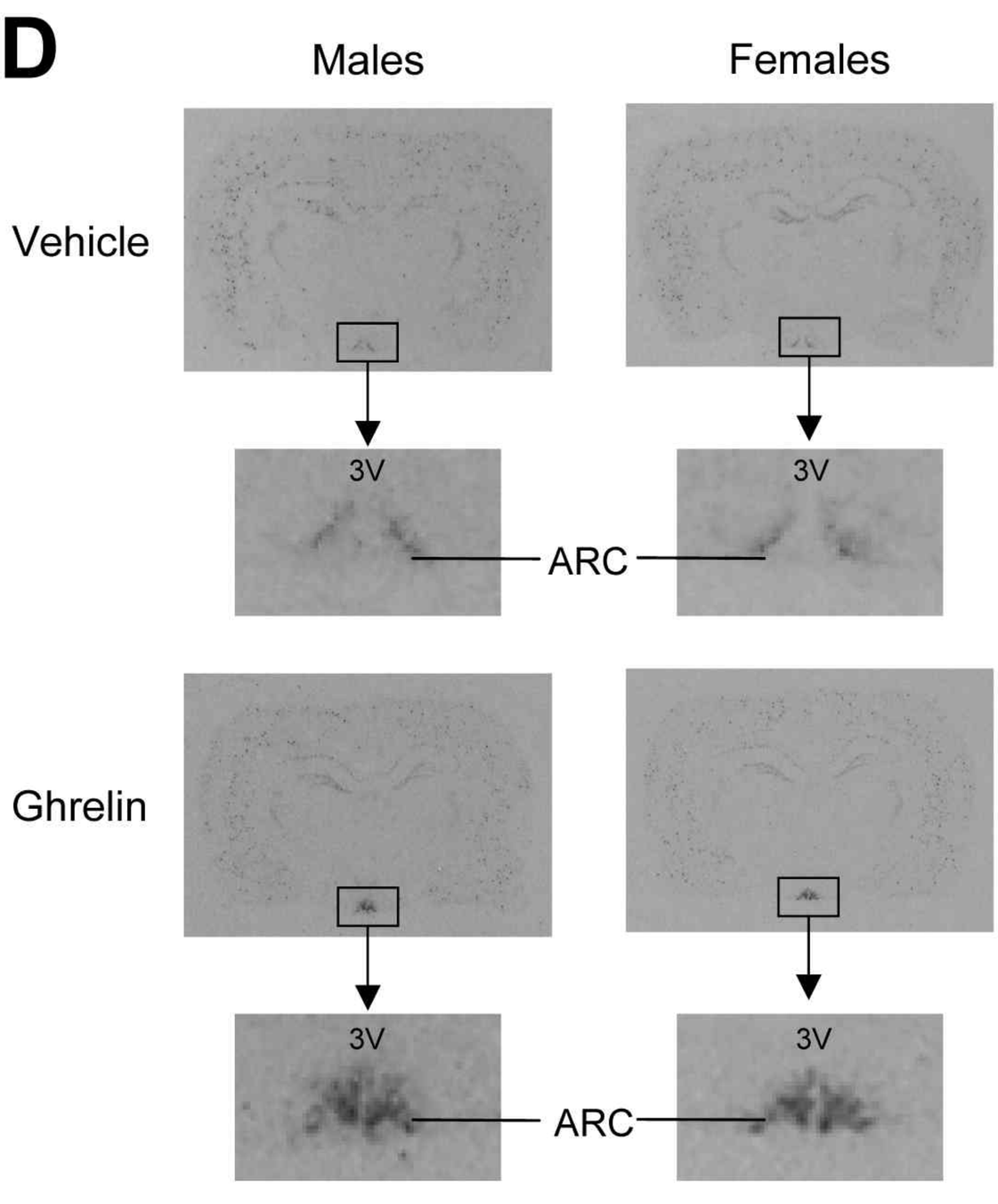
mRNA	GenBank accession number	Sequence
AgRP	AF206017	5’-CGACGCGGAGAACGAGACTCGCGGTTCTGTGGATCTAGCACCTCTGCC-3’
BSX	XM_001064837	5’-CCTCAACGGCTTGGGCTTGTGTAGCAGAATGTCC-3’
FAS	NM_017332	5’-GGGTCCATTGTGTGTGCCTGCTTGGGGTG-3’
NPY	M20373	5’-AGATGAGATGTGGGGGGAAACTAGGAAAAGTCAGGAGAGCAAGTTTCATT-3’

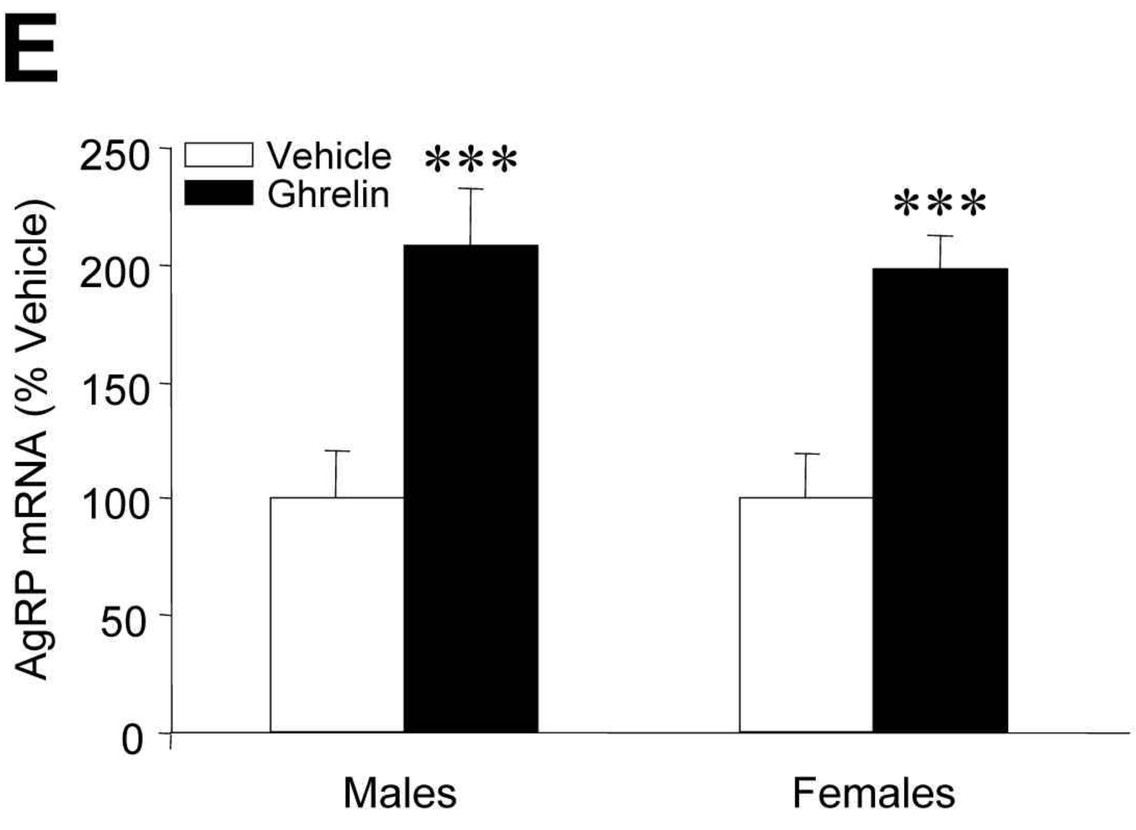
FIGURE 1
(Lage et al)











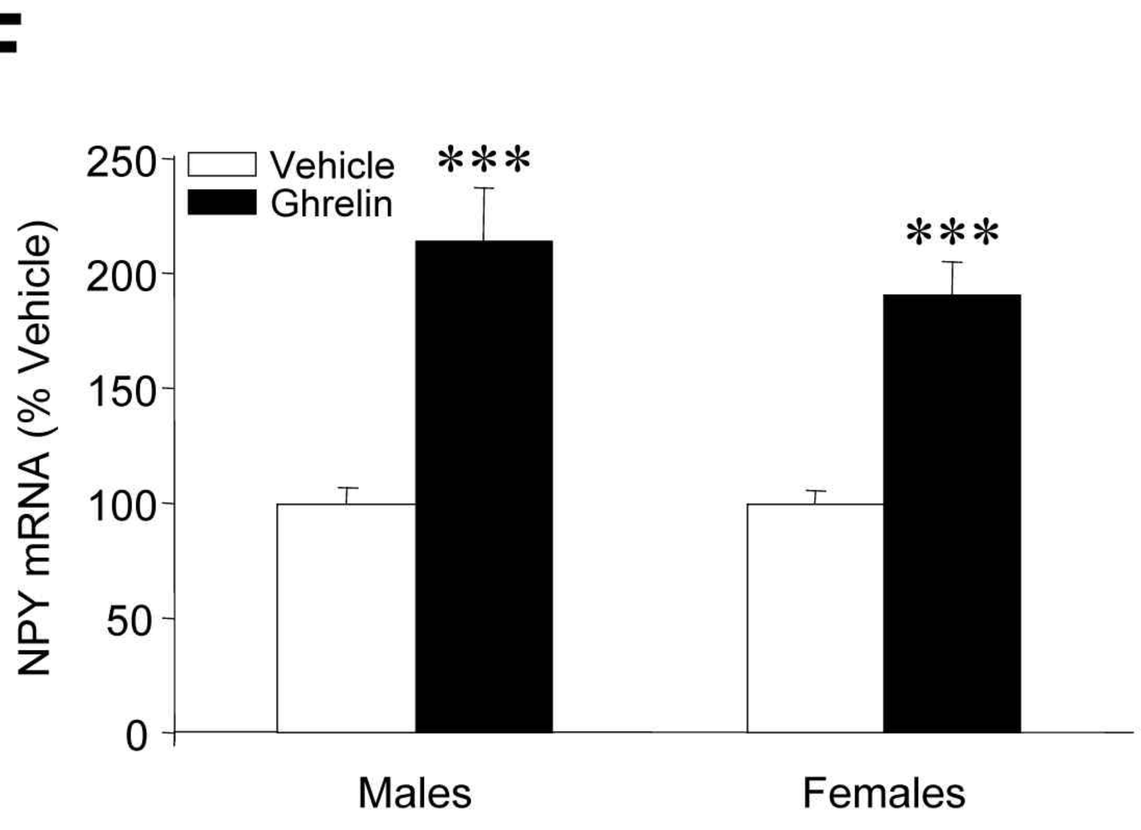


FIGURE 2
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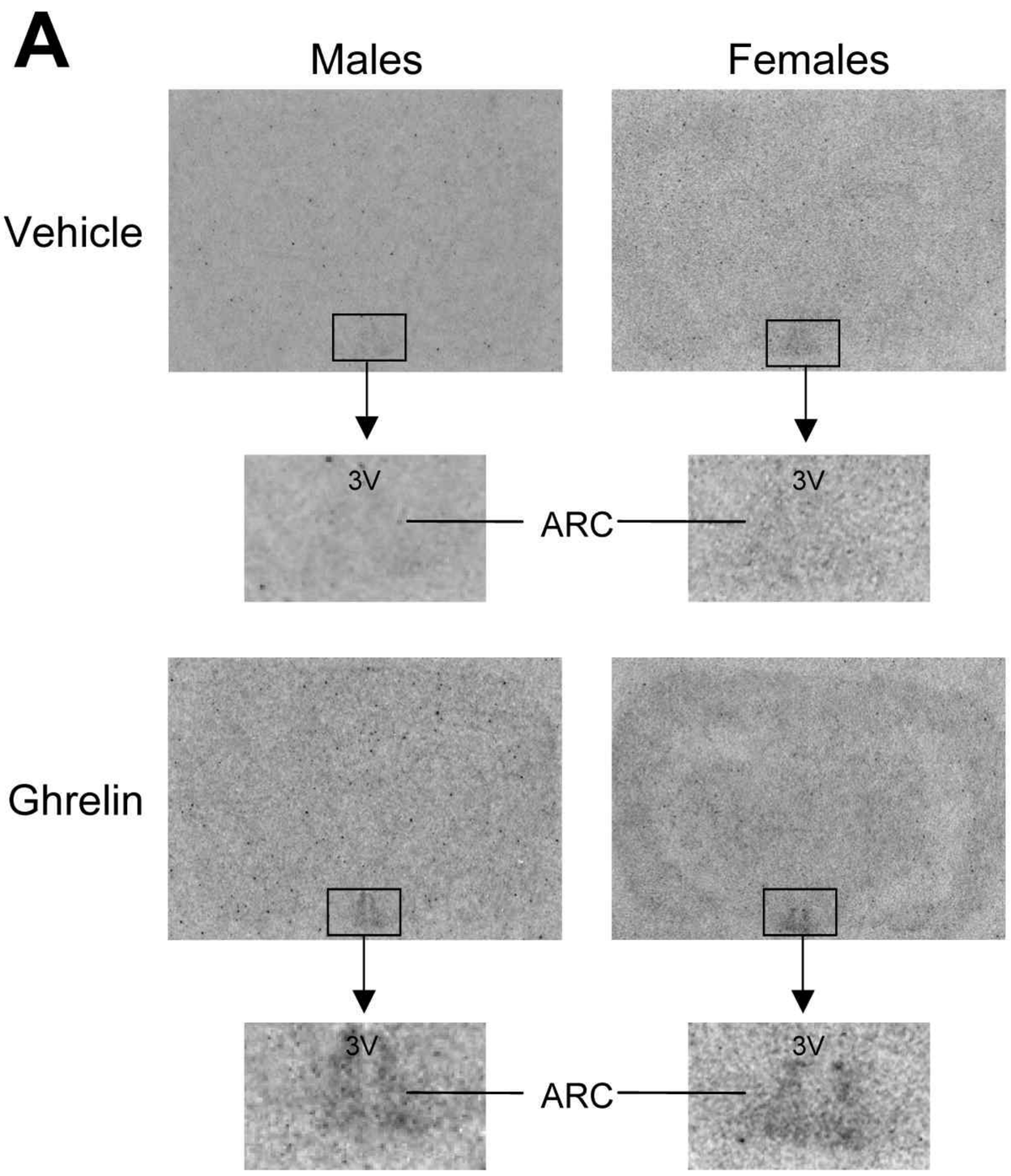

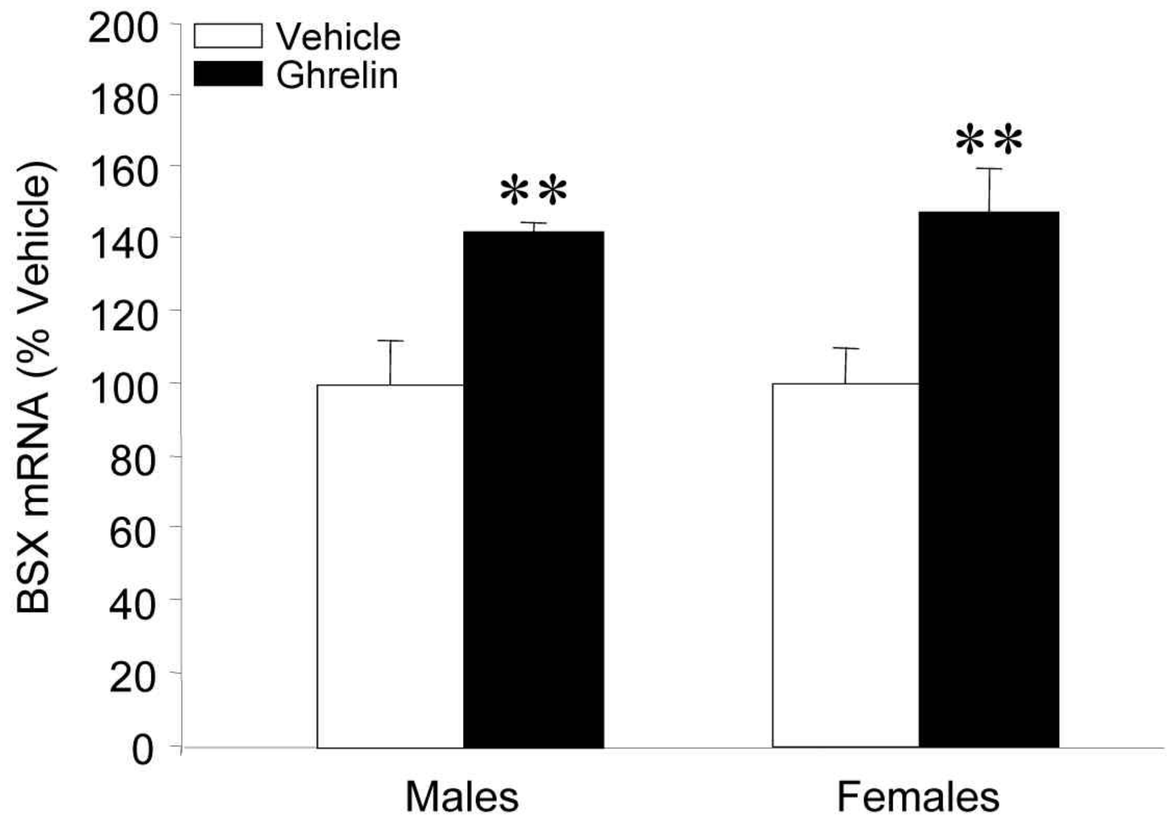

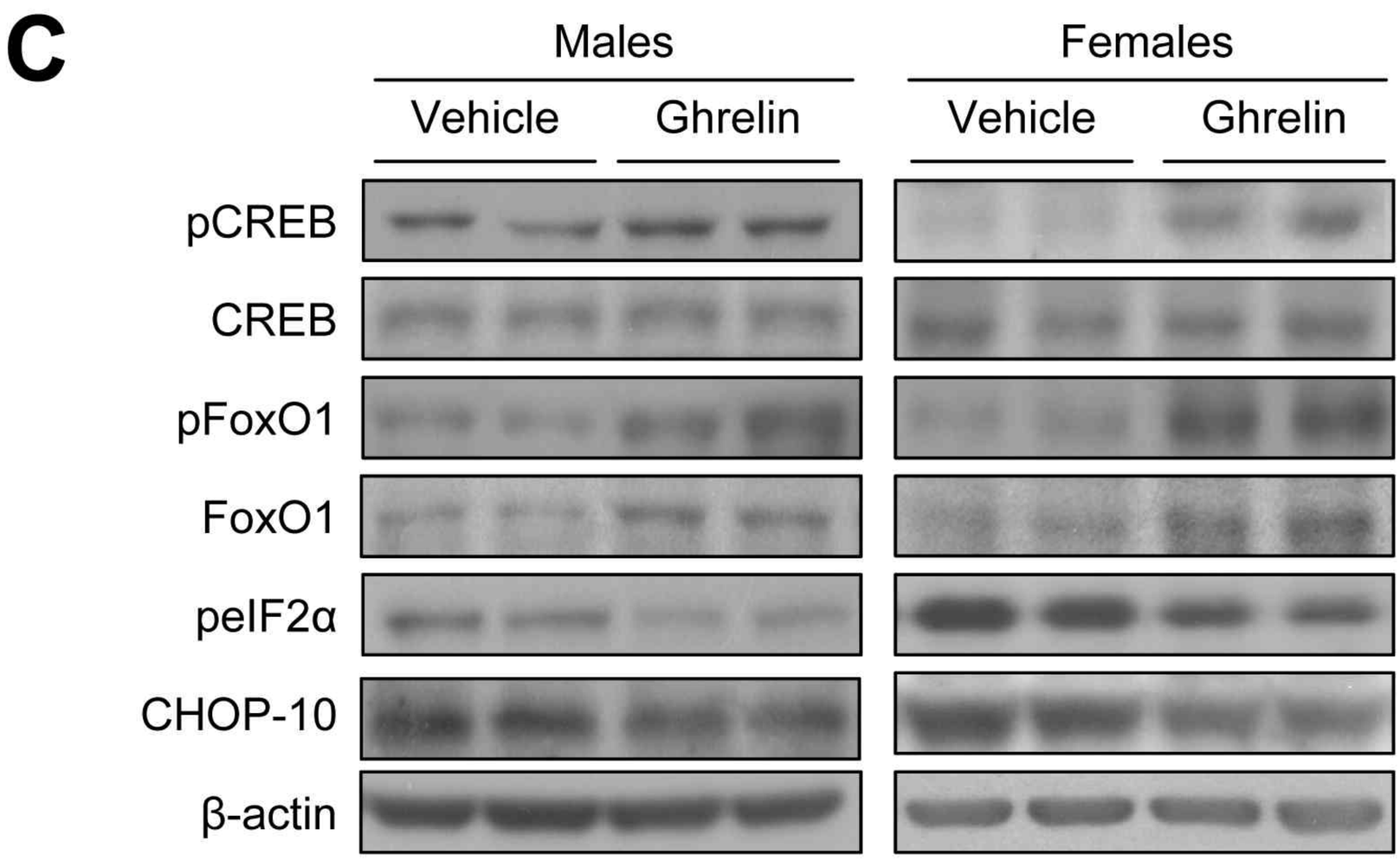

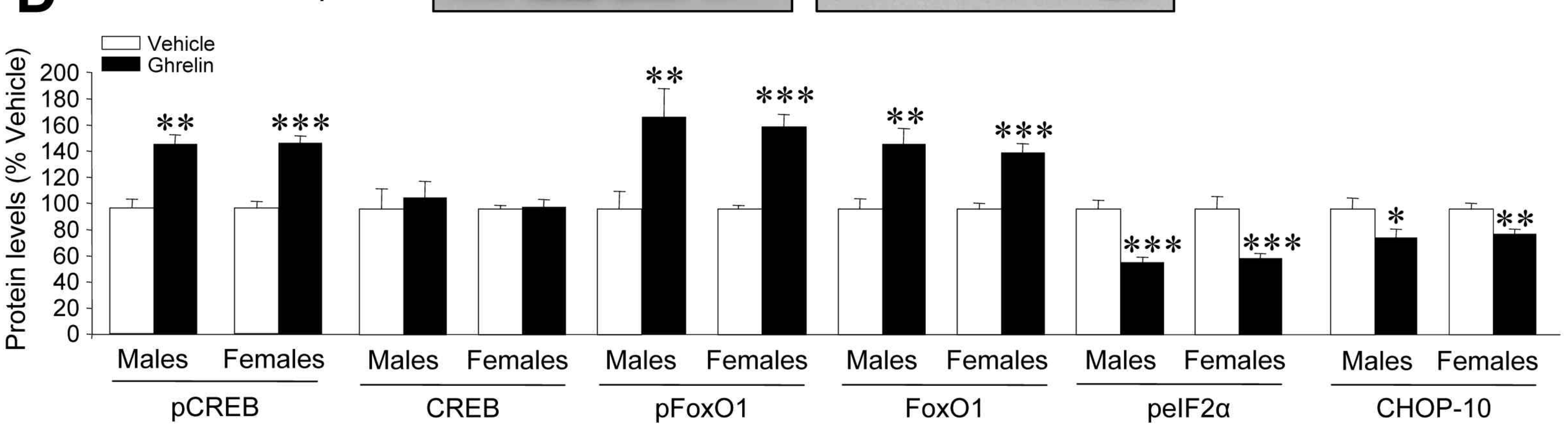

FIGURE 3
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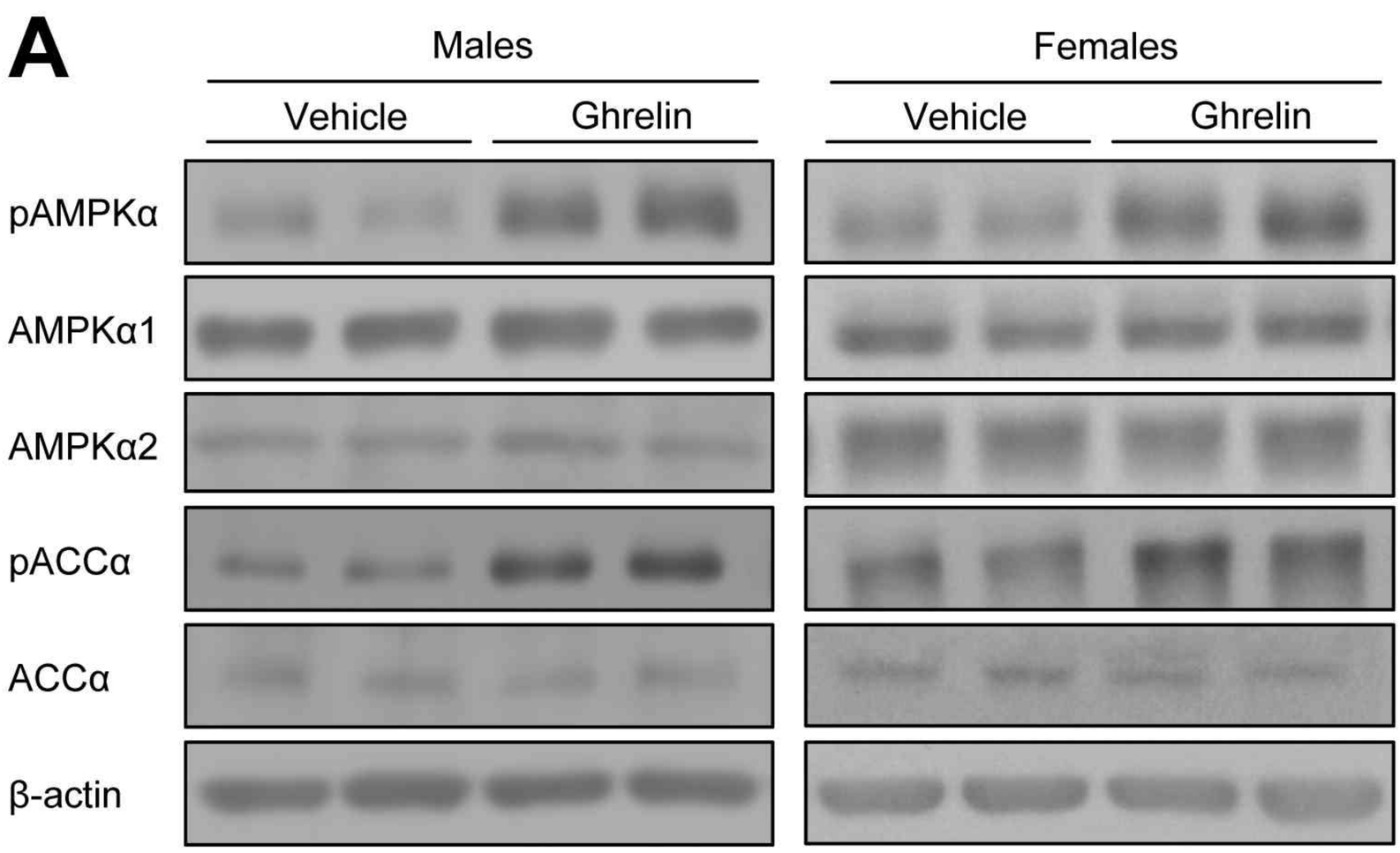

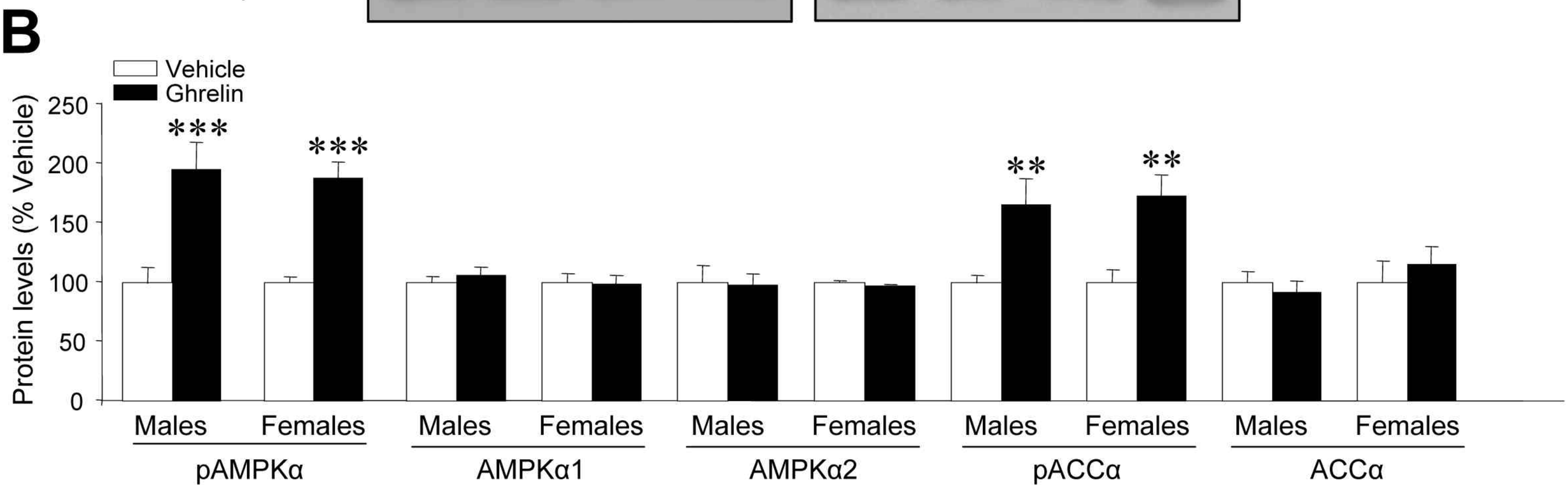

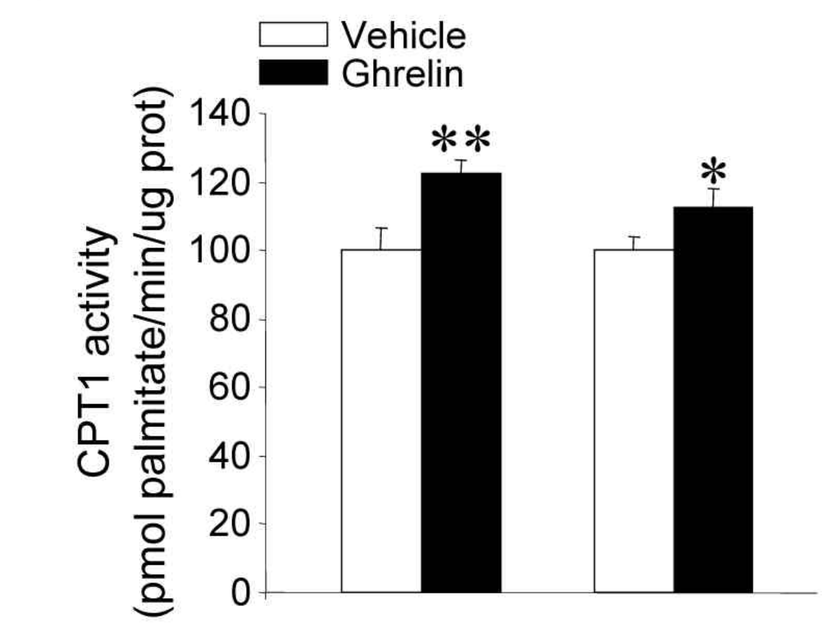

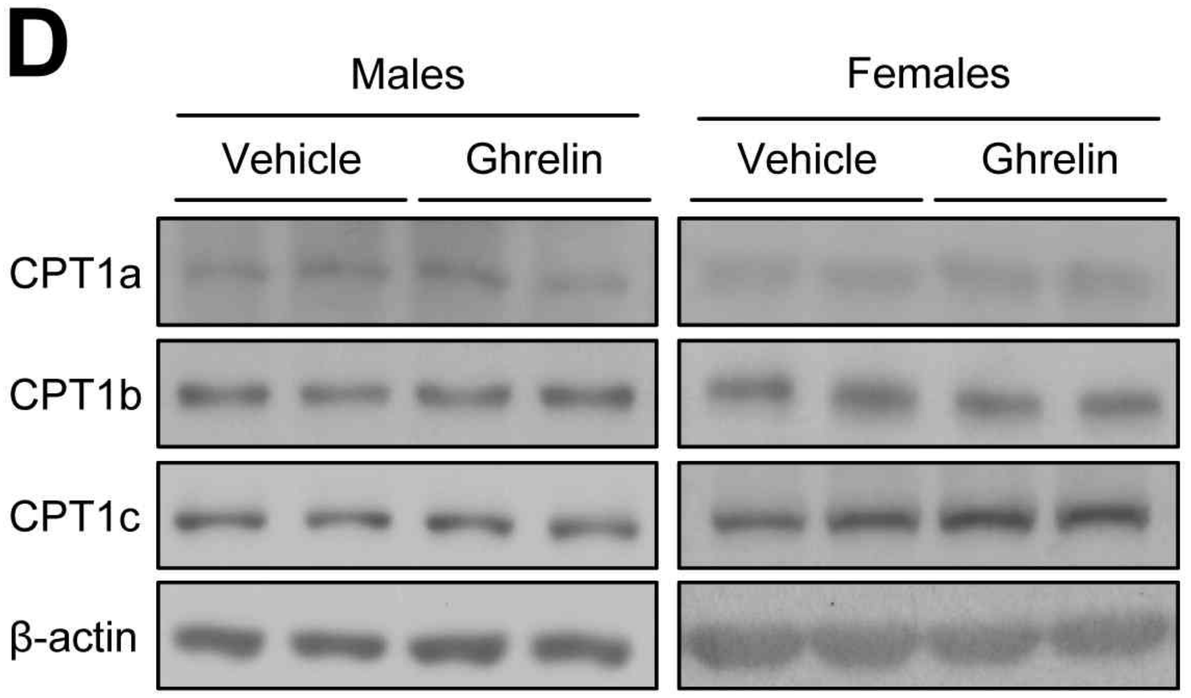

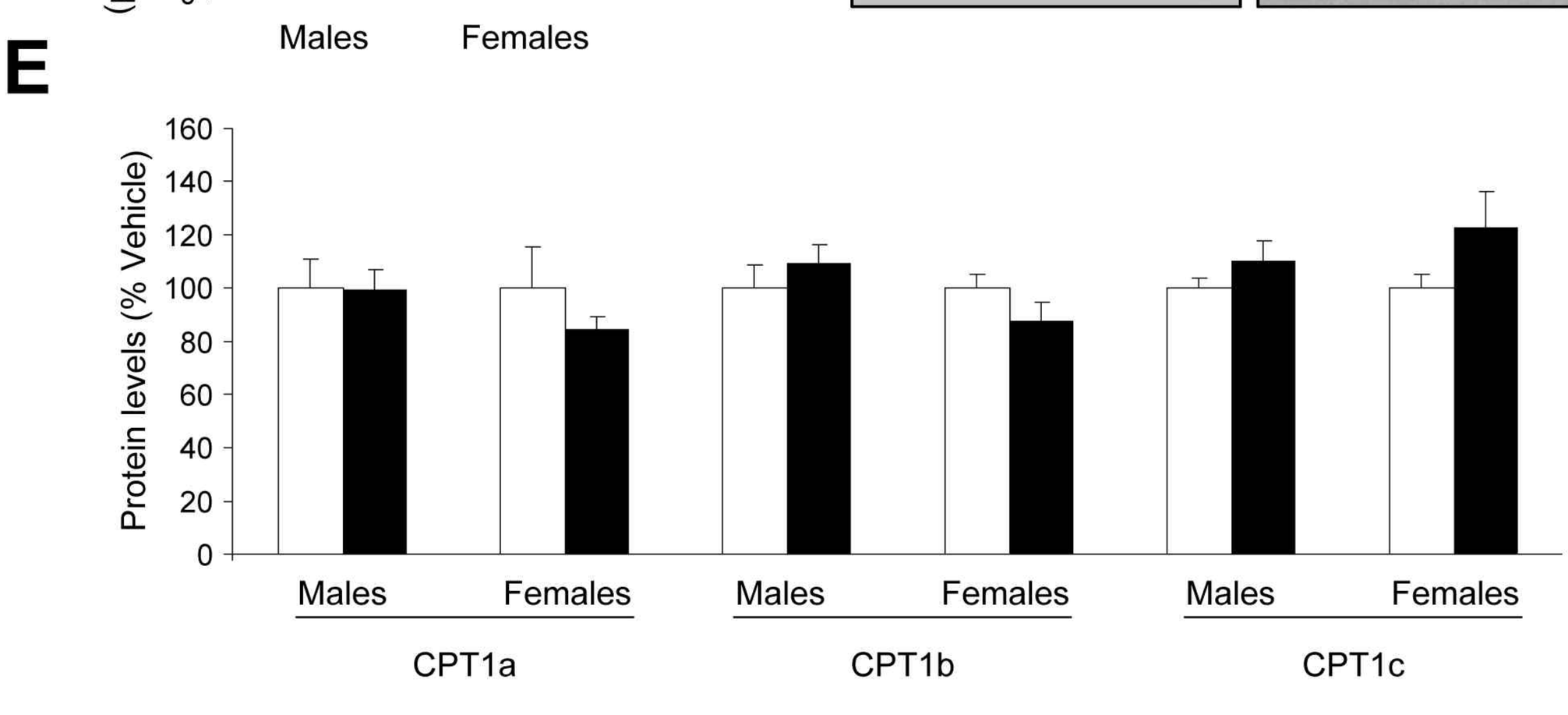

FIGURE 4
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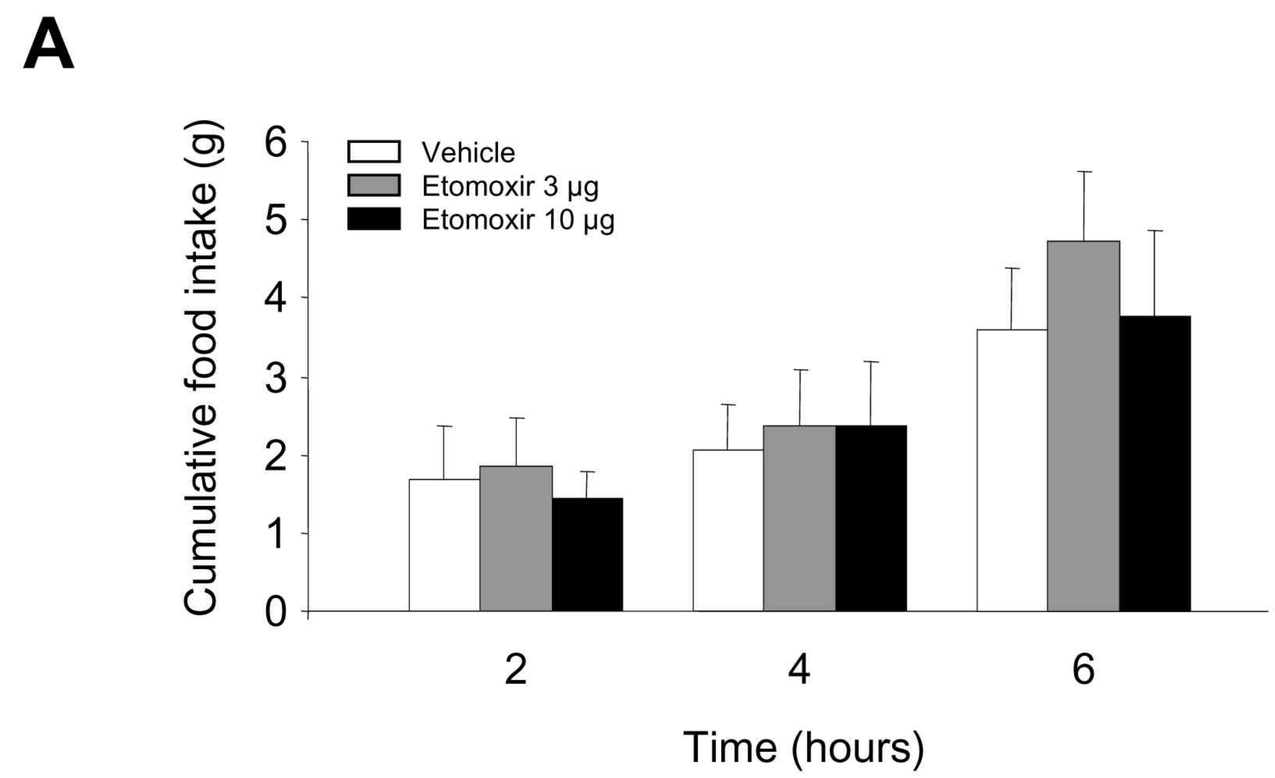
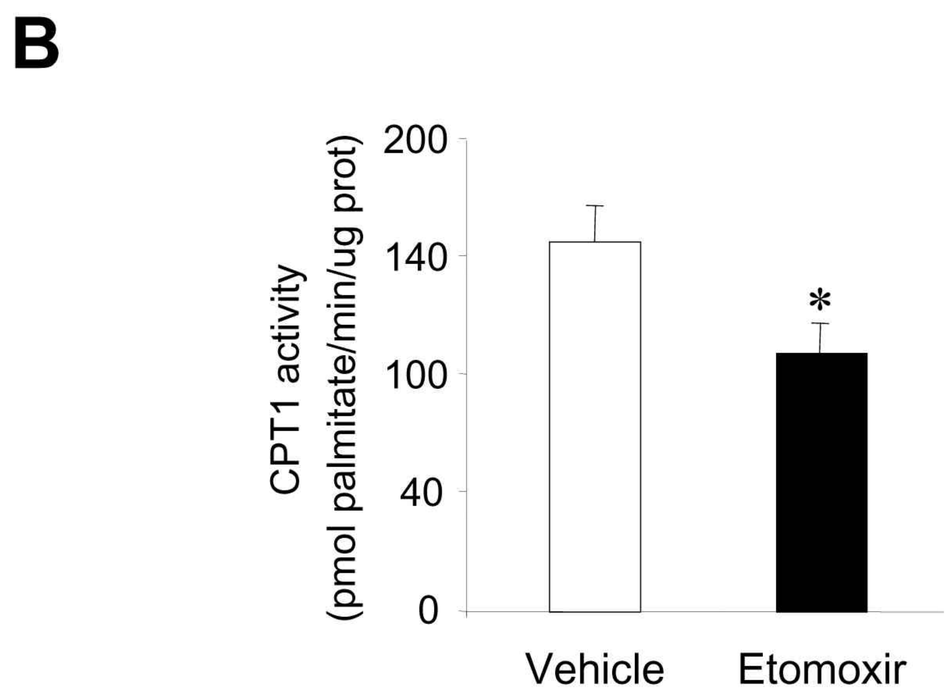
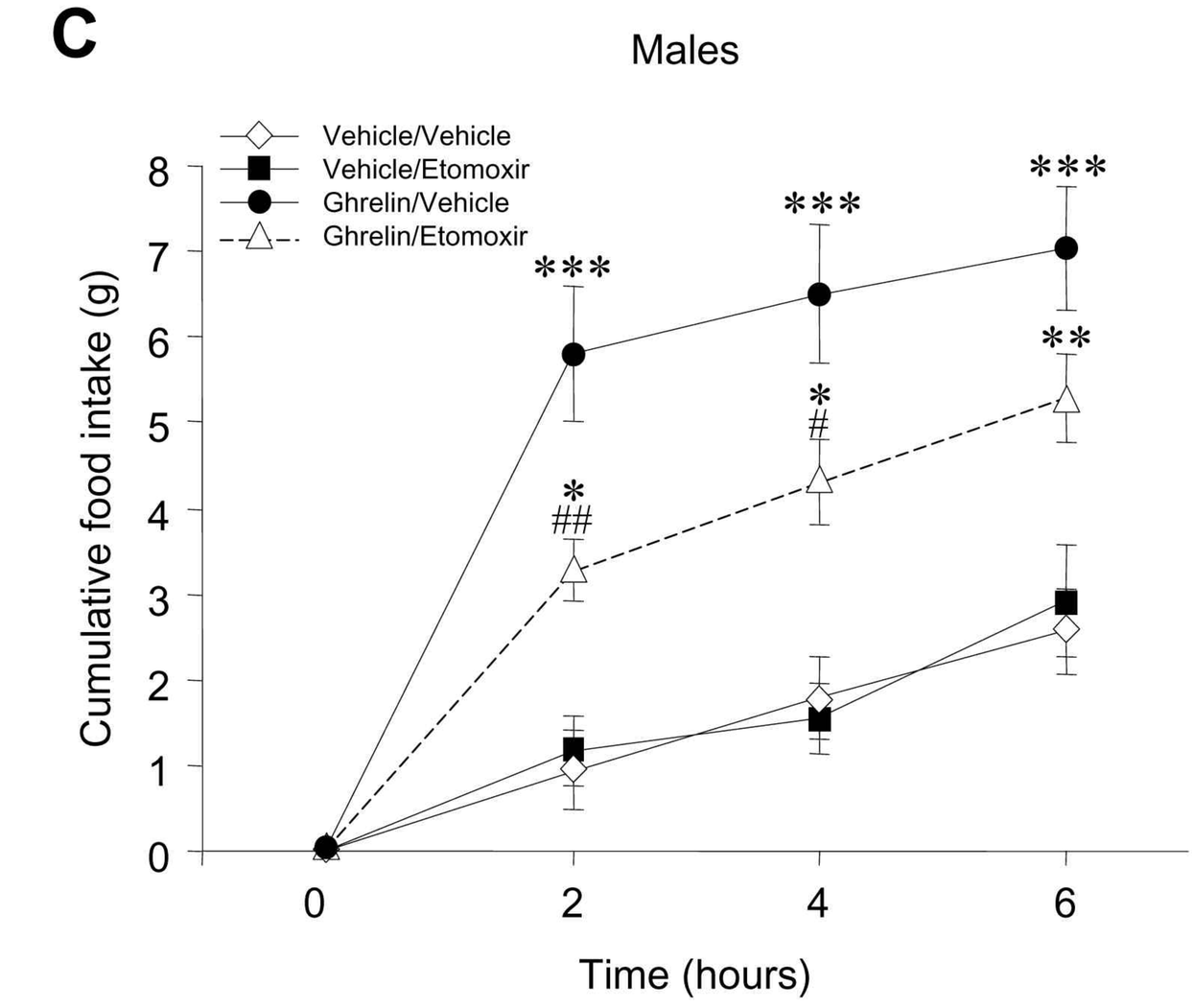






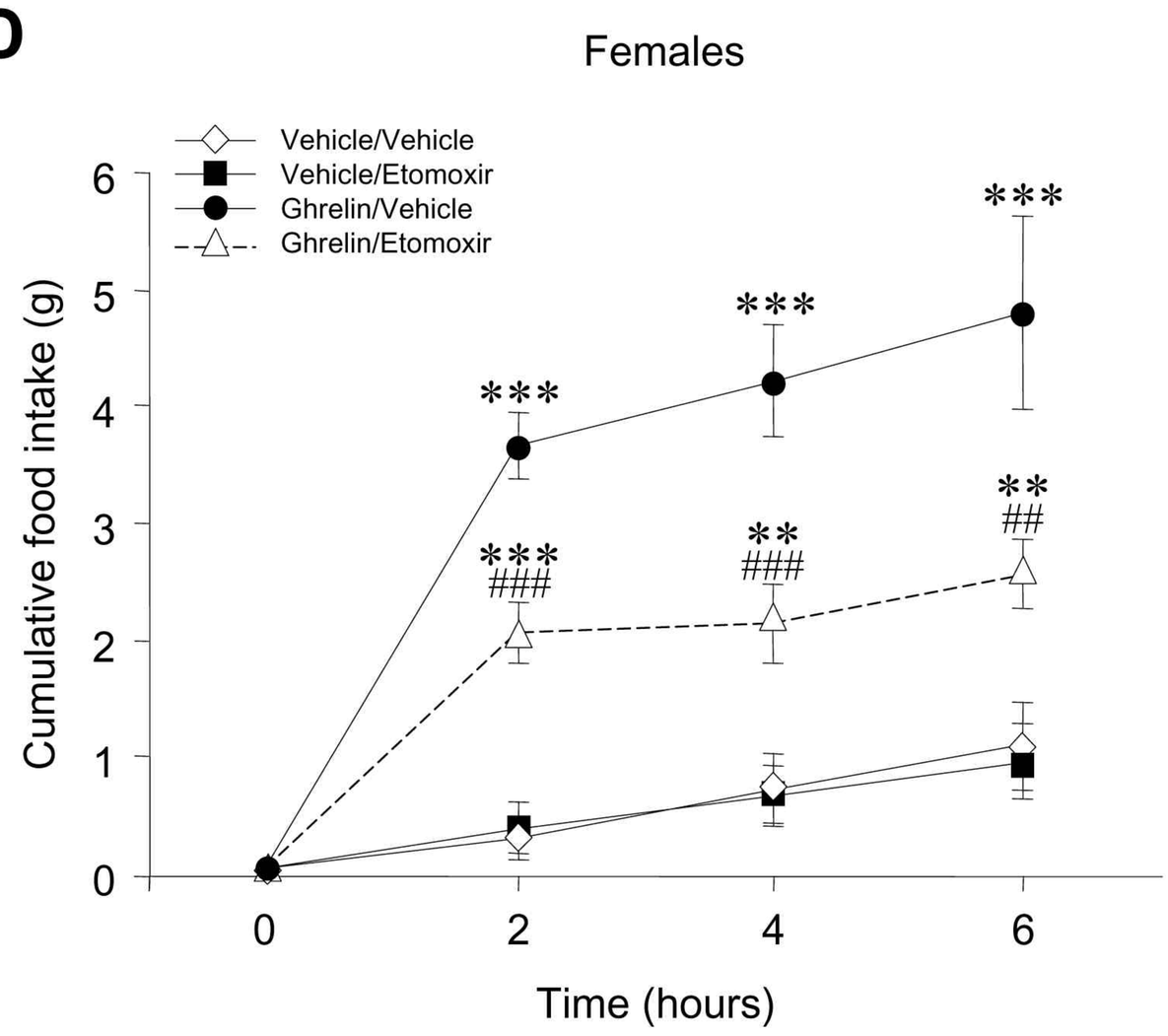
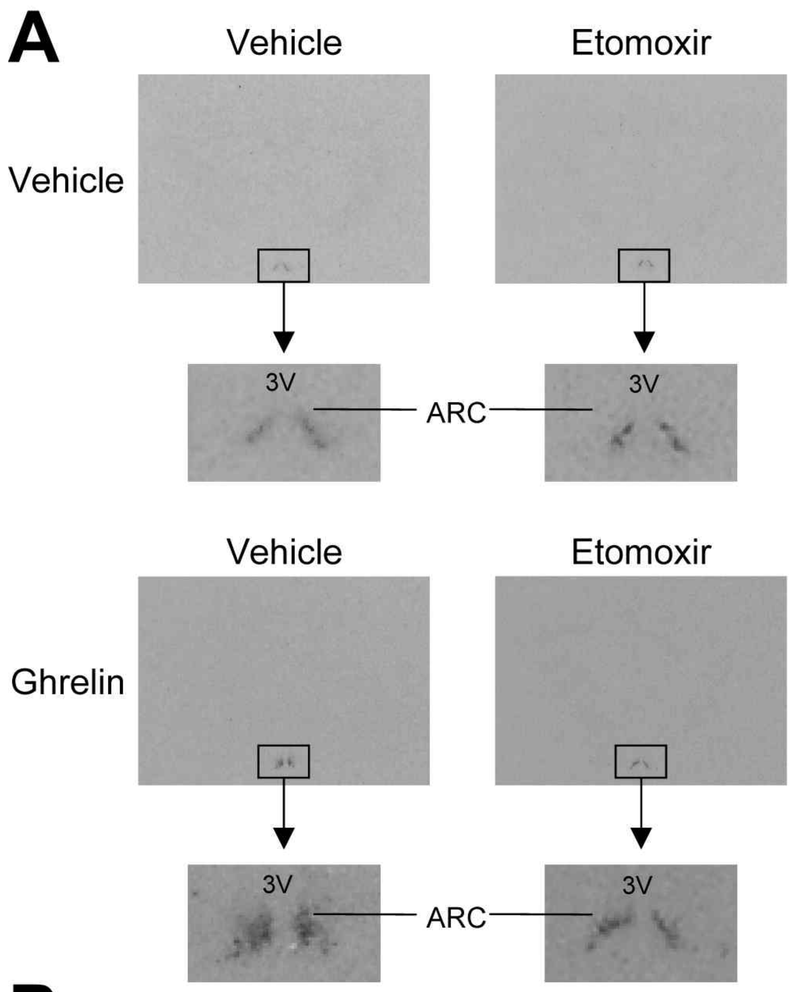
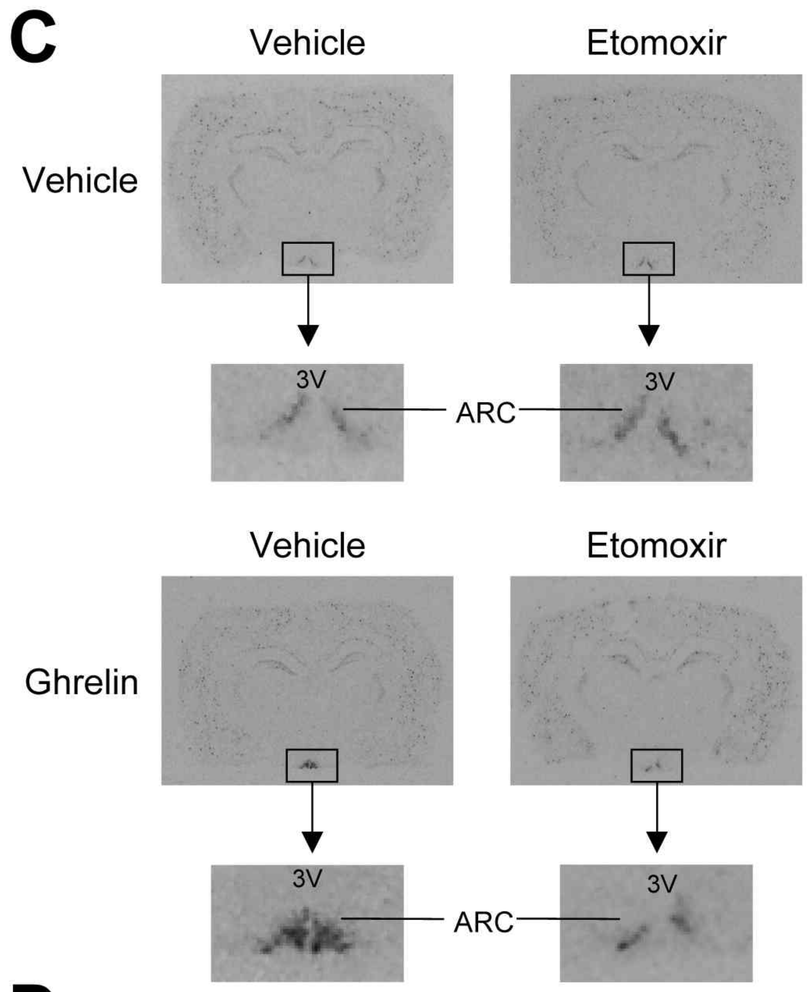
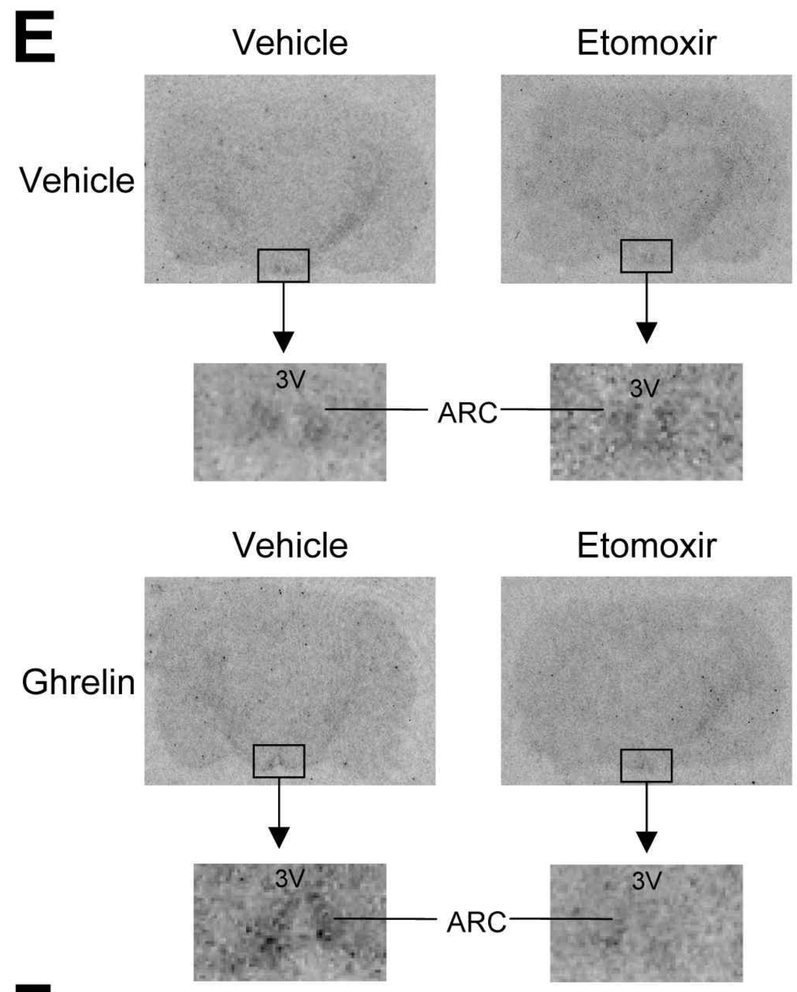


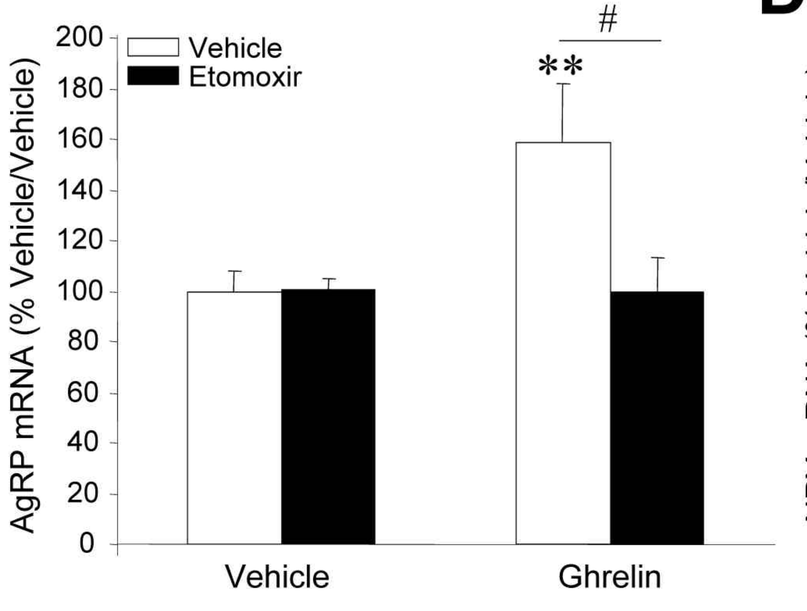
FIGURE 5
(Lage et al)

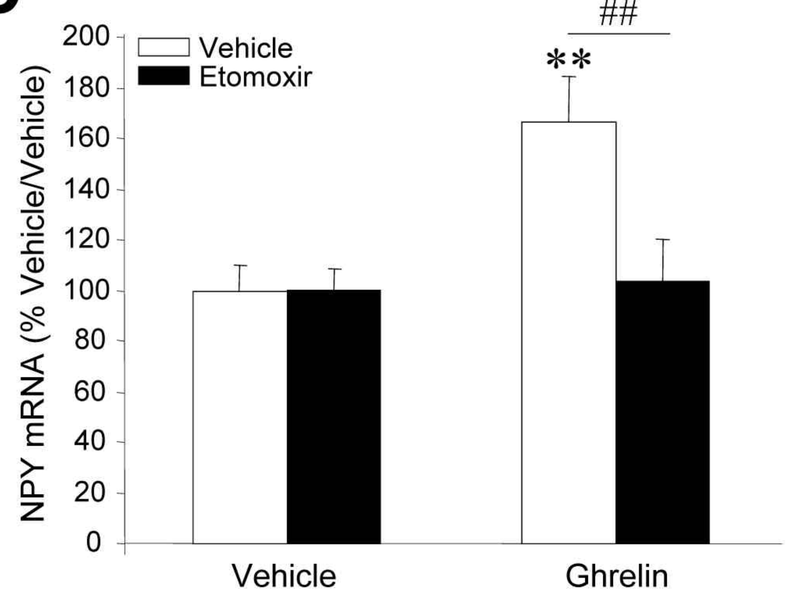
Males











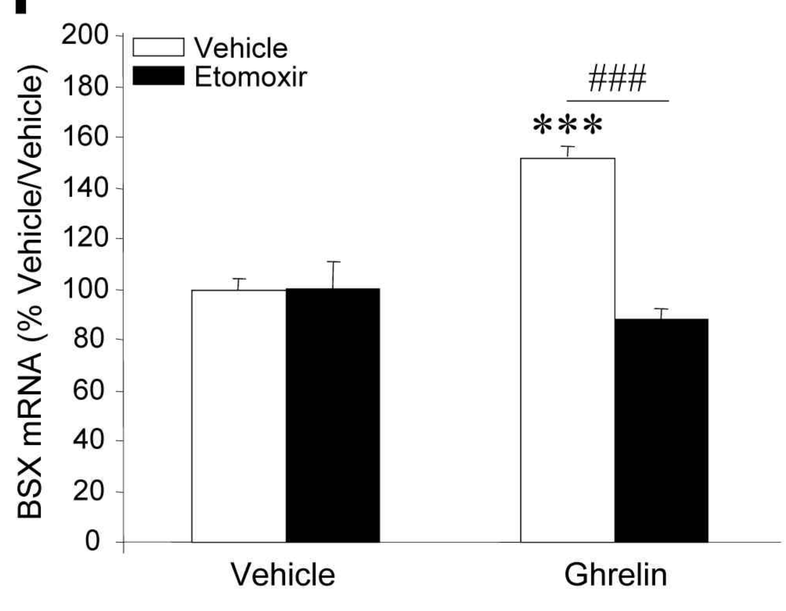


FIGURE 6
(Lage et al)

Females