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By Ashley Kate Castellanos Jankiewicz

**Bile acids signaling as a novel mechanism in the hypothalamic
control of energy balance**

Directed by: Daniela Cota, MD, HDR

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Everyone serves the good wine first, and when people have drunk freely, then the poor wine. But you have kept the good wine until now. - John 2:10

Not that there is good wine and bad wine (at least not when you do your thesis in Bordeaux). But I'd like to finish by thanking the people who are most important to me:

Alicia, my mom, and Arturo, my dad, were the academic adventurers of their time. With their real-life, almost fairytale, stories enriched with *once in an improbable lifetime* experiences, you have been the great inspirers of the path I chose to take. Thank you for your unconditional support from the very start, and for the assurance that, no matter where I go or whatever happens, I will never be too far from home. I am also grateful to have not only one, but two people who are always on my side: thank you **Alec and Lee, my brothers**, for watching out for me, guiding me and listening. I wouldn't have gone very far without your examples. Geographical distance doesn't mean much with a family like ours

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Abstract (English)

Résumé (Français)

Résumé *in extenso* (Français)

ABSTRACT (ENGLISH)

Title: Bile acids signaling as a novel mechanism in the hypothalamic control of energy balance

Introduction: Bile acids (BA) are cholesterol-derived molecules mostly known for their role in digesting lipids. By activating the Takeda G protein coupled receptor 5 (TGR5) in peripheral organs, they can also act as signaling molecules to reduce body weight and improve glucose homeostasis. Notably, TGR5 activation can increase energy expenditure in brown adipocytes, although the metabolic pathways involved in these effects are not yet clear. These outcomes imply an anti-obesity function for TGR5. However, all studies investigating BA in energy balance have exclusively focused on peripheral tissues. Since the major center of convergence of nutrient, hormonal, and environmental cues is the brain, particularly the hypothalamus, we hypothesized a role for TGR5 in this brain structure, suggesting that hypothalamic TGR5 activity may participate in energy balance, specifically under diet-induced obesity.

Objective: To demonstrate the function of the BA δ TGR5 system in hypothalamic populations known to control energy homeostasis, and disentangle its relevance for the treatment of diet-induced obesity.

Methods: C57Bl6/J male mice that were either lean (standard chow) or diet-induced obese (60% high-fat diet; HFD) were implanted with an intra-cerebroventricular (ICV) cannula for the pharmacological delivery of TGR5 agonists. TGR5^{flox/flox} mice were used to target the site-specific deletion of the receptor within the mediobasal hypothalamus (MBH), through the stereotaxic delivery of AAV-Cre. The following metabolic outputs were measured: body weight, food intake, body composition (EchoMRI analyzer), insulin sensitivity, serum and hypothalamic BA (liquid mass spectrometry), and energy expenditure (TSE Phenomaster system). To block sympathetic signaling, we exposed mice to thermoneutrality (30°C) or performed chemical sympathectomy (6-hydroxydopamine; 80mg/kg i.p.). Markers of lipolysis, thermogenesis, and thyroid metabolism were measured in the liver, adipose and hypothalamic tissues by qPCR or western blots. All studies received the approval from the animal ethical committee of the University of Bordeaux.

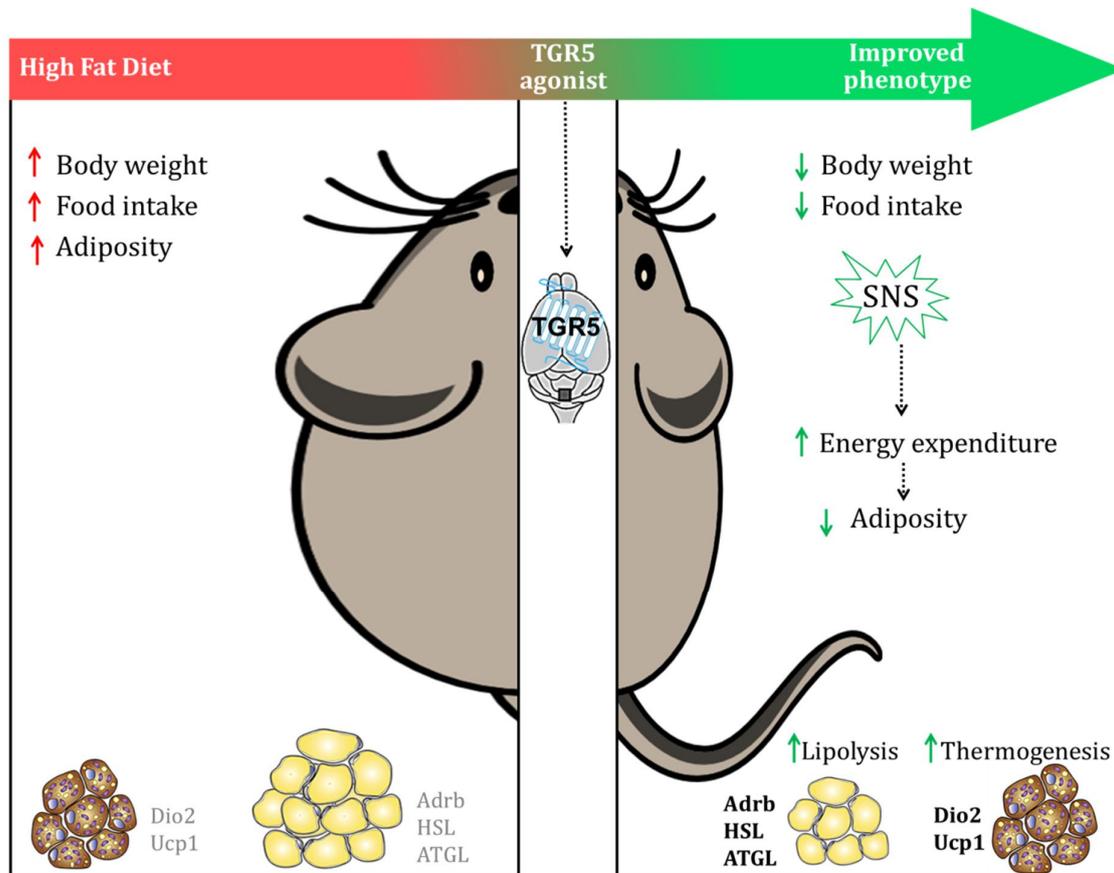
Results: We demonstrate that TGR5 and BA transporters are expressed in the MBH and that diet-induced obese mice have decreased circulating and hypothalamic BA. Acute ICV or

intra-MBH administration of TGR5 agonists reduced food intake and body weight in diet-induced obese mice only, and improved insulin sensitivity. Accordingly, chronic ICV administration of the TGR5 agonist in obese mice reduced their body weight and adiposity, while increasing energy expenditure and mRNA markers of sympathetic activity in the adipose tissue. Indeed, experiments conducted at thermoneutrality or chemical sympathectomy blunted these effects, demonstrating that central TGR5 effects require an enhanced sympathetic tone. By using TGR5^{flox/flox} mice coupled with the delivery of an AAV-Cre, we observed that the deletion of TGR5 in the MBH had no effect in chow-fed mice. However, a HFD switch rapidly increased their body weight, food intake and adiposity. When exposed to the cold (4 h at 4°C), protein levels of lipolysis and thermogenesis markers in the adipose tissue were blunted, implying an interruption in sympathetic signaling to the periphery due to hypothalamic downregulation of TGR5. Lastly, Cre-dependent deletion of TGR5 in the MBH of already obese mice rapidly increased adiposity by inducing hyperphagia, worsening their obese phenotype.

Conclusions: Our work proves the existence of a functional hypothalamic BA ó TGR5 receptor system. We show for the first time that the activation of TGR5 in the MBH decreases body weight and adiposity, while increasing energy expenditure through recruitment of the sympathetic nervous system. Taken together, these results expose a new mechanism of action for potential anti-obesity therapies.

Keywords: Bile acids, TGR5, diet-induced obesity, mediobasal hypothalamus, sympathetic activity, thermogenesis.

GRAPHICAL ABSTRACT



Highlights

- The Takeda G-protein coupled receptor, TGR5, is a specific bile acids receptor that is expressed in the brain
- Acute or chronic central activation of TGR5 improves a diet-induced obese phenotype by decreasing body weight and food intake through a process that likely depends on the sympathetic nervous system
- This will consequently increase the use of energy for promoting lipolysis in the white adipose tissue and thermogenesis in the brown adipose tissue
- Overall, TGR5 can act centrally to participate in whole-body metabolic control

RÉSUMÉ (FRANÇAIS)

Titre : La signalisation des acides biliaires comme nouveau mécanisme dans le contrôle hypothalamique de la balance énergétique

Introduction : Les acides biliaires (AB) sont des molécules connues pour digérer les lipides. En activant le récepteur couplé à la protéine G Takeda 5 (TGR5) dans les tissus périphériques, ils peuvent également servir de molécules de signalisation pour réduire le poids corporel et améliorer le profil glycémique. L'activation de TGR5 peut aussi augmenter la dépense énergétique dans le tissu adipeux, mais les voies métaboliques impliquées dans ces effets sont encore mal connues. Ces observations impliquent une action anti-obésité du TGR5. Cependant, toutes les études sur les AB dans la balance énergétique se sont concentrées exclusivement sur des tissus périphériques. Comme le principal centre de convergence des signaux nutritifs, hormonaux et environnementaux se trouve dans le cerveau, et en particulier dans l'hypothalamus, nous avons émis l'hypothèse que l'activité hypothalamique du TGR5 pourrait moduler la balance énergétique, en particulier dans un contexte d'obésité.

Objectif : Démontrer la fonction du système AB ó TGR5 dans des populations de cellules hypothalamiques connues pour contrôler l'homéostasie énergétique et étudier sa pertinence pour le traitement de l'obésité.

Méthodes : Des canules intra-cérébro-ventriculaires (ICV) ont été implantées sur des souris mâles C57Bl6/J minces (sous régime standard) ou obèses (sous régime riche en graisses) pour permettre l'administration pharmacologique aiguë ou chronique des agonistes du TGR5. Des souris TGR5^{flox/flox} ont été utilisées pour provoquer la délétion du récepteur dans l'hypothalamus médio-basal (HMB), par l'injection *in situ* d'un AAV-Cre. Nous avons mesuré le poids corporel, prise alimentaire, composition corporelle, sensibilité à l'insuline, niveaux des AB hypothalamiques et plasmatiques et dépense énergétique. Pour bloquer la signalisation sympathique, nous avons exposé les souris à un environnement de thermo-neutralité (30°C) ou à une sympathectomie chimique. Des marqueurs de la lipolyse, de la thermogénèse ou du métabolisme thyroïdien ont été mesurés dans le foie, le tissu adipeux et l'hypothalamus par qPCR ou western blot. Toutes les études ont été approuvées par le comité d'éthique en expérimentation animale de l'Université de Bordeaux.

Résultats : Nous montrons que les transporteurs du TGR5 et des AB s'expriment dans l'HMB et que les souris obèses ont une diminution des AB dans la circulation et

l'hypothalamus. L'administration aiguë d'agonistes du TGR5 (ICV ou intra-HMB) réduit la prise alimentaire et le poids corporel chez les souris obèses, tout en améliorant leur sensibilité à l'insuline. De plus, l'administration chronique ICV de l'agoniste réduit le poids corporel et l'adiposité, tout en augmentant la dépense énergétique et les marqueurs de l'activité sympathique dans le tissu adipeux. La thermo-neutralité ainsi que la sympathectomie chimique atténuent ces effets, démontrant que l'activité du récepteur TGR5 nécessite un tonus sympathique accru. La délétion de TGR5 dans le HMB (souris $TGR5^{flox/flox}$) n'a aucun effet chez les souris minces. Cependant, l'exposition à une nourriture riche en graisse augmente rapidement leur poids, prise alimentaire et adiposité. Lors de l'exposition au froid (4 heures à 4°C), l'expression des marqueurs de lipolyse et thermogenèse dans le tissu adipeux était atténuée, suggérant une interruption de la signalisation sympathique. Enfin, la suppression du TGR5 dans le HMB de souris déjà obèses augmente l'adiposité en induisant une hyperphagie, aggravant l'obésité.

Conclusions : Nos résultats prouvent l'existence d'un système fonctionnel du TGR5 hypothalamique, un récepteur des AB. Nous montrons pour la première fois que l'activation du TGR5 dans le HMB induit une myriade d'effets qui améliorent des paramètres métaboliques, et que cela dépend de l'activation du système nerveux sympathique. Ainsi, nous dévoilons un nouveau mécanisme d'action pour des potentiels traitements contre l'obésité.

Mots clés : Sels biliaires, obésité induite par l'alimentation, hypothalamus médio-basal, balance énergétique, thermogenèse.

RÉSUMÉ *IN EXTENSO* (FRANÇAIS)

Contexte de la recherche

L'obésité est une pathologie caractérisée par une accumulation excessive de graisse, sous forme de tissu adipeux, qui présente un risque potentiel pour la santé (Kyle, Dhurandhar, & Allison, 2016). Aujourd'hui, plus de 13% de la population mondiale est obèse (Marques, Peralta, Naia, Loureiro, & de Matos, 2017). Dans le cas de la France, 49% de la population adulte est en surpoids, dont 17,2% d'obèses (Verdot, Torres, Salanave, & Deschamps, 2017).

Le traitement le plus efficace actuellement disponible contre l'obésité sévère est la chirurgie bariatrique. En suivant cette intervention, les patients ont une perte de poids durable ainsi qu'une amélioration de paramètres métaboliques, tels qu'une sensibilité à l'insuline accrue, une meilleure tolérance au glucose (Ashrafian et al., 2010; Kohli et al., 2013) et une diminution des lipides dans la circulation (Douglas, Bhaskaran, Batterham, & Smeeth, 2015; Sjostrom et al., 2004). De plus, la rémission du diabète de type 2 arrive chez une grande proportion de patients et se maintient plus de 14 ans après la chirurgie (Rubino & Gagner, 2002). Néanmoins, notre compréhension des mécanismes biologiques permettant d'obtenir ces effets étonnants sur le métabolisme reste à ce jour très limitée.

Acides biliaires en tant que molécules de signalisation périphériques

Les acides biliaires (AB) sont aujourd'hui reconnus comme l'un des principaux acteurs des bénéfices de la chirurgie bariatrique. Ces sont des molécules amphipathiques synthétisées à partir de molécules de cholestérol, qui participent à la solubilisation des lipides après un repas. Une certaine quantité d'AB peut passer dans la circulation systémique, ce qui suggère qu'ils jouent un rôle au-delà de la simple digestion (Ferrebee & Dawson, 2015).

Dans le cas des patients souffrant d'obésité, leur taux d'AB circulant après un repas est inférieur à celui des sujets de poids normal (Ahmad, Pfalzer, & Kaplan, 2013; Kohli et al., 2013), mais après une chirurgie bariatrique, ils présentent une augmentation marquée d'AB circulants, allant du double (Kohli et al., 2013; Patti et al., 2009) au triple (Spinelli et al., 2016), ce qui est positivement corrélé avec la quantité de poids perdu (Penney, Kinross, Newton, & Purkayastha, 2015). Pour cette raison, le concept classique d'AB est en train de changer, passant des émulsifiants lipidiques à des molécules de signalisation ayant des effets systémiques répandus.

En effet, deux récepteurs spécifiques des AB ont été découverts en dehors du tract gastro-intestinal. L'un d'eux est le récepteur nucléaire farnésioïde X (FXR) (Wang, Chen, Hollister, Sowers, & Forman, 1999), dont la fonction est plutôt d'assurer un rétro-contrôle, car il inhibe le catabolisme du cholestérol, ce qui diminue la biosynthèse des AB. Le second est le récepteur membranaire couplé à la protéine-G Takeda 5 (TGR5), exprimé dans les tissus périphériques et dans le système nerveux central (SNC) (Kawamata et al., 2003; Maruyama et al., 2006). Il répond aussi aux AB, en ayant une fonction métabolique. En effet, son activation entraîne une amélioration de l'homéostasie du glucose, une augmentation de la dépense énergétique et une inhibition de l'expression de marqueurs inflammatoires, ce qui présente un grand intérêt pour le traitement de l'obésité et du diabète du type 2 (Chavez-Talavera, Tailleux, Lefebvre, & Staels, 2017).

Tous ces effets participent à la régulation de l'homéostasie énergétique globale, un processus étroitement contrôlé par le cerveau. Cependant, bien que l'on sache que le TGR5 soit également exprimé dans le cerveau, le rôle qu'il joue dans le SNC reste inexploré.

Contrôle central de la balance énergétique : l'hypothalamus médiobasal

Le cerveau joue un rôle crucial dans l'équilibre énergétique en recevant et en intégrant des signaux provenant de la périphérie. En particulier, l'hypothalamus reçoit des signaux des organes périphériques par le biais de signaux nutritifs, hormonaux et nerveux et est capable d'intégrer ces signaux afin de contrôler les informations relatives à l'utilisation de l'énergie, conduisant ainsi au maintien de l'équilibre énergétique (Cota, Proulx, & Seeley, 2007).

L'hypothalamus médiobasal (HMB) borde le troisième ventricule, permettant ainsi un contact presque direct avec la périphérie. Cette caractéristique distinctive repose également sur l'éminence médiane dans la partie inférieure de l'hypothalamus, qui est située en dehors de la barrière hémato-encéphalique, de sorte que les protéines et les hormones de la périphérie peuvent filtrer dans l'hypothalamus à un taux plus efficace que dans d'autres régions du cerveau (Rizzoti & Lovell-Badge, 2017). Ces caractéristiques neuro-anatomiques confèrent à l'HMB un rôle privilégié pour la détection rapide des besoins énergétiques (Prevot et al., 2018).

Interaction cerveau et périphérie

Le SNC communique constamment avec la périphérie afin d'ajuster les besoins énergétiques immédiats. Pour ce faire, le système nerveux autonome (SNA) est en lien avec les tissus

périphériques qui, à leur tour, peuvent agir sur le SNC (Seoane-Collazo et al., 2015). Le SNA est principalement régulé par l'hypothalamus, et se divise en systèmes nerveux sympathique et parasympathique (SNS et SNP) (Seoane-Collazo et al., 2015).

En particulier, par le biais de l'hypothalamus, le SNS joue un rôle crucial dans la régulation de la lipolyse dans le tissu adipeux blanc (*white adipose tissue*, WAT) et de la thermogénèse dans le tissu adipeux brun (*brown adipose tissue*, BAT) pour réguler la dépense énergétique. Pour ce faire, l'axe hypothalamo-thyroïdien est particulièrement important pour la modulation du SNS liée à la température corporelle. Le produit final de cet axe est la biosynthèse des hormones thyroïdiennes par la glande thyroïde sous forme de thyroxine (T4), qui sera activée en triiodothyronine (T3) par les déiodinases (Nillni, 2010). T3 peut alors agir sur différentes cibles afin de stimuler le SNS, provoquant la synthèse de catécholamines (telles que la noradrénaline). Celles-ci atteindront finalement les tissus périphériques, tels que le WAT, le BAT, le foie et les muscles pour stimuler la dépense énergétique et la production de chaleur en se liant aux récepteurs α -adrénergiques (Blaszkiewicz & Townsend, 2016).

Dans l'ensemble, le contrôle de l'homéostasie énergétique repose sur une stricte régulation de l'apport et de la dépense énergétique. Cette régulation est centralisée au niveau de l'hypothalamus, qui adapte la prise alimentaire et la dépense énergétique selon les besoins de l'organisme. Cette régulation est cruciale pour l'étude de l'obésité et de ses comorbidités associées.

Hypothèse

Nous émettons l'hypothèse d'un rôle du récepteur TGR5 dans l'hypothalamus, une structure cérébrale impliquée dans le contrôle du comportement alimentaire et de la dépense énergétique, suggérant que l'activité hypothalamique de TGR5 soit pertinente pour le contrôle de l'équilibre énergétique, en particulier en situation d'obésité. Nos **objectifs** sont les suivants :

1. Identifier la présence de composants du système d'AB au niveau de l'hypothalamus ;
2. Tester le système *in vivo* par l'activation pharmacologique aiguë et chronique du récepteur TGR5 dans le cerveau, et identifier les voies moléculaires à l'origine de ses effets ;
3. Identifiez les populations cellulaires de l'AMB responsables des effets métaboliques de l'activation du TGR5 central.

Démarche adoptée

Nos études ont été réalisées chez des souris C57Bl6/J minces (sous régime standard) ou en situation d'obésité induite par le régime alimentaire (60% des apports caloriques provenant de matières grasses). Nous avons réalisé des études aiguës et chroniques d'administration pharmacologique intra-cérébro-ventriculaire (ICV) des agonistes du TGR5. Des souris TGR5^{fl^{ox}/fl^{ox}} ont été utilisées pour provoquer la délétion du récepteur dans l'HMB, par l'injection *in situ* d'un AAV-Cre ou d'un AAV-contrôle. Nous avons évalué le poids corporel, la prise alimentaire, la composition corporelle (EchoMRI), la sensibilité à l'insuline, les niveaux des AB hypothalamiques et plasmatiques (chromatographie en phase liquide couplée à la spectrométrie de masse) et la dépense énergétique (Phenomaster TSE). Pour bloquer la signalisation sympathique, nous avons exposé des souris à un environnement en thermo-neutralité (30°C) ou à une sympathectomie chimique (6-hydroxydopamine ; 80 mg/kg i.p.). L'exposition à court terme au froid (4 heures à 4°C) a été utilisée pour augmenter l'activité sympathique chez des souris avec la délétion du récepteur au niveau de l'HMB. Des qPCR ont été réalisées sur du tissu hypothalamique de souris minces et obèses pour mesurer l'expression des composants du système des AB ainsi que du système thyroïdien, après le traitement chronique chez des souris obèses. En plus, des qPCR et des western blots ont été réalisés dans les tissus adipeux (WAT et BAT) de souris recevant le traitement chronique pour mesurer des marqueurs de lipolyse ou de thermogénèse. Toutes les études ont été approuvées par le comité d'éthique en expérimentation animale de l'Université de Bordeaux.

Résultats obtenus

Les composants du système des AB sont présents dans le cerveau et sont dérégulés en situation d'obésité

Nous avons montré que le récepteur TGR5 est exprimé dans le cerveau et plus particulièrement dans l'HMB de souris minces et obèses. En utilisant la chromatographie en phase liquide couplée à la spectrométrie de masse (UPLC-MS/MS), nous avons démontré que les souris obèses ont des niveaux d'AB plasmatiques et hypothalamiques inférieurs comparés aux souris minces, un fait qui a déjà été rapporté chez les humains obèses (Albaugh et al., 2015). En particulier, l'expression des transporteurs des AB dans l'hypothalamus est surexprimée chez les souris minces après une réalimentation faisant suite à un jeûne de 24 heures (*refeeding*), ce qui suggère que les AB peuvent agir comme molécules de signalisation dans le cerveau pour indiquer en temps réel l'état énergétique lié à la consommation de

nourriture. Ce n'est pas le cas dans notre modèle obèse, puisqu'il n'y a pas de changement dans les transporteurs des AB après la réalimentation, impliquant une altération du transport des AB hypothalamiques en situation d'obésité. En effet, des déficits dans les transporteurs hépatiques des AB dans le foie de sujets obèses ont été décrits, où l'expression de transporteurs est en corrélation négative avec l'obésité, ce qui pourrait contribuer à ralentir la circulation entéro-hépatique (Haeusler et al., 2016).

Ainsi, nos données suggèrent que la signalisation centrale des AB est altérée dans l'obésité, puisque les niveaux circulants des AB pourraient ne pas être suffisants pour atteindre le cerveau et moduler l'expression des transporteurs pour engendrer une réponse postprandiale adéquate.

La signalisation centrale aiguë du TGR5 simule un état postprandial

L'administration ICV ou intra-HMB aiguë d'un agoniste sélectif du TGR5 réduit la prise alimentaire et le poids corporel des souris obèses, tout en améliorant leur sensibilité à l'insuline. Ces effets semblent être médiés par l'activité hypothalamique du récepteur, puisque l'administration ciblée de l'agoniste dans l'HMB produit les mêmes résultats phénotypiques que l'administration par voie ICV.

Au niveau moléculaire, nous avons observé que l'activation centrale aiguë du TGR5 diminue considérablement l'expression hépatique du *Cyp7a1* (*cholesterol 7 hydroxylase 1*), la principale enzyme responsable de la biosynthèse des AB (*rate-limiting enzyme*). Il a été démontré que des marqueurs de la biosynthèse des AB sont 2 fois plus élevés chez des sujets obèses (Glicksman et al., 2010). C'est pourquoi nos données peuvent être interprétées comme un mécanisme pour ramener les taux d'AB à la normalité.

Il est maintenant admis que les AB peuvent agir comme molécules de signalisation pour maintenir l'homéostasie métabolique (Chiang, 2013) et réguler la glycémie (Bunnett, 2014; Thomas et al., 2009). Nous avons observé une diminution de l'expression des marqueurs hépatiques du métabolisme du glucose et de la synthèse des AB (*glucose-6-phosphatase*, *G6pc*, et *Cyp7a1*, respectivement), ainsi qu'une augmentation des marqueurs de synthèse des acides gras (*fatty acid synthase*, *FAS*) chez des souris à jeun, suggérant que l'activation centrale du TGR5 est suffisante pour imiter un état postprandial des tissus périphériques, simulant une situation de forte disponibilité énergétique.

L'activation centrale chronique du TGR5 combat l'obésité par l'action du système nerveux sympathique sur le tissu adipeux

Les effets sur la diminution du poids corporel et de la prise alimentaire ont été reproduits dans notre modèle chronique, où la perte de poids est principalement attribuée à une diminution de l'adiposité. De plus, l'efficacité alimentaire, généralement utilisée dans l'élevage pour quantifier la relation entre la nourriture consommée et sa transformation en une production souhaitée, est significativement diminuée chez nos souris traitées par voie ICV aiguë et chronique. Cela signifie que la perte de poids ne peut pas s'expliquer uniquement par la réduction de la prise alimentaire.

Une possibilité est que le traitement provoque une augmentation de la dépense énergétique, ce qui contribuerait à réduire le poids corporel. Il a été démontré que le TGR5 exprimé dans les adipocytes participe à l'augmentation de l'activité du BAT et de la dépense énergétique par stimulation de la thermogénèse (Broeders et al., 2015; Teodoro et al., 2014; Watanabe et al., 2006; Zietak & Kozak, 2016), qui contribue à réduire l'adiposité et le poids corporel. Nous avons observé que l'activation centrale chronique du TGR5 s'accompagne d'une diminution de l'adiposité et d'une augmentation de la dépense énergétique dans notre modèle d'obésité, ce qui suggère que les effets précédemment décrits liés à l'activation du BAT pourraient être engagés en stimulant le récepteur de façon centrale.

La voie canonique par laquelle l'hypothalamus signale au BAT une augmentation de la dépense énergétique est l'activation du SNS, ce qui stimule la libération de catécholamines qui agissent sur α -adrénorécepteurs pour induire la thermogénèse (Bianco & McAninch, 2013). En effet, nous avons observé une augmentation de l'expression des α -adrénorécepteurs dans le tissu adipeux après le traitement chronique central avec un agoniste du TGR5, couplé à une augmentation de la dépense énergétique, suggérant une implication du SNS dans les effets du récepteur.

Pour étudier cela, nous avons réalisé deux expériences afin d'empêcher l'activité sympathique, et avons testé les conséquences de la stimulation centrale du TGR5 dans ce contexte. La première expérience a consisté à placer nos souris soumises au traitement chronique en thermo-neutralité (où la thermogénèse n'est plus nécessaire, bloquant ainsi le SNS). Comme prévu, il n'y a plus eu d'augmentation de la dépense énergétique (puisque à 30°C il n'est plus nécessaire de générer de la chaleur corporelle), et l'expression des α -adrénorécepteurs et de la deiodinase 2 (Dio-2, qui participe à la conversion de T4 à T3) a été

diminuée dans le tissu adipeux. La seconde expérience a consisté à éliminer la connectivité sympathique périphérique par sympathectomie chimique (6-hydroxydopamine par voie i.p.), puis à exposer nos souris au traitement central de l'agoniste du TGR5. Là aussi, en l'absence d'un SNS fonctionnel, les effets précédemment observés ont disparu. Ensemble, ces résultats prouvent que l'activité centrale du récepteur TGR5 protège du gain de poids corporel par l'intermédiaire d'un mécanisme dépendant du SNS, comparable à ce qui serait attendu si les souris étaient exposées au froid.

Il existe des preuves solides présentant les effets de l'hormone thyroïdienne dans l'hypothalamus comme facteur déterminant de l'activation du BAT (Lopez et al., 2010). Nous avons constaté que la stimulation centrale du SNS dépendante du TGR5 augmente l'expression de la Dio-2 dans le BAT. Par conséquent, il est probable qu'il y ait une plus forte conversion de T4 en T3 dans cet organe, ce qui entraînerait une « fuite » de l'hormone dans la circulation. Cela implique que T3 serait augmentée dans la circulation et qu'elle pourrait accéder à l'hypothalamus (Bianco & McAninch, 2013) pour stimuler le SNS suite à l'activation chronique du TGR5. Bien que des mesures des hormones thyroïdiennes soient actuellement prévues afin de confirmer notre hypothèse, nous avons examiné des marqueurs du métabolisme thyroïdien au niveau de l'hypothalamus. Fait intéressant, nous avons constaté que tous les marqueurs sont sous-exprimés suite à l'administration centrale chronique d'un agoniste du TGR5. Ces résultats suggèrent donc que l'activation centrale du TGR5 stimule l'activité du SNS pour favoriser la conversion de T4 en T3 (par la Dio-2 dans le BAT). En retour, les niveaux élevés de T3 libérés dans la périphérie agiraient au niveau du cerveau pour diminuer l'expression ses propres récepteurs et transporteurs au niveau de l'hypothalamus.

Nos résultats impliquent que l'action du TGR5 dans l'augmentation de la dépense énergétique par la production de chaleur ne se produit pas seulement à travers le TGR5 exprimé dans les adipocytes, comme démontré précédemment (Teodoro et al., 2014; Watanabe et al., 2006; Zietak & Kozak, 2016), mais pourrait également être dépendant d'une signalisation d'ordre supérieur, spécifiquement liée à l'activation hypothalamique du TGR5, pouvant entraîner un phénotype similaire à celui observé lors d'une exposition au froid.

La délétion du TGR5 au niveau de l'HMB augmente l'adiposité et aggrave l'obésité

Pour comprendre l'implication hypothalamique du TGR5 dans les effets métaboliques décrits précédemment, nous avons utilisé un modèle génétique nous permettant de supprimer le TGR5 de l'HMB. Comme nous nous y attendions suite à nos expériences aiguës, la

suppression du TGR5 chez des souris minces n'a pas d'effets phénotypiques évidents. Cependant, lorsque les animaux sont passés sous un régime enrichi en graisses, cette approche génétique entraîne un gain de poids très rapide, principalement attribuable à l'augmentation de l'adiposité. De plus, une exposition aiguë au froid (4 heures à 4°C) nous a permis de confirmer que le TGR5 cause effectivement une perte de poids grâce à l'activation du SNS, puisque les marqueurs de lipolyse et de thermogénèse sont sous-exprimés dans le tissu adipeux en absence du récepteur. De plus, la suppression du TGR5 dans l'HMB chez des souris déjà obèses provoque une augmentation rapide de leur poids corporel, prise alimentaire et adiposité, contribuant davantage au phénotype obèse. Dans l'ensemble, ces résultats indiquent une forte implication du TGR5 exprimé dans l'HMB dans la protection contre l'obésité.

Néanmoins, l'approche virale utilisée pour effectuer la délétion du TGR5 dans l'HMB ne nous permet pas de déterminer les types cellulaires ciblés, principalement parce que le récepteur TGR5 est exprimé à des niveaux très faibles dans le cerveau adulte. Par conséquent, nous explorons actuellement la possibilité d'utiliser des modèles génétiques couplés à l'administration d'AAV-Cre avec des promoteurs spécifiques pour cibler des types cellulaires particuliers dans l'HMB afin d'identifier les populations cellulaires exprimant le TGR5 dans cette région du cerveau.

Conclusions

Nos résultats établissent clairement que les AB n'agissent pas seulement comme molécules de signalisation dans les tissus périphériques, mais peuvent aussi atteindre le cerveau pour activer le récepteur TGR5 et déclencher une myriade d'effets métaboliques bénéfiques, particulièrement dans un contexte d'obésité. Plus spécifiquement, nous avons montré que le TGR5 au niveau hypothalamique joue un rôle dans la physiopathologie de l'obésité. Tout d'abord, nous démontrons un dysfonctionnement des composants du système des AB en situation d'obésité. Ensuite, nous montrons que l'activation pharmacologique aiguë ou chronique du récepteur au niveau central entraîne des améliorations métaboliques chez les souris obèses, notamment une diminution de l'adiposité et une augmentation de la dépense énergétique. De plus, nous prouvons que ces effets sont induits par le recrutement du SNS pour augmenter la lipolyse et la thermogénèse dans le WAT et BAT, respectivement. Enfin, nous démontrons que le récepteur TGR5 est en réalité nécessaire pour obtenir une amélioration du phénotype, puisque sa délétion ciblée dans l'HMB augmente l'adiposité en présence d'un régime riche en graisses, bloque le tonus sympathique et aggrave le phénotype

obèse. Nos travaux placent le récepteur TGR5 hypothalamique comme un médiateur clé des effets du SNS qui, *via* sa signalisation dans le tissu adipeux, diminue le poids corporel, dévoilant ainsi un nouveau mécanisme d'action pour de potentielles thérapies anti-obésité. D'autres études seront nécessaires pour identifier les types de cellules impliqués dans les effets médiés par le TGR5 hypothalamique sur le métabolisme.

Scientific output

List of abbreviations

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SCIENTIFIC OUTPUT

Published research articles:

1. André C, Guzman-Quevedo O, Rey C, Rémus-Borel J, Clark S, **Castellanos-Jankiewicz A**, Ladeveze E, Leste-Lasserre T, Nadjar A, Abrous DN, Laye S, Cota D. Inhibiting microglia expansion prevents diet-induced hypothalamic and peripheral inflammation. *Diabetes*. 2017 Apr;66(4):908-919.
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3. Soty M, Vily J, **Castellanos-Jankiewicz A**, Guzman-Quevedo O, Raffin M, Clark S, Vulin F, Silva M, Gautier-Stein A, Cota D, Mithieux G. Brain circuit by which glucose sensing in the portal vein controls energy homeostasis.

Book chapters:

1. González-Franco L.R., **Castellanos Jankiewicz A**. (2019). Riesgos nutricionales y cuidado nutricional en el paciente con enfermedad de Parkinson (1st Ed.). In Entendiendo al paciente con enfermedad de Parkinson (pp. 219-228). México: Minaya Editorial. ISBN: 978-1-5323-9476-8.

Oral communications:

1. **Castellanos-Jankiewicz A**, Guzmán-Quevedo O, Fénelon V, Zizzari P, Clark S, Dupuy N, Bellocchio L, Marsicano G, Cota D. Role of hypothalamic bile acids signaling in the control of energy balance (May, 2019). 21st European Congress of Endocrinology, ESE (Lyon, France). Abstract selected for 2 oral communications: one as part of the European Young Endocrine Scientists (EYES) Symposium.
2. **Castellanos-Jankiewicz A**, Guzmán-Quevedo O, Fénelon V, Zizzari P, Clark S, Dupuy N, Bellocchio L, Marsicano G, Cota D. Role of hypothalamic bile acids signaling in the control of energy balance (April, 2018). 7th Neurocentre Magendie Symposium (Bordeaux, France).
3. **Castellanos-Jankiewicz A**, Guzmán-Quevedo O, Fénelon V, Zizzari P, Clark S, Dupuy N, Bellocchio L, Marsicano G, Cota D. Role of hypothalamic bile acids signaling in the regulation of energy balance (April, 2018). IMPRS retreat 2018, Bordeaux Neurocampus (Bordeaux, France).
4. **Castellanos Jankiewicz A**, Guzmán Quevedo O, Massa F, Mazier W, Cota D. Central bile acids in metabolic control (September, 2017). 42eme Colloque de la Société de Neuroendocrinologie (Dijon, France).
5. **Castellanos Jankiewicz A**, Guzmán Quevedo O, Massa F, Mazier W, Cota D. Unraveling the hypothalamic role of bile acids in metabolic control (March, 2017). 10th meeting of Nutrition and Neuroscience 2017, NutriNeuro (Bordeaux, France).
6. **Castellanos Jankiewicz A**, Guzmán Quevedo O, Cota D. Activación hipotalámica del receptor TGR5 en el balance energético (March, 2017). Simposio de Becarios CONACYT (Strasbourg, France).

Posters:

1. **Castellanos-Jankiewicz A***, Guzmán-Quevedo O*, Fénelon V, Zizzari P, Dupuy N, Clark S, Leste-Lasserre T, Bellocchio L, Marsicano G, Cota D. Bile acids signaling as a novel mechanism in the hypothalamic control of energy balance, Annual Symposium of the Neurocentre Magendie, Bordeaux, France (September, 2019)
2. **Castellanos Jankiewicz A***, Guzmán-Quevedo O*, Fénelon V, Duveau A, Stahlman M, Bäckhed F, Zizzari P, Dupuy N, James S, Bellocchio L, Marsicano G, Cota D. Role of hypothalamic bile acids signaling in the control of energy balance, Journée de l'École Doctorale SVS, Bordeaux, France (April, 2018)
3. **Castellanos Jankiewicz A***, Guzmán-Quevedo O*, Mazier W, Cota D. Role of the bile acids ó TGR5 receptor system in the hypothalamic regulation of energy balance, NeuroFrance 2017, Bordeaux, France (May, 2017)

Science communication:

Articles published in the magazine of the National Academy of Sciences (ACM), Mexico

1. **Castellanos Jankiewicz A.**, Guzmán Quevedo O., Cota D. El origen de la obesidad: ¿está todo en la cabeza? Revista Ciencia (ACM).
<https://www.revistaciencia.amc.edu.mx/index.php/ediciones-anteriores/articulos-fuera-de-los-volumenes-publicados-online/482-el-origen-de-la-obesidad-esta-todo-en-el-cerebro>
2. **Castellanos Jankiewicz A.** (2018 Octubre-Diciembre). Neurobiología del comportamiento alimentario. Revista Ciencia (ACM), vol. 69(4), pp. 56-61.
<https://www.revistaciencia.amc.edu.mx/index.php/ediciones-anteriores?id=282>

Activities

1. Science communication through a radio interview by Radio RCF, program "Qui cherchez-vous?" (What do they search for?) (April, 2019):
<https://rcf.fr/culture/recherches-sur-l-obesite-ashley-castellanos-jankiewicz>
2. Winner of the regional competition (Nouvelle Aquitaine) from the event "Ma thèse en 180 secondes" (My thesis in 180 seconds), and national semi-finalist:
<https://www.u-bordeaux.fr/Portraits-et-temoignages/Ashley-Castellanos-Jankiewicz-son-combat-contre-l-obesite>

3. Participation in other events devoted to the dissemination of science, such as the Fête de la Science (2017), Semaine du Cerveau (Brain Awareness Week, 2017), Déclics (2017, 2018), INSERM Destination Labo (2018), Printemps de la Mixité (April, 2019) and Nuit des Chercheurs (September 2019). Interventions included the organization of workshops and talks for undergraduate students, as well as the general public.

LIST OF ABBREVIATIONS

6-OH-DOPA	6-hydroxydopamine
AAV	Adeno-associated virus
ACC	Anterior cingulate cortex
aCSF	Artificial cerebrospinal fluid
Adrb	Adrenoreceptor
Akt	Protein kinase B
ARC	Arcuate nucleus of the hypothalamus
AVP	Arginine vasopressin
BA	Bile acids
BAT	Brown adipose tissue
BMR	Basal metabolic rate
BSEP	Bile salt export pump
CA	Cholic acid
cAMP	Cyclic adenosine monophosphate
CCDC	A synthetic, potent and specific TGR5 agonist: 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N, 5-dimethylisoxazole-4-carboxamide
CDCA	Chenodeoxycholic acid
CGRP	Calcitonin gene-related peptide
CIT	Cold-induced thermogenesis
CNS	Central nervous system
CYP7A1	Cholesterol 7 α -hydroxylase, rate limiting enzyme in BA biosynthesis
D2	Iodothyronine deiodinase type 2
DA	Dopamine
DCA	Deoxycholic acid
Dio-2	Deiodinase 2
DMN	Dorsomedial nucleus of the hypothalamus
EC	Enterochromaffin cells
EGFR	Epidermal growth factor receptors
FXR	Farnesoid X receptor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein

GLP-1	Glucagon like peptide 1
GPBAR1	G-protein coupled bile acid receptor 1, also known as TGR5
GPCR	G-protein coupled receptor
GRK	G-protein-coupled receptor kinases
G	G protein alpha subunits
G	G protein beta subunits
G	G protein gamma subunits
HFD	High fat diet
ICV	Intracerebroventricular
LCA	Lithocholic acid
LHA	Lateral hypothalamic area
MAPK	Mitogen-activated protein kinases
MBH	Mediobasal hypothalamus
MC4R	Melanocortin 4 receptor
MPFC	Medial prefrontal cortex
mTOR	Mammalian target of rapamycin
NAc	Nucleus accumbens
NF B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPY	Neuropeptide Y
NTCP	Na ⁺ -taurocholate co-transporting polypeptide
NTS	Nucleus of the solitary tract
Ost	Organic solute transporter alpha
PBN	Parabrachial nucleus
PKA	Protein kinase A
POMC	Pro-opiomelanocortin peptide
PVN	Paraventricular nucleus of the hypothalamus
RYGB	Roux-en-Y gastric bypass
SCN	Suprachiasmatic nucleus of the hypothalamus
SEM	Standard error of the mean
SNS	Sympathetic nervous system
SON	Supraoptic nucleus
Srebp1c	Sterol regulatory binding protein 1c
T3	Thyroid hormone tri-iodothyronine

T4	Thyroxine hormone
TGR5	Takeda G protein-coupled receptor 5
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
Ucp-1	Uncoupling protein 1
VMN	Ventromedial nucleus of the hypothalamus
VTA	Ventral tegmental area
WAT	White adipose tissue
-MCA	-muricholic acid
-MCA	-muricholic acid

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I. Introduction

I. Introduction

A. Energy balance and obesity

a. Energy balance: key to survival

The human being is a living system that, like all others, is ruled by the first law of thermodynamics, which states that energy cannot be destroyed or created, but can only be transformed from one form to another (Ho, 2018). Therefore, the amount of energy that we consume (intake) should equal the amount of energy that we use for mechanical work, heat production and biosynthetic processes (expenditure). Losses that occur during the process, such as waste products, should also be considered in the system. Any other remaining energy that is consumed but not expended will be stored (Keith N. Frayn, 2010).

The capacity of individuals to conserve this balance is crucial for survival. Evolutionarily, maintaining a stable body weight (BW) represents an advantage, since individuals whose weight fluctuates significantly over time (particularly in the case of voluntary weight loss, followed by weight regain) carry an increased risk of suffering from cardiovascular disease and new onset diabetes (Bangalore et al., 2017; Cheng, Gao, & Jensen, 2015). In order to maintain this stability, mammals have developed a complex system for controlling their weight. To achieve this, different hardwired mechanisms localized within the brain are responsible for regulating our eating behavior and metabolic responses (use and storage of calories) in peripheral organs. One of them is referred to as homeostatic system, which occurs when there is a decrease in energy substrates within the organism, causing hunger and the subsequent consumption of food. This situation has recently been more precisely referred to as the *endostatic system*, since it detects endogenous levels of energy, and gives indication to the body of replenishing the internal (*endo-ö*) energetic needs (*öI eat when hungryö*) (Piazza, Cota, & Marsicano, 2017). However, the act of eating is not always caused by actual hunger, but can also be triggered by the presence of food, particularly one that is palatable. Hence, animals that are already sated decide to keep eating, although their immediate energy needs have already been fulfilled. This second system is referred to as the non-homeostatic system. Since this system is mostly related to the food that is available in the external (*exo-ö*) environment, it is also referred to as the *exostatic system*, and it mostly relates to push the organism towards food consumption so to stock energy for future energetic needs (*öI eat when food is availableö*) (Piazza et al., 2017).

Both systems participate in controlling energy balance, which depends on both energetic needs and environmental constraints, such as food abundance or scarcity. This balance is comprehensible when thinking in evolutionary terms about the human species in particular, since we evolved in environments where nutrient availability was far from stable, ranging from periods of abundance to dramatic food shortages. However, particularly in Western societies, in a relatively short period of time, our technologies have evolved to provide us with abundant and highly caloric food at any time during the day, having to make little effort to obtain it and consume it (Piazza et al., 2017). Hence, the sophisticated brain circuits regulating food ingestion and energy balance is being challenged, due to the easy availability and consumption of calorie-rich food, consequently leading to obesity and its serious metabolic consequences.

b. Obesity: definition and classification

Obesity is defined as a pathological condition where excessive fat accumulation occurs, in the form of adipose tissue, in a way that carries a potential health risk (Kyle et al., 2016). This increase in body fat typically arises from an imbalance between energy intake (amount of calories ingested) and energy expenditure (amount of energy used as fuel for different purposes, such as maintaining basal metabolism, body heat and performing physical activities). Hence, obesity results when the amount of calories that are ingested surpasses the energy that is actually used by the individual, an effect on metabolism that is referred to as having a positive energy balance (energy intake > energy expenditure) (Krashes, Lowell, & Garfield, 2016).

In order to categorize the severity of obesity and the risk of comorbidities, it is common to use anthropometric parameters that consider the body weight and height of an individual, since tools required to measure body fat in humans are too costly for recurrent use. Hence, the most common tool for this purpose is the body mass index (BMI), which is the ratio resulting from dividing the weight of an individual (kg) by the square of the height (meters). This ratio gives a surrogate measure of body fatness and categorizes an individual as being underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9), obese class I (>30.0-34.9), obese class II (35.0-39.9) and obese class III (>40.0) (WHO, 2000).

Obesity is a preventable disease, and yet, in a relatively short period of time it has become an epidemic, being on the rise since 1997 (Caballero, 2007; Kyle et al., 2016). The World Health Organization has estimated that today, more than 13% of the world population is obese. More

alarming is the fact that, within the countries of the European Union, around 15% of the adult population is obese (Marques et al., 2017). In the particular case of France, it is the first country worldwide where the prevalence has not changed significantly since 2006, affirming stability of tendencies of overweight and obesity within the French population. However, the numbers are still alarming, with 49% of the adult population being overweight, out of which 17.2% are obese (Verdot et al., 2017).

Obesity carries with it a myriad of chronic comorbidities that severely decrease the quality of life and life expectancy itself, such as hypertension, type 2 diabetes, heart disease, respiratory disorders, non-alcoholic fatty liver disease, osteoarthritis, gallstones, reduced fertility, obstructive sleep apnea, several types of cancer and mild cognitive impairment (Kelly, Yang, Chen, Reynolds, & He, 2008). Furthermore, the social perception related to body size also places a heavy burden on the mental health of patients who suffer from obesity, carrying an increased risk of anxiety, low self-esteem and depression (Williams, Mesidor, Winters, Dubbert, & Wyatt, 2015). Obesity also negatively impacts the economy of health systems, making it one of the most common and costly chronic diseases worldwide (M. W. Schwartz et al., 2017).

However, although being obese has received a stigma from society for being associated with negative personal traits (such as a lack of will power), it is nowadays clear that the process of becoming obese is far more complicated and involves fundamental aspects of our biological traits, such as the disruption of circuits that control energy balance, as explained by different central regulatory systems (endostatic and exostatic), together with our evolutionary physiology, which has predisposed us to conserve body fat and gain weight (M. W. Schwartz et al., 2017). In support of this evidence, a recent study has shown that most obesity-associated mutations cluster in the brain, highlighting the role of the central nervous system in understanding and possibly treating this condition (Locke et al., 2015) (**Fig. 1**).

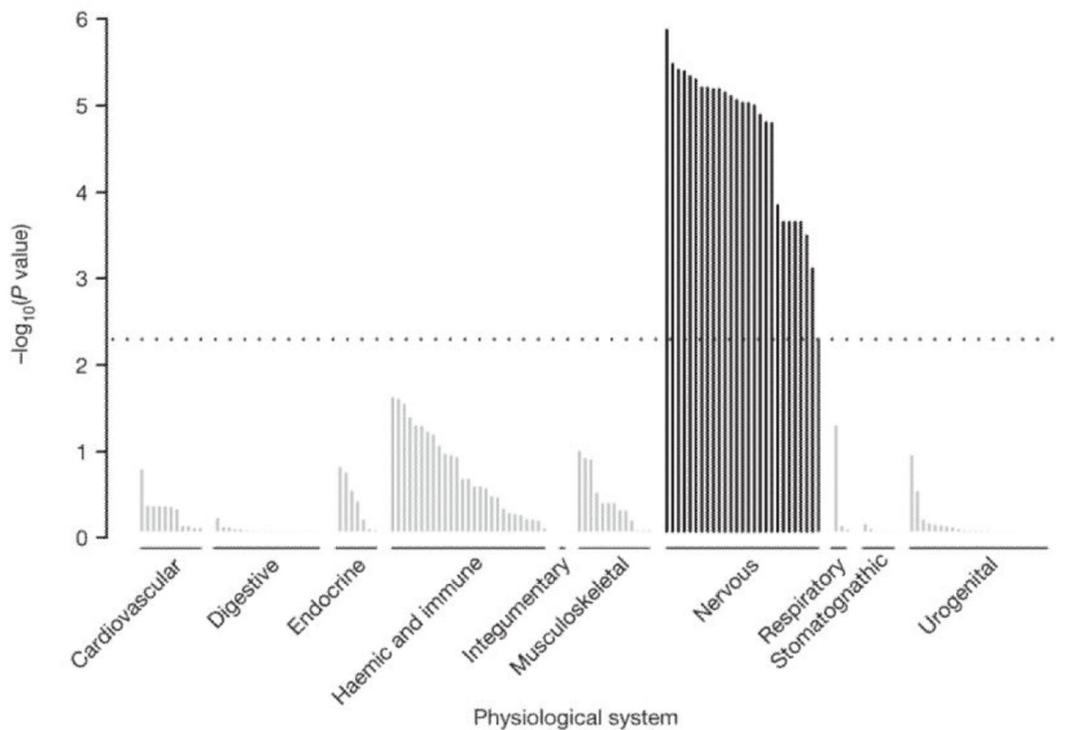


Figure 1. Obesity-associated mutations cluster within the brain. In a recent genome-wide association study, the central nervous system had the most significantly enriched single nucleotide polymorphisms associated with body mass index (BMI; $p < 5 \times 10^{-4}$), indicating that most obesity-associated mutations cluster in the brain. Tissues were sorted by physiological system, where significantly enriched tissues appear in black; surpassing the dotted lines represents statistically significant enrichment [Taken from (Locke et al., 2015)].

In order to tackle the obesity epidemic, public policies worldwide have included the promotion of a healthy lifestyle, education in food choices and nutrition, and have underlined the importance of increasing time spent exercising (Leclerc et al., 2015). However, these strategies often fail to achieve sustained weight loss in the long term, mostly due to the difficulty for patients to adopt lifestyle changes and adhere to specific diets. Hence, much research has focused on developing and testing pharmacological compounds that could treat obesity and its comorbidities from different angles, resulting in five drugs being approved by the US Food and Drug Administration (FDA) to date (Daneschvar, Aronson, & Smetana, 2016). However, these drugs allow for relatively small changes in body weight loss, and most

importantly, all of them present high risks of suffering from serious side effects as a consequence of their use (Krentz, Fujioka, & Hompesch, 2016), so that no drug that is both effective and safe is currently available. Furthermore, adaptive responses to weight loss suggest the existence of a defense system that protects the elevated level of acquired body fat, making it more difficult to lose weight and explaining the poor long-term efficacy of current treatments (Leibel, Rosenbaum, & Hirsch, 1995). For these reasons, a new focus for treating and overcoming obesity is urgently needed.

c. Bariatric surgery: the most successful long-term therapy

The most successful therapy that is currently available to treat severe obesity is bariatric surgery. Within this surgical procedure are grouped many different types of interventions, which are mostly differentiated by the inclusion of a òmetabolic bypassö that causes a re-routing of the gastrointestinal tract, or by procedures that do not have this bypass [Table 1; modified from (Ashrafian et al., 2010; Benaiges et al., 2015; Buchwald et al., 2009; C. K. Huang et al., 2016)].

Type of surgery	Metabolic bypass (Yes/No)	Description	Outcomes
Biliopancreatic diversion/duodenal switch (BPD)	Yes	Partial gastrectomy with an anastomosis of the stomach pouch to the jejunum, forming an alimentary limb and a duodenojejunal biliopancreatic limb connected to the distal ileum.	49.81 kg of body weight lost (73% of excess body weight lost) at ×2 years post-surgery.
Roux-en-Y gastric bypass (RYGB)	Yes	The stomach is divided to create a small pouch (30 to 50mL), whilst the distal jejunum is connected to the stomach pouch, bypassing the duodenum and a part of the proximal jejunum. In doing so, food enters the stomach pouch to directly enter the jejunum. This procedure alters BA and nutrient flow through the gastrointestinal tract.	41.41 kg of body weight lost (63.25% of excess body weight lost) at ×2 years post-surgery.
Jejunioileal bypass	Yes	A segment of the jejunum is anastomosed to the terminal ileum, hence bypassing 90% of the	This procedure is no longer practiced, since it leads to significant

		functioning small intestine (a surgical short bowel syndrome).	comorbidities (renal failure, liver disease, diarrhea, steatorrhea and protein deficiency).
Partial ileal bypass	Yes	It consists of connecting a proximal segment of the ileum 200cm from the ileocaecal valve, which results in shortening the length of the small intestine. It decreases the absorption of cholesterol and bile acids by the ileum.	This procedure is rarely used to treat obesity, since it is mostly indicated for the treatment of hypercholesterolemia.
Duodenojejunal bypass	Yes	The stomach is connected the jejunum, bypassing the duodenum. It can improve diabetes by excluding nutrients from the proximal bowel and is currently used to treat diabetic patients with a BMI $\geq 20\text{kg/m}^2$.	Outcomes are similar to patients that underwent RYGBP. After 1 year post-surgery, 15.7 kg of body weight was lost.
Vertical banded gastroplasty	No	The stomach is partitioned along the smaller curvature and then banded. The procedure is no longer used, since it does not lead to long term weight loss nor metabolic improvements.	36.97 kg of body weight lost (56.48% of excess body weight lost) at $\times 2$ years post-surgery.
Adjustable gastric band	No	An adjustable band is placed around the top portion of the stomach to reduce its storage capacity.	38.30 kg of body weight lost (48.98% of excess body weight lost) at $\times 2$ years post-surgery.
Ileal interposition	No	The ileum is positioned towards the proximal part of the small intestine, without modifying the stomach connectivity.	It is mostly used for the treatment of type 2 diabetes, even when the BMI is $< 20\text{kg/m}^2$.
Sleeve gastrectomy	No	Removal of 70% of the stomach by transecting the greater curvature. Its effects are attributed to increased gastric emptying and intestinal transit, as well as stimulating gut hormone modifications.	Excess body weight loss has been estimated at 62.3% after 5 years post-surgery.

Table 1. Types of bariatric surgery for body weight loss [Modified from (Ashrafian et al., 2010; Benaiges et al., 2015; Buchwald et al., 2009; C. K. Huang et al., 2016)].

Historically, caloric restriction due to a reduction in stomach size or to the bypass of nutrients (malabsorption) into the distal gut was thought to be key in the success of these surgeries (Cummings, Overduin, & Foster-Schubert, 2004). However, nowadays there is a shift in understanding the mechanism of action of these surgical procedures due to the astounding metabolic effects that they carry.

Interestingly, the types of bariatric surgeries that have been most successful in treating obesity and its metabolic consequences in humans belong to interventions that include a metabolic bypass (Buchwald et al., 2009; Kohli et al., 2013). By following these types of surgical interventions, patients achieve sustained weight loss while improving metabolic parameters, such as an increase in glucagon like peptide 1 (GLP-1), a gastrointestinal hormone improving insulin secretion and sensitivity, as well as glucose tolerance (Ashrafian et al., 2010; Kohli et al., 2013), lowering levels of circulating triglycerides, cholesterol and low density lipoprotein levels, all of which remain stable for up to 4 to 10 years after the surgery (Douglas et al., 2015; Sjostrom et al., 2004). Noteworthy, one of the most important clinical consequences that occur shortly after the surgical intervention is the remission of type 2 diabetes in a large proportion of patients, an effect that has been described even after 14 years of follow-up, hence reducing the mortality from diabetes (Rubino & Gagner, 2002). This is especially true for biliopancreatic diversion/duodenal switch (BPD) and Roux-en-Y gastric bypass (RYGB), where body weight loss is maintained even after 2 years post-surgery (73.72% and 63.25% of excess body weight lost, respectively) and where diabetes resolution occurs in 95.1% and 80.3% of patients, respectively. In comparison, interventions such as gastric banding (where no metabolic bypass takes place) achieved an excess body weight loss of only 48.98% after 2 years, with 56.7% of patients achieving diabetes resolution (Buchwald et al., 2009).

The current eligibility criteria for undergoing any type of bariatric surgery is being morbidly obese (BMI $>40\text{kg/m}^2$) or a BMI of $>35\text{kg/m}^2$ with important comorbidities (such as type 2 diabetes) (Fruhbeck, 2015). However, due to all the previously described effects of bariatric surgery, the 2nd Diabetes Surgery Summit (DSS-II) came together in 2015, which hosted diabetes organizations from around the world (including the American Diabetes Association, the International Diabetes Federation, the French Society of Diabetes, among others). In this summit, guidelines were established for clinicians to include these surgeries as a treatment for patients who are diabetic and have a BMI as low as 30kg/m^2 (Rubino et al., 2016). Hence, the concept of bariatric surgery is nowadays shifting from a purely calorie-restriction intervention, to being considered a metabolic surgery that should not only be indicated for

morbidly obese patients, but also for the treatment of non-obese type 2 diabetic patients (Rubino et al., 2006; Rubino et al., 2016).

Nevertheless, there exists a knowledge gap for understanding the biological mechanisms in achieving these astounding effects on metabolism, and due to the strict inclusion criteria for undergoing surgery (and the cost it entails), not all obese or diabetic patients are candidates for this intervention. Moreover, although it is nowadays considered among the safest of available surgical procedures (mortality rate of <0.3%), there are important risks that can rise from this intervention that need to be taken into consideration. Complications include bleeding, leaks and bowel obstruction during surgery, nutritional deficiencies and post-prandial hypoglycemia after surgery, as well as psychological disorders, such as depression, drug and alcohol abuse (Miras & le Roux, 2017).

Therefore, it is in our best interest to better comprehend the underlying mechanisms of bariatric surgery success for finding a less invasive but equally effective solution to obesity.

d. Bile acids and body weight loss after surgery

In this regard, bile acids (BA) are nowadays being recognized as one of the main players in the metabolic benefits of these surgeries. Patients who undergo RYGB have a marked increase in circulating BA levels, going from a 2-fold (Kohli et al., 2013; Patti et al., 2009) and up to a 3-fold increase (Spinelli et al., 2016), an effect that is not observed in non-metabolic bypass interventions, such as gastric banding (Kohli et al., 2013). Moreover, this increase in BA is positively correlated with the amount of lost weight (Penney et al., 2015).

At the experimental level, a study in rats confirmed that the sole diversion of the bile duct from the liver to either the jejunum or the ileum (instead of the duodenum) significantly increased BA in plasma, and mimicked the metabolic effects of bariatric surgery, even during exposure to a high-fat and high-sucrose diet (Goncalves et al., 2015). Moreover, these effects were blunted when providing a diet rich in cholestyramine, a resin that typically prevents BA reabsorption, suggesting that BA play a key role in achieving these metabolic changes. The study further revealed that BA diversion (without any dietary intervention) caused a reduction in BW and food intake (FI). Interestingly, rats also displayed a decreased interest towards palatable foods, effect that was strongly associated with increased plasma BA (Goncalves et al., 2015). These alterations in eating behavior and food preference have also been described in patients who undergo RYGB, suggesting that they do not eat less due to the restrictive

nature of the surgery, but mostly because of the reduction of hedonic hunger (Chakravartty, Tassinari, Salerno, Giorgakis, & Rubino, 2015).

To further understand the metabolic consequences of increased BA after bariatric surgery, it is therefore essential to describe the state of the art in regards to their known metabolic functions.

B. Bile acids

a. What are bile acids?

BA have been amply characterized as amphipathic molecules with detergent properties that are synthesized from cholesterol molecules in the liver, stored in the gallbladder through the common hepatic duct, and are discharged into the duodenum through the common bile duct upon meal ingestion (a process triggered by cholecystokinin, a hormone secreted by enteroendocrine cells), where they participate in lipid solubilization. Recycling of BA is a well-known mechanism for energy optimization (Chiang, 2013; Hofmann & Hagey, 2014), so that 95% of secreted BA are actively reabsorbed in the terminal ileum and carried back to the liver via the portal vein to participate in the next digestive process (Chiang, 2013). This flow of events is termed the enterohepatic cycle (**Fig. 2**) (Gioiello et al., 2014; Mazuy, Helleboid, Staels, & Lefebvre, 2015). In humans, 200 to 600 mg of BA are produced, and the same amount are excreted in the feces. This loss of BA per day is compensated by the synthesis of new BA (*de novo*) in the liver, so that, at any given time, the total BA pool is around 3 g.

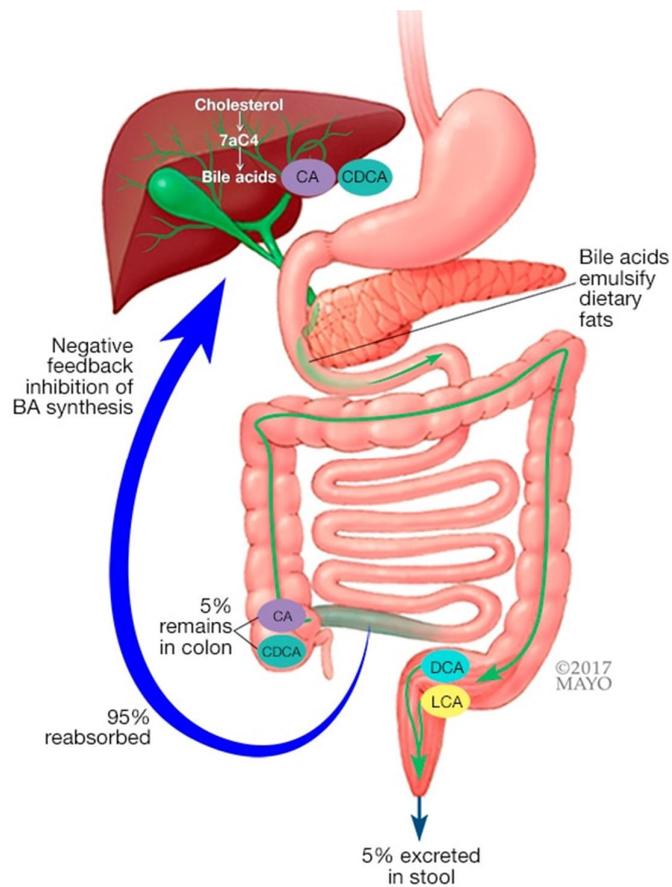


Figure 2. The enterohepatic cycle. BA are synthesized from cholesterol molecules in the liver as primary BA, such as cholic acid (CA) and chenodeoxycholic acid (CDCA). They are then stored in the gallbladder (green) as conjugated primary BA. The presence of food in the stomach stimulates their secretion through the common bile duct into the duodenum in order to participate in the digestion process by emulsifying dietary fats throughout the intestine. Here, they undergo bacterial transformation (deconjugation and 7- -dehydroxylation) to form the secondary BA deoxycholic acid (DCA) or lithocholic acid (LCA) and are either reabsorbed by the small intestine (95%) or lost in feces (5%). Hence, de novo BA synthesis represents 5% of the total BA pool. This pool of BA can cycle between 2 and 3 times per day in humans, depending on the dietary regimen. BA will then travel back to the liver, where they can signal a downregulation of their biosynthesis, completing a negative feedback loop for their regulation (Mazuy et al., 2015) [Taken from (MayoClinic, 2019)].

BA are cholesterol-derived molecules. Therefore, the core molecular structure of BA is a steroid nucleus, consisting of four fused carbon rings (three 6-carbon rings and one 5-carbon

ring). The biosynthesis of BA occurs in the liver, and can be classified in two major pathways: the neutral (or classic) pathway, where building of BA starts with modifications of the core steroid ring, and the acidic pathway, where these modifications are initiated by the cleavage of the side-chains of the steroid molecule. Notably, the classic pathway contributes to about 91% of the total BA that are synthesized in humans (Chiang, 2013). The first step of BA synthesis from cholesterol is initiated by their only rate-limiting enzyme, cholesterol 7 α -hydroxylase (CYP7A1). The initial products from cholesterol conversion are primary bile acids, which in humans are cholic acid and chenodeoxycholic acid, and in mice are cholic acid, α -muricholic acid and α -murocholic acid (Kuipers, Bloks, & Groen, 2014).

Within the liver, primary BA are conjugated with either glycine or taurine, a process that increases their solubility for preventing their passive absorption and protecting them against cleavage by pancreatic enzymes. Upon reaching the distal intestine, BA are deconjugated and undergo further modifications carried out by the gut microbiota that result in secondary BA: cholic acid becomes deoxycholic acid, whilst chenodeoxycholic acid becomes lithocholic acid. These secondary BA are either excreted in the feces or re-circulated back to the liver via the portal system for subsequent excretion into the bile (Chiang, 2013).

Since BA cannot be passively absorbed, they require specific transporters to complete this cycle. Hence, once BA reach the enterocytes in the small intestine, they are exported into the circulation via the organic solute transporter alpha (Ost α). Consequently, the hepatic tissue imports them from the bloodstream through the Na⁺-taurocholate co-transporting polypeptide (NTCP). In contrast, export of BA from the liver takes place through the bile salt export pump (BSEP) and the multidrug resistance associated protein 2 (Ballatori et al., 2009; Mazuy et al., 2015). This complex transport system is able to protect the liver and other tissues from BA accumulation and toxicity (Mazuy et al., 2015).

Of note, during the enterohepatic cycle, a small amount of BA can leak into the systemic circulation, tripling their blood concentrations after a meal, a fact that had been overlooked for many years and suggests sites of action of BA that extend beyond the digestive tract. Nowadays, we know that this spillover can have important metabolic effects (Ferrebee & Dawson, 2015). Moreover, in the particular case of patients who suffer from obesity, it has been shown that their levels of circulating BA after a meal are lower than in normal weight subjects, whilst after following bariatric surgery (particularly after RYGB), these levels were re-established to normal (Ahmad et al., 2013; Kohli et al., 2013). For this reason, the classic

concept of BA is currently shifting, from acting as mere lipid emulsifiers in the intestine to behaving as hormone-like signaling molecules with ubiquitous systemic effects.

b. Bile acids as peripheral signaling molecules

1. The farnesoid X receptor (FXR)

Since BA have detergent properties, this might impose a risk to cells that are exposed to high concentrations of BA, causing inflammation and apoptosis. Consequently, there are protective mechanisms that come to place for the sensing and regulation of BA levels (Kuipers et al., 2014). In 1999, the hypothesis of the existence of a sensing system of BA arose with the discovery of their specific nuclear farnesoid X receptor (FXR) (Wang et al., 1999), which is expressed in the liver, intestine, white adipose tissue, kidney, adrenal glands, stomach, pancreas, endothelial cells, among other tissues (Kuipers et al., 2014) (**Fig. 3**). To the present day, there is no clear evidence of the FXR receptor being expressed within the brain (Forman et al., 1995; X. Zhang et al., 2014), with some authors clearly stating that the receptor is not expressed in the adult mouse brain, as assessed by real-time PCR and *in situ* hybridization (Gofflot et al., 2007).

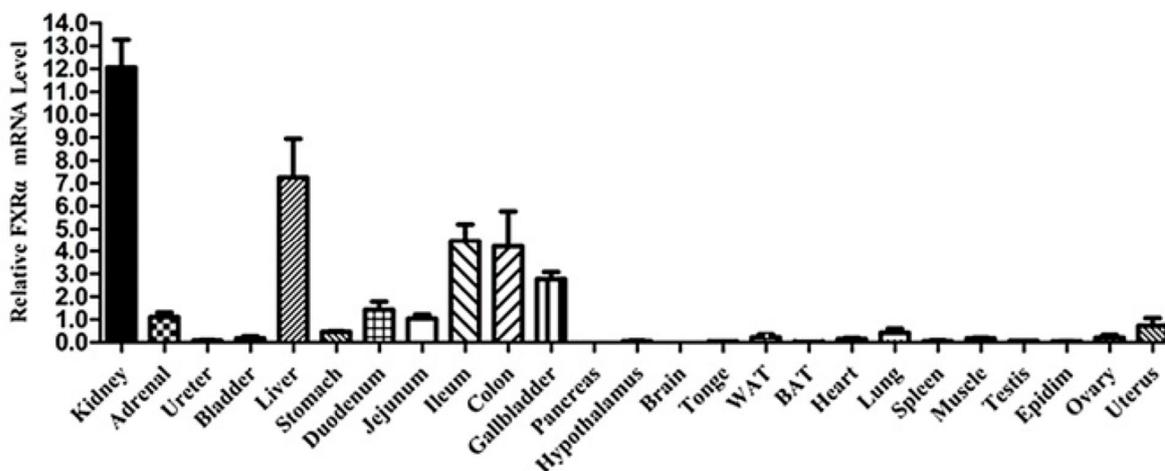


Figure 3. Expression of FXR in the mouse. Mouse tissue distribution of FXR expression (mRNA) relative to standardization by 18S. The receptor is most abundant in the kidney, liver and the gastrointestinal tract [Taken from (X. Zhang et al., 2014)]. BAT: brown adipose tissue; WAT: white adipose tissue.

Since BA can be highly toxic to cells, due to their amphipathic nature, the main role of this nuclear receptor is to tightly regulate their synthesis and transport through tissues. Once BA bind to FXR in the liver, they inhibit BA synthesis by inhibiting cholesterol catabolism. This is achieved through the recruitment of transcriptional factors that will thereafter suppress the expression of the BA biosynthetic enzyme CYP7a1. *In vivo*, activation of BA-FXR signaling inhibits the expression of genes involved in hepatic gluconeogenic pathways, as well as in hepatic lipid metabolism (Ma, Saha, Chan, & Moore, 2006). Therefore, BA can regulate hepatic carbohydrate and lipid metabolism through FXR-induced changes in gene expression (Duran-Sandoval, Cariou, Fruchart, & Staels, 2005; Duran-Sandoval, Cariou, Percevault, et al., 2005). FXR is also implicated in other metabolic functions, such as BA transport towards the liver, by repressing the expression of the NTCIP transporter, and in the regulation of lipogenesis and triglyceride synthesis through the suppression of the sterol regulatory binding protein 1c (Srebp1c). Moreover, studies in FXR knockout mice, as well as in transgenic models, suggest a potential role of this BA receptor as an anti-atherogenic target through lowering plasma cholesterol and reducing aortic plaque formation (Mazuy et al., 2015).

2. *The Takeda G protein-coupled receptor 5 (TGR5)*

Beyond the nuclear receptor FXR, in 2002, a novel G protein coupled receptor (GPCR) was found through searching in an existing GenBank DNA database. In order to understand its function, HEK293 cells that expressed this receptor were established and were exposed to different compounds. Interestingly, levels of cAMP (cyclic adenosine monophosphate, a second messenger that is activated by GPCRs) increased when the cells were exposed to compounds that were similar to cholesterol. By screening for compounds that possess the same core carbon ring structure, the authors found that this receptor was specifically responsive in a dose dependent manner to BA (Maruyama et al., 2002). Hence, for the first time they described the G-protein membrane BA receptor 1 (GPBAR1), also known with the name of Takeda G protein-coupled receptor 5 (TGR5) (Maruyama et al., 2002).

2.1. *G-protein coupled receptor signal transduction*

GPCRs are cell membrane receptors with seven transmembrane domains that are capable of transducing signals from the extracellular space into the cytoplasm and the nucleus, in order to modulate the expression of genes within the cell that will lead to a myriad of different responses (Rajagopal & Shenoy, 2018). They respond to stimuli such as

hormones, neurotransmitters, ions, odorants, and others, for which they play an important role in physiology and represent interesting drug targets. Specific agonists of GPCRs are able to induce a maximal response, while partial agonists and inverse agonists cause submaximal responses or even decrease their activity, respectively (Hilger, Masureel, & Kobilka, 2018).

Upon GPCR agonist binding, different signaling cascades can take place, which can be divided in G-protein dependent and G-protein independent. The first refer to processes that include the activation of heterodimeric G proteins that are composed of three subunits (G_s, G_i and G_q). While G_s can modulate the activity of adenylyl cyclase, the complex of G_i can interact with channels that will allow the entrance of potassium to the cell. G-protein signaling is finalized by the re-association of the proteins to form an inactive heterodimer. The second signaling cascade leads to G-protein-coupled receptor kinases (GRK) phosphorylation and coupling to arrestins, which can either lead to activation of the mitogen-activated protein kinases (MAPK) pathway, promote the internalization of the receptor in endosomes for degradation, or recycling back to the plasma membrane (see **Fig. 4**) (Hilger et al., 2018). Continuous stimulation of GPCRs can eventually lead to the receptor being refractory to the stimulus or to its degradation by endosomes. This process is referred to as desensitization. After chronic exposure to their agonist, the response of the receptor can be significantly reduced and is associated with a decrease in its expression, a process referred to as the downregulation of the receptor (Rajagopal & Shenoy, 2018).

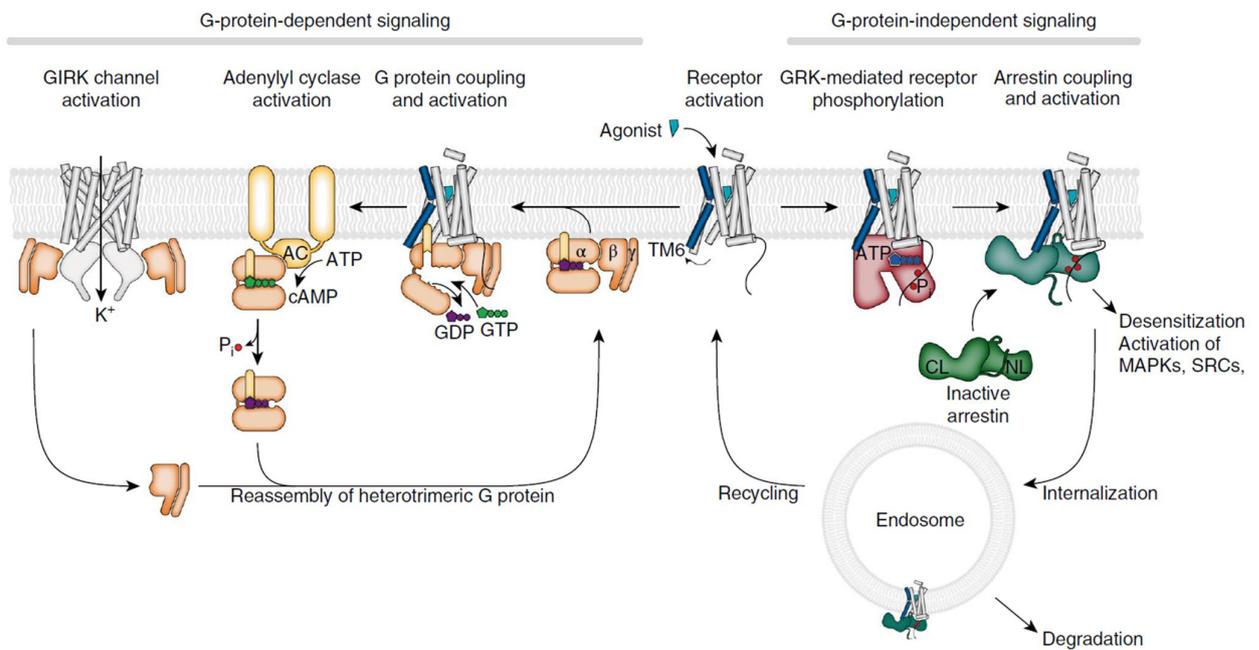


Figure 4. G-protein-coupled receptor signal transduction. This process can either be dependent (left) or not (right) on G-protein signaling. The activated receptor is capable of binding to different intracellular signaling proteins, such as G proteins (orange), GRKs (red), and active (turquoise) or inactive (green) arrestins. G-protein mediated signaling ends by the reassembly of G and G to an inactive complex. G-protein independent signaling by arrestins can also lead to the internalization of the receptor into endosomes to either be recycled back to the membrane, or to its degradation [Taken from (Hilger et al., 2018)].

In the specific case of TGR5, this GPCR responds to different endogenous agonists, particularly to BA, in a different rank order of potency, as follows: lithocholic acid, deoxycholic acid, chenodeoxycholic acid, and cholic acid (Duboc, Tache, & Hofmann, 2014). Its activation results in cAMP signaling at the plasma membrane and in the cytoplasm. It is interesting to note that TGR5 is an unusual kind of GPCR, since it does not interact with arrestins or GRK when it is activated by its endogenous agonists (Jensen et al., 2013). Moreover, even during constant activation by potent agonists (endogenous or synthetic), it does not undergo desensitization nor does it traffic to endosomes for degradation, but leads to continuous cAMP signaling at the level of the plasma membrane and the cytosol. Moreover, continuous stimulation causes the receptor's redistribution in the plasma membrane to form microdomains that are referred to as membrane lipid rafts (Jensen et al., 2013). Here it can

associate with epidermal growth factor receptors (EGFR) in order to mediate MAPK signaling. Although it is not yet clear what the function might be in the particular case of the TGR5 receptor, there is evidence that the formation of membrane rafts allow the enhancement of the signal transduction (Patel, Murray, & Insel, 2008).

The TGR5 receptor is expressed in peripheral tissues and in the central nervous system (CNS) both in humans (Kawamata et al., 2003) and in mice (Maruyama et al., 2006) (**Fig. 5**).

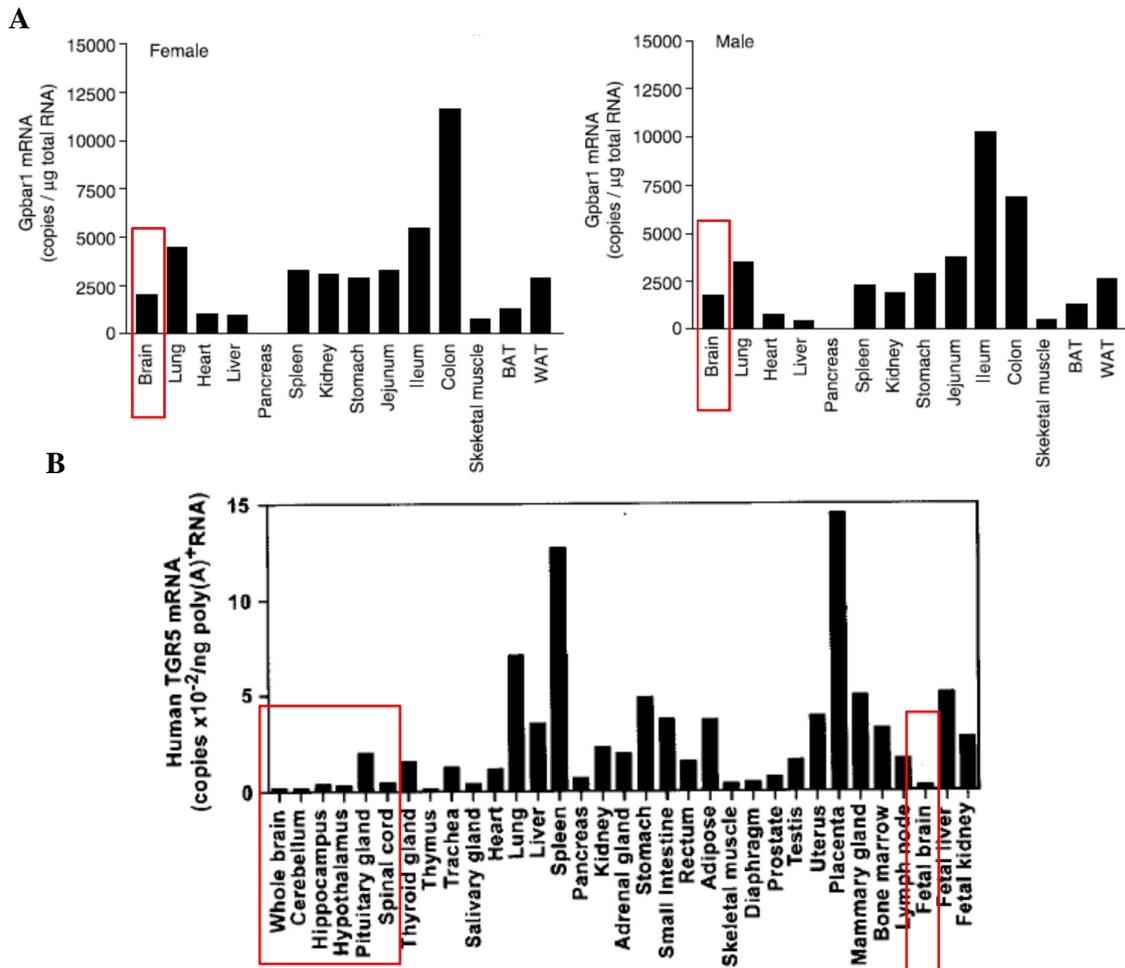


Figure 5. TGR5 receptor expression in mice and humans. The TGR5 receptor (also known as Gpbar1) is expressed in many different tissues in A) female and male mice and B) human samples. Total RNA underwent reverse transcription and quantitative PCR. Each column represents the mean of duplicate values [Taken from (Kawamata et al., 2003; Maruyama et al., 2002)]. BAT: brown adipose tissue; WAT: white adipose tissue. Red rectangles: expression in brain and brain-related structures.

2.2. *Gastrointestinal regulatory functions*

At the time of its discovery, questions arose related to the usefulness of a specific BA receptor in tissues outside of the digestive tract, meaning tissues that are not directly related to the digestive process. However, now it is clear that the ubiquitous expression of TGR5 leads to widespread metabolic effects in the periphery. Since this is essentially a BA receptor, the first experiments to understand its role were focused on the liver and the gastrointestinal tract.

Within the biliary tree, the activation of TGR5 involves the proliferation of cholangiocytes (epithelial cells of the bile duct) by phosphorylation of the MAPK/ERK1/2 pathway, a process that is independent of adenylate cyclase activity. TGR5 is highly expressed in the gallbladder, where its activation promotes its filling with BA and blocks its emptying (T. Chen et al., 2018). Moreover, the stimulation of TGR5 in non-parenchymal cells of the liver promotes choleresis, a process that allows for bile to flow from the liver to the gallbladder (Deutschmann et al., 2018). Within the gastrointestinal tract, it is expressed in either the apical or basolateral membranes of epithelial cells of the intestine, where it exerts different **regulatory functions related to digestive processes** (Bunnett, 2014):

- Enterochromaffin (EC) cells from the colon: BA from the lumen bind to TGR5 on the apical membrane of EC cells to stimulate the peristaltic reflex that is dependent of serotonin secretion;
- Gastric myocytes: The function of TGR5 in these cells is related to the contraction of the stomach muscles to facilitate digestion;
- Colonocytes: The activation of the receptor on the basolateral membrane of colonocytes can inhibit chloride secretion;
- Macrophages: Activation of TGR5 in macrophages that reside in the colon suppresses the secretion of tumor necrosis factor (TNF) to blunt inflammation;
- Primary sensory neurons: BA that reach the basolateral membrane of enterocytes can reach primary sensory neurons to activate the TGR5 receptor, which will transmit irritant signals to the spinal cord.

2.3. *Metabolic effects of TGR5 activation*

Outside of its role within the gastrointestinal tract, TGR5 can also play a role in other peripheral tissues. Notably, it participates in **improving glucose homeostasis**. Its activation in L cells, which are specialized endocrine cells of the gastrointestinal tract, protects against

insulin resistance by stimulating the release of glucagon-like peptide 1 (GLP-1), an incretin that increases insulin secretion while decreasing glucagon, hence controlling glycaemia (Bunnett, 2014; Thomas et al., 2009). Moreover, when activated in pancreatic β -cells, TGR5 can also directly induce insulin secretion (Kumar et al., 2012). Similarly, it has been recently shown that TGR5 activation by a synthetic agonist in the skeletal muscle of genetically obese (ob/ob) or diet-induced obese mice **improves insulin sensitivity** by a mechanism that is dependent of the cAMP/PKA pathway (S. Huang et al., 2019).

Activation of TGR5 within cells of the **immune system** (such as human monocytes and macrophages) leads to a decrease of pro-inflammatory cytokines such as TNF α . Furthermore, in isolated macrophages from mice lacking TGR5 (TGR5 $^{-/-}$), the synthesis of pro-inflammatory cytokines was higher than in wild type mice after a lipopolysaccharide stimulation. These effects are achieved by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which is a pro-inflammatory transcriptional factor (Duboc et al., 2014). Particularly at the level of Kupffer cells of the liver and macrophages, ligands that bind to the receptor inhibit pro-inflammatory cytokine production through inhibition of NF- κ B, as well as through other mechanisms, such as the protein kinase A (PKA) and protein kinase B (Akt) and mammalian target of rapamycin (mTOR) pathways for the suppression of chemokine expression (Keitel & Haussinger, 2018). Thus, TGR5 activation in the peripheral immune system overall plays an anti-inflammatory action through different signaling cascades.

TGR5 has also been found to **increase heat production in the adipose tissue** of rodents (Watanabe et al., 2006). To understand this, it is important to differentiate between the process of heat production, or thermogenesis, in mammals that can be α -obligatory or β -adaptive. The first refers to heat produced as a consequence of inefficient energy transformation during biochemical processes. Instead of being lost and wasted, this heat allows us to increase our body temperature to one that permits optimal biological functions. Obligatory thermogenesis occurs at temperatures ranging from 26-28°C. On the other hand, temperatures lower than this cause a stress that requires additional heat for proper thermoregulation, especially in small mammals, which produce heat in order to adapt to these colder environments (adaptive thermogenesis). Both of these processes are regulated by thyroid hormones (Bianco, Salvatore, Gereben, Berry, & Larsen, 2002), and the sympathetic nervous system (SNS) (see also further below, section **C. Central control of energy balance**), among other mechanisms. Furthermore, adaptive thermogenesis can be sub-

classified in shivering and non-shivering. During cold exposure, the hypothalamus causes an involuntary shivering response that will increase the blood flow throughout the body and hence, lead to heat production. At the same time, cold exposure activates the SNS for the release of catecholamines (such as adrenaline and noradrenaline), in order to increase heat production (non-shivering thermogenesis). Because the brown adipose tissue (BAT) is abundantly innervated by the SNS, it is the key organ responsible for non-shivering adaptive thermogenesis (Bianco et al., 2002). In fact, within the BAT, noradrenaline secreted as a consequence of SNS stimulation, together with the active thyroid hormone tri-iodothyronine (T3), are capable of stimulating uncoupling protein-1 (Ucp-1) gene transcription, and sustaining its expression during longer periods of cold exposure, allowing for an increase in heat production (Bianco et al., 2002).

Studies have shown the implication of TGR5 in **increasing thermogenesis** by affecting the action of thyroid hormones. By looking at the BAT of mice fed a high fat diet (HFD), TGR5 activation by BA lead to an increase in energy expenditure, preventing obesity and insulin resistance. This process was dependent on the activation of iodothyronine deiodinase type 2 (D2), an enzyme responsible of converting thyroxine (T4) into T3. This study has been key in confirming the implication of TGR5 in the BAT as a primary target of BA for increasing energy expenditure through thermogenesis (Watanabe et al., 2006).

In addition to this thermogenic effect of TGR5, this receptor has also been recently implicated in the **beiging of subcutaneous white adipose tissue** (SAT) of mice during cold exposure, as well as during prolonged exposure to a TGR5 agonist-enriched HFD, a process that also leads to increased mitochondrial content in the SAT. This beiging of the adipose tissue is dependent of an increase in lipolysis though increased β -oxidation and thermogenesis (Velazquez-Villegas et al., 2018).

Hence, the activation of the TGR5 receptor in peripheral tissues can have vast effects depending on the tissue where it is expressed, through different signaling cascades (**Fig. 6**), effects that are particularly relevant in the presence of obesity.

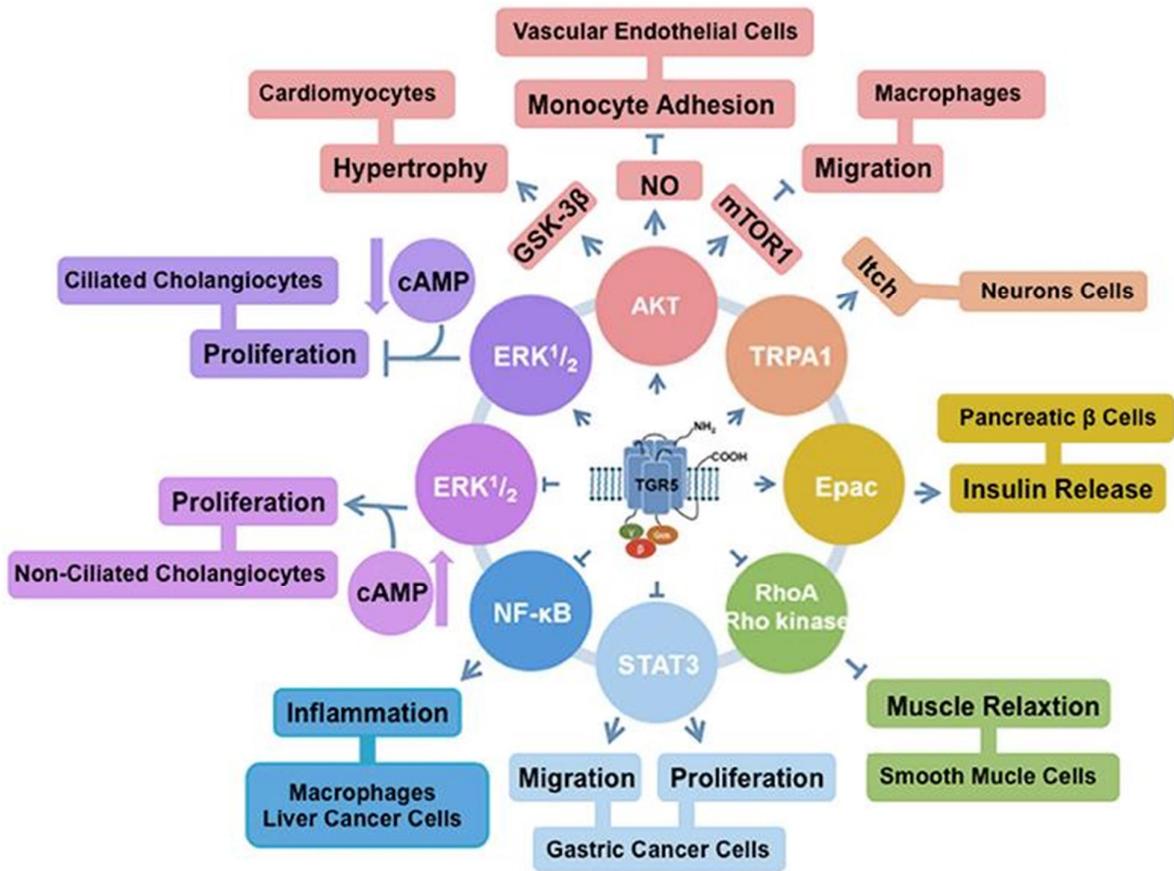


Figure 6. *TGR5* receptor activation participates in different cell signaling pathways. *TGR5* activation can regulate a vast number of metabolic functions, depending on the tissue in which it is expressed [Taken from (Guo, Chen, & Wang, 2016)].

The presence of the **TGR5 receptor within the CNS** is in line with evidence of BA found in the brain, thanks to studies showing their ability to cross the blood-brain barrier (Keene et al., 2001; Parry et al., 2010), as well as showing the presence of BA intermediates that circulate within the cerebrospinal fluid (Ogundare et al., 2010).

Other authors have speculated on the role of the TGR5 receptor within the hypothalamus. Of note, in a rat model of acute cholestasis, circulating BA levels were increased to pathological levels rapidly (within 1 hour) in the hypothalamus, evidencing their ability to cross the blood-brain barrier. The accumulation of these BA within the brain activated TGR5 that was expressed in the hypothalamic supraoptic nucleus (SON), which triggered a neuroendocrine loop regulating BA secretion from the liver. The authors hence suggested that BA may be

involved in an endocrine pathway connecting the liver and the hypothalamus through a TGR5-dependent mechanism (Doignon et al., 2011). To our knowledge, this work has been the first to suggest a hypothalamic role of TGR5 activation through peripheral BA signaling.

Considering the metabolic impact of TGR5 activation in peripheral tissues, particularly in relation to its effects in improving glucose homeostasis, increasing energy expenditure and inhibiting the expression of inflammatory markers, the modulation of this receptor's activity is of great interest in the treatment of obesity and type 2 diabetes (Chavez-Talavera et al., 2017). It is noteworthy that these effects participate to the regulation of overall energy homeostasis, a process that is tightly controlled by the brain. However, although it is known that TGR5 is also expressed within the brain, as mentioned above, the role it plays in the CNS, and potentially within the hypothalamus for participating in energy homeostasis remains unexplored.

C. Central control of energy balance

The brain is an organ that plays a critical role in driving energy balance, by receiving and integrating signals from the periphery of the body's energetic status. In response, the brain can modulate food intake and energy expenditure. Both environmental and endogenous factors can influence our eating behavior, and hence modulate brain networks involved in food intake and thermogenesis. There are three main interacting brain regions that are related to the storage and use of energy, all of which exert particular functions: the hypothalamus and the brainstem, which classically integrate physiological cues (endostatic: the need to eat for energetic purposes), and regions of the forebrain that control the incentive to seek and ingest food (exostatic: wanting and liking food), such as the ventral tegmental area and the nucleus accumbens (Myers & Olson, 2014). However, these systems are not independent. Neurons that are involved in homeostatic responses to feeding are directly linked to neurons that influence the perception of reward and aversion to food. Both these systems are activated during any behavior that is related to feeding and associated control of peripheral metabolism, even though the degree in which each is activated may differ, depending on the food type and the metabolic state of the subject (Rossi & Stuber, 2018).

Although the neural basis of feeding and reward are intimately linked and can be overlapping, it is important to distinguish the main brain regions that regulate each.

c. Non-hypothalamic structures participating in energy balance

1. Brainstem

The nucleus of the solitary tract (NTS) is a caudal region within the brainstem that receives projections from peripheral tissues, such as the gut and the adipose tissue, and in turn projects to hypothalamic and limbic systems within the brain. It is capable of mediating meal-related stimuli and determining food consumption and meal size (Blouet & Schwartz, 2012; G. J. Schwartz, 2006). These meal-related signals are transmitted through visceral afferent feedback signals (chemical, mechanical, hormonal and nutritional), which reach the NTS: during food intake, the distention of the stomach and the presence of nutrients in the digestive tract cause the release of peptide hormones that stimulate the vagus nerve and the area postrema (AP, a circumventricular organ, hence devoid of a complete blood brain barrier), to activate neurons in the NTS. Through a negative feedback loop, the NTS can then stimulate gut movement through the vagus nerve for limiting food intake. Of note, the lesioning of both the NTS and the AP causes sustained hyperphagia. Similarly, the knockdown of leptin receptors in both regions induces hyperphagia (Waterson & Horvath, 2015).

In relation to the emotional aspects linked to feeding, the NTS projects to the amygdala to promote aversion to food, and also specific nuclei in the hypothalamus implicated in the control of ingestion (Myers & Olson, 2014). For this reason, there is a developing idea suggesting that the NTS is an integrator of neural processing related to feeding, the extent of which remains to be determined (G. J. Schwartz, 2006).

2. Reward circuits

Circuits that are involved in the motivation to ingest food are mostly mediated by dopaminergic (DA) neurons within the ventral tegmental area (VTA). These neurons project to the nucleus accumbens (NAc), which is mostly related to wanting food (incentive salience) and taking pleasure in eating it (hedonic attribute, liking), and integrate information such as the taste of food, the environment in which it is consumed and the social stimuli surrounding its consumption. These stimuli reach the hypothalamus for modulating appetitive behaviors. However, they can also project to the amygdala, hence mediating aversive responses to feeding (Myers & Olson, 2014). The insula and the amygdala can, in turn, communicate the overall value of food to the prefrontal cortex (PFC) and ACC through specific neuronal inputs, since these are brain regions that are highly sensitive to reward cues from the

environment. The ventral striatum, and in particular the medial shell of the NAc, classically dictates representations on -liking and -wanting components of behaviors driven by reward seeking through dopaminergic activity (Richard, 2015).

In an environment where highly palatable foods are readily available, the reward circuit is constantly stimulated, and hence contributes to the development of obesity. Moreover, a stressful lifestyle further contributes to the obesogenic context by weakening self-regulation brain circuits (comfort eating) (Richard, 2015).

3. Other structures

The PFC and the anterior cingulate cortex (ACC) drive the conscious decision to engage in eating or in performing physical activity, and mediate the self-discipline to modulate food intake (Richard, 2015). The parabrachial nucleus (PBN), located in the pons and in proximity to the cerebellum, is involved in many biological functions, such as arousal, control of glycaemia, body temperature regulation, breathing, and the perception of taste, pleasure and pain. Neurons of the PBN expressing calcitonin gene-related peptide (CGRP) play a major role in regulating appetite: they are stimulated by peripheral hormones that are released after a meal, which can subsequently promote a starvation phenotype that is either aversive (gastric discomfort) or rewarding (satiety after a meal) (Essner et al., 2017; Palmiter, 2018).

Studies in mice have shown an involvement of the medial PFC (mPFC) in the regulation of feeding. Optogenetic photostimulation of dopamine excitatory neurons (D1) in the mPFC has been shown to increase food intake, while inhibiting these neurons decreases intake. Moreover, D1 neurons in the PFC send projections to the amygdala, and the photostimulation of these axons seems to be sufficient to increase feeding, as observed with mPFC D1 stimulation (Land et al., 2014).

d. Hypothalamic control of energy balance

The first studies demonstrating a hypothalamic role on the control of food intake and body weight date back to the mid-20th century, where lesions in the ventromedial (VMH) hypothalamus of rats caused an increase in adiposity, leading to obesity (Hetherington & Ranson, 1942). Later on, it became clear that the VMH was responsible of causing hyperphagia, whereas another region, the lateral hypothalamus (LH) drove satiety (Anand & Brobeck, 1951). Subsequent studies identified other hypothalamic nuclei, such as the

paraventricular nucleus (PVN), the dorsomedial nucleus (DMN) and the arcuate nucleus (ARC), all of which are involved in controlling energy homeostasis.

The hypothalamus receives signals from peripheral organs through nutrient, hormonal and nervous signals, and is capable of integrating these signals in order to control energy-related information, such as caloric intake, storage and use. Indeed, fuels derived from recently ingested foods (nutrients) or that are secreted by peripheral tissues (ghrelin, leptin, insulin) can be sensed by the hypothalamus, which then integrates this information. The consequent modulation of signaling pathways can regulate the neuronal firing rate and the transcription of other molecules, hence leading to the maintenance of energy balance (Cota et al., 2007).

Due to the presence of the blood-brain barrier surrounding vessels in the brain, including the hypothalamus, these signals can take some time to be received centrally. However, one particular region, the mediobasal hypothalamus (MBH, which includes the arcuate and ventromedial nuclei) is distinctive in that it boards the third ventricle of the brain, allowing for an almost direct and immediate contact with the peripheral environment, such as with hormones and nutrients. This distinctive characteristic of direct exposure to blood-borne molecules also relies on the median eminence in the inferior portion of the hypothalamus (ventral to the third ventricle), which is a circumventricular organ, meaning that it is localized outside of the blood-brain barrier. The median eminence is constituted of fenestrated capillaries from which proteins and hormones from the periphery can filter into the hypothalamus at a more efficient rate than in other brain regions. The cells that line the median eminence are called tanycytes, a type of glial cells which also facilitate the passage of blood borne molecules for hypothalamic input and output (Rizzoti & Lovell-Badge, 2017), hence conveying the MBH a privileged neuroanatomic attribute for sensing real-time energetic needs (Prevot et al., 2018).

1. The arcuate nucleus (ARC)

Neurons located in the ARC have been largely investigated because of their anatomical location and express receptors for many different circulating molecules that are associated to eating behavior and regulation of peripheral metabolic responses, such as hormones. There exist at least two different neuronal populations that have been amply described within the ARC for guiding the use of energy or its storage:

- The catabolic functions are driven by the expression of pro-opiomelanocortin (POMC), which is processed to form other bioactive products, notably α -melanocortin stimulating hormone (α -MSH), which promotes satiety in response to feeding, increases energy expenditure and leads to weight loss by activating the melanocortin-4 receptor (MC4R) (Krashes et al., 2016; Carole M. Nasrallah & Tamas L. Horvath, 2014). POMC neurons can also express β -endorphin and cocaine-amphetamine regulated transcript (CART). POMC neurons express the receptors for leptin (LepRb), which is an adipose tissue derived hormone that will activate the cells to signal satiety. POMC neurons also express ghrelin receptors (GHS-R), a hormone secreted by the stomach that, conversely, will inhibit the satiety signaling of these neurons.
- The anabolic functions are determined by a decrease in caloric reserves, causing a release of agouti-related peptide (AgRP), neuropeptide Y (NPY) and γ -aminobutyric acid (GABA). Neurons that express AgRP are found exclusively in the ARC, and they are activated by fasting and promote feeding. They send projections to the forebrain, other hypothalamic nuclei and the hindbrain (Rossi & Stuber, 2018). Interestingly, AgRP acts as an inverse agonist of the MC4R, suggesting common downstream targets for both anabolic and catabolic cell types. Moreover, AgRP expressing neurons are capable of inhibiting POMC neurons through GABA signaling, demonstrating that both AgRP and POMC neuronal populations differentially influence feeding. AgRP neurons also express LepRb and GHS-R, and their activity have the opposite effect as in POMC neurons. Leptin will bind to its receptor in AgRP neurons to inhibit appetite signaling, while ghrelin will stimulate hunger.

Both POMC/CART and AgRP/NPY expressing neurons in the ARC, together with neurons in the PVN that express melanocortin receptors, constitute the hypothalamic melanocortin system, which is one of the most important neural circuits involved in controlling feeding behavior and metabolism (**Fig. 7**) (Hill & Faulkner, 2017).

Therefore, the overall energetic state of the organism is strongly influenced by the way in which these two distinct cellular populations in the ARC interact with each other in response to peripheral queues: following a period of fasting, the activity of anorexigenic neurons that express POMC and CART diminishes, while the activation of orexigenic neurons expressing AgRP/NPY is increases, leading to an increased appetite, food consumption and energy storage. Conversely, after a meal, the activity of anorexigenic neurons increases, while orexigenic neuron activation is decreases, hence leading to a signal of satiety, reduction of

food intake and an increased energy expenditure (Richard, 2015). However, recent evidence suggests that these classic roles of POMC and AgRP neurons may be outdated. Work in preparation from our own research group confirms that, indeed, there exist subpopulations of POMC neurons that are capable of driving feeding (Saucisse N, Mazier W, Simon V et al., in preparation). Additionally, fiber photometry studies have shown that POMC neurons are immediately activated (increased calcium signaling) as soon as animals perceive food-related cues, implying that POMC neurons may play a major role in physiologically driving feeding and preparing the organism for actual food intake (Brandt et al., 2018), while AgRP neurons seem to be more relevant in the actual search of food and are then immediately switched off as soon as food becomes available (Betley, Cao, Ritola, & Sternson, 2013; Betley et al., 2015; Y. Chen, Lin, Kuo, & Knight, 2015).

2. *The paraventricular nucleus (PVN)*

This hypothalamic nucleus receives output targets from both AgRP and POMC neurons of the ARC. It is located adjacent to the third ventricle and dorsal to the ARC. It is implicated in behaviors such as feeding, drinking and temperature regulation through projections to the hindbrain (Rossi & Stuber, 2018). The MC4R is expressed within this nucleus, and hence, α -MSH and AgRP produced by POMC and AgRP neurons of the ARC act within the PVN for promoting satiety or hunger, respectively, modulating energy expenditure.

Therefore, by receiving input from other hypothalamic nuclei, the PVN participates in recognizing the energetic needs of the body, and can adjust its outputs accordingly, through directing physiological and behavioral responses (Krashes et al., 2016).

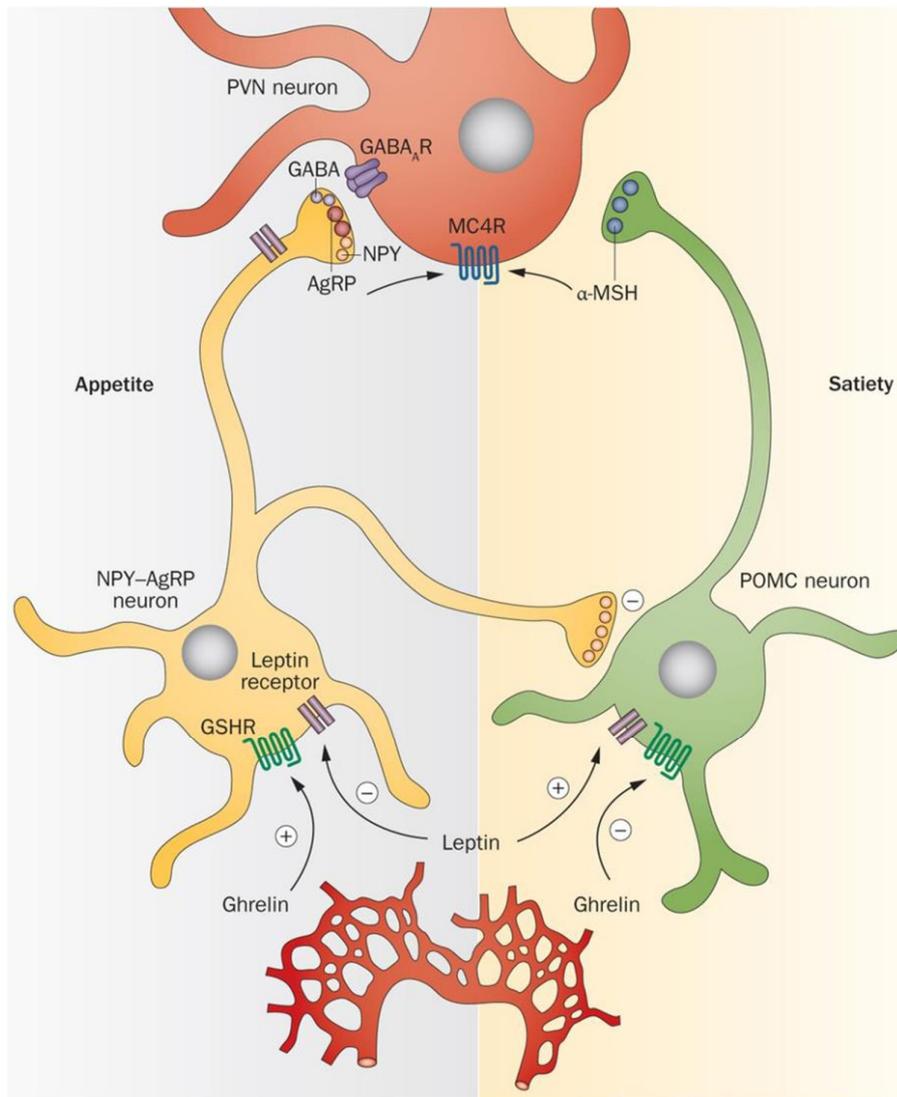


Figure 7. The melanocortin system. Both AgRP and MSH are neuropeptides that bind to the MC4R that are expressed in target neurons, such as those of the PVN of the hypothalamus, causing opposite responses: MSH will activate the MC4R, whose ultimate effect will be to promote satiety. AgRP acts on the same receptor as an antagonist (or biased agonist), leading to an increase in food intake (Krashes et al., 2016). Moreover, neurons expressing NPY/AgRP can also release NPY and GABA to promote orexigenic responses, and inhibit neurons expressing POMC, keeping a balance between this bimodal system. Peripheral hormones such as leptin and ghrelin have differential effects on these neuronal populations [Taken from (C. M. Nasrallah & T. L. Horvath, 2014)]. AgRP: agouti-related protein; GABA: -aminobutyric acid; GABA_AR: GABA receptor; GSHR: growth hormone secretagogue receptor (or ghrelin receptor); MC4R: melanocortin receptor 4; NPY: neuropeptide Y; POMC: proopiomelanocortin; PVN: paraventricular nucleus of the hypothalamus.

3. *The ventromedial nucleus (VMN)*

It classically constitutes a satiety center, which plays a critical role in the sensing of glucose. It is composed of two distinctive neuronal populations that respond directly to changes in extracellular glucose levels: ones that increase their electrical activity (excited) and others that decrease their electrical activity (inhibited) in response to glucose (Fioramonti, Song, Vazirani, Beuve, & Routh, 2011). Hence the VMN is of importance under conditions of hypoglycemia, through receiving and sending projections to other hypothalamic nuclei.

A number of studies suggest that the VMN closely participates in the regulation of energy expenditure (Lopez et al., 2010). The central (ICV or intra-VMN) administration of T3 hormone increased BAT thermogenesis and mitochondrial activity, as well as increased de novo fatty acid synthesis and uptake into the liver. Notably, these effects in the BAT and liver were not observed when T3 was administered into the ARC (Martinez-Sanchez et al., 2017).

4. *The dorsomedial nucleus (DMN)*

It has been implicated in the regulation of body temperature by increasing the thermogenic activity of the brown adipose tissue via the SNS. Certain neurons in this nucleus express leptin receptors, which activation takes to the excitation of the sympathetic nervous system, causing hyperthermia (Enriori, Sinnayah, Simonds, Garcia Rudaz, & Cowley, 2011). The DMN also expresses the MC3R and MC4R and receives projections of POMC and AgRP neurons from the ARC (Mountjoy, Mortrud, Low, Simerly, & Cone, 1994). Moreover, they express the Y1 and Y5 receptors, which are both targets for NPY, demonstrating an involvement of the DMN in the control of eating behavior (Bi, Kim, & Zheng, 2012).

Neurons within the DMN can also have an impact in energy homeostasis by modulating SNS inputs onto WAT and BAT. Notably, knocking down the expression of NPY in the DMN results in an increased peripheral sympathetic tone in discrete fat deposits (inguinal WAT and interscapular BAT), a process that leads to an increase in thermogenesis and energy expenditure (Bi et al., 2012).

5. *The lateral hypothalamic area (LHA)*

This hypothalamic nucleus was originally described as the feeding center, since lesioning studies reported severe hypophagia to the point of starvation, and electrical stimulation promoted food intake even in sated animals. However, it is now clear that the

LHA can modulate a wide variety of systems, including the autonomic, mesolimbic and somatomotor systems to regulate the skeletal and cardiovascular muscles, adrenal gland and BAT, as well as to determine the value of stimuli (food, drugs, stress, etc.). Due to its involvement in these processes, it is thought that the role of the LHA extends to the coordination of motivated behavior through peripheral output (engaging muscles to seek for food, increase energy expenditure through BAT) (Leininger, 2011).

6. *The suprachiasmatic nucleus (SCN)*

Mammals follow a rhythmic environment that is coordinated with the rotation of the Earth around its axis, causing the succession of day and night. Circadian oscillators that follow this 24 hour pattern are found in all mammalian cells, and are coordinated by a master pacemaker that is located in the SCN of the anterior hypothalamus. This is achieved through the transmission of light/dark information to the retina, which then travels through fibers that will leave the optic pathway to reach the SCN (retino-hypothalamic tract). This temporal information is capable of synchronizing the SCN clock to the external environment. Compared to other cells of the body, the rhythm of the SCN is autonomous and self-sustained, and coordinates oscillations in peripheral organs to ensure a phase coherence in the whole body (Kolbe, Brehm, & Oster, 2019). The synchronization is achieved through cellular mechanisms, comprised of nuclear auto-regulatory transcriptional and translational feedback loops which are encoded in specific clock genes (Froy, 2010).

The SCN sends projections to several hypothalamic nuclei in order to participate in energy homeostasis. The DMN and the VMN are responsible of relaying information from the SCN to other cell bodies that are located in the hypothalamus in order to regulate sleep/wake cycles, locomotion, corticosteroid secretion and feeding. The PVN also receives fibers from the SCN, which can control neurons related to the autonomic nervous system (sympathetic and parasympathetic) in order to regulate peripheral tissues (Froy, 2010).

Conversely, the ARC can send projections to the SCN in order to transmit information related to the overall energetic state of the organism, due to its strategic neuroanatomical position boarding the third ventricle and the leaky blood-brain barrier of the median eminence. Hence, this interaction provides the SCN a link with circulating metabolic information (Froy, 2010).

In animal models of diet-induced obesity, it has been shown that perturbations of metabolic pathways are linked to alterations of the circadian oscillations, where cells from peripheral

tissues present a shift in their 24 hour rhythm. Interestingly, it was found that the reprogramming of the peripheral clocks was dependent of the nutritional challenge (a high-fat diet), and not on the presence of an obese phenotype. Moreover, these effects were reversible when mice were returned to a standard chow diet (Eckel-Mahan et al., 2013). The relationship between circadian clock disturbances and associated metabolic complications has also been shown in humans, by observation of subjects that engage in shift work, frequent trans-meridian flights or exposed to continuous levels of stress. These factors exacerbate insulin resistance, elevate cortisol levels, and favor the development of obesity and type 2 diabetes (Albrecht, 2012).

As seen, many different brain structures send and receive projections, either directly or indirectly, to the hypothalamus, so that the circuits that participate in controlling eating behavior and overall energy balance are interconnected (**Fig. 8**) (Myers & Olson, 2014).

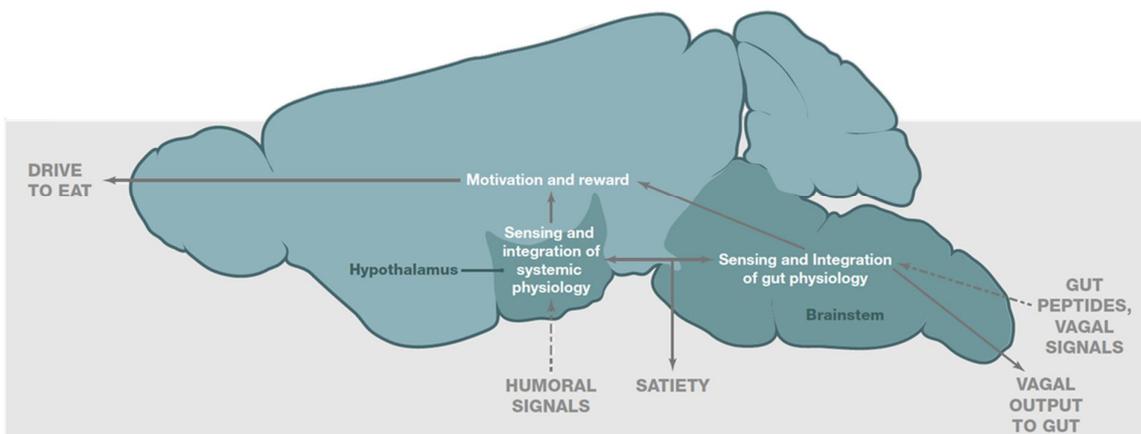


Figure 8. Integrated view of neural pathways that control feeding. The CNS drives the quantity and the timing of food intake. These signals are driven by different interacting brain regions. The brainstem receives information from the gastrointestinal tract, while the hypothalamus receives direct input from the circulation (humoral signals). Both the brainstem and the hypothalamus control the sensations of appetite and satiety, and can relay this information to a third region, the corticolimbic system, which is mostly involved in the motivation to seek food and the reward it entails [Taken from (Myers & Olson, 2014)].

e. **The autonomic nervous system**

The CNS must be in constant communication with the periphery in order to adjust the temporal energetic needs accordingly. For this, the CNS makes use of the autonomic nervous system (ANS) to communicate with peripheral tissues that in turn, can act on the CNS (Seoane-Collazo et al., 2015). The ANS is mostly regulated by the hypothalamus, as previously mentioned when describing the different hypothalamic nuclei, and innervates metabolic organs that play crucial roles in physiological responses. It is divided in the sympathetic and parasympathetic nervous systems (SNS and PSNS) (Seoane-Collazo et al., 2015). The information that the CNS receives from peripheral tissues mainly comes from sensory neurons, the PSNS, and circulating factors, which can all trigger the activation of the SNS to modulate the use of energy (**Fig. 9**).

- **Sensory neurons** are responsible for conveying environmental information to the brain, which will then relay the information to peripheral tissues through SNS innervations. Transient receptor potential channels (TRP), which are found on sensory neurons of the skin and adipose tissues, participate in integrating the sensations of touch, pain, temperature and taste. The activation of such channels can drive the increase of the sympathetic tone through CNS signaling. Moreover, TRP can also act directly on the adipose tissue, where it can inhibit adipogenesis in BAT and WAT (Blaszkiewicz & Townsend, 2016).
- **The SNS** is also involved in this crosstalk, notably through the CNS control of feeding and increased EE, as well as through direct innervation of the WAT for the increase of EE and browning of the adipose tissue (Blaszkiewicz & Townsend, 2016).
- **Circulating factors** from the periphery can further act on the CNS or also directly on the adipose tissues to participate in energy balance (Blaszkiewicz & Townsend, 2016).

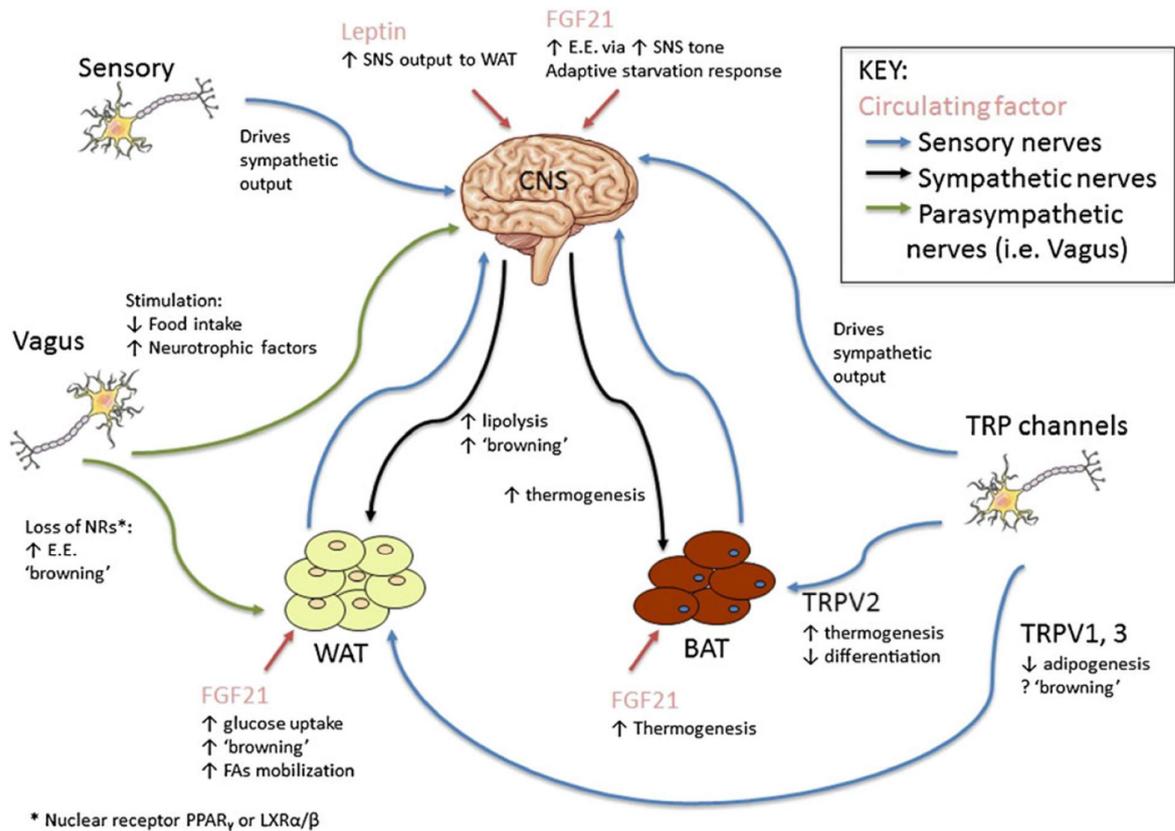


Figure 9. Periphery to brain crosstalk for the regulation of energy expenditure. Sensory neurons transmit environmental queues to the CNS, which can in turn respond to the periphery through the SNS to control the use of energy in the WAT and BAT (thermogenesis, lipolysis, browning). The vagus nerve can either directly target the WAT to control energy use, or do this indirectly through signaling to the CNS. Circulating factors such as hormones can act on the CNS to regulate EE via hypothalamic pathways that will increase the sympathetic tone, or act locally on adipose tissues to directly trigger lipolysis and thermogenesis [Taken from (Blaszkievicz & Townsend, 2016)]. BAT: brown adipose tissue; CNS: central nervous system; EE: energy expenditure; FA: fatty acids; FGF21: fibroblast growth factor 21; SNS: sympathetic nervous system; TRP: transient receptor potential channels; WAT: white adipose tissue.

Independently of the source of the stimulation (sensory neurons, SNS or circulating factors), the contribution of the SNS in dictating the use of energy mostly comes from the modulation

of adipose depots, specifically the BAT for regulating thermogenesis, and the WAT for stimulating lipolysis (Blaszkiwicz & Townsend, 2016).

f. Hypothalamic control of energy expenditure, thermogenesis and lipolysis in the adipose tissue through the sympathetic nervous system

Notably, particular nuclei of the hypothalamus greatly contribute to the control of BAT thermogenesis and WAT lipolysis through modulating the sympathetic drive (**Fig. 10**) (Lage, Ferno, Nogueiras, Dieguez, & Lopez, 2016):

The preoptic area (POA) is known as the thermoregulatory center, since it recognizes cutaneous, hormonal, and central thermal signals through temperature sensitive neurons to regulate adaptive thermogenesis (Boulant, 2000). When stimulated, it can promote adrenergic activity in the BAT and WAT (Lage et al., 2016).

The DMN contains heat-sensitive neurons that project to the brainstem, which will then stimulate thermogenesis in the BAT, which can act as a neuroprotective mechanism against increased environmental heat. Moreover, the DMN also expresses NPY, which in this case modulates the sympathetic tone in WAT and BAT. Under diet-induced obesity conditions, the DMN overexpresses NPY, even in the fed state (as opposed to its lower expression in the ARC), and inhibits the excitation of sympathetic nerves, contributing to the obese phenotype (Chao, Yang, Aja, Moran, & Bi, 2011). In line with this, the expression of NPY in the DMN is increased during cold exposure, which suggests that these neurons would prioritize food intake over thermogenesis in order to cope with the energetic demand (Yang et al., 2009).

Recent work has stressed the involvement of **the VMN** in the regulation of BAT thermogenesis. After central administration of T3 via ICV or directly to the VMN, hypothalamic lipogenesis is heightened, associated with a decrease in adenosine monophosphate-activated protein kinase (AMPK), which leads to an increase in AgRP/NPY expression that activates SNS outflow. As a consequence, central T3 acutely increases thermogenesis markers in the BAT (Lopez et al., 2010; Martinez-Sanchez et al., 2017).

Adaptive thermogenesis is also regulated by **the LHA** through the expression of the orexigenic peptides melanin-concentrating hormone (MCH) and orexin (OX). MCH-knockout mice have a leaner phenotype compared to their wild types, which is mostly attributed to

increased thermogenesis in the BAT (Segal-Lieberman et al., 2003). Conversely, the central injection of OX increases sympathetic flow, which heightens the metabolic rate and body temperature (W. Zhang et al., 2010).

The expression of NPY/AgRP in **the ARC** in response to energetic needs suppresses adaptive thermogenesis in order to preserve energy. Indeed, the expression of NPY in the ARC directly inhibits heat production by suppressing the sympathetic flow to the BAT. Furthermore, it indirectly counteracts thermogenesis by decreasing the expression in the PVN of tyrosine hydroxylase, a key limiting enzyme in the synthesis of catecholamines. On the contrary, a reduction of NPY or AgRP expression in the ARC increases the expression of thermogenesis markers in the BAT (Shi et al., 2013). Similarly, **the PVN** also mediates the NPY-induced decrease of thermogenesis in brown adipocytes. Since the PVN is considered the major autonomic output of the hypothalamus, it is suggested that the effects of the ARC on thermoregulation are mostly mediated by the PVN (Lage et al., 2016).

Hence, there is a growing body of evidence supporting a crucial role for particular hypothalamic nuclei in the regulation of the sympathetic tone towards the BAT for regulating heat production, and the WAT for triggering lipolysis. The fact that these complex processes act together, and are sometimes redundant, allows for a rapid, homeostatic response that will meet the immediate metabolic needs (Lage et al., 2016).

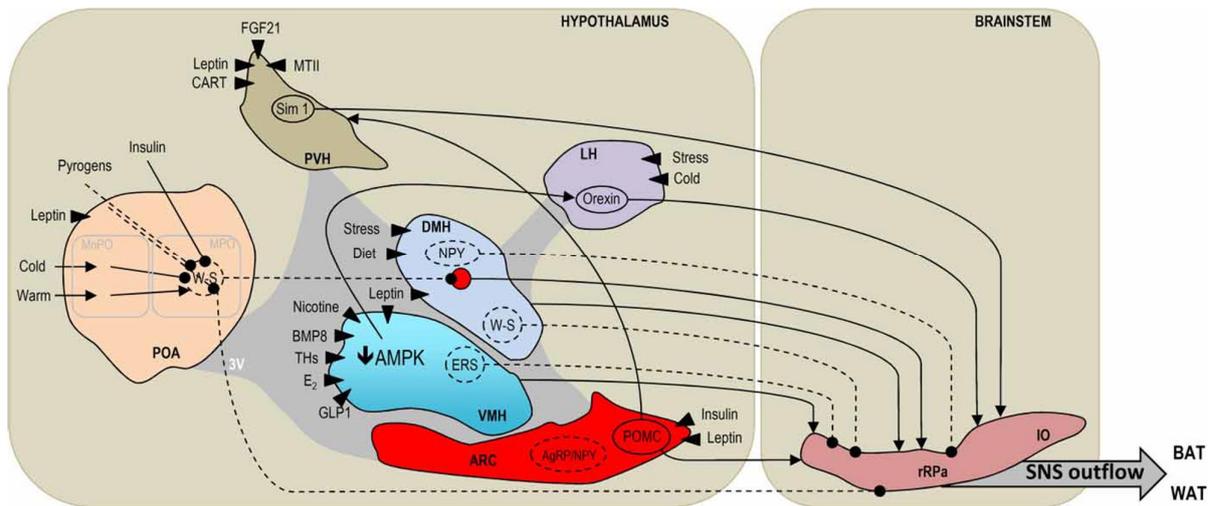


Figure 10. Hypothalamic control of adaptive thermogenesis. Signals from the periphery (hormonal or environmental) are integrated in the hypothalamus to regulate the sympathetic drive towards the BAT and WAT. The POA integrates thermal sensory signals that project information to the DMH that will in turn stimulate the brainstem to increase SNS outflow. Appetite and satiety neurons, such as NPY, AgRP, POMC and orexin neurons in the DMN, the ARC or the LHA also participate in the regulation of body temperature. Lastly, peripheral signals can directly target the VMN to modulate AMPK activity and enhance sympathetic outflow. These effects will collectively have an impact on the thermogenic and lipolytic program of BAT and WAT tissues in the periphery, which are under the control of the SNS. Dashed lines represent inhibited pathways, and rounded ends indicate inhibitory signals [Taken from (Lage et al., 2016)]. 3V: third ventricle; AgRP: agouti-related protein; BAT: brown adipose tissue; DMH: dorsomedial hypothalamus; IO: inferior olivary nucleus; LH: lateral hypothalamus; NPY: neuropeptide Y; POA: preoptic area; POMC: proopiomelanocortin; PVH: paraventricular hypothalamus; rRPa: rostral raphe pallidus; SNS: sympathetic nervous system; VMH: ventromedial hypothalamic nucleus; WAT: white adipose tissue.

The hypothalamic θ thyroid axis is particularly relevant for the temperature-related modulation of the SNS. The CNS responds to changes in external temperatures in order to regulate core body temperature. This is done through the activation of the hypothalamic-pituitary-thyroid axis (HPT axis) that is responsible for synthesizing thyroid hormones. Upon sensing of the environmental cues of temperature (e.g. cold exposure), thermo sensitive

hypothalamic neurons synthesize thyrotropin-releasing hormone (TRH), which will in turn stimulate the synthesis and secretion of thyroid stimulating hormone (TSH) from the pituitary gland. The end product of this axis is the biosynthesis of thyroid hormones by the thyroid gland in the form of T4, which will be modified to active T3 by deiodinases (Nillni, 2010). T3 can then act on different targets in order to activate the SNS for the synthesis of catecholamines (such as norepinephrine). These will finally reach tissues in the periphery, such as the WAT, BAT, liver and muscle to stimulate energy expenditure and heat production through binding to α -adrenergic receptors (Blaszkiewicz & Townsend, 2016).

g. Brown adipose tissue thermogenesis

Compared to other tissues, the thermogenic capacity of the BAT is remarkable, making it the primary site of non-shivering thermogenesis in rodents and accounting for up to 75% of the increased metabolic rate induced by the SNS (Labbe, Caron, Lanfray, et al., 2015). Moreover, it is estimated that the BAT triglyceride pool during an acute cold challenge contributes to up to 84% of heat production (Labbe, Caron, Bakan, et al., 2015). This tissue owes its high thermogenic properties to the production of heat, rather than ATP, as a result from the membrane potential generated from oxygen consumption (Clapham, 2012). Non-shivering thermogenesis is a process that mostly depends on the activation of the SNS. Indeed, brown adipocytes are highly innervated by the SNS (evidenced by their rich expression of thyroxin hydroxylase, the rate limiting enzyme for the synthesis of catecholamines) (Murano, Barbatelli, Giordano, & Cinti, 2009) and abundantly express the α -3 adrenergic receptor, whose activation by catecholamines (such as norepinephrine) stimulates heat production (Blaszkiewicz & Townsend, 2016).

The potential of BAT to participate in heat production is attributed to the expression of Ucp-1, which is a protein that is solely found in the inner membrane of brown adipocytes. Upon stimulation, sympathetic neurons increase their firing and release catecholamines, such as noradrenaline, which in turn activate α -3 adrenergic receptors. This will lead to the expression of Ucp-1, which uncouples oxidative phosphorylation and ATP production in mitochondria. In this way, the energy that is generated in the respiratory chain due to oxygen consumption is used to generate a membrane potential that will be dissipated in the form of heat, rather than ATP synthesis. Therefore, the primary metabolic product in the BAT is heat, rather than ATP, as is the case in most tissues (Clapham, 2012). In order for this process to occur, the

mobilization of free fatty acids is essential for thermogenesis, which is achieved by increasing the hydrolysis of lipids (lipolysis) (Braun, Oeckl, Westermeier, Li, & Klingenspor, 2018).

However, catecholamines that are produced as a direct consequence of SNS activity are not the sole stimulators of heat production in the BAT. In fact, many different activators of BAT have been found that act independently or in association with the SNS (**Fig. 11**) (Villarroya & Vidal-Puig, 2013):

- **The thyroid system:** BAT expresses type II thyroxine 5-deiodinase, which is an enzyme controlled by norepinephrine that is responsible for converting T4 into active T3 hormone. As mentioned previously, intracellular T3 can induce thermogenesis in BAT through inducing the expression of Ucp-1.
- **Muscle activity:** Although it is still unclear if exercise increases BAT activity through browning of WAT depots, it is speculated that a newly discovered hormone, irisin, might play a role in increasing thermogenesis, since it is released from the muscle tissue after exercise (Bostrom et al., 2012).
- **Macrophages:** Cold-induced thermogenesis recruits macrophages that reside in the adipose tissue. These activated macrophages are capable of secreting catecholamines to promote BAT thermogenesis (Nguyen et al., 2011).
- **Natriuretic peptides:** These are hormones produced by the heart that can enhance BAT thermogenesis and the browning of WAT by interacting with their receptors, and can be as potent as α -3 adrenergic receptors in doing so (Bordicchia et al., 2012). This crosstalk is conceivable when considering that BAT activation by a cold environment would need an increased cardiac output to be efficient.
- **Fibroblast growth factor 21 (FGF21):** Produced by the liver, FGF21 can have antidiabetic and weight loss effects in obese rodents. By interacting with its receptor, it can activate thermogenesis in BAT, stimulate the browning of WAT, and induce glucose oxidation (Fisher et al., 2012). Presently, it is thought that FGF21 is an autonomous and direct activator of BAT, since no central effects or interaction with the SNS has been reported.
- **Bile acids:** As mentioned previously, BA are also capable of increasing thermogenesis in the BAT through a process that is dependent of the conversion of T4 into active T3, in order to stimulate Ucp-1 synthesis. Hence, BA can increase the thermogenic

capacity of the BAT (Teodoro et al., 2014; Watanabe et al., 2006; Zietak & Kozak, 2016).

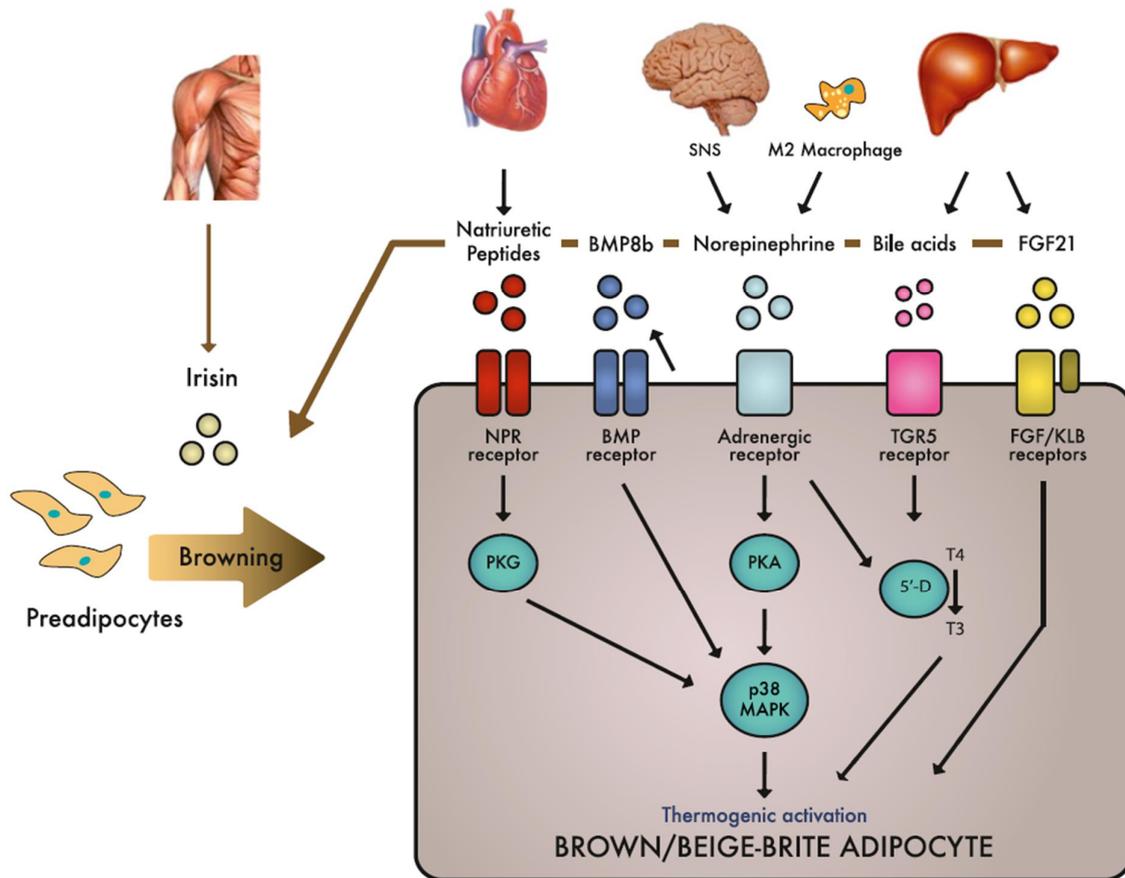


Figure 11. Metabolic factors contributing to BAT thermogenesis. Apart from catecholamines activating adrenergic receptors in the BAT, other molecules are capable of initiating the thermogenic program, which do not necessarily depend on the SNS. Notably, bile acids have been previously reported to participate in this process through the activation of their TGR5 receptor. Irisin, a newly discovered muscle hormone, natriuretic peptides from the heart and FGF21 are other novel activators that promote BAT recruitment and WAT browning to different extents [Taken from (Villarroya & Vidal-Puig, 2013)].

h. White adipose tissue lipolysis

Adipocytes are the main energy storage in mammals, since they are capable of accumulating triacylglycerol (TAG), which are lipids consisting of three fatty acids linked to a glycerol molecule. When energy is required (e.g. during fasting, exercise or cold exposure), the process of lipolysis is initiated, which will lead to a series of intracellular signaling cascades that will culminate in the sequential hydrolysis of TAG to produce free fatty acids. These, eventually, will undergo β -oxidation to generate energy (Bolsoni-Lopes & Alonso-Vale, 2015).

There are three main enzymes that participate in lipolysis: first, TAG is hydrolyzed to diacylglycerol (DAG) and a free fatty acid in a reaction catalyzed by the adipose triglyceride lipase (ATGL). Next, DAG is hydrolyzed to a second free fatty acid and monoacylglycerol (MAG) by the action of hormone sensitive lipase (HSL). Lastly, MAG will be hydrolyzed to glycerol and a third free fatty acid through the action of monoacylglycerol lipase (MGL). These fatty acids will generally be released in the bloodstream and be used as energy substrates by other tissues (Bolsoni-Lopes & Alonso-Vale, 2015). The major enzymes contributing to TAG breakdown are ATGL and HSL, since they are responsible for more than 95% of the hydrolase activity in the WAT (Schweiger et al., 2006).

As occurs in the BAT, there exists a bidirectional communication between the brain and the WAT, which happens through the SNS, and the activation of this pathway is necessary for lipolysis (Bartness, Liu, Shrestha, & Ryu, 2014). Indeed, the tone of sympathetic innervation allows for catecholamines (mostly noradrenalin) output to the WAT, which will bind to β -adrenoreceptors to increase cAMP levels to activate the protein kinase A (PKA). In turn, PKA will lead to the phosphorylation of HSL that will allow its translocation into the lipid droplet to access TAG and initiate lipid mobilization. ATGL, on the other hand, is regulated by an activator protein (/ -fold domain containing protein 5) (Schweiger et al., 2006), and also by an increase in AMPK, which phosphorylates ATGL and increases its activity (Bolsoni-Lopes & Alonso-Vale, 2015). The sympathetic innervation of the WAT has further been evidenced by studies of surgical or chemical sympathectomy, which resulted in an inhibition of lipolysis and the accumulation of TAG within the fat tissue (Vaughan, Zarebidaki, Ehlen, & Bartness, 2014; Youngstrom & Bartness, 1998).

Adipocytes not only accumulate energy, but constantly mobilize large amounts of fatty acids that are stored in TAG droplets. For this reason, the size of the lipid droplet is constantly

changing according to the energy requirements, and as a result of variations in food intake and energy use. Due to the effects of stimulating lipolysis and the potential to decrease body weight, efforts have been made to selectively target the CNS-SNS-WAT through pharmacological treatments of metabolic diseases (Braun et al., 2018).

Apart from catecholamines, other circulating factors are involved in initiating lipolysis either directly, or through an indirect pathway involving the SNS (**Fig. 12**) (Braun et al., 2018):

- **Adenosine:** This purine can either initiate or inhibit lipolysis through binding to distinctive GPCRs. Adipocytes can liberate adenosine into the extracellular space, where it can consequently act in an autocrine fashion.
- **Melanocortins:** Beyond their action in the CNS, melanocortins can also act on melanocortin receptors that are expressed in the periphery. MSH and adrenocorticotrophic hormone (ACTH) display lipolytic functions in rodent adipocytes, although their implication in humans is not yet clear (Kiwaki & Levine, 2003).
- **Natriuretic peptides:** As in the BAT, these peptides can also participate in lipolytic processes. Particularly, the atrial natriuretic peptide (ANP) can stimulate the phosphorylation of HSL to trigger the lipolytic pathway.
- **Parathyroid hormone:** This peptide hormone, which classically regulates circulating calcium levels, can also activate the cAMP ó PKA pathway to initiate lipolysis.
- **Bile acids:** Similar to their actions in the BAT, bile acids can signal through the TGR5 receptor pro-lipolytic effects.

Interestingly, leptin and GLP-1 have been suggested to indirectly participate in this process through hypothalamic signaling that would trigger the activation of the SNS to stimulate WAT lipolysis (Lockie et al., 2012; Zeng et al., 2015). These observations are supported by findings in leptin deficient mice that are obese (ob/ob), where leptin treatment was able to reduce food intake and fat mass (Montez et al., 2005). Conversely, insulin can inhibit lipolysis by decreasing PKA activity, which will hamper HSL phosphorylation and promote adiposity (Bartness & Ryu, 2015; Langin, 2006).

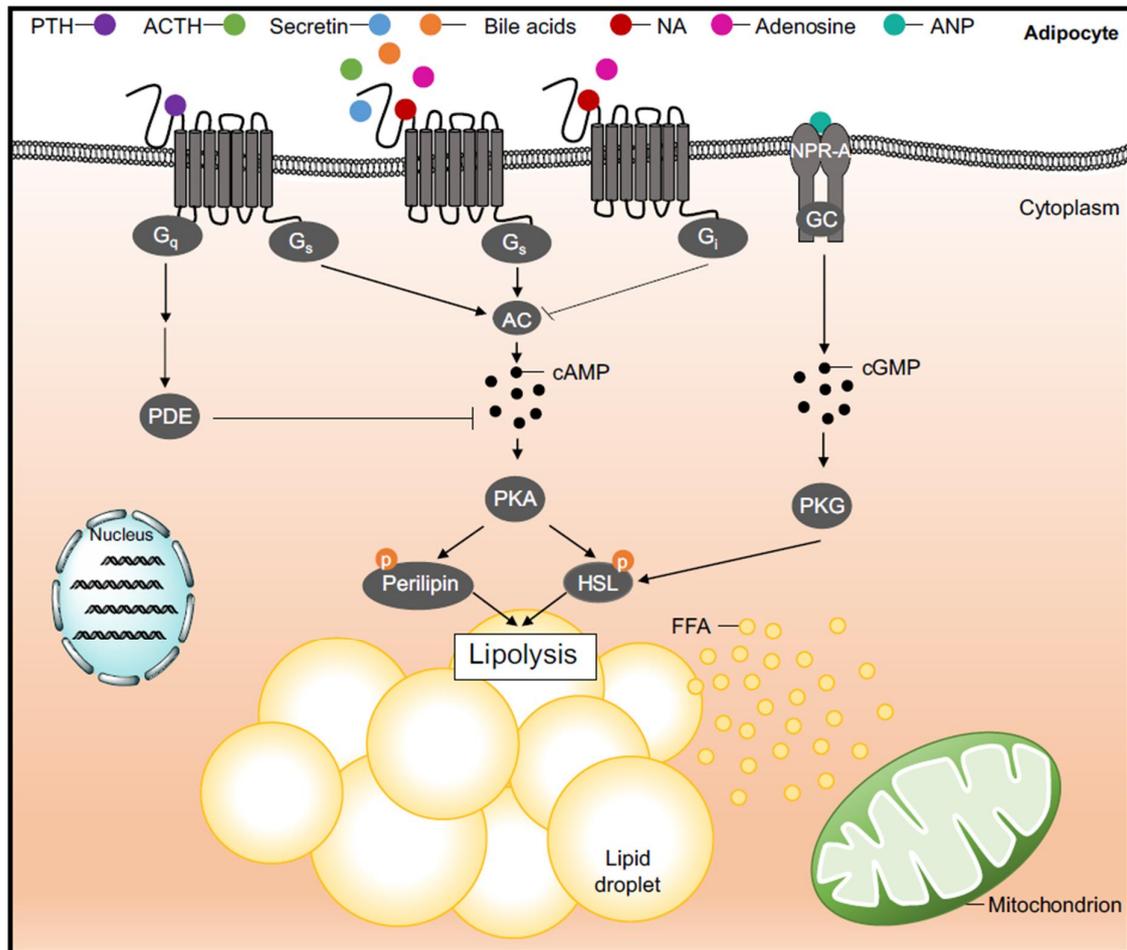


Figure 12. Circulating factors regulating lipolysis in WAT. The action of catecholamines on α -adrenoreceptors is not the only mechanism triggering lipolysis in the WAT. Other circulating factors participate in enhancing the actions of lipolytic enzymes, particularly through the phosphorylation of HSL by G-protein coupled receptor activity. Examples of such receptors are the melanocortin receptor 2 and adrenocorticotropic hormone (ACTH), and the adenosine receptor 2a and adenosine. Interestingly, bile acids are known to participate in this process through their specific G-protein coupled receptor, TGR5 [Taken from (Braun et al., 2018)].

Taken together, the control of energy homeostasis relies on the tight regulation of energy intake (eating behavior) and expenditure (by modulating muscle activity, BAT and WAT energy stores, and thermogenesis). This regulation is dictated by many brain regions that can either participate in the control of executive functions (decision to eat), the hedonic value of food (liking, wanting), or in the autonomic circuits regulating eating behavior (appetite and satiety signals). The activity of these brain circuits are influenced by environmental (temperature) and peripheral (nutrients, hormones, BA) mediators that can inform the CNS of the body's current energetic state. These overlapping and interacting systems have emerged as a central concept for the study of obesity and its associated comorbidities, especially due to the influence of the brain areas involved in the control of BAT and WAT mobilization.

II. Knowledge gap and open questions

III. Hypothesis

IV. Objectives

II. Knowledge gap and open questions

Apart from participating in lipid digestion within the gastrointestinal tract, BA are now recognized as signaling molecules that are involved in a myriad of metabolic effects, the most dramatic being their contribution to the sustained body weight loss and diabetes remission that occur after bariatric surgery (Goncalves et al., 2015; Kohli et al., 2013; McGavigan et al., 2017). The mechanisms by which these effects are achieved are attributed to an increase in circulating BA following surgery, and to their action on peripheral targets. This is achieved through their binding onto their specific receptor, TGR5, whose activation in tissues such as the muscle, gut and adipose tissues reduces body weight, increases energy expenditure and improves glycemic control. These effects are closely related to the control of energy balance and whole body metabolic responses, a process that is tightly regulated by the brain, and in particular by the hypothalamus. However, all studies investigating the metabolic functions of BA through TGR5 activation have so far exclusively focused on their action in peripheral organs, so that the state of the art ignores the role that BA can have in the CNS through the activation of this receptor, and their consequent impact on the regulation of energy balance.

Disentangling the central role of BA through TGR5 activation is of interest for several reasons. First of all, although it is accepted that BA are capable of crossing the blood-brain barrier (Keene et al., 2001; Parry et al., 2010), and that the TGR5 receptor is expressed centrally (Kawamata et al., 2003; Maruyama et al., 2002), the relevance of this system within the brain has not been explored directly and therefore remains to be elucidated. Second, it is not clear how bariatric surgery can lead to such astounding metabolic outcomes. Indeed, the hypothesis that these effects are partly due to an increase in circulating BA is currently being explored, but due to its overall effects on energy metabolism, it mostly hints at the central modulation of energy balance. Furthermore, there is evidence that the regulation of the BA pool size not only occurs in the liver, but is also controlled centrally by the hypothalamus, which might act as a sensor for BA (Doignon et al., 2011). Third, TGR5 activation is considered a therapeutic target against obesity. However, the effects in humans seem to be inconsistent (Hodge et al., 2013), potentially due to off-target effects of TGR5 agonists administered orally. Most important, it is surprising that, given its potential in the treatment of obesity and diabetes, little research has been carried out for understanding the underlying mechanisms by which BA participate in enhancing energy use, a process classically

controlled by the hypothalamus. Lastly, exploring the role of BA within the brain could lead to the discovery of better dietary or pharmacological strategies to treat obesity, mimicking the metabolic benefits of bariatric surgery, while at the same time excluding the risk this intervention carries with it.

III. Hypothesis

We hypothesized a role for the TGR5 receptor within the hypothalamus, a major brain structure involved in the control of eating behavior and energy expenditure, suggesting that the modulation of hypothalamic TGR5 activity is relevant for the control of energy balance, particularly under diet-induced obesity.

IV. Objectives

The general aim of this work was to evaluate the actions of BA within hypothalamic nuclei that participate in energy balance, through the pharmacological activation of their specific receptor TGR5, and to further understand their contribution to metabolic improvements, particularly in the presence of diet-induced obesity. To do this, our objectives were divided in the following axes:

1. Identify the presence of BA system components centrally under different energetic states (lean *vs.* diet-induced obese) at the level of the hypothalamus;
2. Challenge the system *in vivo*, through the acute and chronic pharmacological activation of the TGR5 receptor centrally, and identify the molecular pathways driving its effects;
3. Pinpoint the cellular populations in the MBH responsible for the metabolic effects of central TGR5 activation.

V. Previous results generated in the team

V. Previous results generated in the team

Initial data supporting our hypothesis were generated within Dr. Cota's team by **Omar Guzmán-Quevedo, PhD** (Laboratory "Neuronutrition and Metabolic Disorders", Higher Technology Institute of Tacambaro, Mexico), a postdoctoral researcher at the time who investigated the presence of the components of the BA ó TGR5 receptor system within the hypothalamus of mice under different metabolic conditions (i.e. lean vs. diet-induced obese). These experiments were carried out in C57BL6/J mice (Janvier, France) that were fed either a standard chow diet (Standard Rodent Diet A03, SAFE, France) or a commercial high-fat diet (HFD) (D12492, Research Diets Inc. New Brunswick, NJ, USA). His findings are summarized in the following section.

A. Bile acid receptors are expressed in the hypothalamus

The expression by real-time quantitative PCR (qPCR) of the BA receptors FXR and TGR5 was measured in hypothalamic samples of lean mice that were either fed *ad libitum* (n=6) or fasted (n=7) for 24-h. Hypothalamic and liver tissues were obtained, immediately frozen and kept at -80 °C until further processed. Tissues were taken for isolating total RNA (TRIzol®, Invitrogen, CA, USA), followed by the synthesis of cDNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, France). cDNA of BA receptors TGR5 and FXR were amplified and quantified by qRT-PCR (Applied Biosystems, France) under standard conditions (1x 3 min at 95 °C, 40x 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C). Relative expression was corrected for PCR efficiency and normalized to the reference genes *Ppia* and *Nono*. Expression was quantified using the comparative 2^{-Ct} method. Expression levels are presented as the fold change from the *ad libitum* group after normalization.

These first results show that the TGR5 receptor is expressed within the hypothalamus of C57BL6/J lean mice, whilst the FXR receptor was undetectable, in accordance with previous reports (**Fig. 13**).

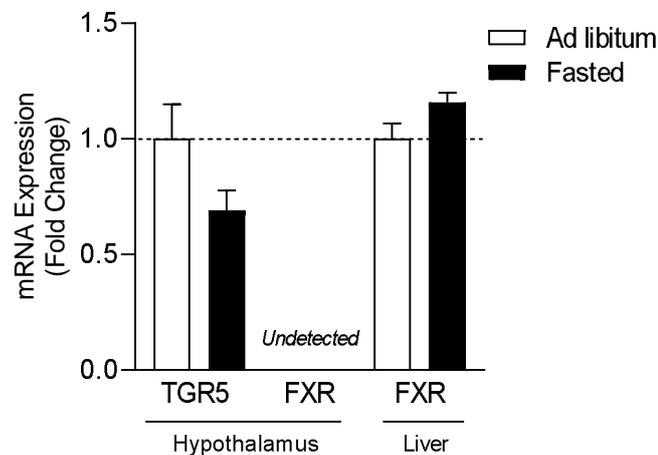


Figure 13. Expression of BA receptors in the hypothalamus. The TGR5 receptor is expressed in the hypothalamus of lean mice; FXR was not detected (expression in the liver is shown as a positive control).

B. Bile acid transporters are found in the hypothalamus and their expression is regulated by the body's energy status

The expression by real-time quantitative PCR (qPCR) of BA transporters responsible for importing and exporting BA from cells was measured in hypothalamic samples of both lean and obese mice that were either fasted (food removed for 24h before killing) or refed (food removed for 24h and replaced for 2h before killing). Hypothalamic tissues (n=7 per group) were obtained and processed as described above. cDNA of BA transporters OST , NCTP and BSEP were amplified and quantified by qRT-PCR as described above with the reference genes SDHA and TUBA4A.

Our data show that BA transporters are expressed in the hypothalamus. Interestingly, mice who were lean responded to refeeding conditions by increasing their BA transporters in the hypothalamus, an effect that was absent in obese mice (**Fig. 14**).

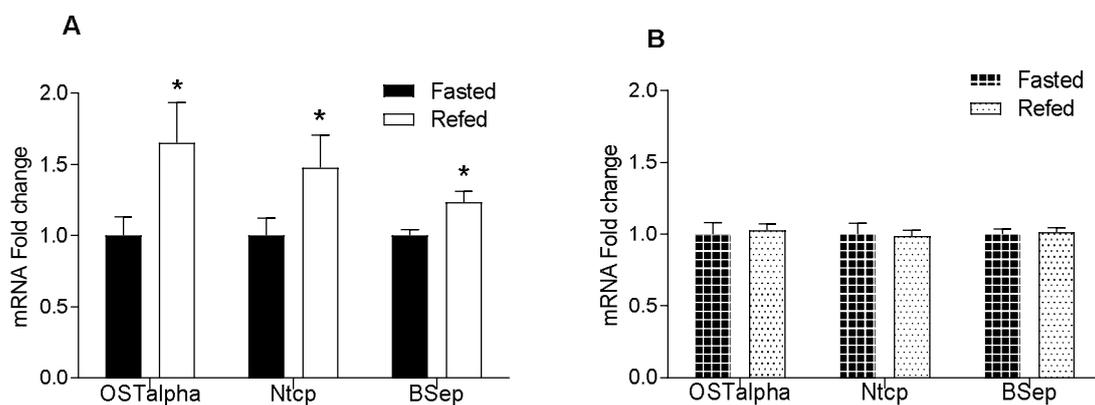


Figure 14. Expression of BA transporters in the hypothalamus. The BA system transporters responsible for importing and exporting BA from cells are expressed both in chow (A) and HFD-fed obese (B) mice. Notably, lean mice show an increase of BA transporters during refeeding, an effect that is not seen in obese mice. Two-tailed Student's *t* tests; **p*<0.05.

C. Bile acid levels are decreased in obesity

In order to confirm the presence of BA within the brain, total BA were measured in the plasma and the hypothalamus of both chow (n=10) and HFD-fed (n=10) mice that were fasted for 24h before killing. Measurements were performed by ultra-performance liquid chromatography, tandem mass spectrometry (UPLC-MS/MS) by collaborators (F. Bäckhed, U. Gothenburg, Sweden).

Notably, BA were lower in mice fed a HFD, both in the circulating plasma and the hypothalamus (**Fig. 15**), suggesting a deregulation of the BA system peripherally and centrally in the presence of diet-induced obesity.

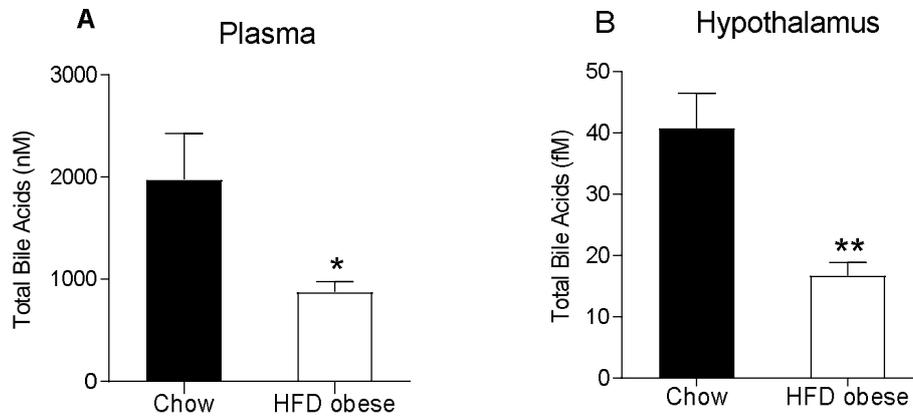


Figure 15. Quantification of BA levels. BA are significantly lower in both plasma (A) and the hypothalamus (B) of obese mice, compared to their lean counterparts. Two-tailed Student's *t* test; * $p < 0.05$; ** $p < 0.01$.

VI. Materials and methods

VI. Materials and methods

A. Ethical statement

All procedures involving live animals were approved and carried out in accordance with the National and European Directives 2013/63/EU, the French Ministry of Agriculture and Fisheries and the Ethical Committee of the University of Bordeaux for Animal Experimentation (authorization #3959, #13394 and #13395). Maximal efforts were made to avoid or reduce any suffering and to reduce the number of animals used.

B. Animals

Male mice starting at 7 weeks old were housed individually in standard plastic rodent cages under a 12:12 h reversed light/dark cycle (lights on at 1:00 h) at 23 ± 2 °C. Mice received a standard chow diet (Standard Rodent Diet A03, SAFE, France; 3.236 kcal/g; 13.5% lipids, 25.2% proteins and 61.3% carbohydrates) and water *ad libitum*, unless otherwise stated. For the diet-induced obesity model, mice were switched to a commercial high-fat diet (HFD) (D12492, Research Diets Inc. New Brunswick, NJ, USA; 5.24 kcal/g; 20% of calories from proteins, 20% from carbohydrates, 60% from lipids) one week after arrival and were maintained on the diet for 12 weeks before the start of experiments unless otherwise stated. Body weight was recorded weekly from all mice throughout the studies and daily when a particular experimental procedure was carried out.

C57BL6/J mice (Janvier, France) and TGR5^{fl_{ox}/fl_{ox}} mice on a C57BL6/J background (obtained from Dr. K. Schoonjans, EPFL, Switzerland) and generated as described in (Thomas et al., 2009) were used. At the end of the *in vivo* experiments, mice were either anesthetized and perfused for neuroanatomical analysis or killed by decapitation and tissues collected, frozen on isopentane chilled on dry ice, and stored at -80°C until needed for molecular and biochemical analysis. Number of animals used in each experiment is indicated in the figure legends.

C. Body composition

Body composition analysis was performed *in vivo* by nuclear echo magnetic resonance imaging whole-body composition analysis (EchoMRI[®] 900; EchoMedical Systems,

Houston, TX, USA) at arrival, after the 12-week exposure to the HFD, and during chronic experiments (at the start, one week after the start of treatment, and at the end of treatment), as described previously (Cardinal et al., 2014; Cardinal et al., 2015). Mice were weighed and placed in the EchoMRI[®] using a movement restrainer to allow for proper measurements. All measures were taken at the same time of day under free-feeding condition. Data for total water, fat mass and lean mass were extracted for analysis.

D. Pharmacological tools

In order to stimulate TGR5 activity, we used two different compounds:

1. Sodium choleate (Sigma-Aldrich): a mix of BA containing taurocholic, glycocholic, deoxycholic and cholic acids, or
2. The specific and potent synthetic TGR5 agonist, 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N, 5-dimethylisoxazole-4-carboxamide (Abcam), commonly referred to as CCDC.

For acute experiments, sodium choleate was diluted in artificial cerebrospinal fluid (aCSF), while CCDC was diluted in 100% dimethylsulfoxide (DMSO). For chronic experiments, CCDC was diluted in 60% DMSO and 40% aCSF. Doses are indicated below (see **Experimental design**).

E. Surgical procedures

i. Intracerebroventricular (ICV) cannula implantation

7. ICV cannula placement for acute pharmacology studies

An ICV cannula was implanted in the lateral ventricle in order to perform pharmacological studies. Both lean and diet-induced obese mice were anesthetized using isoflurane 5% to induce the anesthesia and 1-2% during the surgery. Subcutaneous analgesics were injected to reduce discomfort: buprenorphine (0.1 mg/kg) as an analgesic, and lidocaine (0.1mL at 0.5%) as a local anesthetic. Unconscious mice were placed on a stereotactic frame (David Kopf Instruments, USA). A burr hole for the guide cannula (C313GS-5/SPC, G22; Plastics One[®]) was drilled on the skull [coordinates relative to bregma: ML: -1.2; AP: -0.5; DV: -2.1; (Paxinos, 2013)] and was fixed with dental cement (MajorRepair[®]). Following surgery, mice received an anti-inflammatory and analgesic drug

(meloxicam, 5 mg/kg for 2 days) as well as 0.3 ml of saline solution subcutaneously. After surgery, animals continued to be housed individually and body weight was monitored daily during one week to assess recuperation and well-being.

Correct cannula placement was assessed *in vivo* by ICV infusion of the well-known orexigenic peptide NPY (Phoenix Pharmaceuticals Inc.), as described previously (Bellocchio et al., 2013). Each mouse received 5 μ g of NPY in 1 μ L of 0.1 M PBS (pH 7.4) through an internal injector (projection of 0.5mm below the guide cannula). Body weight and food weight were recorded before and 2 h after NPY injection, which was administered during the light phase. Animals that ingested at least 0.50 g of food after 2 h were included in the experiments.

8. *ICV cannula placement for chronic pharmacology studies*

The same procedure as described for the acute cannula placement was used, with the addition of the subcutaneous implantation of a mini osmotic pump (Alzet® System, model 1004; pump rate 0.1 μ l/h during 28 days) (adjusted coordinates relative to bregma: AP: -0.3; ML: -1.0; DV: -2.5). This pump was filled with either vehicle or CCDC. The subcutaneous pumps were connected to the ICV cannula through a catheter.

j. Intra-MBH cannula implantation

Diet-induced obese mice were anesthetized and placed on a stereotactic frame as described above. A bilateral cannula (C235I-SPC, G33; Plastics One®) targeting the MBH was implanted (AP: -1.1; ML: \pm 0.4; DV: -4.9). Cannula placement was verified at the end of the acute experiments by injection of a blue dye into the cannula. Mice were sacrificed and brains were dissected and sliced using a cryostat (Leica CM 1950) in order to observe the blue dye trace. Animals that did not have the trace within the MBH (arcuate and ventromedial nucleus) were excluded from further data analysis.

k. Intra-MBH viral injections

TGR5^{flox/flox} adult mice were used for the site-specific deletion of TGR5 in the MBH by injecting an adeno-associated virus (AAV) linked to Cre recombinase (AAV1/2-Cre-CAG-hrGFP) or its control (AAV1/2-CAG-hrGFP). The virus was injected bilaterally in the MBH of both chow-fed and HFD-fed mice (coordinates relative to bregma: AP: -1.5; ML: \pm 0.3; DV: -5.8 from the brain surface) using a Hamilton syringe of 10 μ l, an automatic pump at a

rate of 100 nl/min and a 10 mm stainless steel injector (Nanofil® NF33BL, World Precision Instruments, USA). Total volume injected was 500 µl per side. The same pre- and post-operative care procedures as described earlier for cannula placement were followed.

F. Experimental design

l. Experiment 1: Acute ICV effects of TGR5 agonism on food intake and body weight

In order to test the effect of acute central TGR5 activation *in vivo* on body weight and food intake, mice fed chow or HFD were injected ICV after 24 h fast, just before the dark phase (13:00 h):

1. 2 µl aCSF as vehicle, or 2 µl sodium choleate (2.5 µg/µl), or
2. 2 µl 100% DMSO as vehicle, or 2 µl of CCDC (2.5 µg/µl).

Body weight was measured at baseline and after 24 h of injection; food intake was measured at baseline and after 1, 2, 4 and 24 h. Feed efficiency was calculated by dividing the body weight gain into the total calories ingested (3.2 kcal/g of chow or 5.24 kcal/g of HFD), over 24 h food intake and expressing the ratio in percentage. In a separate experiment, an insulin tolerance test was performed after the ICV injection of vehicle or CCDC.

m. Experiment 2: Acute intra-MBH effects of TGR5 agonism on food intake and body weight in diet-induced obesity

Since ICV compound delivery can diffuse throughout the brain, acute intra-MBH administration of CCDC was carried out in diet-induced obese mice in order to more precisely target this brain region. The volumes used were adjusted to 0.2µl per mouse, and each time mice were injected with either vehicle or CCDC (5µg/µl). Body weight was measured at baseline and after 24 h of injection; food intake was measured at baseline and after 1, 2, 4 and 24 h. Feed efficiency was calculated as described above.

n. Experiment 3: Chronic ICV effects of TGR5 agonism on food intake and body weight in diet-induced obesity

Body weight and food intake were followed for 28 days in mice with an ICV cannula that was connected to a mini-osmotic pump. Body composition analysis was performed before surgery, after 1 week of the start of treatment and at the end of the treatment. Mice were placed in calorimetric cages on day 10 of treatment and during 5 consecutive days to evaluate

energy expenditure at 22°C. A separate group of mice receiving chronic infusion of CCDC or vehicle were placed in calorimetric cages at 22°C and then housed at thermoneutrality (30°C) during 48 h to evaluate energy expenditure in the absence of sympathetic activity. In another group of mice, an insulin tolerance test was performed at 4 weeks of treatment. All mice were killed by decapitation either at 22°C or at 30°C, blood was collected and tissues were extracted, immediately frozen in isopentane chilled on dry ice, and stored at -80°C until needed.

o. Experiment 4: Effect of chemical sympathectomy on chronic ICV TGR5 agonism

Diet-induced obese mice were i.p. injected for 3 consecutive days with 80mg/kg of 6-hydroxydopamine (6-OH-DOPA) diluted in saline solution with 0.1% ascorbic acid, as in (Quarta et al., 2010). One group of mice was killed after 1 week of the i.p. injections in order to obtain WAT and BAT tissues for assessing the downregulation of markers the SNS by western blot.

The same procedure for chemical sympathectomy was performed in an independent group of mice, which after 1 week underwent stereotaxic surgery coupled with subcutaneous mini-pump implantation for chronic delivery of either vehicle or CCDC, as described above, in order to assess the effect of central TGR5 agonism in the absence of functional sympathetic connectivity. Body weight and food intake were followed for 28 days. Body composition analyses were performed before sympathectomy, after 1 week of the start of CCDC treatment and at the end of the treatment. Mice were killed by decapitation, blood was collected and tissues were extracted, immediately frozen in isopentane chilled on dry ice, and stored at -80°C until needed (**Table 2**).

p. Experiment 5: Effect of AAV-mediated genetic deletion of TGR5 in the MBH on food intake, body weight and adiposity

To assess the phenotypic effect of genetic TGR5 deletion in the MBH, three groups of mice were created:

1. Chow-fed lean $TGR5^{flox/flox}$ mice were injected in the MBH with AAV-GFP or AAV-Cre;
2. Chow-fed $TGR5^{flox/flox}$ mice were injected in the MBH with AAV-GFP or AAV-Cre and switched to a HFD after 1 week of the viral injection;

3. Diet-induced obese $TGR5^{\text{flox/flox}}$ mice were injected with either AAV-GFP or AAV-Cre and maintained on HFD.

An additional group of chow-fed mice were injected in the MBH with AAV-GFP or AAV-Cre. These mice were perfused after 4 weeks for subsequent neuroanatomical analysis to verify the localization of the expression of Cre recombinase, as well as to verify effective recombination by genotyping in these samples.

In all cases, body weight and food intake were measured for up to 21 days. Mice were killed by decapitation for blood collection and tissue extraction. In the case of chow-fed mice switched to a HFD (group 2 described in the list above), killing was preceded by a 4 h cold challenge at 4°C so to stimulate SNS-related responses (**Table 3**).

G. Pharmacokinetics of TGR5 agonist

The abundance of the TGR5 agonist CCDC was measured after an acute ICV dose of 5µg, and after chronic ICV infusion. Groups of 3 mice were sacrificed at 15, 30, 60, 120 and 240 min after the injection, and the hypothalamus and the rest of the brain were collected and directly frozen for quantification by liquid-chromatography-mass spectrometry. Blood was collected in heparin tubes, centrifuged at 5000 rpm for 10 min at 4°C, and plasma was collected and frozen at -80°C. Blood samples after 28 days of ICV CCDC were also processed for CCDC quantification. Actual measurement of CCDC was carried out by our collaborator Julie Charton (Pasteur Institute, INSERM U1177, Lille) by mass spectrometry analysis.

H. Insulin tolerance test

This procedure was performed as in (Cardinal et al., 2014). Briefly, mice were fasted for 6 h and were injected intra-peritoneally (i.p.) with 0.5 U/kg insulin (Humulin, Lilly, France). Blood samples were collected from the tail vein and glucose was measured using glucose strips (OneTouch, Vita, France) at baseline, 15, 30, 60, 90 and 120 min.

I. Indirect calorimetry

Indirect calorimetry, in-cage locomotor activity and gas exchange analysis were carried out in light, temperature and humidity controlled calorimetric chambers (TSE Systems GmbH, Bad Homburg, Germany), as in (Cardinal et al., 2014). Diet-induced obese mice were

acclimated for 3 days in the chambers before recording. O₂ consumption and CO₂ production were measured every 20 minutes in order to calculate the gas exchange, the respiratory quotient (RQ) and the energy expenditure. At the same time, in-cage locomotor activity was determined using a tridimensional infrared light beam system. Food intake was measured continuously by integration of scales inside the cages. Body weight was measured daily during this time and the temperature was fixed to 22°C. When recording at thermoneutrality, the temperature in the calorimetric chambers was increased to 30°C. For mice that underwent a cold challenge, the temperature of the chambers was dropped to 4°C.

By placing a group of mice under thermoneutral conditions, analysis of energy expenditure components was carried out as in (Abreu-Vieira, Xiao, Gavrilova, & Reitman, 2015) in order to determine the energy expenditure of physical activity (PAEE), the thermic effect of food (TEF), the basal metabolic rate (BMR) and the cold-induced thermogenesis (CIT).

J. BA quantification

In independent experiments, diet-induced obese mice were killed by decapitation after 2 h of ICV infusion of vehicle or CCDC, respectively, or after 28 days of chronic treatment with CCDC. Blood was collected in EDTA-treated tubes placed on ice, centrifuged for 10 min at 10000 rpm at 4°C and plasma was recuperated. Total (free and conjugated) BA were measured by UPLC MS/MS by our collaborators Dr. A. Muhr-Tailleux and Dr. B. Staels (Pasteur Institute, INSERM U1011, Lille).

K. Quantitative real-time PCR (qPCR)

qPCR on epididymal white adipose tissue (WAT), brown adipose tissue (BAT), liver and the hypothalamus were performed with a LightCycler® 480 qPCR System (Roche Diagnostics, Meylan, France). Tissue RNA extraction, qPCR reactions and analysis were performed as described in (Binder et al., 2014). Relative expression analysis was corrected for PCR efficiency and normalized against two reference genes, depending on the tissue (WAT: Gapdh and Ppia; SAT: Gusb and Ppia; BAT: Sdha and Nono; liver: Nono and Eef1a1; hypothalamus: Ppia and Nono). The relative level of expression was calculated using the comparative 2^{-CT} method. Samples were run by duplicates, where the average of both was considered for quantification. mRNA expression levels are expressed as the fold change (FC) from vehicles after normalization.

L. Neuroanatomical analysis of Cre expression in the MBH

At the end of **Experiment 5**, TGR5^{flox/flox} mice receiving the AAV linked to the Cre recombinase or their controls were deeply anesthetized with an i.p. injection of sodium pentobarbital (300 µl/30g Exagon® mixed with 30 mg/kg lidocaine) and then perfused transcardially with ice-cold PBS (pH 7.4), followed by 4% paraformaldehyde (PFA, Sigma-Aldrich, France). Whole brains were extracted and post-fixed in 4% PFA overnight at 4°C, then cryo-protected with a 30% sucrose solution in PBS at 4°C and frozen. Coronal sections (30µm) were cut with a cryostat (CM1950, Leica, Germany), collected, and stored in an antifreeze solution (30% ethylene glycol, 30% glycerol in KPBS) at -20°C until needed.

The neuroanatomical expression of Cre recombinase in the MBH of these samples is ongoing, and hence it will not be included in this manuscript.

M. Assessment of Cre recombination by PCR

Perfused brain slices containing the MBH from TGR5^{flox/flox} receiving the AAV linked to the Cre recombinase or their controls were genotyped by PCR to evaluate recombination in genomic DNA (**Experiment 5**). Brain slices were incubated overnight at 56°C in Proteinase K buffer (100 mM Tris-HCl pH8, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 0,2 mg/mL PK); after 10 min at 13200 rpm, the supernatants were purified by vacuum on silica columns, according to the manufacturer's protocol (Macherey-Nagel kit), on a Zephyr automatic workstation (Perkin-Elmer). PCR assay was carried out on a Bio-Rad C1000 thermal cycler, in a 20 µL volume, using GoTaq G2 Hot Start Green Master Mix (Promega), and 0.2 µM of each primer. PCR conditions were as follows: 1 cycle, 5 min at 95°C; 37 cycles, 30 sec at 95°C, 30 sec at 60°C, 45 sec at 72°C; 1 cycle, 5 min at 72°C. PCR products were analyzed on a Labchip GX microfluidic electrophoresis system (Perkin-Elmer) using the DNA5k kit.

N. Western blot analysis

WAT and BAT were homogenized in radioimmunoprecipitation assay buffer with phosphatase and protease inhibitors (Santa Cruz Biotechnology) for obtaining protein extracts. Proteins were separated in an SDS-polyacrylamide gel (9-13%) by electrophoresis and transferred to nitrocellulose membranes, which were then blocked for 1h with 5% skim milk powder and incubated overnight at 4°C with the following primary antibodies: adipose triglyceride lipase (ATGL, 1:1000, #2439, Cell Signaling), hormone-sensitive lipase (HSL,

1:1000, #4107, Cell Signaling), phospho-HSL ser563 (1:1000, #4139, Cell Signaling), uncoupling protein-1 (Ucp-1, 1:1000, #10983, Abcam), tyrosine hydroxylase (TH, 1:1000, #152, Millipore), and β -actin (1:2000, #4967, Cell Signaling). β -actin was used as loading control. After washing in Tris-buffered saline with Tween (TBST), the membranes were incubated for 1 h at room temperature with secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit; 1:2000; Cell Signaling Technology). After 3 washes in TBST, immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, PerkinElmer) then exposed on a ChemiDoc® MP Imaging System (Biorad). Bands were quantified using ImageJ software (NIH, Bethesda, MA). When necessary, membranes were stripped after protein detection for 10 min at 55°C with a solution containing 62.5 mmol/l Tris-HCl, 100 mmol/l 2-mercaptoethanol, and 2% SDS, blocked, and reblotted with another primary antibody.

O. Statistical analysis

Statistical analysis was carried out using the GraphPad Prism Software version 8.0 for Windows (La Jolla, CA, USA). Data are expressed as mean \pm S.E.M.

When comparing 2 groups, data were analyzed by an unpaired Student's *t*-tests. When comparing 3 or more groups, data were analyzed by one-way ANOVA. Body weight, insulin tolerance test and cumulative food intake over time were analyzed by two-way repeated measures ANOVA considering treatment and time as factors. Bonferroni's *post-hoc* tests were run when applicable for identifying differences amongst groups. In all cases, tests were two-tailed and were considered significant when $p < 0.05$.

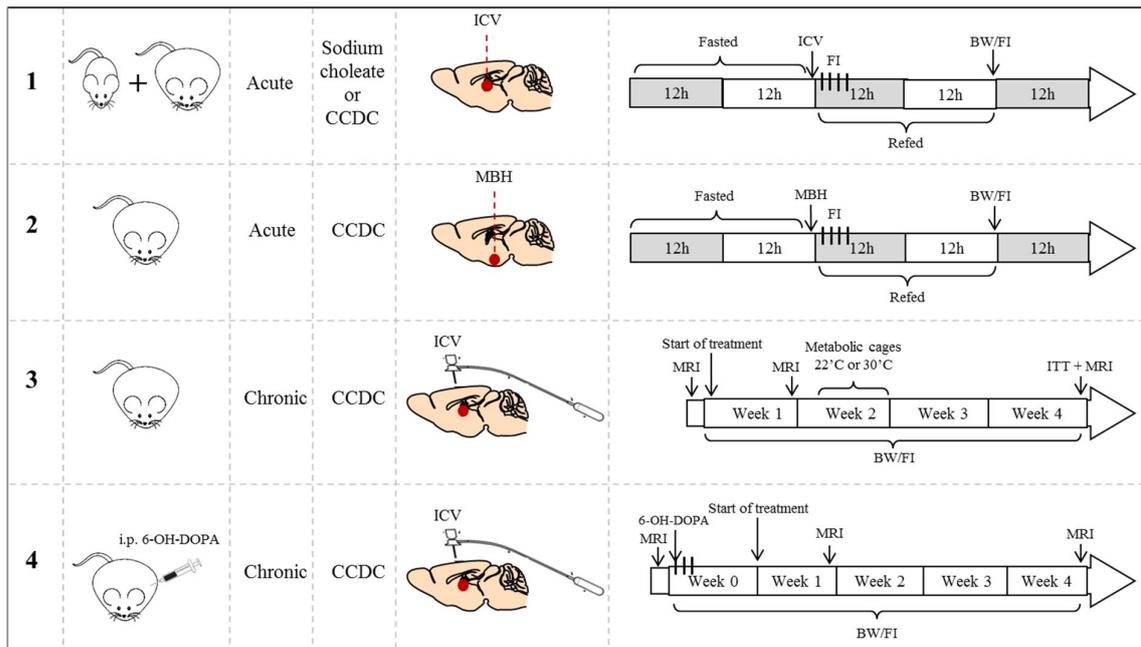


Table 2. Experimental procedures 1 to 4. See Materials and methods, section Experimental design.

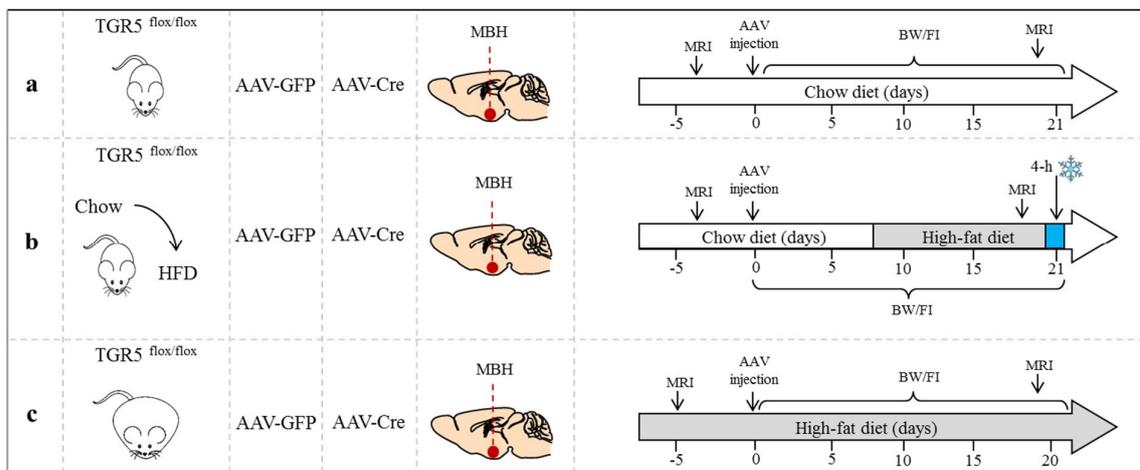


Table 3. Experimental procedure 5. See Materials and methods, section Experimental design.

VII. Results

VII. RESULTS

A. Acute pharmacology experiments in lean and diet-induced obese mice

q. Central administration of TGR5 agonists have no effect on chow-fed lean mice

We first explored under standard chow diet conditions if the central activation of TGR5 had an impact on 24 h food intake or body weight changes. We found that the acute infusion of 5 μ g of sodium choleate (a BA mix) or CCDC (a synthetic, potent TGR5 agonist) *via* ICV did not have an effect in neither of these parameters in chow-fed mice (**Fig. 16A-D**).

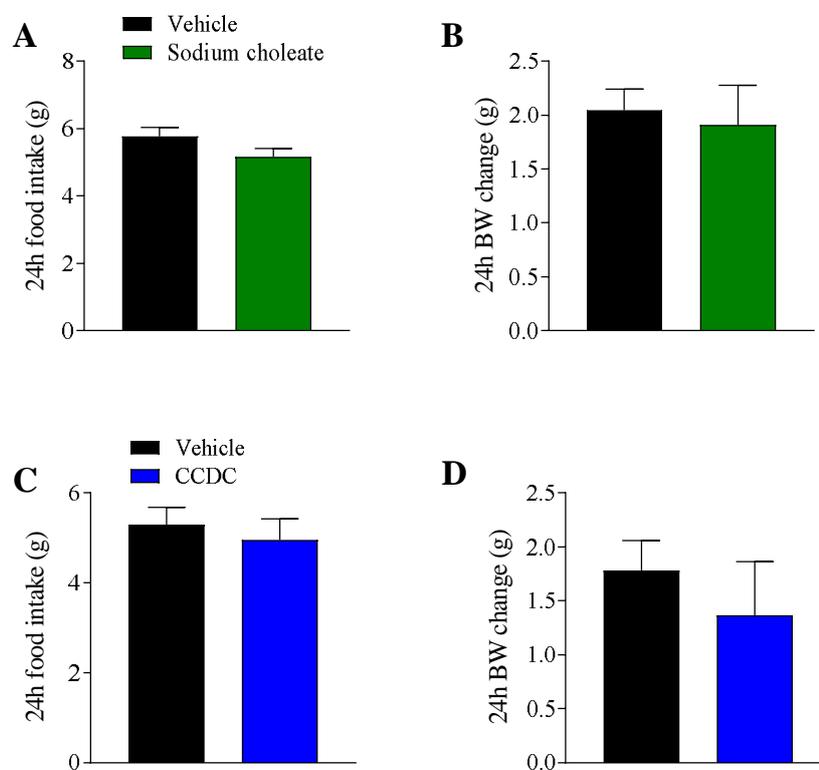


Figure 16. Acute ICV infusion of sodium choleate or CCDC does not affect food intake or body weight in lean mice. 24h cumulative food intake and associated body weight change in chow-fed mice that received an ICV injection of sodium choleate or its vehicle (A, B; $n=9$ per group) or CCDC and its vehicle (C, D; $n=10$ per group), respectively. Data are mean \pm SEM; two-tailed student's t test.

r. Central administration of TGR5 agonists decrease food intake and body weight in diet-induced obese mice

Since TGR5 activity is particularly relevant in the presence of obesity (Chavez-Talavera et al., 2017; S. Huang et al., 2019; Velazquez-Villegas et al., 2018; Watanabe et al., 2006), we performed the same acute ICV experiment in mice that had been fed with a HFD for at least 12 weeks and had developed obesity. Contrary to what we had previously seen in lean mice, the same dose (5 μ g) of sodium choleate or CCDC *via* ICV significantly lowered their food intake, body weight, and their feed efficiency (Fig. 17A-F).

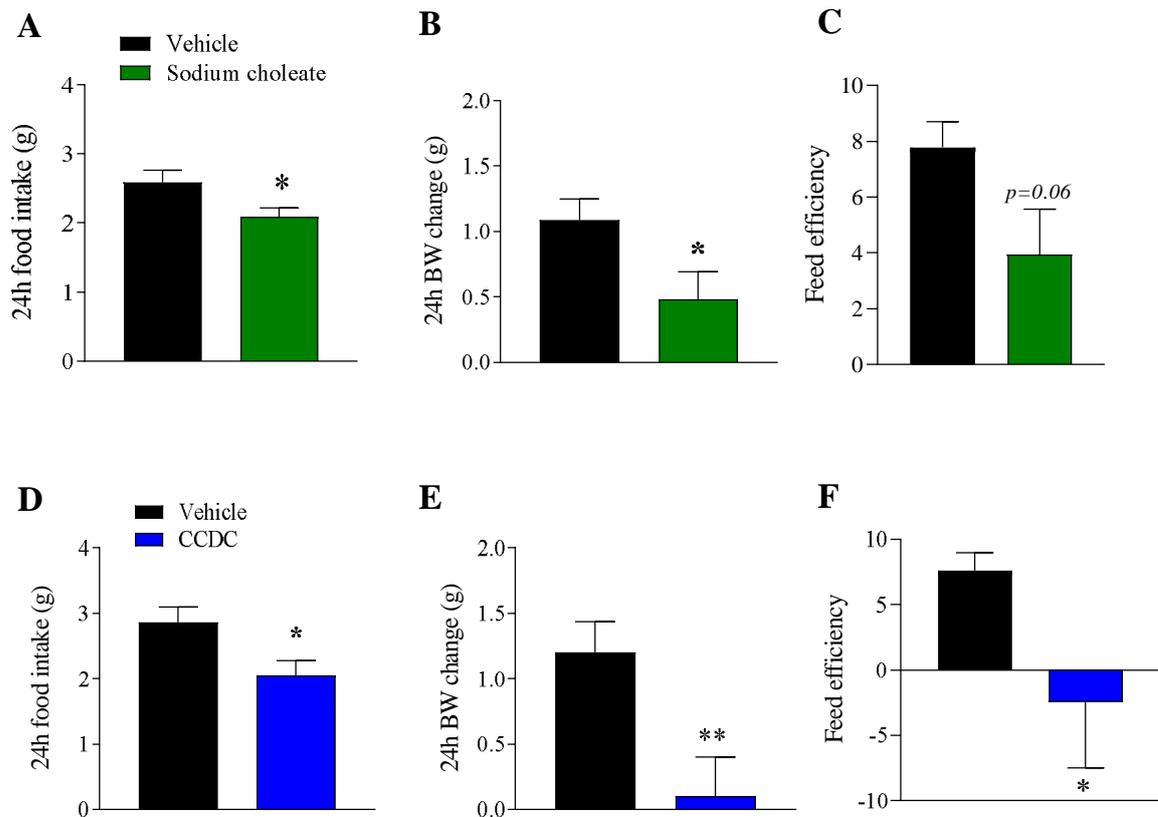


Figure 17. Acute ICV infusion of sodium choleate or CCDC decreases food intake and body weight in diet-induced obesity. 24h cumulative food intake and associated body weight change and feed efficiency in diet-induced obese mice that received an ICV injection of sodium choleate or its vehicle (A-C; n=12 per group) or CCDC and its vehicle (D-F; n=15 per group), respectively. Data are mean \pm SEM; two-tailed student's *t* test: **p*<0.05; ***p*<0.01.

Hence, the acute effects of central TGR5 activation seemed to be dependent upon the metabolic status (i.e. HFD vs. standard chow). For this reason, for our subsequent experiments investigating the central role of TGR5 in metabolic control, we used diet-induced obese animals.

s. MBH infusion of CCDC acutely decreases food intake in diet-induced obese mice

Compounds that are administered *in vivo* via ICV can diffuse throughout the brain. To further confirm that ICV administration of CCDC was modulating food intake and body weight in diet-induced obese mice by acting on hypothalamic circuits, we administered the TGR5 agonist directly in the MBH. CCDC infusion into the MBH of diet-induced obese mice significantly reduced their food intake and their body weight (**Fig. 18A-B**). Representative image of the injection of a blue dye shows correct bilateral cannula placement at the level of the MBH (**Fig. 18-C**).

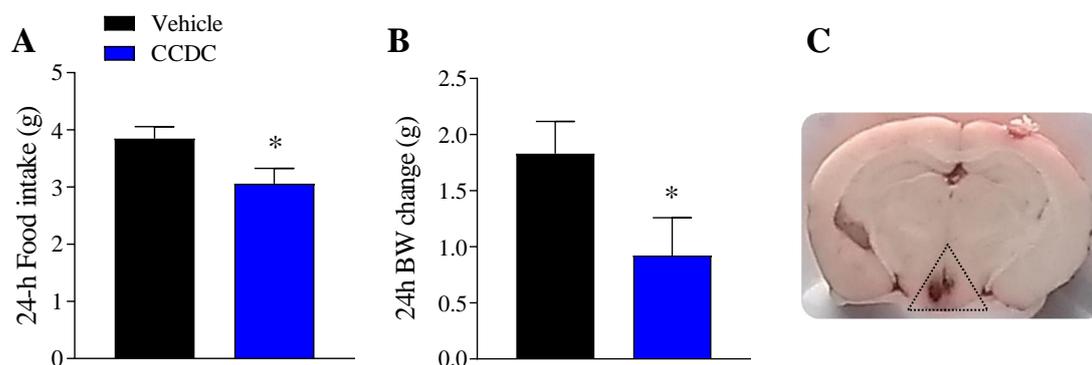


Figure 18. Acute intra-MBH infusion of a TGR5 agonist decreases food intake and body weight in obesity. 24h cumulative food intake (A) and associated body weight change (B) in diet-induced obese mice that received an intra-MBH injection of CCDC and its vehicle (n=16-17), respectively. C) Representative image of correct cannula placement after *in vivo* blue dye injection and tissue collection. Data are mean \pm SEM; two-tailed student's *t* test: * $p < 0.05$.

These results show that both acute ICV and intra-MBH pharmacological activation of the TGR5 receptor by means of endogenous (sodium choleate) or synthetic (CCDC) agonists is capable of decreasing food intake and body weight in a diet-dependent manner, since the strongest outcomes were seen in diet-induced obese mice.

Interestingly, the feed efficiency in obese mice receiving the agonist was reduced, implying that the reduction in body weight could not solely be explained by the reduction in food intake. As mentioned earlier, TGR5 activation in the adipose tissue can drive an increase in energy use through the production of heat (Watanabe et al., 2006). To test the contribution of increased energy expenditure by thermogenesis in the observed phenotype, we went on to evaluate the expression of thermogenic markers in the WAT and BAT of mice receiving CCDC centrally.

t. Central infusion of CCDC acutely increases markers of thermogenesis in peripheral tissues

After 30 min and 2 h of the ICV administration of CCDC, we found a significant increase in markers of thermogenesis in the WAT, particularly in the case of the β -adrenoreceptor 1 (Adrb1), Ucp-3, and Dio-2. Conversely, the β -adrenoreceptor 2 (Adrb-2) and Ucp-2 seemed to be decreased in the BAT at 2 h (**Fig. 19**).

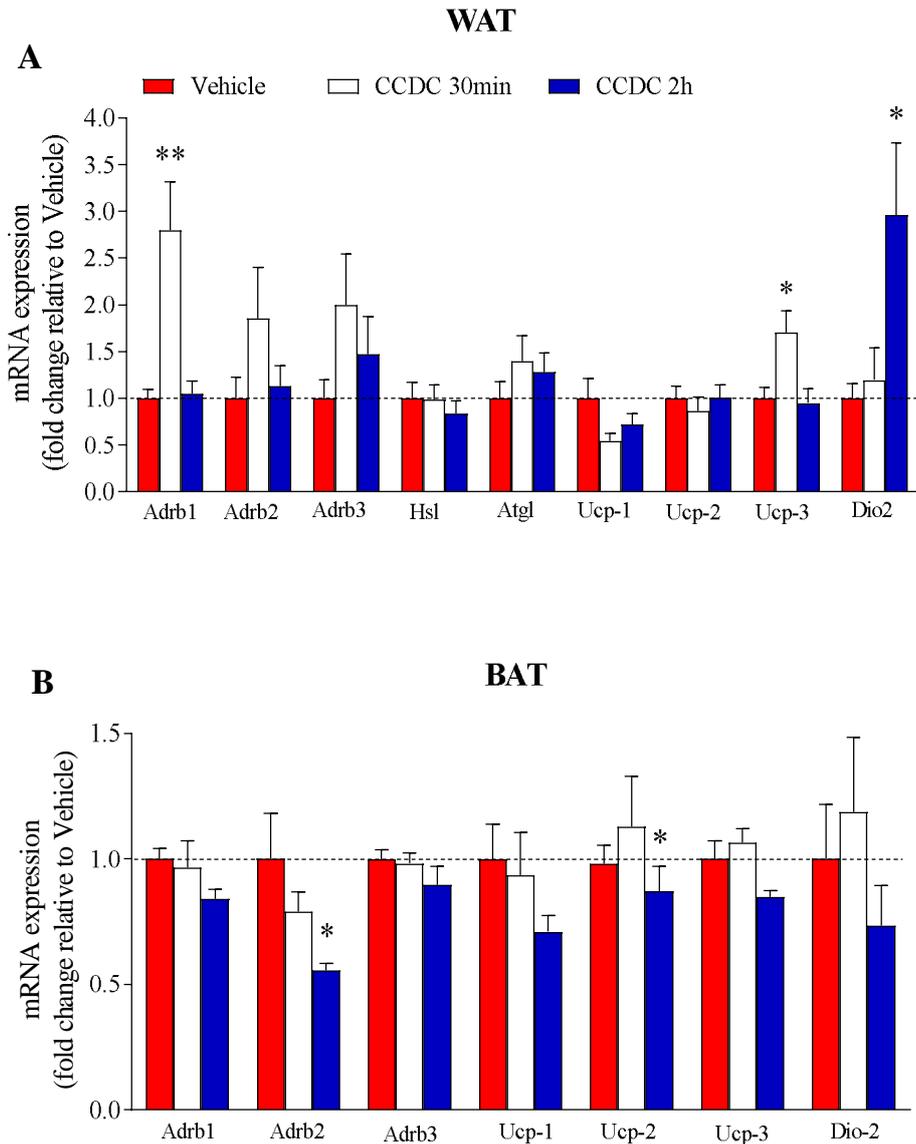


Figure 19. Acute central TGR5 activation induces changes in the expression of markers of thermogenesis in the adipose tissue. mRNA expression levels of markers of thermogenic activity in A) WAT and B) BAT. For both tissues: n=7 vehicle, n=7 CCDC 30min, n=8 CCDC 2h. Data are mean \pm SEM; one-way ANOVA: * p <0.05; ** p <0.01.

Ard1: -adrenoreceptor 1; *Ard2*: -adrenoreceptor 2; *Ard3*: -adrenoreceptor 3; *Hsl*: hormone sensitive lipase; *Atgl*: adipose triglyceride lipase; *Ucp-1*: uncoupling protein 1; *Ucp-2*: uncoupling protein 2; *Ucp-3*: uncoupling protein 3; *Dio2*: deiodinase 2.

Overall, these results suggest that the acute central activation of TGR5 can signal to peripheral tissues in order to modify the body's energetic state. This could initiate a series of metabolic events that would eventually lead to an increased use of energy for heat production and/or changes in the handling of lipids and their storage.

Since it has been shown that peripheral TGR5 activation also leads to better glucose homeostasis by stimulating the release of GLP-1 from the gut (Bianco et al., 2002), we went on to assess if the acute central stimulation of TGR5 could have an impact on modulating the glycaemic response.

u. Acute central TGR5 agonism improves insulin-mediated control of glycaemia in diet-induced obese mice

We therefore carried out an insulin tolerance test in diet-induced obese mice acutely receiving ICV CCDC or its vehicle. We found that CCDC-treated mice had a better insulin-dependent control of glycaemia compared to their controls, as their glucose levels remained significantly lower after 120 min of i.p. insulin injection (**Fig. 20**).

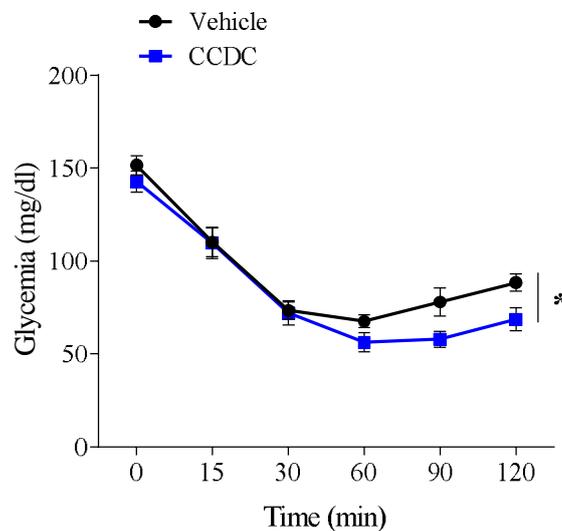


Figure 20. Acute ICV infusion of CCDC improves insulin-dependent glucose control in diet-induced obese mice. Blood glucose levels following an insulin tolerance test in diet-induced obese mice (10 mice per group). Data are mean \pm SEM; two-way repeated measures ANOVA: * denotes effect of treatment at $p < 0.05$.

In order to evaluate the potential underlying mechanisms that were driving the improvement in glycaemia, we evaluated markers in the liver related to glucose metabolism. Furthermore, we looked at the expression levels of markers associated to BA synthesis and transport to assess whether the central activation of TGR5 would have an effect on BA metabolism.

We found that the acute activation of TGR5 caused a significant decrease of glucose-6-phosphatase catalytic subunit (G6pc), which is key in glucose homeostasis, particularly for gluconeogenesis and glycogenolysis. Furthermore, there was an increase in insulin receptor substrate 2 (Irs2), a signaling molecule that mediates the effects of insulin, as well as in the fatty acid synthase (FAS), which catalyzes the synthesis of fatty acids. Lastly, we found a significant decrease in Cyp7a1, which is the rate-limiting enzyme in the synthesis of BA, although no changes were observed in the expression levels of BA transporters (**Fig. 21**).

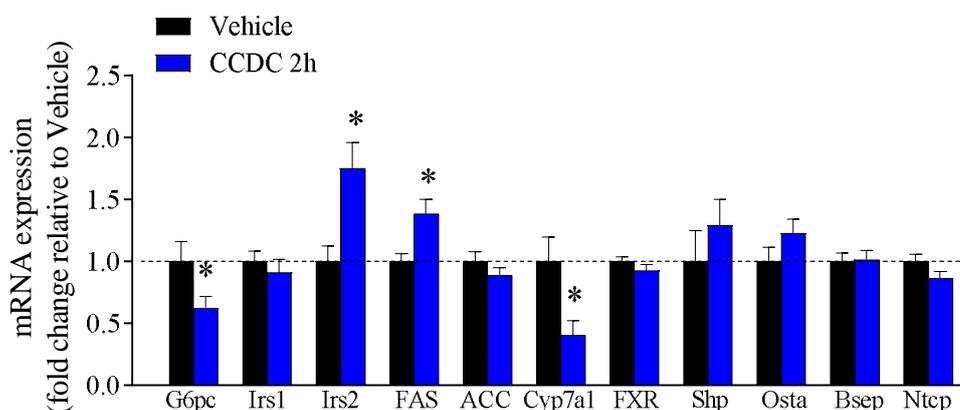


Figure 21. Acute central TGR5 activation induces changes in the expression of markers of the energetic state in the liver. mRNA expression levels of markers of glucose metabolism and BA synthesis in the liver 2 h after ICV injection of CCDC or its vehicle ($n=7$ vehicles, $n=8$ CCDC). Data are mean \pm SEM; two-tailed student's t tests: $*p<0.05$.

ACC: acetyl-CoA carboxylase; Bsep: bile salt export pump; Cyp7a1: cholesterol 7 alpha-hydroxylase; FAS: fatty acid synthase; FXR: farnesoid X receptor; G6pc: glucose-6-phosphatase catalytic subunit; Irs1: insulin receptor type 1; Irs2: insulin receptor type 2; Ntcp: Na⁺-taurocholate co-transporting polypeptide; Osta: organic solute transporter alpha; Shp: short heterodimer partner.

The central activation of the receptor seemed to be triggering a rapid response in peripheral tissues to signal a high energetic state (i.e. as would be expected after a meal), decreasing the availability of glucose through G6pc and Irs2, while activating pathways for the synthesis of fatty acids. Most notably, the dramatic decrease in Cyp7a1 suggested that central TGR5 stimulation might be acting as a negative feedback mechanism that would downregulate the production of BA in the liver. To further explore this possibility, we measured circulating BA levels after an acute ICV administration of CCDC.

v. Acute central TGR5 agonism affects hepatic BA production in diet-induced obese mice

Plasma samples from diet-induced obese mice (24h fasted) were collected during an independent experiment 2h after ICV infusion of vehicle or CCDC, respectively. CCDC-treated mice had lower levels of total free BA compared to their controls. No changes were observed in levels of total, total glyco- or total tauro-conjugated BA (**Fig. 22-A**). Since BA are produced as free species in the liver from cholesterol molecules and are subsequently conjugated, this suggests that the central acute activation of TGR5 signals a high energetic state to peripheral tissues, such as the liver, which in turn responds by downregulating BA biosynthesis.

The species of free BA that were lowest after the intervention were cholic acid and -muricholic acid (**Fig. 22-B**). Interestingly, cholic acid is one of the strongest endogenous TGR5 agonists (Duboc et al., 2014; Kawamata et al., 2003).

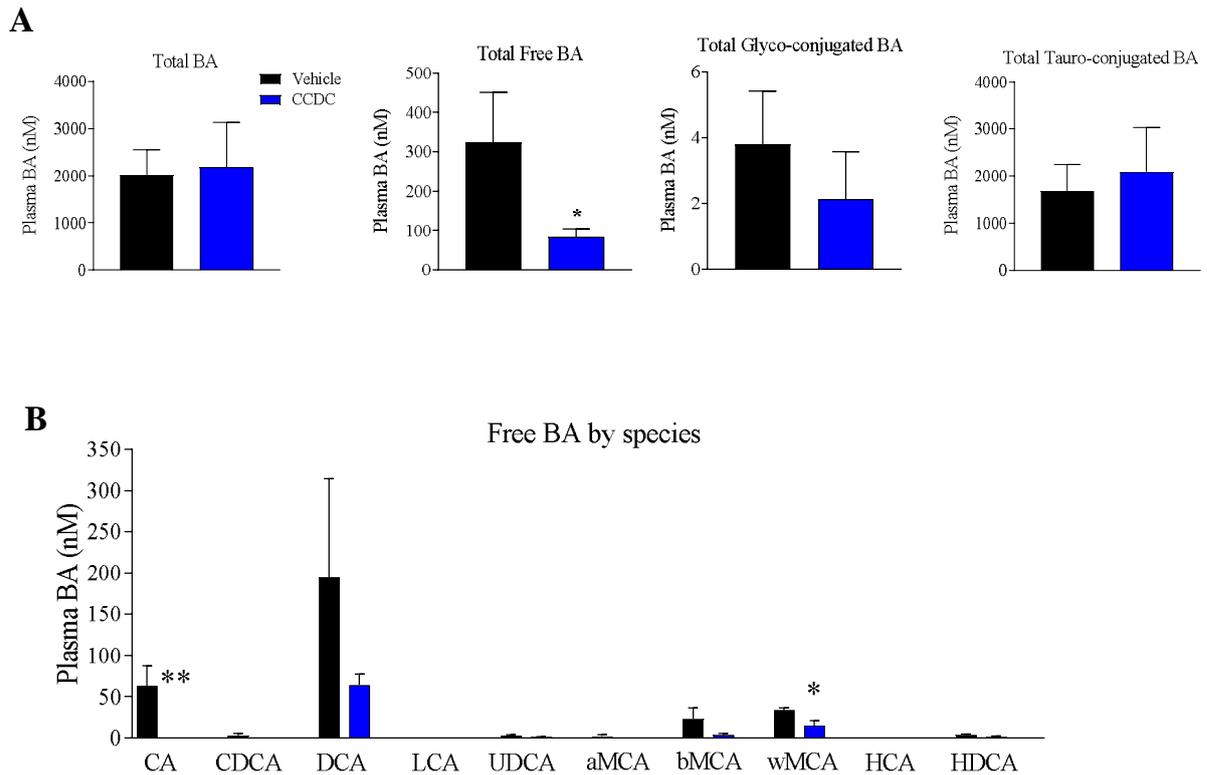


Figure 22. Acute central activation of TGR5 induces changes in BA species in obesity. A) Quantification of total plasma BA and their fractions by liquid-chromatography-mass spectrometry 2 h after ICV administration of CCDC or its vehicle in diet-induced obese mice ($n=7$ vehicles; $n=8$ CCDC). B) Quantification of different free BA species. Data are mean \pm SEM; two-tailed student's t test: * $p<0.05$; ** $p<0.01$.

CA: cholic acid, CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; UDCA: ursodeoxycholic acid; aMCA: α -muricholic acid; bMCA: β -muricholic acid; γ -MCA: γ -muricholic acid; HCA: hyocholic acid; HDCA: hyodeoxycholic acid.

Overall, our results show that the acute central activation of TGR5 decreases food intake and body weight in a diet-dependent manner. Moreover, TGR5 agonism can signal a high energetic state to peripheral tissues, particularly in obese mice, activating a postprandial profile that favors fatty acid synthesis, glucose uptake, downregulation of gluconeogenesis and of BA synthesis. These effects can be observed as early as 30 min or 2 h post-stimulation and are in line with an improvement in insulin sensitivity.

We finally verified that the effects observed were not due to leakage of the compound into the periphery by performing a pharmacokinetic study.

Free-fed diet-induced obese mice were sacrificed immediately after the ICV injection of CCDC at different time points (**Table 4**). After 15 min, CCDC was mostly detected within the hypothalamus and the total brain, while marginal concentrations were found in the plasma. From 30 min onwards, marginal concentrations were found in the hypothalamus and brain only, while it was undetectable in the plasma. The compound was also not detected in the plasma of mice that received CCDC ICV chronically for 28 days, as further detailed in the following section.

		[CCDC] sample (nM)			
		Time	Plasma	Hypothalamus*	Brain*
Acute	15 min		22	9671	3651
			18	4710	1957
			< 3	14	8
	30 min		< 3	13	160
			< 3	7	883
			< 3	< 5	287
60 min		< 3	< 5	11	
		< 3	< 5	49	
		< 3	15	2274	
120 min		< 3	24	31	
		< 3	24	26	
		< 3	6	12	
240 min		< 3	7	21	
		< 3	< 5	7	
		< 3	< 5	8	
Chronic	At 28 days		< 3	-	-
			< 3	-	-
			< 3	-	-

*1g of tissue per 1mL

Table 4. Pharmacokinetics of CCDC shows that the compound does not leak into the systemic circulation. Assessment of CCDC concentrations (nM) by liquid-chromatography-mass spectrometry in the plasma, hypothalamus and brain of diet-induced obese mice receiving either acute or chronic treatment (n=3 per group).

B. Chronic pharmacology experiments in diet-induced obese mice

w. Lateral ventricle infusion of a TGR5 agonist chronically decreases adiposity while improving insulin sensitivity

The greatest challenge for combating obesity is to find successful therapies that can achieve sustained body weight loss that is maintained chronically. In order to test if the observed acute effects of central TGR5 activation could be maintained in the long term, we carried out chronic experiments in diet-induced obese mice. By means of a subcutaneous mini-osmotic pump, we administered ICV the TGR5 agonist CCDC (5µg/day) during 28 days and followed the body weight, food intake and assessed metabolic parameters in this model.

Diet-induced obese mice receiving chronic ICV infusion of CCDC lowered their body weight significantly from day 14 and until the end of treatment compared to their vehicles (**Fig. 23A**). Moreover, they decreased their food intake over time and had a lower feed efficiency towards the end of the treatment (**Fig. 23B-C**). Body composition analysis showed that weight loss was mostly explained by a reduction in their total fat mass, without changing their lean mass content (**Fig. 23D-E**).

Insulin sensitivity was assessed by an insulin tolerance test, where a main effect of treatment was observed. The quantification of the area under the curve evidenced an overall lower glycaemia in mice chronically receiving ICV CCDC (**Fig. 23F**).

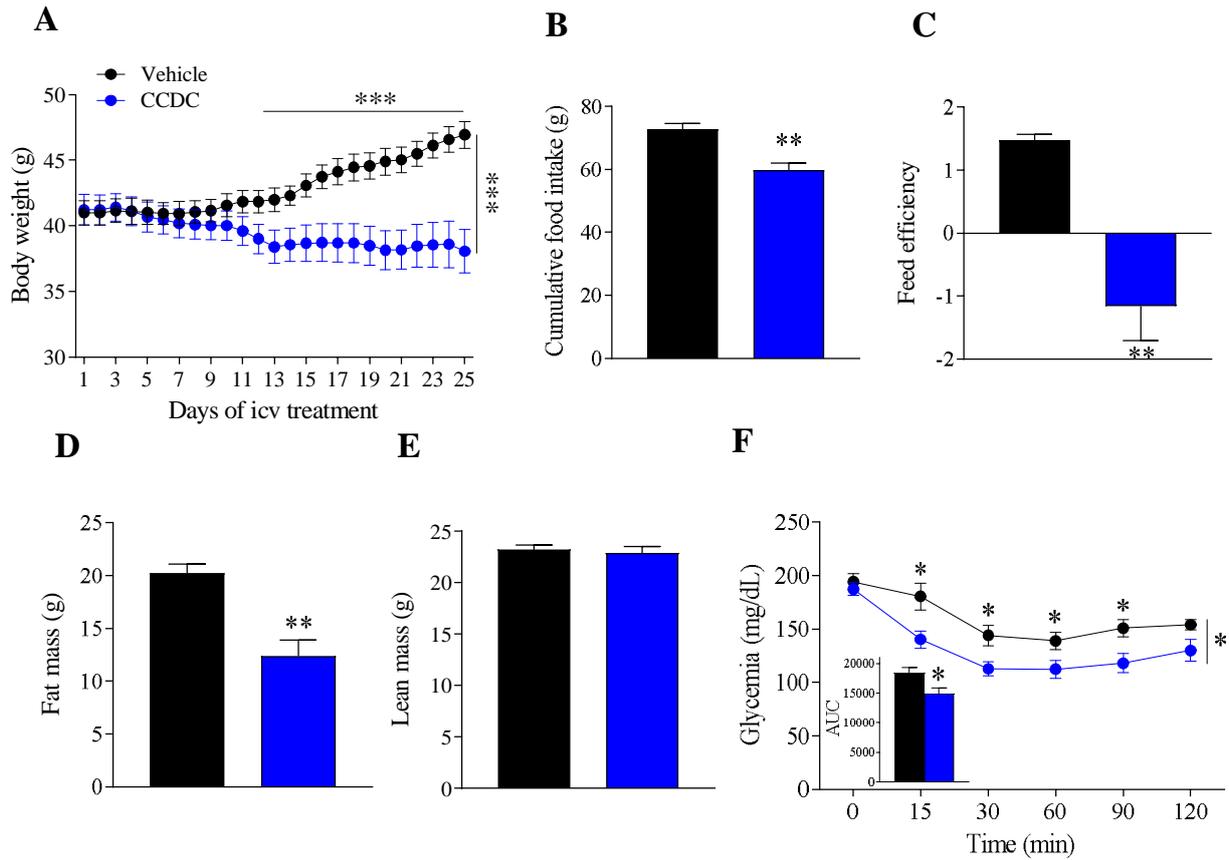


Figure 23. Chronic central TGR5 activation reduces obesity. A) Body weight follow-up, B) cumulative food intake, C) feed efficiency, D) fat mass and E) lean mass of obese mice that underwent chronic ICV CCDC treatment (n=6 vehicles; n=7 CCDC). F) Blood glucose levels following an insulin tolerance test from an independent experiment (n=11 vehicles; n=18 CCDC). Data are mean \pm SEM; analyzed by two-tailed student's *t* test when comparing two groups, or by two-way repeated measures ANOVA when measuring outcomes across time: **p*<0.05; ***p*<0.01; ****p*<0.001.

AUC: area under the curve.

As we previously observed that acute central administration of CCDC was able to alter plasma free BA levels (Fig. 22), we went on to verify the impact of chronic ICV CCDC treatment on circulating BA. We collected plasma samples from an independent chronic experiment, where free-fed diet-induced obese mice were killed after 10 days of ICV infusion

of vehicle or CCDC. No changes were observed in the levels of total, free, total glyco- or total tauro-conjugated BA (**Fig. 24**).

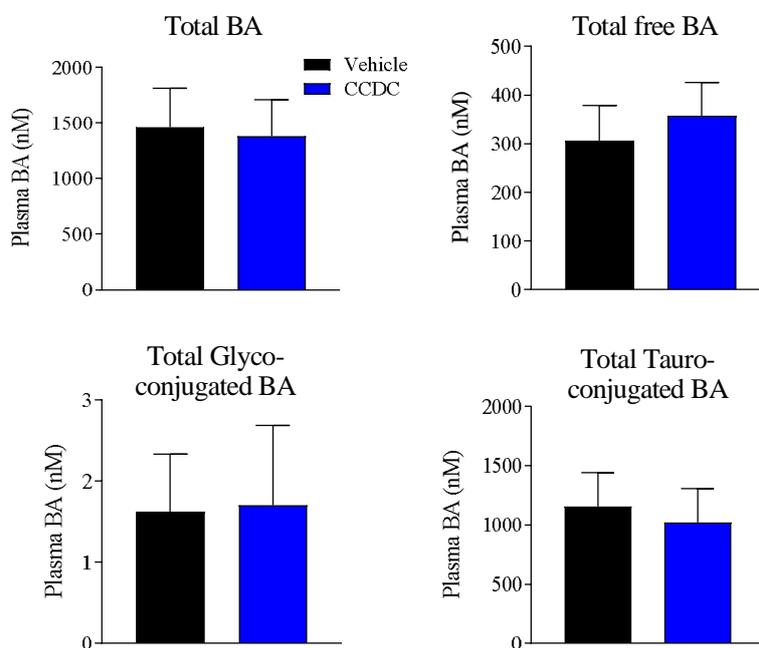


Figure 24. Chronic central TGR5 activation does not induce changes in BA species. Plasma quantification of total, total free and total conjugated (glyco- and tauro-) BA by liquid-chromatography-mass spectrometry in free fed, diet-induced obese mice after 10 days of treatment with CCDC or its vehicle ($n=8$ vehicles; $n=10$ CCDC). Data are mean \pm SEM; two-tailed student's t test.

Although mice receiving chronic CCDC treatment had a lower food intake compared to their controls, the decrease in food consumption did not explain the magnitude of the body weight loss, as evidenced by their lower feed efficiency, an observation that was consistent with our acute pharmacology studies. This suggested other mechanisms involved in improving their phenotype. In particular, one likely possibility was that central TGR5 activity could be stimulating the SNS to enhance energy expenditure in peripheral tissues. This seemed logical, considering we had previously observed changes in thermogenesis markers in the WAT (most notably an increase in Dio-2) after acute central TGR5 stimulation (see **Fig. 19A**). Hence, we

looked for markers of thermogenesis and SNS activity in peripheral tissues, and measured energy expenditure and use of fuel substrates in our chronic model.

x. Chronic central TGR5 agonism stimulates sympathetic activity and increases energy expenditure

WAT and BAT were collected and processed to measure the expression levels of SNS and thermogenesis markers. Notably, in the WAT, all forms of β -adrenergic receptors (Adrb) were upregulated in obese mice receiving CCDC chronic treatment, while adipose triglyceride lipase (ATGL), a marker of lipolysis, had a tendency towards being increased. In the BAT, there was an increased expression of Adrb2, uncoupling protein 3 (Ucp-3) and Dio-2 in mice chronically receiving ICV the TGR5 agonist (**Fig. 25**).

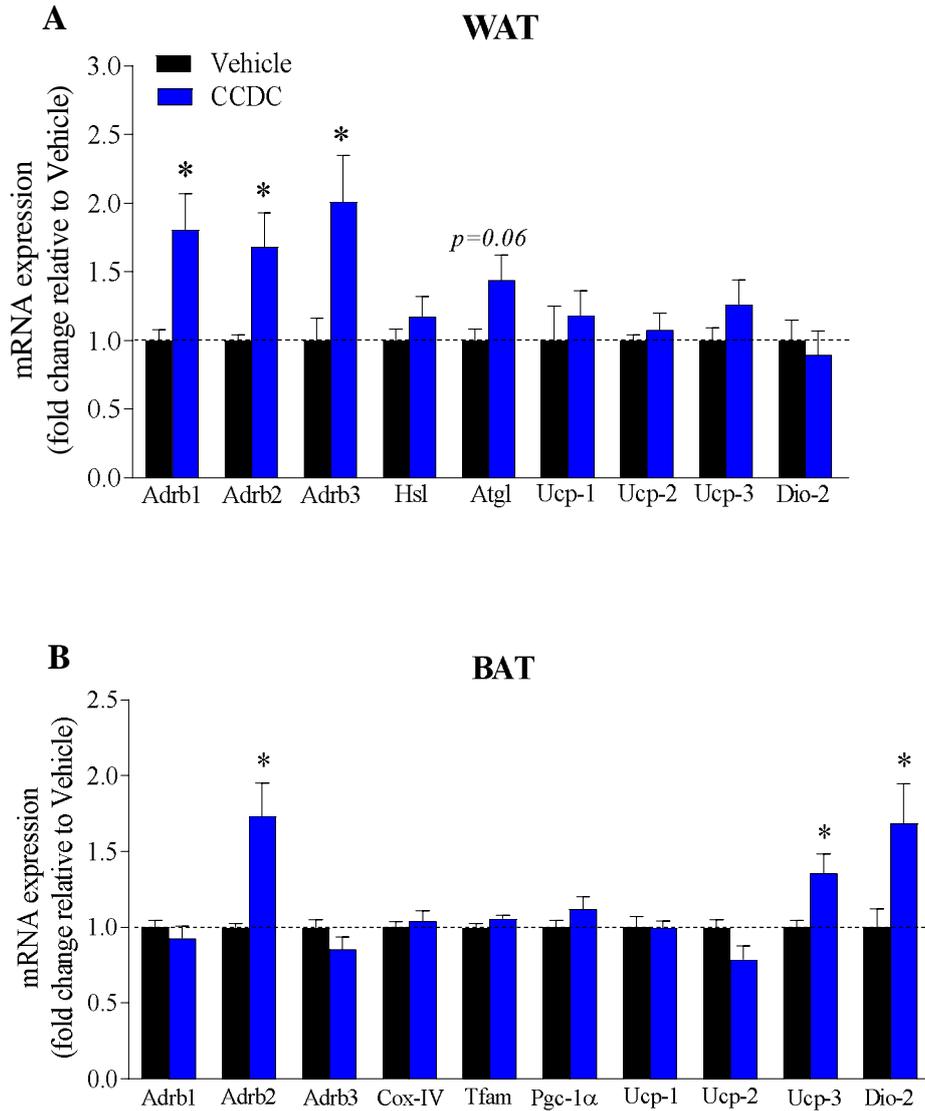


Figure 25. Chronic central TGR5 activation increases markers of thermogenesis in peripheral tissues. mRNA expression levels of thermogenesis markers in WAT (A) and BAT (B) from diet-induced obese mice receiving chronic ICV CCDC (n=6 vehicles; n=7 CCDC); data are mean \pm SEM; two-tailed student's t test: *p<0.05.

Ardb1: -adrenoreceptor 1; Ardb2: -adrenoreceptor 2; Ardb3: -adrenoreceptor 3; Hsl: hormone sensitive lipase; Atgl: adipose triglyceride lipase; Ucp-1: uncoupling protein 1; Ucp-2: uncoupling protein 2; Ucp-3: uncoupling protein 3; Dio2: deiodinase 2; Cox-IV: cytochrome c oxidase IV; Tfam: mitochondrial transcription factor A; Pgc-1 : peroxisome proliferator-activated receptor co-activator 1 .

It is known that the adipocyte TGR5 receptor participates in the regulation of energy expenditure by promoting intracellular thyroid hormone action through Dio-2 activation in adipose tissue as this enzyme transform the pro-hormone T4 in the active form T3 (Watanabe et al., 2006). However, increased expression and activation of the Dio-2 enzyme can be also driven by the SNS (Bianco & McAninch, 2013). Accordingly, we found that at thermoneutrality, when the SNS is not engaged, expression of Dio-2 in BAT is not altered in animals chronically receiving ICV CCDC (see further below in the manuscript, **Fig. 29**). Intra-cellular produced T3 is known to leak into the circulation (Bianco & McAninch, 2013). Thus, a possibility arises that chronic ICV CCDC by stimulating the SNS is inducing T3 production in the BAT, resulting in increased T3 in the plasma, which may feed forward SNS activation and thermogenesis by acting onto the hypothalamus (Lopez et al., 2010). While this hypothesis is being currently tested, as we have collected plasma samples from chronic ICV CCDC treated animals in which we will measure T4 and T3, we assessed whether the mRNA expression of thyroid hormone transporters and receptors was altered in the hypothalamus of ICV CCDC treated mice.

We found that chronic ICV CCDC administration was associated with the downregulation of the solute carrier family 16 member 2 (Slc16a2), which is responsible of transporting T3 hormone into neurons (Friesema et al., 2006), the solute carrier organic anion transporter family member 1C1 (Slco1c1), which mediates the sodium-independent uptake of thyroid hormones in brain tissues, especially at the level of the blood-brain barrier (Roberts et al., 2008), and thyroid hormone receptor (Thra) and (Thrb), which are receptors for T3 (**Fig. 26**). These results, which will need further confirmation, may therefore be the reflection of a negative feedback loop induced by high plasma T3 levels released in the periphery in response to the TGR5-dependent SNS-Dio-2 engagement in the BAT.

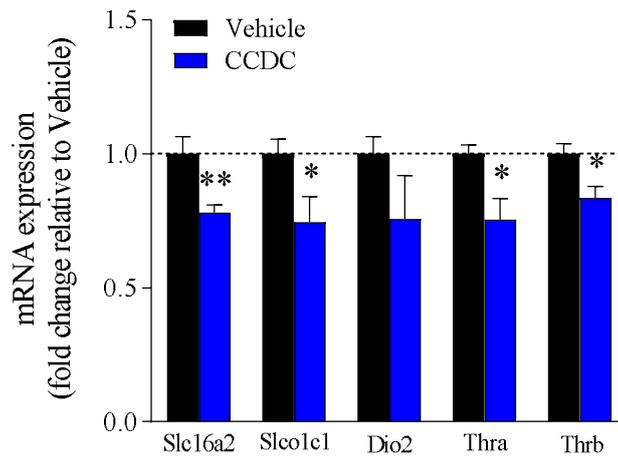


Figure 26. Chronic central TGR5 activation induces hypothalamic changes in thyroid hormone transporters and receptors. mRNA expression levels of markers of thyroid hormone system in the hypothalamus in response to chronic central CCDC treatment in diet-induced obese mice (n=6 vehicles, n=7 CCDC); data are mean \pm SEM; two-tailed student's t test: * $p < 0.05$.

Slc16a2: solute carrier family 16 member 2; *Slco1c1*: solute carrier organic anion transporter family member 1C1; *Dio2*: deiodinase 2; *Thra*: thyroid hormone receptor ; *Thrb*: thyroid hormone receptor .

In order to verify if molecular changes indicating increases SNS activity and thermogenesis in adipose tissue were associated with actual changes in energy expenditure *in vivo*, CCDC-treated mice were housed in calorimetric cages under normal temperature conditions (22°C) at day 10 of chronic treatment, before any significant weight loss was achieved. We observed a significant effect of treatment over time, where the average of 48 h energy expenditure was significantly higher in the diet-induced obese mice chronically receiving ICV CCDC (**Fig. 27**).

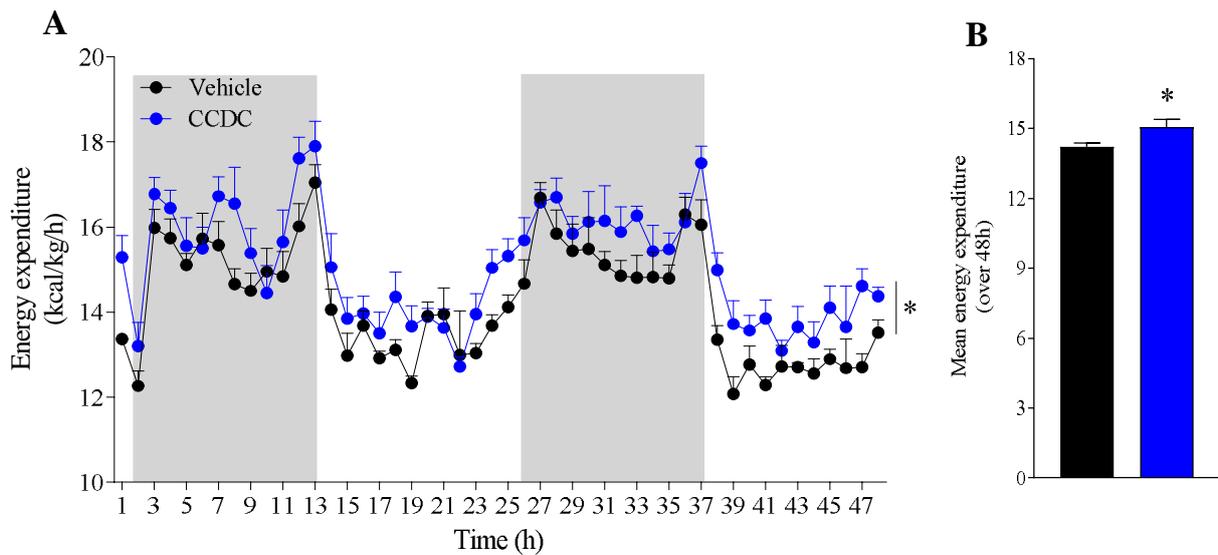


Figure 27. Chronic central TGR5 activation increases energy expenditure in diet-induced obesity. A) Energy expenditure over 48 h collected from day 10 of chronic treatment, before differences in body weight were evident. Grey bars indicate the 12 h dark phase; B) average energy expenditure over 48h (n=6 vehicles, n=6 CCDC); data are mean \pm SEM; analyzed by two-way repeated measures ANOVA when measuring outcomes across time, or by two-tailed student's *t* test when comparing two groups: * $p < 0.05$.

Since the SNS drives increased use of energy for heat production (Labbe, Caron, Bakan, et al., 2015; Labbe, Caron, Lanfray, et al., 2015), and peripheral markers of thermogenesis were upregulated in our chronic model (see **Fig. 25**), we wanted to understand if the SNS was driving the effects on energy expenditure from mice receiving CCDC chronic treatment centrally. For this, we placed the mice in the calorimetric cages, this time at thermoneutrality (30°C) in order to blunt the activity of the SNS. In doing so, the previously observed effects on energy expenditure as a result of chronic CCDC infusion were lost (**Fig. 28A**). By calculating the different components that participate in the use of energy (see Materials and Methods; (Abreu-Vieira et al., 2015)), we obtained the basal metabolic rate (BMR) per group [EE at 22°C ó EE at 30°C]. From this analysis, we were able to calculate the amount of energy that is exclusively used to maintain body temperature at 22°C (cold-induced thermogenesis). Interestingly, mice receiving the TGR5 agonist had an increased cold-

induced thermogenesis during their resting period (light) compared to their controls (**Fig. 28B**) implying that the effect of chronic central TGR5 stimulation on energy expenditure was indeed dependent of the SNS.

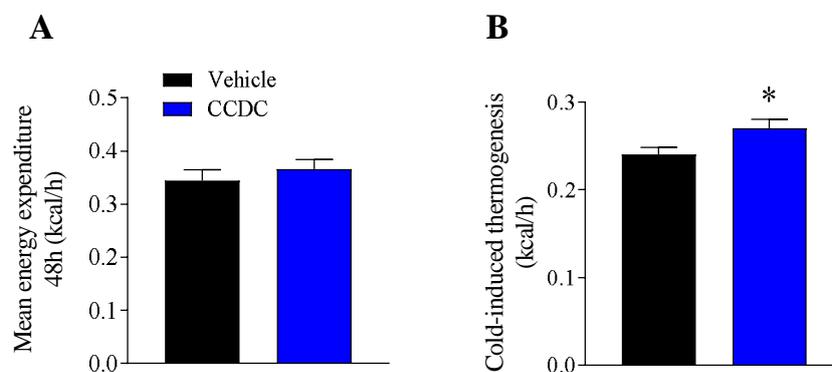


Figure 28. Effects of chronic central TGR5 activation on energy expenditure depend on sympathetic activity. A) 48 h energy expenditure at thermoneutrality (30°C) and B) cold-induced thermogenesis during the light phase at 22°C of obese mice receiving chronic ICV treatment of CCDC (n=6 vehicles, n=6 CCDC); data are mean \pm SEM; Two-tailed student's t test: * $p < 0.05$.

Moreover, the previously seen effects on the increased expression of markers of sympathetic activity and thermogenesis in WAT and BAT (see **Fig. 25**) were lost under thermoneutrality (**Fig. 29**), further suggesting an implication of the SNS in the effects of chronic TGR5 central activation.

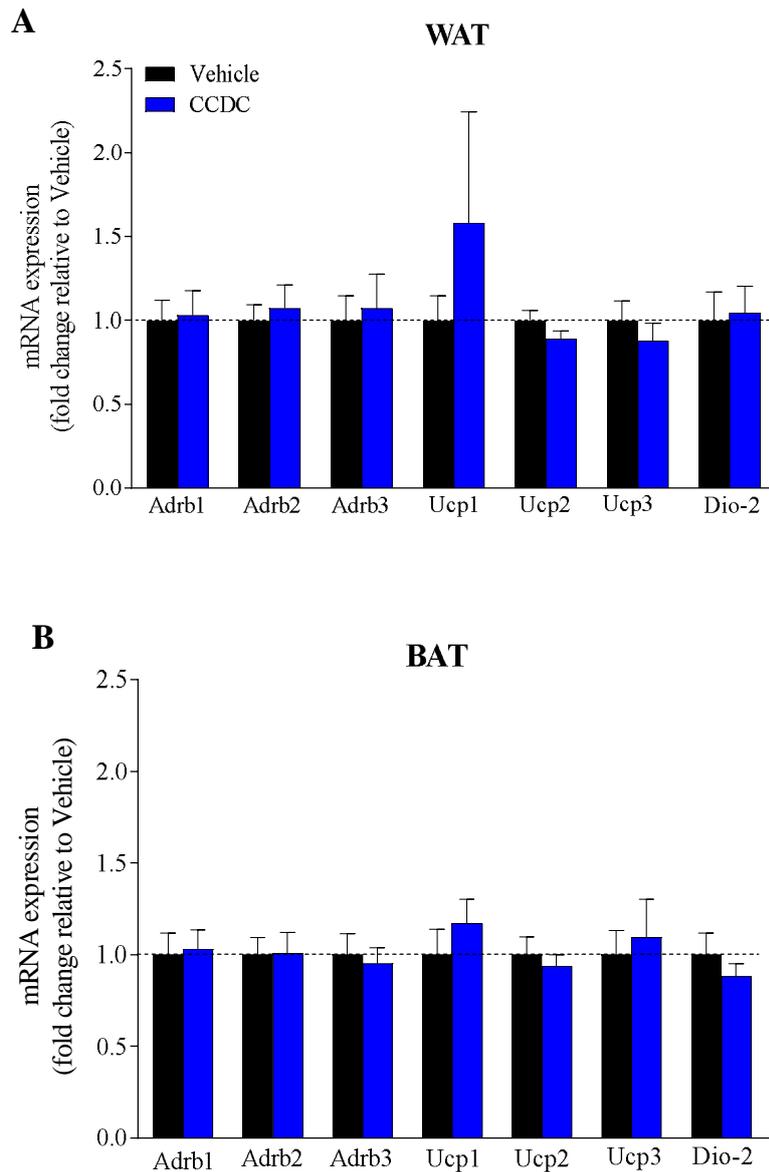


Figure 29. Thermoneutrality blocks the effects of central TGR5 activation on peripheral tissues. mRNA expression levels of markers of sympathetic activity and thermogenesis in WAT (A) and BAT (B) from diet-induced obese mice receiving chronic treatment of CCDC that were placed at thermoneutrality during 6 days (30°C) (n=12 vehicles, n=10 CCDC); data are mean \pm SEM; two-tailed student's *t* test.

Ardb1: α -adrenoreceptor 1; *Ardb2*: α -adrenoreceptor 2; *Ardb3*: α -adrenoreceptor 3; *Ucp-1*: uncoupling protein 1; *Ucp-2*: uncoupling protein 2; *Ucp-3*: uncoupling protein 3; *Dio2*: deiodinase 2.

To further confirm that the observed effects of central TGR5 on body weight and adiposity were indeed attributable to an increase in the sympathetic tone, we performed chemical sympathectomy using peripheral administration of 6-OH-DOPA, a toxic compound that causes the selective destruction of noradrenergic terminals, and hence disrupts the sympathetic innervation towards peripheral tissues (Vaughan et al., 2014). These mice then underwent a chronic ICV treatment with CCDC, as done in previous experiments.

y. Chemical sympathectomy prevents weight loss induced by central TGR5 agonism

Peripheral chemical sympathectomy was achieved by injecting i.p. 6-OH-DOPA (80mg/kg; (Quarta et al., 2010)) in diet-induced obese mice during 3 consecutive days. A first cohort of mice was used to verify successful sympathectomy after 1 week of the i.p. injections. Tyrosine hydroxylase (TH) protein in the WAT and BAT was used as a measure of successful denervation (Vaughan et al., 2014). Indeed, after 1 week from the first i.p. injection, TH was significantly reduced in both tissues with respect to mice receiving vehicle only (**Fig. 30**).

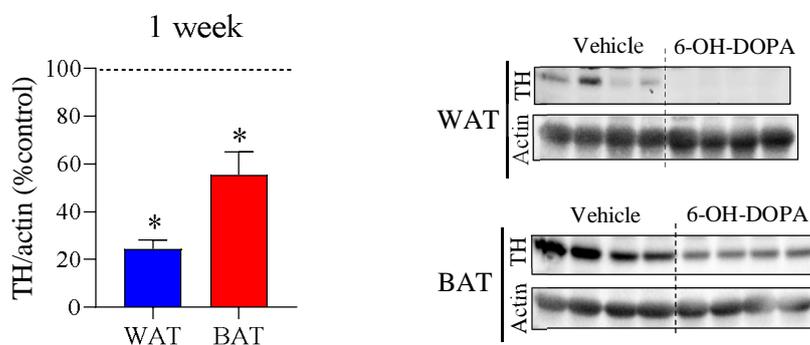


Figure 30. Chemical sympathectomy decreases TH protein content in adipose tissue. Protein quantification by western blot of TH from diet-induced obese mice that were killed after inducing chemical sympathectomy. A) Ratio between TH and β -actin in WAT and BAT; B) representative blots of TH and β -actin in the WAT and BAT. (n=4 per group); data are mean \pm SEM; two-tailed student's t test with respect to their vehicles: * $p < 0.05$.

6-OH-DOPA: 6-hydroxydopamine; TH: tyrosine hydroxylase.

Another group of mice treated with 6-OH-DOPA was implanted with mini-osmotic pumps for chronic central CCDC treatment after 1 week of denervation. We found that there was no significant effect in body weight across time between groups, and no effect in food intake or feed efficiency (**Fig. 31A-C**). Moreover, no changes in body composition were observed (**Fig. 31D-E**). These results show that in the absence of sympathetic connectivity, the phenotypic effects of central TGR5 agonism are blunted.

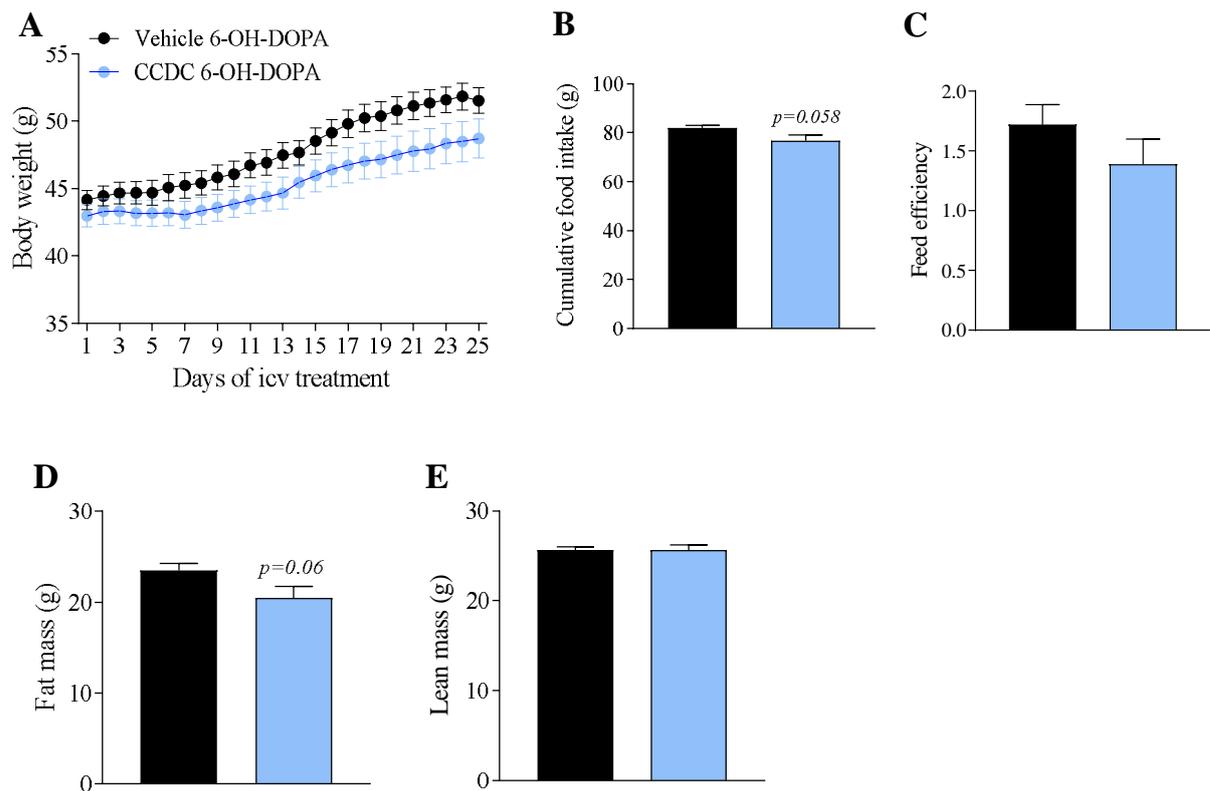


Figure 31. Chemical sympathectomy blocks the beneficial effects of central TGR5 activation. A) Body weight follow-up, B) cumulative food intake, C) feed efficiency, D) fat mass, and E) lean mass of obese mice that underwent chemical sympathectomy, followed by chronic ICV CCDC treatment (n=9 vehicles, n=10 CCDC). Data are mean \pm SEM; analyzed by two-tailed student's *t* test when comparing two groups, or by two-way repeated measures ANOVA when measuring outcomes across time.

At the end of the chronic treatment, WAT and BAT samples were collected in order to assess denervation, since recovery has been reported after several weeks following 6-OH-DOPA treatment (Demas & Bartness, 2001). At 5 weeks post-6-OH-DOPA injections, a significant decrease of TH in the WAT of vehicles remained, while a marginal reduction was still present in the WAT of mice receiving CCDC and in the BAT of both groups (**Fig. 32**), suggesting a partial recovery of SNS innervation.

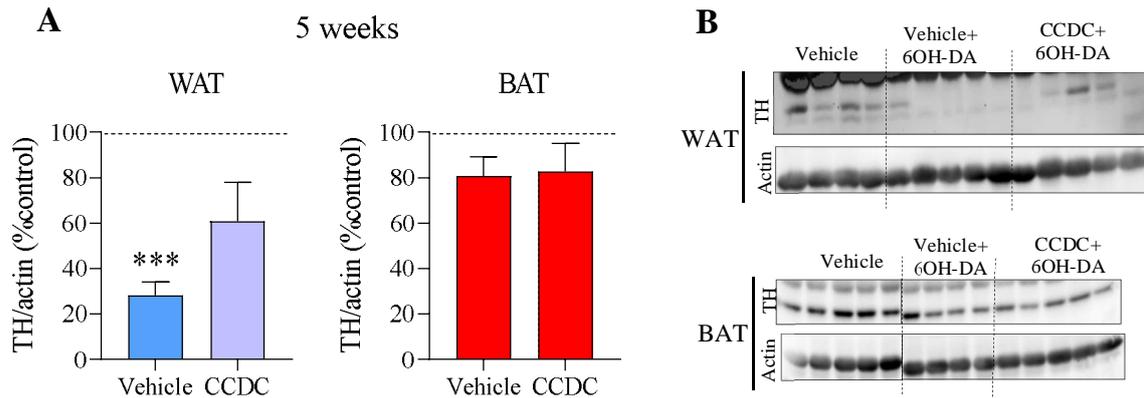


Figure 32. Denervation after 5 weeks post-treatment with 6-OH-DOPA. Protein quantification by western blot of TH from obese mice that were killed after 5 weeks of chemical sympathectomy. A) Ratio between TH and β -actin in WAT and BAT; B) representative blots of TH and β -actin in the WAT and BAT; ($n=9$ vehicles, $n=10$ 6-OH-DOPA for both tissues); data are mean \pm SEM; two-tailed student's t test with respect to their vehicles: *** $p<0.001$.

6OH-DA: 6-hydroxydopamine.

These results indicate that in a diet-induced obesity, chronic central TGR5 activation by CCDC is capable of reducing body weight, food intake, and adiposity through a heightened SNS, which will dictate an increase in metabolic markers of thermogenesis in peripheral tissues, as well as increased energy expenditure. In the absence of a functional sympathetic connectivity, central TGR5-dependent effects are lost.

Taken together, we have shown that chronic central activation of the TGR5 receptor in diet-induced obese mice is capable of sustained body weight loss (mostly attributed to decreased

adiposity), reduced food intake, and improved insulin sensitivity. Moreover, we show that these effects are possible through a heightened SNS, which will stimulate peripheral tissues (WAT and BAT) to increase energy expenditure and thermogenesis.

However, up until this point, we had not proven that the TGR5 receptor was indeed necessary for attaining these effects. To demonstrate this, we made use of a genetic animal model (TGR5^{flox/flox} mice with a B16/J background), which allowed performing a site-specific, Cre-dependent deletion of the receptor within the MBH.

C. Genetic animal models

In the following sections, the deletion of the TGR5 receptor within the MBH was carried out through the stereotaxic injection of a Cre-linked AAV or its GFP-linked control. Although the neuroanatomical expression of Cre recombinase in the MBH of these samples is ongoing, we have genotyped by PCR representative samples per group to evaluate recombination in genomic DNA, as a means to control that the TGR5 gene was indeed excised in this region. In all brain slices originating from mice receiving the Cre-linked AAV, recombination was identified, contrary to what was seen in mice receiving AAV-GFP, where, as expected, no recombination was present.

z. Deletion of TGR5 in the MBH has no effect on lean mice

Chow-fed TGR5^{fl^{ox}/fl^{ox}} mice that were lean were injected in the MBH with either a Cre-linked AAV or its control. There was no effect in terms of their body weight, feed efficiency or body composition, although there was a small increase in their food intake (**Fig. 33**).

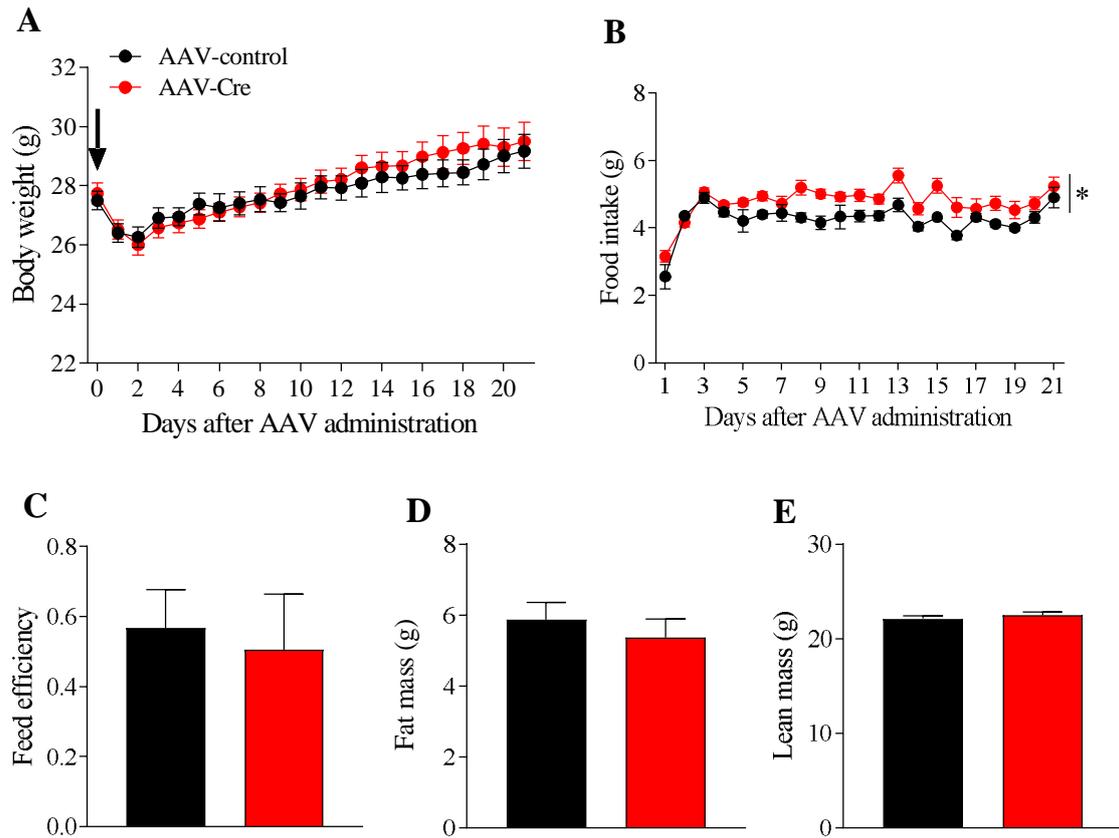


Figure 33. Deletion of TGR5 in the MBH does not affect the phenotype of lean mice. A) Body weight follow-up, B) food intake, C) feed efficiency, D) fat mass, and E) lean mass of lean mice receiving AAV-control or AAV-Cre ($n=5$ and 16 , respectively). The black arrow represents the time of AAV injection. Data are mean \pm SEM; analyzed by two-tailed student's t test when comparing two groups, or by two-way repeated measures ANOVA when measuring outcomes across time: $*p<0.05$.

Since our acute experiments suggested that effects of TGR5 activation on food intake and body weight were diet-dependent, we went on to test if the deletion of the receptor had an impact when mice were exposed to a HFD. For this, we designed an experiment in which lean mice were switched to a HFD one week after the AAV-Cre delivery in the MBH.

aa. Deletion of TGR5 in the MBH favors weight gain in the presence of a HFD

Chow-fed TGR5^{flox/flox} mice were injected into the MBH with either a Cre-linked AAV or their AAV-GFP control and switched to a HFD after 1 week from the viral injection. Animals receiving the Cre-linked AAV rapidly gained more weight, increased their food intake and feed efficiency compared to their controls when exposed to the HFD (**Fig. 34**).

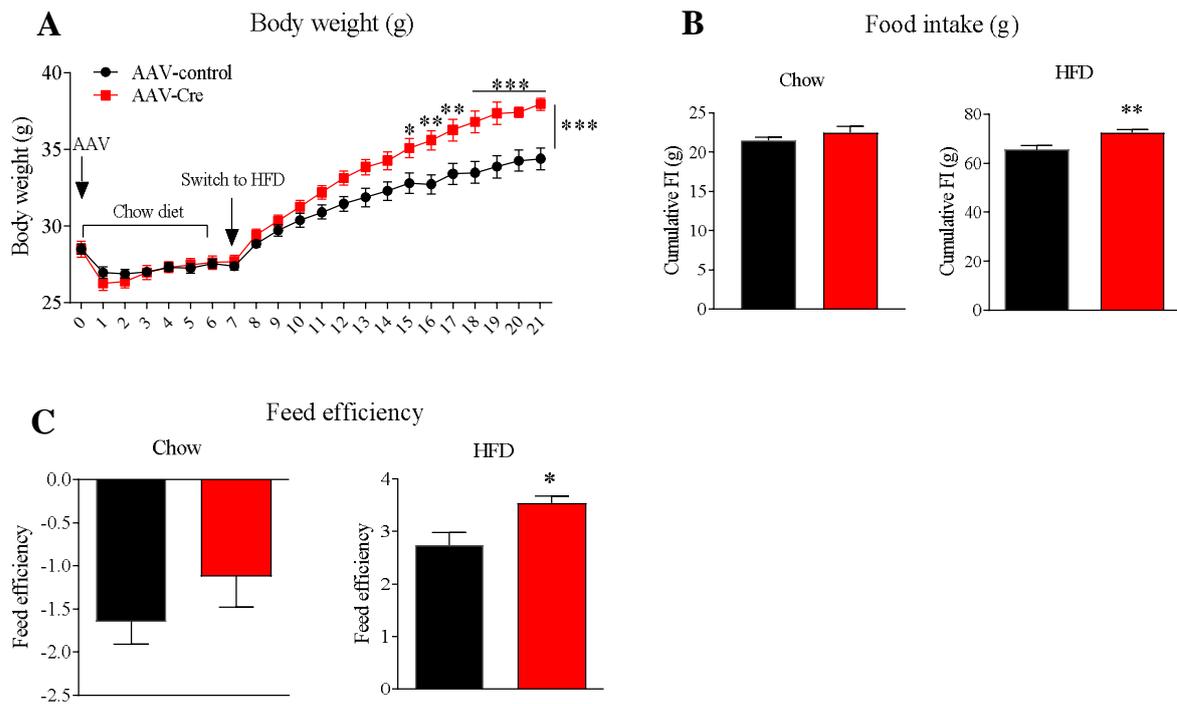


Figure 34. Deletion of TGR5 in the MBH increases body weight in a diet-dependent manner. A) Body weight follow up, B) cumulative food intake, and C) feed efficiency after 1 week of chow or 2 weeks of HFD exposure ($n=7$ AAV-controls; $n=8$ AAV-Cre). Data are mean \pm SEM; repeated measures ANOVA or two-tailed student's t test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

To understand what was causing the increased body mass gain (since in this model, the feed efficiency was higher in HFD-fed mice with the MBH deletion), we carried out a cold challenge. For this, mice were placed at 4°C for 4 h in order to stimulate the SNS. By measuring markers of lipolysis and thermogenesis, we found that mice with the MBH deletion of TGR5 were not as efficient as their controls in coping with a cold challenge (**Fig. 35**),

further implying that TGR5 in the MBH is necessary for cold-induced SNS-dependent lipolysis and thermogenesis.

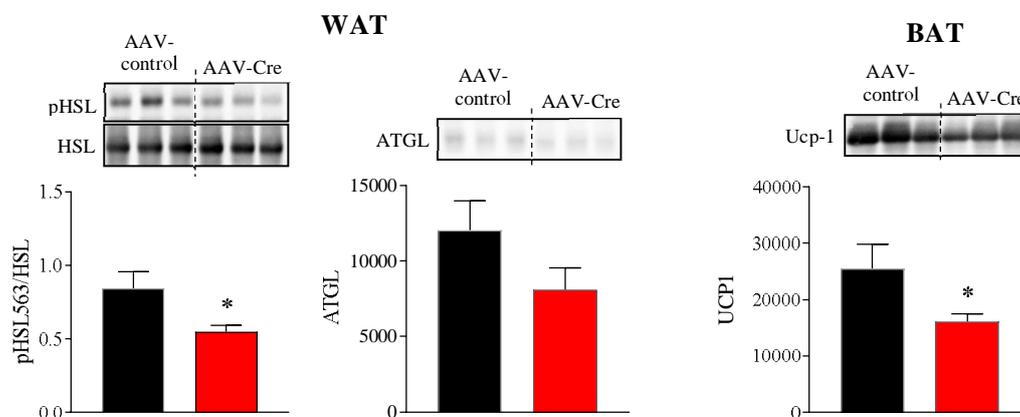


Figure 35. TGR5 in the MBH is necessary for maintaining appropriate cold-induced SNS responses in adipose tissue. Protein quantification by western blot from AAV-control or AAV-Cre treated mice that were killed after a 4 h cold challenge on a HFD. For WAT, pHSL563 and ATGL were used as markers of lipolysis; for BAT, Ucp-1 was used as a marker of thermogenesis (n=7 controls, n=6 Cre for both tissues); data are mean \pm SEM; two-tailed student's t test: *p<0.05.

ATGL: adipose triglyceride lipase; HSL: hormone sensitive lipase; pHSL563: phosphorylated form of HSL at Ser563; Ucp-1: uncoupling protein 1.

Finally, we assessed whether MBH deletion of TGR5 in already obese animals affected their phenotype.

bb. Deletion of TGR5 in the MBH of already obese mice worsens their phenotype

Diet-induced obese $TGR5^{flx/flx}$ mice were injected the AAV-Cre or its control in the MBH. Compared to their controls, AAV-Cre treated mice significantly gained more weight, increased their food intake, feed efficiency and their fat mass content (Fig. 36), hence worsening the obese phenotype.

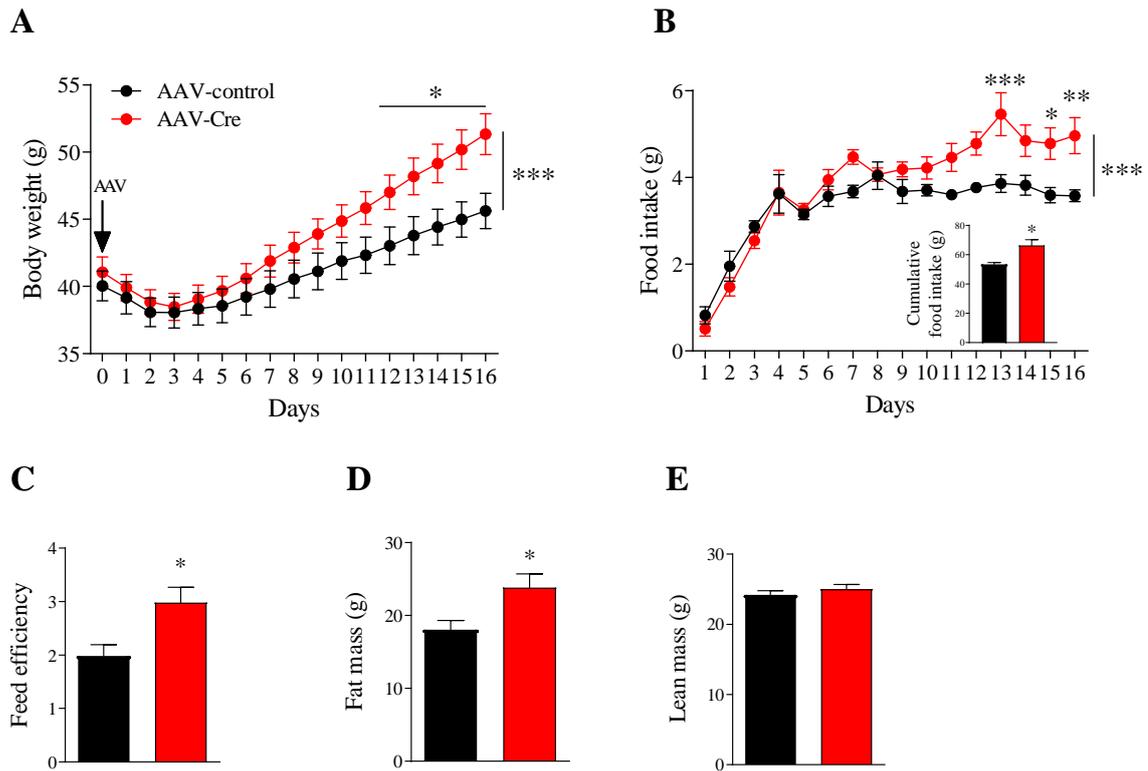


Figure 36. Deletion of TGR5 in the MBH worsens obesity. A) Body weight follow up, B) food intake, C) feed efficiency, D) fat mass, and E) lean mass of $TGR5^{flx/flx}$ diet-induced obese mice receiving AAV-Cre or its control in the MBH ($n=10$ AAV-controls; $n=10$ AAV-Cre); repeated measures ANOVA or two-tailed student's t test (mean \pm SEM); * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Overall, our experiments in $TGR5^{flox/flox}$ mice allowed confirming that the hypothalamic TGR5 receptor is indeed necessary to achieve the previously described metabolic benefits on body weight, food intake, body composition and increased thermogenesis. This is because its site-specific deletion increases adiposity in a diet-dependent manner, blunts sympathetic activity in HFD-fed mice exposed to the cold, and causes a rapid increase in body weight, food intake and fat mass, even in mice that are already obese, worsening their phenotype.

VIII. Discussion and conclusions

VIII. Discussion

BA have been mostly studied in the context of their action in peripheral tissues. Due to their widespread effects on metabolism and energy use, we hypothesized a role for BA at the level of the hypothalamus through the activation of their receptor, TGR5. We first demonstrate that TGR5 and BA system components are present in the hypothalamus, and are deregulated in the presence of an obese phenotype. Next, we show that both acute and chronic ICV pharmacological activation of TGR5 decreases body weight, food intake, adiposity, and improves insulin sensitivity, effects that are evident only in the presence of diet-induced obesity. Moreover, we demonstrate that these effects are coupled to an increase in energy expenditure, which is in turn driven by recruiting the SNS and signaling lipolysis and thermogenesis in the WAT and BAT, respectively. Lastly, we show that the hypothalamic TGR5 receptor is indeed necessary for driving these metabolic effects, since its deletion in the MBH of adult mice rapidly increases food intake, body weight, and adiposity in a diet-dependent manner, worsening the obese phenotype. For the first time, we provide evidence of a hypothalamic BA δ TGR5 receptor system that is particularly relevant in the presence of diet-induced obesity. Therefore, with this evidence, we propose a shift in the current view of BA metabolic actions, which should now include a δ central perspective.

D. The BA system is deregulated in obesity

We have found that the components of the BA system are present within the brain, since the TGR5 receptor and BA transporters were detected at the level of the hypothalamus. Consistent with the literature, we were unable to detect the nuclear BA receptor FXR in our hypothalamic samples (Forman et al., 1995; X. Zhang et al., 2014).

Notably, the expression of BA transporters in the hypothalamus was upregulated in refed, lean mice, suggesting that BA can act as signaling molecules in the brain to indicate the real-time energy status related to meal consumption. This was not the case in our obese model, since there was no change in BA transporters following refeeding. Deficits in hepatic BA transporters have been reported in liver samples of obese human subjects, where NTCP and BSEP were negatively correlated with body mass index, possibly contributing to a slowing down of the enterohepatic circulation during obesity (Haeusler et al., 2016). Our results are therefore in line with the literature. Moreover, the levels of circulating and hypothalamic BA were dramatically lower in obese mice, a fact that has already been reported in the plasma of obese humans (Albaugh et al., 2015).

Thus, our data suggest that the central signaling of BA is impaired in obesity, since BA circulating levels might not be sufficient to reach the brain and modulate the expression of their transporters for an accurate postprandial response.

Central TGR5 signaling emulates a postprandial state

We first focused on testing the relevance of TGR5 centrally, both during acute and chronic exposure to its agonists. We administered sodium choleate, which contains two of the most potent endogenous agonists of the receptor (cholic acid and deoxycholic acid), as well as CCDC, a synthetic agonist which is selective to TGR5 and the most potent stimulant of cAMP formation, without causing the endocytosis of the receptor after continuous exposure (Jensen et al., 2013). Since the selective activation of TGR5 centrally seemed to have an effect only in the presence of diet-induced obesity, we carried out subsequent experiments in an obese model.

Human subjects that are obese have a 2-fold increase in markers of BA synthesis, as well as a blunted response of serum BA following a meal (Glicksman et al., 2010; Haeusler et al., 2016). We observed that the acute central activation of TGR5 in obese mice dramatically decreased hepatic expression of Cyp7a1, perhaps as a mechanism to bring BA levels back to normal. If, in the context of obesity, the *de novo* synthesis of BA is 2-fold higher to compensate for the lack of circulating BA, it seems logical that the activation of central TGR5 by BA would recognize their high availability, hence triggering a negative feedback loop to decrease their biosynthesis. In line with these findings, we found that the acute central stimulation of TGR5 caused changes in the plasma BA pool, notably decreasing levels of one of the most potent endogenous agonists of the receptor, cholic acid, most likely as a mechanism to compensate for the central overstimulation of the receptor.

It is now accepted that BA can act as signaling molecules to maintain metabolic homeostasis (Chiang, 2013) and more particularly to regulate glycaemia (Bunnett, 2014; Thomas et al., 2009). Our results on the downregulation of hepatic markers of glucose metabolism and BA synthesis (G6pc and Cyp7a1, respectively), as well as on the increase of fatty acid synthesis markers (FAS), suggest that the central activation of TGR5, even during fasting, since food was not given back to animals then undergoing these *ex vivo* analyses, is sufficient to emulate a postprandial state in peripheral tissues, mimicking a situation of high energy availability.

Circulating BA levels are known to follow circadian rhythms, in part due to clock genes regulating BA biosynthetic pathways through regulating the expression of Cyp7a1 (Duez et al., 2008; Le Martelot et al., 2009). BA can also induce changes in the expression profiles of circadian genes (Govindarajan et al., 2016), evidencing an intricate regulation system for their availability. In humans, a clear diurnal variation in BA synthesis has been evidenced through measuring a marker of Cyp7a1 activity, 7-hydroxy-4-cholesten-3-one (C4), in plasma samples. The authors showed that, independently of the energetic state (i.e. fasted vs. refed), BA synthesis follows pronounced diurnal rhythms (peaks at 13 h and 21 h), while decreasing their levels during the night (Galman, Angelin, & Rudling, 2005). Mice seem to show the opposite pattern, where total serum BA are lowest at the peak of the light phase, and highest at the beginning and end of the dark phase, suggesting two main episodes of food ingestion during the dark (Y. K. Zhang, Guo, & Klaassen, 2011). Interestingly, when looking into the main BA species driving these concentration peaks in mice, some of the strongest TGR5 agonists (cholic acid and lithocholic acid) seem to have the most significant peaks of secretion at these time points (Y. K. Zhang et al., 2011). However, to our knowledge, no studies of the circadian fluctuations of BA have been carried out in the context of obesity. In our studies, BA sampling was performed just before the dark period. Although we did not explore the 24 h synthesis patterns of BA, it would be of interest to assess if these patterns are altered in our model, and if so, how they would contribute to the phenotype. We can only hypothesize that, since circulating BA are lower in our obese mice, their secretion may not reach the same peak levels as in normal subjects. This would be in line with literature showing that obese subjects have deregulated meal patterns (Aparicio et al., 2018; Baron, Reid, Kern, & Zee, 2011), which may again contribute to defective rhythms in their biosynthesis and secretion.

Effects on insulin sensitivity

TGR5 that is expressed in pancreatic β cells can directly stimulate the release of insulin in order to participate in the control of glucose homeostasis (Kumar et al., 2012). Furthermore, it can also indirectly participate in the control of glycaemia by inducing the release of intestinal GLP-1 from enteroendocrine cells, leading to enhanced glucose tolerance in obese mice (Bunnett, 2014; Thomas et al., 2009). In humans, it seems that the release of insulin following a meal plays a role in the reduction of circulating BA after their postprandial peak, by promoting their uptake from the blood to the liver. This process seems to be blunted in obesity (Haeusler et al., 2016). It is likely that these blunted effects are due to insulin

resistance in the liver, added to the fact that, as mentioned earlier, hepatic transporters of BA are also downregulated in obesity (Haeusler et al., 2016), hence preventing or slowing down their reuptake. Moreover, high hepatic gluconeogenesis can occur in the presence of insulin resistance (Hatting, Tavares, Sharabi, Rines, & Puigserver, 2018). In line with this, we observe that the acute central activation of TGR5 leads to better control of glucose in a manner that is dependent of insulin. Due to the late phase of glucose decrease (i.e. past 30 minutes of insulin administration), this might be reflective of a counterregulatory response driven by other hormones, such as glucagon, adrenaline, cortisol and growth hormone. However, it is also possible that this effect is achieved through the increase in *Irs2* expression in the liver, which is accompanied by a decreased mRNA expression of glucose-6-phosphatase, indicating decreased hepatic gluconeogenesis. Similarly, this decrease in markers of hepatic glucose production has also been reported in animal models undergoing bile diversion, which mimics the metabolic effects of bariatric surgery, where bile-diverted rats had decreased mRNA and protein levels of glucose-6-phosphatase (Goncalves et al., 2015). It remains to be elucidated whether this might lead to better BA reuptake by the liver, and hence a reestablishment of the enterohepatic cycle to bring BA levels back to normal following a meal.

E. Metabolic effects of TGR5 are driven by its central activity

The effects of central TGR5 activity on decreased food intake and body weight loss over 24 h seemed to be driven by the hypothalamic activity of the receptor, since the targeted administration of CCDC into the MBH had the same outcomes as the ICV administration. Moreover, these effects were sustained in our chronic model, where weight loss was mostly attributed to decreased adiposity. Our outcomes are in line with the only published literature that has explored central TGR5 effects on metabolism. By administering tauroolithocholic acid *via* ICV, a recent publication shows that obese mice receiving the chronic infusion centrally decreased their fat mass. However, the authors did not observe changes in body weight nor energy expenditure, despite BAT markers of energy expenditure being upregulated (Eggink et al., 2018). This could be explained by the fact that their compound of choice for ICV administration, tauroolithocholic acid, was acting on other targets within the brain (not a specific TGR5 agonist). Furthermore, tauroolithocholic acid has a much lower signaling capacity than our compound, CCDC, which, as mentioned previously, is the most potent stimulant of TGR5-driven cAMP formation.

Unlike our acute studies, where we observed changes in BA content in the plasma, this was not the case after chronic central activation of the receptor in our obese model. However, we were still able to see a clear phenotype in terms of reduced body weight, adiposity and food intake. This observation, added to the fact that CCDC was not found in the circulation after chronic ICV treatment (i.e. it stays within the brain), suggests that, in fact, these effects were driven by CCDC targeting central TGR5 exclusively, and not by peripheral changes in BA targeting other tissues.

F. Central TGR5 activity increases energy expenditure through the SNS-dependent engagement of the adipose tissue

The feed efficiency, or feed conversion ratio, is an index that is typically used in animal husbandry to quantify the relation between food consumed and its transformation into the desired output (milk, meat, weight) (Koch, Swiger, Chambers, & Gregory, 1963). In the context of our studies, both our acute and chronic models significantly decreased their feed efficiency after central CCDC treatment. This hinted at a differential use of nutrients that was being reflected in a lower conversion of energetic substrates to body mass, meaning that the loss of body weight could not be solely explained by the reduction in food consumed. One likely explanation was that the treatment was causing an enhanced use of energy, which would consequently contribute to further decreasing body weight. As mentioned earlier, TGR5 expressed in adipocytes has been shown to participate in increased brown fat activity and energy expenditure through thermogenesis (Broeders et al., 2015; Teodoro et al., 2014; Watanabe et al., 2006; Zietak & Kozak, 2016), which contributes to a reduction of adiposity and overall body weight. Similarly, we observed that sustained central TGR5 activation was accompanied by a decrease in adiposity and an increase in energy expenditure in our obese model, suggesting that the previously described effects in increased BAT activation could be initiated by stimulating the receptor centrally.

The canonical pathway by which the hypothalamus signals to the BAT for increasing energy expenditure is through the activation of the SNS, increasing the release of catecholamines that act on β -adrenoreceptors to induce thermogenesis (Bianco & McAninch, 2013). Indeed, the upregulation of the mRNA expression of β -adrenoreceptors in the adipose tissue from our model, together with the increased energy expenditure, hinted at the involvement of the SNS in the centrally-driven effects of TGR5. To prove this, we performed two experiments that would hinder sympathetic activity, and tested the relevance of central

TGR5 stimulation. The first experiment consisted on placing our chronically treated mice at thermoneutrality (where thermogenesis is no longer necessary, hence blunting the SNS). As expected, there was no longer an increase in energy expenditure (since at 30°C there is no need to generate body heat), and the expression of β -adrenoreceptors and Dio-2 were blunted in adipose depots. The second experiment entailed eliminating peripheral sympathetic connectivity through chemical sympathectomy, and then exposing our mice to the central treatment with CCDC. Similarly, in the absence of a functional SNS, the previously seen effects on decreased body weight, adiposity and food intake were lost. Although we did observe a tendency towards decreased body weight and adiposity at the end of the chronic treatment, this was most likely due to a partial recovery of SNS innervation (Thureson-Klein, Lagercrantz, & Barnard, 1976). Indeed, such recovery in the innervation was confirmed by the marginal reductions in the TH protein in the WAT and BAT after 5 weeks from 6-OH-DOPA administration as compared to suppression of TH expression observed after 1 week. Together, these results prove that the central activity of the TGR5 receptor protects from body weight gain through an SNS-dependent mechanism leading to enhanced β -adrenoreceptor activity for the thermogenic activation of the BAT, similar to what would be expected if mice were exposed to a cold environment.

It is also known that sympathetic signaling and thyroid hormone pathways are interconnected, since the SNS can drive an increased expression of Dio-2 in the BAT (Bianco & McAninch, 2013), leading to increased T3 production in this organ and resulting increased thermogenesis (Labbe, Caron, Bakan, et al., 2015; Labbe, Caron, Lanfray, et al., 2015). Indeed, in our studies we found that Dio-2 mRNA expression was upregulated in the BAT of mice treated with CCDC centrally, while its expression was not changed when we placed them at thermoneutrality, implying that increased Dio-2 expression depends upon SNS activation. Other studies have actually shown that administration of the BA cholic acid supplemented in a HFD can increase Ucp-1 expression, independently of action on the SNS and engagement of Dio-2 (Zietak & Kozak, 2016). We did not see changes in Ucp-1 expression. However, we did observe an increase in Ucp-3 due to chronic central activation of TGR5, a marker that is being increasingly recognized as an important mediator of thermogenesis (Oliveira et al., 2016).

There is an important body of research linking thyroid effects on the hypothalamus (particularly the VMH) as an important determinant of BAT activation (Lopez et al., 2010). We have found that central TGR5-dependent stimulation of the SNS increases Dio-2

expression in the BAT. Therefore a likely possibility is that there is a higher conversion of T4 to T3 in this organ, leading to the spillover of the hormone in the circulation. This would imply that T3 would be increased in the circulation, and that it could reach the hypothalamus (Bianco & McAninch, 2013) to feed forward the TGR5-induced SNS stimulation chronically. Although measurements of thyroid hormones in the circulation are warranted and are currently planned in order to confirm our hypothesis, we did look at thyroid metabolism markers at the level of the hypothalamus. Interestingly, we found that all markers were downregulated in the presence of the chronic central administration of CCDC. These findings therefore suggest that the central activation of TGR5 would stimulate the activity of the SNS to favor the conversion of T4 to T3 (through Dio-2 in the BAT). In turn, the elevated levels of T3 released in the periphery would then signal back to the brain to blunt the expression of its own receptors and transporters at the level of the hypothalamus.

Our results imply that the action of TGR5 to stimulate energy expenditure through heat production not only happens locally through TGR5 expressed in adipocytes, as previously demonstrated (Teodoro et al., 2014; Watanabe et al., 2006; Zietak & Kozak, 2016), but it could also be dependent on a higher order signaling, specifically on the hypothalamic activation of TGR5, resulting in a phenotype resembling that of being exposed to cold and which may involve subsequent central action of T3. Accordingly, work by Lopez and collaborators has disentangled a T3-dependent system that initiates in the hypothalamic VMN, which takes to enhanced activation of the SNS, and ultimately to an increase in BAT thermogenesis through increased catecholamines (Lopez et al., 2010; Martinez-Sanchez et al., 2017), providing a metabolic loop between the canonical SNS-BAT signaling pathway and hypothalamic thyroid hormone signaling (Villarroya & Vidal-Puig, 2013).

G. MBH deletion of TGR5 increases adiposity and worsens obesity

To understand the hypothalamic implication of TGR5 in all the previously described metabolic effects, we decided to make use of a genetic model that would allow us to delete TGR5 from the MBH. As expected from our acute experiments in chow-fed mice, this deletion in lean mice had no gross phenotypic effects. However, this genetic approach resulted in a rapid weight gain, mostly attributed to increased adiposity when animals were switched to a HFD. Moreover, a cold challenge allowed us to further confirm that TGR5 indeed drives weight loss through enhanced SNS, since markers of lipolysis and thermogenesis were blunted in the adipose tissue of mice with the MBH deletion of the

receptor, which was also in line with our previous results blunting the SNS. Additionally, when the deletion of MBH TGR5 was performed in already obese mice, they rapidly increased their body weight, food intake and adiposity, further contributing to the obese phenotype. Overall, these results strongly point to an involvement of TGR5 expressed within the MBH in protecting from diet-induced obesity. Unfortunately, the viral approach that was used for conducting the MBH deletion of TGR5 does not allow us to determine the specific types of cells that were targeted, mostly because the TGR5 receptor is expressed at very low levels in the adult brain. This stresses the difficulty of imaging the receptor by other tools. In our experience, immunohistochemical approaches to visualize the localization of the receptor have been inconsistent and very much related to the specific batch of primary antibody used to label TGR5. Moreover, we have further tested the possibility of creating a probe that would target the mRNA within the cells. However, in line with its low expression, this strategy also proved to be challenging. Therefore, we are currently exploring the possibility of using genetic models coupled with promoter-specific AAV-Cre delivery that will target discrete cell types in the MBH in order to pinpoint the cellular populations expressing TGR5 within this brain region.

H. Pinpointing the hypothalamic cellular populations expressing TGR5 in the brain that participate in improving obesity

There are several hypothalamic nuclei that are involved in feeding behavior and the control of energy homeostasis (see section **C. Central control of energy balance**). Indeed, it is of interest to dissect the role of TGR5 in each of these nuclei in order to pinpoint where the metabolic effects of its activation are originated.

The ARC, containing at least two distinctive cellular populations mediating appetite and satiety, could very likely be implicated in TGR5-mediated effects. Unpublished work by our collaborators (Dr. Schoonjans, Laboratory of Metabolic Signaling, Ecole Polytechnique Fédérale de Lausanne) performed in lean mice suggest that both $Tgr5^{-/-}$ (full knockout) and $Tgr5^{Syn^{-/-}}$ (neuronal knockout) mice have an increased food intake, as well as an increased expression of AgRP within the ARC compared to their control littermates. We have analyzed samples from these same models, and found that activation of POMC neurons seemed to be inhibited, which would explain the increased food intake in these mice and further suggest that the BA sensing pathway to the MBH is dependent on neuronal TGR5. However, $Tgr5^{Syn^{-/-}}$, which lack the receptor in all neurons, both in the CNS and the PNS, do not seem to have a

deleterious phenotype when exposed to HFD. This could be due to the aspecificity of the neuronal deletion in this animal model. In addition, deletion of POMC neurons does lead to hyperphagia during HFD, but this is compensated for by an increase in energy expenditure. Because of these data generated by our collaborator, we have therefore started to investigate a possible role for TGR5 in other hypothalamic cell populations.

Since SF1 neurons expressed in the VMH have been associated with the hypothalamic effects driving increased BAT thermogenesis through thyroid T3 hormone stimulation (Lopez et al., 2010), we have recently conducted experiments in mice with SF1 constitutive deletion of TGR5 (SF1**TGR5*-Cre+). However, our preliminary data suggest that TGR5 might not be relevant in this cellular population, since no changes were observed in lean or HFD-fed mice carrying this deletion, as compared to their Cre- controls. However, this evidence does not exclude that SF1 neurons may be part of the phenotypic effects of chronic central TGR5 agonism. In order to prove this latter point, we would actually need to delete thyroid hormone receptors from the VMH and evaluate whether metabolic effects of central TGR5 activation are still observed.

Another possibility is that TGR5 may also directly increase hypothalamic T3 and in this way engage SNS responses and improve obesity. In the brain, astrocytes possess the Dio-2 enzyme and are the main source of T3 (Freire-Regatillo, Argente-Arizon, Argente, Garcia-Segura, & Chowen, 2017). This type of cells also express TGR5 (Keith N. Frayn, 2010), like neurons. Therefore, targeting the deletion of TGR5 in astrocytes within the MBH would help answer the question of a possible non-neuronal action of TGR5, which may drive the observed improvements in the obese phenotype via increased central production of T3 and subsequent SNS activation.

Lastly, another unexplored option would be that Sim1 neurons that are particularly expressed in the PVN would be driving the TGR5-dependent effects that we have observed. This is because genetic mouse models with Sim1 ablation limited to the PVN exhibit early onset obesity caused by hyperphagia and decreased thermogenesis and energy expenditure (Xi et al, 2013).

I. Prospective clinical implications of central TGR5-driven metabolic effects

cc. Potential implications of bariatric surgery

As mentioned earlier, the astounding long-lasting metabolic effects that follow bariatric surgeries are increasingly being attributed to a systemic increase in circulating BA following surgery. In particular, interventions that include a rerouting of the gastrointestinal tract seem to have more dramatic effects (in terms of body weight loss and diabetes remission) compared to surgeries that only restrict stomach size (e.g. gastric banding) (Buchwald et al., 2009; Goncalves et al., 2015; Kohli et al., 2013), a fact most likely due to the shorter transit of BA through the gut, allowing them to reach distal parts of the intestine more rapidly, where they would be reabsorbed.

Although the increase in circulating BA following surgery have been amply discussed in the context of targeting the TGR5 receptor in peripheral tissues for promoting improvements in glycaemia, insulinaemia, lipolysis, reduced food intake, among others, it is highly possible that this increase in circulating BA would also reach the brain to target the MBH, especially considering the evidence pertaining to BA crossing the blood-brain barrier (Keene et al., 2001; Parry et al., 2010). It has been reported in experimental models that bile routing modifications, which mimic the BA delivery in the gut following gastric bypass surgery, can cause changes in food preference (Goncalves et al., 2015): when presented with a choice between standard chow or a high fat-high sucrose diet, rats that underwent bile diversion surgery almost exclusively preferred the standard chow, whereas their sham controls would consume 60% of the high fat-high sucrose diet (Goncalves et al., 2015). This observation on food preference has also been reported in patients who undergo bariatric surgery, since they show a marked decrease in hedonic hunger (pleasure associated with consumption of highly palatable foods, as well as cravings in the absence of immediate energetic needs) compared to severely obese patients (Schultes, Ernst, Wilms, Thurnheer, & Hallschmid, 2010). These studies further hint at the involvement of the MBH in the attained effects, considering the role that the distinct cell populations of this brain region have on directing eating behavior, as well as of relaying this information to other brain regions. Moreover, it further suggests an implication of other circuits in these effects, such as the reward system. It is likely that central TGR5 activity might be acting as a lipid sensing mechanism, thus preventing the subject from consuming highly caloric food and limiting the amount of circulating lipids. Although we have not performed food preference experiments in

our models, it would be of interest to test if the central administration of the TGR5 agonist can have effects on food choice, and by which mechanisms this takes place.

dd. Clinical trials administering BA

BA have been long used for treating specific diseases (cholelithiasis, non-alcoholic fatty liver disease, symptoms from neurodegenerative diseases). However, the discovery of their receptors led the way for greater research in their clinical applications, which has translated in the US Food and Drug Administration (FDA) approving the use of cholic acid for the treatment of BA synthesis disorders (Mullard, 2016). Due to their known effects on glucose, lipid and energy metabolism, BA signaling pathways through TGR5 activation have been considered as attractive targets for treating metabolic diseases, particularly obesity. Since BA can activate at least 2 different receptors, efforts have been made to either identify or synthesize novel BA with high specificity towards the TGR5 receptor (Ethanik et al., 2018).

However, to our knowledge, only one clinical trial has been published that administered a selective TGR5 agonist (Hodge et al., 2013). In this trial, patients with type 2 diabetes (with a BMI of 29 kg/m², on average) received a daily oral dose of compound SB-756050, a selective TGR5 agonist that had previously shown improvements in glycaemia and insulinaemia in diabetic animal models. Although authors expected improvements in glucose profiles, patients that received the two lowest doses of the compound (15 and 50 mg) had increased fasting glucose levels, as well as higher levels during an oral glucose tolerance test. The highest doses (100 and 200 mg) did not translate in any metabolic changes (Hodge et al., 2013). Although no adverse effects were reported, the high variability in response to the treatment may be one of the main reasons why clinical trials are lacking. Moreover, it is possible that, due to oral administration of the compound, the targeting of TGR5 was mostly restricted to the proximal gastrointestinal tract. Our work suggests that it is mostly the modulation of central TGR5 activity that would be driving the peripheral effects on metabolism, including control of glucose homeostasis, through either changes in BA biosynthetic pathways or stimulation of the SNS that would lead to adipose tissue mobilization and increased energy use. Finding strategies that could allow selective TGR5 agonists to reach hypothalamic targets for modulating the activity of the receptor would be necessary for implementing pre-clinical protocols that could translate to treatments for human subjects suffering from obesity and its related metabolic complications.

J. Conclusions

Our results clearly establish that BA do not only act as signaling molecules in peripheral tissues, but they can also reach the brain to activate TGR5 and trigger a myriad of beneficial metabolic effects, particularly in the context of obesity. More specifically, we have shown that hypothalamic TGR5 plays a role in obesity pathophysiology. First, we demonstrate a dysfunction of the BA system components in the presence of an obese phenotype. Next, we show that the acute or chronic pharmacological activation of the receptor centrally leads to metabolic improvements in obese mice, including a decrease in adiposity and an increase in energy expenditure. Further, we prove that these effects are driven by recruitment of the SNS to increase lipolysis and thermogenesis in WAT and BAT, respectively. Lastly, we demonstrate that TGR5 is in fact necessary for driving the improved phenotype, since its targeted deletion in the MBH increases adiposity in a diet-dependent manner by blunting the sympathetic tone, and worsens the obese phenotype.

Our work places hypothalamic TGR5 as a key mediator of SNS-driven effects on body weight loss through adipose signaling, unveiling a new mechanism of action for potential anti-obesity therapies. Further studies will be necessary for pinpointing the cell types involved in the central TGR5-mediated effects on metabolism.

IX. List of references

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