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Hypothalamic AgRP-neurons control peripheral substrate utilization and nutrient partitioning

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 May 2012

Thank you for submitting your manuscript to the EMBO Journal. I am very sorry for the delay in getting back to you with a decision, but it has unfortunately taken a bit longer than anticipated to receive the reports. I have now received two of the reports, which are enclosed below. I am still waiting for a third one, but given the present set of comments I can make a preliminary decision now. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so. I would like to give the referee until mid next week to get back to us. If we don't hear by then we will just go with the two reports that we have on hand

As you can see below, both referees find the analysis interesting. However significant revisions are also needed for further consideration here. In particular referee #1 raises many issues that would have to be resolved. S/he finds the analysis remains too descriptive and that the cause of the obesity phenotype needs to be better explored. Given the comments provided, I would like to ask you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. I will forward the comments of the third referee to you as soon as we receive them, together with our final editorial decision.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

Joly-Amado et al examined the effects of ablation of AgRP neurons on energy metabolism. They found that mice lacking AgRP-neurons become obese and hyperinsulinemic on regular chow but reduce body weight gain and paradoxically improve glucose tolerance on high fat diet (HFD). They examined many metabolic parameters including gene expressions and sympathetic nerve activity in peripheral tissues and concluded that the mice favor the utilization of lipids over carbohydrate. The results are very interesting. However, the data are descriptive and premature to understand the mechanism.

1) Fig. 2-5: it is unclear when those experiments were performed. According to the manuscript, those were done at obese stage. If so, almost all results might be secondary effects of obesity, except of hyperinsulinemia.
2) The authors showed many metabolic parameters in mice lacking AgRP neurons and fed regular chow diet. However, what is important for the obese phenotype of the mice? Low locomotor activity, low body temperature, low sympathetic nerve activity or hyperinsulinemia? The reviewer thinks that changes in those parameters cannot explain the obesity phenotype with increase in energy expenditure. "Energy expenditure" includes all those metabolic and locomotor alterations. Furthermore, low temperature in scapular region (Fig. 2) may be due to alteration of heat dissipation but not energy metabolism. In addition, increased lipid utilization cannot directly explain the mechanism of the obesity phenotype.
3) Fig. 3: again, low sympathetic nerve activity in the liver and pancreas does not explain the obesity phenotype directly. In addition, high sympathetic nerve activity in soleus may not be consistent with the obesity. Furthermore, decrease of plasma insulin level with clonidine may not be specific in AgRP neurons-deficient mice. Clonidine probably decreases insulin level in other obese mice as well as AgRP neurons-deficient mice.
4) Fig. 4: Fig. 4C suggests that triglyceride turnover is enhanced in AgRP neurons-deficient mice. Why is no change in Fig. 4E? Moreover, increased plasma glycerol and free fatty acid levels may be due to the decrease of utilization rather than increased utilization.
5) Fig. 5: the reviewer did not understand the mechanism by which Complex I respiration decreased in the mice. It may be secondary effect of obesity rather than the cause of obesity. Furthermore, why is energy expenditure high (Fig. 2A), while mitochondrial function is impaired? Why are they resistant to the impairment of glucose metabolism under HFD feeding (Fig. 6)?
6) Fig. 7: the effect of Bretasenil (mp) may not be specific in AgRP neurons-deficient mice. Bretasenil would decrease fat content in other obese mice too, because GABA regulates many functions of the brain and body. In addition, how does improved carbohydrate utilization by Bretazenil decrease fat content?
7) Cell death by diphtheria toxin may activate microglia and inflammatory response in the brain. Inflammatory response in the hypothalamus and brain would cause obesity and affect many brain functions non-specifically. Negative control may be necessary.
8) The authors must have used post hoc analysis after ANOVA. It should be described.
9) In Page 7, second paragraph: "Fig. 2c" is a mistake.
10) Bar graphs in Fig. 3D and E: which is soleus?

Referee #3

In this paper, Joly-Amado et al. reveal a role for AgRP- neurons in the control of nutrient partitioning. They show that ablation of AgRP neurons leads to a change in autonomic regulation of the liver, muscle, and pancreas to promote lipid metabolism on the expense of carbohydrate utilization. Mice lacking AgRP-neurons become obese and hyperinsulinemic on regular chow but display reduced body weight gain and paradoxical improvement in glucose tolerance on high fat diet. The authors argue that their results establish a role for AgRP-neurons in the coordination of efferent organ activity and nutrient partitioning, providing a mechanistic link between obesity and obesity-related disorders.

Comments:

I think that this is an excellent and timely work. Semantically, I disagree with the characterization of these finding providing a "novel" role for AgRP neurons, because this role has been suspected and considered by many. The novelty of the work lies in the fact that this is the first experimental demonstration of such role and the author deserve great credit for this.
Along the same line, because carbohydrate metabolism is enhanced in AgRP ablated mice, I am not sure whether the finding that they are resistant to high fat diet should be characterized as paradoxical. By eliminating the AgRP system, increased carbohydrate metabolism maybe explained, at least in part, by elevated activity of the POMC neurons. These issues could be discussed a bit more thoroughly citing works delineating the anatomical and functional connectivity between these arcuate nucleus neuronal populations. The authors may also consider recent works that showed that selective impairment of AgRP neuronal excitability appears to shift metabolic profile in agreement with the findings of the present paper (Dietrich MO et al., 2010 JNS).

On the schematic illustration, the 3 axons leaving the AgRP neurons should be changed to 1 which, once leaving the cell body, splits to 3. One cell body-one axon initial segment.

Additional Correspondence 25 May 2012

I have still not received the third report on your paper and at this stage don't think that I will. We will therefore go ahead with the two reports that we have on hand. As noted in my previous email major revisions are needed for consideration here, but should you be able to address the other raised concerns in full then we would be willing to consider a revised manuscript. I should add that it is EMBO Journal policy to allow a single round of major revision only and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 27 July 2012

Point-by-point response to the referees'

Referee #2

Joly-Amado et al examined the effects of ablation of AgRP neurons on energy metabolism. They found that mice lacking AgRP-neurons become obese and hyperinsulinemic on regular chow but reduce body weight gain and paradoxically improve glucose tolerance on high fat diet (HFD). They examined many metabolic parameters including gene expressions and sympathetic nerve activity in peripheral tissues and concluded that the mice favor the utilization of lipids over carbohydrate. The results are very interesting. However, the data are descriptive and premature to understand the mechanism.

1) Fig. 2-5: it is unclear when those experiments were performed. According to the manuscript, those were done at obese stage. If so, almost all results might be secondary effects of obesity, except of hyperinsulinemia.

Fig 1, 2, 4, 5, 6 and 7 described indeed the phenotype of AgRP-ablated mice at a time point where they displayed a significant difference in body weight compared to naïve.

Importantly however, Fig. 3 (a-f) presents the cathecholamine turn over rate in peripheral tissue and clonidine action onto insulin level measured at a time point that precedes obesity. The difference observed in Norepinephrine outflow and hyperinsulinemia can therefore not be attributed to obesity but to a pre-existing metabolic set point created by the lack of AgRP-neurons.

Furthermore, we show that the lack of AgRP neurons leads to a change in preganglionic structures neuronal activity (Fig 3a) that we believe is primarily due to the lack of GABA tone from these neurons. The data presented Fig. 7 and now in Supplementary Fig. 9 support that claim by showing that $GABA_AR$ potentiation restores indeed part of the phenotype.

Taken together Fig 3/7 provide what we believe to be the core physiological mechanism by which the lack in AgRP-neurons will lead to a change in peripheral energy partitioning and to the consequent obesity on carbohydrate diet.

These observations also are, to our knowledge, the first direct experimental demonstration of the role AgRP neurons in the coordinated regulation of peripheral organs ANS outflow and substrate utilization

We agree with the referee #2 that the manuscript is not clear enough in describing the experiments that were done on animals that were not yet obese. We have made changes in the text and figure legend to make that point more evident.

Noteworthy, we would like to stretch out that the goal of our study was to provide experimental demonstration that AgRP neurons have a role in nutrient partitioning that extend beyond their role in the control of food intake. AgRP neurons are positive promoter of food intake, in that regards one would have expected from AgRP neuron ablation a defect in nutrient intake and a lean phenotype. Consistent with the role of these neurons in the positive control of feeding we found that AgRP-ablated mice show defective response to ghrelin. The non-hyperphagic obesity displayed by AgRP-ablated mice is, although counter-intuitive, provided us with a very visible demonstration that these neurons have indeed physiological function that extend beyond the strict regulation of food intake.

In this study we provide an experimental demonstration that lack of AgRP-neurons alters the overall balance between carbohydrate and lipid metabolism. Indeed a difference in body weight gain is observed on carbohydrate diet while, on the contrary, high fat feeding has beneficial consequence in AgRP-ablated (Fig. 6 and Supplementary Fig 7).

In that regards the two opposed phenotypes obtained on RCD and HFD can be considered as an integrated phenotype resulting from that imbalance and illustrates the role of AgRP-neuron as a central cursor of peripheral energy preference.

2) The authors showed many metabolic parameters in mice lacking AgRP neurons and fed regular chow diet. However, what is important for the obese phenotype of the mice? Low locomotor activity, low body temperature, low sympathetic nerve activity or hyperinsulinemia?

We agree with referee#2 that we show different alteration in metabolism but we do not provide one specific tissue that would be solely involved in the phenotype. We believe that the concerted change in ANS output onto efferent organs leads to a global change in nutrient partitioning in which hyperinsulinemia, enhanced liver TG synthesis and increased muscle oxidative capabilities participate altogether in creating a subtle metabolic shift favoring lipids substrate over carbohydrate. It is that subtle change that promotes both body weight gain on RCD but glucose tolerance amelioration on HFD.

The scheme presented Fig. 8 is an attempt to capture what we believe is an integrated and coordinated change in tissue activity leading to altered nutrient partitioning.

The reviewer thinks that changes in those parameters cannot explain the obesity phenotype with increase in energy expenditure. "Energy expenditure" includes all those metabolic and locomotor alterations.

We thank the referee#2 for raising that concern which prompted us to clarify the manuscript.

By dividing the Energy expenditure measured by the lean body mass measured by NMR we provided a value EE/g of LBM that is higher in AgRP-ablated mice. We realize that this way of presenting the data might be misleading since it conveys the idea that AgRP-ablated mice have increased in whole-body energy expenditure (EE) which is not true.

By multiplying the EE/g of LBM by the total LBM gives an absolute value of whole-body EE by animal. When presented that way we do not find a decrease in whole-body energy expenditure in AgRP-ablated mice compared to control Supplementary fig. 1D.

We agree with the referee#2 that we cannot exclude the possibility that a subtle, non-measurable defect in energy expenditure contributed to the body weight gain in AgRP-ablated mice. We believe however that our observation supports the claim that a defect in nutrient partionning rather than hyperphagia or decreased metabolic efficiency is a primary mechanism in the phenotype observed in AgRP-ablated mice.

Indeed, AgRP-ablated mice display a ~10g of FAT (26 to 36) difference compared to naïve animals that they acquired over a 5 months among which 4 are really after weaning. Assuming that 10g of FAT=90kcal it means that AgRP-ablated mice had a differential energy balance of 90kcal over a 90 days period, that is 1kcal per day, a mere 6.6% of the daily energy intake which is 15kcal (Fig. 1d). These differences are at the limit of discriminatory capacity of indirect calorimetry technique (Butler & Kozak, 2010). Butler and Kozak (2010) recently suggested that a ~5% difference in EE can account for a marked accumulation in fat mass over time.

Finally, we also have provided new experimental data comparing in the same session of analysis naïve, obese AgRP-ablated mice and obese *ob/ob* mice. In that experiment whole energy expenditure and total energy intake were measured to provide a daily calories balance (total EE intake/whole body EE expended). This analysis clearly illustrates a state of positive energy balance *ob/ob* mice while daily calories balance between AgRP-ablated mice and naïve are similar.

We have added this result as Supplementary fig. 1d and mentioned this result in the text page 7.

Furthermore, low temperature in scapular region (Fig. 2) may be due to alteration of heat dissipation but not energy metabolism.

We agree with the referee#2, low temperature in the scapular region could indeed be a defect in heat dissipation. A change in ANS output onto vasculature could also change blood accessibility and heat dissipation to the skin which would be consistent with our finding. In any way we agree with the referee that this defect could contribute, but cannot account alone for the overall phenotype.

However, since the temperature defect was corrected by supplementing the animals with $GABA_A$ receptor partial agonist we argued that the defect could be due to the lack of GABAergic tone from AgRP-neurons onto preganglionic structures cold therefore be considered as resulting from the lack of AgRP-neurons GABAergic tone.

In addition, increased lipid utilization cannot directly explain the mechanism of the obesity phenotype.

As for question 2, we believe that the overall energy imbalance in AgRP-mice is rather subtle and we might not be able to quantify accurately the differential with indirect calorimetry techniques. Our aim was to demonstrate that AgRP neuron exert a control onto efferent activity and nutrient partitioning. The change in efferent organ activity that we described as a consequence of AgRP-neuron ablation and that precedes obesity, in our view, support our hypothesis. Indeed, under carbohydrate diet mice lacking AGRP-neurons display enhanced liver lipogenic activity lipid export. We also found that adipose tissue lipogenic activity and insulin sensitivity was maintained in AgRP-ablated mice despite obesity. Indeed that adipose expression of malic enzyme (a lipogenic markers) was significantly increase in perirenal (2.09 ± 0.25 vs. 1 ± 0.29, $p < 0.05$, normalized mRNA level) and subcutaneous adipose depot (2.36 ± 0.53 vs. 1 ± 0.25 $p < 0.05$, normalized mRNA level) in AgRP-ablated compared to naïve mice, respectively. These data, previously referred as not shown are now presented on page 19.

Altogether an increase in carbohydrate transformation and lipid export by the liver will provide TG supply to muscle but also to adipose. In addition, a better adaptation of muscle to lipid oxidation (Fig 5) would help sparing energy for storage in adipose tissue

The overall consequence of a sustained supply of TG associated with retained insulin sensitivity will promote adipose enlargement.

Nonetheless, we understand that the abstract contains two consecutive statements that might seem incompatible: that is "enhanced lipid utilization and…obesity". The abstract has been changed accordingly.

3) Fig. 3: again, low sympathetic nerve activity in the liver and pancreas does not explain the obesity phenotype directly.

We agree that none of the change found in efferent organs, if taken individually, explains by itself the obesity phenotype. Our study shows that AgRP-neuron exerts role efferent organs coordination. The lack of these neurons results in a concerted changes in all organs, at the time point preceding that changes peripheral energy fluxes. These changes are characterized by an Increase in both carbohydrate transformation lipid production in the liver associated with increase efficiency for lipid oxidation in the muscle.

Increased in SNS output in the liver has been shown to control liver glucose production independently from food intake (Nonogaki, 2000). We propose that a decrease in SNS outflow onto liver associated with high insulin will results in a mirror situation shifting the equilibrium between glucose and lipid production in favor of lipid. In data no published yet, we have found that blood glucose level in AgRP-ablated mice were significantly lower than these of naive mice after an overnight suggesting impaired liver neoglucogenesis.

The combination high TG syntheses in the liver and retained insulin sensitivity in the adipose tissue will provide a physiological ground for body weight gain under carbohydrate diet.

In addition, our study brings support the hypothesis that altered ANS output at the level of the brain can be a primary mechanism in obesity through the coordinated change in multiple efferent organs (Buijs & Kreier, 2006).

In addition, high sympathetic nerve activity in soleus may not be consistent with the obesity.

The change in NE turnover rate in soleus muscle and the increase in mitochondrial oxidative capability will result in better metabolism efficacy. Therefore, among the TG that are actively made by the liver a small portion can be spared for adipose storage. In that regards, increase NE tr in the soleus can participate in body weight gains under RCD but should also be instrumental in the better adaptation to high fat diet observed in AgRP-ablated mice of the animals (Fig.6 and Supplementary Fig. 7).

Furthermore, decrease of plasma insulin level with clonidine may not be specific in AgRP neurons-deficient mice. Clonidine probably decreases insulin level in other obese mice as well as AgRP neurons-deficient mice.

In agreement with the comments of referee#2, the literature described a decreased in sympathetic tone associated with most obesity (Bray, 1991; Bray & York, 1998). In that regards we do not dispute the fact that the lack of AgRP-neurons could trigger a change in ANS output that somewhat recapitulates the changes observed in obesity.

However, it is a commonly accepted that the change in ANS is a consequence rather than a cause of obesity.

A main result of our study lies in the fact that the ablation of AgRP neuron induces a change in ANS that precedes obesity indicating that these changes are likely to be casual rather than the consequence of obesity (Fig. 3 a-f). This view is supported recent literature that sees a brain-borne ANS defect at the origin of the metabolic syndrome (Buijs & Kreier, 2006).

Nonetheless, to support that view we felt, in agreement with the comment of referee#2, that we ought to provide experimental data to strengthen the link between the early ANS changes and the phenotype observed.

In complement to Fig 3g we therefore attempted to pharmacology restore insulinemia that exists in AgRP-ablated mice prior to obesity. We have collected new experimental data, inserted in Fig 3f, showing that the a2-agonist clonidine is also able to restore hyperinsulinemia selectively in AgRP-ablated mice at a time point that precedes any body weight difference between naïve and AgRP-ablated mice.

We think that new result support our mechanistic hypothesis in which change in ANS exist prior to obesity and is associated with that lack of AgRP-neurons rather than the secondary consequence of obesity. In that regards the overall outcome of AgRP-neuron depletion might indeed result in obesity on RCD but the mechanism are different from other obesity.

Also, we have added several experiments comparing our model with *ob/ob* mice that further illustrate the difference that existing between obesity displayed by AgRP-ablated and other form of obesity

4) Fig. 4: Fig. 4C suggests that triglyceride turnover is enhanced in AgRP neurons-deficient mice. Why is no change in Fig. 4E?

Here again, we thank the referee for pointing out element in the manuscript that need to be clarified.

AgRP-ablated mice at obese stage have increased level of circulating TG during the post-prandial period but if they are deprived of food the level of TG in the blood normalizes indicating that TG are used (Fig. 4c). In that regards, obese AgRP-ablated mice unlike *ob/ob* mice or high fat fed mice do not display fasted hypertriglycemia, (Kim et al, 2007).

The experiment presented in Fig. 4e, f, g is the result of an oral charge of TG on obese AgRP-ablated mice and naïve. For that experiment animals were fasted for 3 hours prior to oral charge in order to insure a normalization of circulating TG prior to the gavage. Hence, plasmatic TG level at time 0 (after a 3-hrs fast) is identical between naïve and AgRP-ablated as observed in Fig. 4c.

Moreover, increased plasma glycerol and free fatty acid levels may be due to the decrease of utilization rather than increased utilization.

Fig. 4e, f, g represents the plasmatic excursion of TG and the level of glycerol and free fatty acids after an oral charge of lipids a time = 0. In that regards we believe that the changes observed in glycerol and free fatty acids after the gavage are the results of increased TG breakdown since TG level remains similar in both group and only glycerol and FFA are increase. Moreover, FFA level in fed and fasted condition are similar in obese AgRP-ablated mice and naïve (Supplementary Fig. 5) which we believe preclude that a AgRP-neurons display a defect in FFA handling.

Finally, in a similar experiment Kim et al. have shown that oral lipid charge in *ob/o*b mice leads to a sustained increase in circulating TG compared to control (Kim et al, 2007). The result that we provide demonstrates that AgRP-ablated mice have unaltered ability to clear TG after an oral charge despite their obesity.

We agree with the referee that using the term “enhanced utilization” as a strict conclusion for Fig. 4e, f, g might suggest that only muscle TG breakdown is involved in the result obtained whereas it is reasonable to assume TG storage in the adipose tissue might also significantly contribute to the differential pattern observed after TG injection. In the manuscript we have change “enhanced utilization “ by “enhanced storage and utilization”

5) Fig. 5: the reviewer did not understand the mechanism by which Complex I respiration decreased in the mice. It may be secondary effect of obesity rather than the cause of obesity. Furthermore, why is energy expenditure high (Fig. 2A), while mitochondrial function is impaired? Why are they resistant to the impairment of glucose metabolism under HFD feeding (Fig. 6)?

The decrease in complex I activity specifically affect the oxidative-lipid burning soleus muscle and not the white glycolytic muscle. It does correlate with the reciprocal change in norepinephrine turnover rate observed in this tissue prior to the obesity (Fig. 3d, e).

At the level of the muscle, It is Complex I maximum activity that is reduced, meaning the capability of complex I in soleus muscle in condition of maximum substrate, whereas in the muscle the other respiratory complex II (lipid oxidation) is unaffected. These changes associated with enhanced substrate utilization for lipid will translate into a shift in mitochondrial oxidative substrate towards lipids and complex II utilization.

In non-saturating condition (V0) no difference is observed between soleus and white muscle for both complex I and II. In conclusion, the phenotype observed leads to an enhanced capability to utilize lipid substrates in soleus mitochondrion with otherwise unaltered glucose utilization capabilities in complex I in white muscle. In that regards one should expect a better capability to handle lipids under high fat diet which will protect AgRP-ablated mice from deleterious action of ectopic fat deposition.

Thus mitochondrial function is not impaired but there is a remodeling of oxidative muscle towards higher capacity to utilize fatty acids.

The result obtained in Fig 3e show that an increase in NE Turn over rate in soleus oxidative muscle exists prior to the obesity in AgRP-ablated mice. According to the literature, an increase in catecholamine outflow onto muscle leads to increased substrate utilization (Shiuchi et al, 2009).

We therefore suggest that this early change in oxidative muscle ANS output will contribute to increase lipid availability in this muscle together with mitochondrial adaptation towards lipid substrate. Data Fig. 4 brings indirect support to our hypothesis since we found that, unlike obesity of *ob/ob* mice for instance (Kim et al, 2007)), AgRP-ablated mice display and enhance ability to clear triglycerides when injected as an oral bolus (Fig 4. e, f, g)

In addition, although we cannot preclude that an adaptive metabolic remodeling secondary to obesity could also potentiate mitochondrial oxidative change, we have accumulated several argument to support that a metabolic set point in favor of lipid utilization was an integrated consequence of the lack of AgRP-neurons regardless of obesity:

1-AgRP-ablated mice are not only resistant to the impairment of glucose metabolism under HFD feeding; their insulin and glucose profile is actually improved by the high fat regiment.

2-When AgRP-ablated and naïve are subjected to HFD soon after weaning prior to any body weight difference (Supplementary Fig. 7) both group will develop obesity but both O2 consumption and glucose tolerance are increased in AgRP-ablated mice.

In both case animals were tested for metabolic efficacy and glucose tolerance once AgRP-ablated and naïve animals reached a similar degree of obesity.

We therefore promoted the hypothesis that the metabolic set point created by the lack of AgRP neuron, partly through increased muscle lipid preference, participate to the paradoxical beneficial action of the HFD observed in AgRP-ablated mice.

6) Fig. 7: the effect of Bretasenil (mp) may not be specific in AgRP neurons-deficient mice. Bretasenil would decrease fat content in other obese mice too, because GABA regulates many functions of the brain and body.

Bretazenil binds to benzodiazepine site $GABA_A$ receptor and will potentiate the action of $GABA_A$ receptor agonist. $GABA_A$ receptor is mostly expressed in the central nervous system (CNS) in that regards the action of bretazenil should be mostly restricted to the CNS.

Nonetheless, the comment of the referee#3 prompted us to further explore the difference between the obesity displayed by AgRP-ablated mice and other model of obesity.

We have therefore performed new experiments in order to compare the respiratory quotient profile in obese AgRP-ablated mice and other form of obesity: the leptin deficient *ob/ob* mice.

1-Respiratory quotient evolutions during basal condition were acquired on a new group of animals including 5-6 months-old lean naïve animals; obese AgRP-ablated and obese *ob/ob* mice. (n=4-6 in each group). Data are presented as an average of 3 consecutive days in an additional figure in Supplementary Fig. 2a. The results show that this decrease in RQ at the entry of the dark period was a specific feature of AgRP-ablated mice. Indeed, when acquired simultaneously the RQ profiles of AgRP-ablated mice was found to significantly differ from both naïve and o*b/ob* mice at the entry and exit of the dark cycle (Supplementary Fig 2a) while naïve and *ob/ob* displayed similar profile. The value for RQ at the same time point than in Fig. 3d and Fig.6 at the entry of the dark cycles was analyzed using repeated measure ANOVA and shows significant difference between obese AgRP-ablated mice compared to naïve or *ob/ob* mice (Supplementary Fig. 2a).

This result reproduces the data obtained in Fig. 3d and Fig. 6 and supports our claims that the decrease in RQ profile observed is a specific feature of the obesity displayed by mice lacking AgRP-ablated mice. Furthermore, this result is in good agreement with study showing that selective invalidation of the gene encoding the vesicular GABA transporter (VGAT) in AgRP-neurons also display a decrease in RQ (Tong et al, 2008).

2- We also have performed new experiments in order to compare the action of bretazenil of body composition in naïve, obese AgRP-ablated mice and obese *ob/ob* mice. In that experiment, animals

were implanted with subcutaneous minipumps (mp) delivering bretazenil (0.25µl/hr; 3mg/ml), in a similar experiment setting than previously described. Body composition was assessed at day 0 and 21 days after implantation of the mp. The results of that experiment are presented in an additional Supplementary Fig. 9. Using paired test repeated paired student ttest we found that over the course of 21 days bretazenil treatment induced a small but significant decrease in body fat mass selectively in AgRP-ablated mice while body fat content in naïve and *ob/ob* mice remained unchanged (Supplementary Fig. 9b).

Interestingly we also found that *ob/ob* mice actually gained body weight during the treatment (Supplementary Fig. 9a)

This result shows that GABA tone restoration has beneficial consequences selectively on the obesity displayed by AgRP-ablated mice and not *ob/ob* mice. We believe that this result strengthens our claim that GABA release by AgRP neurons is a core mechanism in the defect in nutrient partitioning observed in AgRP-ablated mice.

In addition, how does improved carbohydrate utilization by Bretazenil decrease fat content?

The measure of respiratory quotient provided us with an integrated view of overall energy substrate utilization. We believe that bretazenil treatment, by restoring, at least partially, a missing GABA tone to preganglionic structures impacted on the ANS outflow to downstream tissue.

We agree with the referee#2 that we do not provide here any precise description of the mechanism by which bretazenil treatment leads to reduced adipose store. We believe that what we observed implies a global change in inter-organ communication and activities, and it would be difficult for us to identify one precise mechanism in the present study. We suspect that central restoration of GABA tone by restoring ANS output would change liver carbohydrate transformation and TG production. This would reduce TG availability for adipose storage. Since we found that adipose tissue lipolysis remained unaltered in AgRP-ablated mice (Supplementary Fig. 5) we assume that the overall consequence would be a decrease in adipose store. We have made a change in the manuscript to clearly state that mechanistic hypothesis in the result of Fig 7.

7) Cell death by diphtheria toxin may activate microglia and inflammatory response in the brain. Inflammatory response in the hypothalamus and brain would cause obesity and affect many brain functions non-specifically. Negative control may be necessary.

Acute ablation of AgRP-neurons in adult animals are indeed associated with increased inflammatory response locally in the arcuate nucleus (Wu et al, 2008a; Wu et al, 2008b), but the starvation that resulted from this manipulation could be rescued by bretazenil injection in the parabrachial nucleus (Wu et al, 2009) regardless of the local inflammation created.

Moreover, similar cell-knock out strategy were used to selectively ablate the POMC neurons in adult animals and resulted in increased body weight rather than starvation (Gropp et al, 2005). It is likely that in both case a similar local inflammation was created by cell death but the general output being the opposite it is reasonable to believe that inflammation was not determinant in the phenotype.

Finally and more importantly, the model we present in this study results from the ablation of AgRP-neurons achieved during post-natal period (5-8 days after birth). We agree with the referre#2 that local inflammation might occur at that time but we believe that 3-6 months after the injection, when we performed the study on adult animals the local inflammation is gone.

Comparative DNA array from hypothalamic punches were performed in animals in which AgRP-neurons were ablated during neonatal period or at adult stage. Gene expression analysis clearly showed an increase in transcript associated with inflammation in the hypothalamus of mice in which AgRP neurons were killed at adult stage (that is in the last week) but in animals lacking AgRP neurons from birth there were no sign of increased inflammation compared to control animals.

Although we feel that adding these result to the present manuscript might be beyond the scope of this study we can provide these results if referee#2 believes it is necessary.

8) The authors must have used post hoc analysis after ANOVA. It should be described.

The referee#2 is right, we have used post hoc test, it is now clearly stated in figure legend and Statistical Methods.

9) In Page 7, second paragraph: "Fig. 2c" is a mistake.

This is indeed a mistake; Fig. 2c was replaced by Fig. 2d

10) Bar graphs in Fig. 3D and E: which is soleus?
Figure 3d, e, the histogram were indeed misplaced and were inverted this is now corrected. The histogram showing soleus cTR is now on the far right (Fig. 3e).

Referee #3

In this paper, Joly-Amado et al. reveal a role for AgRP- neurons in the control of nutrient partitioning. They show that ablation of AgRP neurons leads to a change in autonomic regulation of the liver, muscle, and pancreas to promote lipid metabolism on the expense of carbohydrate utilization. Mice lacking AgRP-neurons become obese and hyperinsulinemic on regular chow but display reduced body weight gain and paradoxical improvement in glucose tolerance on high fat diet. The authors argue that their results establish a role for AgRP-neurons in the coordination of efferent organ activity and nutrient partitioning, providing a mechanistic link between obesity and obesity-related disorders.

Comments:
I think that this is an excellent and timely work. Semantically, I disagree with the characterization of these finding providing a "novel" role for AgRP neurons, because this role has been suspected and considered by many. The novelty of the work lies in the fact that this is the first experimental demonstration of such role and the author deserve great credit for this.
We thank the referee#3 for these helpful comments on the manuscript.
We understand the aforementioned comment and we have modified the statement in the abstract, introduction and discussion.

Along the same line, because carbohydrate metabolism is enhanced in AgRP ablated mice, I am not sure whether the finding that they are resistant to high fat diet should be characterized as paradoxical.

We agree with the referee#3 that the statement is matter of discussion. We would like to stretch out that AgRP-ablated mice are not only resistant to the impairment of glucose metabolism under HFD feeding; their insulin and glucose profile is actually improved by the high fat regimen. In that regards, it is the beneficial action of a HFD on glucose metabolism that we considered to be “paradoxical”. In the manuscript, we were unclear in that statement and it has been corrected.

By eliminating the AgRP system, increased carbohydrate metabolism maybe explained, at least in part, by elevated activity of the POMC neurons. These issues could be discussed a bit more thoroughly citing works delineating the anatomical and functional connectivity between these arcuate nucleus neuronal populations.

We agree with referre#3 that we cannot dispute that increase melanocortin tone might also be operant in the phenotype we observed. We have implemented the discussion in that regards according to the suggestion of referee#3.

Facing the counter-intuitive result that mice lacking the endogenous melanocortin antagonist AgRP become obese, we proposed that the lack of GABA release (and may be some other molecules) might also contribute to the phenotype that we observed. This view is consistent with the recent literature (Dietrich & Horvath, 2009a; Dietrich & Horvath, 2009b; Wu & Palmiter, 2011). Furthermore, selective activation of AgRP neurons using optogenetics demonstrated that inhibition of the a-MSH signaling cascade might not be required for AgRP-neurons to initiate the complex behavioral sequence that leads to food intake (Aponte et al, 2011).

Finally, we have tested experimentally whether lack of AgRP-neuron could have triggered any hypersensitivity to the melanocortin system that would have resulted in increasing the sensitivity to low doses of AgRP injected centrally. In our hands, low doses of AgRP (0.1nmol) resulted in a

similar increase in food intake in both naïve and AgRP-ablated mice, suggesting that loss of AgRP neurons did not lead to AgRP hypersensitivity.

The authors may also consider recent works that showed that selective impairment of AgRP neuronal excitability appears to shift metabolic profile in agreement with the findings of the present paper (Dietrich MO et al., 2010 JNS).

This study is indeed in great accordance with the concept we are trying to promote. The discussion was modified in that regards together with more thorough reference to the literature.

On the schematic illustration, the 3 axons leaving the AgRP neurons should be changed to 1 which, once leaving the cell body, splits to 3. One cell body-one axon initial segment.

We thank the referee for that input; we have corrected that mistake in Figure 8

REFERENCES

Aponte Y, Atasoy D, Sternson SM (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nature neuroscience* 14: 351-355
Bray GA (1991) Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. *The Journal of nutrition* 121: 1146-1162
Bray GA, York DA (1998) The MONA LISA hypothesis in the time of leptin. *Recent progress in hormone research* 53: 95-117; discussion 117-118
Buijs RM, Kreier F (2006) The metabolic syndrome: a brain disease? *J Neuroendocrinol* 18: 715-716
Butler AA, Kozak LP (2010) A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. *Diabetes* 59: 323-329
Dietrich MO, Horvath TL (2009a) Feeding signals and brain circuitry. *Eur J Neurosci* 30: 1688-1696
Dietrich MO, Horvath TL (2009b) GABA keeps up an appetite for life. *Cell* 137: 1177-1179
Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, Plum L, Balthasar N, Hampel B, Waisman A, Barsh GS, Horvath TL, Bruning JC (2005) Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci* 8: 1289-1291
Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G, Jelicks LA, Mehler MF, Hui DY, Deshaies Y, Shulman GI, Schwartz GJ, Scherer PE (2007) Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest* 117: 2621-2637
Nonogaki K (2000) New insights into sympathetic regulation of glucose and fat metabolism. *Diabetologia* 43: 533-549
Shiuchi T, Haque MS, Okamoto S, Inoue T, Kageyama H, Lee S, Toda C, Suzuki A, Bachman ES, Kim YB, Sakurai T, Yanagisawa M, Shioda S, Imoto K, Minokoshi Y (2009) Hypothalamic orexin stimulates feeding-associated glucose utilization in skeletal muscle via sympathetic nervous system. *Cell Metab* 10: 466-480
Tong Q, Ye CP, Jones JE, Elmquist JK, Lowell BB (2008) Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nat Neurosci*
Wu Q, Boyle MP, Palmiter RD (2009) Loss of GABAergic signaling by AgRP neurons to the parabrachial nucleus leads to starvation. *Cell* 137: 1225-1234
Wu Q, Howell MP, Cowley MA, Palmiter RD (2008a) Starvation after AgRP neuron ablation is independent of melanocortin signaling. *Proc Natl Acad Sci U S A* 105: 2687-2692
Wu Q, Howell MP, Palmiter RD (2008b) Ablation of neurons expressing agouti-related protein activates fos and gliosis in postsynaptic target regions. *J Neurosci* 28: 9218-9226
Wu Q, Palmiter RD (2011) GABAergic signaling by AgRP neurons prevents anorexia via a melanocortin-independent mechanism. *Eur J Pharmacol*

Detailed description of the changes made
Manuscript correction
1-Figure 3D, E, the histogram were indeed misplaced and were inverted this is now corrected
2-line 159, (Fig. 2c was replace by Fig. 2d)

3-line 521 (50nmol/kg)
4-line 334 (Fig. 2d was changed in Fig.2d)
5-line 340 “the” was inserted before treatment
6-line 380 “increases locomotors” was replaced by increased locomotor
7-line 382 (Supplementary Fig e) was replace by (Supplementary Fig 7e)
8-line 440 “the ANS has be distinct” was replaced by “the ANS has distinct”
9-line 451 “is result” was replaced by “results”
10-line 462 “in ? glucose” was replaced by “in glucose”
11-line 584 “temperatue” was replaced by “temperature”
12-line 615 “R. V” was replaced by “R. C”
13-line 891 “distribution and” was replaced by “distribution”
14-line 896 (red bars) was replaced by (red circles)
15- Line 607: The sentence “We thank Lydia Danglot for helpful input in statistical analysis of body composition data” was added to the acknowledgment.
Response to referee #2
1-we have changes the manuscript in order to state more clearly that ANS changes preceded obesity
2- Fig. 3f was changed with new experimental data showing clonidine action onto insulin level in non-symptomatic AgRP-ablated mice
3-line 264, “enhanced utilization “ was replaced by “enhanced storage and utilization”
4-Supplementary Figure 9 was created to incorporate new experimental data comparing RER profile in obese *ob/ob* mice and AgRP-ablated mice and the impact of bretazenil treatment onto body weight and body fat content.
5-Supplementary information. Line 65: the legend was modified by “lean naïve (black bars) and obese AgRP-ablated.
6-Line 879, Figure 3 legend was modified for “Plasma insulin levels after 5-hr food deprivation were measured prior (time=0) and 30 min after an intraperitoneal injection of the a2-adrenergic receptor agonist clonidine (50 nmol/kg of body weight) (f, g). (a-f) All measurement were acquired on lean naïve (black bar & black circles) and lean AgRP-ablated mice (red bar & red circles) at a time point that preceded obesity (n = 5-8 in each group) while Fig 3g present plasma insulin change after clonidine injection in lean naïve and obese AgRP-ablated mice after obesity has established (g), (n=6-8 in each group). Displayed values are means ± SEM. * P <0.05.”
7-Page 26, line 581: Statistical Methods was changed for “Data were analyzed by unpaired student’s t-test or, where noted, paired student ttest or repeated measures ANOVA followed by post hoc Tukey test (using Sigmplot 11 software, Systat). Analysis of body temperature was performed using a general linear model. In all tests a p value < 0.05 was considered significant. All data are presented as means +/- standard error of the mean or unless specified otherwise.”
8-line 351, a paragraph was added to state our hypothesis regarding the potential mechanism by which bretazenil treatment could affect adipose store:
“The change in body fat store induced by bretazenil treatment could be the indirect consequence of central restoration of ANS output to the liver, which in turn would decrease TG production. A sustained lipolytic activity of the adipose tissue combined with a reduced supply in TG availability will *in fine* lead to a reduced adipose storage.”
9-line 158 Fig. 2c was corrected for Fig. 2d.
10- Page 7, line 150 this sentence was added “In addition, control experiments including *ob/ob* mice and AgRP-ablated mice at a comparable level of obesity clearly showed a positive energy balance in *ob/ob* mice (consumed/expended) that was about twice that of both naïve and AgRP-ablated mice (supplementary Fig. 1d)”.
11-Supplementary Fig. 1 lwas completed by Fig1.d and the legend changed accordingly by adding line 30 “A group of *ob/ob* mice (blue), obese AgRP-ablated mice (red) and naïve (black) were also analyzed for whole energy expenditure a daily energy balance (total calories intake/whole energy expended) (d). (n=3-4) in each group).”
12-page 20, line 456 the sentence “malic enzyme (a lipogenic marker) in perirenal and subcutaneous adipose tissue of AgRP-ablated compared to. naïve mice (2.09 ± 0.25 vs. 1 ± 0.29, p < 0.05, and 2.36 ± 0.53 vs. 1 ± 0.25 p < 0.05, normalized mRNA level respectively) “ was added
13-Page 10, line 222 the text was changed to introduce the new result obtained with clonidine injection. The following sentence was added “In order to evaluate if the decreased SNS outflow is a primary contributor of hyper-insulinemia, we attempted to pharmacologically correct this phenotype in AgRP-ablated mice prior to, and after the establishment of obesity.. We found indeed that injection of the a2AR agonist clonidine normalized plasma insulin levels in AgRP-ablated mice in both lean (Fig. 3f) and obese condition (Fig. 3g)”

14-Pge 10, line 229 the sentence "Note that the injection of a muscarinic receptor antagonist atropine methyl bromide had no effect (data not shown). These results strongly support our hypothesis that increased plasma insulin levels were an early consequence of decreased SNS outflow (but not PNS) onto pancreas and not simply a secondary adaptation to obesity. "was added.
15- A sentence was modified in the abstract, Page 2, line 36 "ablation of AgRP-neurons…that favors the utilization of lipids over carbohydrates, and…as consequence mice lacking AgRP neurons become obese" was changed for : "We report that ablation of AgRP neurons leads to a change in autonomic output onto liver, muscle, and pancreas affecting the relative balance between lipids over and carbohydrates metabolism".
16- Page 19, line 415: "Moreover, the modulation of transcription 3 (Stat3) signaling specifically in AgRP-neurons alters locomotor activity and promotes resistance to diet-induced obesity independently of AgRP regulation (Mesaros et al, 2008)" was added.
17-Page 19, line 434: "For instance, selective inactivation of the insulin receptor in AgRP-neurons but not in POMC-Neurons results in altered hepatic insulin sensitivity (Konner et al, 2007) demonstrating a central dichotomy in the action of insulin that will affect both neuronal population but with different peripheral outcome (Konner et al, 2007) }" was added.
18-page 18, line 405a sentence was added "This concept is further substantiated by recent studies showing that Sirt 1 invalidation selectively in AgRP-neurons results in the impairment of the metabolic adaptation induced by a fast and change electrophysiological excitability to ghrelin (Dietrich et al, 2010).
19-page 15, line 325 A sentence was modified: Along that line, we found that intracerebroventricular (ICV) injection of a low dose of AgRP peptide (0.1 nmol) produced a similar increase in feeding in both naïve and AgRP-ablated mice, suggesting that loss of AgRP neurons did not lead "to a hypersensitivity of AgRP that could have resulted from the overall increase in the melanocortin system activity AgRP hypersensitivity" (not shown).
20-Page 36, line 937 GABAa was replaced by Bretazenil
21-Figure 7 the coma on the Y-axis of the RER graph were replaced by dot.
22- Page 15, line 342; the sentence "Importantly, we could show that Bretazenil action onto body fat store was specific to the obesity displayed by AgRP-ablated mice. Indeed, subcutaneous replacement of bretazenil in obese *ob/ob* mice resulted in body weight gained but no loss in body fat while inducing a small but significant decrease in adiposity selectively in obese AgRP-ablated mice (supplementary Fig.9)" was added
23-Page 8, line 163 the sentence "In addition, this decrease in RQ at the entry of the dark period was a specific feature of AgRP-ablated mice. Indeed, when acquired simultaneously the RQ profiles of AgRP-ablated mice was found to significantly differ from both naïve and o*b/ob* mice at the entry and exit of the dark cycle (Supplementary Fig 2a)." Was added
24-page 8, line 168 (Supplementary Fig. 2) was replaced by (Supplementary Fig. 2a)
25-Page 8, line 168 "In addition" was replaced by "Finally"
Response to referee #3
1-Page 2, Line 40 abstract: "These results establish a novel role for AgRP-neurons in.."was replaced for "These results provide a direct demonstration of a role for AgRP-neurons in…"
2-Page 5, line 111: the end of the introduction was modified "novel function" was replaced by "key function"
3-Page 21, line 472: end of the discussion, "We thus define a novel role" a novel" was replaced by "We thus demonstrate a role.."
5-Page 3-4, line 69 new references were added (Cowley et al, 2001; Horvath et al, 1992; Pinto et al, 2004) and (Dietrich & Horvath, 2009; Wu et al, 2009; Wu et al, 2008; Wu & Palmiter, 2011).
6-Page 14, line 318- the sentence "AgRP-neuron also makes GABA (Horvath et al, 1997)" was added
7-The summary scheme Figure 9 has been changed according to the comments of referre#3
New experimental data
Supplementary Figure 1d
A new experimental experiment was performed to assess the daily calories balance between two different models of obesity: the *ob/ob* leptin deficient mice and the AgRP-ablated mice. Using automated measure of calories intake and indirect calorimetry technics this experiment shows that, while total energy intake/energy expended is similar between naïve and AgRP-ablated mice, the *ob/ob* mice present a clear positive balance.
Supplementary Figure 2 was modified to incorporate a new experiment
Supplementary Fig. 2A shows the result of a new experiment that was performed to compare the RER profile in naïve, obese AgRP-ablated mice and obese *ob/ob* mice. Representative distribution

of respiratory quotient (VCO_2/VO_2) acquired by indirect calorimetry on the course of several days in naïve (black) and obese AgRP-ablated mice (red) and obese *ob/ob* mice (blue).
Figure 3f
A new experiment of clonidine injection was performed on a group of lean naïve and AgRP-ablated mice prior to any body weight difference. In this experiment the hyperinsulinemia in AgRP-ablated mice could also be corrected by clonidine injection. These result replaced the initial Fig. 3F.
Supplementary figure 9
A new experiment was also added to evaluate the action of bretazenil onto body composition in different model of obesity: the *ob/ob* leptin deficient mice and the AgRP-ablated mice. A group of lean naïve, obese AgRP-ablated mice and obese *ob/ob* mice were implanted with subcutaneous minipump delivering bretazenil and body composition was assessed at day 0 and 21 days after implantation in similar experimental setting as presented in Figure 7c.

REFERENCES

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411: 480-484

Dietrich MO, Antunes C, Geliang G, Liu ZW, Borok E, Nie Y, Xu AW, Souza DO, Gao Q, Diano S, Gao XB, Horvath TL (2010) Agrp neurons mediate Sirt1's action on the melanocortin system and energy balance: roles for Sirt1 in neuronal firing and synaptic plasticity. *J Neurosci* 30: 11815-11825

Dietrich MO, Horvath TL (2009) GABA keeps up an appetite for life. *Cell* 137: 1177-1179

Horvath TL, Bechmann I, Naftolin F, Kalra SP, Leranth C (1997) Heterogeneity in the neuropeptide Y-containing neurons of the rat arcuate nucleus: GABAergic and non-GABAergic subpopulations. *Brain Res* 756: 283-286

Horvath TL, Naftolin F, Kalra SP, Leranth C (1992) Neuropeptide-Y innervation of beta-endorphin-containing cells in the rat mediobasal hypothalamus: a light and electron microscopic double immunostaining analysis. *Endocrinology* 131: 2461-2467

Konner AC, Janoschek R, Plum L, Jordan SD, Rother E, Ma X, Xu C, Enriori P, Hampel B, Barsh GS, Kahn CR, Cowley MA, Ashcroft FM, Bruning JC (2007) Insulin Action in AgRP-Expressing Neurons Is Required for Suppression of Hepatic Glucose Production. *Cell Metab* 5: 438-449

Mesaros A, Koralov SB, Rother E, Wunderlich FT, Ernst MB, Barsh GS, Rajewsky K, Bruning JC (2008) Activation of Stat3 signaling in AgRP neurons promotes locomotor activity. *Cell Metab* 7: 236-248

Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, Horvath TL (2004) Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 304: 110-115

Wu Q, Boyle MP, Palmiter RD (2009) Loss of GABAergic signaling by AgRP neurons to the parabrachial nucleus leads to starvation. *Cell* 137: 1225-1234

Wu Q, Howell MP, Cowley MA, Palmiter RD (2008) Starvation after AgRP neuron ablation is independent of melanocortin signaling. *Proc Natl Acad Sci U S A* 105: 2687-2692

Wu Q, Palmiter RD (2011) GABAergic signaling by AgRP neurons prevents anorexia via a melanocortin-independent mechanism. *Eur J Pharmacol*

2nd Editorial Decision 09 August 2012

Thank you for submitting your revised MS to the EMBO Journal. Your study has now been seen by the original referee #2 and the comments are provided below. As you can see the referee appreciate the introduced changes and support publication here. The referee has comments regarding the

interpretation of some the results, but that can be resolved with appropriate text changes. Once we get these last issues resolved then we will proceed with the acceptance of the paper for publication here.

Thank you for submitting our interesting paper to the EMBO Journal

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #2

This paper improved. Although the data are still descriptive and does not explain the mechanism, the results are interesting and important. The reviewer only has some comments regarding the interpretation of the results, as follows:

1) The last paragraph in the result section (page 16) is overstating. There is no evidence that bretazenil changed ANS output to the liver and decrease TG production. Furthermore, this paper does not show any data of lipolysis.

2) Fig. 4F and G: The authors demonstrate that the results of Fig. 4F and G mean enhanced storage and utilization of TG in periphery. However, many researchers including the reviewer interpret that the results are due to the decrease of utilization of FFA and glycerol. The authors should interpret and discuss more carefully.

2nd Revision - authors' response 15 August 2012

Detailed description of the changes made
Manuscript correction
1- Page 16, line 350. According to the comment of referee #2 the last chapter of page 16 has been deleted.
2-Page 12, line 262-268 were modified so as to propose an alternative hypothesis in which decrease utilization of glycerol and FFA could also explain the result obtained in **Fig. 4.f, g.** The section has been modified as follow:

"These observations show that, despite their obesity, mice lacking AgRP neurons retained the ability to store TG. Both free plasma glycerol and FFA, the products of TG breakdown, were significantly increased in mice lacking AgRP-neurons (**Fig. 4f,g**). The latter result could point towards an enhanced TG breakdown that would be consistent with enhanced utilization of TG in the periphery. However, this result could also be explained by a decrease utilization of FFA and glycerol. "

