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Study of the involvement of brain tryptophan metabolism in obesity-induced memory deficits

Carla Elena Mezo Gonzalez

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Carla Elena Mezo Gonzalez. Study of the involvement of brain tryptophan metabolism in obesity-induced memory deficits. Médecine humaine et pathologie. Nantes Université, 2022. Français. NNT : 2022NANU1015 . tel-03881850

HAL Id: tel-03881850

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THESE DE DOCTORAT DE

NANTES UNIVERSITE

ECOLE DOCTORALE N° 605

Biologie Santé

Spécialité : Neurosciences et Nutrition

Par

Carla Elena MEZO GONZÁLEZ

Étude de l'implication du métabolisme cérébral du tryptophane dans les déficits de mémorisation induits par l'obésité

Thèse présentée et soutenue à Nantes, le 28 octobre 2022

Unité de recherche : UMR 1280 Physiologie des Adaptations Nutritionnelles (PhAN)

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This thesis is dedicated to my beloved family.

Acknowledgments

To the *National Council of Science and Technology (CONACYT)* of Mexico for having granted me a scholarship to carry out my doctoral studies in France.

To *Francisco Bolaños Jiménez, Ph.D.* for having accepted me to be part of his work team and for guiding me with such passion in the completion of this thesis. Thank you for all the advice and teachings you gave me, for sharing with me your experiences throughout your career, as well as your scientific and technical knowledge. Thank you for showing me the importance of teamwork and being multidisciplinary, for helping me to find solutions to technical and theoretical difficulties I encountered in the realization of this project, for giving me the pleasure of listening to your opinion on various topics, not only scientific, but mainly, for your enormous human quality. You have inspired me not only to be a better professional but also a better person. It has been a great honor for me to be your student and I will always remember you fondly.

To *Patricia Parnet, PhD* and *Hervé Blottière, PhD* for kindly accepting me as a member of the UMR-PhAN and for supporting me to attend congresses.

To all the members of my jury for the kindness of having accepted to be part of it and for dedicating their valuable time to read my thesis. Thank you to *Nathalie Castanon, PhD* and *José Carlos Jiménez Chillarón, PhD* for evaluating this work, and to *José Alonso Fernández Guasti, PhD*, *Elaine Cristina Gavioli, PhD*, and *Khadija Ouguerram, PhD* for their valuable comments and observations.

To *Rosa María Guéant-Rodríguez, PhD* and *Doo-Sup Choi, PhD* for their encouraging words to continue my research, and for the suggestions they gave me as members of my individual monitoring committee.

To all the *engineers and researchers of the UMR-PhAN* for their valuable comments and suggestions, as well as for having inspired me with each of their lines of research. Special thanks to *Bertrand Kaeffer, PhD* for having taught me how to perform cell culture and for having solved my doubts with great kindness.

À *Agnès David*, merci pour l'amabilité avec laquelle tu m'as toujours traité, et pour toujours trouver des solutions là où il y a des problèmes. *Blandine Castellano*, merci d'avoir eu la gentillesse de m'apprendre différentes techniques de manipulation des animaux et pour ton support lors des séances d'abattage. *Geneviève Fleury*, merci de m'avoir aidé dans toutes les démarches administratives avec beaucoup de bienveillance. *Isabelle Grit*, merci de m'avoir appris à faire de l'immunofluorescence, et de m'avoir aidé à faire de multiples déterminations chimiques ainsi que des RT-PCRs, mais surtout, merci de m'avoir aidé en tout point depuis mon arrivée à Nantes, je n'oublierai jamais ton soutien.

À *Amandine Lefebvre*, *Sandrine Suzanne*, et *Alexis Gandon* pour votre énorme appui dans la prise en charge de mes animaux, de m'avoir aidé avec enthousiasme chaque fois que j'en avais besoin pour réaliser mes expériences, d'avoir partagé avec moi de longues conversations et de m'avoir fait passer des moments très agréables à l'intérieur de l'animalerie. J'ai beaucoup apprécié votre compagnie.

To *Mikaël Croyal, PhD*, *Mathilde Gourdel* and *Audrey Aguesse* for allowing me to work at the Mass Spectrometry Core Facility, and for enthusiastically supporting me in conducting my experiments and analyzing my data.

To *Sandra Olvera Hernández* and *Luis Antonio Reyes Castro* for sharing with me their experiences and technical skills which were of great relevance for me to carry out my project.

To *Amran Daher Abdi* and *Juan Antonio García Santillán* for their friendship and companionship. You both were always a great support to me, and I really enjoyed working as a team with you.

To *Marina Habib*, *Gabriel Araujo Tavares*, *Chloé Guillaume*, *Paul Bobin* and *Morgane Frapin* for their companionship, support and shared laughter. Thanks to *Maëlle Debock* for having supported me in the development of my experiments during her research stay in the laboratory.

A mis padres *María de la Paz González Márquez* y *Carlos Mezo Peña* por su apoyo y amor incondicional. Esta tesis, así como cualquier logro que tenga a lo largo de mi vida siempre será el resultado del gran esfuerzo que ustedes hicieron por darme una buena educación y un hogar feliz. Mamá, gracias por la dedicación con la que me educaste y por impulsarme a ser una buena estudiante. Tú me enseñaste a tener disciplina y a esforzarme para cumplir mis metas, gracias a esas bases he podido continuar por este camino científico. Papá, gracias por ser mi guía y mi cómplice, y por impulsarme a hacer todo lo que me proponga sin tener miedo. Me doy cuenta de que siempre tuviste razón en todos los consejos que me diste, incluso cuando en mi juventud no los entendía, ahora esos consejos me sirven para afrontar todos los retos que la vida me impone. Ambos son un gran ejemplo para mí y me siento muy orgullosa de ustedes.

A mi hermano *Juan Carlos Mezo González* por ser un gran ejemplo de disciplina y pasión por su profesión. Gracias por el apoyo incondicional que me has brindado siempre, por ser la persona en la que siempre encuentro las respuestas que necesito, por ser mi mejor amigo, mi cómplice y mi pilar. Siento una profunda admiración por ti, por tu capacidad de sobresalir en todas las actividades que realizas, por cumplir todas las metas que te propones y por ser una excelente persona. Siempre estaré muy orgullosa de ti.

À *Johann Pintault* pour son amour et son soutien. Merci pour le temps que tu as consacré à m'aider à améliorer mes présentations et mon écriture en français, mais surtout, merci de me pousser à réaliser mes objectifs, de célébrer mes accomplissements comme s'ils étaient les tiens, de croire toujours en moi et de m'inspirer avec tes énormes qualités humaines pour devenir une meilleure personne. Pendant ces quatre années, tu as été un grand soutien émotionnel pour moi et je te serai toujours très reconnaissant pour ça.

À la famille *Pintault* pour leur amour. Vous avez été d'un grand soutien pour moi au cours des dernières années. Je suis très chanceuse de vous avoir dans ma vie.

A *Diana Rubio*, *Marisol Rojas* y *Paolo Salvatore Frascà* por su amor y amistad. Gracias por creer en mí y apoyarme emocionalmente siempre. Atesoro su presencia en mi vida.

A la *Dra. Cynthia Ordaz Pichardo* por haber sido un gran apoyo y una influencia importante en mi vida. Siempre guardaré con cariño tu recuerdo y tus enseñanzas.

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List of abbreviations

3-hydroxykynurenine	3-HK
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptophan	5-HTP
α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Anthranilic Acid	AA
Blood-Brain Barrier	BBB
Body Mass Index	BMI
Brain-Derived Neurotrophic Factor	BDNF
Central Nervous System	CNS
Developmental Origin of Health and Disease	DOHaD
Estradiol	E2
Estriol	E3
Estrone	E1
Estrogen Receptors α	ER α
Estrogen Receptors β	ER β
Follicle Stimulating Hormone	FSH
free-choice High Fat High Sugar	fc-HFHS
Free Fatty Acids	FFA
High Fat Diet	HFD
High Sugar Diet	HSD
Hydroxyanthranilic acid	3-HAA
Indoleamine 2,3-dioxygenase	IDO
Ionotropic glutamate receptors	iGluRs
Interferon γ	IFN γ
Interleukin 1 β	IL-1 β
Interleukin 6	IL-6
Kynurenic Acid	KA
Kynureninase	KYNU
Kynurenine	KYN

Kynurenine-3-monooxygenase	KMO
Kynurenine aminotransferase	KAT
Kynurenine Pathway	KP
Large Neutral Amino Acid	LNAA
Long-term Depression	LTD
Long-Term Memory	LTM
Long-term Potentiation	LTP
Metabotropic Glutamate Receptors	mGluRs
microRNA	miRNAs
N-methyl-D-aspartate	NMDA
Nicotinamide Adenine Dinucleotide	NAD ⁺
Novel Object Location	NOL
Novel Object Recognition	NOR
Paternal Origins of Health and Disease	POHaD
Postsynaptic Density	PSD
Progesterone	P4
Quinolinic Acid	QA
Serotonin	5-HT
Short-Term Memory	STM
Toll-like receptors	TLRs
Tryptophan	TRP
Tryptophan dioxygenase	TDO
Tryptophan hydroxylase	TPH
Tumor Necrosis Factor α	TNF α
Ultra-performance liquid chromatography-tandem mass spectrometry	LC-MS/MS
Xanthurenic Acid	XA
World Health Organization	WHO

Summary in French

L'obésité est une maladie chronique, complexe et hétérogène (Guerreiro et al., 2022) qui a été définie par l'Organisation Mondiale de la Santé (OMS) comme une accumulation excessive de graisse susceptible de nuire à la santé. En raison de l'augmentation accélérée des taux d'obésité dans le monde, l'OMS l'a déclarée en 1977 comme un problème majeur de santé publique (James, 2008). Cette pathologie est considérée comme le principal facteur de risque d'un groupe de maladies non transmissibles telles que les maladies cardiovasculaires, le cancer et le diabète sucré, représentant plus de 70 % des décès précoces dans le monde (Blüher, 2019). En plus de provoquer des problèmes métaboliques, l'obésité est également liée au développement de problèmes psychiatriques et cognitifs.

L'obésité affecte les fonctions cognitives à tous âges. Chez les enfants, l'obésité affecte la mémoire de travail (Wu et al., 2017) et altère l'attention, la rétention, l'intelligence et la flexibilité cognitive (Meo et al., 2019). Chez les jeunes adultes, l'obésité provoque une baisse des performances dans les tâches de mémoire faisant appel aux notions "quoi-où-quand", l'identification d'objets, la mémoire de localisation et la mémoire d'ordre temporel (Cheke et al., 2016). Chez les adultes d'âge moyen, l'obésité a été associée à des troubles cognitifs légers (Rochette et al., 2016), et aussi à la démence et à la maladie d'Alzheimer (Fitzpatrick et al., 2009). La présence de problèmes mentaux ou cognitifs à tout âge affecte la qualité de vie et peut représenter des facteurs invalidants. Il est donc nécessaire de mieux comprendre la physiopathologie de l'obésité et son effet sur le cerveau afin d'établir des mesures préventives ou d'éventuels traitements.

Un élément clé dans la compréhension du lien entre l'obésité et le développement de déficits cognitifs est l'étude du métabolisme du tryptophane (TRP). Le TRP est un acide aminé essentiel qui contribue au processus de synthèse des protéines, mais il agit également comme un précurseur de plusieurs composés bioactifs qui contrôlent la réponse immunitaire et les capacités cognitives (Palego et al., 2016; Friedman, 2018). Le TRP est métabolisé au niveau périphérique et centrale en suivant trois voies cataboliques qui sont la voie de la kynurénine, de la sérotonine et de l'indole. Plus de 95% du TRP qui n'est pas utilisé pour la synthèse des protéines, est catabolisé par la voie de la kynurénine, tandis que seulement 1-2% et 2-3% du TRP sont convertis, respectivement, en sérotonine

et en indoles (Palego et al., 2016; Hsu and Tain, 2020). Ici, nous ne décrivons pas la voie des indoles car elle est régulée par le microbiote intestinal et celui-ci n'a pas été l'objet de la présente étude.

La voie de la kynurénine (KP) est initiée par l'action de l'enzyme indoleamine 2,3-dioxygénase (IDO) ou par l'enzyme tryptophane dioxygénase (TDO). Ces deux enzymes convertissent le TRP en composés appelés "kynurénines". Le groupe des kynurénines comprend : la kynurénine (KYN), l'acide kynurénique (AK), l'acide quinolinique (AQ), l'acide xanthurénique (AX), l'acide anthranilique (AA) et le nicotinamide adénine dinucléotide (NAD⁺). Le TRP est également métabolisé par la voie de la sérotonine (5-HT) qui est débuté par l'action de l'enzyme tryptophane hydroxylase (TPH) (Palego et al., 2016; Hsu and Tain, 2020; Savitz, 2020). En matière de cognition, l'AK et l'AQ sont deux des kynurénines les plus importantes car elles sont impliquées dans la régulation de la plasticité synaptique médiée par le glutamate. Alors que l'AK agit comme un antagoniste de tous les récepteurs ionotropes du glutamate, y compris les récepteurs NMDA (acide N-méthyl-D-aspartique) et AMPA (acide α -amino-3-hydroxy-5-méthyl-4-isoxazolepropionique), l'AQ agit uniquement comme un agoniste des récepteurs NMDA (Richard et al., 2009). Par conséquent, les deux métabolites sont impliqués dans le contrôle des processus de potentialisation à long terme et la dépression à long terme, et donc dans la formation de la mémoire.

Bien que l'AK soit considéré comme neuroprotecteur, une augmentation excessive d'AK peut aussi affecter négativement le fonctionnement du cerveau. En fait, les dérégulations des concentrations de l'AK et de l'AQ ont été associées à plusieurs maladies (Modoux et al., 2021). Des niveaux élevés d'AK ont ainsi été associés à la schizophrénie, tandis qu'une augmentation des niveaux d'AQ a été associée aux maladies d'Alzheimer (Busse et al., 2018), de Huntington (Schwarcz et al., 2010) et de Parkinson (Chang et al., 2018), à la sclérose en plaques (Lim et al., 2017), à l'autisme (Lim et al., 2016) et à la dépression (Savitz et al., 2015). Les effets négatifs induits par l'AQ sont dus au fait qu'il augmente les concentrations cytosoliques de Ca²⁺ dans les neurones en surstimulant les récepteurs NMDA, et produit un dysfonctionnement mitochondrial (Pérez-De La Cruz et al., 2012), conduisant à une production accrue des espèces réactives

de l'oxygène (Santamaría et al., 2001), à de l'excitotoxicité, à une altération du métabolisme énergétique et à la mort neuronale (Lima et al., 2017; Bansal et al., 2019). L'hippocampe, qui est une région clé pour les processus de mémoire et d'apprentissage, est très sensible à l'effet de l'AQ (Vezzani et al., 1991). D'autre part, de par sa fonction en tant qu'antagoniste des récepteurs NMDA, AMPA du glutamate et des récepteurs nicotiques $\alpha 7$ de l'acétylcholine ($\alpha 7$ nAChR), l'AK peut modifier les concentrations de différents neurotransmetteurs (y compris le glutamate, la dopamine, l'acétylcholine et l'acide γ -aminobutyrique) qui sont essentiels au neurodéveloppement, à la plasticité, à la cognition, au comportement et aux processus de mémorisation (Plitman et al., 2017; Ramos-Chávez et al., 2018).

La production d'AK et d'AQ est médiée par les cellules gliales. Les astrocytes expriment la Kynurénine Aminotransférase (KAT), l'enzyme qui catalyse la conversion du KYN en AK, et la microglie exprime la kynurenine-3-monooxygénase (KMO), l'enzyme qui produit l'AQ (Guillemin et al., 2005). Dans un état de neuro-inflammation, les astrocytes et la microglie passent à une forme activée qui favorise la libération de cytokines pro-inflammatoires telles que l'interféron gamma ($IFN\gamma$), le facteur de nécrose tumorale α (ou $TNF\alpha$, de l'anglais tumor necrosis factor), l'interleukine- 1β ($IL-1\beta$) ou l'interleukine 6 ($IL-6$). En activant l'expression de l'IDO, l'enzyme qui catalyse la transformation du TRP en kynurénine (Robinson et al., 2005; Zunszain et al., 2012; Urata et al., 2014), ces interleukines peuvent conduire à une surproduction de kynurénines. En fait, dans certaines maladies comme la maladie d'Alzheimer (Willette et al., 2021), la maladie de Huntington (Forrest et al., 2010) et la dépression (Steiner et al., 2011; Achtyes et al., 2020), il a été déjà démontré l'existence d'un lien entre l'état inflammatoire des patients et l'activation de la voie de la kynurénine. Ceci est intéressant car les patients souffrant d'obésité développent aussi un état inflammatoire chronique de bas grade (Choe et al., 2016) accompagné des niveaux élevés de KYN, AK et AQ dans le sérum (Mangge et al., 2014; Favennec et al., 2015), et de la surexpression des enzymes IDO1, KYNU, KMO et KAT III (Favennec et al., 2015). Cependant, aucune étude n'a cherché jusqu'à présent à déterminer si l'obésité pouvait entraîner un état inflammatoire dans le cerveau susceptible de déclencher des modifications du métabolisme du TRP et donc des problèmes cognitifs.

L'objectif principal de ce travail a été de déterminer si l'obésité induite par une alimentation hypercalorique provoque un état inflammatoire dans le cerveau qui favorisera la surproduction de kynurénines entraînant ainsi des altérations des processus de la mémoire et de l'apprentissage. Pour atteindre cet objectif, nous avons utilisé un modèle de rat Wistar nourri avec un régime hypercalorique consistant en une alimentation à volonté riche en graisses et en sucres. Pour mesurer la capacité d'apprentissage des animaux, nous avons utilisé deux types de tests. A savoir, le test de Reconnaissance de Nouveaux Objets (RNO), et un paradigme d'apprentissage plus complexe qui combine le test de RNO avec le test de Localisation de Nouveaux Objets (LNO) (RNO-LNO). Pour quantifier la concentration des métabolites du TRP, nous avons fait appel à la chromatographie liquide ultra-performante-spectrométrie de masse en tandem (ou LC-MS/MS, de l'anglais ultra-performance liquid chromatography-tandem mass spectrometry). Enfin, pour évaluer l'expression des gènes, nous avons utilisé la technique de Transcription inverse de l'amplification en chaîne par polymérase (ou RT-PCR, de l'anglais Reverse Transcription Polymerase Chain Reaction).

Les résultats de nos premières investigations ont montré, que l'obésité provoque effectivement des déficits de mémorisation chez les rats mâles, mais pas chez les rats femelles et que ces déficits cognitifs sont associés à des altérations du métabolisme des TRP dans le cerveau. Plus précisément, les rats mâles obèses ont présenté une capacité réduite à acquérir de nouvelles informations, mais pas à se rappeler de l'information préalablement acquise dans le test RNO-LNO. Ils ont présenté aussi des niveaux accrus de KYN dans le tronc cérébral, d'AX, AQ, Nicotinamide (Nam) et NAD dans l'hippocampe et de NAD dans le cortex frontal. De plus, nous avons constaté une expression accrue de KMO, dans les trois régions du cerveau. En revanche, KAT n'a été surexprimé que dans l'hippocampe et le cortex frontal. Bien qu'il y ait eu une expression accrue de TPH2 dans le tronc cérébral, cela ne s'est pas traduit par une augmentation des niveaux de 5-HT. Nous avons aussi constaté une augmentation de l'expression génétique de cytokines pro-inflammatoires telles que l'IL-6, IL-1 β , TNF α , et des récepteurs de type Toll 2 et 4 (TLR2 et TLR4, de l'anglais Toll-like receptors) dans le tronc cérébral, et de TLR2 dans l'hippocampe et le cortex frontal. Comme l'expression des enzymes IDO et

TDO est stimulée par l'IL-6, le TNF α et l'IL-1 β (O'Connor et al., 2009; Zunszain et al., 2012; Urata et al., 2014), l'augmentation de la synthèse de KYN pourrait s'expliquer par l'expression exacerbée de ces interleukines. Par ailleurs, TLR4 et TLR2 sont impliqués dans l'activation des macrophages et de la microglie (Lee et al., 2015; Jin et al., 2016) dont l'activation par les cytokines inflammatoires favorise l'expression de l'enzyme KMO qui fait partie de la voie métabolique qui conduit à la production de l'AQ à partir de la KYN (Guillemin et al., 2005). Aussi, nous pouvons supposer que l'augmentation de la production de QA dans les cerveaux des animaux obèses, est dû à une suractivation de la microglie par des cytokines pro-inflammatoires. En conclusion, l'ensemble des résultats obtenus pendant la première partie de la thèse, montrent que, chez des rats mâles, l'obésité favorise le métabolisme du TRP par la voie de la kynurénine, et particulièrement via la branche "neurotoxique", et que cette altération neurochimique est associée à une déficience de mémorisation à court terme

Nous n'avons pas détecté de problèmes de mémoire et de capacité d'apprentissage chez les ratte femelles obèses au cours de notre première étude. Ce résultat est en accord avec différentes études indiquant que l'obésité n'affecte pas les capacités cognitives des femelles en raison de l'effet protecteur des œstrogènes. Ces hormones ont un effet bénéfique sur la mémoire et l'apprentissage (Frye et al., 2007; Patel et al., 2022) car elles modulent la neurogènes en augmentant la densité des synapses et des épines dendritiques (Sheppard et al., 2019). Cependant, étant donné que pendant le cycle œstral il y a des fluctuations d'œstrogènes qui commencent à augmenter dans le proœstrus, atteignent leur maximum en œstrus et diminuent pendant le métœstrus et le dioœstrus (Le et al., 2020; Schmalenberger et al., 2021; Abo et al., 2022), nous nous sommes demandé si l'effet protecteur des œstrogènes se maintient tout au long du cycle. D'autre part, sachant que les œstrogènes favorisent l'expression de la TPH, et que la déplétion en œstrogènes augmente l'expression de l'IDO (Xu et al., 2015; Hiroi et al., 2016), dans la deuxième partie de la thèse, nous avons analysé la capacité de mémorisation et le métabolisme cérébral du TRP au cours cycle œstral chez des femelles saines et obèses. Les résultats de cette étude ont montré que, les ratte non-obèses en proœstrus/œstrus ont passé plus de temps à explorer l'objet nouveau que l'objet familier dans le test de RNO en comparaison à leurs congénères contrôles en métœstrus/dioœstrus. Cependant, le groupe des ratte

obèses en proœstrus/œstrus a présenté, tous comme les rats mâles obèses, une diminution de la capacité d'apprentissage. Ce résultat suggère que l'augmentation naturelle de la production d'œstrogènes pendant les phases de proœstrus/œstrus ne protège pas les rats femelles contre les effets délétères de l'obésité sur la cognition.

Nous avons par ailleurs constaté que l'obésité chez les femelles entraîne une diminution des niveaux de TRP, KYN, AX, NAD dans l'hippocampe et le cortex frontal ainsi qu'une réduction de la concentration de 5-HT et 5-HIAA dans cette dernière région cérébrale. Ces résultats suggèrent que les femelles obèses ont en premier lieu une disponibilité réduite de TRP dans le cerveau qui entraîne donc une diminution de la production de métabolites dérivés des voies de la sérotonine et de la kynurénine. En termes d'expression des gènes codant pour les enzymes du métabolisme du tryptophane, l'obésité a induit la surexpression de l'IDO dans l'hippocampe et le cortex frontal uniquement pendant les phases de métœstrus/diœstrus. Ceci est en accord avec le fait que l'obésité produit une inflammation et que les cytokines pro-inflammatoires régulent à la hausse l'expression de l'IDO (O'Connor et al., 2009; Zunszain et al., 2012; Urata et al., 2014). Cependant, l'augmentation d'expression de l'IDO, ne concorde pas avec la réduction de la concentration de KYN observée chez les rattes obèses. A la lumière de ces résultats et des données de l'étude précédente, nous pouvons donc conclure que l'obésité diminue les capacités de mémorisation aussi bien chez les rats mâles que chez les rats femelles et que dans les deux cas, les effets délétères de l'obésité sur la cognition sont associés à des modifications du métabolisme du tryptophane par la voie de la kynurénine. Il est également évident que, chez les rats femelles, les effets de l'obésité sont étroitement liés au cycle œstral.

Dans la dernière partie du travail, l'effet de l'obésité paternelle et maternelle sur le métabolisme du TRP dans le cerveau de la progéniture a été analysé. Cette étude a été entreprise sur la base des données de la littérature démontrant que l'état nutritionnel de la mère pendant la gestation et/ou la lactation a un impact sur le métabolisme cérébral du TRP chez la progéniture. En effet, les rats adultes nés de mères malnutries présentent des niveaux altérés de sérotonine et des métabolites dérivés de la kynurénine dans le cerveau (Honório de Melo Martimiano et al., 2017). D'autre part, l'obésité maternelle modifie l'expression des enzymes impliquées dans la voie de la kynurénine (Winther et al., 2018).

Cependant, on ne sait pas si cela se traduit par des niveaux altérés de kynurénines qui pourraient entraîner des problèmes cognitifs chez la progéniture. En outre, on ignore si l'obésité paternelle pourrait influencer le métabolisme des kynurénines. Les résultats que nous avons obtenus, montrent que l'obésité maternelle ou paternelle modifie le métabolisme du TRP dans le cerveau de la progéniture à l'âge adulte et que ces altérations sont liées à des troubles de la mémoire et de l'apprentissage. Notamment, les descendants de mères obèses ont montré des difficultés pour acquérir et consolider de nouvelles informations dans le test du RNO-LNO et ce déficit cognitif s'est accompagné d'une augmentation des niveaux d'AK dans l'hippocampe et le cortex frontal et des niveaux élevés d'AQ dans l'hippocampe. De leur côté, les descendants de pères obèses ont présenté une déficience de mémorisation à long terme qui a été associée à une augmentation des niveaux d'AK dans le cortex frontal. Dans l'ensemble, ces résultats indiquent que le métabolisme du tryptophane par la voie de la kynurénine est influencé par la programmation métabolique induite par l'obésité maternelle ou paternelle, et que des modifications de la concentration cérébrale de métabolites dérivés de la KP sont associées à une altération des différentes étapes de la construction de la mémoire.

Dans une autre série d'expériences, nous avons constaté que, en plus d'avoir des problèmes de mémorisation et d'apprentissage, la progéniture de mères et de pères obèses présente une expression altérée des sous-unités des récepteurs NMDA (les sous-unités GluN) et d'autres marqueurs de plasticité synaptique (PSD95, zif-268 et Arc), dans le cerveau. En particulier, l'obésité maternelle a entraîné une diminution de l'expression de GluN1, GluN2B, PSD95 et zif-268 dans l'hippocampe et de GluN2A, PSD95 et Arc dans le cortex frontal. L'obésité paternelle, quant à elle, a provoqué une réduction de l'expression hippocampique de GluN1 et GluN2B ainsi qu'une augmentation du niveau des mRNAs codant pour le GluN2B associée à une réduction de l'expression de PSD95 dans le cortex frontal. Bien que d'autres études aient déjà rapporté que l'obésité maternelle provoque des déficiences cognitives associées à des altérations de la signalisation glutaminergique, cette étude est la première à montrer que l'obésité paternelle entraîne des effets similaires.

En conclusion, l'ensemble des résultats rapportés dans ce travail de thèse, montre que l'obésité induite par la consommation d'une alimentation hypercalorique, modifie le métabolisme cérébral du TRP et que cette modification est associée à des déficiences de mémorisation et d'apprentissage. Ces effets sont dépendants du sexe et, chez les rats femelles, sont étroitement liés au cycle œstral. De manière générale, chez les rats mâles, l'obésité favorise le développement d'un état neuro-inflammatoire qui a comme résultat la surproduction de plusieurs kynurénines dont celle de l'AQ qui est connu pour ces actions neurotoxiques. Pour ce qui est de rattes femelles obèses, les troubles de mémorisation qu'elles présentent, ne s'accompagnent pas d'une stimulation de la voie de la kynurénine, mais plutôt une diminution de la disponibilité cérébrale de TRP dans le cerveau, ce qui conduit à une diminution de la production de sérotonine et des métabolites de la kynurénine. Cet effet négatif de l'obésité est modulé dynamiquement par les fluctuations naturelles des œstrogènes au cours du cycle œstral. Enfin, nous avons mis en évidence pour la première fois, que l'obésité maternelle et paternelle, conduisent à des altérations du métabolisme de TRP dans le cerveau de la progéniture ainsi que à une dérégulation de l'expression de plusieurs marqueurs de plasticité synaptique, dont des sous-unités des récepteurs NMDA du glutamate. Ces altérations neurochimiques sont concomitantes aux déficits de mémorisation observés chez les rats de mères et de pères obèses suggérant une relation de cause à effet entre ces deux phénomènes.

Introduction

In recent decades we have observed an acceleration in the onset of chronic degenerative diseases at a younger age and this is a consequence of the change in our habits and behaviors. Nowadays, most of the people have a detrimental lifestyle that includes the consumption of unhealthy foods, having a sedentary life, being exposed to stress, smoking, drinking alcohol, among other negative factors that promote the development of disease and disability (Farhud, 2015). In particular, the consumption of unhealthy diets and a sedentary lifestyle (along with other factors) have promoted a significant global increase in worldwide obesity rates in the last 50 years (Lin and Li, 2021).

The World Health Organization (WHO) has defined obesity as the abnormal or excessive fat accumulation that may impair health. The World Obesity Federation and the American and Canadian Medical Associations have even declared it a chronic progressive disease. This pathology is considered the major risk factor for a group of noncommunicable diseases such as cardiovascular diseases, cancer and diabetes mellitus, which account for approximately more than 70 % of early deaths worldwide (Blüher, 2019). Furthermore, obesity is associated with cognitive disabilities and several types of dementia (including Alzheimer's disease) which is the main cause of disability in the old age (Lisko et al., 2021).

Considering that in the last century the life expectancy has increased (Crimmins, 2015), as well as the obesity rates (Jackson et al., 2020), then we can imagine that in the near future the health system in most of the countries will be facing a large part of the population with severe metabolic and mental problems. People will not only have a poor quality of life but also will be dependent on other people to cover their basic needs. Additionally, people will stop working at an early age, which causes a weakening of the labor force in each country. For these reasons, it is essential to better understand the pathophysiology of obesity and its effects on the brain (Nguyen et al., 2014) with the aim of establishing preventive measures or possible treatments.

An important factor that could be key to understanding the link between obesity and the development of cognitive deficits is tryptophan (TRP) metabolism. TRP is an essential amino acid that contributes to the process of protein synthesis, but it also acts as

a precursor of several bioactive compounds that control the immunomodulatory response and the cognitive capacity (Palego et al., 2016; Friedman, 2018). TRP is metabolized peripherally and centrally, and it can follow three catabolic pathways, which are the kynurenine, serotonin, and indole. The principal is the Kynurenine Pathway (KP) which is initiated by the action of the indoleamine 2,3-dioxygenase (IDO) enzyme or by the tryptophan dioxygenase (TDO) enzyme. Both enzymes convert TRP into compounds called “kynurenines”. The group of kynurenines include: kynurenine (KYN), kynurenic acid (KA), quinolinic acid (QA), xanthurenic acid (XA), anthranilic acid (AA), and nicotinamide adenine dinucleotide (NAD⁺) (Hsu and Tain, 2020; Savitz, 2020). TRP can also be metabolized through the serotonin pathway that is mediated by the action of the tryptophan hydroxylase (TPH) enzyme (Palego et al., 2016; Hsu and Tain, 2020).

In terms of cognition, KA and QA are two of the most important kynurenines because they are involved in the regulation of glutamate-mediated synaptic plasticity. While KA act as an antagonist of all glutamate ionotropic receptors, QA act as an agonist of N-methyl-D-aspartate (NMDA) receptors (Richard et al., 2009). Maintaining an equilibrium in the levels of both kynurenines is essential for proper brain functioning. Actually, deregulations in the concentrations of KA and QA have been related with several brain diseases (Schwarcz et al., 2012), such as Alzheimer, Parkinson, (Tanaka et al., 2020) and Huntington disease, multiple sclerosis (Lovelace et al., 2016), depression (Raison et al., 2010), schizophrenia (Pedraz-Petrozzi et al., 2020) and cognitive impairment (Heisler and O’Connor, 2015).

Scientific evidence has shown that TRP metabolism can be modulated by different factors such as obesity, female sex hormones and metabolic programming. In the first place, obesity causes the development of a chronic low-grade systemic inflammatory state characterized by the release of proinflammatory cytokines (Hammarstedt et al., 2018; Hafidi et al., 2019a) that enhance the activity of IDO. This is why obese patients have increased KYN levels and KYN/TRP ratio in the serum (Mangge et al., 2014; Groer et al., 2018). However, it has never been described whether obesity can also alter TRP metabolism in the brain. If that were to happen, then there would probably be an increase in the production of kynurenines, mainly KA and QA. This change could lead to

alterations in synaptic plasticity and thus trigger cognitive impairments, but this has not yet been determined.

On the other hand, evidence has shown that estrogens, the ovarian sex hormones, have a beneficial effect on memory, because when they activate their receptors in the brain, they promote the expression of genes related to neuronal growth, neuroprotection, and memory function (Kumar and Foster, 2020). Moreover, there is a close relationship between estrogens and the TRP metabolism. Indeed, estrogens promote the expression of TPH, but under estrogen depletion IDO is upregulated (Xu et al., 2015; Hiroi et al., 2016). Additionally, women with low estrogen and low TRP levels develop cognitive failure (Epperson et al., 2012; Helmbold et al., 2013). This is quite interesting because during the female reproductive cycle there are natural fluctuations of estrogens, and so far it has never been described how this could impact the production of serotonin (5-HT) or kynurenines and then the memory efficiency. Finally, we know that obesity promotes the adipose tissue expansion, this organ has several functions and one of them is to produce estrogens. Paradoxically, obesity is related with cognitive impairment, and it would be easy to imagine that the increase in estrogens produced by the adipose tissue would have a protective effect. However, it is necessary to analyze with caution whether obesity indeed have a protective effect and whether this effect is maintained during all stages of the reproductive cycle.

Finally, the last factor that could modulate TRP metabolism is metabolic programming. This concept refers to the fact that environmental factors to which individuals were exposed during the early age (including the pre-gestational, gestational and the early postnatal periods), could represent risk factors for the development of future diseases. These factors can be transmitted to the offspring from mothers or fathers. These statements have given rise to the hypotheses of the ‘Developmental Origin of Health and Diseases’ (Barker and Osmond, 1986) and the ‘Paternal Origins of Health and Disease’ (Soubry, 2018) hypotheses. For example, there is evidence showing that maternal obesity predisposes the offspring to metabolic diseases at adulthood, as well as to neurodevelopmental and psychiatric diseases. In the case of obese fathers, evidence is less abundant; however, it shows that they could exert the same negative effect in their offspring than obese mothers. An important point is that the TRP metabolism can be

metabolically programmed by the nutritional status of mothers. In fact, the offspring of malnourished mothers present altered levels of kynurenines in the brain (Honório de Melo Martimiano et al., 2017). On the other hand, maternal obesity alters the expression of enzymes involved in the kynurenine pathway (Winther et al., 2018), however, it is not known whether this translates into altered levels of kynurenines that could lead to cognitive problems in the offspring. Moreover, there is any report showing whether paternal obesity could influence the TRP metabolism in the same way.

The aim of this thesis is to answer the enquiries stated before, but first, I will start by presenting the scientific evidence that sustains each question. In the first chapter, I will give a brief review about the mechanisms that produce the memory construction. In the second chapter, I will describe the general features of TRP, including its metabolic pathways and its impact on memory. Then, in the third chapter I will show the evidence about the negative impact of obesity on mental health and how it can alter TRP metabolism. Next, in the fourth chapter, I will present how estrogens influence the cognitive ability and their relationship with TRP metabolism. Thereafter, in the fifth chapter, I will display the evidence that shows that metabolic programming is also a modulating factor of TRP metabolism. Finally, the results in this thesis are presented as published articles or as manuscripts that will be sent for review for subsequent publication.

Chapter 1.

Memory construction

Memory is defined in psychology as the faculty of encoding, storing, and retrieving information (Zlotnik and Vansintjan, 2019). This capacity allows us to connect experiences, learn and make sense to our lives. Memory is one of the elements of cognitive function which is conceived as the ability to think, learn and remember, thus forming the basis for an individual's capacity for perception, reasoning, creativity, problem solving and possibly intuition (Antony et al., 2017).

Types of memory

There are three major classifications of memory: the Sensory memory, Short-Term Memory (STM), and Long-Term Memory (LTM). The sensory memory allows us to briefly retain the large amounts of information that we encounter daily and that we perceived with our senses of sight (iconic memory), audition (echoic memory) and touch (haptic memory). STM is the ability to keep a small amount of information for a short period of time (Camina and Güell, 2017). This kind of memory is processed by the working memory, defined as the ability to hold information in consciousness for its adaptive use. In other words, it maintains information and then it manipulates it (Harvey, 2019). Finally, LTM refers to the mechanism by which acquired memories gain stability or are strengthened over time and become resistant to interference. This memory is maintained for long periods of time, even for life. (Brem et al., 2013).

There are two types of LTM, implicit or non-declarative memory and explicit or declarative memory. Implicit memory encompasses all unconscious memories, such as certain abilities or skills. It includes the procedural memory, associative memory, non-associative memory, and priming. On another hand, explicit memory refers to information that can be consciously evoked. It comprises semantic and episodic memory. Semantic memory stores verbal information, "concepts and meanings". This ability allows us to disseminate conceptual knowledge to others, and to manipulate, associate, and combine concepts. Semantic memory appears to remain intact over the lifespan and continues to accumulate new information even when people get old (Camina and Güell, 2017; Harvey, 2019).

Episodic memory refers to the conscious recollection of personal experiences that contains information about what, where and when an event happened (Pause et al., 2013). Episodic memory has three components: encoding, storage, and retrieval, all of which are required for successful memory performance. The encoding stage of memory consists of obtaining information from working memory and process it for longer term storage. The storage stage refers to the process of retention of information after encoding, if the information is successfully encoded it can be recalled at long post encoding time periods. Finally, retrieval depends on the recollection of encoded contextual features of a past event, such as time, place, people, sights, thoughts, and emotions (Brem et al., 2013; Camina and Güell, 2017; Harvey, 2019).

Brain structures involved in memory consolidation

Memory has been conceptualized as the set of interconnected cortical neuron networks that contain our experiences in their connectional structures. It is believed that memory networks overlap and interact in a manner that one cellular assembly can be engaged in many memory networks. These networks are integrated by specific anatomical structures found in the neocortex which includes the frontal, temporal, parietal, and occipital lobes, as well as the subcortical structures, including the amygdala, hippocampus, cerebellum, entorhinal cortex, and the basal ganglia (Figures 1 and 2) (García-Lázaro et al., 2012). In this work we will only focus on the hippocampus and the frontal cortex since both maintain a very active bidirectional communication that is key for the construction of memories. (Preston and Eichenbaum, 2013).

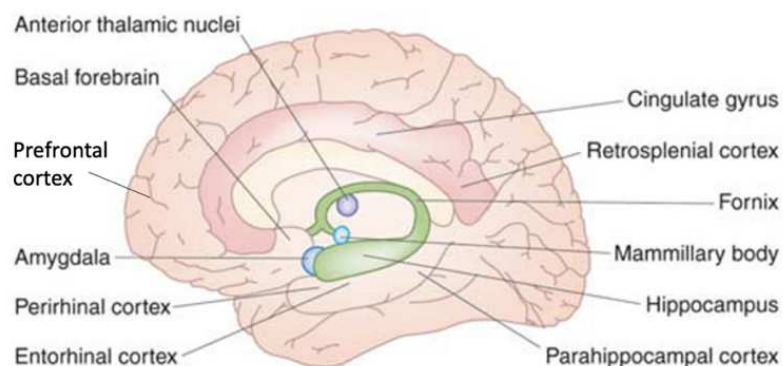


Figure 1. Brain anatomy. Image adapted from Nadel and Hardt (Nadel and Hardt, 2011).

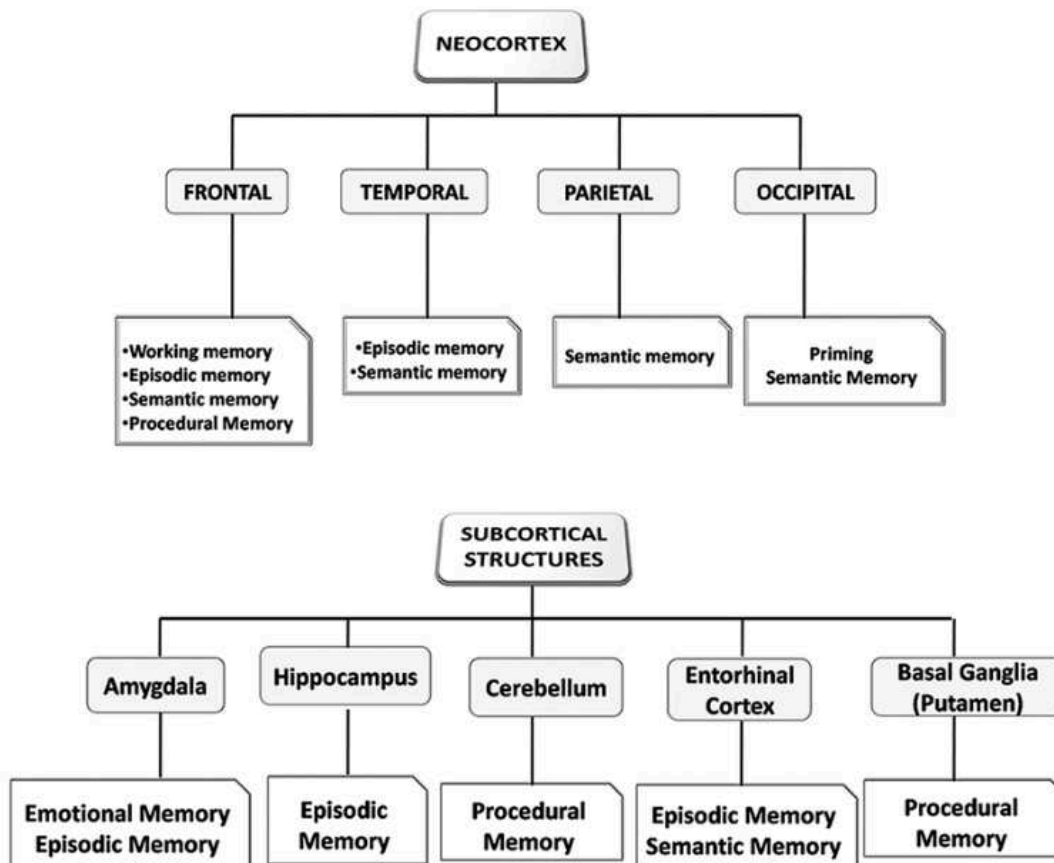


Figure 2. Brain regions involved in memory processing. Memory networks are integrated in several anatomical brain structures, either in the neocortex or in the subcortical structure, and they can overlap and interact between them. Image obtained from García-Lázaro *et al.*, (García-Lázaro *et al.*, 2012).

The hippocampus is an extension of the temporal part of cerebral cortex. It can be distinguished externally as a layer of densely packed neurons, which curls into an S-shaped structure on the edge of the temporal lobe. According to the traditional anatomical model, the hippocampus has different regions, including the *cornu ammonis* (CA) subfields, the dentate gyrus, and the subiculum. The CA field is further subdivided in four subfields; CA1, CA2, CA3, and CA4, however, CA2 and CA4 are not universally accepted as distinct subfields (Genon *et al.*, 2021) (Figure 3). The hippocampus is surrounded and connected with the entorhinal, parahippocampal, and perirhinal cortices, and also with several subcortical and cortical structures (García-Lázaro *et al.*, 2012) (Figure 3). The hippocampus plays a crucial role in the consolidation of information from

short-term memory to long-term memory, the retrieval of memories, and the control of spatial navigation. In addition, since it is part of the limbic system which is considered “the primitive brain” (for its functions related with hunger, motivation, sex drive, mood, pain, pleasure, appetite, and memory), it is also involved in emotional behavior because it shares connections with the amygdala. Further, it participates in the regulation of hypothalamic functions (Anand and Dhikav, 2012; García-Lázaro et al., 2012; Camina and Güell, 2017; Pant et al., 2022).

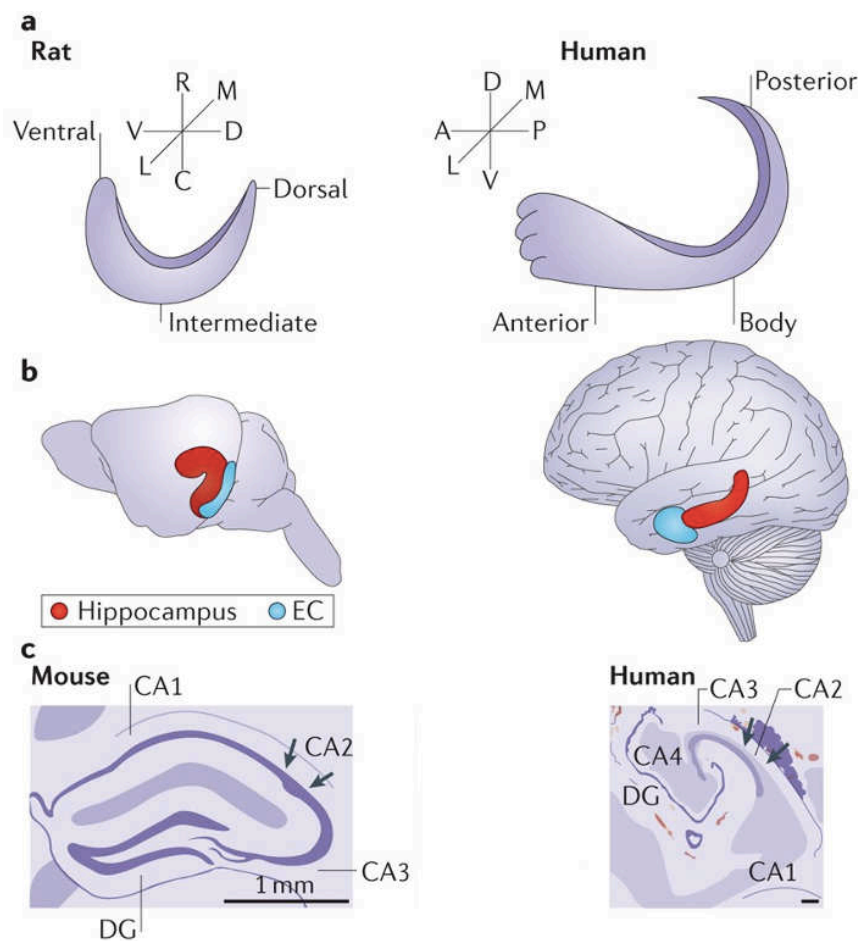


Figure 3. Schematic illustrations of the rodent and human hippocampus. A) Hippocampal long-axis in rat and human. The longitudinal axis is described as ventro-dorsal in rodents, and antero-posterior in humans. It is necessary to perform a 90° rotation of the rat hippocampus to have the same orientation as in humans. B) Anatomical position of the long-axis of the hippocampus (red) and the entorhinal cortex (blue) in the brain. C) Nissl cross-section of the hippocampus in mouse and humans. Image adapted from Strange *et al.*, (Strange et al., 2014).

The prefrontal cortex (PFC), refers to the cortical region of the anterior pole of the mammalian brain (Figure 1). The infrastructure of the PFC is constituted by an interconnected set of neocortical areas which have an overlapping pattern of connectivity with all sensory neocortical and motor systems, and a wide range of subcortical structures. These interconnections provide the perfect infrastructure for performing complex behaviour that includes emotional and social behavior, and cognitive functions such as attention, salience detection, working memory, and inhibitory control. Thanks to these abilities we are capable of store and update new evidence, and (re-)evaluate our choices. Thus, this region is critical for our adaptation and survival to a permanently changing environment.(Miller, 2000; Chini and Hanganu-Opatz, 2021).

Episodic memory construction

Explaining how the brain encodes, organizes, and retrieves memories is not totally understood, however, the functional anatomy of the brain permits to get an insight. In the first place, different regions of the cerebral cortex permit to acquire information by sensory pathways (vision, touch, hearing, and so on) that provide us the identity of an object, or “what” to remember, and then that information converge onto multimodal cortical ‘association’. Moreover, the parahippocampal cortex and the medial entorhinal areas situated in the medial temporal lobe (MTL) provide us information of “where” happened the event (Figure 1). Within the MTL, information of “what” and “when” converge in the hippocampus. Outputs of the hippocampus return to the perirhinal–lateral entorhinal cortex, and parahippocampal–medial entorhinal cortex. These feedback pathways allow the hippocampus to support the retrieval of information.

Furthermore, the hippocampus (the ventral part in rats and the anterior in humans), sends outputs to the medial prefrontal cortex (mPFC), which accumulates information about the context of interrelated memories. Outputs of the mPFC are sent back to the perirhinal and lateral entorhinal cortex, by which the mPFC may select the retrieval of event information in the ‘what’ stream. In general terms, the hippocampus is the responsible for forming and reproducing memories while the prefrontal cortex is responsible for making representations of the meaningful contexts in which memories occur (Preston and Eichenbaum, 2013).

Synapses

To better understand how memories are created, it is necessary to first analyze the cellular and molecular events that occur at the level of neurons, which are the fundamental units of the brain. Neurons communicate to each other through synapses which are considered as the fundamental information processing units. Synapses receive, process, and transmit information, through the release of excitatory and inhibitory neurotransmitters from the presynaptic neuron that act in the postsynaptic neuron (Serrano et al., 2022). Synaptic transmission occurs specifically in the dendritic spines which are small membranous protrusions from neuron dendrites. In response to stimuli, dendrites change their volume and shape which permits the strength and/or changing of existing synapses as well as the formation of new synapses (Middei et al., 2014) (Figure 4).

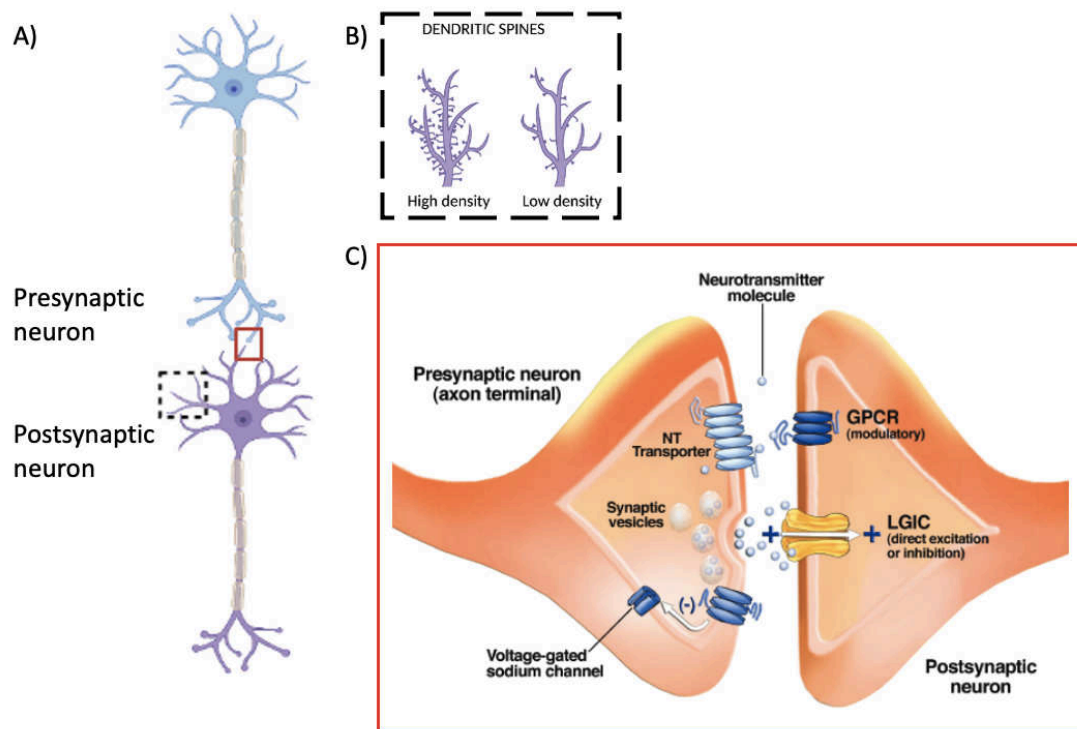


Figure 4. Schematic illustration of a synapse. A) Synapse is formed by three elements, the presynaptic membrane in the terminal button of an axon, the dendrites in the postsynaptic membrane, and the space between these structures called the synaptic cleft. B) Schematic illustration of dendrites with different density of dendritic spines. C) The presynaptic neuron has synaptic vesicles that contain neurotransmitters (NT), these

molecules cross the synaptic cleft and bind to receptors which can be ligand-gated ion channels (LGICs) or G-protein–coupled receptors (GPCRs) on the postsynaptic neuron. Image adapted from Lovinger and Serrano *et al.*, (Lovinger, 2008; Serrano et al., 2022).

In the early life of an individual, including the embryonic, neonatal, and adolescent stages, new synapses are continually formed. This process is known as synaptogenesis, and permits synapses either strengthened or to be eliminated through synaptic pruning when they are no longer useful. Synaptogenesis can continue throughout life, but the main assemblage of neural connections is completed by the end of adolescence (Serrano et al., 2022).

Synaptic plasticity

At the level of neuronal networks, there is a dynamic change in the synaptic connections known as synaptic plasticity, which refers to the capacity of the neural activity generated by an experience to modify neural circuit function and, thereby, modify subsequent thoughts, feelings, and behavior (Citri and Malenka, 2008). Synaptic plasticity allows us to learn and hence, it permits to create memory traces (Brem et al., 2013). These functional and morphological neuronal changes were first proposed by Donald Hebb in 1949 (Cheyne and Montgomery, 2020). According to him, long-term memory formation and learning are processes that require a new organization of synaptic connectivity through the strengthen of crucial synapses and weakening of un-needed synapses (Middei et al., 2014).

In the 1970 decade, synaptic plasticity was discovered in the hippocampus. There, researchers established that repeated, near-synchronous activation of both pre- and postsynaptic neurons produce an increase in the strength of the synaptic input that occurred only in the stimulated connections. This phenomenon became known as long-term potentiation (LTP), and its counterpart was called long-term depression (LTD) (Magee and Grienberger, 2020). Although most of the studies have analyzed the hippocampus as the place where long-term synaptic plasticity occurs, little is known about how memories are stored long-term in the cerebral cortex (Südhof and Malenka, 2008).

Synaptic plasticity mediated by glutamate

Synaptic plasticity occurs under the control of excitatory and inhibitory neurotransmitters (Kim et al., 2020). Glutamate is an excitatory neurotransmitter and the most abundant amino acid in the mammalian brain. It regulates most of the excitatory signals (Kim et al., 2020) and it is involved in most aspects of neurophysiology (Schwarcz, 2016), such as cognitive, motor, sensory and autonomic activities. Keeping extracellular glutamate levels in a physiological range is crucial to ensure proper neuronal transmission and viability (Iovino et al., 2020), in the contrary, hypo- and hyperfunction of glutamate could trigger psychiatric diseases (Schwarcz, 2016).

Glutamate activates glutamate receptors found in neurons and glial cells. There are two categories of glutamate receptors, the ionotropic and metabotropic receptors (iGluRs and mGluRs). iGluRs are ligand gated ion channels permissive to cation flux across the cell membrane, they mediate fast excitatory synaptic signaling and have a key role in synaptic plasticity. However, their excessive activation could provoke excitotoxicity (Kim et al., 2020). On another hand, mGluRs are G protein-coupled receptors that act as modulators of neuronal activity via their effects on intracellular cyclic adenosine monophosphate (cAMP) levels and other second messengers (Reiner and Levitz, 2018; Kim et al., 2020). Here, we will focus only on the activity of ionotropic receptors.

The iGluRs assemble as tetramers that form a non-selective cation channel and they are often colocalized on dendritic spines (Reiner and Levitz, 2018). This family of receptors includes the N-methyl-D-aspartate (NMDA) receptors, and the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Kim et al., 2020). NMDA receptors are the assemble of two obligatory GluN1, and two GluN2 and/or GluN3 subunits, forming together a transmembrane pore for $\text{Na}^+/\text{Ca}^{2+}$ influx and K^+ efflux. GluN1 and GluN3 subunits are activated by glycine or D-serine while GluN2 subunits by glutamate. The receptor subunits are encoded by seven genes. GluN1 is encoded by one gene (GluN1), GluN2 by four genes (the most abundant GluN2A and GluN2B, and 2C, 2D) and GluN3 by two (GluN3A, 3B) (Reiner and Levitz, 2018; Kim et al., 2020).

AMPA receptors are composed of four types of subunits; GluA1, 2, 3, and 4. AMPA receptors are dimers of dimers, composed of a GluA2 dimer and one of the other dimers (Kim et al., 2020). Unlike NMDA receptors, each subunit of the AMPA receptor can bind its agonist, and the binding of two or more subunits is necessary to open its pore. The GluA2 subunit imparts to the channel a low permeability to Ca^{2+} , so the open channel only allows Na^+/K^+ flux (Kim et al., 2020).

Ionotropic glutamate receptors and their role in the LTP and LTD

LTP and LTD are processes that describe enduring changes in synaptic strength, induced by specific patterns of synaptic activity. While LTP enhance, LTD depress synaptic transmission. Although both mechanisms are the actual synaptic processes underlying learning and memory, they have not been definitively understood, however, they can occur in various sets of synapses in the brain (Citri and Malenka, 2008; Bliss and Cooke, 2011).

AMPA and NMDA participate actively in LTP and LTD processes. AMPA receptors are the pillar players of synaptic plasticity, because its activation mediates a fast component with rapid rise time and decay (Hansen et al., 2017). Once activated, AMPA receptors allow an influx of Na^+ and a small K^+ efflux which provokes the depolarization of the postsynaptic neuron (Lüscher and Malenka, 2012). In turn, the molecule of Mg^{2+} that was blocking the ion channel of NMDA receptors is released, this event allows a Ca^{2+} and Na^+ influx through NMDA receptors (Citri and Malenka, 2008) (Figure 5).

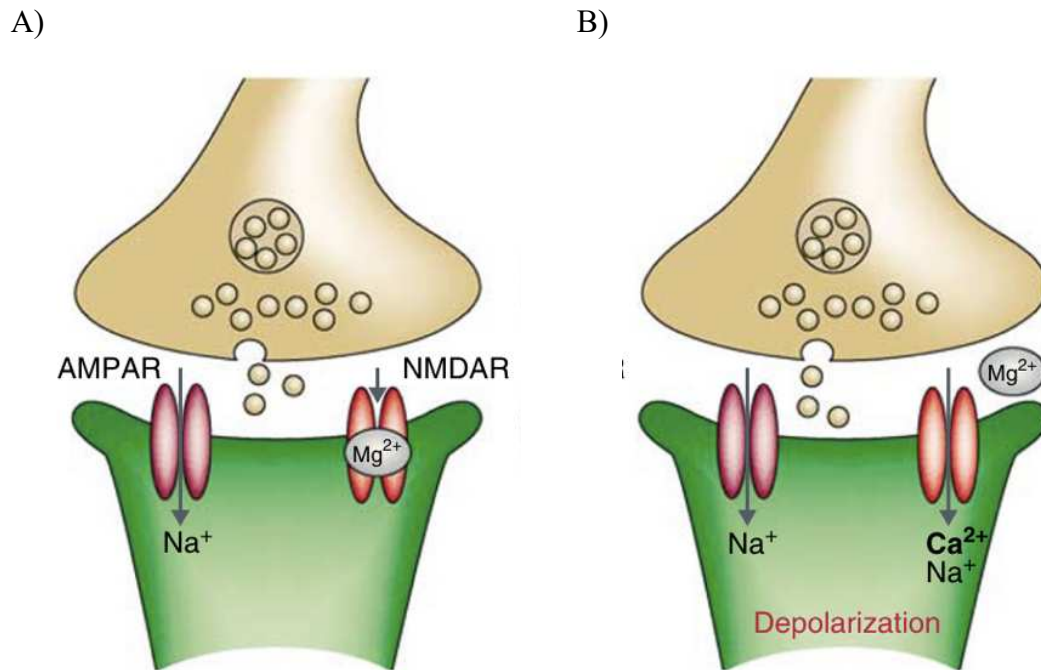


Figure 5. Involvement of AMPA and NMDA receptors in LTP and LTD. A) AMPA receptors are activated and permit the entrance of Na⁺ in the postsynaptic neuron. The ion channel of NMDA receptors is blockaded with a molecule of Mg²⁺. B) Entrance of Na⁺ provokes the depolarization of the membrane releasing the Mg²⁺ molecule from the ion channel of NMDA receptors, this event allows a Ca²⁺ and Na⁺ influx, necessary for LTP. Image obtained from Citri and Malenka (Citri and Malenka, 2008).

LTP in the hippocampus necessarily requires the activation of NMDA receptors (Citri and Malenka, 2008) because the increased concentration of Ca²⁺ activates protein kinases and phosphatases promoting synaptic changes through a variety of pathways including CaMKII, PKC, PKA, and MAPK. One of the consequences of these events is that the overexpression of AMPA receptors will promote a major sensitivity of the membrane to glutamate (Middei et al., 2014; Camina and Güell, 2017; Hansen et al., 2017; Cheyne and Montgomery, 2020; Kim et al., 2020). NMDA receptors promote the active insertion or removal of AMPA receptors in the membrane through endocytosis and exocytosis processes. During LTP there is a longer presence of AMPA receptors at synapses, on the contrary, during LTD they are removed (Cheyne and Montgomery, 2020) (Figure 6).

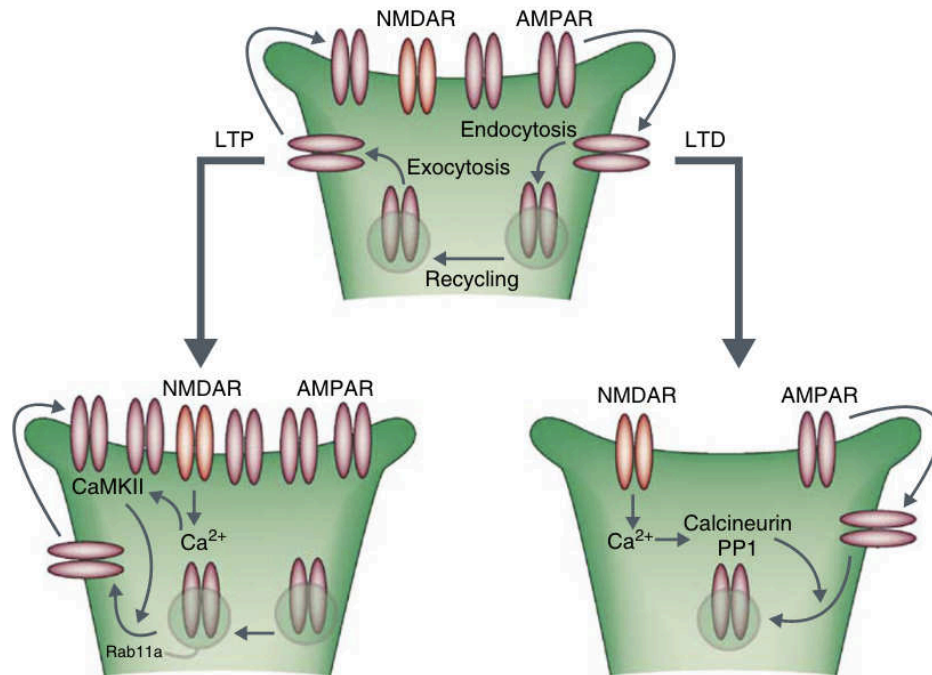


Figure 6. AMPA receptors trafficking during LTP and LTD. AMPA receptors are recycled through process of endocytosis and exocytosis. When LTP occurs, there is enhanced AMPA receptors exocytosis. This process is stabilized by the action of a calcium-driven process which involves CAMKII and fusion of recycling endosomes mediated by Rab11a. In LTD, AMPA receptors undergo endocytosis which involves the action of protein phosphatases, primarily calcineurin and protein phosphatases 1 (PP1). Image obtained from Citri and Malenka (Citri and Malenka, 2008).

Synaptic vesicles and postsynaptic density proteins

Excitatory glutamatergic synapses are characterized by an enrichment of synaptic vesicles at the axon terminal of the presynaptic neuron and by the presence of a presynaptic electron-dense membrane (referred as Postsynaptic density (PSD)) at the top of dendritic spines in the postsynaptic neuron (Feng and Zhang, 2009) (Figure 7). Neurotransmitters are released from synaptic vesicles and regulated via endocytosis. These vesicles are composed of several molecules, among which, the synaptobrevin-II, synaptophysin, synapsin, synaptotagmin and SV2A. (Thiel, 1993; Kokotos et al., 2019).

The PSD is a protein complex composed by postsynaptic receptors, cytoplasmic scaffold proteins, signaling enzymes and cytoskeletal structural elements. The main function of PSD is to anchor and stabilize glutamate receptors. In addition, the PSD proteins regulate trafficking, targeting and insertion of receptors and ion channels along dendrites and into synapses (Vyas and Montgomery, 2016; Levy et al., 2022). The two major PSD protein families are the guanylate kinases (MAGUKs) and the Src homology 3 (SH3) domain and ankyrin repeat proteins (Shanks). The Shank proteins interact directly and indirectly with AMPA- and NMDA receptors, signaling molecules, and cytoskeletal actin to modulate synapse structure, and increase synaptic strength and maturation. They form the focal point of the postsynaptic scaffold (Vyas and Montgomery, 2016).

The discs large (DLG) is a subfamily of the MAGUKs family that comprises four proteins, the SAP97, PSD-93, SAP102, and PSD-95 (encoded by DLG1, DLG2, and DLG3, DLG4, respectively). Proteins of this group are highly enriched in the PSD (Levy et al., 2022) and share a common multi-domain structure with three PDZ domains, an SH3 domain, and a GUK domain through which they interact with AMPA and NMDA receptors (Vyas and Montgomery, 2016), as well as with other components such as the Shaker-type voltage-gated potassium (Kv1) channels, the cell adhesion molecules neuroligins (NLGNs), and the transmembrane protein ADAM22 (Levy et al., 2022). PSD-95 is one of the most studied PSD proteins. It is an essential regulator of the plasticity and strength of excitatory signaling. (Levy et al., 2022)

The role of glial cells in the regulation of glutamatergic signaling

Glutamatergic signaling is regulated by glial cells which constitute between 33 to 66% of the mammalian brain mass. This population of cells comprises astrocytes, microglia, oligodendrocytes, and their progenitors NG2-glia. Astrocytes are multi-branched stellate cells derived from neural stem cells belonging to the embryonic, ectodermal plate, and neural crest, and they represent the most abundant glial cells (Haroon et al., 2017; Jäkel and Dimou, 2017). They are part of the “tripartite synapse”, a concept that demarks a bidirectional communication between astrocytes and neurons forming a functional unit (Perea et al., 2009). Here, astrocytes secret factors that target pre and postsynaptic sites

in neurons, thereby modulating their structure and function. Further, the neurotransmitters released from neurons bind receptors in astrocytes triggering the activation of signaling pathways that modulate synaptic behavior (Farhy-Tselnicker and Allen, 2018).

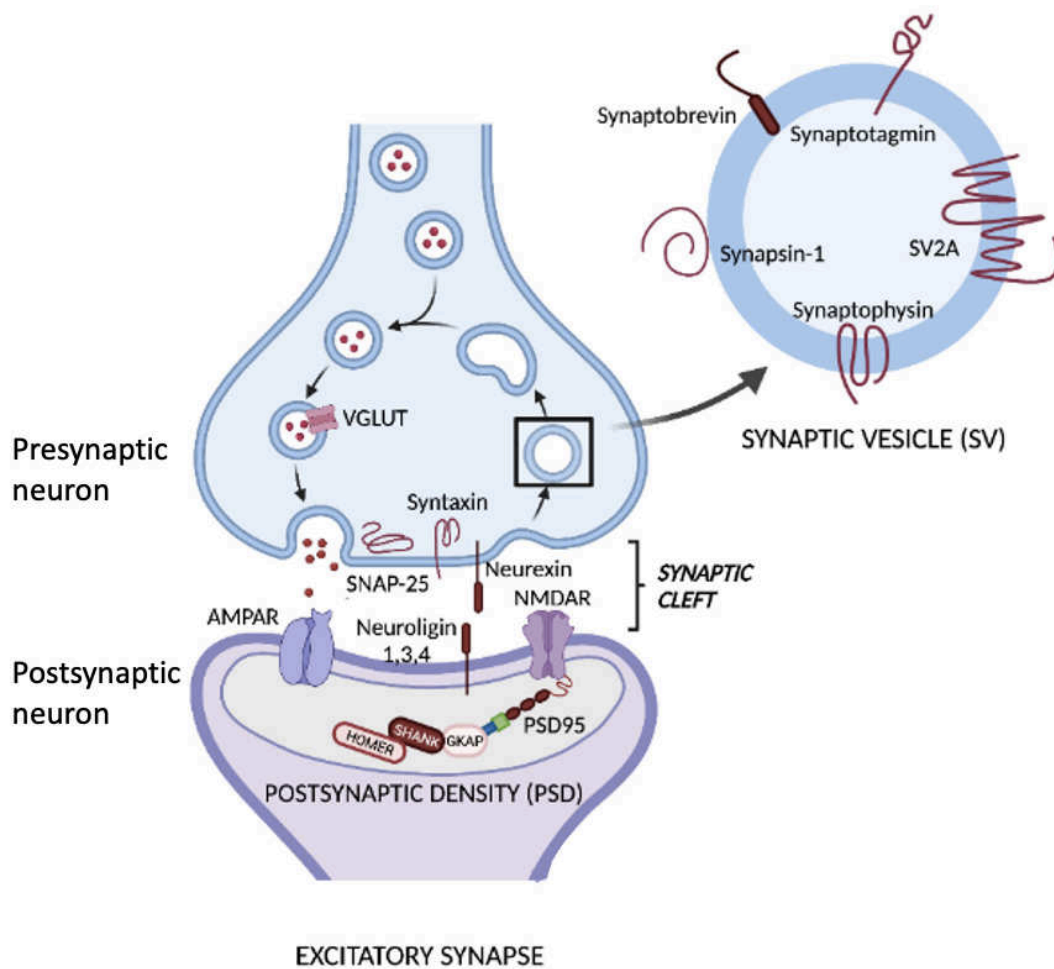


Figure 7. Synaptic vesicles and postsynaptic density proteins that support the synapse. Presynaptic and postsynaptic neurons are separated by a synaptic cleft. In the presynaptic neuron, the release of neurotransmitters is produced by the synaptic vesicles (SV). In the postsynaptic neuron, the elements in the postsynaptic density allow the glutamate ionotropic receptors to anchor to the membrane. Image adapted from Serrano *et al.*, (Serrano *et al.*, 2022)

Astrocytes accomplish several functions, among them, they maintain synaptic homeostasis and integrity, regulate synaptic pruning, participate in neuron–glia signaling, and modulate glutamate metabolism (Haroon et al., 2017). After LTP occurs, glutamate is rapidly cleared by astrocytes to avoid that it continues stimulating the postsynaptic receptors, or to scape and activate unintended synapses. It also prevents overactivation of NMDA receptors which could provoke cytotoxicity and cell death due to the excessive Ca^{2+} influx. Astrocytes take up glutamate via the excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) which use gradients of Na^+ , H^+ , and K^+ to drive glutamate into the cell. Inside the astrocyte, glutamate is either converted into α -ketoglutarate by glutamate dehydrogenase (GDH) or by transaminases and shunted into the astrocytic Tricarboxylic Acid Cycle (TCA), or it can be converted into glutamine by the enzyme glutamine synthetase (GS) and be extruded by the SNAT3 glutamine transporter into the extra synaptic space. Then glutamate can be taken up by neurons and converted into glutamate via phosphate-activated glutaminase (PAG). This glutamate recycling pathway is referred to as the glutamate-glutamine cycle (Todd and Hardingham, 2020) (Figure 8).

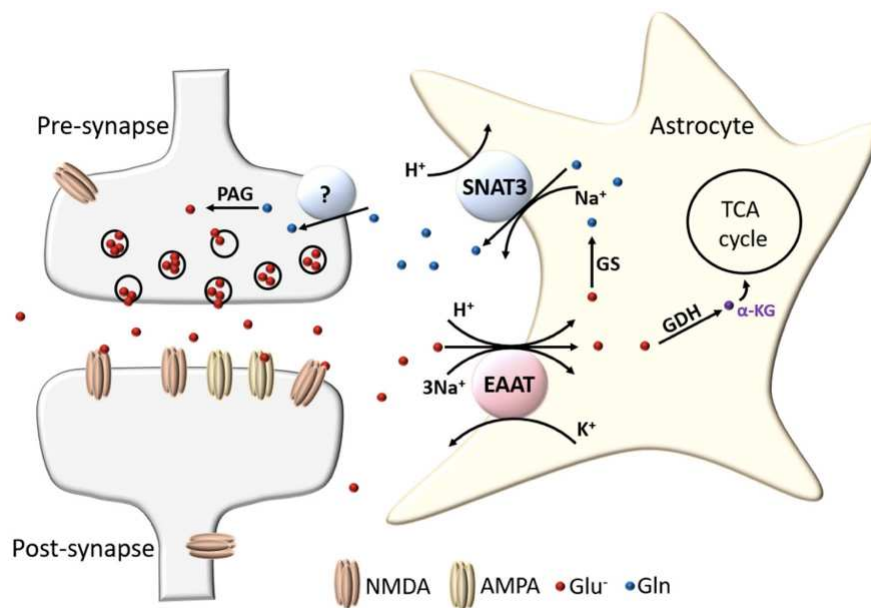


Figure 8. Glutamate-glutamine cycle. Astrocytes are part of the “tripartite synapse”, which demarks a bidirectional communication between astrocytes and neurons. After LTP astrocytes clear glutamate from the synaptic cleft to prevent cytotoxicity and cell death. Image obtained from Todd and Hardingham (Todd and Hardingham, 2020).

Another important glial cell are microglia which are the resident immune cells of the brain. As macrophages in the peripheral blood, microglia arise from primitive myeloid cells in the embryonic mesoderm of the yolk sac but migrate into the neural plate before vascularization or definitive hematopoiesis. Unlike macrophages, microglia reside and function within the brain parenchyma outside the limits of peripheral circulation. As they are mobile cells, they can scan the entirety of the brain parenchyma and with their fine filopodia enriched with immune receptors, they look for immune signals. If they encounter signs of damage or alarm, they shift to an activated state that can be recognized due to a change in their morphology from stellate to amoeboid. When microglia are activated, they release proinflammatory cytokines such as Interleukin 1 β , 6, and 12 (IL-1 β , IL-6, IL-12), tumor necrosis factor α (TNF α), and Interferon γ (IFN- γ) as well as chemokines such as CCL2 that attract peripheral monocytes into the brain (Haroon et al., 2017; Jäkel and Dimou, 2017) (Figure 9).

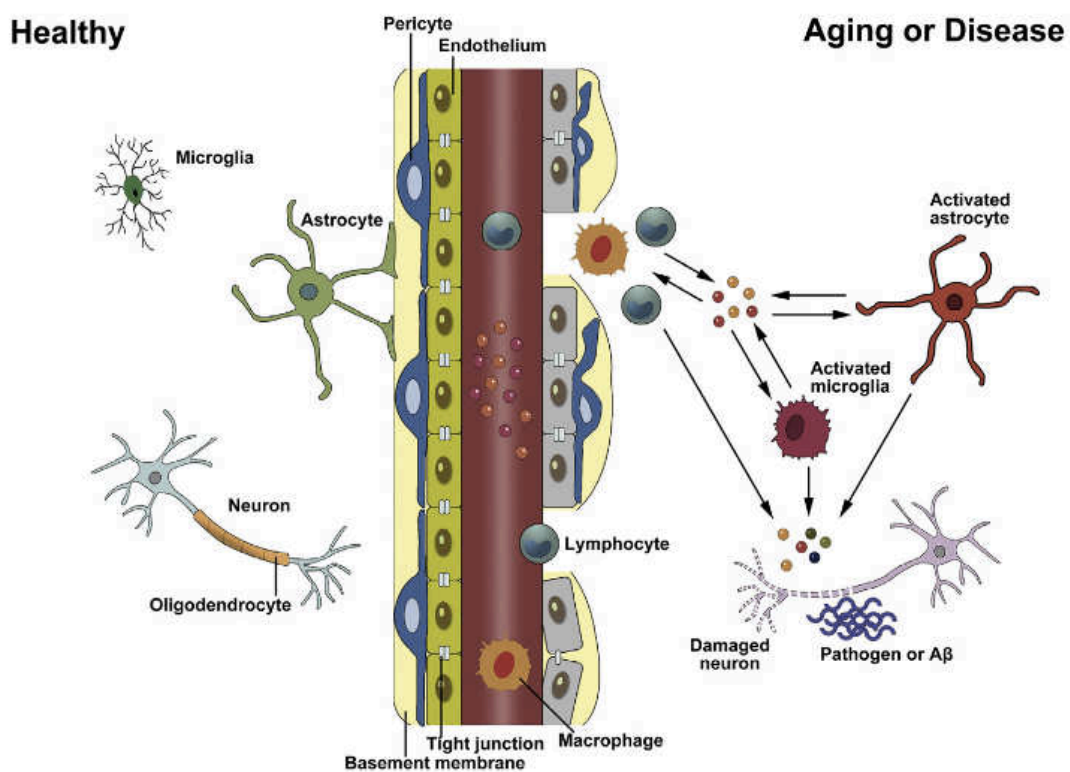


Figure 9. Activation of astrocytes and microglia. Under healthy conditions the capillarity of the blood brain barrier (BBB) remains intact. This structure is composed of endothelial cells, pericytes, astrocytes, and microglia. During aging or disease, the

permeability of the BBB is disrupted which leads to the entrance of neurotoxic molecules and macrophages and, consequently, to the activation of astrocytes and microglia which release proinflammatory cytokines. Image obtained from Pan *et al.*, (Pan et al., 2021).

An important fact is that astrocytes and microglia produce metabolites called kynurenines that modulate the glutamatergic signaling and hence, memory construction. Kynurenines result from the catabolism of TRP, an essential amino acid necessary for mammalian health. TRP is catabolized via the kynurenine pathway to give rise to several bioactive compounds, but particularly kynurenic and quinolinic acids which are of high relevance in the control of glutamatergic signaling. While kynurenic acid acts as an antagonist of all glutamate ionotropic receptors, quinolinic acid is an NMDA agonist (Richard et al., 2009). Therefore, both metabolites have the capacity to control LTP and LTD. In the following chapter we will analyze the properties of TRP, its metabolic pathways and how it enters into the brain. In addition, we will further discuss the relevance of kynurenic and quinolinic acids in glutamatergic signaling.

Chapter 2.

*Role of tryptophan-derived metabolites in
the regulation of cognitive function and
glutamatergic signaling*

Among the twenty amino acids that play a fundamental role in human nutrition and health, only nine are considered as essential because they cannot be endogenously synthesized and need to be supplied from food. TRP is one of these essential amino acids (Palego et al., 2016; Friedman, 2018). Although of all the amino acids tryptophan is the one with the lowest concentration in the body, it serves a fundamental physiological role as it is the precursor for the synthesis of several bioactive and pleiotropic compounds that act in a multitude of essential metabolic pathways. (Richard et al., 2009). Particularly in the brain, TRP is closely related with the processes of plasticity, cognition, memory, energetic homeostasis, mood regulation, sleep regulation and neurologic development (Ramos-Chávez et al., 2018).

Chemical structure of tryptophan

TRP was first discovered by Hopkins and Cole in the early 1900s who isolated it from the casein protein. Then, Ellinger and Flamand determined its molecular structure (Richard et al., 2009). TRP is a large neutral amino acid (LNAA) and the only one deriving from indole, a bicyclic ring formed by a benzene and a pyrrole group, linked to the α -carbon by a $-\text{CH}_2$ -group (Figure 10). The presence of the indole ring in the chemical structure of TRP provides it high hydrophobic properties (Palego et al., 2016).

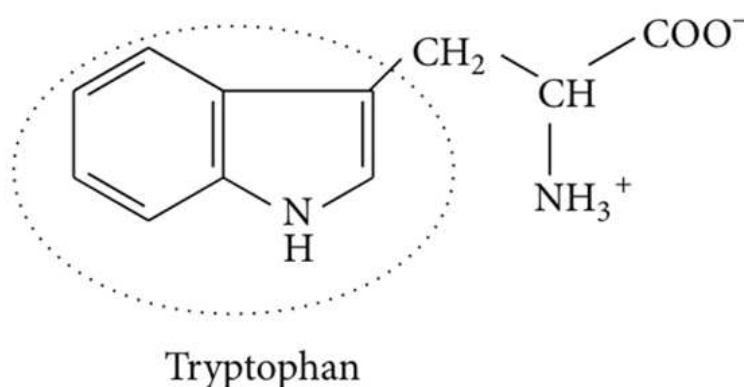


Figure 10. Chemical structure of tryptophan. TRP is composed of an indole group linked to the α -carbon by a $-\text{CH}_2$ -group. Image obtained from Palego *et al.*, (Palego et al., 2016).

Tryptophan metabolism via the kynurenine and serotonin pathways.

TRP is metabolized through three major pathways, the kynurenine, the serotonin and the indole pathways (Palego et al., 2016; Hsu and Tain, 2020). From the TRP that is not used for protein synthesis, over the 95% is catabolized via the kynurenine pathway, while only 1–2% and 2–3% of dietary TRP are converted into serotonin and indoles, respectively (Hsu and Tain, 2020). Here we will not discuss the indole pathway because it is produced by the gut microbiota which is out of the scope of this work.

The kynurenine pathway (KP) plays a critical role in the generation of cellular energy, the regulation of the immune response, and the production neuroactive compounds. The initial and rate-limiting step of the KP involves tryptophan dioxygenase (TDO)2 and the indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2). TDO2 acts principally in the liver as well as in the brain, prevalently in astrocytes. IDOs are expressed in most peripheral tissues, in immune system cells and in the central nervous system, prevalently in the microglia. The product of IDO/TDO-catalyzed reaction, is N-formylkynurenine, that is then hydrolyzed to kynurenine (KYN) (Palego et al., 2016; Hsu and Tain, 2020; Savitz, 2020).

KYN is metabolized through three different pathways. 1) By the action of the kynureninase (KYNU), KYN is converted to anthranilic acid (AA). 2) By the action of kynurenine aminotransferase (KAT), KYN is converted into kynurenic acid (KA). 3) By the action of kynurenine-3-monooxygenase (KMO), KYN is converted into 3-hydroxykynurenine (3-HK), which can be further transformed by KAT to xanthurenic acid (XA) or by the KYNU to 3-hydroxyanthranilic acid (3-HAA). The catabolism of 3-HAA leads to the generation of picolinic acid, quinolinic acid (QA), nicotinamide adenine dinucleotide (NAD⁺) and nicotinamide adenine dinucleotide phosphate (NADP) (Hsu and Tain, 2020) (Figure 11). Under physiological conditions, KYN preferentially produces 3HK, 3-HAA, QA, and ultimately NAD⁺. The remaining balance of KYN is converted into KA (Savitz, 2020). KA, QA and XA are of higher relevance in the regulation of synaptic plasticity and their properties will be explained later.

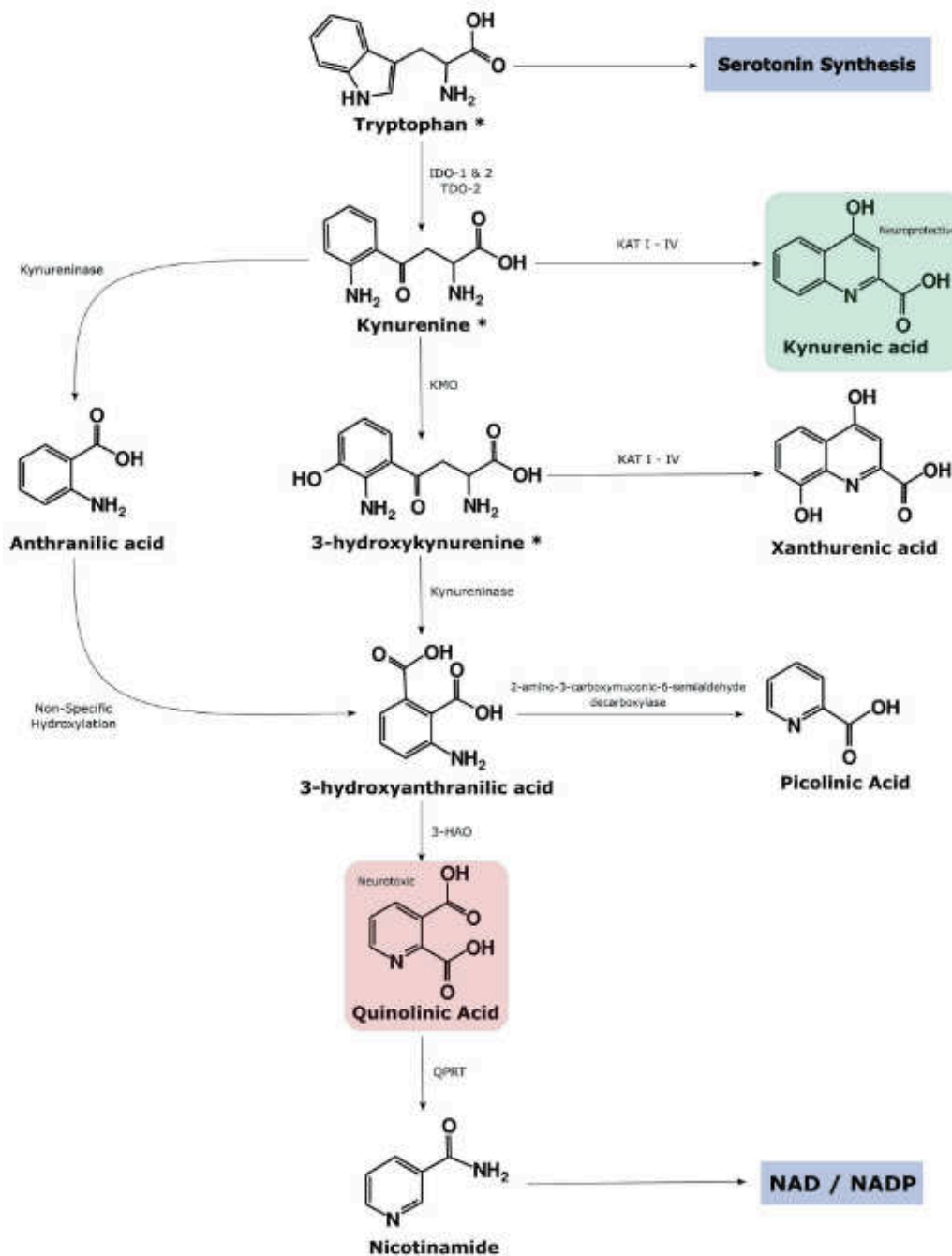


Figure 11. Kynurenine pathway. A little portion of TRP is used to produce serotonin and the rest is metabolized through the kynurenine pathway. Through this pathway are produced kynurenic acid, considered as a neuroprotective compound, and quinolinic acid, considered as neurotoxic. The final product is NAD⁺/NADP, which are essential coenzymes for electron transfer reactions that play a critical role in generating cellular energy. Image obtained from Savitz (Savitz, 2020).

As its name implies, the serotonin pathway produces serotonin as well as other important components such as melanin. Serotonin (5-HT), otherwise known as 5-hydroxytryptamine is a molecule with diverse effects in the central nervous system (CNS) and in the periphery. It functions as a hormone, a neurotransmitter, and a mitogen. Within the CNS, serotonin is synthesized and stored in presynaptic neurons (in serotonergic neurons, pineal gland, and catecholaminergic neurons). Moreover, the raphe nuclei are the major core with both, ascending serotonergic fibers projecting to the forebrain and descending fibers that extend to the medulla and spinal cord. In the periphery, serotonin is produced in the enterochromaffin cells and stored in the platelets (Mohammad-Zadeh et al., 2008).

Serotonin production starts by the action of the enzyme tryptophan hydroxylase (TPH) which converts TRP into 5-hydroxytryptophan (5-HTP). There are two TPH isoforms, TPH1 and TPH2, the first one is prevalent in the periphery and pinealocytes, while TPH2 is expressed in the raphe nuclei. The second step of the serotonin pathway is the decarboxylation of 5-HTP to 5-HT, a reaction catalyzed by the enzyme aromatic amino acid decarboxylase. 5-HT can be released as a neurotransmitter in CNS or as a modulator in the periphery. 5-HT can also be metabolized by the monoamine oxidase (MAO-A) to 5-hydroxyindoleacetic acid (5-HIAA) (Figure 12) (Palego et al., 2016; Hsu and Tain, 2020).

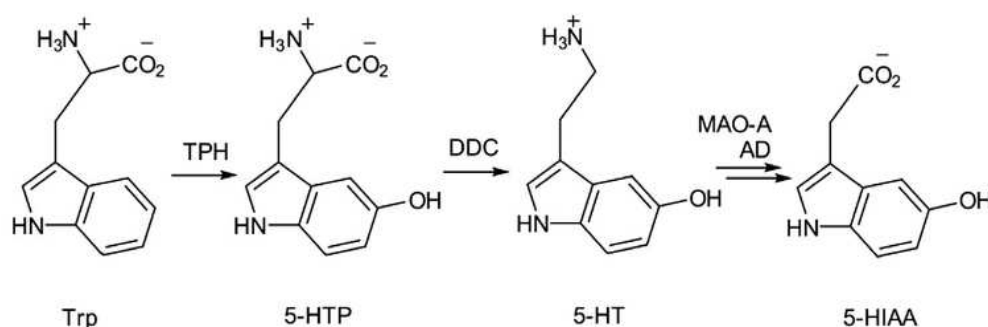


Figure 12. Serotonin pathway. Only a little portion of TRP is used to produce 5-HT and 5-HIAA. Image obtained from Comai *et al.*, (Comai et al., 2020)

5-HT is a neurotransmitter that regulates body temperature, sleep, appetite, pain, motor activity, cognition, and emotional behavior. It is involved in the processes of memory and learning because there is a high density of serotonergic projections in the hippocampus and prefrontal cortex. In particular, in the hippocampus serotonin is involved in memory, spatial navigation, decision making and social relationships, while in the prefrontal cortex it regulates working memory, attention, decision-making and reversal learning (Švob Štrac et al., 2016). Despite the relatively low concentration of serotonin in the brain compared to that in the rest of the body, it is implicated in numerous psychiatric conditions (Richard et al., 2009) such as depression, anxiety, schizophrenia and attention deficit hyperactivity disorder (Lin et al., 2014).

There is another product of the serotonin pathway called melatonin whose production is regulated by the blue light spectrum in both artificial and sun light. It is secreted by the pineal gland, and it regulates diurnal rhythms. It also influences the reproductive and immune systems, as well as digestive processes and gastrointestinal motility (Richard et al., 2009).

Tryptophan intake, absorption and transport into the brain

TRP must be ingested from diet, either from animal or from vegetal foods. Aliments from animal origin with higher TRP content include milk, cheese, and dairy products, eggs (white), meat, chicken, turkey, and seafood (fish and crustaceous). In the case of vegetal aliments, TRP is found in potatoes, chickpeas, soybeans, cocoa beans, nuts, oats, bananas, dried prunes. Lower TRP amounts can be found in some varieties of cereals and maize (Richard et al., 2009; Palego et al., 2016).

Once ingested, TRP is absorbed by the gut, passes into the bloodstream, and is mainly absorbed in the periphery by the muscles and liver. It is important to note that TRP is highly lipophilic and scarcely soluble in aqueous solutions at physiological pH, hence, it circulates in the periphery bound to molecules of albumin. Approximately 75% to 95% of TRP in blood is bound to albumin and only in its free form can cross the blood-brain barrier (BBB). The ratio between free and albumin-bound TRP strongly impacts the

passage of this amino acid into the brain (Figure 13). Interestingly, several conditions can modify this ratio, including sport activity, activation of the sympathetic nervous system, lipolysis, and increased plasma levels of free fatty acids (Palego et al., 2016). Once in the bloodstream, TRP competes with other large neutral amino acids (histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine, and valine) to cross the BBB (Richard et al., 2009). All this amino acids are transported by a complex of amino acids carriers of type “L,” LAT1, and the cell surface antigen CD98 (heavy chain 4F2) that is an accessory protein, highly expressed in the barrier capillary endothelium (Palego et al., 2016).

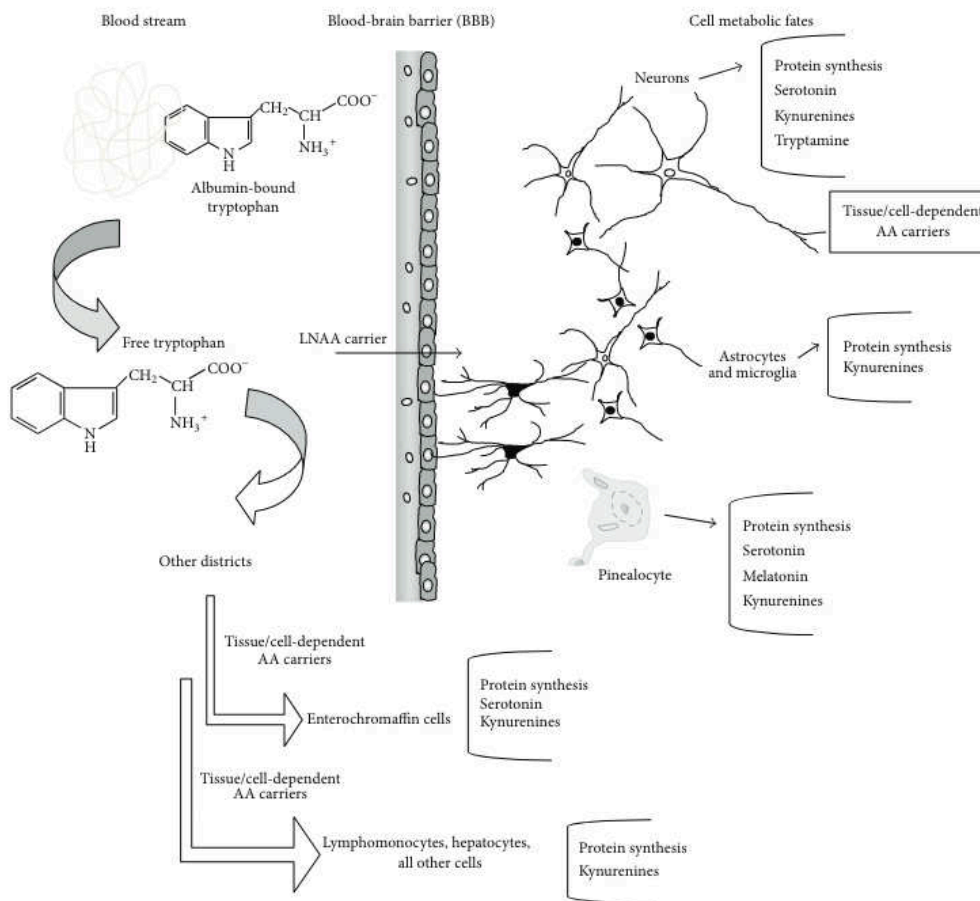


Figure 13. Peripheral and central tryptophan uptake. Only the TRP that is not bound to albumin can cross the BBB and be used by neurons, astrocytes, microglia and pinealocytes to produce serotonin or kynurenines. In the periphery, TRP is used by other cells such as enterochromaffin cells, lymphomonocytes, hepatocytes, among others. Image obtained from Palego *et al.*, (Palego et al., 2016).

Kynurenine pathway metabolites and glutamatergic signaling

Concerning synaptic plasticity, the three most important molecules produced by the kynurenine pathway are KA, QA and XA. While KA and QA interact with glutamate ionotropic receptors, XA interacts with glutamate metabotropic receptors. KA is considered as a neuroprotective metabolite that acts as an antagonist of all the glutamate ionotropic receptors (including AMPA and NMDA), while QA is considered as a neurotoxic metabolite that acts as a NMDA agonist (Modoux et al., 2021). In the case of XA very little is known but evidence suggest that it has also neurotoxic effects.

Even though KA and QA are designated as neuroprotective and neurotoxic compounds, both are necessary for proper synaptic signaling, and under adverse conditions both can have neurotoxic effects. Indeed, increased concentrations of either KA or QA metabolites provokes cognitive impairment (Kozak et al., 2014; Cathomas et al., 2021). Moreover, an imbalance in the concentration of these metabolites has been described in several psychiatric and neurodevelopmental disorders (Modoux et al., 2021). In schizophrenia the serum levels of KA are increased while in Alzheimer (Busse et al., 2018), Huntington (Schwarcz et al., 2010), and Parkinson diseases (Chang et al., 2018), Multiple sclerosis (Lim et al., 2017), Autism (Lim et al., 2016), and depression (Savitz et al., 2015) there is an increase in QA concentration (Figure 14).

The production of KA and QA is mediated by glial cells. While astrocytes express KAT, the enzyme that directs the conversion of KYN into KA, microglia expresses KMO the enzyme that produces QA (Guillemin et al., 2005) (Figure 14). As mentioned in the previous chapter, under pathological conditions, astrocytes and microglia shift into an activated form that promotes the release of proinflammatory cytokines such as IFN γ , IL- β and TNF α . These cytokines induce the expression of IDO, the enzyme that promotes the catabolism of TRP through the kynurenine pathway, and, therefore, induce a deregulation in the concentrations of KA and QA (Robinson et al., 2005; Zunszain et al., 2012; Urata et al., 2014).

Many of the psychiatric diseases mentioned above, are characterized by a proinflammatory state that is related to the overactivation of IDO and therefore, to adysregulation in the balance of kynurenines. However, there is another pathology called obesity that also produces an inflammatory state, and whose relationship with the regulation IDO starts to be explored. In the following chapter we will discuss the generalities about the pathophysiology of obesity and how it produces alterations in the peripheral kynurenine pathway that lead us to believe that it could exert the same effect on the brain, and therefore compromising the cognitive ability.

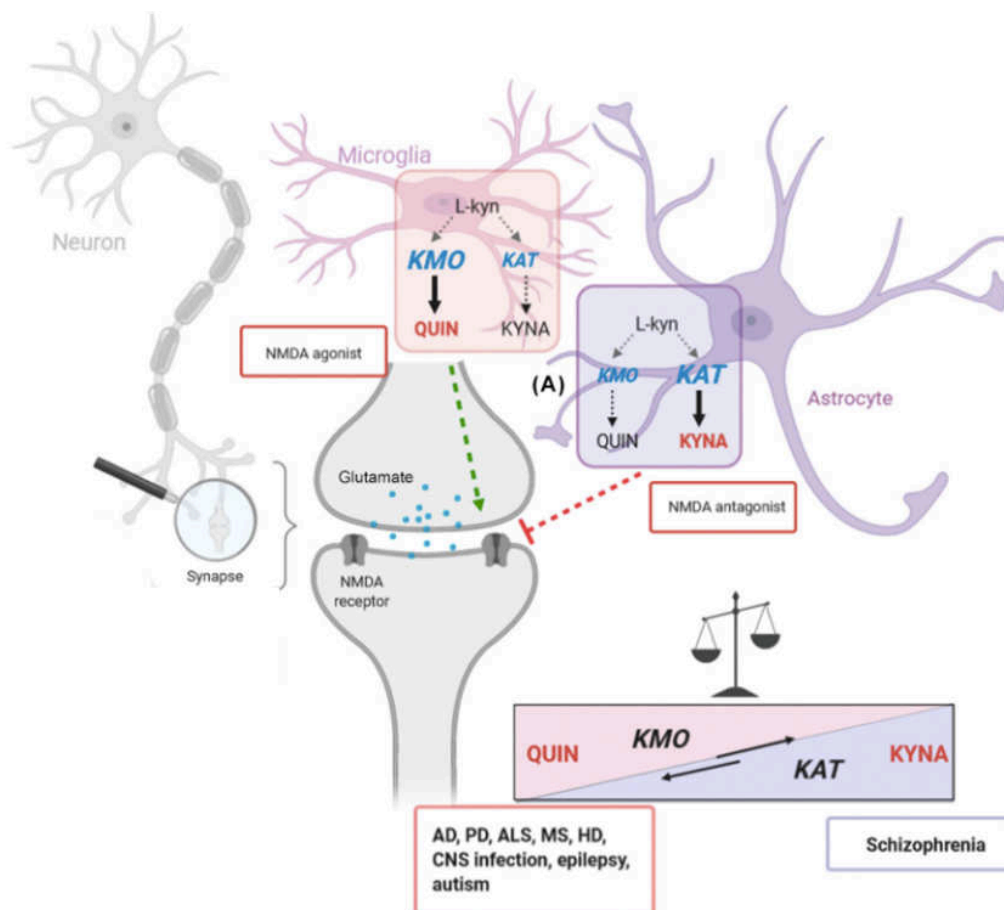


Figure 14. Synthesis of kynurenines by astrocytes and microglia. Microglia produces quinolinic acid and astrocytes kynurenic acid. Under pathological conditions, such as in psychiatric and neurodevelopmental disorders there is an overactivation of KMO or KAT which produces deregulated levels of QA and KA. Image adapted from Modoux (Modoux et al., 2021).

Chapter 3.

The deleterious effects of obesity on cognition

During aging, it is normal to experience a decline in memory capacity. The mildest form of memory impairment associated with age is characterized by self-perception of memory loss and a decline in objective memory performance. In the United States about 40% of people aged 65 or older have an age-associated memory impairment, and only about 1% of them will progress to dementia each year. Mild cognitive impairment represents a more severe form of memory loss, and it is often defined by important memory deficits without functional impairments. About 10% of people aged 65 years or older have mild cognitive impairment, and nearly 15% of them develop Alzheimer's disease each year (Small, 2002). Besides age, memory impairment can be induced by several environmental factors such as alcoholism (Fama et al., 2021), cigarette smoking (Richards et al., 2003), Vitamin B12 deficiency (Jatoi et al., 2020), drugs use (Chavant et al., 2011), traumatic brain injury (Flynn, 2010), as well as some diseases such as type 2 diabetes mellitus (Saedi et al., 2016), cardiovascular disease (Leritz et al., 2011) and obesity (Anjum et al., 2018).

Definition of obesity

Obesity is a chronic, complex, and heterogeneous disease (Guerreiro et al., 2022) that was defined by the WHO as an excessive fat accumulation that might impair health. Due to the accelerated increase in obesity rates worldwide, in 1977 the WHO declared it as a major public health problem (James, 2008). In general terms, the fundamental cause of obesity is the long-term energy imbalance between too many calories consumed and too few calories expended (Blüher, 2019). However, the pathogenesis is more complex than that, and there are other factors contributing to its etiology such as environmental, sociocultural, physiological, medical, behavioral, genetic, epigenetic, among other factors (Gadde et al., 2018).

Obesity substantially increases the risk of metabolic diseases (Type 2 Diabetes and Fatty liver disease), cardiovascular diseases (hypertension, myocardial infarction, stroke and atherosclerosis), musculoskeletal diseases (osteoarthritis), hyperlipidemia, some types of cancer (breast, endometrial, ovarian, esophageal, pancreatic, liver, kidney, prostate, and colon), and other chronic diseases such as subfertility and asthma. (Blüher, 2019; Guerreiro et al., 2022). Besides, obesity is commonly accompanied by low self-esteem, mood disorders, eating problems, impaired body image, interpersonal

communication problems (Djalalinia et al., 2015). Additionally, growing evidence shows that obesity promotes cognitive impair, dementia (Ma et al., 2020) and Alzheimer disease (Lloret et al., 2019).

Diagnosis

In clinical practice obesity is traditionally determined based on the calculation of the body mass index (BMI) that is obtained by dividing a person's weight by the square of its height (kg/m^2). A patient is considered as overweighted if her or his BMI ranges between 25.0 to 29.9, or obese if the BMI is greater or equal to 30. In addition, the patient can be categorized into three classes of obesity: class I (BMI 30.0 to 34.9), class II (BMI 35.0 to 39.9) and class III (BMI ≥ 40.0), the last one is considered as morbid obesity (Lin and Li, 2021) (Figure 15).

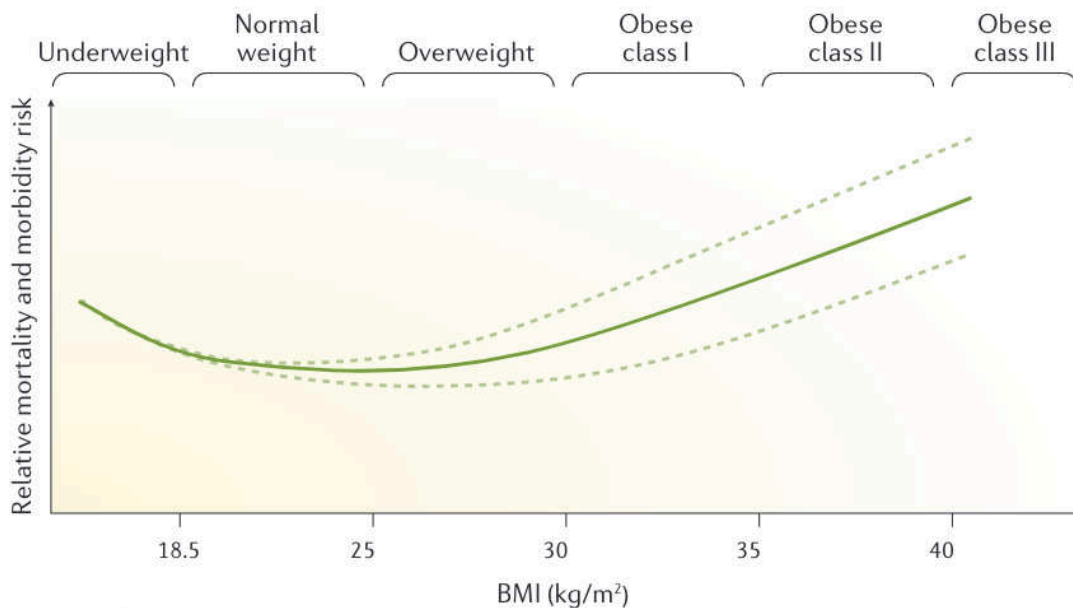


Figure 15. Relationship between body mass index and mortality and morbidity risk. Obesity is defined when the BMI is superior or equal to $30\text{kg}/\text{m}^2$. The higher the BMI, the higher the risk of mortality and morbidity. Image obtained from González-Muniesa *et al.*, (González-Muniesa et al., 2017).

It is important to note that the BMI has important limitations since it does not distinguish between fat mass and lean muscle mass. In contrast, there are other measures such as the waist circumference or the waist-to-hip ratio that give us an approach of the percentage of fat, also called adiposity (Gadde et al., 2018; Nimptsch et al., 2019). In addition, there are other sophisticated techniques that can more accurately quantify the amount of fat in specific areas of the body. These techniques are the bioimpedance analysis, dual-energy X-ray absorptiometry, computed tomography and magnetic resonance imaging or magnetic resonance spectroscopy scans (Nimptsch et al., 2019).

Epidemiology

The worldwide prevalence of obesity has increased at an alarming rate. Since 1989 it has doubled in more than 70 countries. Nowadays, around one third of the global population is considered obese or overweight. (Lin and Li, 2021). When analyzing the prevalence of obesity by sex, it is observed that from 1975 to 2014, the incidence of obesity increased from 3.2% to 10.8% in men and from 6.4% to 14.9% in women (Figure 16). In addition, in 2014, 0.64% of men and 1.6% of women had morbid obesity. Likewise, from 1975 to 2016 the obesity rates in children passed from 0.7% to 5.6% in boys and from 0.9% to 7.8% in girls (Blüher, 2019). If present trends continue, by 2025, global obesity prevalence will reach 18% in men and 21% in women, and severe obesity will surpass 6% in men and 9% in women (NCD Risk Factor Collaboration (NCD-RisC), 2016).

In the past, it was considered that obesity represented a problem only for high-income countries, but now it is also affecting low- and middle-income countries, especially in urban areas (Lin and Li, 2021). This phenomenon varies from country to country ranging from 3.7% in Japan to 38.2% in the United States. Furthermore, the increase in the incidence of obesity has occurred at different rates across countries. The regions where it was observed an accelerated increase were south Asia (including Bangladesh, Bhutan, India, Nepal, and Pakistan), southeast Asia (Indonesia, Malaysia, Philippines, Sri Lanka, Thailand, and Vietnam), the Caribbean (Belize, Cuba, Dominican Republic, Jamaica, and Puerto Rico) and Latin America (Argentina, Brazil, Colombia, Chile, El Salvador, Guatemala, Mexico, Paraguay, Uruguay, and Venezuela). In contrast, in some countries of eastern Europe (Belarus, Latvia, Lithuania, Russian Federation and

Ukraine) the increase in BMI has not occurred at an accelerated rate (NCD Risk Factor Collaboration (NCD-RisC), 2016; Blüher, 2019). Particularly in France, there is a study showing that from 2013 to 2016 the prevalence of obesity has ranged from 14.2 to 15.2% in women and from 14 to 15.3% in men, and the most affected were young adults aged 18–29 years from both sexes. In addition, authors found that the highest prevalence of obesity was among individuals with lower education and income (Czernichow et al., 2021).

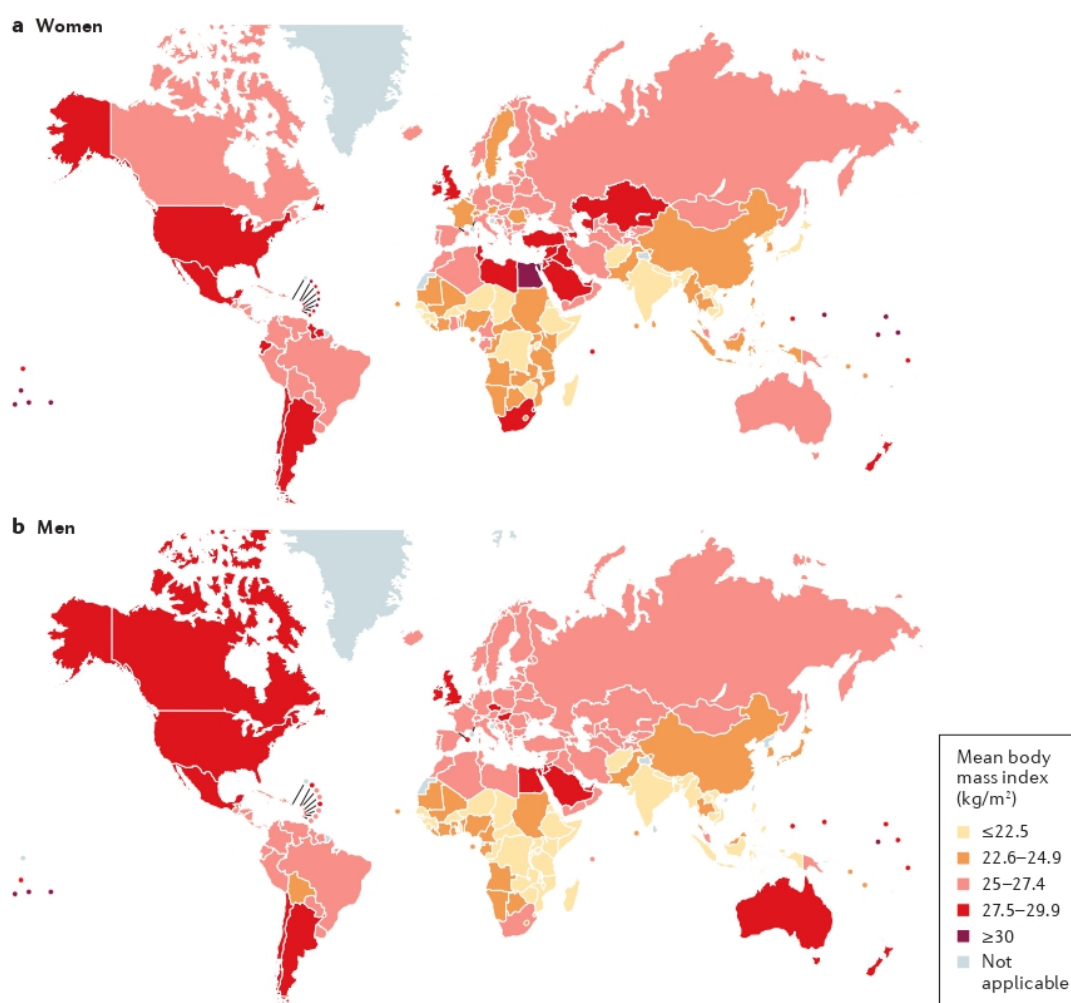


Figure 16. Worldwide prevalence of obesity according to the BMI measure. A) Age-standardized global body mass index for women and B) men. In 2014, the incidence of obesity increased to 10.8% in men and 14.9% in women worldwide. Image obtained from González-Muniesa *et al.*, (González-Muniesa et al., 2017).

Adipose tissue function and composition

To understand the pathophysiology of obesity it is first necessary to understand how adipose tissue functions. Adipose tissue is considered as the most variable and complex organ in the body whose primary function is to control energy homeostasis. It has a mesenchymal origin, and it is mostly composed by cells called adipocytes, but it also contains preadipocytes, mesenchymal stem cells, vascular cells, and inflammatory cells. In particular, adipocytes act as a lipid reservoir preventing lipids to accumulate in other tissues, a phenomenon called lipotoxicity (Hafidi et al., 2019; Kahn et al., 2019). In the case of excessive nutrient intake, free fatty acids (FFAs) are stored in the adipocytes as triglycerides. In contrast, when there is a nutritional deficit or energy need, triglycerides are hydrolyzed into FFAs to provide energy (Choe et al., 2016). In addition to acting as energy repertoire, adipocytes also secrete a large number of factors with hormonal, autocrine, and paracrine properties (Kahn et al., 2019; Longo et al., 2019) that control the immune response, blood pressure, hemostasis, bone mass, and thyroid and reproductive function (Rosen and Spiegelman, 2006).

Types of adipose tissue

The adipose tissue (AT) has been traditionally classified according to its anatomic location and major cell type constituents. There are three major types of AT: white AT (WAT), brown AT (BAT) and beige AT. WAT represents more than 95% of total adipose mass, and BAT only represents 1% to 2% of fat. Regarding beige AT, it is complicated to quantify it because its cells are interspersed within the WAT (Kahn et al., 2019). Adipocytes from WAT and BAT derived from different cell lineages. BAT adipocytes share a common precursor with myocytes (the skeletal muscle cells) that are the Pax7+/Myf5+ stem cells. In another hand, WAT and beige adipocytes derive from Pax7- / Myf5- stem cells (Rosen and Spiegelman, 2006) (Figure 17).

Adipocytes from BAT and beige AT have multilocular droplets and high mitochondrial density. BAT is specialized in the use and dissipation of the energy derived from lipids to produce heat via the action of uncoupled protein 1 (UCP-1) located in the inner membrane of the mitochondria. BAT appears brown because of the high mitochondrion content and dense vascularization. On the other hand, beige AT has

interesting features because after cold exposure or adrenergic stimulation, it adopts features of BAT, including increased UCP-1 expression, mitochondrion density and vascularization. This process is called adaptive thermogenesis, or ‘browning’ (Kwok et al., 2016; Kahn et al., 2019).

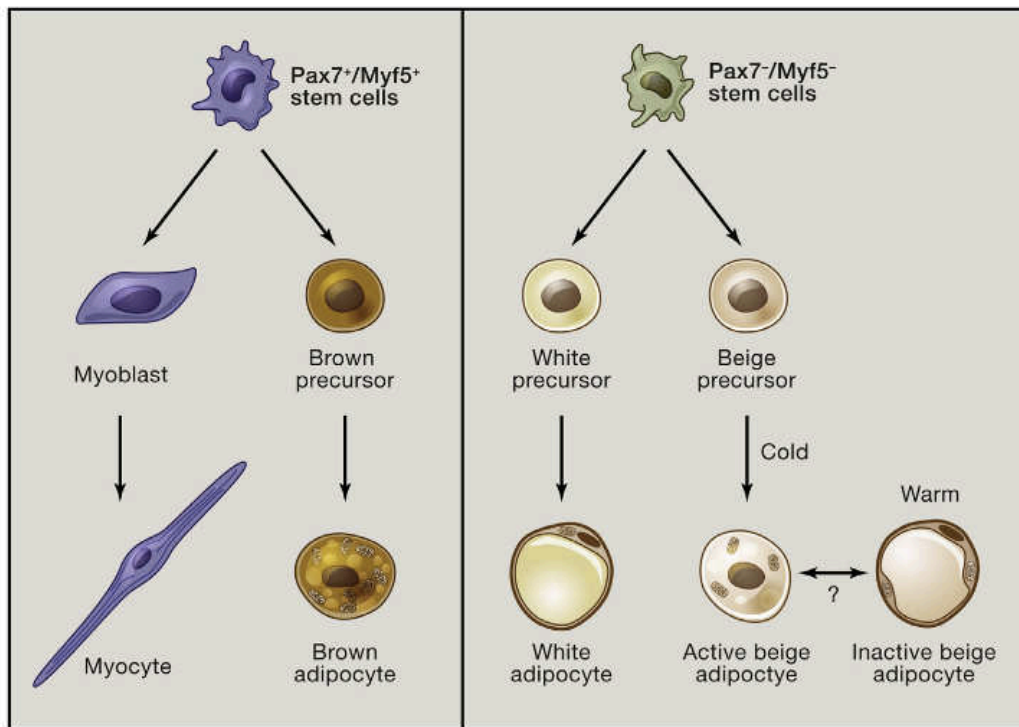


Figure 17. Cellular lineage of the different types of adipocytes. Adipocytes from BAT derived from Pax7⁺/ Myf5⁺ stem cells as myocytes. Adipocytes from WAT beige AT derived from Pax7⁻/ Myf5⁻ stem cells. Image obtained from Rosen and Spiegelman (Rosen and Spiegelman, 2006).

WAT is characterized for having adipocytes with a large unilocular lipid droplet (Kahn et al., 2019). This tissue comprises two major depots, the visceral (VAT) and subcutaneous (SAT) adipose tissue. VAT is concentrated in the abdominal cavity (Choe et al., 2016) and is further subdivided according to its anatomical localization into: perirenal, pericardial, omental and mesenteric. SAT is subdivided into cranial, facial, upper body, abdominal, gluteal, and femoral. In addition, it also exists the pink-SAT in the mammary glands. During lactation and pregnancy, these adipocytes transdifferentiate to milk-producing cells and return to adipocytes after the post-lactation period (Hafidi et al., 2019; Kahn et al., 2019) (Figure 18).

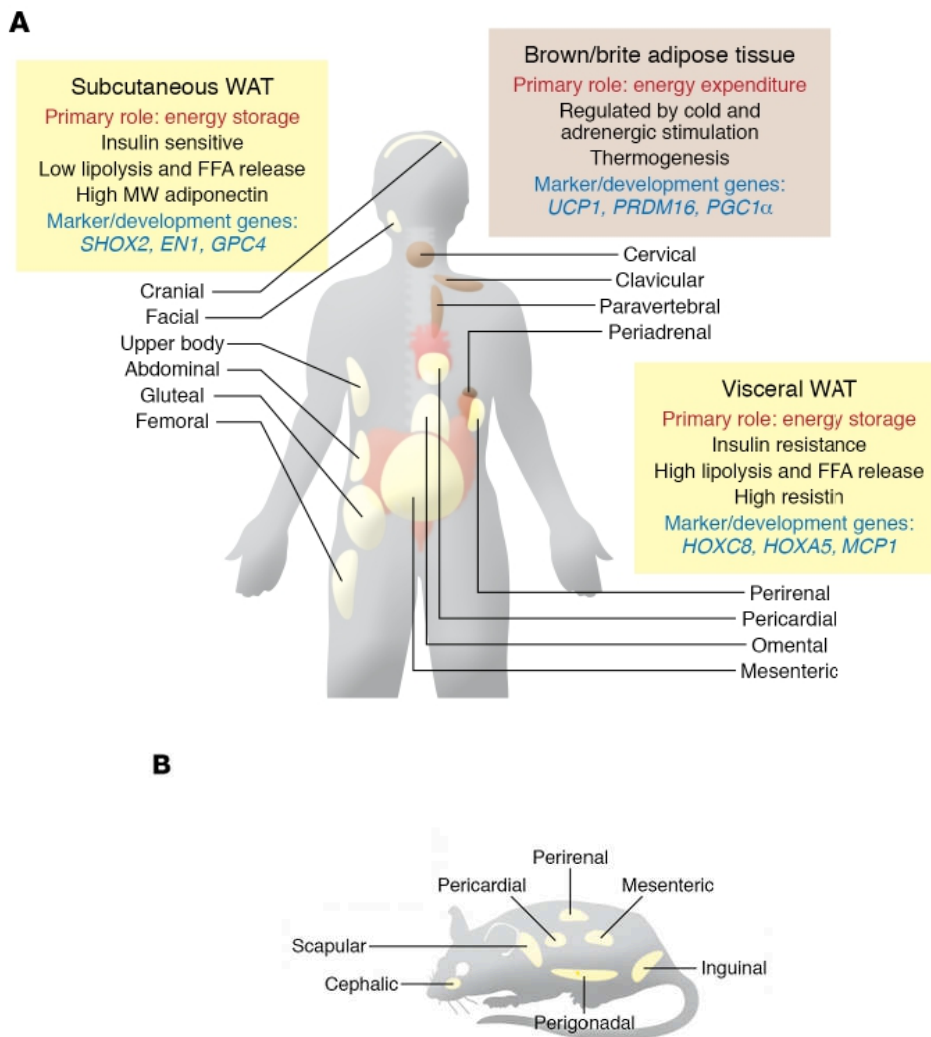


Figure 18. Heterogeneity of adipose tissue in humans and rats. A) Location of SAT, WAT and beige AT beige in the human. Each tissue has a different function and the cells that comprise them can be recognized by different cell markers. B) In rodents, WAT is also classified according to its location. Image adapted from Kahn *et al.*, (Kahn et al., 2019).

Adipose tissue as an endocrine organ

The adipose tissue is an important endocrine organ that secretes many factors with hormonal, autocrine, and paracrine properties. These factors are called adipokines and the most studied of them is Leptin, a protein produced primarily by white adipocytes which acts in the hypothalamus to suppress feeding and increase energy expenditure. Another adipokine is adiponectin, produced in the WAT and BAT, it acts in the muscle and the

liver to improve insulin sensitivity and to suppress hepatic glucose output. In addition, adiponectin can act in the CNS to stimulate appetite, reduce energy expenditure, and perhaps, affect neurodegeneration. In addition, adipose tissue produces resistin which, in mice, is produced mainly by VAT and induces insulin resistance. In contrast, in humans resistin is produced mainly by macrophages and its role in insulin resistance is less clear. There are other adipokines such as apelin, which is involved in the regulation of cardiovascular function; omentin, an insulin-sensitizing peptide produced by non-adipocyte cells in adipose depots; vaspin, thought to act as an insulin sensitizer; nesfatin-1 suggested to potentiate glucose-induced insulin secretion from β cells; DPP4 that degrades GLP-1; and asprosin that stimulates hepatic glucose release (Kahn et al., 2019).

Adipose tissue remodeling during obesity

Obesity occurs because the adipose tissue expands due to the excess fat that is stocked in mature adipocytes. This process is called hypertrophy and provokes adipocytes to develop and increase in size. When adipocytes have reached the critical cell size they get to a point of no return and present an abnormal function, which leads to insulin resistance, necrosis, cell death and inflammation. The adipose tissue then attempts to repair these metabolic alterations by enhancing the number of adipocytes, a process called hyperplasia (Choe et al., 2016; Hafidi et al., 2019; Longo et al., 2019) (Figure 19). Hyperplasia allows adipocytes to continue functioning in normal way, which is why it is the preferred way of adipose tissue expansion, because it protects against metabolic disease (Hammarstedt et al., 2018). It is important to note that hyperplasia permits some obese people to maintain a temporal “healthy” metabolic status, because they present an expansion of subcutaneous adiposity but not visceral. Nevertheless, it seems that this is a transient condition that will finally be directed to the typical metabolic complications of obesity (Longo et al., 2019).

During the development of obesity, the first place where fat accumulates is the subcutaneous adipose tissue, which is the largest adipose tissue depot in the body, the second is the visceral adipose tissue. When fat accumulation in these depots becomes excessive, fat stores in ectopic tissues such as the liver, skeletal muscle, heart, pancreas, and kidneys leading to lipotoxicity. The liver is particularly prone to the accumulation of

ectopic lipids which provokes the development of Non-alcoholic fatty liver disease (NAFLD) (Hammarstedt et al., 2018). NAFLD covers a range of liver disorders that are distinguished by excessive fat accumulation in this organ. It starts as hepatic steatosis, then non-alcoholic steatohepatitis (NASH) and it can progress to liver fibrosis, cirrhosis, and/or hepatocellular carcinoma (Vancells Lujan et al., 2021). Ectopic fat also accumulates in the pancreas where it contributes to β -cell dysfunction and to the development of type 2 diabetes (Longo et al., 2019).

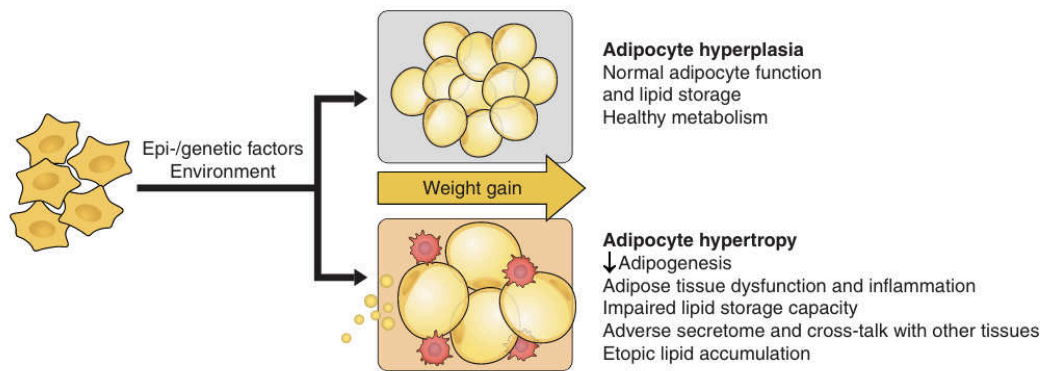


Figure 19. Mechanisms of adipose tissue expansion. Adipose tissue expansion is promoted by environmental (overnutrition) and genetic/epigenetic factors. Healthy expansion through hyperplasia protects against metabolic complications. In contrast, unhealthy expansion produced by hypertrophy promotes several metabolic complications like lipotoxicity. Image obtained from Hammarstedt *et al.*, (Hammarstedt et al., 2018).

Obesity-associated inflammation

A characteristic feature of adipose tissue expansion through hypertrophy is the development of a low-grade chronic inflammatory state. Inflammation is a biological defense response against harmful stimuli, such as pathogen invasion and cell damage (Choe et al., 2016). Inflammation in obesity is initiated when adipocytes produce an abnormal amount of adipokines and proinflammatory cytokines such as $\text{TNF}\alpha$, IL-6, MCP-1, iNOS, TGF- β 1, as well as procoagulant proteins (Hammarstedt et al., 2018; Hafidi et al., 2019). Moreover, there is a participation of innate (macrophages, mast cell, eosinophils, neutrophils) and adaptive (CD4^+ T cells and CD8^+ T cells) immune cells that are found in adipose tissue and contribute to the inflammatory response. Macrophages

(the most abundant innate immune cells) are the major contributors to the inflammatory process. In obesity conditions, macrophages constitute up to 40% of all adipose tissue cells (Zatterale et al., 2019). There are two types of macrophages, the proinflammatory M1 and the anti-inflammatory M2. M1 macrophages are the dominating population in inflamed adipose tissue, whereas the anti-inflammatory M2 macrophages are the principal population in metabolically healthy adipose tissue. Macrophages clean death adipocytes but while performing this task, they deliver proinflammatory cytokines, ROS and reactive nitrogen species, which contribute to tissue damage and promote fibrosis (Hammarstedt et al., 2018; Hafidi et al., 2019).

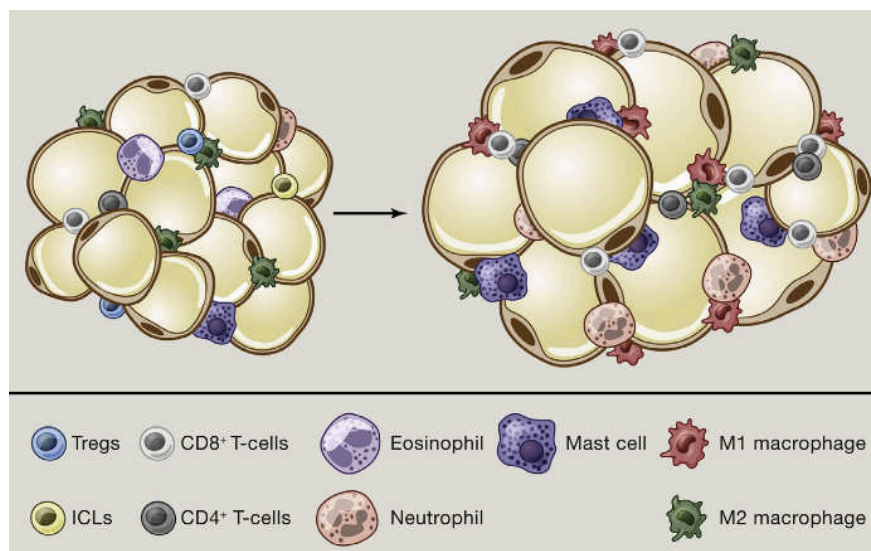


Figure 20. Cellular composition of adipose tissue in lean and obese conditions. Under healthy conditions (left) adipose tissue is composed of adipocytes, and innate and adaptative immune cells. There is a dominant presence of M2 macrophages, eosinophils, and Tregs cells. On the contrary, in obese conditions (right), there is a greater dominance of M1 macrophages, mast cells, and various T lymphocyte classes. Image obtained from Rosen and Spiegelman (Rosen and Spiegelman, 2006).

Hypertrophic adipocytes have elevated basal levels of lipolysis which promotes the release of free fatty acids (FFAs) that activate Toll-like receptors (TLRs) of type 2 and 4 (TLR2 / TLR4). In this process there is a release of proinflammatory cytokines and the activation of the c-Jun NH2-terminal kinase (JNK) and the IKB-kinase/nuclear factor-

κ -light chain enhancer of activated b-cell (IKK- β /NF- κ B) signaling pathways. Both JNK and IKK inhibit insulin action through the serine phosphorylation and inhibition of the insulin signaling adapter proteins, IRS-1/2, thus avoiding IRS-insulin receptor coupling and reducing downstream insulin signaling and glucose uptake. FFAs release also induces endoplasmic reticulum (ER) stress that triggers cell apoptosis/autophagy and induction of inflammation. Mechanistically, FFAs affect the ER by altering the composition of the ER membrane and by blocking the ER-specific Ca²⁺-ATPase pump, which results in accumulation of misfolded proteins (Hammarstedt et al., 2018).

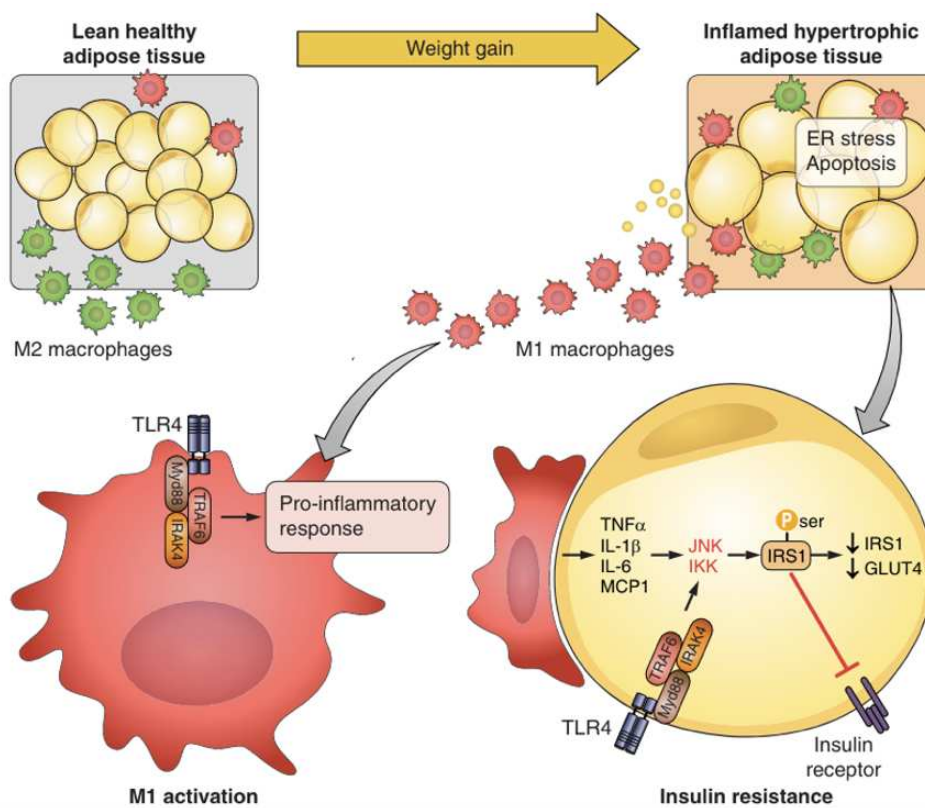


Figure 21. Role of inflammatory signals produced by adipocytes and macrophages in insulin resistance. Hypertrophic adipose tissue releases free fatty acids (FFA) that activate TLR receptors on macrophages, this leads to an exacerbation of the release of proinflammatory cytokytes, endoplasmic reticulum stress, activation of JNK and IKK signaling pathways and finally insulin resistance. Image adapted from Hammarstedt *et al.*, (Hammarstedt et al., 2018).

Adipocyte hyperplasia requires the formation of new vessels, which in turn allow adipocyte differentiation. However, the hypertrophic expansion stage of the adipose tissue is not accompanied by a similar expansion rate of angiogenesis (Hammarstedt et al., 2018). The deficiency of vasculature induces local hypoxia which is produced by the activation of the transcription factor called hypoxia-inducible factor (HIF)1 α (Choe et al., 2016). Hypoxia leads to lower mitochondrial respiration and biogenesis, a rise in the concentration of leptin and reduced adiponectin levels (Hafidi et al., 2019). All this abnormalities promote a necrotic-like phenotype in hypertrophic adipocytes which impairs adipose tissue function and increases inflammation (Choe et al., 2016). Finally, adipocytes, as other cells, release microvesicles that can contain RNA, proteins, and lipids. It has been reported that some microRNA (miRNAs) contained in these microvesicles are able to promote M1-like polarization and to inhibit cell insulin signaling adipose tissue (Hammarstedt et al., 2018). In addition, miRNAs can be transferred to small adipocytes and stimulate lipogenesis and adipocyte hypertrophy (Kahn et al., 2019).

Cognitive decline induced by obesity

The brain represents only 2 % of a human body's weight but it accounts for 20 % of its resting metabolism. In particular, the neural processing of information is metabolically expensive (Attwell and Laughlin, 2001) and to maintain a correct functioning it is necessary to ingest a correct quantity and quality of nutrients, including macronutrients and micronutrients. In the case of macronutrients, the WHO recommends a carbohydrate intake of 55-75%, a protein intake of 10-15%, and a fat intake of 15-30% (Muth and Park, 2021). In obese conditions, this energy requirement is not covered because people consume a greater amount of saturated fats and refined sugars which have serious consequences on the functioning of the brain.

Obesity affects the cognitive capacity of people of different ages. In children obesity affects working memory (Wu et al., 2017) and impairs cognitive functions, especially attention, retention, intelligence, and cognitive flexibility (Meo et al., 2019). In young adults obesity induces lower performance in memory tasks including the what–where-when memory test, the object identification, location memory, and temporal order

memory (Cheke et al., 2016). In midlife adults, obesity has been related with Mild Cognitive Impairment (Rochette et al., 2016), dementia, Alzheimer disease (no vascular dementia) and vascular dementia (with or without Alzheimer disease) (Fitzpatrick et al., 2009). The risk of developing dementia, Alzheimer disease and vascular dementia increases when obesity is presented in the midlife, but not during late life (Fitzpatrick et al., 2009). In fact, when using BMI as parameter, obesity at the age of 50 years is associated with a risk of dementia but not at 60 or 70 years (Singh-Manoux et al., 2018). Surprisingly, some reports pointed out that obesity protects against cognitive impairment in old age (Zhang et al., 2017). However, it is suggested that this discrepancy could be explained because of an underestimation of adiposity. Aged people tends to have less lean body mass and enhanced white adipose tissue without weight gain, but this cannot be differentiated when using the BMI as a tool for classifying obese aged people (Monda et al., 2017).

How the brain is affected by obesity is not completely understood, however, it has been observed that obese people have a decreased brain volume (Ward et al., 2005). This volume reduction particularly affects the frontal lobes, anterior cingulate gyrus, hippocampus, thalamus (Raji et al., 2010), precuneus, cingulate and orbito-frontal gyri (Driscoll et al., 2011). Remarkably, obese patients who already have mild cognitive impairment or Alzheimer's disease also present volume deficits in the frontal, temporal, parietal, and occipital lobes (Ho et al., 2010).

To better understand the mechanisms by which obesity impairs brain functioning, researchers have used rodent models of diet-induced obesity. These diets can be enriched in fat, High Fat Diet (HFD), in sugar, High Sugar Diet (HSD), or in both. Those diets result in increased adiposity and several types of neurodysfunction. For example, HFD produces impaired spatial learning (Valladolid-Acebes et al., 2011; Wahid et al., 2021) and object recognition memory (Gainey et al., 2016). HSD impairs spatial memory (Hernández-Ramírez et al., 2021), and a high caloric diet including sugar and fat results in recognition memory impairment (Paulo et al., 2021; Garcia-Serrano et al., 2022). In general, it has been observed that the hippocampal and pre-frontal cortex (PFC)-dependent tasks are negatively impaired by these diets, whereas amygdala-dependent

function seems to be enhanced. In addition, cognitive impairments are accompanied by addictive behaviors driven by the dopaminergic reward system which includes direct neuronal projections from the ventral tegmental area (VTA) to the amygdala, to the PFC and to the nucleus accumbens (NAc), Direct projections from the VTA to the hippocampus are on current debate (Morin et al., 2017) (Figure 22).

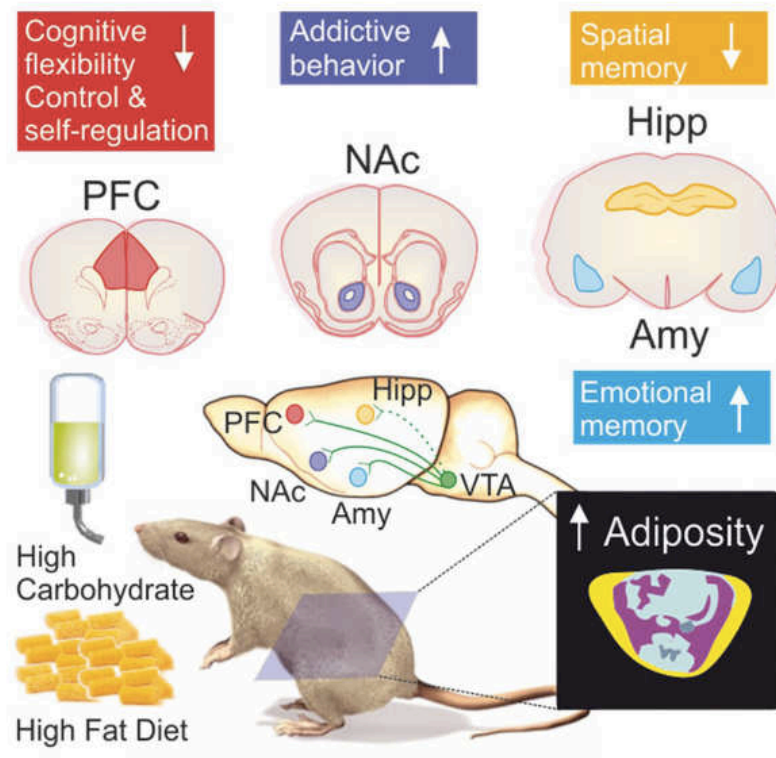


Figure 22. Brain regions areas affected by obesity in rodent models. Rodent models fed with hypercaloric diets present reduced cognitive flexibility, self-regulation and spatial memory, in addition they have increased addictive behavior and emotional memory. Image obtained from Morin *et al.*, (Morin et al., 2017)

Through the use of rodent models of obesity, key points have been identified that could be underlying obesity-associated cognitive deficiencies. The hippocampus is highly sensitive to the negative effects of obesity. In the hippocampus it has been observed a decrease in neuroplasticity as evidenced by the downregulated expression of synapsin and Brain-derived neurotrophic factor (BDNF), a key molecule involved in plastic

changes that mediates cell proliferation and survival (Miranda et al., 2019)) (Wahid et al., 2021), as well as by a decreased density of dendritic spine and synaptic plasticity (Morin et al., 2017). In the hippocampus there is also an activation of microglia, referred as gliosis that promotes neuroinflammation and deteriorate memory (Wahid et al., 2021) (Figure 23).

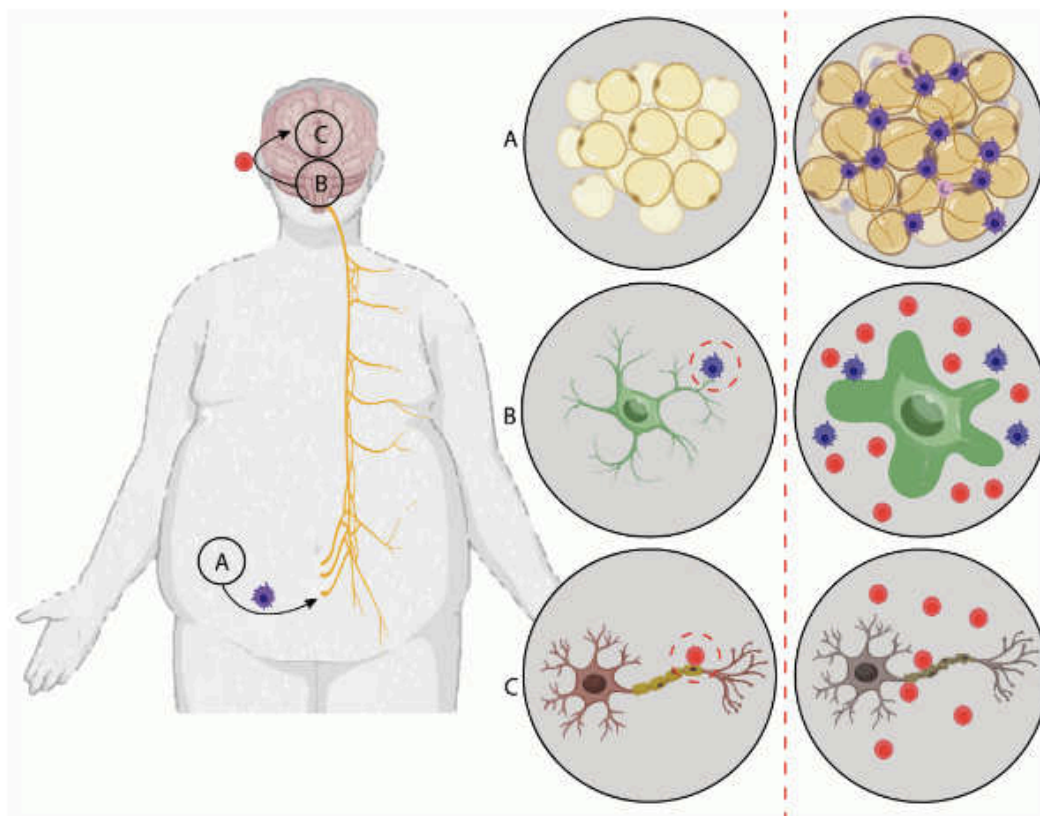


Figure 23. Stages in the process of obesity-induced neurodegeneration. A) During obesity, adipose tissue expands by hypertrophy which promote the release of proinflammatory cytokines that will circulate through the vagus nerve and cross the blood brain barrier. B) In the brain, inflammatory cytokines activate microglial cells which will secrete more proinflammatory cytokines. C) Finally, chronic neuroinflammation triggers neuronal damage. Image obtained from de A Boleti *et al.*, (de A Boleti et al., 2023).

How likely is it that obesity-induced memory deficits are underpinned by impaired tryptophan metabolism?

Several clinical reports have pointed out that obese patients display altered peripheral TRP metabolism which is reflected in high levels of KYN, KA, and QA in serum (Favennec et al., 2015) as well as an increased KYN/TRP ratio. The enhanced KYN levels are more evident in obese adults than in young people (Mangge et al., 2014). In addition, obese patients have decreased levels of 5-HT, increased levels of 5-hydroxyindoleacetic acid and decreased levels of several indole metabolites (Cussotto et al., 2020). The incremented levels of TRP metabolites has been associated with the overexpression of the enzymes IDO1, KYNU, KMO, and KAT III. These enzymes are expressed in both, macrophages and adipocytes and are induced by proinflammatory cytokines. (Favennec et al., 2015). Overactivation of IDO is of particular interest as it promotes TRP metabolism via the kynurenine pathway. IDO expression is stimulated primarily by IFN γ , but also by TLR4, IL-1 β and TNF α (Savitz, 2020).

As discussed in Chapter 2, increased levels of KYN, KA and QA in the brain have been observed in various psychiatric diseases and this has been associated with a state of neuroinflammation. If we consider that obesity produces a peripheral inflammatory state that is subsequently reflected in the brain, and if we consider that under inflammatory conditions microglia are activated, then we could expect that obesity will induce the overproduction of kynurenines in the brain that would probably trigger cognitive decline. An additional and interesting fact is that this effect could be regulated in a sex dependent manner. Indeed, obese women do present an inflammatory state as men, however, they also have an additional element that could modulate TRP metabolism. That is, the production of estrogens in the adipose tissue. In the following chapter we will analyze the combined effect of estrogens and obesity in the regulation of TRP metabolism.

Chapter 4.

*Role of estrogens in the regulation of
tryptophan metabolism and cognitive
function*

Males and females differ in their reproductive system, behavior, stress response, immune response, metabolic and cardiovascular functions, and brain functioning. Moreover, the physiological responses in females are dynamically modulated by their reproductive cycle (Abo et al., 2022) which is a natural process that leads to fertilization and pregnancy. In addition to its primary function, the reproductive cycle can also modulate the emotional, cognitive, and behavioral functioning (Sato et al., 2016; Schmalenberger et al., 2021). During the reproductive cycle there are fluctuations of estrogens which are the ovarian hormones. Some studies have reported that estrogens enhance cognitive performance in certain tasks because they promote synaptic transmission (Le et al., 2020). In the brain, glial cells are highly sensitive to estrogenic activity (Hidalgo-Lanussa et al., 2020) and under an inflammatory state, estrogens can affect TRP metabolism (Xu et al., 2015), which could probably have a negative impact in cognitive function.

The human menstrual cycle

The menstrual cycle is the physiological process in which sex hormone fluctuations alter the uterus lining and drive ovarian maturation of eggs so fertilization and pregnancy can occur (Le et al., 2020). In women, this process repeats monthly from menarche (the first menstrual bleed during puberty) to menopause. The average menstrual cycle length is 28 days, starting the first day of menses and ending the day before the subsequent bleeding onset (Schmalenberger et al., 2021).

The hypothalamus–pituitary–gonadal (HPG) axis controls the menstrual cycle. First, the hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). Both LH and FSH stimulate the ovaries to produce estrogen and progesterone which in turn will negatively feed back to the hypothalamus and pituitary to decrease the secretion of GnRH, LH, and FSH (Le et al., 2020). The feedback between the HPG axis and the ovaries triggers fluctuations of estrogens and progesterone during the menstrual cycle (Schmalenberger et al., 2021) that will promote the cyclical changes of the mucosal lining of the uterus (endometrium) (Chumduri and Turco, 2021) (Figure 24).

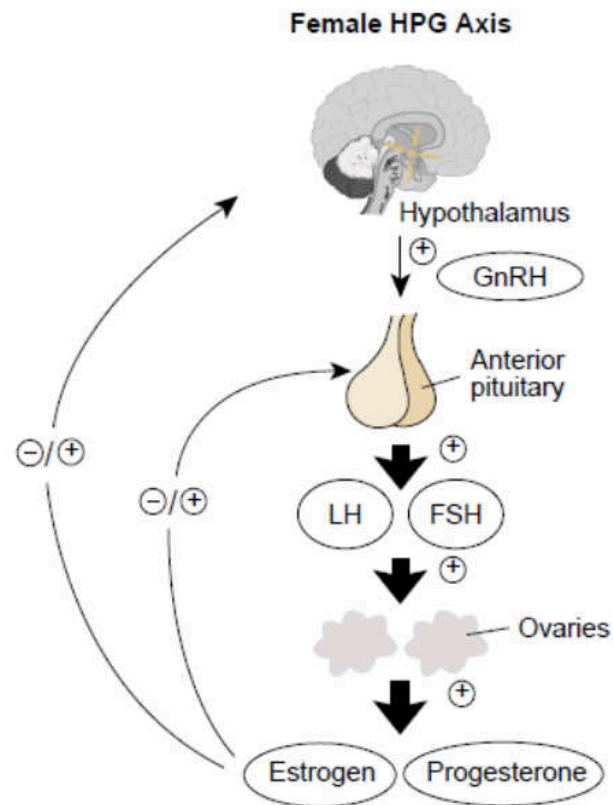


Figure 24. Communication between the hypothalamus–pituitary–gonadal (HPG) axis and the ovaries. The hypothalamus secretes GnRH, that stimulates the anterior pituitary to produce LH and FSH. Then, LH and FSH stimulate the ovaries to produce estrogen and progesterone which will negatively feed back to the hypothalamus and pituitary to decrease the secretion of GnRH, LH, and FSH. Image obtained from Kong *et al.*, (Kong *et al.*, 2014)

The menstrual cycle is divided in follicular and luteal phases which lasts approximately 16.9 days and 12.4 days, respectively. However, some variations in the duration could depend on the woman's body weight and age (Bull *et al.*, 2019; Abo *et al.*, 2022). The follicular phase begins with the onset of menses (which means the shedding of the proliferated endometrium) and finishes the day of ovulation (when an oocyte is released by the dominant follicle). At the beginning and during the mid-follicular phase, progesterone (P4) levels remain consistently low, in contrast, estrogens (estradiol-(E2))

rises gradually through the mid-follicular phase reaching its peak at ovulation (Le et al., 2020; Schmalenberger et al., 2021; Abo et al., 2022). During ovulation, the follicle ruptures to release the oocyte from the ovarian surface epithelium (OSE) (Chumduri and Turco, 2021). One day after ovulation, it starts the luteal phase and will continue until the day before menses. In this period, the dominant follicle that remains transforms into the corpus luteum. The corpus luteum produces P4 and E2; thus, there is a gradual augmentation of those hormones. The mid-luteal phase is characterized by peaking of P4 and a secondary peak of E2. If there is not fertilization of the oocyte, the corpus luteum involutes, causing rapid perimenstrual withdrawal of E2 and P4 and triggering menstruation. With this subsequent menstrual onset, the cycle begins again (Schmalenberger et al., 2021; Abo et al., 2022) (Figure 25).

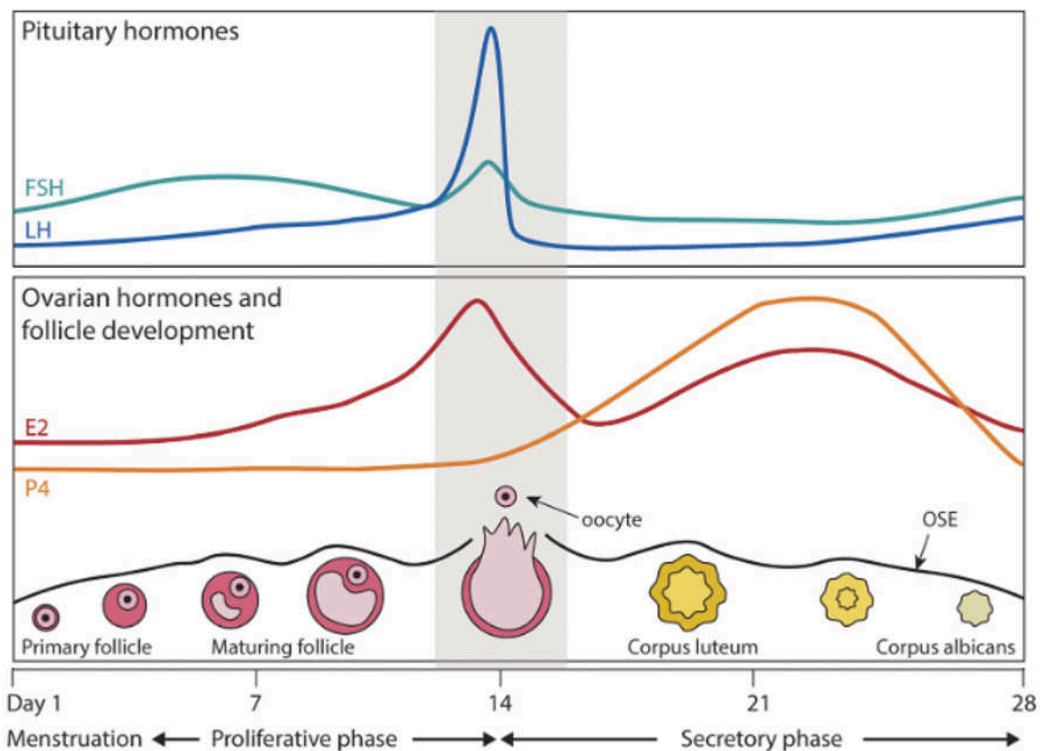


Figure 25. The menstrual cycle in women. In the follicular or proliferative phase, FSH promotes the growth of ovarian follicles that will increase the estrogen (E2) levels. E2 rises their maximum peak in the middle of the cycle resulting in a surge of LH and the release of the oocyte, an event known as ovulation. After this event, it starts the luteal or

secretory phase, where the levels of progesterone (P4) augment, provoking the differentiation of the endometrium to prepare for implantation. In the absence of implantation, P4 levels drop triggering menstruation. Image adapted from Chumduri and Turco (Chumduri and Turco, 2021).

The oestrous cycle in rats

As for women, other mammalian animals have a similar reproductive cycle that varies among species, depending on their environment or reproductive strategy. In rodents, the reproductive cycle is known as oestrous cycle, a name derived from the Latin adaptation of the Greek word “oistros” which means “sexual season”, “frenzy”, or “madness”. The oestrous cycle repeats with a constant period of four to five days, and it starts since puberty (the 4th postnatal week). In contrast to women, in rodents both ovaries exhibit the same morphological changes throughout the cycle and ovulate bilaterally. Since the ovulation number is relatively large (approximately 12 to 14 overall), a large number of follicles and corpora lutea can be observed in the ovaries (Sato et al., 2016; Ajayi and Akhigbe, 2020).

The rodent oestrous cycle is divided in four phases: proestrus, estrus, metestrus and diestrus. The first two phases are equivalent to the follicular phase in women, while the next two are like the luteal phase. During proestrus, the follicles mature rapidly until ovulation. At this stage, two to four large follicles can be observed, and estrogen levels increase. The subsequent phase is estrus that starts at ovulation triggered by a surge in LH. After ovulation, FSH reaches a maximum peak which is associated with the rapid decline in the estrogen levels. Then, as in women, follicles are transformed into corpora lutea. These corpora lutea begin to regress at metestrus and continue their regression into diestrus (Sato et al., 2016; Ajayi and Akhigbe, 2020; Abo et al., 2022) (Figure 26).

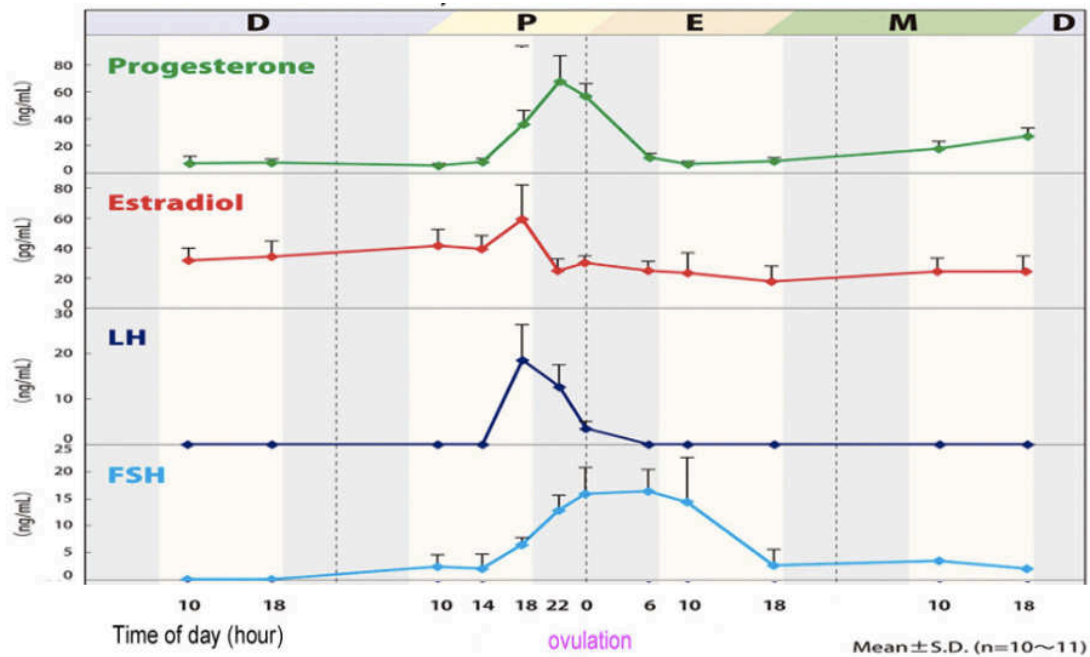


Figure 26. Fluctuation of pituitary and ovarian hormones during the oestrous cycle. D=diestrus, P=proestrus, E=estrus, M=metestrus. Image adapted from Sato *et al.*, (Sato *et al.*, 2016).

Cytological evaluation of the oestrous cycle in rats

When performing animal experimentation with female rats, it is necessary to learn to differentiate in which phase of the oestrous cycle they are, because their physiological responses may depend on it. There are several techniques to characterize each phase, however, the cytological analyze is the more practical because it only requires doing a vaginal lavage with water. When analyzing these samples under the microscope, three different populations of cells are observed: lymphocytes, round nucleated epithelial cells, keratinized squamous cells and leucocytes. The appearance and proportions of these cells permit to determine the four stages of the oestrous cycle (Lovick and Zangrossi, 2021). In proestrus there will be numerous round nucleated cells grouped in clusters or individually, and probably a few anucleated cornified epithelial cells. Then in estrus, there will be an abundance of anucleated cornified epithelial cells with a granular cytoplasm and irregular shape, and occasionally some nucleated epithelial cells. In metestrus, there will be a lot of leucocytes and a few non-granular and anucleated cornified epithelial

cells. Some nucleated epithelial cells may also be present. Finally, in diestrus there will be prominent polymorphonuclear leukocytes and a few epithelial and cornified cells (Ajayi and Akhigbe, 2020) (Figure 27).

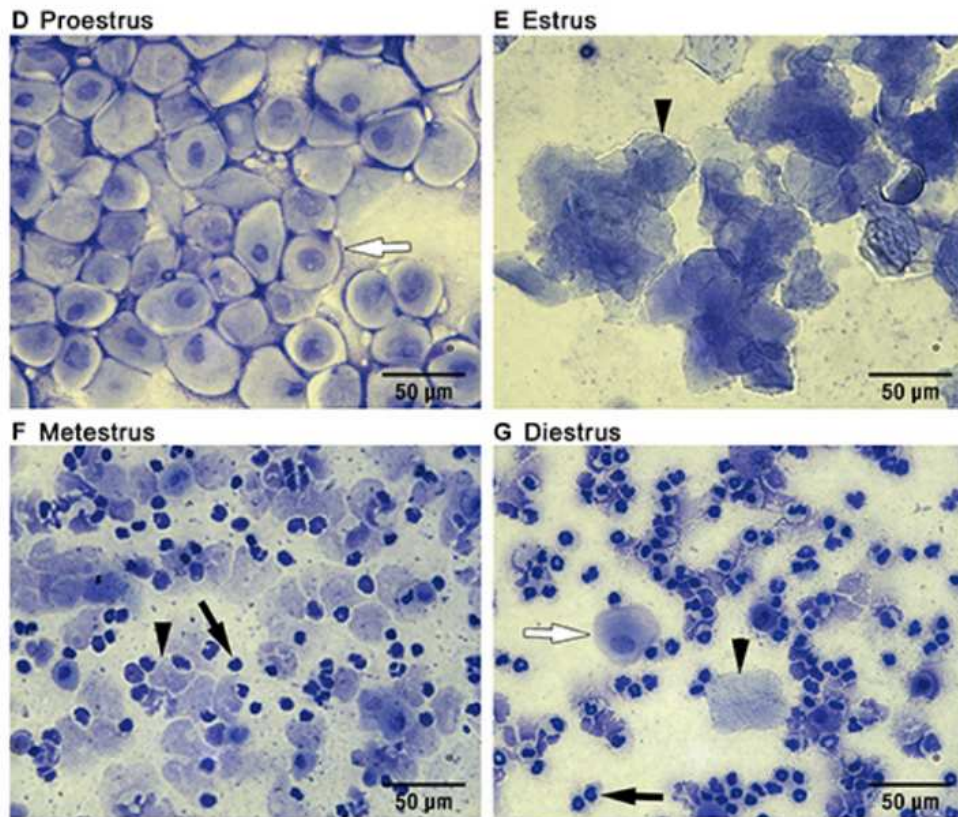


Figure 27. Cell populations characteristic of each stage of the oestrous cycle. Black arrowheads in estrus, metestrus and diestrus point to representative cornified squamous epithelial cells. Black arrows in metestrus and diestrus point to representative leukocytes. White arrows in proestrus and diestrus highlight representative nucleated epithelial cells. Image obtained from McLean *et al.*, (McLean *et al.*, 2012).

Estrogen synthesis

Once described how estrogens are modulated during the female reproductive cycle, we will now delve deeper into how these hormones are produced. There are three major forms of estrogens in females: estrone (E1), estradiol (E2, or 17 β -estradiol), and estriol

(E3). E2 is the major product from the whole estrogen biosynthesis, and it is produced by the ovaries, it is also the most potent estrogen in the premenopausal period. E1 is synthesized in the adipose tissue and plays a larger role after menopause. E3 is the least potent estrogen and is formed from E1 through a 16 α -hydroxylation, it is produced by the placenta and plays a larger role during pregnancy. The synthesis of estrogens depend on the reproductive status, reaching the highest levels during the reproductive years and decreasing in the postmenopausal period.

E2 can be produced in the ovaries and the brain. In the ovaries, estradiol synthesis needs the collaboration of thecal and granulose cells. The thecal cells cannot produce estrogen, but do produce androgens; in contrast, granulose cells do not produce androgens, but converts them into E2 by the action of the P450aromatase, then E2 are released into circulation. Even though estrogens can pass across the blood brain barrier, they are also synthesized within the brain from testosterone or cholesterol. This process is only performed by neurons and astrocytes, but not by microglia or oligodendrocytes. The process starts by the action of the enzyme P450 side chain cleavage (P450scc) which converts cholesterol to pregnenolone that is then converted by the aromatase in E2 (Figure 28).

In the brain, P450scc is present in the hippocampus, hypothalamus, amygdala, caudate nucleus, thalamus, cerebral cortex, cerebellum, and some circumscribed limbic areas (Cui et al., 2013). On the other hand, aromatase is found in the hypothalamus, amygdala, pons, thalamus, hippocampus, temporal cortex, frontal cortex, preoptic area, putamen, cerebellum and white matter. In the case of the rat, P450scc is found in the hippocampus and aromatase in the hypothalamus, amygdala, preoptic area, hippocampus, bed nucleus of the stria terminalis, cingulate cortex, brain stem and cerebellum. Aromatase is principally located in pyramidal neurons of CA1-CA3 regions, and in granule neurons in the dentate gyrus, more precisely in the pre- and post-synaptic compartments (Brann et al., 2022).

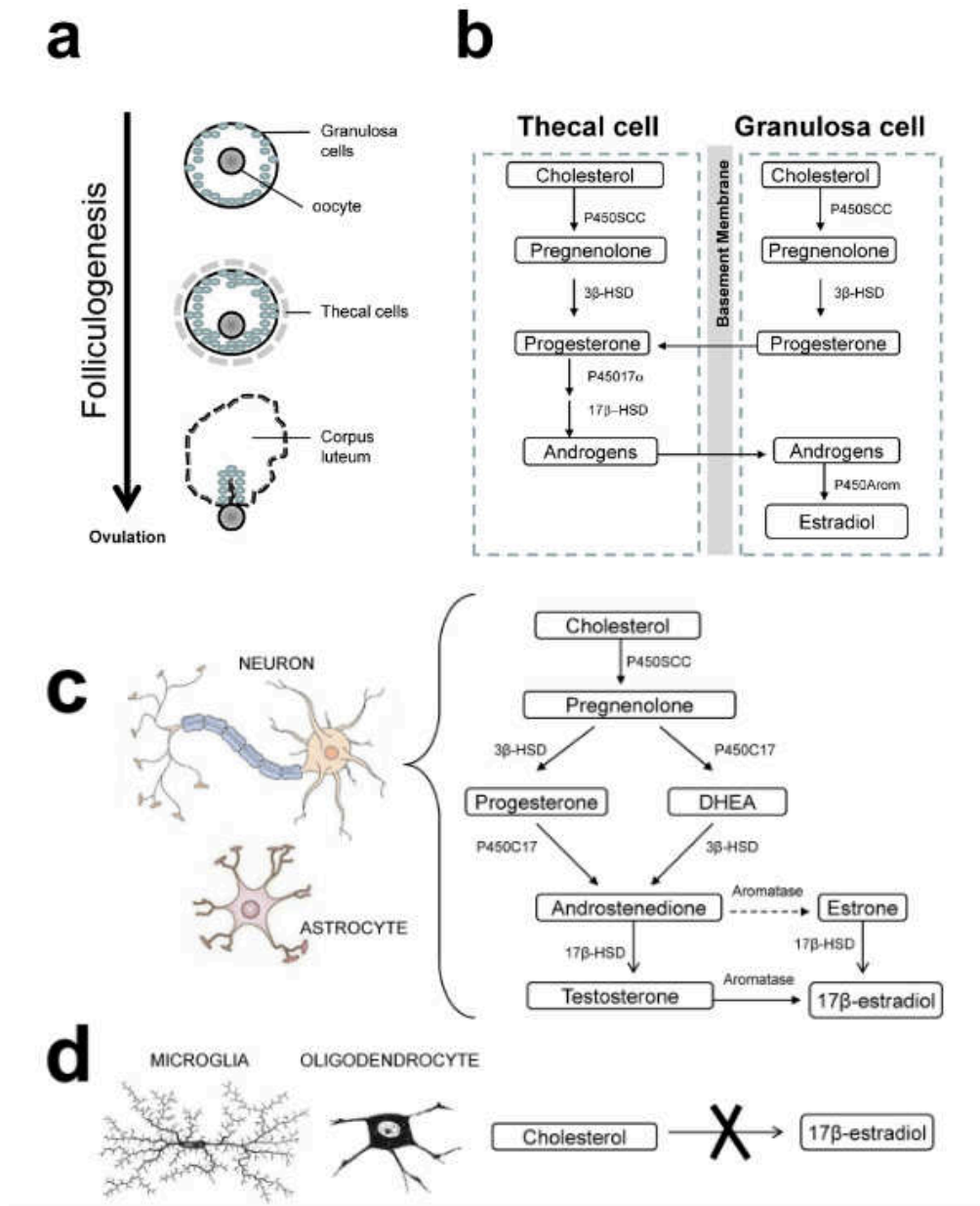


Figure 28. Estrogen synthesis in the ovaries and brain. A) During folliculogenesis the thecal cells form a layer surrounding the granulosa cells when the follicle is activated. Then thecal cells luteinize to form the corpus luteum after ovulation. B) The production of estrogens is catalyzed by the action of the cytochrome P450 side chain cleavage enzyme (P450scc). C) Neurons and astrocytes express all enzymes required for estrogen synthesis but not Microglial cells or oligodendrocytes (D). Image obtained from Cui *et al.*, (Cui *et al.*, 2013).

Estrogen's receptors and their relevance in cognition

Estrogens bind to two types of receptors, the classical nuclear receptors, ER α and ER β (Estrogen receptors α and β) and the novel cell surface membrane receptors GPR30 and ER-X (G protein-coupled receptor 30, and estrogen receptor X) (Cui et al., 2013). ER α receptors are predominantly expressed in the uterus and the pituitary gland, but they are also expressed in the liver, hypothalamus, bone, mammary gland, cervix, and vagina. On the other hand, ER β is expressed in the ovary, lung, and prostate. ER β expression is especially high in the ovary and is found exclusively in the granulosa cells (Hamilton et al., 2017). In the brain, ER α and ER β are expressed in the hippocampus and the cortex (Orikasa et al., 2000; Scudiero and Verderame, 2017)

In the brain the activation of estrogen receptors stimulates the transcription of genes linked to neuronal growth, neuroprotection, and the maintenance of memory function (Kumar and Foster, 2020). All estrogen receptors including the classical nuclear receptors, and G protein-coupled estrogen receptors produce beneficial memory effects. This has put in evidence by using specific agonists which rapidly activate kinase-signaling and have complex dose-dependent effects (Bean et al., 2014). In particular, ER β increases the production in vivo of key synaptic proteins including PSD-95, synaptophysin, the AMPA receptor subunit GluN1 (Liu et al., 2008), CREB and BDNF (Koyuncuoğlu et al., 2019). The activation of ER β receptors enhances LTP in the hippocampus and increase dendritic branching and spines density (Liu et al., 2008). On the other hand, activation of the G protein-coupled estrogen receptor-1 (GPER1) increases the potentiation of synaptic transmission in the entorhinal cortex which is a major source of cortical sensory and associational input to the hippocampus (Batallán Burrowes et al., 2021) (Figure 29).

Estrogen depletion and cognitive impairment

Under healthy conditions it is normal that women present a decrease in estrogen levels during the menopause transition. In fact, the serum levels of E2 decrease by 85–90% and those of E1 by 65–75% in menopausal women (Cui et al., 2013). Estrogen depletion in natural or surgical menopause is potentially associated with increased risk of depressive

disorders (Weber et al., 2014) and cognitive impairment. This is the reason why women suffer more frequently than men from Alzheimer's disease (Le et al., 2020), the most common neurodegenerative disease. Alzheimer's disease not only impacts a greater number of women but also causes more aggravated symptoms than in men, leading women to have poorer cognitive profiles than men at the same stage of the disease. For example, men have better performance than women when analyzing language and semantic abilities, visuospatial abilities and episodic memory (Laws et al., 2016). Although the loss of ovarian estrogens at menopause has been presumed to account for the increased Alzheimer's disease susceptibility in women, recent studies suggest that depletion of brain-derived estrogen is a more direct and significant risk factor for developing Alzheimer's disease (Cui et al., 2013).

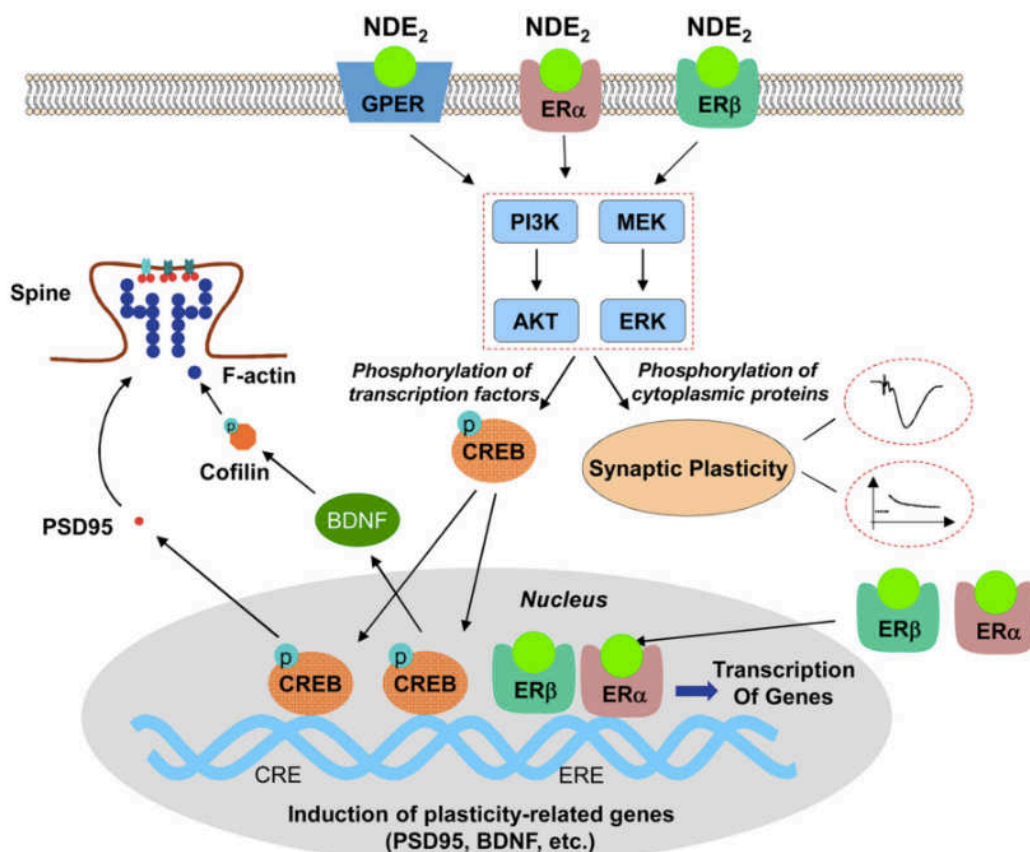


Figure 29. Role of neuron-derived estradiol (E₂) (NDE₂) in the regulation of synaptic plasticity. NDE₂ binds to ER α , ER β , and G-protein coupled estrogen receptor

(GPER) which activates the PI3K/AKT and MEK/ERK kinase signaling and promotes the expression of genes involve in regulation of spines, synapses and LTP. The activation of intracellular kinase signaling phosphorylates CREB (a transcriptional factor) to induce the expression of BDNF and PSD95. Moreover, BDNF signaling activates cofilin which is required for F-actin assembly and dendritic spine formation. The intracellular ER α and ER β can act as transcriptional factors that will activate the estrogen response elements (ERE) and promote the expression of genes involved in synaptic plasticity. Image obtained from Brann *et al.*, (Brann et al., 2022).

Interaction of obesity and estrogens in the regulation of cognition

Evidence shows that obesity aggravates the deleterious effect of estrogen depletion in cognition. This was put it evidence in obese-ovariectomized rats that presented cognitive impairment earlier than the ovariectomized-non obese rats or the obese-non ovariectomized rats. Estrogen deprivation and obesity appears to individually cause peripheral insulin resistance, increased brain oxidative stress, hippocampal synaptic dysfunction, brain mitochondrial dysfunction and brain insulin resistance, contributing to cognitive impairment (Pratchayasakul et al., 2015). Moreover, there is another study showing that both, aging and the consumption of a HFD induce differential expression patterns of ER α and ER β receptors in the hippocampus and the cortex. Aging produces a downregulation of ER α and ER β in the cortex whereas in the hippocampus the expression of ER α increases and that of ER β decreases. In the case of consumption of a HFD, only after 4 weeks of eating this diet, ER β is downregulated in the cortex, and after 12 weeks, both ER α and ER β are upregulated. On the other hand, in the hippocampus ER α is upregulated and downregulated after the consumption of a HFD for, respectively, 4 and 12 weeks (Scudiero and Verderame, 2017).

The levels of estrogens can increase due to obesity. This event has extensively been studied in the context of breast cancer. Inflammatory mediators and adipokines enhance the expression of the aromatase gene promoting the increase in estrogen levels. One of them is prostaglandin E2 (PGE2). This inflammatory mediator inhibits p53 which is a negative regulator of the aromatase expression resulting in the elevated production of

this enzyme. The action of PGE2 can be exacerbated by the presence of IL-6 in the serum of obese patients as has been shown in breast cancer cells. Furthermore, adipokines can play an important role. Adiponectin which activates the liver kinase B1/ AMP-activated protein kinase (LKB1/AMPK) pathway triggers the suppression of aromatase transcription. Since obese patients have decreased levels of adiponectin, there is an overproduction of aromatase (Mair et al., 2020).

Regulation of tryptophan metabolism by estrogens

Estrogens modulate the enzymes that initiate the TRP catabolism. Overexpression of IDO induced by estrogens has been observed in the placenta, this effect protects the embryos against mother's immune system by inhibiting the T-lymphocyte reaction (Li et al., 2020). In addition, IDO is upregulated in samples of human breast cancer where there is also an overexpression of estrogen receptors (Soliman et al., 2013; Ghafouri-Fard et al., 2020).

In the same line, animal studies show that estrogens modulate TRP metabolism in the brain. Estradiol increases the expression of TPH2 in the dorsal raphe nucleus, bringing beneficial behavioral effects in rats (Hiroi et al., 2016). In contrast, estrogen depletion in ovariectomized rats provokes a decrease in the concentration of 5-HT and a depression-like behavior accompanied by an upregulation of IDO and inflammatory markers (IFN- γ , IL-6, TLR4) in hippocampus. Interestingly estradiol administration counteracts these effects (Xu et al., 2015).

In women, estrogen levels have fluctuations during the reproductive cycle that may impact brain TRP metabolism and cognitive capacity. First, a study showed that healthy young women (20–31 years) who were submitted to a procedure of acute dietary TRP depletion (in order to reduce serotonin levels) during the early follicular phase of their menstrual cycle (when the levels of estrogens are low), presented impaired short-term verbal memory, suggesting that this period is more vulnerable to serotonergic dysfunction (Helmbold et al., 2013). In another study, the same acute dietary TRP depletion procedure applied to healthy menopausal women, induced cognitive and

affective tasks failure, however, a treatment with estradiol counteracted these effects (Epperson et al., 2012).

Women's cognitive ability largely depends on maintaining adequate levels of estrogen and TRP. However, if there is not an adequate level of TRP and this happens at the same time as a decline in estrogen levels, the consequences on cognition are negative. If we consider that estrogens modulate the expression of IDO and TPH, then we could suppose that one of the causes of cognitive impair is due to alterations in the levels of kynurenines in the brain. Another factor that could influence this response is the presence of obesity since it increases estrogen levels at the peripheral level, but we do not know what their effect is at the central level.

In chapters 3 and 4 we have seen that obesity greatly affects cognitive ability and that this is linked to alterations in the metabolism of TRP. An interesting aspect is that obese mothers or fathers also compromise the cognitive capacity of their offspring even if they are not obese. The molecular and cellular mechanisms involved in this phenomenon are not fully understood, however, evidence shows that alterations in TRP metabolism could be involved. We will address this topic in the next chapter.

Chapter 5.

*Metabolic programming as a tryptophan
modulating factor*

In the recent years, hundreds of studies have reported that the metabolic state of mothers before, during pregnancy or during the breastfeeding period leave traces in their offspring that will last throughout their lives. This phenomenon is called metabolic programming. According to this concept, the positive or adverse conditions to which an individual is exposed during the first stages of his life will program him/her metabolically (Fall and Kumaran, 2019; Zhu et al., 2019). Mainly, maternal obesity predisposes the offspring to develop metabolic diseases and cognitive troubles during infancy and at adulthood. Although it may be obvious that maternal obesity contributes the most to the offspring's health, the possible negative effect that paternal obesity may exert should not be ignored (Billah et al., 2022). Indeed, new evidence indicates that paternal obesity may predispose the offspring to develop the same metabolic diseases than those induced by maternal obesity. So far, several mechanisms have been described by which maternal and paternal obesity can metabolically program the offspring, however, in this work we are particularly interested in the effect of metabolic programming on TRP metabolism, because this could trigger cognitive impairment.

Developmental origins of health and disease (DOHaD)

Some decades ago, scientists pointed out that environmental factors to which individuals were exposed during the early age, including the gestational stage, could represent risk factors for the development of future diseases. This proposal gave rise to the hypothesis of the "Developmental Origins of Health and Disease" (DOHaD) also called "The fetal programming hypothesis" (Alfaradhi and Ozanne, 2011; Shrestha et al., 2020). The DOHaD hypothesis has its roots in the findings of David Barker who analyzed a cohort in the United Kingdom and observed a relationship between reduced body weight at birth and the occurrence of ischemic heart disease in the adulthood (Barker and Osmond, 1986). Based on these observations, he proposed the concept of "thrifty phenotype" which states that poor nutrition during fetal development and the early infancy affects the development and function of the beta cells of the islets of Langerhans which could provoke Type 2 diabetes at adulthood (Hales and Barker, 1992). After him, several reports in humans and animals have enlarged and corroborated his findings and now it is clear that overnutrition and undernutrition, among others factors, alter the physiology, metabolism, and the epigenome of the offspring in a long-term (Billah et al., 2022).

Nowadays, it is more important to study the adverse effects due to maternal obesity than to maternal undernutrition because the rate of obesity has increased alarmingly. Actually, in 2009 approximately 30% of women in reproductive age were obese, since then this percentage has continued to increase (Schoonejans and Ozanne, 2021). Obesity during pregnancy is a risk factor for gestational diabetes, preeclampsia, thromboembolic events, cardiac, respiratory, and cerebrovascular morbidity, among other adverse events. In addition, in the obese pregnancy the fetus are at risk of miscarriage, intrauterine fetal death, preterm birth, among others (Stubert et al., 2018). Additionally, maternal obesity is a risk factor for the development of several diseases in the descendance (Shrestha et al., 2020). Actually, clinical reports show that a high BMI in the pregnant mother sensitizes the offspring to increased weight and adiposity during infancy (Olson et al., 2010; Chiavaroli et al., 2021), adolescence (Laitinen et al., 2012), and adulthood (Mamun et al., 2009; Tequeanes et al., 2009), leading to an increased risk of Type 2 diabetes, cardiovascular diseases (Eriksson et al., 2014), asthma (Harpsoe et al., 2013), and of premature death at adulthood (Reynolds et al., 2013).

The observations made in humans have been reproduced by studies in animal models who have shown that the offspring of obese dams display higher body weight and adiposity, higher systolic blood pressure, higher levels of triglycerides, cholesterol, glucose and insulin (Samuelsson et al., 2008; Sen and Simmons, 2010; Menting et al., 2019), than offspring from lean dams and a predisposition to develop type 2 diabetes, cardiovascular pathologies (Samuelsson et al., 2008; Schoonejans and Ozanne, 2021), dyslipidemia and hypertension (Menting et al., 2019). Moreover, maternal obesity accelerates the process of aging in the offspring, reduces locomotor activity and skeletal muscle mass (Samuelsson et al., 2008), decreases bone mineral density, induces osteoporosis (Liang et al., 2009), and accelerates immunological aging (Imai et al., 2019). Interestingly, it has been observed that the effects of maternal obesity could vary in a sex-dependent manner with males displaying a greater predisposition to develop type 2 diabetes than females (Samuelsson et al., 2008). Metabolic programming could be transgenerational transmitted, and this was observed when maternal obesity promoted an increased in the left ventricular mass in the offspring, and provoke cardiac mitochondrial abnormalities that affected the F3 generation (Ferey et al., 2019).

The principal factors by which maternal obesity impacts the development of the fetus, are the increased levels of lipids and glucose, hyperinsulinemia, alterations in the adipokine release, inflammation, placental dysfunction (Schoonejans and Ozanne, 2021) and microbiota dysbiosis. The sequence of adverse events begins in the placenta where there is an accumulation of lipids, activation of M1 macrophages, and alterations in the mitochondrial metabolism. These factors increase placental oxidative and endoplasmic reticulum stress (Brombach et al., 2022). Also, obesity decreases microbial diversity and shifts the microbial composition (Dreisbach et al., 2022), which could indirectly affect the development of the infant and the establishment of its microbiota (Zhou and Xiao, 2018) (Figure 30).

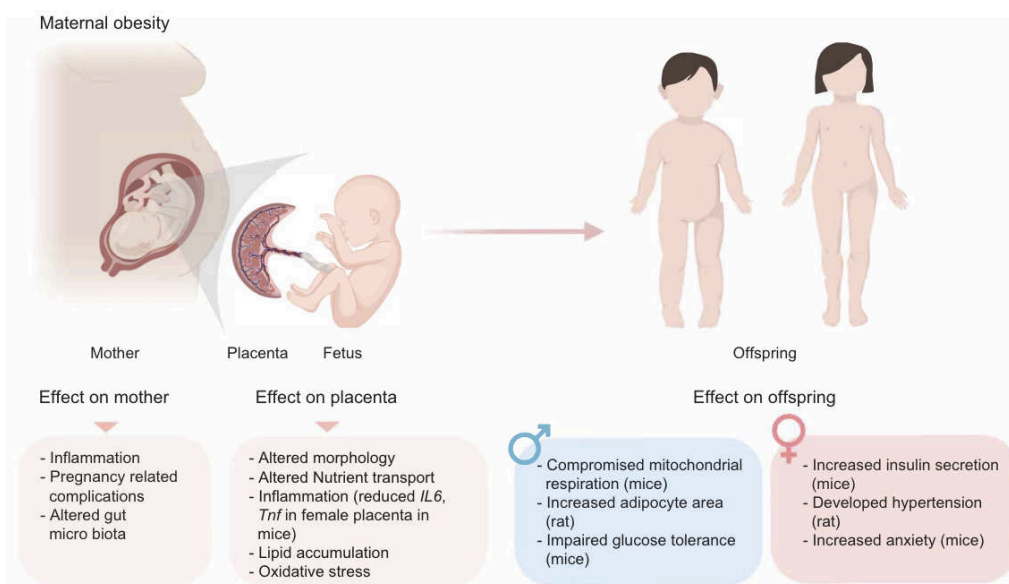


Figure 30. Adverse effects of maternal obesity during pregnancy. Obesity during pregnancy puts the mother and her offspring at risk. In addition, obesity causes metabolic changes in the placenta that will affect the metabolism of the fetus. Clinical and animal studies have shown that the offspring of obese mothers are prone to develop lipid dysfunction, type 2 diabetes, obesity, cardiovascular disease, among other diseases, which could be sex dependent. Image obtained from Shrestha *et al.*, (Shrestha et al., 2020).

Paternal origins of health and disease (POHaD)

In the recent years it was proposed a new concept called “Paternal Origins of Health and Disease” (POHaD) which emerged as an extension of the DOHaD paradigm (Soubry, 2018). According to this concept, the father’s health can also impact the development of the embryo as well as the metabolic and reproductive health of future generations. Most of this evidence has been obtained from animal studies because in humans it is almost impossible to study the effect of paternal health as an independent factor of maternal health in the offspring (Billah et al., 2022).

In humans, it was observed that the offspring of prenatally undernourished fathers who were born during the 1944–1945 famine in the Netherlands, were heavier and more obese than the offspring of fathers who were not undernourished prenatally (Veenendaal et al., 2013). Other research found that a higher paternal BMI had a negative impact on embryo development and pregnancy health (Billah et al., 2022), because it is associated with decreased blastocyst development and lower pregnancy rate (Bakos et al., 2011). Then, at birth, paternal obesity is associated with higher birth weight (Noor et al., 2019) and changes in biparietal diameter, head circumference, abdominal diameter, abdominal circumference, and pectoral diameter in children (Chen et al., 2012). It is interesting to note that men’s health has transgenerational effects. For example, a study showed a relationship between an increased mortality due to diabetes if the grandfather was exposed during puberty to a surfeit of food (Kaati et al., 2002).

In animals, the health status of the father influences offspring’s weight in different ways. While some studies have reported enhanced body weight in adult offspring of obese fathers (Shi et al., 2017), others have associated paternal obesity with reduced birth weight, reduced postweaning growth, less body weight at adulthood, and smaller fat pads and skeletal muscle mass (Lecomte et al., 2017). Moreover, paternal obesity is associated with impaired glucose tolerance and insulin resistance in the offspring (Wei et al., 2014; Shi et al., 2017). Interestingly, in some cases paternal obesity did not provoke glucose intolerance or altered levels of insulin, leptin or triglycerides in serum, but it increased muscle triglyceride content (Lecomte et al., 2017). In other cases, paternal obesity triggered a reduction in pancreatic beta-cell mass (de Castro Barbosa et al., 2016) or

induced impaired glucose metabolism, and exacerbated lipogenesis (Ornellas et al., 2015). Paternal obesity also provoked premature aging in the female offspring as shown by differential expression in retroperitoneal adipose tissue of genes involved in mitochondrial and cellular response to stress, telomerase signaling, cell death and survival, cell cycle, cellular growth and proliferation, and cancer (Ng et al., 2014). Like obese mothers, obese fathers can also influence the development of several generations provoking defects in glucose and lipid metabolism in the F2 generation (Cropley et al., 2016), or triggering obesity and insulin resistance in the F1 and F2 generations (Fullston et al., 2013) (Figure 31).

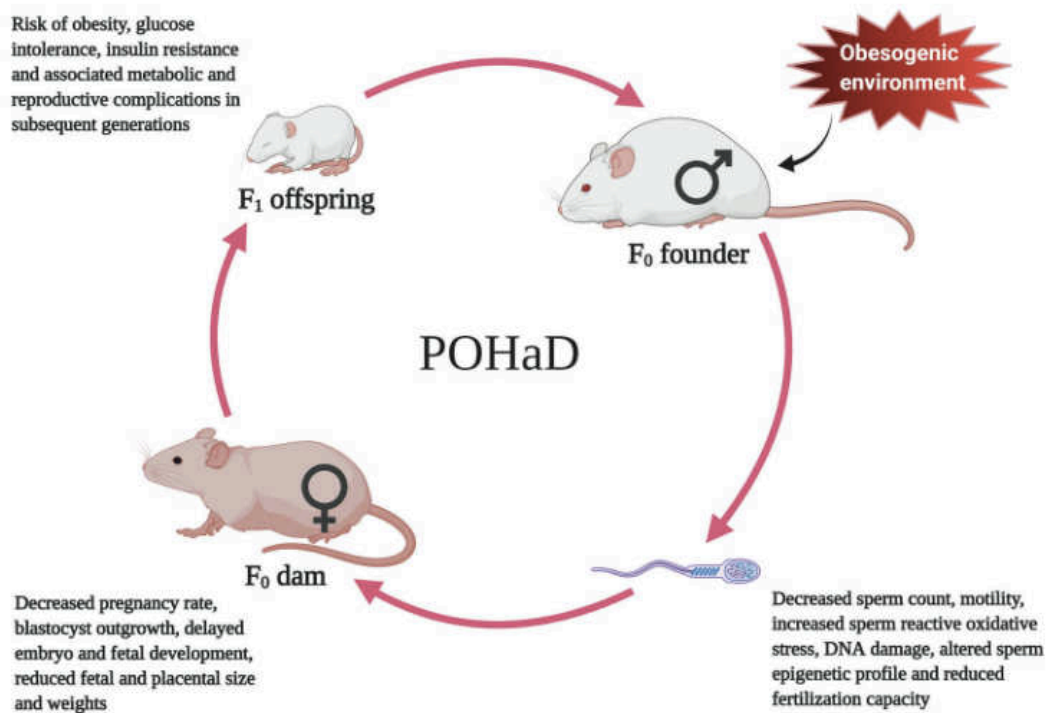


Figure 31. Paternal obesity has negative consequences on fertility, pregnancy and compromises the health of offspring. Obese males (F0) have less quality of sperm which affects pregnancy and predisposes the offspring (F1) to develop diseases at adulthood. Image obtained from Billah *et al.*, (Billah et al., 2022).

Elucidate how paternal obesity influences the development of his offspring is still challenging but evidence points out that epigenetic markers may play an important role. DNA methylation patterns transferred from parents to the offspring is one of the most studied epigenetic markers in the field of metabolic programming. For example, in humans, there was an association between an increased paternal BMI with a methylation pattern in the offspring that remained from 3 to 7 years of age (Noor et al., 2019). Another study showed that methylation patterns were also found in several imprint regulatory regions in children of obese parents (Soubry et al., 2015). In animals, paternal hyperglycemia induced impaired glucose tolerance and insulin resistance in the offspring, and authors found a relationship between the methylome patterns in sperm and changes in cytosine methylation in insulin signaling genes in the pancreatic islets of the offspring (Wei et al., 2014).

On the other hand, small RNA species have also been largely studied. For example, in one study, it was found that the transfer of several small RNA species from obese male fathers to their offspring was associated with defects in glucose and lipid metabolism in the F2 generation (Copley et al., 2016). In some studies, it has been found a combined effect of methylation patterns and transfer of microRNAs. For example, in mice it was found that obese fathers presented modifications in the content of microRNAs in sperm compared to healthy fathers in addition to a 25% reduction in global DNA methylation. These effects were associated with the development of obesity and insulin resistance in the offspring. (Fullston et al., 2013). It appears that once these epigenetic signals are present in the spermatozoa, they are transmitted to the offspring. This was put in evidence by a study that showed that the DNA methylation pattern and the profile of expression of some small non-coding RNAs in the sperm of rats fed with a HFD, were also present in the offspring's sperm (de Castro Barbosa et al., 2016) (Figure 32).

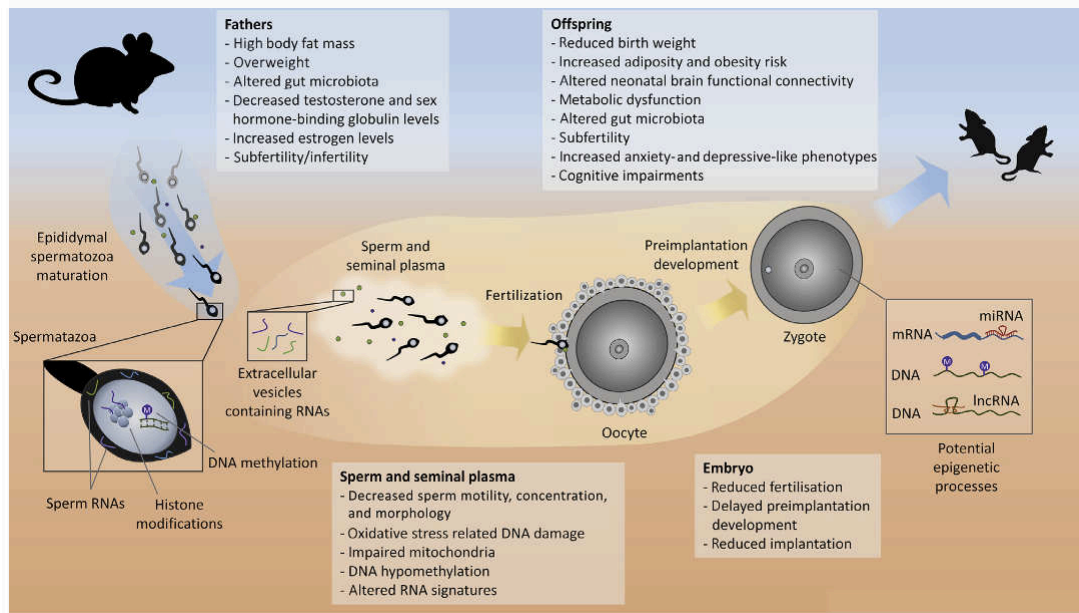


Figure 32. Paternal obesity predisposes offspring to developing metabolic and cognitive alterations via epigenetic modifications. Spermatozoa from obese fathers transfer different epigenetic markers such as microRNAs, histone modifications, and DNA methylation that predispose the offspring to develop metabolic and cognitive problems. The transfer of microRNAs can also occur through extracellular vesicles found in the seminal fluid. Moreover, the alterations produced in both spermatozoa and seminal fluid derived from obesity affect fertilization efficiency as well as zygote implantation. Image obtained from Bodden *et al.*, (Bodden et al., 2020).

Elucidate how does the sperm epigenome is affected by obesity is still challenging, nevertheless, it is believed that an unbalanced of ROS is the responsible of sperm alterations (Soubry, 2018). Obesity induces ROS and sperm is particularly susceptible to it. Spermatozoa need small amounts of ROS to acquire the ability of nuclear maturation regulation and condensation to fertilize the oocyte, but an unbalance can promote lipid peroxidation, DNA fragmentation, axonemal damage, denaturation of the enzymes, over generation of superoxide in the mitochondria, lower antioxidant activity and finally abnormal spermatogenesis (Sabeti et al., 2016) which has negative impact on energy balance of spermatozoa, sperm motility and capacity for fertilization (Soubry, 2018).

Early origins of neuropsychiatric and cognitive disorders

Nowadays, many scientists believe that there is an early origin of neuropsychiatric and cognitive disorders. Indeed, several lines of evidence indicate that maternal obesity impacts negatively the development of the offspring's brain. During pregnancy, the neural cells of the fetus proliferate, migrate, and generate functional neuronal circuits, this period is extremely sensitive to environmental factors which will have the capacity to shape and prime brain development (Monk et al., 2019; Davis and Mire, 2021). In humans, maternal obesity increases the risk in the offspring to develop autism spectrum disorder, developmental disorders (Li et al., 2016; Matias et al., 2021), attention deficit hyperactivity disorder (Chen et al., 2014), and mood disorders (Jo et al., 2015). It has also been observed that children born to obese mothers present less scores in verbal cognitive tests (Casas et al., 2017; Widen et al., 2018), lower reading and mathematics scores (Tanda et al., 2013) and poorer executive functioning affecting their attention, inhibitory control, and working memory (Mina et al., 2017; Fuemmeler et al., 2019). Animal studies have confirm these findings by showing that the offspring of obese mothers display impaired reference memory, working memory (Wolfrum and Peleg-Raibstein, 2019), object recognition memory and spatial memory (Tozuka et al., 2010; Cordner et al., 2019; Mizera et al., 2021).

Maternal obesity impacts several brain regions and in particular the hippocampus. Actually, reduced expression of insulin and leptin receptors (Ding et al., 2018; Cordner et al., 2019), insulin resistance (Schmitz et al., 2018), increased lipid peroxidation, reduced BDNF expression (Tozuka et al., 2010), microglial activation (Kang et al., 2014) and increased expression of the proinflammatory cytokines (Winther et al., 2018) have been observed in the hippocampus of the offspring of obese dams. In addition, maternal obesity disrupts the composition and regulation of the NMDA receptors in the hippocampus but also in the medial prefrontal cortex (Mizera et al., 2021). Moreover, it produces a decrease of excitatory and inhibitory amino acid levels and this can also be present in the F2 and F3 generations (Sarker and Peleg-Raibstein, 2018) (Figure 33).

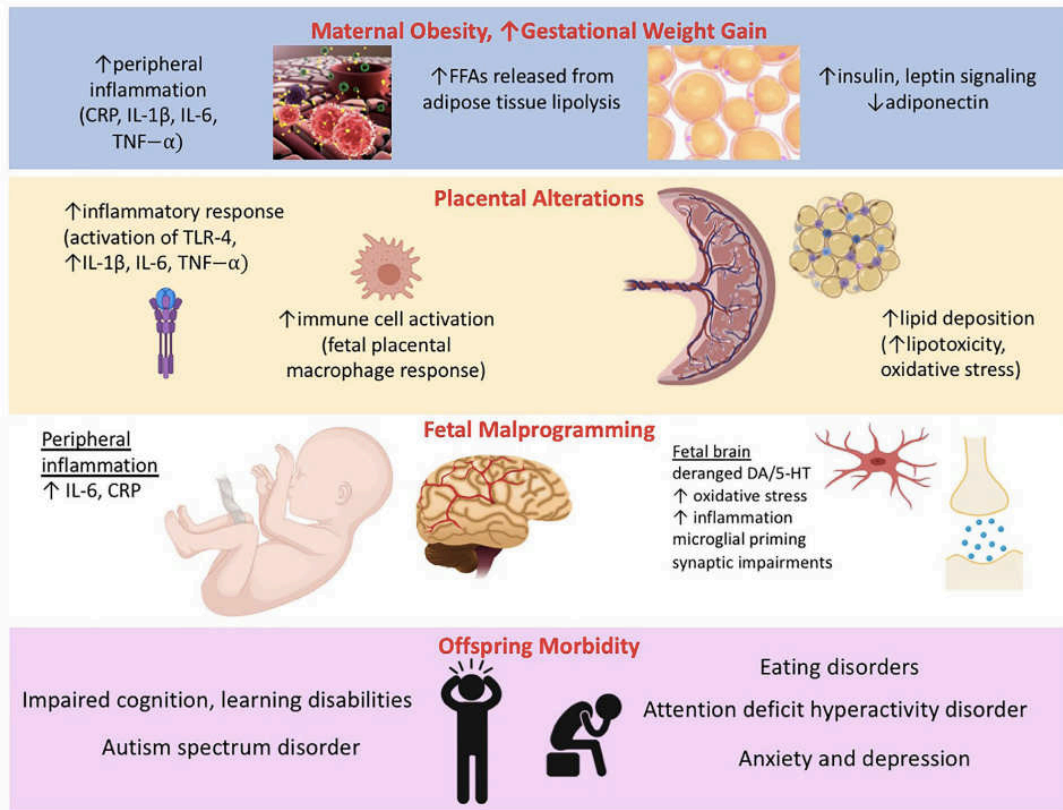


Figure 33. Effect of maternal obesity on the brain of the offspring. Maternal obesity increases peripheral inflammation which promotes the release of proinflammatory cytokines, free fatty acids, and enhance insulin and leptin signaling pathways. These alterations provoke an inflammatory state in the placenta which activates macrophages. In the brain fetus, proinflammatory signals activate microglia compromising the correct synaptic signaling in the hippocampus, which finally results in propension to develop cognitive and learning disabilities, among other neurodevelopmental and psychiatric diseases. Image obtained from Shook *et al.*, (Shook *et al.*, 2020).

Even though there is a growing body of knowledge showing that paternal obesity sensitises the offspring to develop metabolic diseases, little is known about the impact of paternal obesity on offspring’s behavior or cognitive function (Bodden *et al.*, 2020) Indeed, there is only one study which has showed that paternal obesity impairs offspring’s learning and memory abilities at adulthood (Zhou *et al.*, 2018).

Information about the mechanism by which paternal obesity affects offspring's brain is scarce, but evidence suggest that epigenetic markers in the sperm and the seminal plasma could be implicated (Bodden et al., 2020), principally the transfer of small and long noncoding RNAs and methylation patterns in some genes. Actually, the offspring of obese fathers presented impair hippocampal neurogenesis associated to increased methylation of the BDNF gene in spermatozoa (Zhou et al., 2018). In contrast, physical exercise results in demethylation of the DNA promoter region of BDNF in sperm (Mega et al., 2018), and improved spatial memory capacities in the offspring. Interestingly, there is a study showing that the offspring born to obese mothers display reduced spatial learning ability, reduced BDNF expression and decreased cell proliferation and differentiation in the hippocampus and that paternal treadmill exercise counteracted all these negative effects. These results suggest that paternal exercise enhances hippocampal neuroplasticity (Park and Kim, 2017). Likewise, when fathers are exposed to an enriched environment that includes physical and mental exercise, they transfer the microRNA212/132 to their offspring which enhances their synaptic plasticity and cognitive abilities (Benito et al., 2018). In contrast, the transfer of microRNAs has also been related to the stress and depression. If parents are submitted to stress they promote the offspring to displayed significantly reduced HPA stress axis responsivity and this is due to the transfer of some microRNA in the sperm that are upregulated by stress (Rodgers et al., 2013). On the other hand, the microRNAs transfer from depressive fathers to the offspring provokes the offspring to be susceptible to depression-like symptoms at the molecular, neuronal, and behavioral levels (Wang et al., 2021).

Could tryptophan metabolism be programmed?

TRP intake and metabolism depend on both the nutritional and physiological state (Strasser et al., 2015). During pregnancy, it is highly important to maintain the correct levels of TRP because the needs for serotonin and kynurenine in the fetus are highly dependent on TRP metabolism in the placenta. During mid-gestation serotonin is principally produced by the placenta and then, it switches to fetal production. In contrast, the production of kynurenine depends almost entirely on the placenta and not on the fetal organs (Abad et al., 2020). Nutritional interventions during pregnancy can affect the homeostasis of TRP metabolism in the fetus and leave long-term traces.

Honório de Melo Martimiano *et al.*, (Honório de Melo Martimiano *et al.*, 2017) described for the first time that early malnutrition due to protein restriction during pregnancy reduces the levels of TRP, 5-HT and KA in the embryos and shifts to an increase in these components in the adult rat brain. Thereafter, some authors explored the effect of maternal obesity in the hippocampus of the offspring and found an enhanced expression of TPH2, the enzyme that converts TRP into 5-HT, that was not associated with an increase in 5-HT levels (Dias *et al.*, 2020). In the same way, maternal obesity respectively increased and decreased the expression of KMO and KAT1 in the hippocampus of the offspring and this was associated with an anxious behavior (Winther *et al.*, 2018).

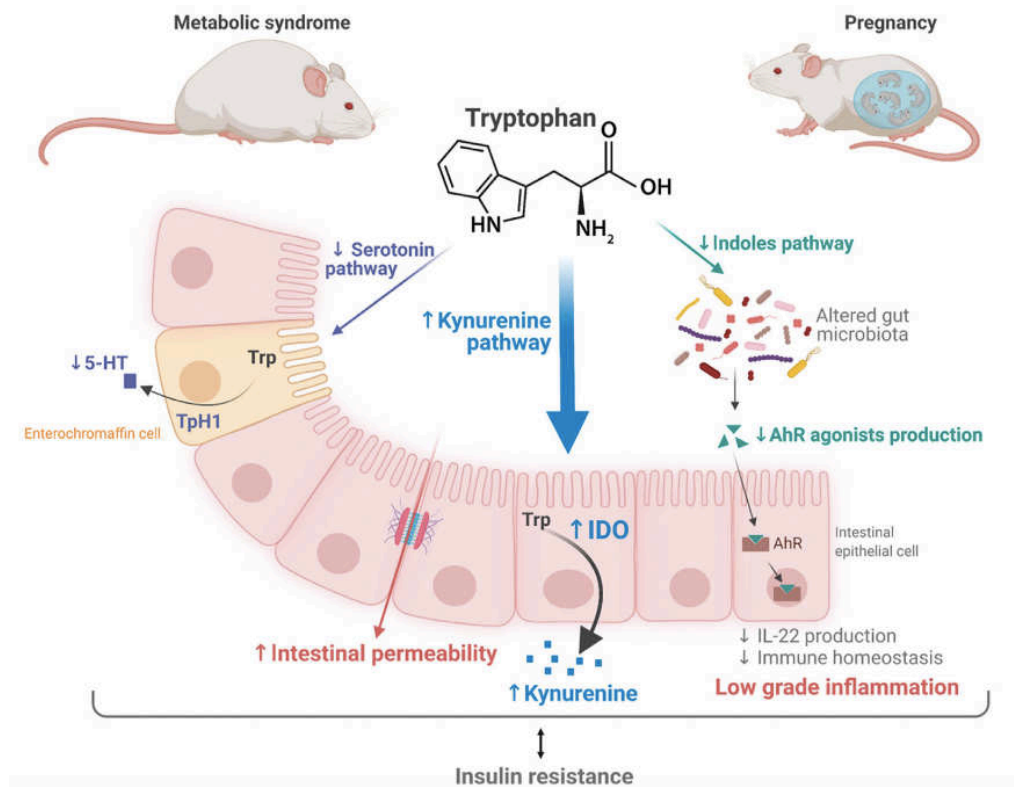


Figure 34. Effect of obesity on the tryptophan metabolism. Obesity induces a decrease in 5-HT levels, increases the production of kynurenines, and reduces the production of indoles in the periphery. Collectively, these alterations favour the development of an inflammatory state and insulin resistance. Those events are also presented in pregnancy. Image obtained from Benech *et al.*, (Benech *et al.*, 2022).

How maternal malnutrition modulates the expression of the enzymes involved in TRP metabolism is not totally elucidated. In addition, the existent evidence shows that maternal obesity does increase the expression of IDO, but we do not know whether this is reflected in an increased production of kynurenines in the brain. In the case that kynurenines were elevated, then we could associate this with the development of cognitive impairment in the offspring. An interesting point is that until now, no study has explored the effect of paternal obesity on TRP metabolism. Hence, the purpose of this study is to compare the effect of maternal and paternal obesity on the memory and learning capacities of the offspring and to assess the concentration of TRP-derived metabolites in their brain.

Thesis problem statement

Evidence has shown that obesity in humans provokes alters the peripheral metabolism of TRP leading to increased levels of metabolites derived from the kynurenine pathway such as KYN, KA and QA. This effect is the result of the overexpression of the enzymes that participate in the kynurenine pathway under the action of proinflammatory cytokines released from the adipocytes and macrophages of the adipose tissue. Besides, patients suffering from psychiatric diseases enhanced levels of KYN, as well as a dysregulation in the equilibrium of KA and QA in the brain, and this effect has been related to the existence of an inflammatory state. Although in different psychiatric diseases it has been observed that obesity-derived inflammation is related to a dysregulation of the production of kynurenines, this phenomenon has never been explored in the field of cognitive impairment.

Here, we propose that obesity-induced neuroinflammation will activate the kynurenine pathway leading to a deregulation of the levels of some kynurenines (including the KA and QA), and this phenotype will trigger memory decline. In addition, we believe that this effect will be more complex in females due to the action of estrogens that have an extra modulatory effect on TRP metabolism. Finally, we claim that those deleterious effects can be transmitted to the first filial generation.

Objective

To demonstrate in a rodent model that obesity induced by the consumption of a hypercaloric diet provokes neuro-inflammation and triggers alterations of TRP metabolism in the brain along with learning deficits at adulthood.

Specific objectives:

1. To examine if the fluctuations of estrogens during the oestrous cycle promote changes in brain TRP metabolism and to determine whether these changes impact cognitive capacity.
2. To determine if obesity alters the fluctuations of estrogens during the oestrous cycle and the impact of these alterations on learning and memory.
3. To determine whether maternal and paternal obesity can compromise the metabolism of TRP in the brain of the offspring and to evaluate the impact of these alterations on the memory and learning capacities of the offspring.
4. To analyze the mechanisms underpinning the deficits in memory and learning in the offspring of obese mothers or obese fathers.

General methodology

The following is a general description of the methodology used in the experimental series that it will be described in detail in each article included in the results section.

Animal model

We decided to use Wistar rats to produce a model of obesity induced by diet. When Wistar rats are exposed to hypercaloric diets they develop more rapidly and more pronounced obesity-related disorders than other rodents as the Sprague-Dawley rats (Marques et al., 2015). In addition, they have been extensively used for assessing memory and learning capacities (Boitard et al., 2014; Hernández-Ramírez et al., 2021; Paulo et al., 2021).

Free choice – High Fat High Sugar diet

To induce obesity we used a free-choice High Fat High Sugar (fc-HFHS) diet that is similar to the one reported by La Fleur et al., (la Fleur et al., 2010). This kind of diet let us get an approach of what happens in the modern-day dietary environment of humans since it considers the animal's choice between food components, that vary in texture, taste, and caloric content. In addition, this model promotes a persistent hyperphagia contrary to animals subjected to a no-choice hypercaloric diet. Moreover, fat consumption increases meal size and sugary water increases meal frequency. Finally, the simultaneous access to both components in a separate manner, produces profound adaptations in the reward-related brain circuitry, rapidly increases adiposity, the development of insulin resistance, glucose intolerance, and neuroinflammation (Slomp et al., 2019).

We used two types of fc-HFHS diets. The first fc-HFHS diet (fc-HFHS-type 1) was composed of tap water, standard chow diet, powdered standard chow diet mixed with pig fat (30 g of fat per 70 g of food), and a sweetened condensed milk (30 % sugar solution). The second fc-HFHS diet (fc-HFHS-type 2) was composed of tap water, standard chow diet, tapioca mixed with pig fat (30 g of fat per 70 g of food), and a sweetened solution using only sugar (30 % solution). The choice to change the diet was made in an attempt to aggravate the obesogenic phenotype and to facilitate the practical work in the laboratory.

The nutritional composition of standard diets used for feeding pregnant or lactating rats (SAFE ® A03), or for daily maintenance of rats (SAFE ® A04), is shown below (Table 1).

Table 1. Nutritional composition of the standard chow diet

	SAFE ® A03	SAFE ® A04
Nitrogen Free Extract	52 g / 100 g	60.4 g / 100 g
of which Starch	33.5 g / 100 g	43.5 g / 100 g
of which Sugars	4.4 g / 100 g	3.2 g / 100 g
Crude Protein	21.4 g / 100 g	16.1 g / 100 g
Crude Fat	5.1 g / 100 g	3.1 g / 100 g
Crude Ash	5.4 g / 100 g	4.6 g / 100 g
Crude Fiber	4.0 g / 100 g	3.9 g / 100 g
Moisture	12.1 g / 100 g	11.9 g / 100 g
	Amino acids	
Arginine	14 000 mg / 1kg	9 000 mg / 1kg
Cystine	3 200 mg / 1kg	2 500 mg / 1kg
Lysine	11 500 mg / 1kg	7 200 mg / 1kg
Methionine	8 800 mg / 1kg	2 800 mg / 1kg
Tryptophan	2 600 mg / 1kg	1 900 mg / 1kg
Glycine	12 000 mg / 1kg	8 100 mg / 1kg

NOR and NOR-NOL tests

We used two behavioral tests to evaluate the learning and memory capacities of rats, either the Novel Object Recognition (NOR) test or a more complex paradigm that combines the NOR with the Novel Object Location test (NOR-NOL). The NOR test is divided in three sessions: habituation, training, and testing. During the habituation session, rats are allowed to freely explore an open-field arena (50 x 50 x 40 cm) for 20 minutes during three consecutive days. The fourth day, the training and testing sessions start. First, in the training session two identical objects are placed into the arena before introducing the rat and each animal is allowed to explore the objects for 7 minutes. After

2, and 24 hours, testing sessions are performed to evaluate, respectively, short-term memory (STM) and long-term memory (LTM). During the testing section one of the familiar objects is exchanged for a new one. Animals are allowed to explore the objects for 7 minutes. In the NOR-NOL test the procedure is similar, the only difference is that during the testing session, one of the familiar objects is exchanged for a new one and both, the new and the familiar objects, are placed in a different location from the one they occupied in the previous session. Moreover, we added another stage of memory to evaluate memory retrieval after 7 days (Figure 35).

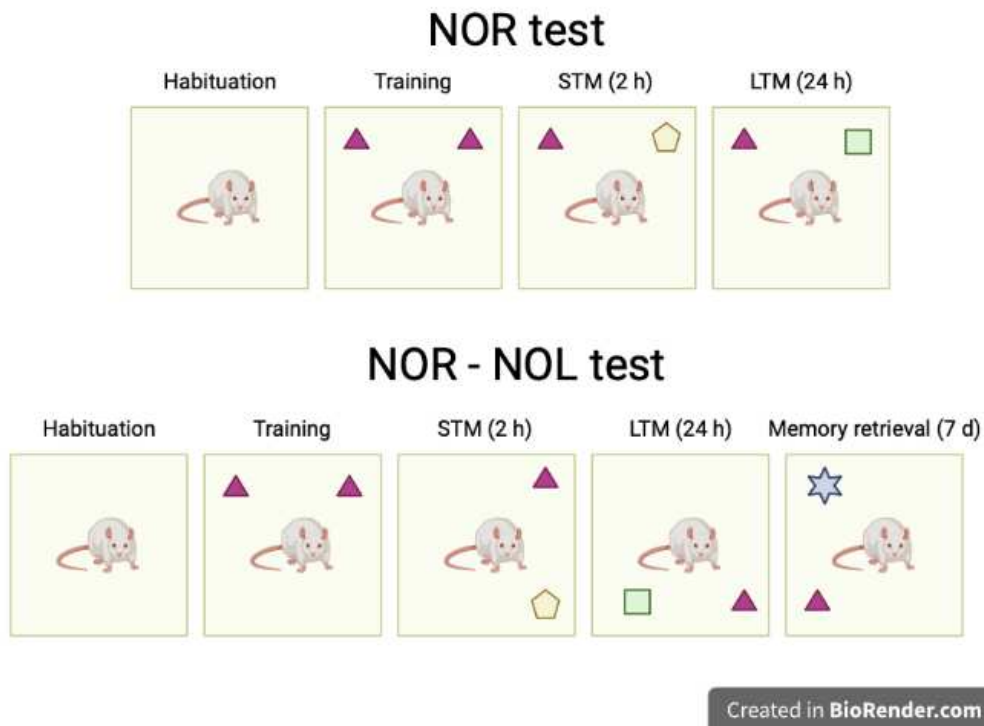
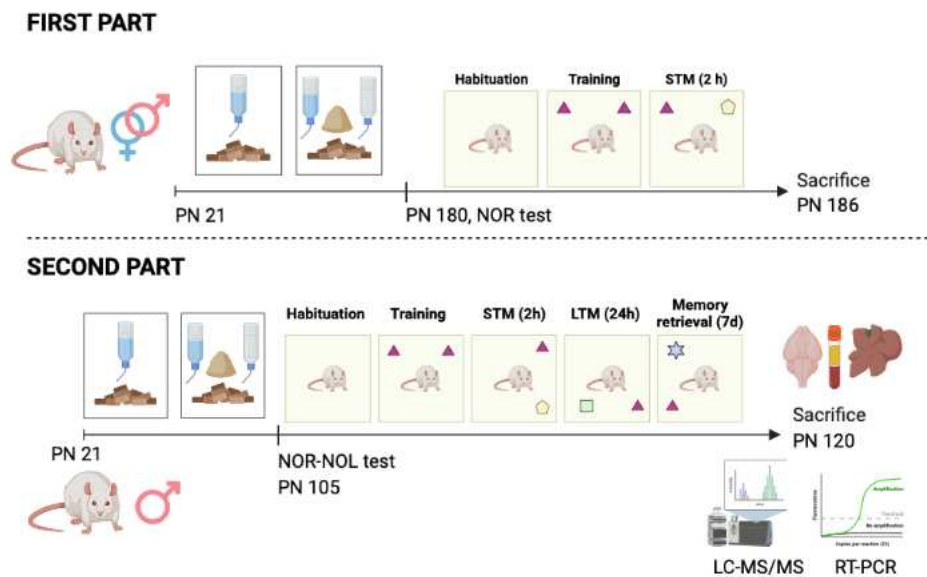


Figure 35. NOR and NOR-NOL tests. Image created with BioRender.com by Carla Mezo.

Methodology for the first article. “Learning deficits induced by High Calorie Feeding in the Rat are associated with impaired Brain Kynurenine Pathway Metabolism”

This work was divided in two parts. In the first one, we used female and male rats who were fed a fc-HFHS-type 1 diet from weaning (PN 21) until adulthood (PN 180). At this age, animals performed the NOR test in order to analyze their memory and learning capacities, then they were sacrificed. Since only males presented memory deficits, in the second part of the study we only used male rats. These animals were fed with the fc-HFHS-type 1 diet from weaning (PN 21) through adulthood, but this time we evaluated memory in the early adulthood (PN 120) and we used the NOR-NOL test. Finally, at sacrifice we collected samples of brain (including the brain stem, hippocampus and frontal cortex), liver, and serum to quantify by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) the concentration of TRP as well as the metabolites derived from the serotonin and kynurenine pathways. Finally, we evaluated by RT-PCR the expression of the enzymes involved in TRP metabolism in the brain (Figure 36).

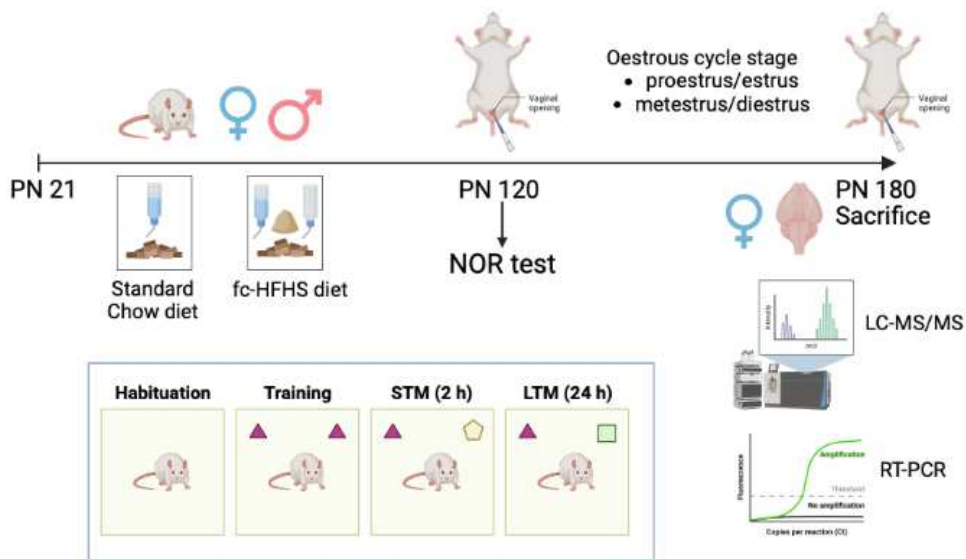


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Figure 36. Flowchart of the first experimental series. Image created with BioRender.com by Carla Mezo.

Methodology for the second article. "Obesity-induced learning deficits are oestrous cycle-dependent and are associated with impaired kynurenine pathway metabolism"

In the first part obese female rats did not present memory deficits, hence, in this work we performed a more detailed analysis by controlling the stages of the oestrous cycle. The experimental series started by using female and male rats who were fed a fc-HFHS-type 2 diet from weaning (PN21) until adulthood (PN120). Learning abilities were evaluated at PN 120 using the NOR tests. We checked the stage of the oestrous cycle by analyzing the vaginal cytology immediately after each female rat performed the two sessions in the NOR test (STM and LTM). Females were divided in two groups for the analysis of the results: proestrus/estrus and metestrus/diestrus. At sacrifice we also determined the stage of the oestrous cycle and collected the hippocampus and the frontal cortex to quantify by LC-MS/MS the levels of TRP, as well as the metabolites derived from the serotonin and kynurenine pathways. Finally, we performed a gene expression analysis of the enzymes involved in the TRP metabolism in the brain (Figure 37).



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Figure 37. Flowchart of the second experimental series. Image created with BioRender.com by Carla Mezo.

Methodology for the third article. “Both maternal and paternal obesity in the rat induce learning deficits in offspring linked to impaired brain kynurenine pathway metabolism”.

The purpose of this work was to evaluate the effect of maternal and paternal obesity on TRP metabolism in the brain of the offspring; hence, female, and male rats were fed with a fc-HFHS-type 1 diet from weaning (PN 21) until adulthood (PN 120). Then, animals were mated in the following way: 1) Control female x control male, 2) Obese female x control male, and 3) Control female x obese male. After weaning, male offspring were fed until adulthood a standard chow diet. At adulthood (PN 120), they performed the NOR-NOL test. Finally, we used the hippocampus and the frontal cortex to quantify by LC-MS/MS the levels of TRP as well as the metabolites derived from the serotonin and kynurenine pathways (Figure 38).

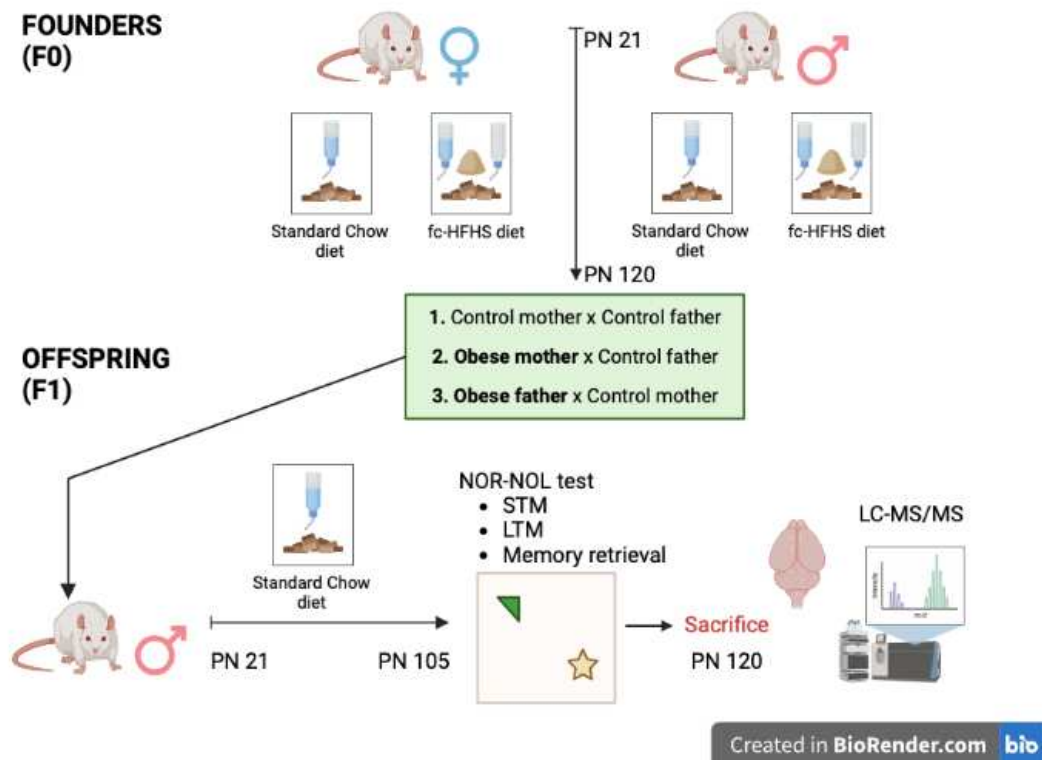


Figure 38. Flowchart of the third experimental series. Image created with BioRender.com by Carla Mezo.

Methodology for the fourth article. “Maternal and paternal obesity trigger learning and memory deficits in the adult offspring along with impaired expression of NMDA receptors in the brain”.

In the final part of this research, we repeated the same methodology described in the third part to obtain a first filial generation of animals of obese mothers or of obese fathers. Once again, we used only male offspring who was fed from weaning until adulthood a standard chow diet. Starting at PN 105 animals performed the Elevated Plus Maze to evaluate their levels of anxiety, the Open Field test for analyzing the locomotor activity and the NOR test to evaluate memory. At sacrifice we collected the hippocampus and the frontal cortex to analyze the expression of markers involved in the synaptic plasticity mediated by glutamate (Figure 39).

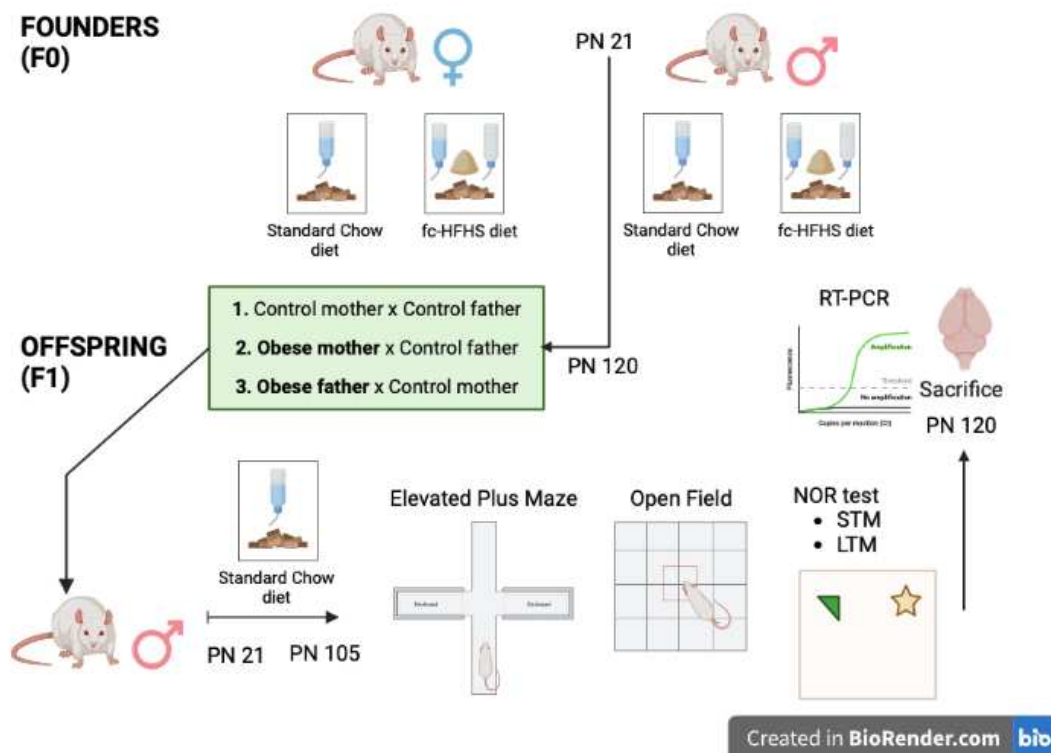


Figure 39. Flowchart of work the fourth experimental series. Image created with BioRender.com by Carla Mezo.

Results

ARTICLE 1.

Learning deficits induced by High Calorie Feeding in the Rat are associated with impaired Brain Kynurenine Pathway Metabolism.

Mezo-González CE, Daher-Abdi A, Reyes-Castro LA, Olvera Hernández S, Almeida C, Croyal M, Aguesse A, Gavioli EC, Zambrano E, Bolaños-Jiménez F. Learning Deficits Induced by High-Calorie Feeding in the Rat are Associated With Impaired Brain Kynurenine Pathway Metabolism. *Int J Tryptophan Res.* 2022.



Learning Deficits Induced by High-Calorie Feeding in the Rat are Associated With Impaired Brain Kynurenine Pathway Metabolism

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International Journal of Tryptophan Research
Volume 15: 1–13
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DOI: 10.1177/11786469221111116



ABSTRACT: In addition to be a primary risk factor for type 2 diabetes and cardiovascular disease, obesity is associated with learning disabilities. Here we examined whether a dysregulation of the kynurenine pathway (KP) of tryptophan (Trp) metabolism might underlie the learning deficits exhibited by obese individuals. The KP is initiated by the enzymatic conversion of Trp into kynurenine (KYN) by indoleamine 2,3-dioxygenase (IDO). KYN is further converted to several signaling molecules including quinolinic acid (QA) which has a negative impact on learning. Wistar rats were fed either standard chow or made obese by exposure to a free choice high-fat high-sugar (fCHFHS) diet. Their learning capacities were evaluated using a combination of the novel object recognition and the novel object location tasks, and the concentrations of Trp and KYN-derived metabolites in several brain regions determined by ultra-performance liquid chromatography-tandem mass spectrometry. Male, but not female, obese rats exhibited reduced learning capacity characterized by impaired encoding along with increased hippocampal concentrations of QA, Xanthurenic acid (XA), Nicotinamide (Nam), and oxidized Nicotinamide Adenine Dinucleotide (NAD⁺). In contrast, no differences were detected in the serum levels of Trp or KP metabolites. Moreover, obesity enhanced the expression in the hippocampus and frontal cortex of kynurenine monooxygenase (KMO), an enzyme involved in the production of QA from kynurenine. QA stimulates the glutamatergic system and its increased production leads to cognitive impairment. These results suggest that the deleterious effects of obesity on cognition are sex dependent and that altered KP metabolism might contribute to obesity-associated learning disabilities.

KEYWORDS: Tryptophan, kynurenine, brain, obesity, learning, memory

RECEIVED: March 17, 2022. **ACCEPTED:** June 14, 2022.

TYPE: Original Research Article

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Carla Mezo González is supported by an international PhD fellowship from the Mexican National Council of Science and Technology (CONACYT). Amran Daher Abdī was the recipient of a doctoral fellowship from the Ministry of Research and Innovation of the Government of the Republic of Djibouti. Sandra Olvera Hernández was supported by the programme “Food for Tomorrow” of the French Region Pays de la Loire and the European Regional Development Fund (FEDER). Luis Reyes Castro received support from the EU in the

framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship. This work was supported by the French National Research Agency (ANR, grant ANR-16-CE21-0007-01) and CONACYT (award number 2015-16-273510).

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Obesity is a current worldwide public health issue that affects the quality of life and increases the risk of developing metabolic, cardiovascular and musculoskeletal diseases as well as some types of cancer.¹ Mounting evidence indicates that obesity has also a detrimental impact on cognitive function.² In particular, several clinical studies have shown that individuals with a high body mass index display poor cognitive performances including reduced memory abilities and impaired decision making.^{3–5} Consumption of high-calorie diets, either rich in fat, sugar or both, is also positively associated with deficiencies in prospective memory and memory recall.^{6,7} Experimental studies in rodents have confirmed that obesity is accompanied by cognitive impairment and have further shown that hippocampal-dependent memory

tasks are particularly sensitive to diet-induced obesity^{8,9,10} (see Abbott et al¹¹ for review).

Obesity is characterized by a low-grade inflammatory state which is assumed to be at the heart of the metabolic and cardiovascular comorbidities experienced by obese individuals. Multiple lines of evidence show that obesity-related inflammation also underlies the cognitive impairments associated with this pathological condition. Actually, high-fat feeding results in the disruption of the blood-brain barrier (BBB) leading to neuroinflammation via the entry of pro-inflammatory cytokines and peripheral immune cells into the brain and the activation of microglia, the resident immune cells of the brain.^{12–16} Moreover, a positive association has been found between neuroinflammation and learning impairment in genetic or diet-induced experimental models of obesity.^{17–20} In addition, the activation of the



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immune response by the administration of lipopolysaccharide (LPS), results in reduced learning.^{21,22} The existence of a causal link between neuro-inflammation and cognitive dysfunction, is also supported by data showing that the intracerebral administration of high concentrations of the pro-inflammatory cytokines IL-1 β or IL-6 impairs memory formation in several learning paradigms.²³⁻²⁵ Similarly, transgenic mice overexpressing tumor necrosis factor alpha (TNF- α) in brain, exhibit impaired memory acquisition in the Morris water maze and passive avoidance tests,^{26,27} and IL-1 β , IL-6 and TNF- α inhibit long-term potentiation (LTP), a cellular model of memory.²⁸⁻³³

However, although the correlation between neuroinflammation and cognitive impairment is well established, the mechanisms by which an increase in the cerebral concentration of pro-inflammatory interleukins leads to learning and memory deficits are far from being fully established. In this respect, it is worth mentioning that IL-1 β and TNF- α enhance the expression of indoleamine 2,3-dioxygenase (IDO).³⁴⁻³⁷ This enzyme catalyses the conversion of tryptophan (Trp) to kynurenine (KYN) which is the first and rate-limiting step of the kynurenine pathway (KP) of Trp metabolism. The subsequent transformation of KYN through a series of enzymatic reactions leads to the production of several neuroactive compounds and eventually to the synthesis of nicotinamide adenine dinucleotide (NAD⁺). Among the different products generated by the kynurenine pathway, quinolinic acid (QA), and kynurenic acid (KA) stand out for their impact on learning and memory as a result of their respective agonist and antagonist actions at the ionotropic glutamate and $\alpha 7$ nicotinic acetylcholine receptors.³⁸⁻⁴²

Cerebral biosynthesis of KYN, takes place from Trp transported to the brain from the bloodstream where it is bound to albumin or in free form. Only in its free form Trp is able to cross the blood-brain barrier. Non-esterified fatty acids (NEFAs) bind to albumin at the same site as Trp so that an increase in plasma concentrations of NEFAs results in the displacement of Trp from its site of interaction with albumin, leading to an increase in the proportion of free Trp in the circulation and increased transport of Trp into the brain.⁴³⁻⁴⁵ The concentration of free tryptophan also increases as a result of its dissociation from albumin within the blood-brain barrier.⁴⁶ In addition to diet, this process is modulated by physiological and metabolic factors.⁴⁷

On the basis of all above observations, it can be hypothesized that obesity-related cognitive alterations involve a signaling cascade triggered by the exacerbated production of pro-inflammatory interleukins in the brain that would lead to the activation of tryptophan metabolism through the KP and eventually to the impairment by KA and QA of the memory process mediated by cholinergic and glutamatergic receptors. This signaling pathway would be favored by an enhanced entry of Trp into the brain resulting from an elevation of free Trp in blood as consequence of the displacement of Trp from albumin by NEFAs. Here we have tested this hypothesis by examining the learning capacities and the concentrations of tryptophan and kynurenine-derived metabolites in several brain regions of obese rats.

Materials and Methods

Animals and diet-induced obesity model

We used the free-choice high-fat high-sugar diet (fc-HFHS) obesity model as described by La Fleur et al.⁴⁸ with minor modifications. This model has the advantage of simulating the modern-day dietary environment of humans and of leading to a rapid increase in adiposity.^{48,49} Male and female Wistar rats (Janvier Labs, Le Genest Saint Isle, France), were fed either standard chow (controls), or exposed from weaning to high-fat food and to a 30% sugar solution in addition to standard chow and tap water (obese). High-fat food consisted in powdered standard chow mixed with pig fat in the proportion of 30 g of fat per 70 g of food. Sweetened condensed milk was used as a base to prepare the 30% sugar solution. Animals were housed 4 per cage until they reached 300 g of body weight and 2 per cage thereafter and maintained under an inverted 12-/12-hour dark-light cycle (lights off at 7:00 AM), and at $21 \pm 1^\circ\text{C}$ with food and water ad libitum through all the experiment.

Behavioral analyses

Memory tests were performed under red light 2 hours after the beginning of the dark phase of the light-dark cycle in a laboratory adjacent to the housing room of the animals and recorded using the Viewpoint Videotrack system (Point Grey Research Inc., Richmond B.C. Canada), for offline analysis by 2 investigators blind to the nutritional status of the animals. In a first set of experiments, the learning capabilities of 180-day-old male and female rats of both experimental groups (control and obese), were assessed using the novel object recognition test (NOR) as described by Ennaceur and Delacour.⁵⁰ Subsequently, the impact of obesity on the different components of the memory process was evaluated in a different set of 105-day-old male rats using the combination of the NOR and the novel object location (NOL) tasks.⁵¹ The NOR test was divided into 3 sessions: habituation, training and testing. During the habituation session, rats were allowed to freely explore an open-field arena (50 \times 50 \times 40 cm) for 20 minutes during 3 consecutive days. The fourth day, 2 identical objects were placed into the arena before introducing the rat and each animal was allowed to explore the objects for 7 minutes. 2 hours after this training phase, animals were returned to the arena in which one of the familiar objects has been exchanged for a new one. The time exploring each object was recorded and the learning capacity determined from the difference in time spent exploring the novel versus the familiar object. Preferential exploration of the novel object versus the familiar one indicates memory acquisition whereas an identical or similar time spent exploring the 2 objects means no learning. Object exploration was defined as sniffing or touching the object with the vibrissae or when the animal's head was oriented toward the object with the nose placed at a distance of less than 2 cm from the object. The NOR-NOL test was carried out in the same way excepting that 2 additional testing sessions were carried out 24 hours

and 7 days after the first test session to evaluate, respectively, long term (LTM) and consolidated (CM) memories. In addition, in each of the test sessions, including the 2-hour session to assess short term memory (STM), both the new and the familiar objects were placed in a different location from the one they occupied in the previous session (see Pérez-García et al⁵¹ for a complete description of the test).

Biological samples collection and processing

The day following the last NOR or NOR-NOL testing session, animals were deeply anesthetized with isoflurane 2 hours after the beginning of the dark phase of their light-dark cycle to obtain a blood sample by cardiac puncture and sacrificed immediately after by cervical dislocation. The frontal cerebral cortex, brainstem and hippocampus were dissected and the serum separated from blood was split into 2 fractions. One of them was filtrated using an ultrafiltration device endowed with an Ultracel YM-T membrane with a pore size of 30 kDa (Millipore, France), to obtain serum free tryptophan. All brain samples were immediately frozen after dissection in liquid nitrogen and stored at -70°C together with serum samples until analysis. Mediastinal and abdominal (omental, perirenal, retroperitoneal, epididymal, periovarian, perivesical, and parametrial), fat depots were dissected, weighed and summed to provide a measure of body fat.

Metabolite determinations

Serum was assayed for insulin using an assay kit from Linco Research Inc. Triglycerides, cholesterol and fatty acids were analyzed by enzymatic methods (Triglycérides enzymatiques PAP 150, BioMérieux; Cholesterol RTU, Biomérieux; NEFA FS, DiaSys). Serum glucose concentrations were determined with a blood glucose monitor (Accu-Check[®], Roche Diagnostics).

Analyses of tryptophan metabolism

Tryptophan and the metabolites generated by its catabolism through the serotonin (5-HT) and KYN pathways were quantified in the brain-stem, hippocampus, frontal cortex and serum by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the methodology described in detail by Zheng et al and Martimiano et al.^{52,53} In brief 50 mg of tissue or 50 μL of serum were homogenized in 200 μL of an ice-cold solution of 2.7 mM EDTA containing 1% formic acid and mixed with 10 μL of a pool of exogenous internal standards (500 μM D₅-TRP, 2000 μM cafeic acid and 50 μM 2-CAD). Subsequently, 200 μL of the homogenate were mixed with 500 μL of ice-cold acetonitrile and, after centrifugation at 15 000g for 15 minutes at 4°C , the supernatant was recovered and evaporated to dryness under a stream of nitrogen at room temperature. Calibration curves were generated using serial

dilutions of Trp, QA, 5-HT, KYN, 5-HIAA, KA, and Xanthurenic acid (XA), in 2.7 mM EDTA containing 0.1% formic acid which were processed in the same way that the tissue and serum samples. At the end, all dried samples were dissolved in 100 μL of EDTA 2.7 mM containing 0.1% formic acid and 5 μL of each sample were injected into the LC-MS/MS system for analysis. Compounds were separated on a reverse Acquity HSS T3 column (2.1 \times 100 mm, 1.7 μm , Waters Corporation, Milford, MA, USA) at 30°C with a linear gradient of the mobile phase B (acetonitrile containing 0.5% formic acid and 2.5 mM ammonium acetate) in the mobile phase A (water containing 0.5% formic acid and 2.5 mM ammonium acetate) and at a flow rate of 400 $\mu\text{L}/\text{min}$. The electrospray interface of the mass spectrometer was operated in positive ion mode and the multiple reaction monitoring mode was chosen for metabolite detection. Data acquisition and analysis were performed using MassLynx[®] and TargetLynx[®] software, respectively (version 4.1; Waters). Compound concentrations were calculated using calibration curves plotted from standard solutions.

Real time quantitative RT-PCR

A conventional Trizol extraction method was used to obtain total RNA from a fraction of the same tissue homogenate that was used for the quantification of Trp metabolites by mass spectrometry. Thereafter, 1 μg of purified RNA was treated with a DNase and reverse transcribed using the MultiScribe[™] Reverse Transcriptase kit from Thermo Fisher according to the manufacturer's instructions. The resulting cDNA was diluted 40-fold in DNase and RNase free water and 5 μL of each cDNA diluted sample were used as template for amplification using an iCycler iQ Real-Time PCR detection system and SYBR Green as fluorogenic intercalating dye. Relative gene expression differences between control and obese animals were determined by the comparative $2^{-\Delta\Delta\text{Ct}}$ method,⁵⁴ using 18 S, $\beta 2$ -microglobulin or GAPDH transcripts as housekeeping genes. The applicability of the $2^{-\Delta\Delta\text{Ct}}$ method was first validated by determining how the amplification efficiencies of the different transcripts, including those of the reference genes, varied with template dilution. These experiments showed that the efficiency of the PCR amplification was the same for all the genes and that the expression of 18 S, $\beta 2$ -microglobulin or GAPDH was not influenced by obesity. The sequences of the primers used for the amplification are as follows: *Tryptophan hydroxylase 2 (TPH2)*, forward: ATC CCA AGT TCG CTC AGT TTT CTC, reverse: ACG CCC GCA GTT GAC CTT C; *Indoleamine 2,3-Dioxygenase 1 (IDO1)*, forward: CGT TGG TGA AGG ATC TGC TG, reverse: AGG GCT CTT CTG ACT TGT GG; *Indoleamine 2,3-Dioxygenase 2 (IDO2)*, forward: GGT GAC AGT CTT GGT GGA GAA G, reverse: GGT GTC CTG GCT GTG TTG C; *Tryptophan 2,3-Dioxygenase (TDO2)*, forward: CAA GGT GAT GAC TCG GAT GC,

Table 1. Anthropometric characteristics and serum metabolic profile of male and female 180 days-old rats exposed to the FcHFHS diet.

	MALES		FEMALES	
	CONTROL	OBESE	CONTROL	OBESE
Body weight (g)	620 ± 11	770 ± 19****	341 ± 6	454 ± 11****
Adiposity Index	6.35 ± 0.43	9.80 ± 0.38****	6.45 ± 0.38	10.25 ± 0.39****
Leptin (ng/mL)	13.56 ± 2.02	24.28 ± 2.34**	7.27 ± 0.96	22.69 ± 3.03***
Insulin (ng/mL)	1.58 ± 0.21	2.99 ± 0.67	1.36 ± 0.45	3.41 ± 0.76*
Glucose (mg/dL)	163 ± 10.76	193 ± 19.97	175 ± 4.61	183 ± 10.06
Cholesterol (ng/dL)	73.62 ± 5.55	74.83 ± 2.90	63.98 ± 4.28	85.11 ± 5.23*
Triglycerides (ng/dL)	101 ± 10.96	151 ± 20.08*	137 ± 15.72	251 ± 84.09**

* $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$ compared to their respective male or female control group as determined by Student's *t*-test.

reverse: GGA ACT GAA GAC TCT GGA AGC; *Kynurenine aminotransferase (KAT)*, forward: GAA TTA CTC AAG GTT CCT CAC TG, reverse: *GAT GGT GCT TCC GTT CTC C*; *Kynurenine mono-oxygenase (KMO)*, forward: GCT TCC AAC GCA TAC TGA TG, reverse: GGC AGG CAA CAG AAA GAA ATC; *3-hydroxyanthranilate 3,4-dioxygenase (HAAO)*, *IL-6*, forward: GCC CTT CAG GAA CAG CTA TGA, reverse: TGT CAA CAA CAT CAG TCC CAA GA; *IL-1 β* , forward: AAT GCC TCG TGC TGT CTG ACC, reverse: GGG TGG GTG TGC CGT CTT TC; *TNF- α* , forward: CCA CCA CGC TCT TCT GTC TAC TG, reverse: GGC TAC GGG CTT GTC ACT CG; *TLR2*, forward: GTC TCC AGG TCA AAT CTC, reverse: CAG CAT CAC ATG ACA GAG, *TLR4*, forward: GAT TGC TCA GAC ATG GCA GTT TC, reverse: CAC TCG AGG TAG GTG TTT CTG CTA A; *18 S*, forward: GAT GCG GCG GCG TTA TTC C, reverse: CTC CTG GTG GTG CCC TTC C; *β 2-microglobulin*, forward: GAT GGC TCG CTC GGT GAC, reverse: CGT AGC AGT TGA GGA AGT TGG; *GAPDH*, forward: CAG TAT GAC TCT ACC CAC GGC A, reverse: ATC TCG CTC CTG GAA GAT GGT G.

Statistical analysis

Experimental results were analyzed using software GraphPad Prism version 9. Data were first checked for normality by the Shapiro-Wilk normality test and the statistical differences assessed by unpaired Student's *t*-test or 2-way ANOVA. Statistical significance was set at $P < .05$. Data are expressed as means \pm SEM.

Results

Obesity impairs learning in male but not female rats

One hundred eighty day-old male and female rats exposed to the fc-HFHS diet developed a clearly obese phenotype characterized by increased body weight and body fat as well as by

enhanced serum levels of leptin and triglycerides (Table 1). In addition, obese females displayed higher serum concentrations of insulin and cholesterol in comparison to their respective control group (Table 1).

To address whether fc-HFHS feeding-induced obesity was affecting learning, animals were exposed to the NOR test using the standard protocol described by Ennaceur and Delacour.⁵⁰ This test assesses the animal's capacity to distinguish a novel from a familiar object therefore providing a measure of recognition memory. We chose this learning paradigm because it does not require the use of positive or negative reinforcements and it does not rely on the achievement of a physical activity that could be compromised by obesity. As illustrated in Figure 1, obese male rats showed a reduced ability to distinguish the novel object from the familiar one, indicating a decreased learning capacity, during a single test session performed 2 hours after the training phase of the NOR test. In contrast, no difference in exploration time for the novel versus the familiar object was observed in obese female rats compared to their control counterparts.

Fc-HFHS feeding enhances the expression of proinflammatory cytokines in brain

With the aim of identifying the components of the memory construction affected by the fc-HFHS diet, we performed a second experiment using only male rats. In addition, we decided to assess learning skills in younger animals (105-day-old) in order to distinguish the effects of obesity from those induced by aging. Indeed, aging entrains the decline of certain cognitive abilities, such as memory, visuospatial, and executive function abilities,⁵⁵ and obesity accelerates the rate of aging.⁵⁶

Similar to the 180-day-old animals, the 105-day-old rats that consumed the fc-HFHS diet, showed an obese phenotype characterized by enhanced body weight and increased adiposity index in comparison with control rats (Figure 2A and B). In addition, they presented elevated levels of leptin (Figure 2C),

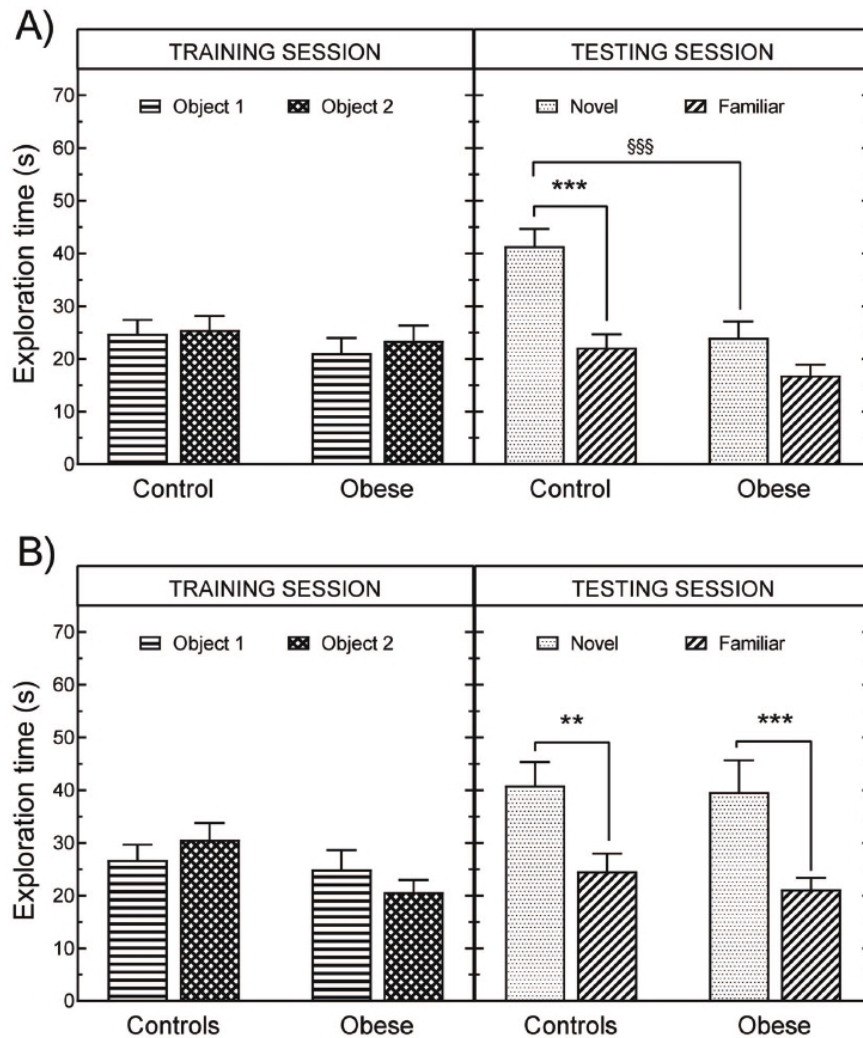


Figure 1. Impact of obesity on the cognitive skills of 6-month-old male (A) and female (B) rats. The learning capacity of the animals was tested using the standard NOR test. Bars correspond to the exploration time of each of the objects during a 7-minute period. No difference in object exploration time was observed between the different groups during the training session. In contrast, obese male animals were unable to distinguish the novel object from the familiar one as shown by the almost identical exploration time of the 2 objects. Learning tests were performed at the beginning of the dark phase of the animal's dark-light cycle with a total number of 8 to 12 animals per experimental group. ** $P < .01$; *** $P < .001$ (Student's *t*-test). \$\$\$ $P < .001$ (2-way ANOVA).

insulin (Figure 2D), triglycerides (Figure 2E) and glucose (Figure 2F). In contrast, there was no difference in the circulating levels of free fatty acids between obese (29.43 ± 8.42 mg/dL) and control animals (33.48 ± 8.25 mg/dL). fcHFHS fed rats displayed also increased levels of mRNAs encoding for IL-6, IL-1 β , TNF- α , TLR2, and TLR4 receptors in the brain stem (Figure 3). In contrast, only the expression of TLR2 mRNAs was enhanced in the hippocampus and frontal cortex of obese rats (Figure 3).

Obesity delays the acquisition of the memory trace but does not impair memory retrieval

To assess the learning and memory skills of young male rats, we used a behavioral paradigm based on the combination of the NOR and the Novel Object Location (NOL) tests (NOR-NOL). This test allows the analysis of the 3 main stages of the memory process, that is, encoding, consolidation and retrieval (see Pérez Garcia et al.⁵¹ for a detailed description of the test and of its theoretical ground).

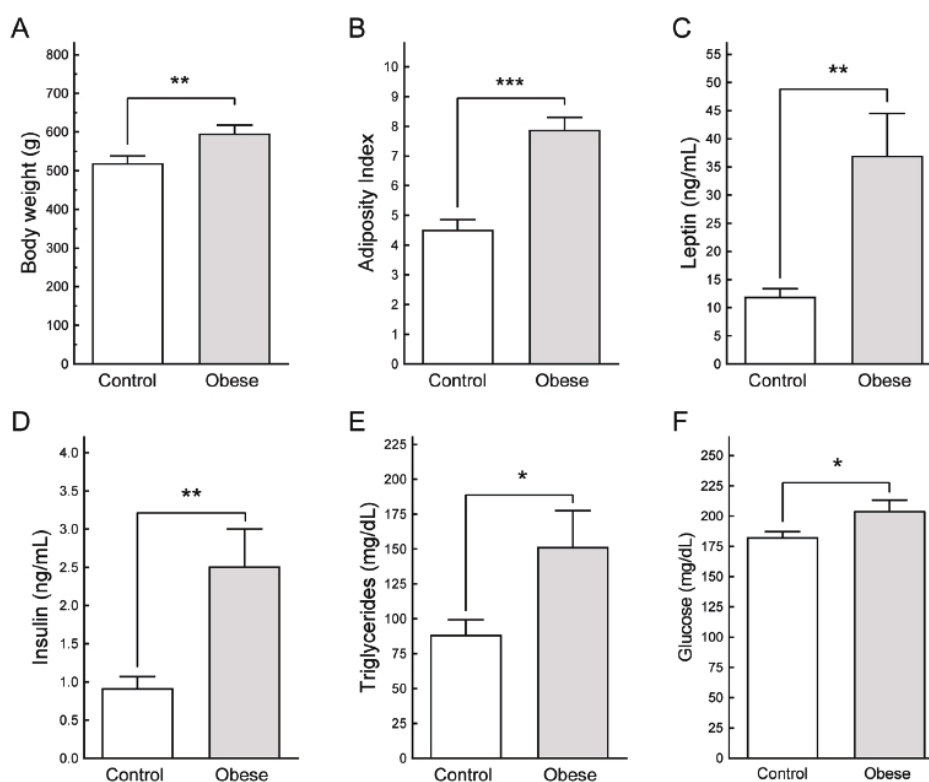


Figure 2. Body weight (A), adiposity index (B), and serum levels of leptin (C), insulin (D), triglycerides (E) and glucose (F) exhibited by 105-day-old control and obese male rats. Animals were made obese by exposure to a FcHFHS diet from weaning. Data represent the mean \pm SEM from a total number of 8 animals per experimental group. * $P < .05$; ** $P < .01$; *** $P < .001$ as determined by Student's *t*-test.

As illustrated in Figure 4, obese rats displayed reduced ability to distinguish the novel object from the familiar one 2 hour after the encoding session. In contrast, clear differences in exploration time for the novel versus the familiar object were observed in both control and obese animals during the test sessions performed 24 hours and 7 days after training. This result indicates that, at least under our experimental conditions, obesity impairs the capacity to encode new information but not the capacity to remember a previously encoded and stored memory trace.

Obesity impairs brain kynurenine pathway metabolism

With the aim of determining the potential contribution of Trp metabolites to the learning deficits observed in obese animals, Trp and several products spanning its metabolism through the 5-HT and KYN pathways were quantified by LC-MS/MS in the brain stem, hippocampus and frontal cortex of control and obese rats. Quantified metabolites included 5-HT and 5-HIAA (5-HT pathway); KYN, KA, XA, QA, nicotinic acid (NA) nicotinamide (NA_m), and NAD⁺ (KYN pathway).

The results of these analyses showed that the concentrations of XA, QA, Nam, and NAD⁺ in the hippocampus are enhanced by fc-HFHS feeding-induced obesity (Figure 5A). We also found increased levels of KYN in the brain stem (Figure 5B) and of NAD⁺ in the frontal cortex (Figure 5C) of obese rats in comparison to the control group. Otherwise, there were no differences between control and obese rats in the levels of all other quantified tryptophan metabolites (Supplemental Table 1).

To get insight into the mechanisms underlying the enhanced concentration of KYN breakdown products in the brain of obese animals, the expression of mRNA transcripts encoding key enzymes of Trp and KYN metabolism was evaluated by quantitative real-time PCR. We observed an enhanced expression of TPH2 and of kynurenine 3 monooxygenase (KMO), which is involved in the metabolic pathway leading the conversion of KYN to QA, in the brain stem of obese rats as compared to controls (Figure 6). Likewise, mRNA levels encoding for KAT—the enzyme that catalyses the catabolism of KYN into KA- and KMO were enhanced in the hippocampus and frontal cortex of obese rats (Figure 6).

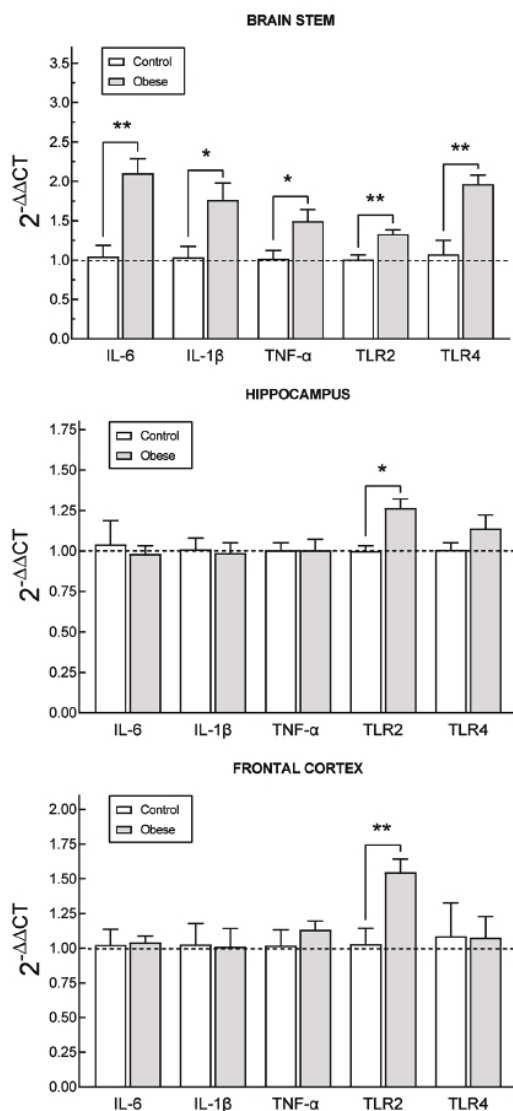


Figure 3. Brain expression levels of mRNAs encoding for genes involved in inflammatory responses. Male rats were fed standard chow or rendered obese by exposure to a fcHFHS diet. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression of control animals as a calibrator. Data represent the mean \pm SEM from a total number of 8 animals per experimental group. * $P < .05$; ** $P < .01$ (Student's *t*-test).

Impact of obesity on peripheral kynurenine pathway metabolism

The metabolism of KYN within the brain, is in part dependent on the availability of free Trp in blood as well as on the peripheral concentration of KYN which can readily enter into the brain. We therefore evaluated the concentrations of total and free Trp as well as the levels of KYN and its metabolites in

serum. Consumption of the fcHFHS diet did not alter neither the total nor the free circulating levels of Trp (Supplemental Table 2). Likewise, no differences were detected in the serum levels of KYN pathway metabolites with the exception of a decrease in the concentration of QA (Figure 7A), which was paralleled by a decrease in the expression levels of IDO2 and of HAAO mRNAs in liver (Figure 7B and C).

Discussion

A large number of epidemiological studies and animal investigations show that obesity is associated with learning and memory impairment. However, the mechanisms underlying this cognitive alteration remain largely to be determined. The results presented herein provide compelling evidence for the involvement of the kynurenine pathway of tryptophan metabolism in obesity-induced cognitive deficits. Namely, adult rats that became obese by exposure to a fc-HFHS diet, showed learning difficulties characterized by a decreased ability to acquire new information but not to remember a previously encoded memory trace. These alterations were associated enhanced production of QA, XA, Nam, and NAD⁺ in the hippocampus along with upregulated expression of mRNAs coding for KMO, an enzyme involved in the transformation of KYN into XA and QA.

QA is known for its neurotoxic action and its deleterious effects on learning,^{41,57} resulting from its action as an agonist of glutamate NMDA receptors. Indeed, overstimulation of these receptors by QA, leads to massive calcium entry into the cell which results into free radical production, cytoskeleton disorganization, apoptosis, and mitochondrial dysfunction.⁵⁸ XA has also been shown to modulate the glutamatergic system via its interaction with the metabotropic receptors mGlu2 and mGlu3 and the inhibition of vesicular glutamate transporters (see Fazio et al⁵⁹ for review). Although the effects of XA on memory are yet to be determined, it has been shown that administration of this compound reduces the amplitude of field excitatory postsynaptic potentials (fEPSP) in the hippocampus,⁶⁰ suggesting that an increase in the brain concentration of XA could inhibit the neuronal plasticity which underlies the learning process.

Both QA and XA are by products of the neurotoxic branch of KYN metabolism. This pathway is initiated by the conversion of KYN to 3-hydroxykynurenine (3-HK), by KMO. 3-HK is then converted to XA by kynurenine amino transferase and, subsequently, to 3-hydroxyanthranilic acid, QA and NAD⁺ by, respectively, kynureninase, 3HAAO and quinolinate phosphoribosyl transferase. Consistent with the increase in the concentration of QA, the hippocampal content of NAD⁺ and of Nam, a by-product of the KP which serves as a precursor for NAD⁺ synthesis via the salvage pathway, was also enhanced by obesity. Supplementation with Nam^{61,62} or NAD⁺^{63,64} in animal models of Alzheimer's disease, aging or diabetes, have been shown to restore the cognitive defects that are associated

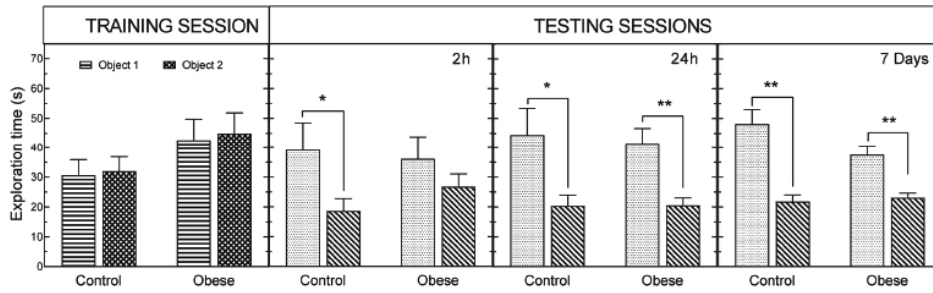


Figure 4. Evaluation of the impact of obesity on the different components of the memory process. The learning capacity of 105-day-old male rats, was tested using a combination of the novel object recognition and novel object location tests. Bars represent the capacity of the animals to encode (2 hours), consolidate (24 hours), and retrieve (7 days), new information. Learning tests were performed at the beginning of the animal's dark-light cycle. Data represent the mean \pm SEM of the data from a total number of 9 to 12 animals per experimental group. * $P < .05$ as determined by Student's *t*-test.

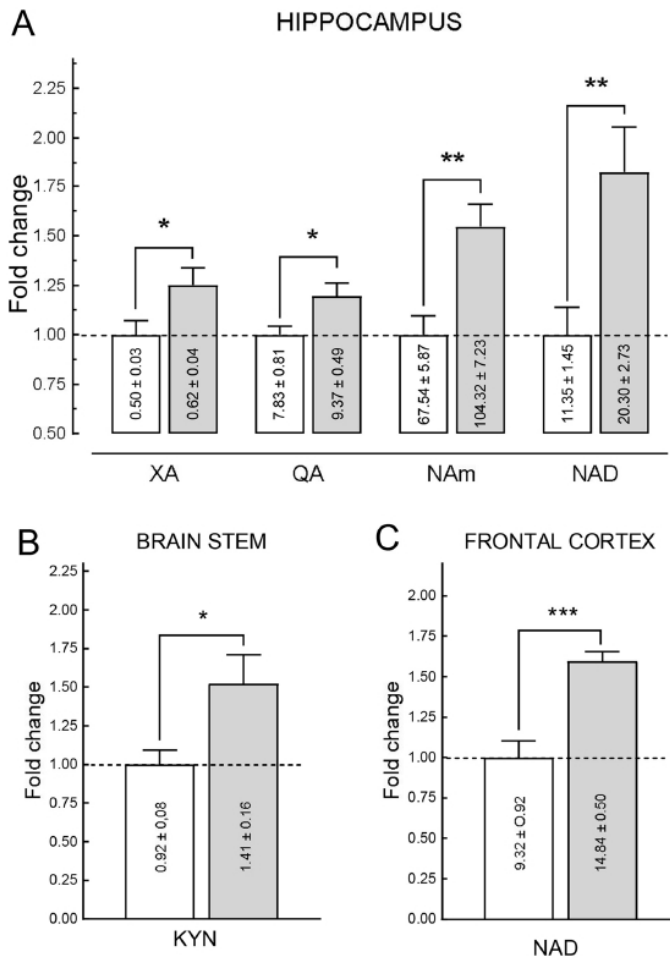


Figure 5. Concentration of several metabolites derived from the metabolism of tryptophan through the kynurenine pathway in the hippocampus (A), brain stem (B) and frontal cortex (C) of 105-day-old control (white bars), and obese (grey bars), male rats. The concentration of the different metabolites was determined by liquid chromatography–tandem mass spectrometry using brain samples from 7 to 8 animals per group. Data are expressed in percentage of values in adult animals fed a standard diet. Absolute values in pmol/g of tissue of xanthurenic (XA) and quinolinic (QA) acids as well as of kynurenine (KYN) and NAD⁺, for control animals in the different brain regions are indicated in the corresponding white bars. * $P < .05$; ** $P < .01$; *** $P < .001$ as determined by Student's *t*-test.

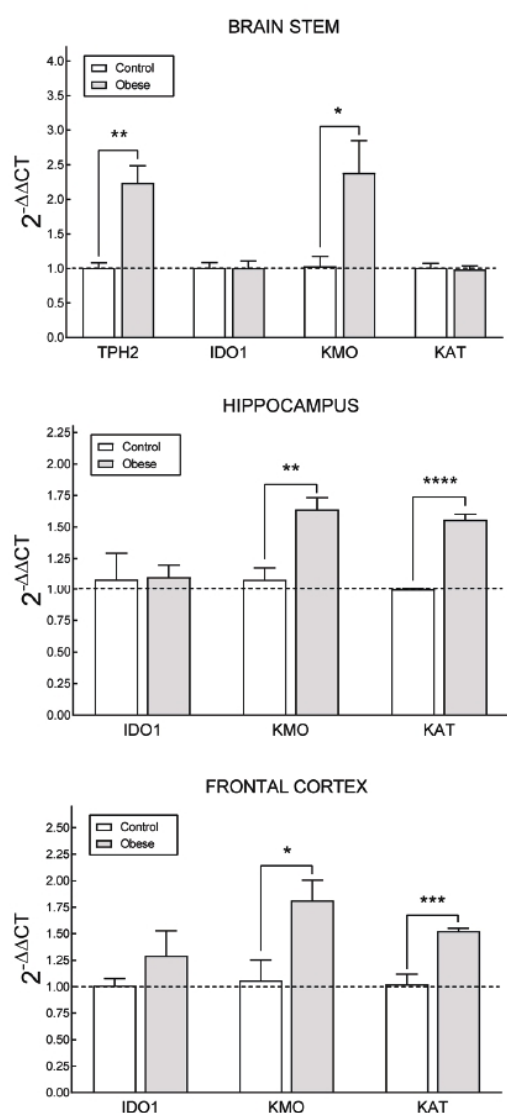


Figure 6. Expression levels of mRNAs encoding for key enzymes of tryptophan metabolism through the kynurenine pathway in the brain of control and obese 105-day-old male rats. The expression of TPH2, IDO1, KMO, and KAT, was determined by real-time quantitative PCR. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression of control animals as a calibrator. Data represent the mean \pm SEM from a total of 8 animals per experimental group. White bars, control group; Grey bars, obese group. * $P < .05$. ** $P < .01$. *** $P < .001$ (Student's *t*-test).

with these pathological conditions. It may therefore seem paradoxical that the concentration of Nam and NAD⁺ is increased in obese rats while they show learning deficits. Nevertheless, negative effects on brain function have also been reported following the administration of these compounds.^{65,66} In particular, the degeneration of dopaminergic neurons and the motor deficit characteristic of Parkinson's and Huntington's disease

are exacerbated by Nam injection.^{67,68} Similarly, in vitro studies in cell cultures or brain slices have shown that exposure to NAD⁺ results in astrocyte cell death.⁶⁹ Further research is needed to establish the relationship between increased brain NAD⁺ and Nam concentration and the learning deficits identified in the obese animals in this study.

The results of the present study also show that the changes in brain KYN metabolism in response to obesity differ by brain region. Thus, in contrast to the increase in QA, XA, Nam, and NAD⁺ concentrations in the hippocampus, only the content of NAD⁺ was increased in the brain stem of obese animals. On the other hand, consumption of the high-calorie diet increased the concentration of KYN only in the frontal cortex. This differential response pattern, can be explained by the phenotypic and functional heterogeneity of microglial cells. Indeed, in the brain, the transformation of KYN into KA takes place predominantly in astrocytes, whereas the synthesis of 3-HK and other metabolites resulting from its transformation, including QA, is carried out mainly in the microglia.^{70,71} The gene expression profile and physiological characteristics of microglial cells vary from one brain region to another, including in response to an immunological challenge.^{72,73} In this respect, an observation of particular relevance to the present study, is that stimulation of the immune response by peripheral administration of LPS increases the proliferation, and thus density, of microglial cells in the dentate gyrus and subventricular zone of the hippocampus but not in the cortex.⁷⁴

In order to determine the mechanisms underlying the alterations in the brain concentration of Trp metabolites in obese animals, the expression level of the genes coding for the main enzymes regulating its metabolism was assessed by RT-PCR. The results of these analyses showed that obesity increases the expression of mRNAs coding for TPH2 in the brain stem along with those of KAT in the hippocampus and frontal cortex. The increased levels of XA, QA and NAD⁺ in the hippocampus, could therefore be consecutive to the enhanced KMO expression detected in this brain region. In contrast, obesity enhanced the expression of TPH2 in the brain stem without affecting the concentration of 5-HT. Similarly, there is no correlation between the increased expression of KAT in the hippocampus and frontal cortex and the absence of change in KA levels in these structures or between the increase in KYN concentration and the lack of change in KAT expression in the brain stem. It must be considered, however, that there are several levels of regulation between the synthesis of a given mRNA and its translation into a functional protein. These include the control of mRNA stability and translation efficiency as well as the post-translational modifications and the level of degradation of the protein it encodes. Consequently, there is not always a direct correlation between the expression levels of an mRNA and the concentration and/or functional activity of the protein which results from its translation. Another factor to consider is the lack of cellular resolution of our analyses. Indeed, TPH2 expression takes

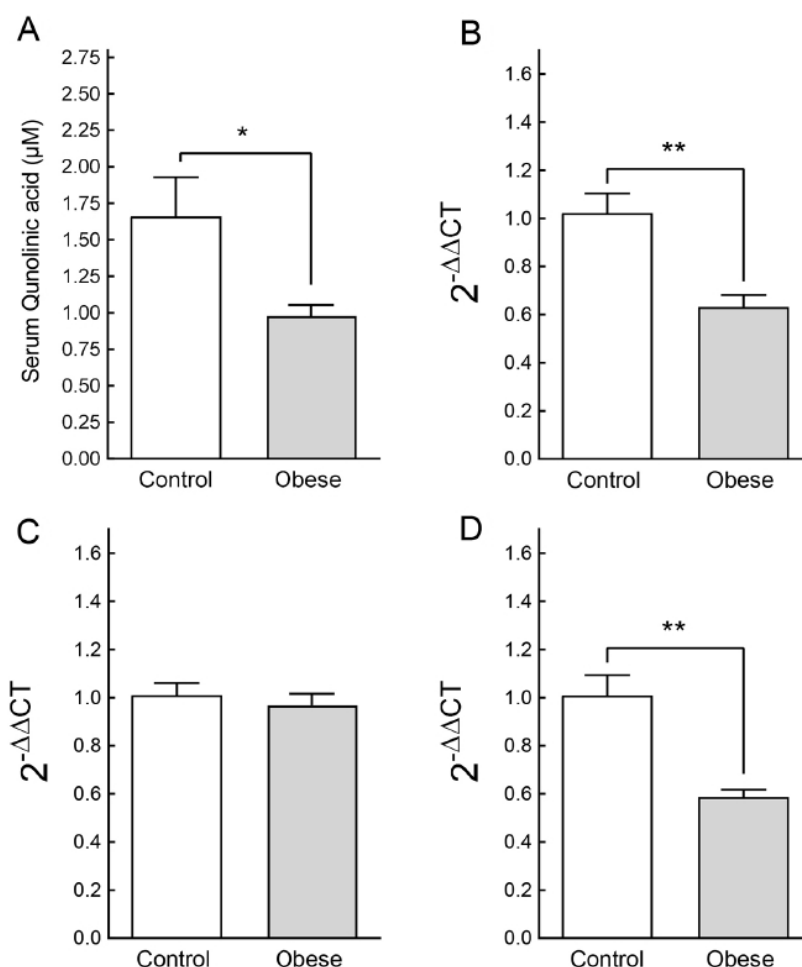


Figure 7. Concentration of quinolinic acid in serum (A) and gene expression levels of IDO2 (B), TDO2 (C) and HAAO (D) mRNAs in liver of control and obese 105-day-old rats. The concentration of QA was determined by liquid chromatography–tandem mass spectrometry whereas the expression of IDO2, TDO2, and HAAO mRNAs was determined by real-time quantitative PCR. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression of control animals as a calibrator.

Data correspond to the mean \pm SEM from a total number of 7 to 8 animals per experimental group * $P < .05$. ** $P < .01$ (Student's *t*-test).

place in serotonergic neurons located in the raphe nuclei which are part of, but that do not constitute, the entire brainstem. On the other hand, Trp metabolism via the KP in the brain takes place in glial cells, which are present in a slightly lower proportion than neurons.⁷⁵ Hence, the mixture of cell types on which the analyses were performed is a limitation of the present study. To overcome this limitation, it would be necessary to perform experiments on enriched preparations or primary cultures of glial cells. However, the separation of glial cells from neurons and their subsequent segregation into astrocytes and microglia in the adult brain, remains a highly challenging task.

We conducted analyses in the brain stem because both positive and negative actions on learning have been described for 5-HT^{76,77} and because the raphe nuclei located within this brain region are the seat of serotonergic neurons which innervate the whole brain. Concerning the hippocampus and

the prefrontal cortex, these regions are considered to be the main areas underlying the memory process. It is indeed accepted that the encoding and retrieval of information relies on a bi-directional communication network between the hippocampus and the prefrontal cortex. Within this network, the acquisition and consolidation of new information is carried out in the hippocampus while the retrieval of a memory trace is done in the frontal cortex (reviewed by Preston⁷⁸). The increased concentration of QA in the hippocampus but not in the frontal cortex in obese animals, is therefore consistent with their cognitive deficit characterized by a difficulty in encoding new information. On the other hand, the fact that there were no differences between obese and control animals in the cerebral content of 5-HT or 5-HIAA, indicates that the cognitive disturbances linked to obesity result mainly from the alteration of Trp metabolism through the kynurenine pathway.

It should be noted, however, that in contrast to our observations, Haleem and Mahmood,⁷⁹ recently reported an increase in the concentration of Trp and 5-HIAA in the hippocampus of obese rats. Several factors may explain these discrepancies. First, the housing conditions of the animals and the obesity model differed significantly between the 2 studies. Thus, while in our case the animals were made obese by exposure to a free-choice high-fat, high-sugar diet and were always housed at least 2 per cage and at a controlled temperature of 21°C, in Haleem and Mahmood's study the rats were fed exclusively with a high-fat diet and were housed individually at a temperature of 24 ± 2°C. Another important difference concerns the phase of the circadian cycle during which the animals were sacrificed, that is, during the dark phase in the present study versus the light phase in the study by Haleem and Mahmood. Interestingly, despite these experimental differences and the use of 2 distinct learning tests, the NOR-NOL test in the present study and the water maze test in Haleem and Mahmood, in both studies obese animals showed reduced memory encoding. The analysis of other functional parameters of the serotonergic system, such as the quantification of the expression and the evaluation of the sensitivity of the 5-HT receptors involved in the regulation of the memory process, should allow to draw a definitive conclusion as to the involvement of 5-HT in the cognitive alterations induced by obesity.

The brain concentration of KYN is determined by its synthesis in microglial cells from Trp and by the amount of kynurenine transported into the brain from the bloodstream since, unlike 5-HT, KYN can cross the blood-brain barrier.⁸⁰ At the peripheral level, 85% to 95% of Trp is bound to albumin and the remaining 15% to 5% flows in free form.⁸¹⁻⁸³ Only free Trp can cross the blood-brain barrier via the large neutral amino acids (LNAA) transporter. The binding site of Trp on albumin is common to free fatty acids⁸⁴ and, in fact, NEFAs inhibit, *in vitro*, the binding of Trp to albumin, thereby increasing the concentration of free Trp.^{43,44} Furthermore, it has been shown that the administration of insulin increases the cerebral transport of Trp.^{85,86} This effect would be the result of a decrease in the blood concentration of LNAA, whose uptake by the muscle is stimulated by insulin, and thus of a greater availability of the LNAA carrier for the transport of free Trp into the brain. On the basis of these data from the literature, we hypothesized that the neuroinflammation triggered by the consumption of a high-calorie diet would activate the KP and that this process would be favored by an increased passage of Trp to the brain due to the stimulatory action of insulin on this process and the increase in free Trp in the bloodstream resulting from its displacement from albumin by free fatty acids.

However, contrary to our expectations, there was no difference in serum free Trp levels between control and obese animals despite the hyperinsulinemia of the latter. Obese animals also showed the same circulating levels of KYN and NEFAs than control animals. Several arguments can be advanced to explain these results. First, we deliberately euthanized the animals at the same time of the day and in the same nutritional status at which the memory tests were performed. That is, 2 hours after the

beginning of the dark phase of their light-dark cycle and under fed conditions. However, the blood concentration of NEFAs decreases after each meal so that to observe any variation in the circulating level of these lipids, a period of food deprivation of at least 12 hours is necessary even in obese individuals. On the other hand, the deregulation of lipid metabolism induced by obesity is directly related to its severity. Actually, more than 8 weeks of exposure to a hyperlipidic diet were required to observe an increase in serum NEFAs levels after an overnight fast in rats.⁸⁷ Finally, the correlation between increased NEFAs concentration at the peripheral level and enhanced brain Trp^{88,89} as well as the stimulatory effect of insulin on brain Trp transport,⁸⁶ have been documented only in fasted animals. Therefore, the results obtained in this study lead us to conclude that Trp metabolism at the peripheral level is not involved in the cognitive alterations induced by obesity.

The first experimental series that was performed showed that obesity reduces learning capabilities in male animals but not in female rats. This observation is in agreement with several reports in the literature indicating that there are sex differences in how new information is processed within the hippocampus,⁹⁰ and with the results of Hwang et al⁹¹ and Lord et al⁹² who showed that male mice or male rats made obese by exposure to a high-fat diet show learning deficits while their female counterparts exposed to the same nutritional insult do not display any decrease in their memory capacity as compared to control animals. However, learning deficits in obese female rats have also been reported.⁹³ These discordant results could be explained by the impact of sex hormones on memory. Indeed, estrogens positively impact learning such that female rats generally exhibit better cognitive performances during the estrous phase of the estrous cycle when estrogen levels are at their highest.⁹⁴⁻⁹⁷ Unfortunately, the phase of the estrous cycle during the memory tests was not documented either in the present investigation nor in the aforementioned studies. In consequence, it remains to be determined whether the negative effects of obesity on learning in female rats are attenuated or exacerbated as a function of the phase of their estrous cycle.

In conclusion, the results of the present study show that, in rats, the learning deficit induced by obesity is characterized by a reduction of the capacity to acquire new information. This cognitive deficit is associated with a dysfunction of the cerebral metabolism of tryptophan through the kynurenine pathway which results in increased concentrations of QA, XA, Nam, and NAD⁺ in the hippocampus. Given the major role of the hippocampus in the encoding process of memory and the negative effects of QA on learning, it can be reasonably proposed that altered kynurenine pathway metabolism contributes to obesity-induced learning disabilities. The reduction of QA synthesis in obese animals through the blocking of IDO1 or KMO enzymatic activity with specific inhibitors, would add further support to this proposal and could pave the way for pharmacological treatment of obesity-related cognitive alterations. The deleterious effects of obesity on cognition

were observed only in male animals indicating that they are sex dependent. Additional studies are needed to determine the extent to which the effect of obesity on cognition in female rats is modulated by sex hormones.

Acknowledgements

The authors are grateful to Amandine Lefebvre, Sandrine Suzanne and Alexis Gandon for the care of the animals.

Author Contributions

FBJ conceived the study and designed the experiments. CEMG, ADA, LARC, SOH and CA performed the experiments and analysed the data. MC and AA performed the mass spectrometry analysis. ECG and EZ contributed with conceptual ideas. CEMG and FBJ wrote the original draft. All authors revised the manuscript and agreed to publish it.

Supplemental Material

Supplemental material for this article is available online.

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Supplemental Table 1

Tryptophan metabolite concentrations in the brain of control and obese male rats at 120 days of age

Metabolite	Brain-Stem		Hippocampus		Frontal Cortex	
	Controls	Obese	Controls	Obese	Controls	Obese
Trp	53.01 ± 2.78	59.40 ± 3.59	53.22 ± 2.59	59.22 ± 3.24	35.18 ± 2.06	33.24 ± 1.43
5-HT	59.93 ± 7.17	63.57 ± 7.27	33.87 ± 4.44	37.55 ± 5.07	20.32 ± 2.33	20.75 ± 2.03
5-HIAA	22.28 ± 3.14	21.28 ± 2.67	15.31 ± 2.22	19.47 ± 2.82	9.31 ± 1.15	9.04 ± 0.94
Nam	83.01 ± 7.10	96.45 ± 9.92	67.54 ± 5.87	104.32 ± 7.23	67.26 ± 6.12	56.43 ± 9.3

Supplemental Table 2

Tryptophan metabolite concentrations in serum of control and obese 105 days-old male rats

Metabolite	Controls	Obese
Total-Trp	56.93 ± 4.92	53.73 ± 4.16
Free-Trp	3.51 ± 0.46	3.10 ± 0.37
Bound-Trp	58.19 ± 4.71	50.63 ± 4.32
5-HT	2.38 ± 0.48	2.38 ± 0.39
5-HIAA	0.17 ± 0.02	0.16 ± 0.02
KYN	1.74 ± 0.17	1.64 ± 0.15
KA	0.03 ± 0.003	0.03 ± 0.01
XA	0.61 ± 0.05	0.55 ± 0.02

ARTICLE 2.

*Obesity-induced learning deficits are
oestrous cycle-dependent and are
associated with impaired kynurenine
pathway metabolism.*

Carla Elena Mezo-González, Juan Antonio García Santillán, Luis Antonio Reyes-Castro, Mathilde Gourdel, Mikaël Croyal, Francisco Bolaños-Jiménez. (Submitted to Neuroendocrinology).

Obesity-induced learning deficits in the female rat are oestrous cycle-dependent and are associated with impaired kynurenine pathway metabolism

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Short title: Kynurenine pathway and obesity-induced memory deficits.

Key words: Obesity, Learning, Kynurenine pathway, Estrous cycle.

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Funding: This work was supported by the French National Research Agency (ANR, grant ANR-16-CE21-0007-01) and CONACYT (award number 2015-16-273510).

Disclosures

All authors declare that there is no conflict of interest in this study.

Abstract

Objectives: Obesity induces cognitive deficits affecting males more than females. It is believed that females are protected from cognitive decline by oestrogens. The concentration of these hormones, peaks during proestrus and gradually declines through oestrus, dioestrus and metestrus. Oestrogens enhance the expression of tryptophan hydroxylase-2, the rate-limiting enzyme in the transformation of tryptophan (Trp) to serotonin. This neurotransmitter plays a significant role in learning and memory. However, several learning/memory-regulating compounds arise also from Trp metabolism through the kynurenine (KYN) pathway including xanthurenic acid (XA) and NAD⁺. The aim of the present study was to determine the involvement of the kynurenine pathway of Trp metabolism in the regulation of learning in control and obese female rats.

Methods: The learning capabilities of control and obese female rats were evaluated using the Novel Object Recognition test. Tryptophan and Trp-derived metabolites were quantified in the hippocampus and frontal cortex by Ultra-performance liquid chromatography-tandem mass spectrometry.

Results: Control rats in proestrus/oestrus performed better than their control mates in dioestrus/metestrus. In contrast, while there were no differences in learning between control and obese rats in dioestrus/metestrus, obese rats in proestrus/oestrus displayed decreased memory capacity along with reduced production of XA and NAD⁺ and upregulated expression of mRNAs coding for the enzyme Indoleamine 2,3-dioxygenase, that catalyses the transformation of tryptophan to kynurenine.

Conclusions: The deleterious effects of obesity on learning are closely linked to the oestrous cycle and are mediated, at least in part, by an impairment of the kynurenine pathway of tryptophan metabolism.

Introduction

In addition to be a primary risk factor for developing diabetes, cardiovascular diseases and other metabolic disorders, obesity also impairs brain function. In particular, several studies have evidenced the existence of memory and attention deficits in obese adults and children (1–5), as well as in animal models of obesity (6,7). It has also been suggested that obesity increases the risk of senile dementia and of neurological disorders characterised by the progressive loss of memory and cognitive abilities such as Alzheimer's disease (8–11). However, the hypothesis that obesity in adulthood predisposes to the development of cognitive disorders in old age has been challenged by various authors who have observed, on the contrary, a reduction in the incidence of senile dementia in obese patients (12).

Though it is clear that obese patients display impaired brain function, it remains controversial whether the cognitive alterations associated with obesity show sexual dimorphism. Thus, while some studies report a decrease in memory capacity in obese females but not in obese males (13–16), other reports show better cognitive performance in obese females compared to their male counterparts (17,18). Discordant results have also been obtained in studies in rats and mice with either a decrease in cognitive abilities only in obese male animals (19–21), or in both sexes (22,23). One factor that could explain these contradictory results is the impact of the oestrous cycle of female individuals at the time of the cognitive tests. Indeed, ovarian hormones modulate memory and learning processes in a significant way (24,25). However, very few studies to date have investigated the possible influence of the oestrous cycle on obesity-induced cognitive impairments.

With the aim of providing some insight into this question, here we have assessed the learning capacity of control and obese female rats during the oestrous cycle. In addition, in order to determine the mechanisms that might underlie possible differences in the effects of obesity during the oestrous cycle, the metabolism of tryptophan (Trp) through the serotonin (5-HT) and kynurenine (KYN) pathways was also examined. Oestrogens promote brain 5-HT synthesis through their ability to increase the expression of tryptophan hydroxylase 2 (TPH2), the first and rate-limiting enzyme in the transformation of Trp into 5-HT (26–29). Estrogens also up regulate the gene expression of indoleamine 2,3-dioxygenase (IDO), the limiting enzyme of Trp catabolism via the kynurenine pathway (30,31). This metabolic pathway leads to the production of several neuro-active compounds which, like serotonin, are involved in the regulation of learning and memory processes.

Materials and Methods

Animals and diet-induced obesity model

We used a free-choice high-fat high-sugar diet (fc-HFHS) obesity model which has the advantage of simulating the modern-day dietary environment of humans and of leading in a short time to an obese phenotype (32,33). Male and female Wistar rats (Janvier Labs, Le Genest Saint Isle, France), were fed either standard chow (controls), or exposed from weaning to high-fat food and to a 30% sugar solution in addition to standard chow and tap water (obese). High-fat food consisted in powdered standard chow mixed with pig fat in the proportion of 30 g of fat per 70 g of food. Sweetened condensed milk was used as a base to prepare the 30% sugar solution. Animals were maintained under an inversed 12-

/12-hour dark-light cycle (lights off at 7:00 AM), and at $21 \pm 1^\circ\text{C}$ with food and water ad libitum through all the experiment.

Behavioural analyses

Memory tests were performed under red light 2 hours after the beginning of the dark phase of the animal's light-dark cycle in a laboratory adjacent to the housing room of the animals and recorded using the Viewpoint Videotrack system (Point Grey Research Inc., Richmond B.C. Canada), for offline analysis by two investigators blind to the nutritional status of the animals. The learning capabilities of 120-days-old male and female rats of both experimental groups (control and obese), were assessed using the novel object recognition test (NOR) as described by (Ennaceur y Delacour 198). The NOR test was divided into three sessions: habituation, training and testing. During the habituation session, rats were allowed to freely explore an open-field arena (50 x 50 x 40 cm) for 20 minutes during three consecutive days. The fourth day, two identical objects were placed into the arena before introducing the rat and each animal was allowed to explore the objects for 7 minutes. Two and 24 hours after this training phase, animals were returned to the arena in which one of the familiar objects has been exchanged for a new one to evaluate, respectively, short term (STM) and long term (LTM) memory. The time exploring each object was recorded and the learning capacity determined from the discrimination index (DI). This corresponds to the difference in exploration time of the novel object versus the familiar one divided by the total amount of time spent exploring both objects. A positive DI indicates memory acquisition whereas a DI equals to or below zero suggest no learning. Object exploration was defined as sniffing or touching the object with the vibrissae or when the animal's head was oriented toward the object with the nose

placed at a distance of less than 2 cm from the object. The stage of the oestrous cycle of each female rat, was determined from the structural changes of the vaginal epithelial cells collected each day at two o'clock from 15 days before the NOR experiments until the sacrifice of the animals.

Biological samples collection and processing

The day following the last NOR testing session, animals were deeply anesthetized with isoflurane two hours after the beginning of the dark phase of their light-dark cycle to obtain a blood sample by cardiac puncture and sacrificed immediately after by cervical dislocation. The frontal cerebral cortex and hippocampus were dissected, and the serum was separated from blood. All samples were immediately frozen after dissection in liquid nitrogen and stored at -70°C until analysis. Retroperitoneal fat depots were dissected and weighed to provide a measure of body fat.

Metabolite determinations.

Triglycerides, cholesterol and fatty acids were analysed in serum by enzymatic methods (Triglycérides enzymatiques PAP 150, BioMérieux; Cholestérol RTU, Biomérieux; NEFA FS, DiaSys). Serum glucose concentrations were determined with a blood glucose monitor (Accu-Check®, Roche Diagnostics). Oestradiol quantification was performed using the Estradiol Parameter Assay Kit from Bio-Techne.

Analyses of tryptophan metabolism.

Tryptophan and the metabolites generated by its catabolism through the serotonin (5-HT) and KYN pathways were quantified in the hippocampus and frontal cortex of male rats

by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the methodology described in detail by Martimiano et al.2017 (35). In brief, 50 mg of tissue were homogenized in 200 μ L of an ice-cold solution of 2.7 mM EDTA containing 1% formic acid and mixed with 10 μ L of a pool of exogenous internal standards. Subsequently, 200 μ L of the homogenate were mixed with 500 μ L of ice-cold acetonitrile and, after centrifugation at 15,000 g for 15 minutes at 4 °C, the supernatant was recovered and evaporated to dryness under a stream of nitrogen at room temperature. Calibration curves were generated using serial dilutions of Trp, QA,5-HT, KYN, 5-HIAA, KA and Xanthurenic acid (XA), in 2.7 mM EDTA containing 0.1% formic acid which were processed in the same way that the tissue samples. At the end, all dried samples were dissolved in 100 μ L of EDTA 2.7 mM containing 0.1% formic acid and 5 μ L of each sample were injected into the LC-MS/MS system for analysis. Compounds were separated on a reverse Acquity HSS T3 column (2.1 x 100 mm, 1.7 μ m, Waters Corporation, Milford, MA, USA) at 30°C with a linear gradient of the mobile phase B (acetonitrile containing 0.5% formic acid and 2.5 mM ammonium acetate) in the mobile phase A (water containing 0.5% formic acid and 2.5 mM ammonium acetate) and at a flow rate of 400 μ l/min. The electrospray interface of the mass spectrometer was operated in positive ion mode and the multiple reaction monitoring mode was chosen for metabolite detection. Data acquisition and analysis were performed using MassLynx® and TargetLynx® software, respectively (version 4.1; Waters). Compound concentrations were calculated using calibration curves plotted from standard solutions.

Real time quantitative RT-PCR

A conventional Trizol extraction method was used to obtain total RNA. Thereafter, 1 µg of purified RNA was treated with a DNase and reverse transcribed using the MultiScribe™ Reverse Transcriptase kit from Thermo Fisher according to the manufacturer's instructions. The resulting cDNA was diluted 40-fold in DNase and RNase free water and 5 µl of each cDNA diluted sample were used as template for amplification using an iCycler iQ Real-Time PCR detection system and SYBR Green as fluorogenic intercalating dye. Relative gene expression differences between control and obese animals were determined by the comparative $2^{-\Delta\Delta C_t}$ method (36), using 18S, β 2-microglobulin or GAPDH transcripts as housekeeping genes. The sequences of the primers used for the amplification are as follows : *Tryptophan hydroxylase 2 (TPH2)*, forward: ATC CCA AGT TCG CTC AGT TTT CTC, reverse: ACG CCC GCA GTT GAC CTT C; *Indoleamine 2,3-Dioxygenase 2 (IDO2)*, forward: GGT GAC AGT CTT GGT GGA GAA G, reverse: GGT GTC CTG GCT GTG TTG C; *Kynurenine aminotransferase (KAT)*, forward GAA TTA CTC AAG GTT CCT CAC TG, reverse GAT GGT GCT TCC GTT CTC C; *Kynurenine mono-oxygenase (KMO)*, forward: GCT TCC AAC GCA TAC TGA TG, reverse: GGC AGG CAA CAG AAA GAA ATC; *18 S*, forward: GAT GCG GCG GCG TTA TTC C, reverse: CTC CTG GTG GTG CCC TTC C; *β 2-microglobulin*, forward: GAT GGC TCG CTC GGT GAC, reverse: CGT AGC AGT TGA GGA AGT TGG; *GAPDH*, forward: CAG TAT GAC TCT ACC CAC GGC A, reverse: ATC TCG CTC CTG GAA GAT GGT G.

Statistical analysis

Experimental results were analysed using software GraphPad Prism version 9. Data were first checked for normality by the Shapiro-Wilk normality test and the statistical differences assessed by unpaired Student's t-test or two-way ANOVA. Statistical significance was set at $p < 0.05$. Data are expressed as means \pm SEM.

Results

Metabolic alterations induced by the consumption of the FcHFHS diet

In agreement with our previous observations (37), both male and female animals exposed to the FcHFHS diet developed a clear obesity phenotype characterised by increased body weight and adipose tissue as well as by enhanced concentration of circulating free fatty acids (**Table 1**). In addition, obese male rats showed elevated serum cholesterol and triglyceride levels compared to their normal weight counterparts.

The oestrous cycle determines the learning capacity of female rats both under basal conditions and in response to hypercaloric feeding

In order to check if the obesity model used in this study induce learning deficits in both males and males, a group of male rats of the same age which were exposed to the same nutritional conditions as the female rats, were included as controls in the NOR tests. The results of these experiments show that male animals exposed to the FcHFHS diet have a deficit in short-term memory as indicated by their inability to distinguish the novel object from the familiar one 2h after the training session (**Figure 1A**). In contrast, there were no differences in the capacity to distinguish the two objects between obese and control female rats as determined by two-way ANOVA analysis of the data which showed a

significant interaction between sex and diet ($F(1,33) = 5.80$ $p = 0.02$). However, when the results of the NOR test were grouped according to the phase of the oestrous cycle, it became clear that the discrimination index of obese rats in proestrus/oestrus is also decreased compared to that of control rats that are at the same stage of the cycle (**Figure 1B**). By contrast, the discrimination index of obese and control rats in metestrus/dioestrus is almost identical. Actually, the learning ability of control rats in metestrus/dioestrus is comparable to that of obese female rats in proestrus/oestrus (**Figure 1B**).

Similar to the observations made during the assessment of short-term memory, a significant decrease in the learning ability of obese male animals compared to their control counterparts was observed 24 hours after the training phase with a significant effect of sex ($F(1,23) = 4.72$; $p = 0.04$; **Figure 1C**). In contrast, no differences in long-term memory were observed between control and obese female rats. However, a closer inspection of the data revealed that female control rats have a reduced capacity to distinguish the novel object from the familiar one in comparison to male control animals during the 24h testing session (**Figure 1C**). The learning differences displayed by female control rats between the two testing sessions, were not associated to a reduction in total exploration time (**Figure 1D**), indicating that, under the present experimental conditions, they are not able to consolidate or retrieve a previously encoded memory trace.

Hormonal determinations

In order to verify the reliability of the determinations of the oestrous cycle stage from the cytological analysis of vaginal smears, oestradiol levels were quantified in serum samples collected at the time of sacrifice and compared with the results of cytological analyses performed that same day. This comparative analysis showed a positive relationship

between the concentration of oestradiol in serum and the stage of the oestrous cycle determined by vaginal smear analysis. That is, a high concentration of oestradiol during proestrus/oestrus and lower levels during metestrus/dioestrus (**Figure 2**). Furthermore, no differences in oestradiol concentration were found between control and obese rats neither during the proestrus/oestrus stage nor during metestrus/dioestrus.

The deleterious effects of obesity on learning are associated with impaired brain tryptophan metabolism.

With the aim of determining to what extent the differences in the learning abilities of female rats across the oestrus cycle could result from an altered brain production of 5-HT and KYN, the concentration of these neurotransmitters and other Trp-derived metabolites were quantified by mass spectrometry in the hippocampus and prefrontal cortex of obese and control female rats. The results of these analyses showed a decreased concentration of Trp in the hippocampus of obese rats in comparison to their control counterparts (**Figure 3A**). Similarly, the hippocampal levels of KYN and of their downstream metabolites xanthurenic acid (XA) and NAD⁺ were reduced in obese rats (**Figure 3B**). The levels of Trp, 5-HT, KYN, XA and NAD⁺ were also reduced in the frontal cortex of obese rats together with those of 5-HIAAA (**Figure 4A, 4B**).

It is noteworthy that all changes in tryptophan metabolism induced by obesity were closely related to the oestrous cycle. Indeed, the reduction in the concentration of Trp, KYN and XA in the hippocampus of obese rats, was evident only in rats in proestrus/oestrus, whereas in the frontal cortex these neurochemical alterations occurred exclusively during metestrus/dioestrus. The two-way ANOVA analysis of the data also showed a reduction in the hippocampal concentration of Trp, KYN and XA in control

rats while in metestrus/dioestrus in comparison to their congeners in proestrus/oestrus (**Figure, 3A, 3B**) with a significant effect of diet and oestrus cycle on tryptophan ($F(1,15) = 11.91$; $p = 0.003$; $F(1,15) = 11.91$; $p = 0.003$) and kynurenine ($F(1,15) = 9.16$; $p = 0.008$; $F(1,15) = 8.31$; $p = 0.01$) levels and a significant effect of diet on the concentration of XA ($F(1,15) = 6.58$; $p = 0.02$) and NAD⁺ ($F(1,15) = 9.19$; $p = 0.007$).

Obesity impairs the expression of Trp metabolizing enzymes in a regional and oestrus cycle dependant manner.

To get insight into the mechanisms underpinning the aforementioned neurochemical alterations induced by obesity, the mRNAs levels encoding the enzymes regulating the main steps of Trp metabolism via the kynurenine pathway, i.e. IDO2, KAT and KMO, were determined by RT-PCR. As illustrated in Figure 5A, obesity induced an up-regulation of IDO2 expression in the hippocampus only in females in metestrus/dioestrus (**Figure 5A**). Regarding KAT expression, an increase in the level of mRNAs encoding this enzyme was observed in obese animals in proestrus/oestrus but not in the rats in metestrus/dioestrus (**Figure 5B**). This was determined by two-way ANOVA analysis of the data which showed a significant effect of the diet ($F(1,16) = 1.90$; $p = 0.004$), of the oestrus cycle ($F(1,16) = 7.84$; $p = 0.01$) and a significant interaction between the two factors ($F(1,16) = 5.61$; $p = 0.03$). Moreover, the levels of the mRNAs coding for KMO were enhanced in control rats in metestrus/dioestrus in comparison to control rats in proestrus/oestrus (**Figure 5C**, oestrous cycle $F(1,14) = 11.18$; $p = 0.004$).

Finally, similar to what was observed in the hippocampus, obesity increased the expression of IDO2 in the frontal cortex only in rats in metestrus/dioestrus with a significant effect of diet ($F(1,13) = 10.01$; $p = 0.007$), and estrus cycle ($F(1,13) = 8.87$,

0.01) and a significant interaction between both factors ($F(1,13) = 6.42; 0.02$). However, unlike in the hippocampus, no differences in the expression of KAT and KMO were observed between control and obese rats (**Figure 6B, 6C**).

Discussion

A large number of epidemiological studies and animal investigations indicate that obesity causes cognitive impairment (1–5,38,39). However, whereas various studies show that obesity-induced learning deficits exhibit sexual dimorphism (14–17,40,40), other reports show no difference in learning abilities between obese male and female individuals (22,23,41).

The results of the present study show that, in rats, obesity reduces learning ability independently of sex. However, in female animals these effects are closely related to the oestrous cycle. Indeed, when the ability to distinguish the novel object from the familiar object 2h after the training session in the NOR test was assessed (short-term memory), only obese rats in proestrus/dioestrus showed impaired learning compared to control rats that were in the same phase of the oestrous cycle. It should be noted, however, that control rats in metestrus/dioestrus displayed a reduced memory capacity that was, in fact, of the same order as that of their obese counterparts in proestrus/dioestrus. This observation is in agreement with the results of previous experiments in mice and rats in which a decreased ability to distinguish the novel from the familiar object during metestrus/dioestrus was also found (42–45). Therefore, the fact that there were no differences in learning ability between control and obese female rats in metestrus/dioestrus indicates that the deleterious effect of obesity on learning does not

add to the reduction in cognitive ability occurring naturally during this phase of the oestrus cycle.

Furthermore, when assessing long-term memory (24 hours after the training session), it was found that female control rats had substantial difficulties to distinguish the novel object from the familiar one, regardless of the phase of their estrous cycle. Consequently, no difference in long-term memory was observed between control and obese female rats, whereas the obese male animals displayed significantly lower learning ability than their control counterparts. The observation that male control animals have better learning skills than female control rats is in agreement with the results of Frick and Gresik, 2003 in mice (46), but is at odds with several studies reporting, on the contrary, better cognitive performances of female rodents in the NOR test (47–50). However, these last observations have not been confirmed by other studies (44,51–53). Several arguments can be put forward to explain these contradictory results, including the use of animals of different strains, the timing of the circadian cycle during which the learning experiments were carried out and the fact that the estrous cycle of female animals was not systematically taken into account. Therefore, which of the two sexes has better cognitive performances in the NOR test remains controversial.

We then asked whether the variations in learning ability displayed by female animals during the estrous cycle, and therefore in response to obesity, could be related to an alteration in brain tryptophan metabolism. The transformation of this amino acid by the enzyme tryptophan hydroxylase (TPH) leads to the production of serotonin, whose involvement in the regulation of learning and memory is well documented (54,55). In addition, the expression of TPH is stimulated by estradiol (28,29,56). We therefore hypothesised that the fluctuations in oestradiol production during the oestrous cycle could

lead to variations in the cerebral concentration of serotonin which might interfere with the learning processes. However, serotonin is not the only neurotransmitter derived from tryptophan. In fact, more than 95% of tryptophan is catabolised via the kynurenine pathway. The first step of this metabolic pathway is the conversion of Trp by IDO to N-formyl kynurenine, which serves as an intermediate for the production of KYN. Subsequently, KYN follows two catabolic pathways. On the one hand, it is transformed into KA by the action of KAT and, on the other hand, it is hydroxylated by KMO to produce 3-hydroxy kynurenine, the transformation of which leads to the synthesis of xanthurenic acid (XA) and QA. The latter compound serves as a precursor for the de novo synthesis of NAD⁺. KA and QA induce negative cognitive effects by acting, respectively, as agonist and antagonist of glutamate NMDA receptors (57–60). In contrast, NAD⁺ administration has been shown to improve cognitive deficits in animal models of Alzheimer's disease or diabetes (61–64). Since IDO expression is enhanced by pro-inflammatory interleukins such as IL-1 β , TNF α and interferon- γ (65,65–68), we hypothesised that obesity-induced cognitive deficits may be underpinned by the exacerbated production of KYN metabolites, the synthesis of which is enhanced by the state of low-grade inflammation characterising this metabolic dysfunction. Interestingly, estradiol has been reported to increase IDO expression in splenic dendritic cells (69) and in placenta (30,70), suggesting that there might also be a link between the variations in the production of estradiol during the estrous cycle and the synthesis of kynurenine metabolites.

The quantification of Trp and Trp-derived metabolites by mass spectrometry, showed that there are regional variations in the brain concentration of Trp in control rats as a function of the estrous cycle. Namely, Trp levels in the hippocampus decreased but

increased in the frontal cortex during metestrus/dioestrus. Despite these differences, obesity resulted in reduced Trp levels in both brain regions. Consistent with this neurochemical alteration, the concentrations of KYN, XA and NAD⁺ were decreased in both the hippocampus and the frontal cortex of obese rats. Obesity also reduced the hippocampal concentration of 5-HT.

Tryptophan depletion or decreased levels of 5-HT in the brain, result in memory deficits both in humans and in experimental animals (54,71,72). Likewise, reduced cognitive abilities and other age-related neurological impairments have been associated with decreased NAD⁺ levels (73,74). Conversely, NAD⁺ administration improves the cognitive alterations associated with Alzheimer's disease including memory deficits (64,75,76). Recently, several studies have also reported a positive association between low XA levels and the cognitive impairments of Alzheimer's disease (77–79), and the memory deficits induced by diabetes (80), or by chronic stress (81). In the light of these data from the literature, the results of the present study therefore indicate that the memory impairments associated with obesity are the consequence, at least in part, of the reduced synthesis in the brain of 5-HT, XA and NAD⁺.

Regarding the mechanisms underlying the obesity-induced alterations in the synthesis of tryptophan metabolites, analyses of gene expression by RT-PCR showed that the levels of mRNAs encoding the main enzymes involved in the kynurenine pathway also vary depending on the estrous cycle and the brain region. Thus, the hippocampal expression of KMO in control rats increased during the metestrus/dioestrus while no changes in the levels of mRNAs coding for this enzyme were observed in the frontal cortex. Conversely, obesity enhanced the expression of IDO mRNAs in the frontal cortex but not in the hippocampus of control rats while in proestrus/diestrus. In contrast, KAT

expression during the latter stages of the oestrous cycle was enhanced by obesity in both the hippocampus and cortex. The same applied to IDO2 expression, which was increased during the metestrus/dioestrus in obese rats in both the cortex and hippocampus. These observations support the results obtained by mass spectrometry indicating that the metabolism of tryptophan via the kynurenine pathway is also regulated by the oestrous cycle. However, it is difficult at the present time to establish a causal relationship between the changes in gene expression and the oscillations in the levels of tryptophan metabolites occurring during the estrous cycle.

In summary, the present study provides clear evidence that obesity induces memory deficits in both male and female rats. In female rats, the deleterious effects of obesity are dependent on the estrous cycle and are associated with the impaired production of several compounds derived from the metabolism of tryptophan via the serotonin and kynurenine pathways. These results underline the need not only to study individuals of both sexes but also to analyse, under the same experimental conditions, the influence of the estrous cycle in order to draw clear conclusions on the existence of sexual dimorphism for a given physiological effect.

Acknowledgements

Carla Mezo González and Juan Antonio García-Santillán are supported by an international PhD fellowship from CONACYT. Luis Reyes Castro received support from the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship. The authors are grateful to Amandine Lefebvre, Sandrine Suzanne, Alexis Gandon and Blandine Castellano for the care of the animals.

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[Mezo-González et al., Table 1]

Anthropometric characteristics and serum metabolic profile of male and female 120 days-old rats exposed to the FcHFHS diet

	Males		Females	
	Control	Obese	Control	Obese
Body weight (g)	510 ± 12	567 ± 15*	318 ± 4	346 ± 6**
Liver weight (g)	13.57 ± 0.57	16.09 ± 0.71*	9.24 ± 0.19	11.41 ± 0.37****
Adipose tissue (g)	10.06 ± 0.67	18.71 ± 2.01**	13.96 ± 1.38	23.81 ± 2.42**
Glucose (mg/dL)	198 ± 5.84	215 ± 6.34	172 ± 3.94	174 ± 5.83
Cholesterol (ng/dL)	53.74 ± 2.88	65.54 ± 3.17*	63.28 ± 2.35	62.14 ± 3.57
Triglycerides (ng/dL)	45.82 ± 3.04	102.19 ± 16.77*	10.62 ± 1.85	13.95 ± 3.33
Free fatty acids (mg/dL)	11.25 ± 1.97	27.66 ± 2.68**	27.12 ± 2.45	40.90 ± 5.97*

*p < 0.05; **p < 0.01; ***p < 0.001 versus control animals of the same sex as determined by Student's t-test with 8 animals par experimental group.

LEGENDS TO FIGURES.

Figure 1. Learning performances of control and obese male and female rats in the novel object recognition test. The animals' capacities to discriminate a novel object from a familiar one was assessed 2 h (A, B) and 24 h (C) after the training session. Figure (D) shows the total exploration time and the discrimination index of control female rats in proestrus/dioestrus during the two test sessions in which short term memory (STM) and long-term memory (LTM) were evaluated. Note that when the data of the discrimination index of female rats are separated according to the oestrous cycle, it is observed that the learning ability of control animals decreases naturally during metestrus/dioestrus and the negative impact of obesity on short-term memory becomes evident in proestrus/oestrus rats (B). Also noteworthy is the impairment of long-term memory in control rats in proestrus/diestrus (D). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as determined by two-way ANOVA analysis using diet and sex (A,C) or diet and estrous cycle stage ((B) as factors.

Figure 2. Concentrations of estradiol in serum as a function of the estrous cycle in female rats at sacrifice. Circulating estradiol levels are significantly lower during the phases of the estrous cycle characterised cytologically as metaestrus/diestrus. Obesity had no impact on the concentration of serum estradiol. ** $p < 0.01$; *** $p < 0.001$ (Student's t-test).

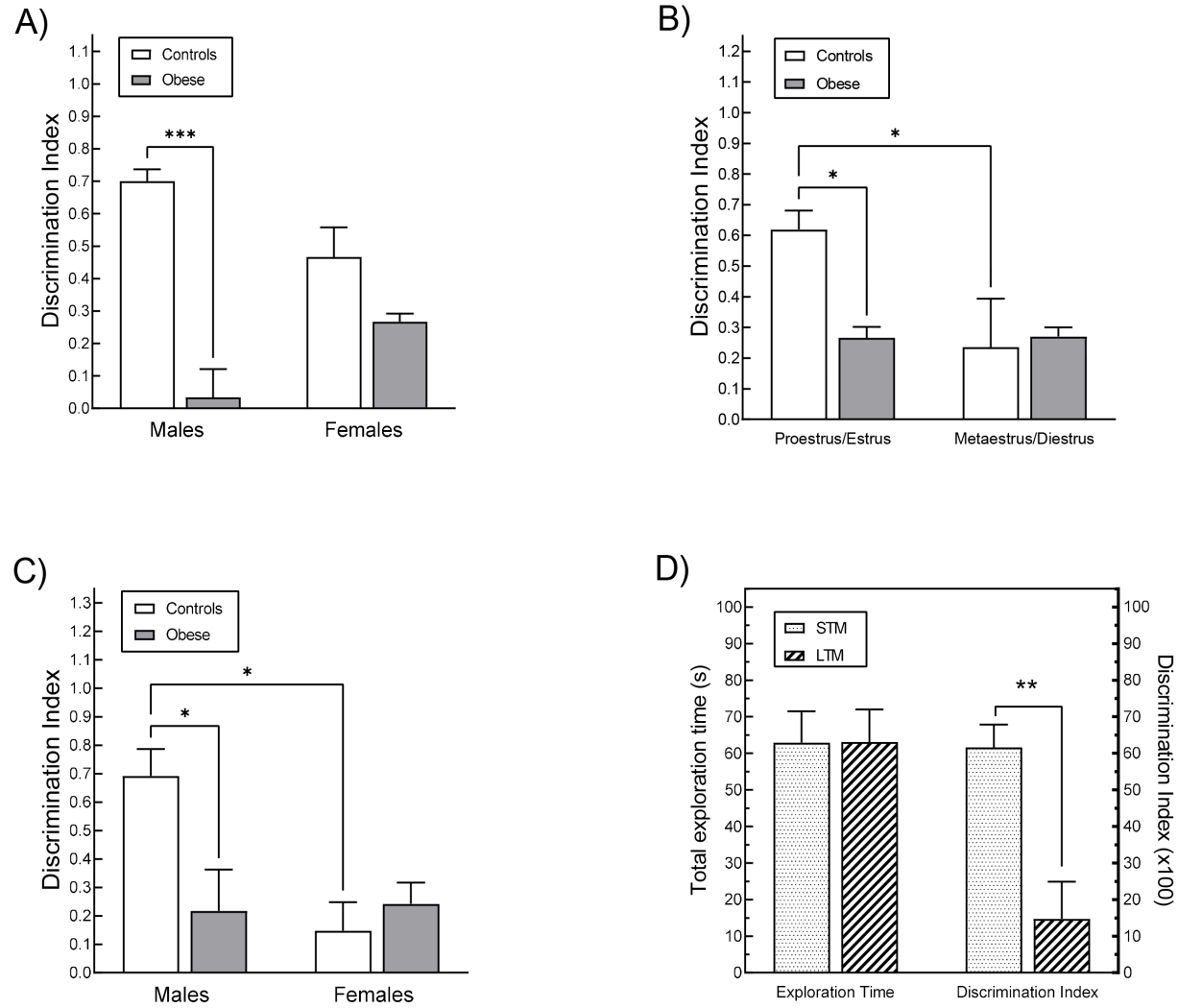
Figure 3. Impact of obesity on tryptophan metabolism through the serotonin (A) and kynurenine (B) pathways in the hippocampus. Bars represent the metabolite concentrations determined by ultra-performance liquid chromatography-tandem mass spectrometry. * $p < 0.05$ as determined by two-way ANOVA analysis.

Figure 4. Impact of obesity on tryptophan metabolism through the serotonin (A) and kynurenine (B) pathways in the frontal cortex. Bars represent the metabolite concentrations determined by ultra-performance liquid chromatography-tandem mass spectrometry. * $p < 0.05$ as determined by two-way ANOVA analysis.

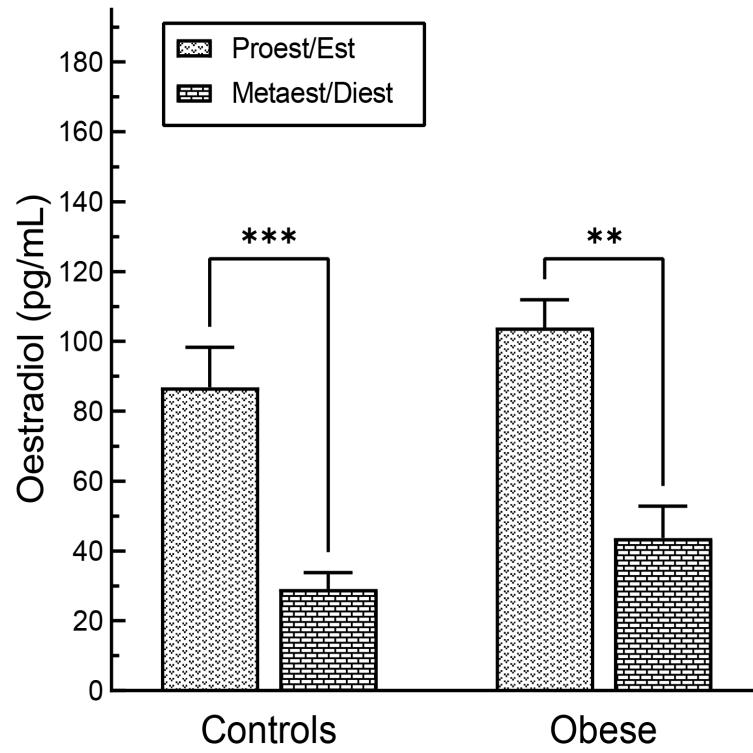
Figure 5. Effect of obesity on the expression in the hippocampus of genes coding for key regulatory enzymes of the kynurenine pathway. The levels of the mRNAs encoding for indoleamine 2,3-dioxygenase (IDO), kynurenine 3-monooxygenase (KMO) and kynurenine amino-transferase (KAT), were determined by real-time quantitative PCR. * $p < 0.05$; ** $p < 0.01$ as determined by two-way ANOVA analysis.

Figure 6. Effect of obesity on the expression in the frontal cortex of genes coding for key regulatory enzymes of the kynurenine pathway. The levels of the mRNAs encoding for indoleamine 2,3-dioxygenase (IDO), kynurenine 3-monooxygenase (KMO) and kynurenine amino-transferase (KAT), were determined by real-time quantitative PCR. * $p < 0.05$; ** $p < 0.01$ as determined by two-way ANOVA analysis.

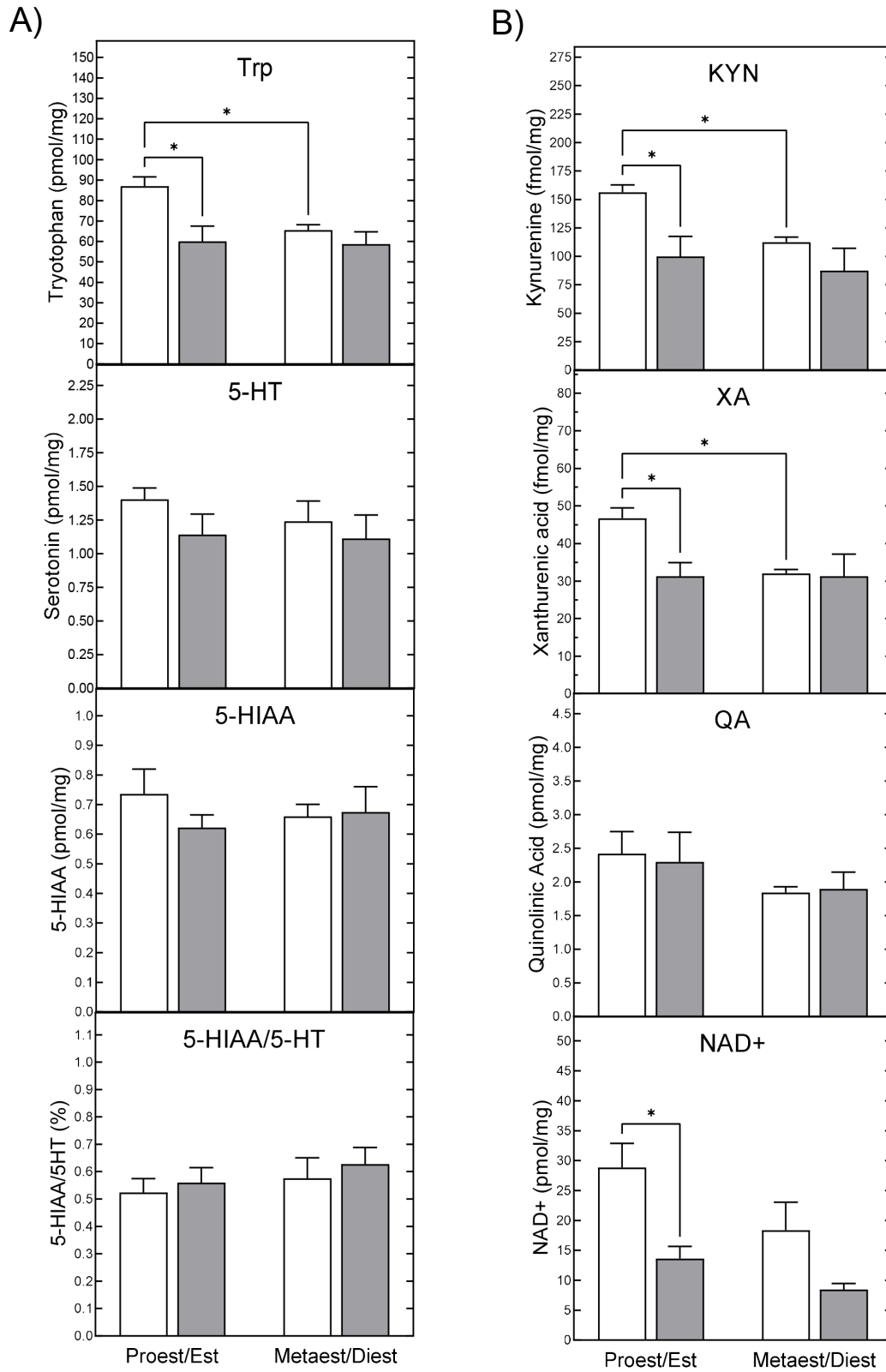
Mezo-González et al., Figure 1



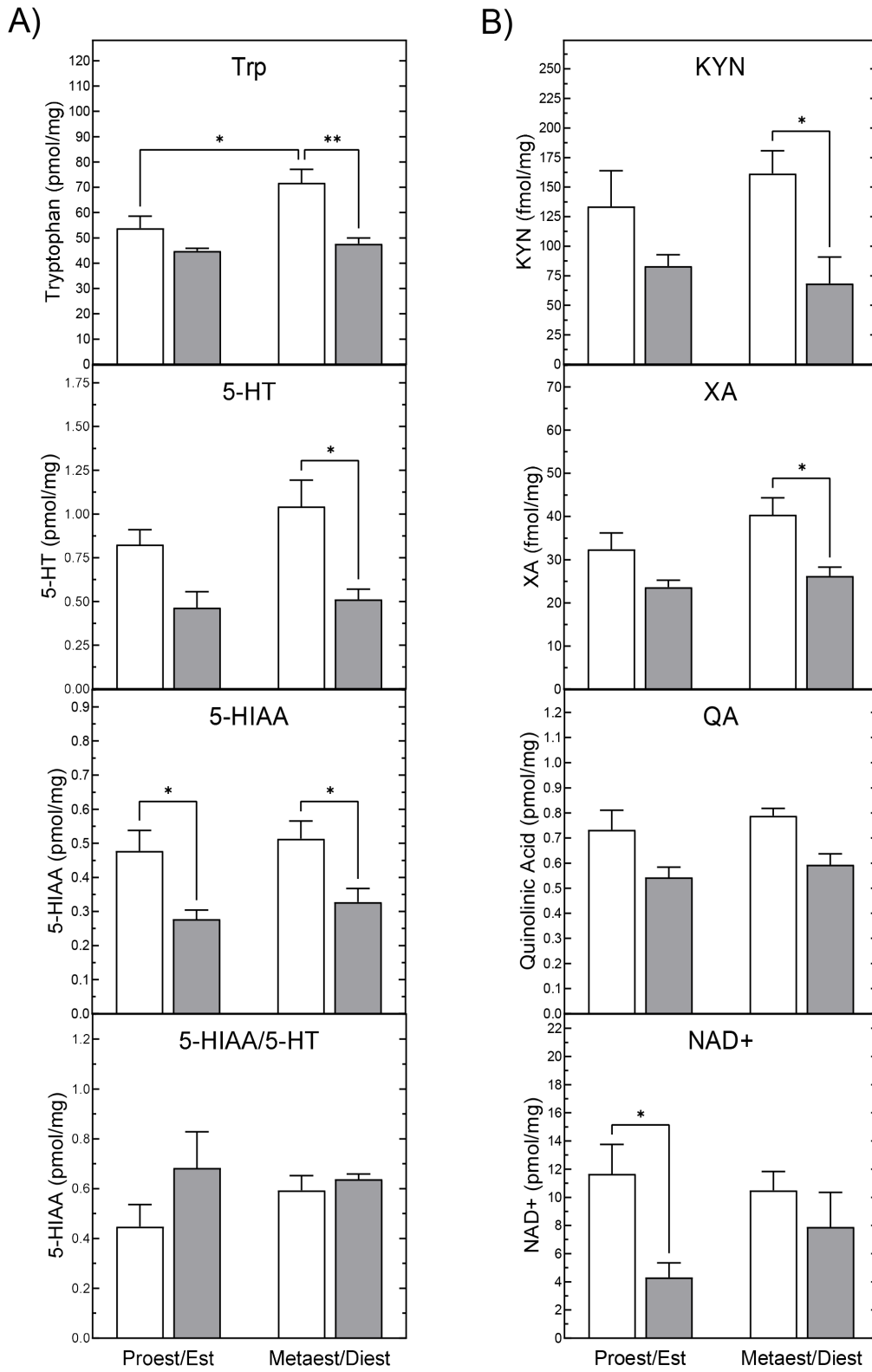
Mezo-González et al., Figure 2



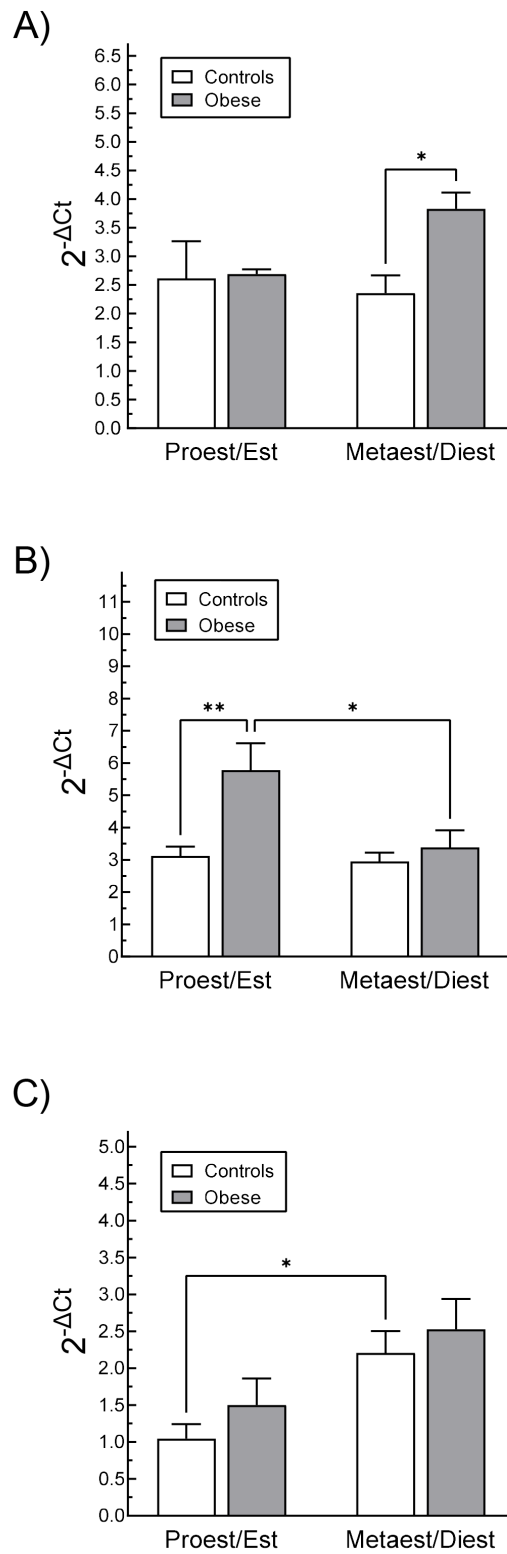
Mezo-González et al., Figure 3



Mezo-González et al., Figure 4

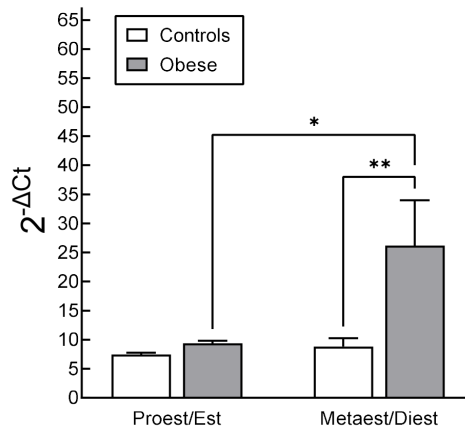


Mezo-González et al., Figure 5

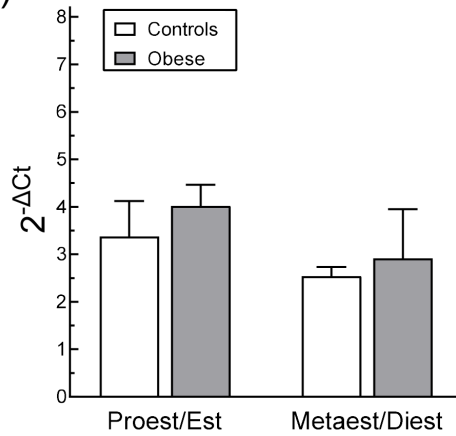


Mezo-González et al., Figure 6

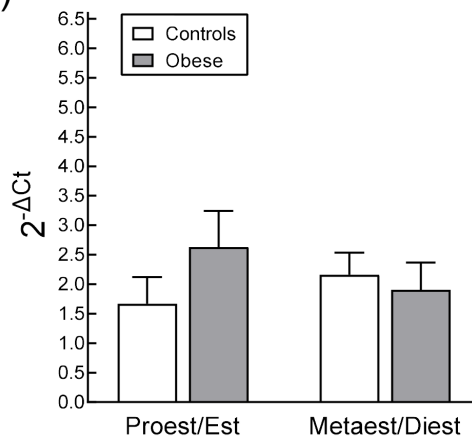
A)



B)



C)



ARTICLE 3.

Both maternal and paternal obesity in the rat induce learning deficits in offspring linked to impaired brain kynurenine pathway metabolism.

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Both maternal and paternal obesity in the rat induce learning deficits in offspring linked to impaired brain kynurenine pathway metabolism

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Short title: Obese parents' offspring memory deficits.

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ABSTRACT

Aim: Offspring of obese mothers are at high risk of developing metabolic syndrome and cognitive disabilities. Impaired metabolism has also been reported in offspring of obese fathers. However, whether brain function can be affected by paternal obesity has been barely examined. This study aimed to characterise the memory impairments induced by maternal and paternal obesity in the offspring and the involvement of the kynurenine pathway in this cognitive deficit. **Methods:** Founder control and obese female and male Wistar rats were mated to constitute 3 first generation (F1) experimental groups: control mother/control father; obese mother/control father; obese father/control mother. All F1 animals were weaned onto standard chow and underwent a learning test at four months of age, after which kynurenine and its metabolites were quantified in the hippocampus and frontal cortex by ultra-performance liquid chromatography-tandem mass spectrometry. **Results:** Maternal obesity induced severe learning deficiencies by impairing memory encoding. In contrast, offspring of obese fathers exhibited only reduced memory consolidation resulting in impaired long-term memory formation. Memory deficits in the offspring born to obese dams, were associated with enhanced hippocampal and frontal cortex levels of kynurenic acid together with increased quinolinic acid in the hippocampus. Offspring of obese fathers exhibited also an upregulation of kynurenic acid levels in the frontal cortex. **Conclusion:** Paternal and maternal obesity impair offspring's learning abilities by affecting different processes of memory formation. Nevertheless, in both cases the cognitive deficits are associated with enhanced production of kynurenic acid and quinolinic acid known for their deleterious effects on learning.

KEYWORDS: Developmental programming, kynurenine pathway, learning, maternal obesity, paternal obesity, tryptophan.

1. INTRODUCTION

The fetal and neonatal periods of development are highly sensitive to environmental factors that can shape and durably modify brain structure and function. Observational and experimental studies indicate that many mental disorders, including autism, schizophrenia and intellectual and learning disabilities, are related to adverse nutritional or psychological conditions experienced by the mother during pregnancy¹⁻³. These observations fall within the hypothesis of the Developmental Origins of Health and Disease (DOHaD), also known as developmental programming. According to this hypothesis, exposure to malnutrition, infection, stress and other adverse events during in utero development and/or neonatal life, leads to a high risk of developing metabolic and mental disorders via epigenetic changes that translate effects of early, and transient, environmental stimuli leading to long-term changes in brain function and energy homeostasis^{6,7}.

In light of the current obesity epidemic, particular attention has been devoted during the last years to the analysis of the consequences of maternal obesity on the health of the offspring. A large number of clinical and experimental studies have thus reported that maternal obesity sensitises the offspring to the development of obesity, insulin resistance, stroke and other metabolic diseases⁸⁻¹⁰. Likewise, maternal obesity impairs the cognitive function of the offspring. Namely, children born to obese mothers present reduced scores in verbal cognitive tests^{11,12}, lower reading and mathematical skills¹³ and poor executive

functioning affecting their attention, inhibitory control, and working memory^{14,15}. Similarly, animal studies have shown that the offspring born to obese dams display reduced working, spatial and associative memory^{16–20}. There is also a large body of evidence demonstrating that paternal obesity predisposes the offspring to metabolic diseases^{21–28}. However, whether brain function can be affected by the obese state of the father, has been barely examined²⁹. The present study aimed at clarifying whether paternal obesity induces alterations of the memory process in the offspring and if these alterations are common to those induced by maternal obesity. We also searched to determine whether the learning deficits in the offspring of obese parents can be underpinned by an impairment of brain tryptophan (Trp) metabolism. Trp is an essential amino acid that serves as a precursor for several bioactive compounds via its catabolism through the serotonin (5-HT) and kynurenine (KYN) pathways. 5-HT is known for its enhancing or inhibitory effects on memory depending on the type of 5-HT receptor activated and the experimental paradigm used to assess learning^{30,31}, while kynurenic acid (KA) and quinolinic acid (QA), two by products of KYN metabolism, inhibit the learning and memory processes mediated by glutamate and acetyl choline. Indeed, KA blocks the actions modulated by $\alpha 7$ nicotinic acetylcholine ($\alpha 7$ nAChR) and N-methyl-D-aspartate (NMDA) receptors whereas QA is an NMDA receptor agonist³².

2. RESULTS

2.1. *Metabolic alterations induced by the fc-HFHS diet in founder (FO) rats.*

Since our principal objective was to determine whether maternal and paternal obesity can compromise the cognitive function of the offspring, we first ensured that consumption of the

fc-HFHS diet does induce an obese phenotype. Examination of F0 rats before mating, showed that male animals exposed to the fcHFHS diet displayed enhanced body weight (520 ± 19 g vs 597 ± 22 g, $p < 0.01$) and serum glucose (182.50 ± 4.87 ng/dL vs 204.30 ± 8.63 ng/dL, $p < 0.01$) in comparison to male rats fed standard chow. F0 obese males displayed also reduced glucose tolerance as indicated by the higher area under the curve of the GTT (controls = 4085 ± 85 vs obese = 7717 ± 394 , $p < 0.0001$). Likewise, F0 female rats of the fcHFHS diet group were heavier (293 ± 9 gr vs 369 ± 25 gr, $p < 0.05$) and presented an increased concentration of glucose in serum (87.60 ± 3.42 ng/dL vs 100.20 ± 2.67 ng/dL, $p < 0.05$) in comparison with their control counterparts.

2.2. Memory deficits induced by maternal and paternal obesity on the offspring

There were no differences between the different experimental groups in the exploration time of the two objects during the sample phase of the NOR-NOL test (Figure 1A). However, clear memory deficits were observed in animals with an obese parent. Thus, the offspring from obese mothers were unable to distinguish the novel object from the familiar one during the three testing sessions performed 2h, 24h and 7 days after the sample phase (Figure 1B). In contrast the offspring from obese fathers, showed reduced memory capacity only during the test session carried out 7 days after the training session (Figure 1B). These results indicate that, maternal obesity impairs the encoding stage of memory processing whereas only the consolidation stage of memory is affected by paternal obesity leading to impaired long-term memory formation.

2.3. Effect of the maternal and paternal obesity on offspring's brain tryptophan metabolism

With the aim of determining whether the aforementioned memory deficits could be underpinned by an impairment of the cerebral metabolism of Trp, its concentration and that of several metabolites derived from its catabolism through both the 5-HT and KYN pathways, were determined in the hippocampus and frontal cortex of the adult offspring of control and obese parents. We focused the analysis on these two brain regions because of their prominent role in the regulation of learning and memory processes.

To our surprise, neither maternal nor paternal obesity induced any alteration of Trp metabolism via the serotonin pathway in the hippocampus (Figure 2). In contrast, reduced 5-HT and 5-HIAA levels were observed in the frontal cortex of rats from obese fathers (Figure 2D, 2F). In addition, maternal and paternal obesity enhanced the ratio of 5-HIAA to 5-HT (Figure 2H) indicating enhanced serotonin turnover.

Concerning the metabolism of Trp via the KYN pathway, maternal obesity resulted in higher KA levels in the hippocampus and the frontal cortex (Figure 3A, 3B), as well as in increased hippocampal concentration QA (Figure 3C). Offspring of obese fathers exhibited also an upregulation of KA levels in the frontal cortex (Figure 3A). Moreover, maternal and paternal obesity enhanced the levels of nicotinamide in the hippocampus and, conversely, reduced the concentration of this metabolite in the frontal cortex (Figure 3E, 3F). Otherwise, we observed no differences in the concentration of the other quantified metabolites (data not shown).

3. DISCUSSION

The results of this study show that both, maternal and paternal obesity impair the learning abilities of the offspring by disrupting different steps of the memory process. More precisely, offspring born to obese mothers were unable to distinguish a novel object from a familiar one during the three learning sessions designed to measure the short and long-term storage and retrieval processes of memory. These findings show that maternal obesity impairs memory encoding. In contrast, animals born from the mating of an obese male rat with a non-obese female, did distinguish the novel object from the familiar object during the first two test sessions performed 2h and 24h after the training session but not during the last one performed 7 days later, indicating that their capacity to consolidate a memory trace and, therefore, to form long-term memories is impaired but that their ability to encode and retrieve new information remains intact. Similarly, maternal and paternal obesity induced different effects on the concentration of QA and KA in the hippocampus but not in the frontal cortex. Notably, while the concentrations of QA and KA in the hippocampus were enhanced exclusively in the offspring born to obese mothers, the levels of the latter metabolite in the frontal cortex were increased in F1 rats from both obese mothers and obese fathers. Likewise, both, maternal and paternal obesity induced opposite effects on the concentration of nicotinamide in the hippocampus and the prefrontal cortex. i. e. an increase in the former region and a reduction in the latter. Overall, these results show that maternal and paternal obesity induce learning deficits in the offspring that are concomitant with an impairment of brain tryptophan metabolism through the kynurenine pathway.

It is generally accepted that the acquisition of new information is mediated primarily by the hippocampus, while long-term memory relies on the structural and functional integrity of the prefrontal cortex. In other words, the hippocampus plays an essential role in the encoding and consolidation of memory and the prefrontal cortex in memory retrieval ^{33,34}. The increased concentration of QA and KA in the hippocampus and frontal cortex in the offspring of obese mothers, is therefore in agreement with their inability to distinguish, from the first testing session, the novel and familiar objects. Likewise, the enhancement of KA levels exclusively in the frontal cortex of animals of obese fathers is consistent with their difficulty to distinguish the two objects 7 days after the encoding session.

Elevated brain levels of KA or QA have been associated with cognitive impairment in previous studies ³⁵⁻³⁸. Conversely, reducing the brain concentration of KA has been shown to improve memory performance ^{39,40}. Several lines of evidence indicate that the negative effects of these metabolites on learning are related to their ability to modulate the glutamatergic system. Indeed, KA blocks the actions of glutamate through its binding at the glycine site of NMDA receptors whereas QA produces neurotoxic effects by overstimulating this same type of receptors ^{32,41,42}. Moreover, the administration of an NMDA glutamate receptor antagonist prevents the learning deficits induced by the intracerebral infusion of QA ³⁸. Similarly, the reduction of the brain concentration of KA via the pharmacological or genetic inactivation of the kynurenine aminotransferase II (KAT II) gene, leads to enhance glutamate levels and to improved cognitive performance ^{39,43}. The causal link between the cognitive deficits and the modifications of the glutamatergic system induced by KA, is further reinforced by the results of the literature showing that the intra-cerebral

administration of KYN or KA leads to a reduction in the extracellular concentration of glutamate in the hippocampus along with decreased learning performance and that these two actions are blocked by the pharmacological inhibition of KA synthesis^{44,45}. Furthermore, enhanced glutamate levels together with impaired NMDA receptor expression have recently been described in the brain of offspring born to dams fed a high-sugar diet²⁰. On the basis of these observations, one can reasonably suggest that the cognitive deficits in the offspring of obese parents described in the present study, result from the disturbances of the glutamatergic system following the enhanced production of KA and QA. To fully substantiate this hypothesis, it would be necessary to determine whether maternal and paternal obesity lead to concomitant alterations in the brain concentration of glutamate, KA and QA, and to assess to what extent the memory deficits exhibited by animals of obese parents are improved by the pharmacological blockade of KA synthesis and/or QA production.

Concerning the dual, positive and negative, effects of maternal and paternal obesity on nicotinamide levels, further analyses are also needed to understand the relationship of these changes with the cognitive impairment displayed by F1-OM and F1-OF rats. Nicotinamide is a water-soluble amide form of vitamin B3 which acts as precursor of NAD⁺. It can be provided both by the diet and by the endogenous metabolism of Trp through the kynurenine pathway. Nicotinamide promotes neuronal differentiation and neuronal survival⁴⁶. However, in high concentrations it can exert negative effects such as the inhibition of the poly(ADP-ribose) polymerases (PARPs), which protect genome integrity. Excess nicotinamide can also increase the intracellular NAD⁺ pool leading to impaired energy metabolism and altered methylation patterns of DNA and proteins (see Hwang⁽⁴⁷⁾ for review).

In the brain, Trp metabolism via the kynurenine pathway takes place in astrocytes and microglia. In a first step common to both cell types, Trp is transformed to KYN by the enzyme indoleamine 2,3-dioxygenase (IDO). Subsequently, KYN is converted to KA in astrocytes and to 3-hydroxykynurenine (3-HK) in microglia through the action of, respectively, kynurenine aminotransferase (KAT) and kynurenine-3-monooxygenase (KMO) ^{48,49,49,50}. 3-HK serves as intermediate for the production of xanthurenic acid, quinolinic acid, nicotinic acid and ultimately NAD⁺. In a previous study, maternal obesity was shown to up regulate the expression of KMO and, conversely, reduce the mRNAs levels coding for KAT in the hippocampus of the offspring and these gene expression changes were associated with anxious behavior⁵¹. Unfortunately, in the present study it was not possible to assess the expression of the genes encoding for these enzymes. Therefore, it remains unclear whether the herein observed elevated levels of QA and KA reflect an overexpression of KMO and KAT.

Maternal obesity during pregnancy increases the number of astrocytes in the hypothalamus of the offspring both during the fetal period and after birth ⁵², Likewise, enhanced expression of microglia activation markers in the hippocampus along with anxiety behavior and reduced spatial learning have been observed in the offspring born to obese dams ⁵³. The increased levels of KA and QA in the brain of the offspring documented in the present study, could therefore result from a reactive gliosis process triggered by the exposure during fetal development and neonatal life to inflammatory agents derived from maternal obesity. In addition, considering that rats start consuming food at the age of 18-days ⁵⁴, and that, in our experiments, they were weaned at 21 days, the effects of consuming the FcHFHS diet

short before weaning could accumulate with those of maternal obesity to trigger an overactivation of microglia that endured until adulthood. Alternatively, or in addition to, the alterations produced by maternal obesity on the offspring's kynurenine pathway, could be mediated by epigenetic mechanisms comprising DNA methylation and post-translational histone modifications. The alteration of the epigenetic regulation of gene expression is the most common mechanism advanced to explain the long-term effects induced by the temporary exposure to an adverse environmental stimuli during early development and, indeed, variations in DNA methylation levels or histone binding at the promoter site of several genes have been described in the brain of offspring born to obese mothers ⁵⁵⁻⁵⁸. Changes in the amount of microRNAs (miRNAs) in diverse tissues of animals born to obese dams have also been found ⁵⁹⁻⁶². miRNAs are small non-coding RNAs that negatively modulate gene expression including that of genes belonging to the epigenetic machinery. However, to the best of our knowledge, no study to date has documented the brain epigenetic modifications related to the cognitive deficits observed in offspring born to obese mothers.

The above arguments can provide an explanation for the deleterious effects of maternal obesity, but they are obviously not relevant to the impaired cognitive and neurochemical phenotype produced by paternal obesity since, in this latter case, the offspring was not exposed to an obese environment neither during intrauterine development nor during the first days of postnatal life. The only mechanism that can explain the effects of paternal obesity are alterations produced by the consumption of FcHFHS diet on semen. Three mechanisms have been proposed to underlie the process of developmental programming via the paternal lineage: sperm DNA damage, impaired sperm epigenetic profile and altered

seminal plasma composition. As for the first mechanism, several studies have shown that obesity induces increased sperm oxidative stress^{63–65}. This metabolic insult leads in turn to DNA fragmentation which is traditionally associated with impaired fertility^{66,67}. However, reduced glucose tolerance and elevated insulin levels were also observed in female mice born to dams inseminated with sperm whose oxidative stress was enhanced by *in vitro* exposure to H₂O₂⁶⁸. Increased oxidative stress and DNA sperm fragmentation correlated with enhanced adiposity and high circulating levels of cholesterol and free fatty acids in offspring, were also reported after a severe restriction of food supply to the father⁶⁹. Therefore, the association between obesity-induced sperm DNA damage and the cognitive deficits in the offspring is quite likely but has yet to be ascertained.

Regarding developmental programming through epigenetic mechanisms, several studies have demonstrated altered DNA methylation patterns^{29,70–73}, impaired miRNAs expression levels^{28,70,74,75} and of other forms of non-coding RNA^{76,77}, as well as differential histone occupancy^{78,79} in the sperm of obese individuals. In some cases, the same type of epigenetic changes identified in the sperm have been detected in tissues of the offspring^{29,70}. Clear evidence of the fundamental role of sperm RNAs in generating a dysfunctional metabolic phenotype in the offspring, was provided by studies showing that the microinjection of transfer RNAs or of miRNAs RNAs extracted from sperm of mice fed a high-fat diet into naive oocytes⁷⁷ or into one-cell embryos⁸⁰, results in offspring exhibiting impaired metabolic phenotype. Adding to this evidence, obesity-induced alterations of the histone H3 trimethylated lysine 4 (H3K4me3) DNA occupancy pattern in sperm, coincide with the expression profile of genes regulating energy metabolism in the embryo⁷⁹. However,

with the exception of the study by Zhou et al. 2018, all investigations conducted to date on obesity-induced epigenetic changes in spermatozoa have focused on the relationship between these alterations and the metabolic phenotype of the offspring. A causal relationship between the epigenetic changes found in the sperm of obese fathers and the cognitive deficits observed in their offspring remains therefore to be established.

The last mechanism thought to mediate inter- and trans-generational transmission of pathological susceptibility via the paternal line, is the modification of seminal fluid composition. In keeping with this idea, experimental studies show that the male progeny sired by mice in which the seminal vesicle gland was excised, exhibit increased body fat along with enhanced circulating levels of leptin, glucose tolerance, and hypertension⁸¹. Similarly, artificial insemination with sperm from normal mice followed by mating with vasectomised males fed a low protein diet, results in offspring with high body weight and fat mass that, in addition, exhibit glucose intolerance and impaired vascular function^{82,83}. This demonstrates that seminal fluid factors other than spermatozoa influence also the postnatal health outcomes of the offspring. Impaired concentrations of leptin, insulin, estradiol and interleukins have been found in the seminal fluid of obese animals^{84,85}, but it is not known how these compounds and other components of the seminal fluid might contribute to paternal developmental programming.

In summary, both maternal and paternal obesity impair offspring learning ability but to different degrees and by interfering in different ways with the process of memory formation. Specifically, maternal obesity impairs the encoding step of memory as indicated by the inability of rats born to obese mothers to distinguish a novel object from a familiar

one during the three learning sessions designed to measure short and long-term memory. In contrast, only the ability to retain in the long term a previously encoded information is affected by paternal obesity. Despite these differences, cognitive deficits induced by maternal and paternal obesity are associated with increased brain production of KA and QA, two by-products of the kynurenine pathway known to have deleterious effects on learning and memory.

4. MATERIAL AND METHODS

4.1. Animal model of maternal and paternal obesity

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the principles of laboratory animal care (NIH publication no. 85–23, revised 1985), and were approved by the Ethical Committee for Animal Experimentation of the Pays de la Loire (authorization number 2017012013318453/APAFIS 8215).

A group of founders (F0) female and male animals was first constituted by feeding Wistar rats (Janvier Labs, Le Genest Saint Isle, France) from weaning (21 days old) either with a standard chow diet (control parents), or with a free choice-High Fat/High Sugar diet (fc-HFHS) to promote an obese phenotype (obese parents). The fc-HFHS was composed of four separated elements: 1) A 30% sugar/water solution prepared with sweetened condensed milk, 2) powdered standard chow mixed with pig fat in the proportion of 30 g of fat per 70 g of food, 3) standard chow and 4) tap water. Animals were maintained under an inversed 12-/12-

hour dark-light cycle (lights off at 7:00 AM), and at $21 \pm 1^\circ\text{C}$ with food and water *ad libitum* through all the experiment. When animals reached the age of 100 days, they were mated to constitute the following experimental groups: 1) F1-Control Parents (F1- CP, born from the mating of a control male with a control female, 2) F1- Obese Mother (F1-OM, born from the mating of a control male with an obese female and 3) F1- Obese Father (F1-OF, born from the mating of an obese male with a control female). Female F0 rats continued consuming the same diet (standard chow or fc-HFHS diet) during pregnancy and lactation. In total we used 12 control and 6 obese female rats that were mated with an identical number of control and obese male animals. All animals, including pregnant rats, were housed 4 per cage until they reached 300 g of body weight and 2 per cage thereafter. Three days before delivery, pregnant rats were housed individually and one day after birth, litters were homogenized to eight pups. Only two males from each litter of the F1 generation were kept after weaning and all animals from the three groups were fed standard chow until the end of the experiment.

4.2. Glucose Tolerance Test (GTT)

A GTT test was performed in F0 animals before mating to search for metabolic disturbances. The test was performed during the dark phase of the 12-/12-hour dark-light cycle after six hours of fasting. In brief. A drop of blood was taken by making a small incision in the tip of the tail immediately before the oral administration of glucose (2 g of glucose/kg of body weight), and 20, 40, 80 and 120 min after. Blood glucose was determined with a blood glucose monitor (Accu-Check® Active, Roche Diagnostics, Vercors, Meylan, France).

4.3. *Novel Object Recognition – Novel Object Location test*

To analyze the memory and learning capabilities of F1- animals, a paradigm combining the Novel Object Recognition test (NOR) and the Novel Object Location tests was used⁸⁶. The test was divided into three sessions: habituation, training and testing. During the habituation session, rats were allowed to freely explore an empty open-field arena (50 x 50 x 40 cm) for 20 minutes during three consecutive days. The fourth day, two identical objects were placed into the arena before introducing the rat and each animal was allowed to explore the objects for 7 minutes. It is during this sample phase that memory encoding takes place. The consolidation and retrieval processes of memory were determined in three testing sessions performed 2h, 24h and 7 days after the initial training session. During the testing sessions, one of the familiar objects was exchanged for a new one and both, the new and the familiar objects were placed in a different location from the one they occupied in the previous session (see⁸⁶ for a complete description of the test). As during the training, animals were allowed to explore the objects for 7 minutes. The time exploring each object was recorded and the capacity to remember the familiar object was determined from the discrimination index (DI). This was calculated by dividing the difference in exploration time of the novel object versus the familiar one over the total exploration time. A positive DI indicates the existence of recognition memory whereas a DI equals to or below zero suggest no memory recognition. Memory tests were performed under red light, 2 hours after the beginning of the dark phase of the animal's light-dark cycle (09:00 am) in a laboratory adjacent to their housing room. All sessions were recorded using the Viewpoint Videotrack system (Point Grey Research Inc., Richmond B.C. Canada) for offline analysis by two investigators blind to the

experimental group of the animals. Object exploration was defined as sniffing or touching the object with the vibrissae or when the animal's head was oriented toward the object with the nose placed at a distance of less than 2 cm from the object.

4.4. Biological samples collection and processing

The day following the last NOR-NOL testing session, F1 animals were deeply anesthetized with isoflurane two hours after the beginning of the dark phase of their dark-light cycle. The hippocampus and frontal cerebral cortex were collected and immediately frozen in liquid nitrogen and stored at -70 ° C until analysis.

4.5. Quantification of tryptophan and its derived metabolites in the brain

Tryptophan and the metabolites generated by its catabolism through the serotonin and kynurenine pathways were quantified in the hippocampus and frontal cortex by ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the methodology described in detail by Martimiano *et al.*, (Honório de Melo Martimiano *et al.* 2017). In brief, 50 mg of tissue were homogenized in 200 µL of an ice-cold solution of 2.7 mM EDTA containing 1% formic acid and mixed with 10 µL of a pool of exogenous internal standards (500 µM D₅-TRP, 2000 µM cafeic acid and 50 µM 2-CAD). Subsequently, 200 µL of the homogenate were mixed with 500 µL of ice-cold acetonitrile and, after centrifugation at 15,000 g for 15 minutes at 4 °C, the supernatant was recovered and evaporated to dryness under a stream of nitrogen at room temperature. Calibration curves were generated using serial dilutions of TRP, QA, 5-HT, KYN, 5-hydroxyindoleacetic acid (5-HIAA), KA and

Xanthurenic acid (XA), in 2.7 mM EDTA containing 0.1% formic acid which were processed in the same way that the tissue samples. At the end, all dried samples were dissolved in 100 μ L of EDTA 2.7 mM containing 0.1% formic acid and 5 μ L of each sample were injected into the LC-MS/MS system for analysis. Compounds were separated on a reverse Acquity HSS T3 column (2.1 x 100 mm, 1.7 μ m, Waters Corporation, Milford, MA, USA) at 30°C with a linear gradient of the mobile phase B (acetonitrile containing 0.5% formic acid and 2.5 mM ammonium acetate) in the mobile phase A (water containing 0.5% formic acid and 2.5 mM ammonium acetate) and at a flow rate of 400 μ l/min. The electrospray interface of the mass spectrometer was operated in positive ion mode and the multiple reaction monitoring mode was chosen for metabolite detection. Data acquisition and analysis were performed using MassLynx® and TargetLynx® software, respectively (version 4.1; Waters). Compound concentrations were calculated using calibration curves plotted from standard solutions.

4.6. *Statistical analysis*

Experimental results were analyzed using software GraphPad Prism version 9. Data were first checked for normality by the Shapiro-Wilk normality test and the statistical differences assessed by Student's t-test or one-way ANOVA followed by Dunnett's multiple comparisons test. Statistical significance was set at $p < 0.05$. Data are expressed as means \pm SEM of the data.

ACKNOWLEDGEMENTS

Carla Mezo González was supported by an international PhD fellowship from the Mexican National Council of Science and Technology (CONACYT). Amran Daher Abdi was the recipient of a doctoral fellowship from the Ministry of Research and Innovation of the Government of the Republic of Djibouti. Luis Reyes Castro received support from the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreeSkills fellowship. This work was supported by the French National Research Agency (ANR, grant ANR-16-CE21-0007-01) and CONACYT (award number 2015-16-273510).

CONFLICT OF INTEREST

The authors have nothing to declare.

AUTHOR CONTRIBUTIONS

F.B-J conceived the study, designed the experiments and analysed the data. C.E.M-G, A.D-A, L.R-C, and C.A performed the experiments and analysed the data. M.C. and A.A. performed the mass spectrometry analysis. E. Z. and E.G. contributed with conceptual ideas. C.E.M-G and F.B-J wrote the original draft. All authors revised the manuscript and agreed to publish it.

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FIGURE LEGENDS

Figure 1. Impact of maternal and paternal obesity on the learning skills of the offspring.

The learning abilities of adult control rats (F1-FP), or born to obese mothers (F1-OM), or from the mating of an obese male with a female of normal body weight (F1-OF), were assessed using a combination of the Novel Object Recognition and Novel Object Location tests. (A) Object exploration time during the training phase. (B) Discrimination index during the test sessions conducted at 3 different time intervals after the training session to measure short term (2h), long term (24h) and long-lasting (7 days) memories. Note that rats born to obese mothers do not distinguish the novel object from the familiar one in any of the test sessions despite the fact that they spent the same amount of time than control animals exploring two identical objects in the sample phase during which the encoding of the information takes place. Learning tests were performed at the beginning of the dark phase of the animal's dark-light cycle. Data represent the mean \pm SEM of the data from a total number of 9 to 12 animals per experimental group. * $p < 0.05$; ** $p < 0.01$ as determined by one-way ANOVA.

Figure 2. Impact of maternal and paternal obesity on tryptophan metabolism through the serotonin pathway in the hippocampus and frontal cortex of the offspring.

Bars represent the mean \pm SEM of the concentration of tryptophan (A, B), serotonin (C, D) and 5-HIAA (E, F) in the hippocampus and frontal cortex of adult control rats (F1-FP), or born to obese mothers (F1-OM), or from the mating of an obese male with a female of normal body weight (F1-OF). The 5-HIAA/5-HT ratio is also provided as an index of serotonin activity (G, H). The concentration of the different metabolites was determined by liquid chromatography–tandem mass spectrometry using brain samples from 7 to 8 animals per group. * $p < 0.05$ as determined by one-way ANOVA.

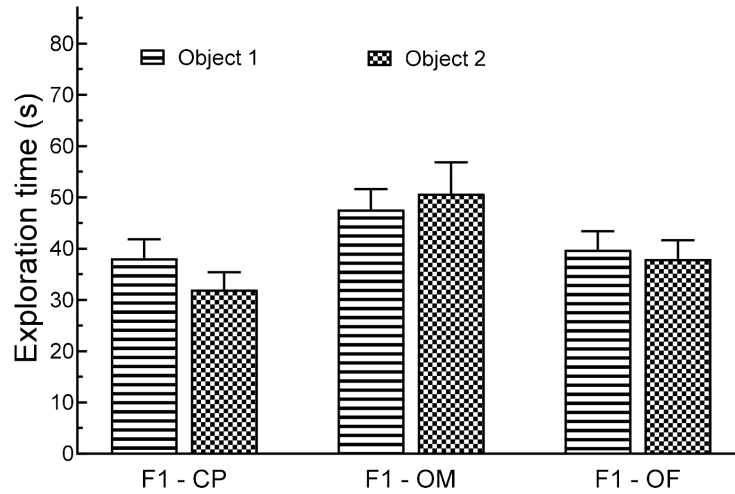
Figure 3. Impact of maternal and paternal obesity on tryptophan metabolism through the kynurenine pathway in the hippocampus and frontal cortex of the offspring.

Bars represent the mean \pm SEM of the concentration of kynurenic acid (A, B), quinolinic acid (C,

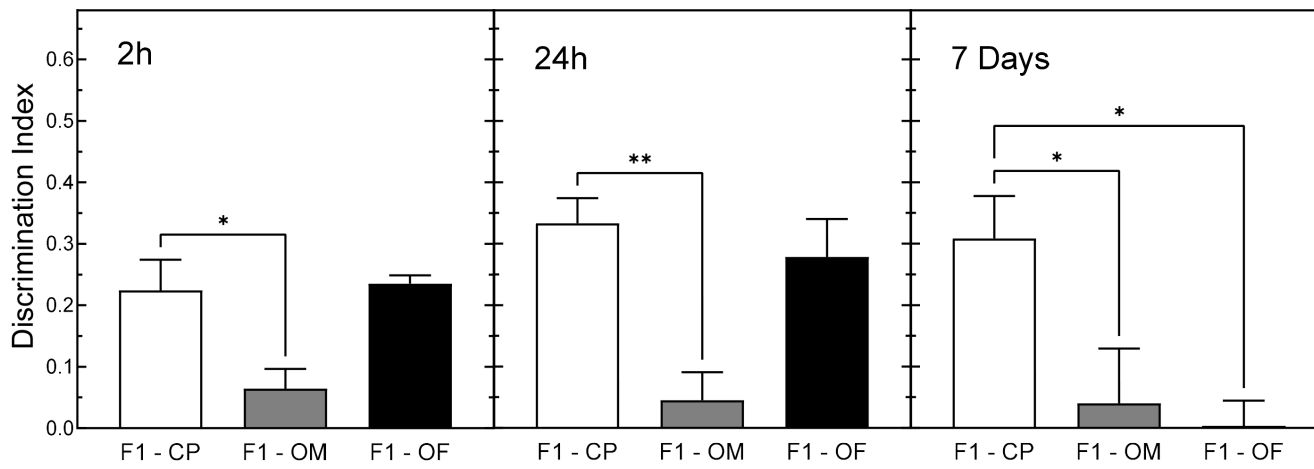
D) and nicotinamide (E, F) in the hippocampus and frontal cortex of adult control rats (F1-FP), or born to obese mothers (F1-OM), or from the mating of an obese male with a female of normal body weight (F1-OF). The concentration of the different metabolites was determined by liquid chromatography–tandem mass spectrometry using brain samples from 7 to 8 animals per group. (G, H). * $p < 0.05$; $p < 0.01$ as determined by one-way ANOVA.

Mezo-González et al., Figure 1

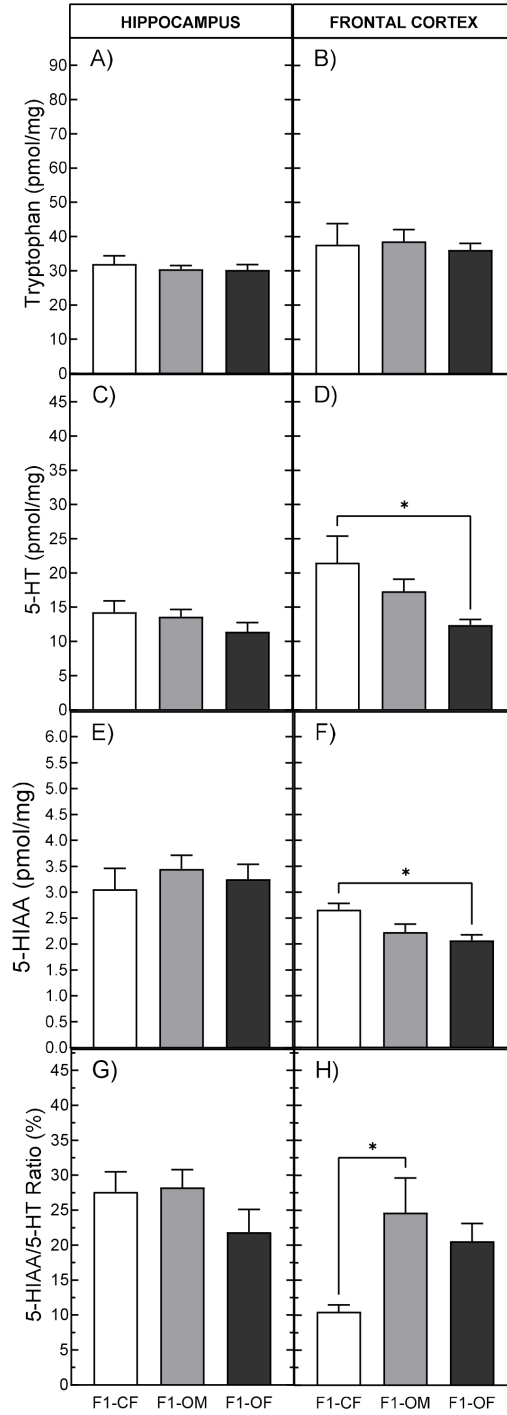
A)



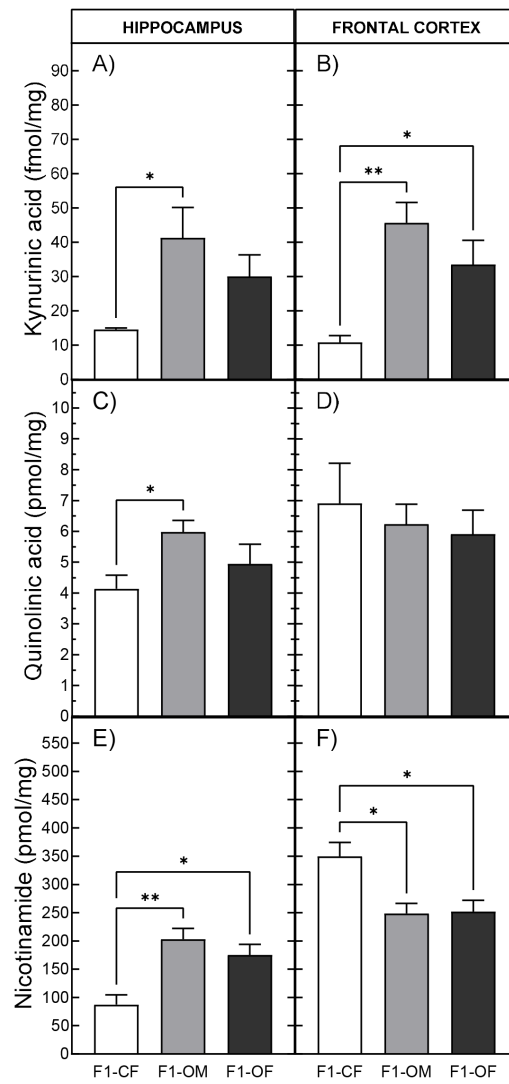
B)



Mezo-González et al., Figure 2



Mezo-González et al., Figure 3



ARTICLE 4.

Maternal and paternal obesity trigger learning and memory deficits in the adult offspring along with impaired expression of NMDA receptors in the brain.

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Maternal and paternal obesity trigger learning and memory deficits in the adult offspring along with impaired expression of NMDA receptors in the brain.

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ABSTRACT

Evidence points out that maternal and paternal obesity negatively affect the memory and learning capacities of the offspring, but the mechanisms that triggered this effect are not totally understood. The glutamatergic signaling is an important component of synaptic plasticity and it is essential for the consolidation of memories. Maternal obesity alters the expression of ionotropic receptors that are essential in the glutamatergic signaling, hence, prompting an impaired cognitive function in the descendance, however, it has never been reported whether paternal obesity exerts the same effect. **Aim:** The purpose of this study is to compare the effect of maternal and paternal obesity in the cognitive function of the offspring and establish whether they produce a differential gene expression of glutamatergic signaling markers. **Methodology:** An F0 generation of female and male Wistar rats were fed with a control chow diet (control group) or a free-choice High Fat/High Sugar diet (obese group). At adulthood, rats were mated producing the following F1 generation: 1) offspring of control founders (F1-Control founders), 2) offspring of obese mother and control father (F1-Obese mothers), and 3) offspring of control mother and obese father (F1-Obese fathers). The F1 generation was submitted to the Elevated Plus Maze, Open Field, and Novel Object Recognition tests to evaluate the

anxiety behavior, locomotor activity and recognition memory, respectively. Thereafter, the expression of glutamatergic signaling markers in the hippocampus and frontal cortex was evaluated through RT-PCR. **Results:** Maternal and paternal obesity promoted impair memory capacities in the offspring. While maternal obesity affected the Short-Term memory (STM) and Long-Term memory (LTM), paternal obesity only affected STM. Through gene expression analyses we observed that both, maternal and paternal obesity triggered a downregulation of GluN1 and GluN2B in the hippocampus, as well as a decreased of PSD95 in the frontal cortex. Only maternal obesity provoked a downregulation of PSD95 and zif-268 in the hippocampus and GluN2A and Arc in the frontal cortex. Finally, paternal obesity upregulated GluN2B in the frontal cortex of the offspring. **Conclusion:** Maternal and paternal obesity induced by the consumption of a fc-HFHS diet caused memory and learning impairment in the adult offspring and this was accompanied by altered mRNA expression of NMDA receptor subunits, and other synaptic markers.

KEY WORDS: Maternal obesity, paternal obesity, memory, learning, NMDA receptors.

INTRODUCTION

Memory is the capacity to encode, store, and retrieve information (1). This capacity depends of learning, a process that relays on synaptic plasticity (2). Synaptic plasticity (SP) is the brain capacity to modify a neural circuit function due to neural activity generated by an experience (3). SP is mediated by processes of Long-Term Potentiation (LTP) and Long-Term Depression (LTD), two types of activity-dependent long-term changes in synaptic efficacy (4). While LTP increases synaptic strength, LTD decreases it (5) and these effects are triggered by increases or decreases of neurotransmitters released (6).

Glutamate is the major excitatory neurotransmitter in the mammalian brain (7). When glutamate is released, it activates metabotropic and ionotropic receptors (mGluR, iGluR) (8). The iGluR are structures assembled as tetramers of intertwined subunits that form a non-selective cation channel (9). This family includes the α -amino-3-hydroxy5-

methyl-4-isoxazolepropionic acid (AMPA) and the N-methyl-D-aspartate (NMDA) receptors. AMPA receptors are composed by GluA1, 2, 3, and 4 subunits, that are assembled as dimers of dimers, composed of a GluA2 dimer and one of the other dimers (10). In another hand, NMDA receptors are tetraheteromers of two obligatory GluN1 subunits, and two GluN2 and/or GluN3 subunits (9). At resting membrane potential, the NMDA ion channel is blocked by physiological levels of extracellular Mg^{2+} (11).

After the activation of AMPA receptors there is a Na^+ influx, and a small K^+ efflux which provokes the depolarization of the postsynaptic neuron (4), triggering the release of the voltage dependent Mg^{2+} block of NMDA receptors which in turn lead the influx of Ca^{2+} . The increased in Ca^{2+} upregulates AMPA receptor expression and hence, the sensitivity of the membrane to glutamate. Thus, increases in Ca^{2+} influx lead to LTP of synaptic efficacy, and decreases provoke LTD (10,11).

Keeping the extracellular glutamate levels in a physiological range is crucial to ensure proper neuronal transmission and viability (12), and the hypo- and hyperfunction of glutamate is linked to psychiatric diseases (13). While overactivation of NMDA receptors is related with Huntington (14) and Alzheimer diseases (15), hypofunction is related with schizophrenia (16), autism (17,18), frontotemporal dementia (19), memory and cognitive impairment (20,21). Notably, obesity also affects the glutamatergic signaling. Obese animals with cognitive impairment present decreased expression of GluA1 (22) and GluA2 (22,23) in the cortex and hippocampus. Likewise, obesity reduces the protein expression of GluN2B and it makes difficult the LTD process (24).

Compelling evidence suggest that glutamatergic signaling could be impacted by metabolic programming. Evidence resulting from the Developmental Origins of Health and Disease (DOHaD), and the Paternal Origins of Health and Disease (or POHaD) concepts points out that the pre-conceptional and the early stage of life are highly sensitive periods to environmental factors, such as obesity. Parental obesity compromises the metabolic health of the descendence, and it could also shape and prime its neurodevelopment predisposing them to develop cognitive or psychiatric diseases (25–27). Indeed, the offspring of obese mothers presents differential expression patrons of the

NMDA subunits in the hippocampus and the prefrontal cortex, accompanied with memory deficiencies (28–30). It also affects the protein expression of the Postsynaptic density (PSD) complex (30) that anchor iGluR in the membrane (31). In the case of paternal obesity little is known its effect in the brain of the offspring, however, a study showed that depressed fathers transfer microRNAs through the sperm that trigger depression in the offspring and modulate the AMPA and NMDA receptors (32).

It is important to note that in a previous study performed by our research team we found that maternal and paternal obesity induced memory and learning deficits in the adult offspring, and this was accompanied by increase concentrations of quinolinic acid (QA) and kynurenic acid (KA) in the brain. Both metabolites derived from the tryptophan metabolism through the kynurenine pathway (33) and modulate the glutamatergic signaling (34). While KA is an antagonist of NMDA and AMPA receptors, QA is an NMDA agonist. Alterations in the concentration of those components are involved with several psychiatric diseases and cognitive impairment (35). Hence, the present study is based on the hypothesis that if parental obesity causes an increase in the concentration of QA and KA, there is probably a change in the expression of the iGluR causing cognitive deficits. So far, the evidence has already probed that maternal obesity do modulate the expression of iGluR triggering cognitive impairment, but it has never been described for paternal obesity.

MATERIAL AND METHODS

Animal model and induction of maternal and paternal obesity

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the principles of laboratory animal care (NIH publication no. 85–23, revised 1985), and were approved by the Ethical Committee for Animal Experimentation of the Pays de la Loire (authorization number 2017012013318453/APAFIS 8215). Female and male 21-days-old Wistar rats (Janvier Labs, Le Genest Saint Isle, France) were fed from weaning (PN 21) until adulthood (PN120) either with a standard chow diet (control animals) or with a free choice-High Fat/High Sugar diet (fc-HFHS) in order to promote an obesogenic profile (obese animals). The fc-HFHS diet was a modification of the diet used by La Fleur (36) and it was

composed of four separated elements: 1) A 30% sugar solution, 2) starch (tapioca) mixed with pig fat in the proportion of 30 g of fat per 70 g of starch, 3) standard chow diet and 4) A tap water. Animals were housed 4 per cage until they reached 300 g of body weight and 2 per cage thereafter. They were maintained under an inversed 12-/12-hour dark-light cycle (lights off at 7:00 AM), and at $21 \pm 1^\circ\text{C}$ with food and water *ad libitum* through all the experiment. When animals were 100-day old, they were mated in the following way: 1) control male x control female, 2) control male x obese female and 3) obese male x control female. Pregnant rats continued consuming the same diet (the standard or the fc-HFHS diet) during pregnancy and lactation. The offspring or F1 generation of each matting group was determined as: 1) F1-Control founders, 2) F1-Obese mothers and 3) F1-Obese fathers, respectively. Three days before delivery, pregnant rats were housed individually and one day after birth, litters were homogenized to eight pups. After weaning, we only kept two males from each litter and the three groups were fed with standard chow diet until adulthood.

Behavioral tests

All behavioral tests were performed during the dark phase of the light cycle and experiments started at 09:30 (2 hours after starting the dark phase of the 12-/12-hour dark-light cycle). Animals were handled by the same people who always had contact with them to avoid stress. Mazes and arenas were placed in an experimental room adjacent to the housing room with a one dim red-light bulb. A camera was placed on the top of the equipment to record all sessions using the Viewpoint Videotrack system (Point Grey Research Inc., Richmond B.C. Canada). Equipment and objects were always cleaned with ethanol 5% after they were used by each animal.

Elevated Plus Maze

To evaluate the anxiety levels of rats, we used the Elevated Plus Maze (EPM). This equipment is a maze of four elevated arms, two of them open and two closed, which radiate from a central platform forming a plus shape (37). In only one session, animals were putted in the center of the EPM, and they were allowed to freely explore the maze for seven minutes. During the test, movements or noisy sounds that could dismay the animal were avoided. The number of entries and the time spent in each arm was

registered. The test is based on the rodents' natural tendency to avoid open spaces; therefore, it is expected that they will spend more time in the closed arms, however, due to their curious nature, they will momentarily go out to explore open arms. In theory, a less anxious animal will visit the open arms more frequently, whereas an anxious animal will tend to spend more time in the closed arms (37).

Novel Object Recognition test and Open Field test

To analyze the memory and learning capabilities of animals we used the Novel Object Recognition test (NOR) described by Ennaceur and Delacour (38). The NOR test was divided into three sessions: habituation, training and testing. During the habituation session, rats were allowed to freely explore an open-field arena (50 x 50 x 40 cm) for 20 minutes during three consecutive days. The first day of habituation, the time and the distances traveled during the first five minutes were registered to evaluate locomotor activity (Open Field test). The fourth day, the NOR test started. First, it was performed the training session where two identical objects were placed into the arena before introducing the rat and each animal was allowed to explore the objects for 7 minutes. The same day it started the testing session that was carried out 2 and 24 hours after the training to evaluate, respectively, short-term memory (STM) and long-term memory (LTM). Each time, one of the familiar objects was exchanged for a new one. As during the training, animals were allowed to explore the objects for 7 minutes. The time exploring each object was recorded and the learning capacity was determined from the difference in time spent exploring the novel versus the familiar object. Preferential exploration of the novel object versus the familiar one indicates memory acquisition whereas an identical or similar time spent exploring the two objects means no learning. Object exploration was defined as sniffing or touching the object with the vibrissae or when the animal's head was oriented toward the object with the nose placed at a distance of less than 2 cm from the object.

Glucose Tolerance Test (GTT)

The day of the test, we took out food at 07:15 in all cages and we only leave tap water. After seven hours of fasting, we made an incision in the tail of each rat to obtain a blood sample and for monitoring the glucose levels on basal state. Then, we administered via orogastric a glucose solution 40 % (2 g of glucose/ kg body weight) and we measured the

glucose levels each 30 minutes after receiving the solution for two hours. For obtaining measures, the blood drops samples were deposited on test strips that were inserted into a glucometer for reading. We always discarded the first blood drop.

Biological samples collection and processing

The day following the NOR testing session, animals were deeply anesthetized with isoflurane two hours after the beginning of the dark phase of their light- dark cycle to obtain a blood sample by cardiac puncture and sacrificed immediately after by cervical dislocation. The frontal cerebral cortex and hippocampus were collected and frozen after dissection in liquid nitrogen and stored at -70 ° C together with serum samples until analysis. Mesenteric, retroperitoneal, pancreatic, gonadal and sternal fat depots were dissected, weighed and summed to provide a measure of body fat.

Metabolite determinations

Serum was assayed for insulin using an assay kit from Linco Research Inc. Triglycerides, cholesterol and fatty acids were analyzed by enzymatic methods (Triglycérides enzymatiques PAP 150, BioMérieux; Cholesterol RTU, BioMérieux; NEFA FS, DiaSys). Serum glucose concentrations were determined with a blood glucose monitor (Accu-Check®, Roche Diagnostics).

Real time quantitative RT-PCR

We performed a conventional Trizol RNA extraction method using the hippocampus and frontal cortex of rats. Then, 1 µg of purified RNA was treated with a DNase at 37 °C for 30 minutes and thereafter reverse transcribed by the Multiscribe Reversed Transcriptase in a total volume of 20 µL. The RT parameters on the thermal cycler were the following: incubation at 25 °C/10 minutes, 37 °C/120 minutes, 85 °C/5 minutes and 4 °C. The resulting cDNA was then diluted 40-fold in DNase and RNase free water in order to have a final concentration of 2 ng/µL. Thereafter, the cDNA was used an iCycler iQ Real-Time PCR detection system instrument to perform a PCR amplification. We used 5 µL of each cDNA-diluted sample as template and the SYBR Green as fluorogenic intercalating dye. The PCR parameters were the following: initial denaturation step at 95 °C for 5 minutes, then 45 cycles of 95 °C for 30 seconds and 30 seconds for 60 °C. The

relative expression levels were calculated using the comparative $2^{-\Delta\Delta C_t}$ method (39) and the CT geometric media of 18S and B-microglobulin transcripts was used as housekeeping gen. The primers used for the amplification of genes were the following: 18S (Forw): 5' GAT GCG GCG GCG TTA TTC C 3', 18S (Rev): 5' CTC CTG GTG GTG CCC TTC C 3', B-microglobulin (Forw): 5' GAT GGC TCG CTC GGT GAC 3', B-microglobulin (Rev): 5' CGT AGC AGT TGA GGA AGT TGG 3', GluA1 (glutamate ionotropic receptor AMPA type subunit 1) (Forw): 5' ACA ACT CAA GCG TCC AGA ATA GG 3', GluA1 (Rev): 5' GCC CTC ATA GCG GTC ATT GC 3', GluA2 (glutamate ionotropic receptor AMPA type subunit 2) (Forw): 5' GAG AAA GAA TAC CCT GGA GCA CAC 3', GluA2 (Rev): 5' TCA TCA CTT GGA CAG CAT CAT ACG 3', GluN1 (glutamate ionotropic receptor NMDA type subunit 1) (Forw): 5' GGT GGC AGA TGG CAA GTT TGG 3', GluN1 (Rev): 5' CGC TCA TTG TTG ATG GTC AGT GG 3', GluN2A (glutamate ionotropic receptor NMDA type subunit 2A) (Forw): 5' TTG GTG ATG GTG AGA TGG AGG AG 3', GluN2A (Rev): 5' AGA TGA AGG TGA TGA GGC TGA GG 3', GluN2B (glutamate ionotropic receptor NMDA type subunit 2B) (Forw): 5' TTC CAT CGT CAC CAC CTA CTT CC 3', GluN2B (Rev): 5' CTT AGA GTC GCC ATC GTC CAG AG 3', Arc (activity-regulated cytoskeleton-associated protein) (Forw): 5' CCG AAG TGT CCA AGC AGG TG 3', Arc (Rev): 5' CAT AGC CGT CCA AGT TGT TCT CC 3', zif-268 (early growth response 1) (Forw): 5' GGG TAG TTT GGC TGG GAT AAC 3', zif-268 (Rev): 5' TAC GAG CAC CTG ACC ACA GA 3', PSD95 (discs large MAGUK scaffold protein 4) (Forw): 5' GAC AGT GAG ACC GAC GAC ATT G 3', PSD95 (Rev): 5' GAT GAT GAT GGG ACG AGC ATA GTG 3'.

Statistical analysis

Experimental results were analyzed using software GraphPad Prism version 9. Data were first checked for normality by the Shapiro-Wilk normality test and the statistical differences assessed by one-way ANOVA and Tukey's multiple comparisons test. Statistical significance was set at $p < 0.05$. Data are expressed as means \pm SEM of the data.

RESULTS

Effect of the fc-HFHS diet consumption in founder rats (F0 generation)

We first verified that the consumption of the fc-HFHS diet promoted an obesogenic profile in the founder rats (F0 generation). Male and female rats who were fed with the fc-HFHS diet presented augmented body weight, liver weight and adiposity index compared with their respective control groups. In addition, only male obese rats presented glucose intolerance, elevated basal glucose levels, triglycerides, and cholesterol levels (Table 1).

	Males		Females	
	Control	Obese	Control	Obese
Body weight (g)	509.7 ± 12.84	590.5 ± 7.615 ***	318.3 ± 4.438	346.5 ± 6.477 **
Liver weight (g)	13.57 ± 0.6260	15.46 ± 0.4840 *	9.241 ± 0.1974	11.41 ± 0.391 ****
Adiposity index	5.167 ± 0.3073	7.500 ± 0.6268 *	4.40 ± 0.4761	7.00 ± 0.7071 **
GTT (AUC)	15292.0 ± 599.4	17896.0 ± 632.4 *	15917 ± 483.4	16131 ± 617.8
Glucose (ng/dL)	96.85 ± 3.039	107.7 ± 3.245 *	86.83 ± 2.033	88.23 ± 3.275
Triglycerides	91.71 ± 7.050	153.9 ± 22.17 *	49.25 ± 2.684	53.00 ± 4.295
Free Fatty Acids (ng/dL)	20.71 ± 6.274	25.43 ± 3.359	30.12 ± 3.020	37.28 ± 6.605
Cholesterol (ng/dL)	62.84 ± 2.659	53.74 ± 3.074 *	63.28 ± 2.425	62.14 ± 3.821

Effect of parental obesity in the metabolic status of the offspring (F1 generation)

When we compared the metabolic status of the offspring at adulthood, we observed that neither maternal nor paternal obesity changed any parameter of total body, liver weight, or any metabolic determination in the serum (except for the free fatty acids in the offspring of obese mothers). There was not any difference in the adipose tissue index between groups, however, when we compared the weight of the mesenteric, retroperitoneal, pancreatic, and gonadal and sternal fat, we observed that maternal obesity promoted an increment in the pancreatic adipose tissue weight, and both, maternal and paternal obesity increased the gonadal adipose tissue weight. Those results are interesting

since there was no difference in the total body weight when comparing with the offspring of control founders, in addition, all animals from the F1 generation were fed only with standard chow diet (Table 2). Thus, we can state that under our experimental conditions, maternal and paternal obesity metabolically programmed specific adipose tissue regions to suffer increased expansion.

Table 2. Body weight and serum metabolic determinations of 150-days old founder rats (F1 generation).			
	F1-Control founders	F1-Obese mothers	F1-Obese fathers
Body weight (g)	510.1 ± 9.535	532.8 ± 9.661	542.5 ± 9.107
Liver weight (g)	15.14 ± 0.7346	14.93 ± 0.333	14.62 ± 0.3679
Adiposity index	4.077 ± 0.4731	4.643 ± 0.3075	3.00 ± 0.00
Mesenteric fat (g)	6.482 ± 0.869	7.140 ± 0.463	5.671 ± 0.258
Retroperitoneal fat (g)	14.72 ± 2.217	16.25 ± 1.553	10.27 ± 0.495
Pancreatic fat (g)	0.8539 ± 0.073	1.362 ± 0.092 ***	0.9347 ± 0.067
Gonadal fat (g)	4.380 ± 0.113	5.819 ± 0.353 **	5.834 ± 0.280 **
Sternal fat (g)	0.6567 ± 0.043	0.6148 ± 0.086	0.4966 ± 0.042
GTT (AUC)	17905 ± 580	17911 ± 610	18154 ± 620
Glucose (mg/dL)	90.08 ± 1.979	93.78 ± 1.496	89.59 ± 1.692
Triglycerides (mg/dL)	60.16 ± 5.522	73.89 ± 4.837	67.49 ± 4.008
Free Fatty Acids (mg/dL)	16.39 ± 0.723	9.638 ± 1.351 **	12.95 ± 1.317
Cholesterol (mg/dL)	60.33 ± 2.135	62.61 ± 2.188	61.45 ± 3.306

Parental obesity did not promote anxiety-behavior in their offspring

We used the elevated plus maze (EPM) in order to assess whether maternal or paternal obesity trigger anxiety-behavior in their offspring. The EPM is a widely behavioral assay validated to assess anxiety. Animals are placed in the junction of a maze that has two open and two closed arms. An augmentation of activity either by the number of entries or by the time that animals spent in the open arm reflects anti-anxiety behavior (37,40). According to our results, neither maternal nor paternal obesity provoked any difference between the time or numbers of entries that the offspring spent in the open and closed arms compared to the offspring of control founders (Figure 1). Moreover, we measured the time animals spend practicing self-grooming because is an innate behavior that can

reflect the levels of anxiety in rodents (41), we observed a little increment in the time of self-grooming in animals from both, obese mothers and obese fathers but it was not statistically significant different (data not shown).

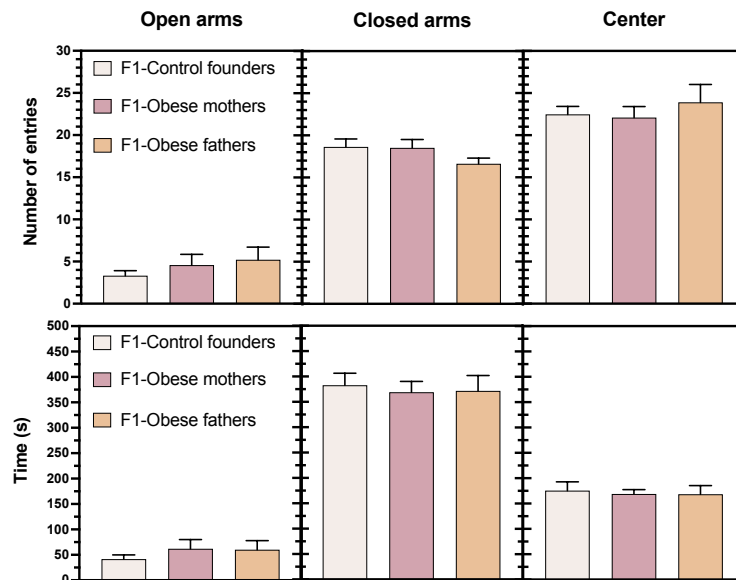


Figure 1. Adult offspring of obese mothers and obese fathers did not present anxiety behavior in the Elevated plus maze. Bars represent the number of entries and the total time spend in the a) open and b) closed arms, and in the c) center of maze. The anxiety behavior was evaluated when animals were 105-days old and during the beginning of the dark phase of the animal's dark-light cycle. Data represent the mean \pm SEM of the data from a total number of 10 to 14 animals per experimental group.

Parental obesity did not promote impaired locomotor activity in their offspring

The Open Field (OF) test is used to analyze locomotion and anxiety in rodents (42). We let animals to explore an empty arena during five minutes and we observed that neither maternal nor paternal obesity promoted a decrease in the total activity time or the distances traveled by the offspring when compared with the offspring of control founders. Through the OF test, it is also possible to identify anxiety when animals avoid exploring the center of the arena, but we did not observe this behavior in any group (Figure 2). Therefore, under our experimental conditions, neither maternal obesity nor paternal obesity causes impairment of locomotor activity or anxiety in the adult offspring.

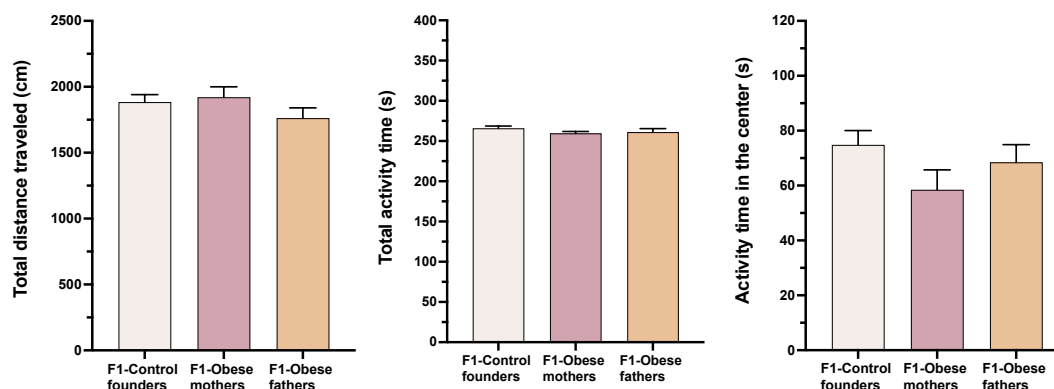


Figure 2. Adult offspring of obese mothers and obese fathers did not present impaired locomotor activity in the Open Field test. Bars represent the total distance traveled, total activity time and the time rats spend in the center of the arena. The locomotor activity was evaluated when animals were 110-days old and during the beginning of the dark phase of the animal's dark-light cycle. Data represent the mean \pm SEM of the data from a total number of 10 to 14 animals per experimental group.

Parental obesity promoted learning and memory impairment in their offspring

In order to evaluate the memory and learning capacities of rats, we used the Novel Object Recognition test that was first described by Ennaceur and Delacour (38). This test is based on the spontaneous behavior of rats to explore novelty. It does not require positive or negative reinforcement such as food or electric shocks, hence, the stress experienced by animals is minimal (38,43). According to our results, the offspring of obese mothers and obese fathers present lower values in the Discrimination index, it means that they could not differentiate between the novel and the known objects. While offspring of obese mothers were affected when we evaluated the STM (2 hours after training) and during the LTM (24 hours after training), offspring of obese fathers were only affected during the STM. Under our experimental conditions we can state that maternal and paternal obesity compromises the capacity of their offspring to learn and remember.

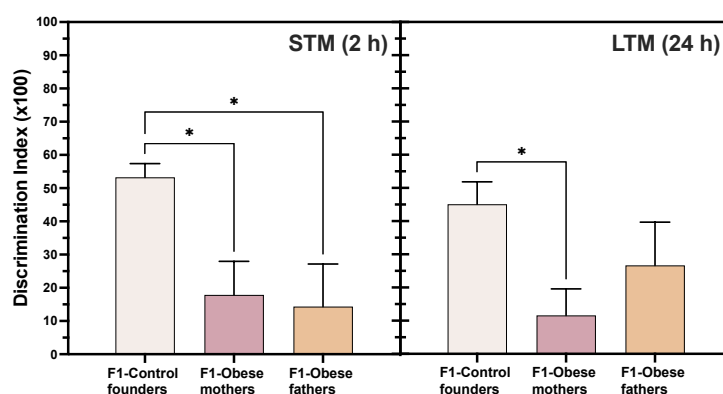


Figure 3. Maternal and paternal obesity compromises the learning and memory recognition capacities in the offspring in the Novel Object Recognition test. Bars represent discrimination index (x100) when evaluating STM (2 hours after training) and LTM (24 hours after training). The learning and memory capacities were evaluated when animals were 113-days old and during the beginning of the dark phase of the animal's dark-light cycle. Data represent the mean \pm SEM of the data from a total number of 10 to 14 animals per experimental group. * $p < 0.05$ was determined by one-way ANOVA.

Parental obesity triggered differential gene expression of synaptic plasticity

Once established that offspring of obese mothers and obese fathers presented memory and learning deficits, we wanted to analyze some markers of synaptic plasticity to establish a possible route by which the cognitive function of animals was affected. We analyzed the expression of subunits that form the ionotropic glutamate receptors. Including the AMPA receptor subunits GluA1 and GluA2, as well as the NMDA receptor subunits GluN1, GluN2A and GluN2B. In addition, we analyzed the expression of the anchoring protein for ionotropic receptors, PSD95, the neuron-expressed activity regulated immediate early gene product, Arc, as well as the transcription factor zif-268. According to our results, both, maternal and paternal obesity altered the expression of NMDA receptor subunits but not AMPA. Specifically, we observed that in the hippocampus, maternal and paternal obesity downregulated GluN1 and GluN2B, and only maternal obesity downregulated PSD95 and zif-268. In the frontal cortex maternal obesity downregulated GluN2A, PSD95 and Arc, while paternal obesity upregulated GluN2B and downregulated PSD95 (Figure 4).

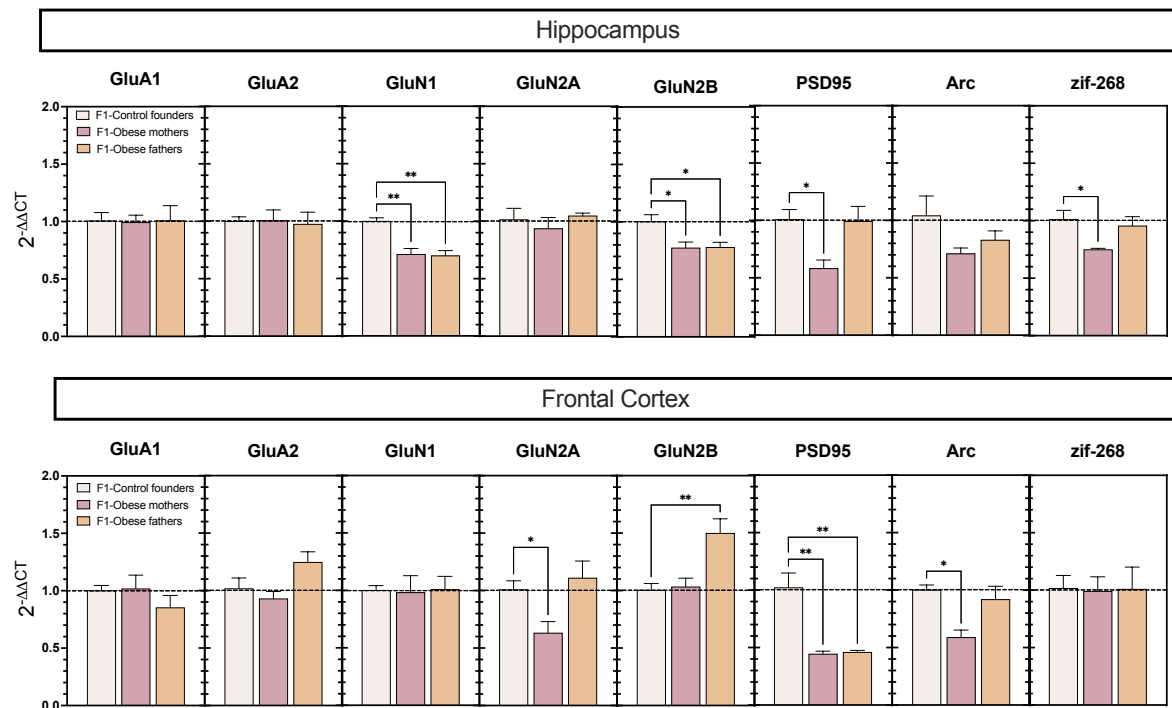


Figure 4. Expression levels of mRNAs that encode for ionotropic receptor subunits and synaptic markers in the brain of the offspring of obese mothers and fathers. The expression of GluA1, GluA2, GluN1, GluN2A, and GluN2B, PSD95, Arc and zif-268 was determined by real-time quantitative PCR in the hippocampus and the frontal cortex. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression of control animals as a calibrator. Data represent the mean \pm SEM of the data from a total number of 6 animals per experimental group. * $p < 0.05$; ** $p < 0.01$ were determined by one-way ANOVA.

DISCUSSION

Here we have demonstrated that maternal and paternal obesity induced by the consumption of a fc-HFHS diet provokes the offspring to have learning and memory impairment at adulthood, and this is accompanied with an altered expression of NMDA receptors subunits and other synaptic markers in the brain. Maternal obesity triggered a downregulation of GluN1, GluN2B, PSD95 and zif-268 in the hippocampus, and a downregulation of GluN2A, PSD95 and Arc in the frontal cortex. In another hand, paternal obesity caused a downregulation of GluN1 and GluN2B in the hippocampus, and

an upregulation of GluN2B and downregulation of PSD95 in the frontal cortex. Although other studies have already reported that maternal obesity provokes similar alterations in the glutamatergic signaling, this is the first study that describes the same effect in the offspring of obese fathers.

In general, we observed that maternal and paternal obesity altered the expression of NMDA receptor subunits but not AMPA. This is a noteworthy result because even if AMPA receptors are considered as the pillar players of the LTP and LTD, it is the activation of NMDA receptors which promotes the strengthening of synapses and then the consolidation of memories (10,11,44). Hence, we can assume that the learning and memory impairment presented by the F1 generation was in fact due to the altered regulation of NMDA receptors. Indeed, similar alterations have already been described in rodent animals with impaired object recognition memory (45), spatial memory (30) and social recognition memory (46).

The altered expression of NMDA receptor subunits was observed in the hippocampus and the frontal cortex, both, critical regions for the consolidation and retrieval of memories (47). According to our results, maternal and paternal obesity downregulated GluN1 and GluN2B in the hippocampus. Moreover, maternal obesity downregulated GluN2A in the frontal cortex. This is in accordance with other studies where mothers fed with hypercaloric diets predisposed their offspring to have cognitive impairment and a downregulation of GluN2B (28,29,48), and GluN2A (29) in the hippocampus. Interestingly, in another study there was a downregulation of GluN1, GluN2A and GluN2B in the frontal cortex in the early age of the offspring, and then, an upregulation of GluN1 and GluN2A at adulthood (30). In another hand, we found an upregulation of GluN2B in the frontal cortex of the offspring of obese fathers, which is a similar result observed in the hippocampus of Alzheimer models (49). Remarkably, an upregulation of GluN2B was observed in the offspring of fathers with depression that developed the same behavior at adulthood (32). In the same line, maternal obesity triggered an upregulation of GluN2B in the hippocampus (30) and the amygdala (50), provoking memory impairment and anxiety-behavior, respectively. In general, our results confirm that the expression of NMDA receptors subunits is altered not only by maternal

obesity but also paternal obesity. Even though we have some variations comparing with other studies, we should consider that we all used different hypercaloric diets for nourishing the F0 generation, in addition, the age at which markers were evaluated was different in all the studies, with ranges that varied from the early age until 18-months old, and, as we will discuss later, age is an important factor that determines the differential expression patterns of NMDA receptors throughout life. In addition, it is important to note that the downregulation of GluN1 in the hippocampus of the offspring of obese mothers is in accordance with the downregulation of zif-268, this molecule is a member of the immediate early gene family of transcription factors and it controls the expression of GluN1 among other genes (51).

Remarkably, we note that GluN2B is susceptible of suffering downregulation due to maternal obesity, and a combined effect of down and upregulation due to paternal obesity, either way, both cases produce deleterious effects. GluN2B subunit is absolutely necessary for permitting LTP (52), however, the overactivation of receptors that contain it impairs the synaptic plasticity (53). In another hand, GluN1 and GluN2A are mostly essential for memory acquisition, in fact, its expression rises some minutes after acquiring new information and then it decreases in a short period of time, as it has been observed in rats who were submitted to the Open Field and the NOR tests (54). Under our experimental conditions, GluN1, and GluN2B, were downregulated by maternal and paternal obesity, hence, we would have imagined that all the F1 generation would present memory impairment during the STM and the LTM, but that was only showed by the offspring of obese mothers. The interpretation of this result is then complex, and it is important to note that we did not sacrifice animals immediately after performing the NOR test, hence, further analyzes are necessary to know how under our experimental conditions these subunits are expressed when animals are exposed to a learning paradigm.

Maternal, and paternal obesity not only affected the expression of NMDA receptor subunits, but also the structure that stabilizes them in the presynaptic membrane called Postsynaptic density (PSD). The PSD is a protein complex composed by postsynaptic receptors, cytoplasmic scaffold proteins, signaling enzymes and cytoskeletal structural elements whose main function is to anchor glutamate receptors (31). PSD-95 is

fundamental in the PSD architecture, and it is essential for the regulation of plasticity and strength of excitatory signaling (55). Depletion of PSD95 provokes smaller postsynaptic densities and impairs the localization of NMDA receptors in the presynaptic membrane (19). According to our results, maternal obesity triggered a downregulation of PSD95 in the hippocampus and the frontal cortex of the offspring, while paternal obesity downregulated it only in the frontal cortex. This is partially in accordance with another study where maternal obesity downregulated PSD95 in the frontal cortex, but not in the hippocampus. The downregulation was observed in the early age but it shift to an upregulation at adulthood (30). Something interesting to remark is that PSD95 expression normally correlates with the GluN2A expression (56), however, we only observed this relationship in the frontal cortex of offspring of obese mothers. Nevertheless, there are other PSD proteins such as PSD93 that are also associated with GluN2A but we did not analyze (56). In the main, we can state that the downregulation of PSD95 surely had an important negative impact on the cognitive ability of the F1 generation, probably by avoiding the establishment of NMDA receptors in the dendritic synapse.

Something to note is that in accordance with the downregulation of PSD95, maternal obesity triggered a downregulation of Arc (or Arg3.1) in the frontal cortex. Arc is a neuron-expressed activity regulated immediate early gene product considered as a master regulator of long-term synaptic plasticity (57) and it is a critical component for storage and consolidation of memories (58). Arc is a multifunctional molecule that interacts with several proteins in the synapse, and it regulates the AMPA receptors trafficking to maintain them in a homeostatic manner, which means that when there are high levels of Arc there is a decrease function of AMPA receptors (59). It is important to note that Arc necessarily requires PSD95 to assemble in super-complexes in the synapse (60). When analyzing our results, we observed that neither maternal nor paternal obesity affected the expression of AMPA receptors, moreover, only maternal obesity triggered a downregulation of Arc in the frontal cortex, a result that is in accordance with other study where maternal obesity provoked the same effect but in the hippocampus (48). With a decrease of Arc, we would expect higher expression of AMPA receptor subunits, but it was not like that. However, it is important to note that the induction of Arc is transient and it dissipates within hours upon learning (61). Another important fact is that Arc is

targeted to activated synapses in a process dependent of NMDA receptors activation (62), hence, as we had a downregulation of NMDA receptor subunits, it is quite logical to observed a downregulation of Arc. This effect was not observed in the brain of the offspring of obese fathers which could explain why only the offspring of obese mothers presented a LTM impairment.

Explaining how maternal and paternal obesity provokes an altered expression of NMDA receptors is challenging, however, we believe that there are least three possible factors influencing this effect. 1) The transfer of epigenetic markers such as microRNAs from the F0 to the F1 generation that could modulate the expression of glutamatergic markers. 2) The increased expansion of visceral adipose tissue observed in the offspring even if they were nourished with a standard chow diet. 3) A possible altered tryptophan metabolism. In the first line, it has already been described the relationship between metabolic programming and modulations of the glutamatergic signaling. In a model of paternal depression, the offspring develop the same behavior, as well as modulation of NMDA receptor subunits that was associated with microRNAs transferred via the sperm (32). In another hand, the offspring of obese mothers that presented altered expression of NMDA receptor subunits was accompanied with modulations of the miR-132, miR219, and miR-223, these changes were dependent of age and sex of the offspring (30).

Exploring the second hypothesis, we observed that under our experimental conditions the offspring of obese mothers and obese fathers presented an increased weight of gonadal adipose tissue and only the offspring of obese mothers an increased pancreatic adipose tissue. Remarkably, those animals did not present an increased total body weight compared with the offspring of control founders, which let us imagine that they could probably had a loss of muscle mass. This phenomena has already been proposed by other authors and it has been called sarcopenic obesity (63,64). This process is indeed a characteristic of aging (65), hence, if our animals were developing an obesogenic profile, we could imagine that it came with an accelerated process of aging (66). Age is an important factor for glutamatergic signalization. In fact, in the hippocampus of rats, NMDA receptors densities change throughout life, showing an increased from birth until PN30 and then a decreased in PN90, which authors propose could represent a process of

synapse elimination that redefines neuronal networks (67). In conjunction with this fact, it is known that the expression of PSD95 and GluN2A increases gradually from birth throughout adulthood, in the contrary, GluN2B is abundant at birth and then it declines with age (56,68). Since we are observing an altered expression of those markers, we could imagine that this is in part due to the accelerated process of aging, but this is only a risky assumption since we did not measure any marker of aging or muscle degeneration.

Finally, we believe that metabolites derived from the tryptophan (TRP) metabolism could be related with the altered expression of NMDA receptor subunits. In previous research that we performed, we used the same model of maternal and paternal obesity that we used here (with some little modifications in the diet composition), and we found that the F1 generation presented increased levels of kynurenic acid (KA) and quinolinic acid (QA) in the brain. KA and QA participate in the regulation of glutamatergic signaling, while KA is an antagonist of NMDA and AMPA receptors, QA is an NMDA agonist (34). As we are using the same experimental conditions, we suppose that in the present experiment animals could have presented the same increased concentration of both metabolites. In that case, excess KA could have blocked the activation of AMPA and NMDA receptors, preventing both depolarization of the postsynaptic membrane and hence, the strengthening of synapses. On the other hand, excess QA would have overactivated NMDA receptors so that there would be cytotoxicity due to excess calcium influx and thus cell death. Under our experimental conditions, it would have been more probable that the QA acid exerted its action because no differences were observed in the expression of AMPA receptors but differences in the expression of NMDA receptors were observed. However, further studies are required to establish a direct relationship between the altered concentration of TRP-derived metabolites and the expression of NMDA receptor subunits.

CONCLUSION AND PERSPECTIVES

Maternal and paternal obesity induced impair memory and learning in the offspring at adulthood and it was associated with an altered expression of NMDA receptor subunits as well as other synaptic markers in the brain. We do not know the mechanisms by which this occurred; however, it is necessary to evaluate epigenetic markers that could be transferred from obese parents to their offspring. Moreover, since there is a variation in the pattern of gene expression of NMDA receptors throughout life, it would be interesting to perform an analysis at different points in the rat lifespan, from infancy to the old age, to know whether the cognitive capacity of the offspring is compromised since the development of the central nervous system, or whether it occurs during the dendritic pruning processes that then reinforce the neuronal networks in adulthood.

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General discussion

General findings

In this work we showed that obesity impairs the synthesis of TRP metabolites in the brain in a sex-dependent manner and that these alterations are associated with the memory and learning deficits displayed by obese individuals. Namely, in male rats, obesity induced a neuroinflammatory state that was related to an overexpression of key enzymes of the kynurenine pathway and to an enhanced production of neurotoxic kynurenines. In female rats, obesity decreased the brain concentration of TRP leading to reduced levels of kynurenines and serotonin. The neurochemical and gene expression changes as well as the cognitive deficits displayed by obese female rats, varied among the different stages of the oestrous cycle. Finally, we provided compelling evidence showing that maternal and paternal obesity results in impaired TRP metabolism in the brain of the male offspring along with memory deficits and altered expression of glutamate receptors and synaptic plasticity markers. Each of these points will be discussed below.

Sex differences induced by obesity on brain TRP metabolism

The principal goal of this work was to determine if the memory and learning deficits induced by obesity could be underpinned by an alteration of brain TRP metabolism. We found that obesity modulates the levels of TRP metabolites in a sex-dependent manner. Actually, obese males exhibited increased levels of KYN in the brain stem, and of XA, QA, NAm and NAD in the hippocampus together with an enhanced concentration of NAD⁺ in the frontal cortex. Moreover, there was an up regulation of the mRNAs coding for KMO in the three brain regions and an overexpression of KAT in the hippocampus and the frontal cortex. Male obese rats displayed also increased expression of TPH2 in the brain stem in comparison to their control counterparts. However, this change in gene expression did not result in an increase of 5-HT levels. Altogether, these results indicate that in the case of males, obesity favors the metabolism of TRP via the neurotoxic branch of the kynurenine pathway.

In female rats, the effects of obesity were completely different. Indeed, obese rats displayed decreased levels of TRP, KYN, XA and NAD⁺ both in the hippocampus and frontal cortex. There was also a decrease in 5-HT and 5-HIAA in the frontal cortex. These

results suggest that in obese females there is in a first instance a reduced availability of TRP in the brain which leads to a decreased production of serotonin and kynurenine-derived metabolites. These neurochemical alterations are accompanied by an overexpression of IDO in the hippocampus and frontal cortex, and of KAT in the hippocampus (see the second part in the results section). Nevertheless, the effects of obesity on both the cerebral content of TRP metabolites and the expression of genes coding for key enzymes of TRP metabolism, are highly modulated by the stages of the oestrous cycle. This will be discussed later.

A limitation of our study is that the quantification of TRP metabolites in males and females was performed in two independent experimental series using animals of different age. Yet, our results provide a good appraisal about the sex-dependent effects of obesity on brain TRP metabolism. Under our experimental conditions, obese males did not display impaired brain TRP levels although there they exhibited an overproduction of some kynurenines derived from this amino acid. On the other hand, obesity in females decreases the availability of TRP which leads to a decreased production of kynurenines and serotonin. In the case of males, we know that the peripheral levels of TRP had no influence on brain TRP because we did not detect any difference between control and obese rats in the serum concentrations of total or free TRP. Unfortunately, we did not quantify the TRP concentration in the serum of obese females such that we ignore if the reduced brain levels of TRP observed in these animals result from a decreased passage of TRP to the brain from the periphery.

Previous studies have reported sex differences in the levels of TRP in the serum of obese humans, but, to our knowledge, there are no data about possible gender differences in the effect of obesity on the brain concentration of TRP. Moreover, the human results are controversial. Indeed, whereas a study showed that overweight/obese women had lower TRP levels than overweight/obese men, (Raheja et al., 2015), Roca *et al.*, (Roca et al., 1999) found lower levels of TRP in lean women than in lean men, and no differences between overweight/obese men and women. However, Roca et al., also found that as the BMI increased, the TRP/LNAA ratio drops, and that this effect was more pronounced in men than in women (Roca et al., 1999). This is an interesting result

because the TRP/LNAA ratio reflects the availability of TRP in the brain. Therefore, the observation of Roca et al., suggests that the availability of TRP in the brain is decreased under obese conditions as we observed in female rats. However, we do not have enough experimental evidence to assure that this is happening under our experimental conditions.

*What promoted an increased concentration of kynurenines in the brain of obese males?
Probably neuro-inflammation.*

We found an increased expression of genes coding for IL-6, IL-1 β , TNF α , TLR2 and TLR4 in the brain stem, and of TLR2 mRNAs levels in the hippocampus and frontal cortex. Enhanced expression of these genes is a hallmark of inflammation. Moreover, it is known that the expression of both IDO and TDO genes is induced by IL-6, TNF α , and IL-1 β (O'Connor et al., 2009; Zunszain et al., 2012; Urata et al., 2014). In addition, TLR4 and TLR2 are involved in microglial activation (Lee et al., 2015; Jin et al., 2016) and it is known that activation of microglia by inflammatory cytokines triggers the expression of KMO and the production of QA (Guillemin et al., 2005). As we found an increased production of QA, we can reasonably propose that this effect is due to an overactivation of microglia by proinflammatory cytokines.

Is it possible that obesity could provoke microglial activation in female rats?

It is complicated at this moment to provide a clear answer to this question because, according to the literature, under a HFD or a HFHS diet, males are more prone than females to develop microglial activation (Dorfman et al., 2017; Daly et al., 2022). Moreover, males have higher microglial density in the hippocampus and cortex than females (Guneykaya et al., 2018), and more highly activated microglia during early development (Schwarz et al., 2012; Han et al., 2021). Furthermore, the responses of microglia appear to be under the control of sex hormones in an age-dependent manner. Male neonatal microglia exposed to LPS produced greater IL-1 β mRNA than female microglia, but when microglia are co-incubated with estradiol, an anti-inflammatory effect is observed in male microglia but a pro-inflammatory effect in female microglia. On the contrary, at adulthood, LPS-induced IL-1 β mRNA expression is attenuated by estradiol in females but not males (Loram et al., 2012). The overexpression of IDO in the hippocampus and frontal cortex and the enhanced expression of KMO in both brain

regions, is in line with the idea that the fc-HFHS diet did produce microglial activation in our female rats. However, we did not observe a concomitant increase in the brain concentration of KYN, or QA. This can be explained by the fact that obesity reduces brain TRP availability.

Do estrogens modulate the obesity-induced memory deficits in female rats?

Estrogens have a beneficial effect on memory and learning capacities in women and female rodents (Frye et al., 2007; Patel et al., 2022). They modulate neurogenesis by increasing synapse and dendritic spines density (Sheppard et al., 2019), and enhance LTP by promoting the expression of key synaptic plasticity genes including PSD-95, synaptophysin, AMPA receptor subunit GluN1, CREB and BDNF (Liu et al., 2008; Koyuncuoğlu et al., 2019; Batallán Burrowes et al., 2021). During the menstrual and oestrous cycles, estrogen levels reach their maximum peak in estrus and decrease through metestrus and diestrus (Le et al., 2020; Schmalenberger et al., 2021; Abo et al., 2022). These fluctuations impact cognition, female rats in estrus had better performance in the NOR test than female rats in diestrus. The improvement in learning was concomitant with the increase in serum estradiol (Walf et al., 2006). Our results are consistent with these observations. In addition, we demonstrated that obese rats display decreased learning even if they are in the proestrus-estrus stage. This suggests that the natural increase in the production of estrogens during proestrus-estrus does not protect female rats against the deleterious effects of obesity on cognition.

How obesity affects the ability to learn even if rats are in proestrus-estrus?

According to the literature, this could be due to the modulation of gene expression by estrogen receptors. Indeed, it has been reported that the consumption of a HFD for 4 weeks induces a downregulation of ER β receptors in the cortex and an upregulation of ER α receptors in the hippocampus. In contrast, after 12 weeks, ER β is upregulated in the cortex and ER α is downregulated in the hippocampus (Scudiero and Verderame, 2017). The variations in the expression pattern of these receptors could affect the way in which synaptic genes are expressed and therefore affect the learning and memory capacities. Moreover, obesity accelerates aging (Santos and Sinha, 2021), and aging has been shown to decrease the expression of estrogen receptors in the hippocampus (Chamniansawat and

Sawatdiyaphanon, 2018). Besides, obesity induces inflammation that activates microglia (Wahid et al., 2021), activation of microglia induces the expression of IL-6 that inhibits the synthesis of hippocampal estradiol. Finally, it has been observed that serum estradiol levels do not correlate always with cognitive performance since aged rats, display reduced spatial memory performance though they have higher levels of serum estradiol but lower levels of hippocampal estradiol (Chamniansawat and Sawatdiyaphanon, 2018). In our study, obese rats did not have lower levels of estrogens in serum, but we did not measure the concentration of estrogens in the brain. This represents a limitation in our study because we do not know whether obesity affected estrogen levels in the brain.

Concerning the relationship between estrogens and TRP, previous studies have shown that administration of estrogen increases the expression of TPH2 in the dorsal raphe nucleus and ameliorates the memory and learning capacities of female rats (Hiroi et al., 2016). Conversely, estrogen depletion induced by ovariectomy decreased the brain concentration of 5-HT and induced depression-like behavior that was accompanied by an overexpression of IDO and inflammatory markers (IFN- γ , IL-6, TLR4) in the hippocampus (Xu et al., 2015). These results show that estrogens have a beneficial impact on the serotonin pathway, but when estrogens levels are downregulated, the kynurenine pathway is stimulated together with the development of a proinflammatory state.

Our contribution in this area is that both, obesity and the stage of the oestrous cycle modulate brain TRP metabolism in a different manner. Indeed, obesity enhanced the expression of IDO in the hippocampus and frontal cortex but only during the metestrus-diestrus phases. This is in accordance with the fact that obesity produces inflammation and that proinflammatory cytokines upregulate IDO expression (O'Connor et al., 2009; Zunszain et al., 2012; Urata et al., 2014). Likewise, depletion of estrogens produces overexpression of IDO and inflammatory markers (Xu et al., 2015) which could explain why we observed enhanced IDO expression only during metestrus-diestrus. A different expression pattern was observed for the expression of KMO and KAT. The expression of KMO in the hippocampus was enhanced by obesity only in rats in proestrus/oestrus while the expression of KAT was increased in rats metestrus/diestrus. When analyzing the complete dataset, we can conclude that under adverse conditions

such as obesity and estrogen depletion, the brain has compensatory elements that try to promote the correct production of serotonin and kynurenines, however, these mechanisms are still unknown.

Which are the specific consequences of the increased or decreased production of serotonin or kynurenines?

The effect of each element will be analyzed separately below.

Reduced serotonin levels in the female brain.

As already mentioned, obese female rats displayed decreased TRP levels in the hippocampus, and decreased TRP, 5-HT and 5-HIAA levels in the frontal cortex. These effects were not observed in obese males. Decreased brain levels of TRP, 5-HT and 5-HIAA have already been reported in genetically male obese Zucker rats (Finkelstein et al., 1982). On the other hand, results obtained in diet-induced obesity models are contrasting. One study reported that HFD reduced the concentration of 5-HT in the whole brain (Labban et al., 2020), whereas another one reported enhanced 5-HT and 5HIAA levels in the hippocampus of obese rats (Haleem and Mahmood, 2021). These differences may be due to the specific analysis in the hippocampus versus the whole brain.

Our results are in accordance with other studies where decreased 5-HT levels have been associated with spatial working memory deficits (Haider et al., 2005), and memory recognition (Haider et al., 2012; Fernandez et al., 2017). Interestingly, Fernandez *et al.*, (Fernandez et al., 2017) observed that this effect affected the LTP in the hippocampus, and when they administrated a 5-HT_{1A} agonist (8-OHDPAT) that is one of the 5-HT receptors it rescued the memory deficits. It is not surprising that decreased 5-HT levels induces memory deficits as its presence in the hippocampus is essential for memory processes, spatial navigation, decision making and social relationships, while in the prefrontal cortex it plays a major role in working memory, attention, decision-making and reversal learning. But the modulatory effect of 5-HT depends on the specific subtypes of receptors (5-HTR), and their localization (Švob Štrac et al., 2016).

Quinolinic acid, a kynurenine induced by inflammation and by metabolic programming in male rats.

We found that QA was overproduced in the hippocampus of obese males (see the first part in the results section) and in the hippocampus of male rats from obese mothers (see the third part in the results section). Although both groups of animals presented memory and learning impairment, the animals directly exposed to the fc-HFHS diet displayed reduced short-term memory (STM), the animals born to obese mothers exhibited also decreased long-term memory. These results are in agreement with previous reports in which increased QA levels have been associated to impaired STM in the Morris water maze (Rahman et al., 2018) and the short-term working memory in the T-Maze (Misztal et al., 1996).

QA is a neurotoxic compound that binds to the glycine site of the NMDA receptors with agonistic properties. The excessive activation of NMDA receptors increases the concentration of cytosolic Ca^{2+} and produces mitochondrial dysfunction, cytochrome c release (Pérez-De La Cruz et al., 2012), increased production of ROS (Santamaría et al., 2001), glutamate excitotoxicity, impaired energy metabolism and neuronal death (Lima et al., 2017; Bansal et al., 2019). Furthermore, even though QA is produced by the microglia, several lines of evidence suggest that it could over activate both, microglia, and astrocytes. First, since microglia expresses NMDA receptors and QA is produced by microglia, the activation of microglial NMDA receptors by QA may initiate a potential vicious circle leading to its overproduction (Kaindl et al., 2012). Second, QA stimulates the expression of IL-1 β in astrocytes, promotes astrogliosis and reduces the activity of glutamine synthetase (Ting et al., 2009) which could also affect the availability of glutamate. The consequences of these effects are highly deleterious because the constant exposure to increased levels of QA provokes neurodegeneration. Indeed, in the hippocampus QA can provoke neuronal death specifically in the CA4 region (Vezzani et al., 1991).

Kynurenic acid, a kynurenine induced by metabolic programming in the male rats.

During the development of this work, we had technical problems in detecting KA in the brain samples. In fact, we were only able to quantify KA in the offspring of obese parents

(third part in the results section). Therefore, we do not know whether obesity directly induces modulations of this metabolite in the brain, but we can assure that KA can be metabolically modulated because the adult offspring from obese fathers or mothers had elevated levels of KA in the hippocampus and frontal cortex. Although KA is widely considered as a neuroprotective component, increased levels of KA have been found in the central nervous system of patients with schizophrenia (Plitman et al., 2017). . Increased KA have also been associated with lower cognitive performance both in humans (Huang et al., 2020) and rats (Chess et al., 2007). Hence, these effects are consistent with our results because rats with higher levels of KA presented impaired memory and learning.

Several reasons could explain the increase KA levels in the brain of the offspring. First, we know that during pregnancy and under normal conditions the placental production of KYN is enhanced (Abad et al., 2020). We did not quantify KA in the placenta, but we can suppose that in obese mothers there is a bigger increment of KYN due to their inflammatory state. Interestingly, it has been observed that KYN supplementation during pregnancy elevates the levels of KA in the hippocampus and frontal cortex of the offspring who also show impaired memory acquisition (Buck et al., 2020). Moreover, maternal obesity enhances the number of astrocytes in the brain of the offspring which could also increase KA levels (Kim et al., 2016).

There are several mechanisms by which KA could trigger deleterious effects. First, KA is produced by astrocytes, which convert KYN into KA but not into QA. However, astrocytes enhance the production of KYN due to the influence of activated macrophages and/or microglia, and these cells will then convert KYN into QA (Guillemin et al., 2001). In our case, only maternal obesity enhanced the production of both, KA, and QA in the hippocampus of the offspring. This difference may be due to the fact the offspring from obese mothers were directly exposed to an inflammatory environment that activated glial cells. On the other hand, the deleterious effects of KA could be due to its interaction with several receptors in the brain. KA is an antagonist of all ionotropic glutamate receptors, including NMDA and AMPA receptors, as well as of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChR). Given that KA interacts with $\alpha 7$ nAChRs, NMDA

and AMPA receptors, it affects the extracellular concentrations of glutamate, dopamine, acetylcholine and γ -aminobutyric acid (GABA). All these neurotransmitters are critical for the regulation of neurodevelopmental, plasticity, cognition, behavior, and memory processes. Another target of KA is the aryl hydrocarbon receptor (AHR), which is considered a xenobiotic receptor whose activation is associated with the suppression of the cellular immune response. Stimulation of AHR by KA enhances the expression of IL-6 (Plitman et al., 2017; Ramos-Chávez et al., 2018) which could exacerbate the inflammatory response. In the case of glutamatergic receptors an increase of KA levels could then impair the processes of LTP and LTD.

Xanthurenic acid, a kynurenine induced by inflammation in male rats.

Enhanced levels of XA were observed in the hippocampus of obese male rats (see the first part in the results section). In terms of memory, it is still difficult to explain the effect of XA because information is scarce. It is believed that it may impair glutamatergic neurotransmission because it acts as an endogenous ligand of Group II (mGlu2 and mGlu3) metabotropic glutamate (mGlu) receptors (Copeland et al., 2013) and inhibits vesicular glutamate transporters (VGLUT). It has been observed that when the hippocampus is exposed to XA, the amplitude of field excitatory postsynaptic potentials is decreased (Neale et al., 2013). Moreover, XA activates G-protein-coupled receptors and increases the intracellular concentration of Ca^{2+} (Taleb et al., 2012). In addition, in the past it was observed that XA promotes the formation of unfolded proteins (Malina, 1999) and apoptotic-like cell death due to the activation of caspases, cytochrome C release, nuclear DNA cleavage, and destruction of mitochondria and nuclei (Malina et al., 2001) by the translocation of proapoptotic Bcl-2 proteins (Malina and Hess, 2004). Taken together, these data lead us to believe that the excessive accumulation of this metabolite in the brain could affect synaptic plasticity or produced neuronal death. Finally, it should be mentioned that although elevated levels of XA in serum have been found in patients with type 2 Diabetes (Oxenkrug, 2015), we did not observe this alteration in obese rats. Furthermore, it is known that XA crosses the BBB (Gobaille et al., 2008) and since we did not find a decrease in serum XA levels, we are certain that its overproduction took place at the central level.

Which are the possible consequences of having increased/decreased concentrations in the TRP-derived metabolites in the process of synaptic plasticity?

Another limitation in this work is that we did not perform electrophysiology or pharmacological experiments to analyze the effect of increased levels of QA and KA induced by obesity in the process of synaptic plasticity. Nevertheless, as mentioned before, evidence has already proven that QA and KA interact with glutamate ionotropic receptors (Modoux et al., 2021). According to the literature, activation of NMDA receptors potentiates glutamatergic signaling and hence, LTP. Building memories requires LTP, thus, one can imagine that increasing LTP would ameliorate the learning and memory capacities of rats, but this does not seem to be the case. Indeed, there is a huge number of articles reporting that increasing LTP paradoxical impairs memory and learning (Pineda et al., 2004; Rutten et al., 2008; Kim et al., 2009; Dilekoz et al., 2015; Garcia-Alvarez et al., 2015). On the other hand, increased levels of KA would in theory block the activation of AMPA and NMDA receptors and, hence, reduce synaptic plasticity. In our case, we only detected increased QA levels in the hippocampus of obese males, and we do not know whether there was an increased in KA levels because of technical problems to quantify it. Nevertheless, we observed an increased expression of KAT, the enzyme that catalyzes the synthesis of KA. We also found enhanced levels of QA in the hippocampus of rats born to obese dams, and an increase of KA in rats whose mother or father were obese. As the altered levels of these kynurenines were associated with impaired memory and learning, one can reasonably assume that the cognitive deficits induced by obesity are related to alterations in the process of synaptic plasticity mediated by glutamate.

Actually, in the final part of our study, we precisely wanted to analyze the expression of some markers that are key in the process of synaptic plasticity, and we observed that both, maternal and paternal obesity induce alterations in the expression of genes coding for NMDA receptors. As a matter of fact, maternal obesity triggered a downregulation of GluN1 and GluN2B, in the hippocampus, and a downregulation of GluN2A in the frontal cortex. On the other hand, paternal obesity induced a downregulation of GluN1 and GluN2B in the hippocampus, and an upregulation of GluN2B in the frontal cortex. In the light of these observations, it is possible to imagine

two possibilities, the first one is that maternal and paternal obesity induce a reduction in the amount of NMDA receptors present in the postsynaptic neurons. The second possibility is that NMDA receptor assembly is simply not correct to produce effective synaptic signaling. In any case, LTP and LTD processes would be affected. Therefore, we can suppose that the excessive production of kynurenines is the consequence of a homeostatic mechanism intended to stimulate synaptic plasticity. However, this is only a speculation since we do not have any element that could support this statement. In any case, under our experimental conditions we observed that maternal and paternal obesity lead to excessive production of KA and QA together with impaired expression of several markers of synaptic plasticity. Further studies are needed to find the mechanistic link between both results.

*Conclusions and
perspectives*

This work confirmed our initial hypothesis that diet-induced obesity promotes the development of a neuroinflammatory state that leads to the overexpression of enzymes of the kynurenine pathway and brings as result the overproduction of several kynurenines that are associated with memory and learning impairment.

In addition, we found that the obesity-induced alterations on TRP metabolism are sex dependent. Female obese rats do not present increased kynurenine pathway metabolism but rather a decreased availability of TRP in the brain. This results in a reduced production of metabolites derived from the kynurenine and the serotonin pathways. This negative effect of obesity is dynamically modulated by the natural fluctuations of estrogens during the oestrous cycle. Molecular mechanisms that remain to be identified, possibly compensate the negative combined effect of obesity and estrogen depletion by overexpressing the enzymes that promote the production of serotonin and kynurenines, even though, at the end obese females present cognitive deficiency and impaired TRP metabolism.

Finally, we found that both, maternal and paternal obesity program TRP metabolism in the brain of the offspring. While maternal obesity increases the production of both QA and KA, paternal obesity increases the production of only KA. These neurochemicals changes are accompanied by memory deficits. The offspring of obese mothers or of obese fathers, presented also an altered expression of the subunits that conform NMDA receptors and of other important markers of glutamate-mediated synaptic plasticity. Both, the alterations on TRP metabolism and on the expression of NMDA receptors and synaptic markers are linked and contribute to the memory deficiencies observed in obese animals. Nevertheless, our results do not allow to establish a cause and effect relationship between these two phenomena.

Based on the data from the literature and our results, we realize that TRP metabolism is regulated by several factors, including the diet, the parental nutritional status, sex, and fluctuations in the levels of estrogens. In addition, the glial cells that produce kynurenines are also under the control of different factors, such as the individual's age and sex. In order to perform an in-depth analysis in the future, it would

be necessary to evaluate the metabolism of TRP at different ages and in both sexes at the same time. In addition, the effect of maternal and paternal obesity on brain TRP metabolism in female rats remains to be analyzed. Likewise, it is necessary to explore the epigenetic markers transmitted from both fathers and mothers that could modulate the production of kynurenines in the offspring. Finally, it would be necessary to perform multidisciplinary experiments comprising immunohistology, pharmacology and electrophysiology to clearly identify the mechanisms by which the modulation of the production of kynurenines impacts the synaptic plasticity mediated by glutamate.

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*Additional publications and
scientific communications*

Publications

- **Mezo-González CE**, Daher Abdi A, Reyes-Castro LA, Olvera Hernández S, Almeida C, Croyal M, Aguesse A, Gavioli EC, Zambrano E, Bolaños-Jiménez F (2022). Learning Deficits Induced by High-Calorie Feeding in the Rat are Associated With Impaired Brain Kynurenine Pathway Metabolism. *Int J Tryptophan Res.*
DOI: [10.1177/11786469221111116](https://doi.org/10.1177/11786469221111116)
- Sandra Olvera Hernández, Luis Antonio Reyes Castro, Amran Daher Abdi, **Carla Elena Mezo-González**, Armando Arredondo, Elena Zambrano & Francisco Bolaños-Jiménez (2021): Adult rats from undernourished dams show sex-dependent impaired expression in taste papillae and hypothalamus of genes responsible for sweet and fat detection and signalling. *Nutritional Neuroscience.*
DOI: [10.1080/1028415X.2021.1920678](https://doi.org/10.1080/1028415X.2021.1920678)
- Daher-Abdi, A.; Olvera Hernández, S.; Reyes Castro, L.A.; **Mezo-González, C.E.**; Croyal, M.; García-Santillán, J.A.; Ouguerram, K.; Zambrano, E.; Bolaños-Jiménez, F. Maternal DHA Supplementation during Pregnancy and Lactation in the Rat Protects the Offspring against High-Calorie Diet-Induced Hepatic Steatosis. *Nutrients* 2021, 13, 3075.
DOI: [10.3390/nu13093075](https://doi.org/10.3390/nu13093075)

Scientific communication by poster

- **Carla Elena Mezo-González**, Amran Daher Abdi, Sandra Olvera Hernández, Luis A. Reyes-Castro, Clarissa Almeida, Mikaël Croyal, Audrey Aguesse, Elaine C. Gavioli, Elena Zambrano, Francisco Bolaños-Jiménez. “*Learning Deficits in Offspring of Obese Mothers or Obese Fathers are Associated with Impaired Tryptophan Metabolism Via the Kynurenine Pathway*”. Latin American DOHaD Chapter (October 2020). Brazil (online)
- **Carla Elena Mezo-González**, Amran Daher Abdi, Sandra Olvera Hernández, Luis A. Reyes-Castro, Clarissa Almeida, Mikaël Croyal, Audrey Aguesse, Elaine C. Gavioli, Elena Zambrano, Francisco Bolaños-Jiménez. “*Alterations in the kynurenine pathway as potential mechanisms underpinning obesity-induced cognitive impairment*”. Journées scientifiques Ecole Doctorale Biologie Santé (December 2020). Nantes (online), France. Chosen as one of the five best posters.
- **Carla Elena Mezo-González**, Juan Antonio García-Santillán, Mathilde Gourdel, Mikaël Croyal, Francisco Bolaños Jiménez. “*Obesity-induced learning deficits in the female rat are oestrous cycle-dependent and are associated with impaired tryptophan metabolism through the kynurenine pathway*”. Federation of European Neuroscience Societies Forum (July 2022). Paris, France.

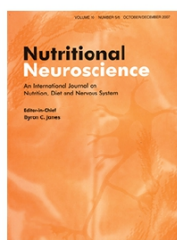
Scientific communication for the general public

- Ma thèse en 180 secondes (March 8, 2022). « J'ai mangé quoi hier soir ? L'obésité comme facteur de risqué pour le développement de problèmes de mémoire » (What did I eat last night ? Obesity as a risk factor for the development of memory problems). Nantes, France. Winner of the 4th place in the competition, chosen by the jury.
<https://www.youtube.com/watch?v=KINUOTrYhpo&t=2725s>
- Ma thèse en 180 secondes (March 16, 2022). « J'ai mangé quoi hier soir ? L'obésité comme facteur de risqué pour le développement de problèmes de mémoire » (What did I eat last night ? Obesity as a risk factor for the development of memory problems). Interregional Final Bretagne and Pays de la Loire. Vannes, France.
https://www.youtube.com/watch?v=n0_NQ8NANpQ&t=6646s
- eDeclics (May 12, 2021). “Effet de la malnutrition sur la mémoire et la cognition” (Effect of malnutrition on memory and cognition). Nantes, France.
- Comptoir des Sciences (May 4 and May 9, 2022). “L'effet de la malnutrition sur la mémoire et l'apprentissage” (The effect of malnutrition on memory and learning).
 - Lycée des Métiers Louis-Antoine de Bougainville. Nantes, France.
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ARTICLE.

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Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System



ISSN: (Print) (Online) Journal homepage: <https://www.tandfonline.com/loi/ynns20>

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To cite this article: Sandra Olvera Hernández, Luis Antonio Reyes Castro, Amran Daher Abdi, Carla Elena Mezo-González, Armando Arredondo, Elena Zambrano & Francisco Bolaños-Jiménez (2021): Adult rats from undernourished dams show sex-dependent impaired expression in taste papillae and hypothalamus of genes responsible for sweet and fat detection and signalling, *Nutritional Neuroscience*, DOI: [10.1080/1028415X.2021.1920678](https://doi.org/10.1080/1028415X.2021.1920678)

To link to this article: <https://doi.org/10.1080/1028415X.2021.1920678>



Published online: 30 Apr 2021.



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Adult rats from undernourished dams show sex-dependent impaired expression in taste papillae and hypothalamus of genes responsible for sweet and fat detection and signalling

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ABSTRACT

Aim: Individuals undernourished in utero or during early life are at high risk of developing obesity and metabolic disorders and show an increased preference for consuming sugary and fatty food. This study aimed at determining whether impaired taste detection and signalling in the lingual epithelium and the brain might contribute to this altered pattern of food intake.

Methods: The preference for feeding fat and sweet food and the expression in circumvallate papillae and hypothalamus of genes coding for sweet and fat receptors and transducing pathways were evaluated in adult rats born to control or calorie-restricted dams. Expression in the hypothalamus and the brain's reward system of genes involved in the homeostatic and hedonic control of food intake was also determined.

Results: Male and female undernourished animals exhibited increased expression in taste papillae and hypothalamus of T1R1, T1R2, CD36, gustducin, TRMP5 and PLC-β2 genes, all of which modulate sweet and fat detection and intracellular signalling. However, the severity of the effect was greater in females than in males. Moreover, male, but not female, undernourished rats consumed more standard and sweetened food than their control counterparts and presented increased hypothalamic AgRP and NPY mRNAs levels together with enhanced dopamine transporter and dopamine receptor D2 expression in the ventral tegmental area.

Conclusions: Maternal undernutrition induces sex-specific changes in food preferences and gene expression in taste papillae, hypothalamus and brain reward regions. The gene expression alterations in the male offspring are in line with their preference for consuming sugary and fatty food.

KEYWORDS

Metabolic programming; taste receptors; food preferences; hypothalamus; taste papillae; sex differences; maternal undernutrition; rat

Introduction

Maternal undernutrition leading to foetal undernutrition and low birth body weight occurs widely throughout the world. Foetal undernutrition also results from placental insufficiency, a major obstetric problem. Human epidemiological and carefully controlled experimental animal studies show that maternal undernutrition increases the risk of developing obesity and metabolic syndrome in the offspring via a metabolic programming process (see [1–3] and therein cited references for a complete description of this concept). The enhanced risk of metabolic disease exhibited by in utero malnourished individuals becomes evident under exposure to high-calorie diets, often evolves with age, is sex-dependent, may be transgenerational in nature and has been

associated with a preference for consuming fatty and sugary food [4–6]. Experimental studies in rodents have further shown that, in addition to favouring the ingestion of high-calorie food [7,8], in utero/neonatal undernutrition induces hyperphagia which is associated with enhanced hypothalamic expression of the orexigenic peptides AgRP and NPY and a reduction in the expression levels of mRNA coding for the anorexigenic peptide POMC [9–11]. Early undernourished animals exhibit also increased motivation for food reward together with an altered expression pattern of opioid receptors and other reward-related genes in the nucleus accumbens (NAc) and other structures of the brain reward system [8,12–14]. Altogether, these latter findings have led to suggest that early malnutrition-induced preference for the consumption of foods high in fat

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and sweet, results from the dysfunction of the neuronal pathways involved in the hedonic control of food intake.

However, the hedonic value of food, and therefore, the choice of what to eat, is also determined by the gustatory system which detects and differentiates the five basic taste qualities – sweet, sour, bitter, salty, and umami – in addition to fat (see [15,16] for review). The first step in taste perception is the interaction of sapid components of food with specific receptors expressed in chemosensory cells of taste buds found in the oral cavity. In particular, sweet substances are detected by a heterodimer composed of the type 1 member 2 (T1R2) and type 1 member 3 (T1R3) taste receptors belonging to the family of G protein-coupled receptors. The binding of a sweet molecule to the T1R2/T1R3 heterodimer activates the associated G protein gustducin leading to the stimulation of phospholipase C-beta 2 (PLC- β 2), to the release of intracellular Ca^{2+} and the opening of the transient receptor potential cation subfamily M member 5 (TRPM5). This results eventually in membrane depolarization and the release of ATP which activates gustatory fibres responsible for transmitting the taste signal to taste Central Nervous System (CNS) areas which mediate taste perception and behaviour. A similar intracellular signalling pathway is activated upon the binding of free fatty acids to the free fatty acid receptor GPR120 and the fatty acid translocase CD36 as well as by the stimulation by umami of the heterodimer composed of type 1 member 1 taste receptor (T1R1) and T1R3 [15,16]. Notably, in addition to being expressed in the lingual epithelium, sweet taste receptors are present in the hypothalamus where their stimulation contributes to glucose sensing in leptin-responsive neurons [17].

Variations in receptor-mediated detection of sweet and fat as well as genetic polymorphisms in sweet and fat receptor genes have been associated with differences in food preferences and predisposition to weight gain and metabolic disorders [18–22]. On the basis of these observations, we hypothesized that impaired taste perception might contribute to the preference for consuming sweet and fatty induced by early malnutrition. To test this hypothesis, this study evaluated the preference for the intake of fat and sweet food of adult rats born to control or calorie-restricted dams and compared the impact of maternal malnutrition on the expression of genes regulating fat and sweet detection and signalling in taste buds and hypothalamus versus the expression of genes involved in the homeostatic and hedonic control of food intake.

Material and methods

Animals and nutritional manipulations

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Principles of laboratory animal care (NIH publication no. 85–23, revised 1985), and were approved by the Ethical Committee for Animal Experimentation of the Pays de la Loire (authorization number 2017012013318453/APAFIS 8215). Three-month-old female and male Wistar rats were obtained from Janvier Labs (Le Genest Saint Isle, France), and maintained under an inverted 12-/12-hour dark/light cycle (lights off at 7:00 AM), and at $23 \pm 1^\circ\text{C}$ with food and water *ad libitum*. After an adaptation period of 10 days, females in natural proestrus were allowed to copulate with a male until at least two ejaculations were displayed. This day was considered as gestational day 0. Twelve hours after coitus, mated female rats were housed individually and fed either *ad libitum* (C) or were undernourished (UN) from gestation through the end of lactation by reducing 50% their daily food ration in relation to the daily amount of food consumed by the control dams. To avoid infanticide behaviour and cannibalism of undernourished dams during the early post-parturition phase, UN mothers were fed *ad libitum* from gestational day 20 until 48 h after delivery when their daily ration of food was again reduced by 50% in relation to the amount of food consumed by control dams. At birth, litter size was adjusted to eight pups per litter maintaining a 1:1 male to female ratio. Pups were weaned at 21 days and fed standard chow (SAFE, Augy, France) until the end of the experiment. At the end, a total of six controls and six undernourished dams were used from which two pups of each sex were selected at random for analysis giving a total of 24 control pups and 24 UN pups.

Evaluation of food preferences

The preference for the consumption of fatty and sugary food was evaluated at 3 and 6 months of age during a series of test meals. Animals were food-deprived in their housing cages at the beginning of the dark phase of their dark/light cycle and transferred 6 h later to individual cages located in a dark room in which they had free access to three types of food made up of chow or chow enriched with 30% of fat or 30% of sugar. One hour later, animals were returned to their housing cages where they continued to be fed standard chow *ad libitum*. The amount of chow and palatable food (fat and sweet), consumed during the test meal, was determined by subtracting the amount of food remaining in the feeder from the amount of food offered at the

start of the meal. Care was taken to inspect the bottom of the cage in detail at the end of the meal to detect any spilled food. Animals were submitted to this test meal during five consecutive days. The averaged quantity of each food consumed during these five test meals was used as a parameter of their food preferences. Foods were prepared every day before the test using powdered standard chow as a base mixed with water, with sugar plus water or with lard to obtain food with a similar consistency. Animals had free access to water during food deprivation and test meals.

Tissue collection

Two days after the last test meal, 6 months-old rats were fasted for 2 h at the beginning of the dark phase of their light/dark cycle and sacrificed by cervical dislocation under isoflurane anaesthesia. The tongue was excised and the circumvallate papilla carefully dissected under a stereoscopic microscope. After extraction of the brain from the skull, the whole hypothalamus was dissected and the rest of the brain was transferred to a rat brain matrix to obtain 1 mm thick sections from which punches of the nucleus accumbens and ventral tegmental area (VTA) were collected. All tissue samples were immediately frozen after dissection in liquid nitrogen and stored at -70°C .

Real-time quantitative RT-PCR experiments

Total RNA was extracted using the Trizol reagent (Invitrogen) and treated with DNase/RNase-free for 30 min at 37°C . Afterwards, $1.5\ \mu\text{g}$ of purified RNA was reversed transcribed using Superscript II RNaseH-Reverse-Transcriptase (Invitrogen) in a total volume of $20\ \mu\text{l}$ and the resulting cDNA was diluted 40-fold in DNase and RNase free water. Thereafter, $5\ \mu\text{l}$ of each cDNA diluted sample was used as template for PCR amplification using SYBR Green (Biorad, France) as fluorogenic intercalating dye and the iCycler iQ Real-Time PCR detection system instrument from BioRad Laboratories (Hercules, CA, USA). PCR parameters were an initial denaturation step of 5 min at 95°C followed by 45 cycles of 30 s at 95°C , and 30 s at 60°C . Primers used for the amplifications are presented in Supplemental Table 1.

Data analysis

Due to inter-individual behavioural variability, food intake data of each animal were considered as a single point. In contrast, for the analyses of RT-PCR results, the Ct values of the two animals from the same litter and from the same experimental group were averaged to yield a final number of 12 points per sex corresponding to 6 controls and to 6 undernourished dams. The

relative expression levels of the mRNAs in the different samples were calculated using the $2^{-\Delta\Delta\text{CT}}$ method and β -actin RNA as housekeeping gene. Prism 6 Software (GraphPad, Inc., CA, US), was used to perform all statistical analyses. Data were first checked for normality using Shapiro–Wilk normality test. Statistical differences between C and UN animals of the same sex were assessed by unpaired Student's *t*-test or Mann–Whitney test. Differences in food intake between male and female animals were analysed by two-way ANOVA using maternal diet and sex as factors followed by Sidak's multiple comparison test. Experimental results are expressed as mean \pm S.E.M. Statistical significance was set at $p < .05$.

Results

Phenotypic characteristics and serum metabolic profile

Dams allocated to C and UN groups exhibited the same body weight at the beginning of gestation ($C = 241 \pm 2\ \text{g}$ vs $UN = 240 \pm 2\ \text{g}$). Nevertheless, while the body weight of control dams gradually increased during pregnancy, the body weight of undernourished mothers remained relatively stable so that at the end of gestation they weighed 27% lower than control dams ($C = 397 \pm 3.98\ \text{g}$ vs $UN = 291.25 \pm 4.37\ \text{g}$, $p < .0001$). These weight differences were more pronounced during the lactation period due to higher weight loss after delivery in malnourished rats (-25%) than in controls (-19%). Otherwise, neither the number of offspring per mother nor the male/female offspring ratio was affected by calorie restriction.

The anthropometric and metabolic characteristics of control and undernourished offspring at 6 months of age are presented in Table 1. Both female and male UN rats exhibited reduced body weight and adipose tissue as well as lower circulating levels of leptin compared with control animals. In utero and neonatal malnutrition induced also a significant reduction of serum triglycerides in males but not in female rats. Conversely, female, but not male, UN rats presented higher levels of serum cholesterol in relation to their control counterparts.

Food preference test

When exposed for the first time to a new food, humans and animals often display taste neophobia which is reflected as a reluctance to eat the unfamiliar food. Furthermore, the amount of food intake varies from day to day even though food consumption is assessed at the

Table 1. Anthropometric and metabolic characteristics of 6-month-old male and female rats born to ad libitum fed or food-restricted dams.

	Males		Females	
	Controls	Undernourished	Controls	Undernourished
Body weight (g)	620.73 ± 10.96	551.67 ± 14.89**	343.64 ± 5.77	298.91 ± 4.28***
Adipose Index	6.35 ± 0.41	4.52 ± 0.22**	6.52 ± 0.41	4.90 ± 0.28**
Leptin (ng/mL)	13.56 ± 1.84	8.51 ± 0.98*	7.27 ± 0.88	4.58 ± 0.51*
Insulin (ng/mL)	1.58 ± 0.19	1.53 ± 0.30	1.36 ± 0.41	0.92 ± 0.24
Triglycerides (ng/dL)	101.44 ± 10.01	73.33 ± 5.80*	136.57 ± 14.35	120.35 ± 25.52
Cholesterol (ng/dL)	73.62 ± 5.07	77.66 ± 9.05	63.98 ± 3.91	79.03 ± 3.10*
Glucose (ng/dL)	163.07 ± 9.82	177.26 ± 20.19	174.76 ± 4.21	169.71 ± 5.63

* $p < .05$; ** $p < .01$; *** $p < .001$ compared to control animals of the same sex.

same time of the day and under the same conditions. To avoid the bias that could be introduced by these phenomena, food preference tests were performed over five consecutive days and the amount of food consumed during each test over the 5-day-period was averaged and used as a readout of food preference. The coefficient of variation for food intake across the test meals among animals in the same group ranged from 7% to 37% in both control and malnourished animals regardless of sex. Likewise, C and UN male and female animals displayed the same feeding pattern during the test meals with a reduced food intake on the first day compared to the following days. Otherwise, food consumption remained stable in each group from the second day until the end of the tests.

During the 1 h test meals, all animals showed a marked preference for the consumption of sweet food versus chow and high-fat food independently of maternal diet, sex and age (Figure 1). In fact, the quantity of sugary food consumed by control male animals represented on average 70% of their total amount of food intake and this percentage was 57% in the case of female rats. However, clear sex-dependent differences in the choice of the other taste categories were observed. Thus, female rats from control and UN groups consumed significantly more chow (Figure 1(A,E)) and high-fat food (Figure 1(C, G)) than their male counterparts at both three and six months of age as revealed by the two-way ANOVA analysis of the data (Table 2). At six months, control female rats consumed also more sweet food than control males (Figure 1(F)). Consequently, female rats ingested more calories in relation to their body weight than males (Figure 1(D, H)).

Concerning the impact of maternal undernutrition, male UN rats ate significantly more chow and sweet food than control males and showed reduced intake of fatty food at three months compared with controls (Figure 1(A–C)). In contrast, there were no differences in preference for consuming chow, fatty or sugary food between C and UN female rats (Figure 1(E–G)). Of notice, however, ageing favoured the consumption

of sweet (95.82 ± 4.08 kcal/kg vs 79.64 ± 8.14 kcal/kg) and fat (50.69 ± 3.55 kcal/kg vs 29.02 ± 2.05 kcal/kg), food in malnourished female animals as determined by two-way ANOVA for repeated measures using maternal diet and age as factors [F (age) = 16.89, $p < .0007$ for sweet; F (age) = 36.71, $p < .0001$ for fat].

Gene expression changes of taste receptors and signalling pathways

There was a significant increase in the relative expression levels of sweet taste receptor T1R2 as well as in the levels of T1R1 and fatty acid receptor CD36 mRNAs in the circumvallate papillae of undernourished animals compared with controls (Figure 2 top graphs). The relative expression of the genes encoding gustducin, TRMP5 and PLC- β 2 proteins, which are all involved in sweet and fat intracellular signalling, was also enhanced by maternal undernutrition. In contrast, control and undernourished rats exhibited the same levels of sweet T1R3 receptor mRNA. The alterations induced by maternal undernutrition on the expression levels of sweet and fat receptors and signalling molecules were common to male and female rats (Figure 2 top graphs). However, the magnitude of the effect was much greater in females than in males excepting for T1R1 and CD36 genes.

Maternal undernutrition enhanced also the expression of genes encoding proteins involved in taste detection and transduction in the hypothalamus but to a lesser extent in males than in females (Figure 2 bottom graphs). Moreover, in contrast to the changes observed in taste papillae, the hypothalamic levels of gustducin and PLC- β 2 mRNAs were decreased in undernourished males (Figure 2 left bottom graph).

Gene expression changes of genes involved in the hedonic control of food intake

The impact of maternal undernutrition on the expression of several genes involved in food reward

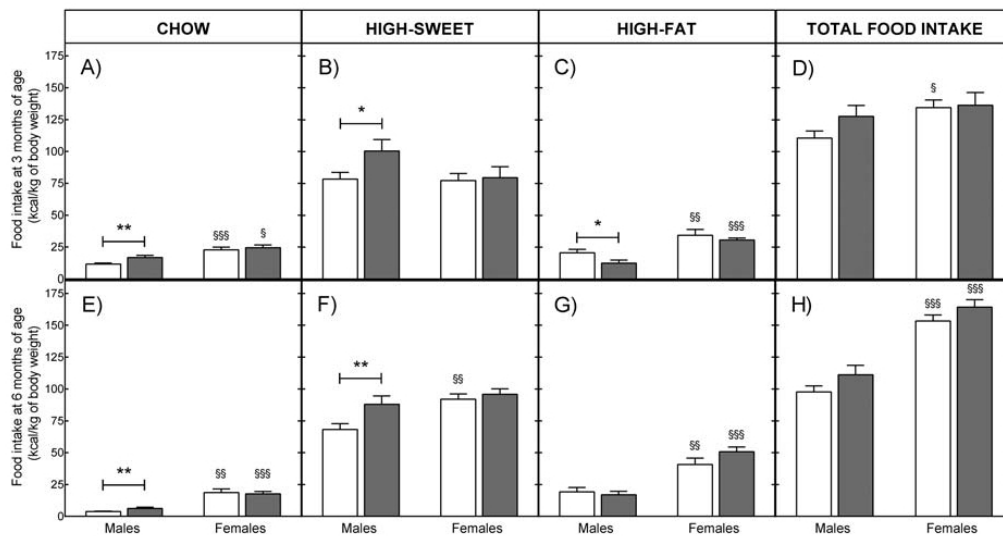


Figure 1. Food preferences of male and female rats born to control (white bars) or undernourished (grey bars) dams. Each bar represents the average amount of chow, sweet or fatty food ingested per test meal during five consecutive days at 3 (top graphs) and 6 (bottom graphs) months of age. Food intake is expressed in Kcal in relation to body weight. Data correspond to the mean \pm SEM with $n = 12$ for control and UN animals of each sex issued from 6 different litters (two animals of each dam). * $p < .05$; ** $p < .01$ (Student's *t*-test). § $p < 0.05$; §§ $p < .01$; §§§ $p < .001$ compared with male animals of the same experimental group as determined by two-way ANOVA analysis using maternal diet and sex as factors followed by Sidak's multiple comparison test.

was evaluated in the nucleus accumbens and ventral tegmental area. These experiments showed that the expression of the dopamine transporter (DAT) in the NAc was decreased by malnutrition in male animals but increased in female rats (Figure 3 top graphs). Conversely, whereas the expression of DAT in the VTA was increased in male undernourished rats in relation to their controls, the levels of DAT mRNA in undernourished females tended to decrease (Figure 3 bottom graphs). The effects of undernutrition varied also according to the brain region. Thus, compared to their control counterparts, female undernourished rats exhibited decreased and increased expression of, respectively, dopamine receptor D2 and mu opioid receptor mRNAs in the Nac (Figure 3 right top graph), but not in the VTA (Figure 3 right bottom graph), whereas the expression of mu opioid receptors decreased in the VTA but not in the NAc of male rats (Figure 3 left top and bottom graphs).

Gene expression changes of genes involved in the homeostatic control of food intake

UN males exhibited increased levels of mRNAs encoding the orexigenic peptides AgRP and NPY but these changes were not observed in female animals (Figure 4). In contrast, maternal undernutrition

did not modify the expression of the anorexigenic genes CART and POMC neither in male nor in female rats.

Discussion

Since the concept of the developmental origins of health and disease was proposed, a large number of experimental studies have been performed with the aim of determining the role of the neuronal circuits regulating food intake on the increased disease susceptibility to metabolic disorders resulting from maternal undernutrition. Feeding behaviour, that is, the choice of what to eat and how much to eat, is modulated by complex regulatory mechanisms categorized as homeostatic or non-homeostatic [23]. The former mechanism relies on the action at the brain of several peptide neurotransmitters released from the periphery in response to the energetic status of the body. These peptides act directly to regulate food intake or modulate feeding by controlling the expression within the hypothalamus of NPY, AgRP, POMC, CART and other neurotransmitters. NPY and AgRP promote food intake (orexigenic effect), while increased POMC and CART expression decrease food intake (anorexigenic effect). The non-homeostatic control of food intake is driven by signals associated with

Table 2 Results of the two-way ANOVA analyses of food intake data represented in Figure 1.

	Source of variation	Chow intake		Sweet intake		Fat intake		Total intake	
		F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
3-Month-old	Interaction	0.9838	.3324	1.935	.1721	0.4918	.4873	0.9859	.3267
	Sex	29.12	.0001	2.411	.1286	24.81	.0001	4.557	.039
6-Month-old	Maternal diet	3.819	.0581	2.966	.093	3.455	.0706	1.492	.2291
	Interaction	0.6219	.437	2.669	.1132	1.93	.1757	0.05349	.8188
	Sex	36.32	.0001	10.72	.0027	39.52	.0001	91.61	.0001
	Maternal diet	0.1246	.7267	5.873	.0218	0.7494	.394	4.575	.0413

Note: Statistics analyses were performed using GraphPad prism software version 6.0 with Sidak's multiple comparison test as *post hoc* test.

cognitive processes, such as decision making and learning, engaged by the sensory systems in response to environmental factors and the reward and reinforcing properties of food. The analysis and processing of these signals is performed by a group of brain regions designed collectively as the brain reward system among which the mesolimbic dopaminergic pathway that connects the ventral tegmental area to the nucleus accumbens, amygdala, and hippocampus. Stimulation of μ -opioid receptors in the VTA blocks the release of GABA onto DA neurons leading to elevated dopamine levels in the NAc and enhanced motivation to consume or 'wanting', whereas the activation of μ -opioid receptors located within the NAc increases

food 'liking'. That is, the perceived palatability of a food or the pleasure derived from the consumption of food [24].

Previous studies have shown that protein or calorie restriction during pregnancy and/or lactation induces hyperphagia [9–11] and a high preference for consuming sweet and fatty food [7,8,14] in the offspring. These altered patterns of food intake are associated with increased expression of the orexigenic peptides NPY and AgRP in the hypothalamus and enhanced expression of tyrosine hydroxylase, dopamine transporter genes and μ -opioid receptors in the nucleus accumbens and ventral tegmental area [12,14,25]. Here, we show that early malnutrition-induced preference for

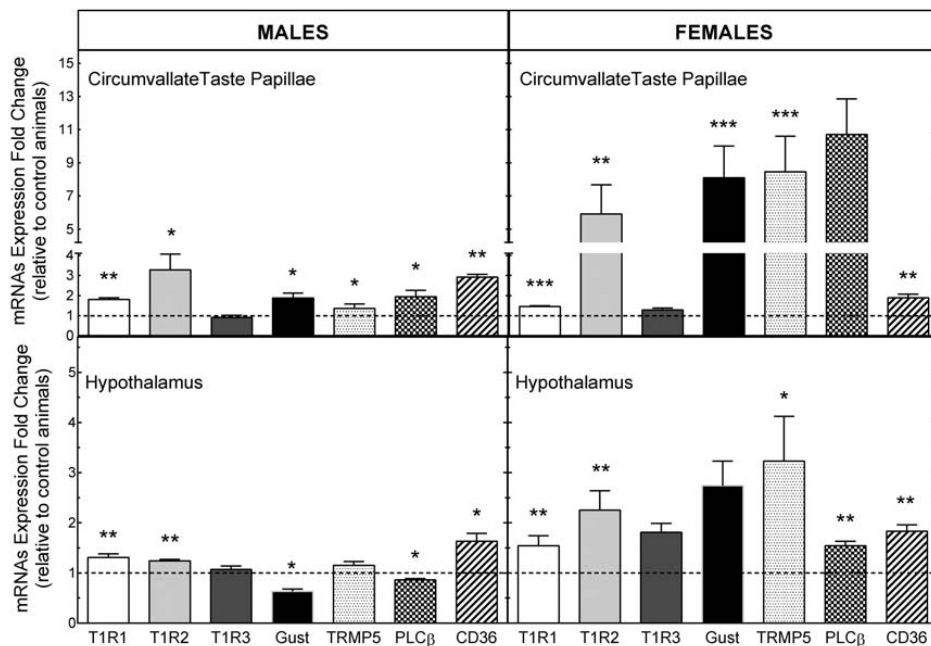


Figure 2. Relative abundance of mRNAs encoding sweet and fat taste receptors and transducing pathways in circumvallate papillae and hypothalamus of male and female 6-month-old rats born to control or calorie-restricted dams. Data correspond to the fold change in mRNA expression levels in relation to those of the control group (expression level 1), as determined by the $2^{-\Delta\Delta CT}$ method using β -actin RNA as housekeeping gene. Expression levels from two animals of the same litter and from the same experimental group were averaged to yield a final number of 6 points per group and per sex. * $p < .05$; ** $p < .01$; *** $p < .001$ versus control rats of the same sex.

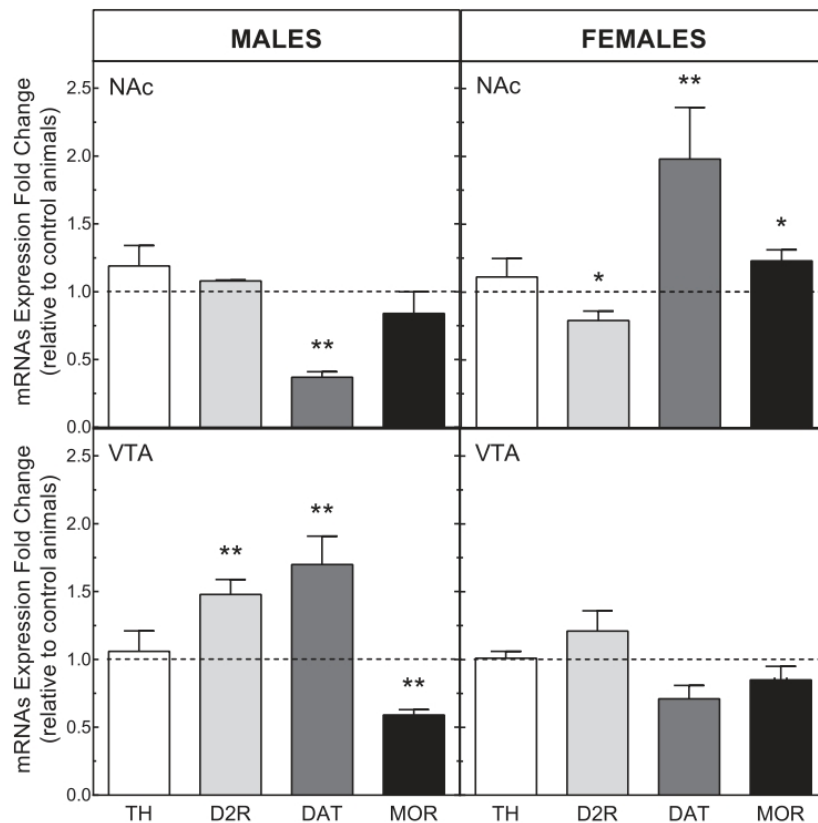


Figure 3. Relative abundance of genes encoding Tyrosine hydroxylase (TH), Dopamine receptor D2, Dopamine transporter (DAT) and Opioid receptor mu (MOR) in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of male and female 6-month old rats born to control or calorie-restricted dams. Data correspond to the fold change in mRNA expression levels in relation to those of the control group (expression level 1), as determined by the $2^{\Delta\Delta CT}$ method using β -actin RNA as housekeeping gene. Expression levels from two animals of the same litter and from the same experimental group were averaged to yield a final number of 6 points per group and per sex. * $p < .05$; ** $p < .01$ versus control rats of the same sex.

the consumption of palatable food is also paralleled by the upregulation in taste papillae and hypothalamus of mRNAs encoding for genes involved in sweet and fat taste detection and signalling. Therefore, in addition to the impairment of the homeostatic and hedonic neuronal circuits regulating food intake, maternal undernutrition results in alterations of the gustatory system.

Oral sucrose stimulates the brain reward system by enhancing the release of dopamine in the NAc [26] and the firing rate of nucleus accumbens neurons [27,28]. Increased dopamine levels in the NAc are also observed following exposure to a high-fat diet [29]. On the other hand, several lines of evidence from the literature indicate that there is a positive relationship between the preference for the consumption of sweet or fat food and the expression levels of sweet and fat taste receptors in the lingual epithelium. Namely, rats made obese by a high-fat diet exhibit both reduced

expression of T1R3 receptor mRNA in the circumvallate papillae and decreased preference for the consumption of saccharine solutions [30]. Similarly, taste receptor cells isolated from the circumvallate papillae of obese mice display reduced responsiveness to sucrose and saccharine as well as decreased expression of gustducin and PLC- β 2 [31]. Worth noting, these latter changes in sweet-sensing and gene expression at the cellular level were correlated to the decreased unconditioned licking responses to saccharine and sucrose exhibited by the same animals. Conversely, overnight fasting increased the preference for the consumption of high concentrations of linoleic acid which was associated with enhanced expression of CD36 mRNA in taste buds [32]. On the basis of these observations, it can be reasonably proposed that the herein reported enhanced expression of sweet and fat receptors and transducing pathways in the circumvallate papillae of male

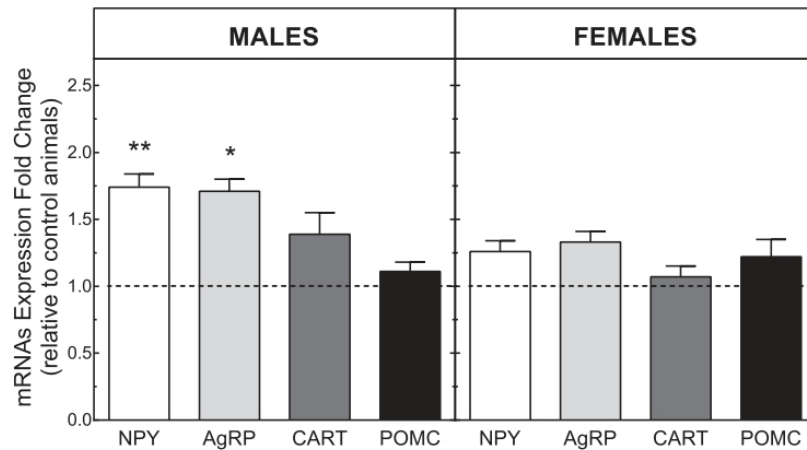


Figure 4. Relative abundance of mRNAs coding for orexigenic (NPY and AgRP) or anorexigenic (POMC and CART) peptides in the hypothalamus of male and female 6-month-old rats born to control or calorie-restricted dams. Data correspond to the mRNA expression levels in relation to those of the control group (expression level 1) amplified within the same PCR run and under the same experimental conditions. Expression levels from two animals of the same litter and from the same experimental group were averaged to yield a final number of 6 points per group and per sex. * $p < .05$; ** $p < .01$; * $P < .01$ compared with control males.

malnourished rats leads to an overstimulation of the brain reward system, therefore, explaining their enhanced preference for consuming sweet and high-fat foods in comparison with control animals. This overstimulation of the reward system would be concomitant with the activation of food intake via non-homeostatic regulatory mechanisms driven by the increased expression in the hypothalamus of the orexigenic peptides NPY and AgRP.

It should be mentioned, however, that though the employed protocol allows the assessment of food preference, it does not provide a direct estimate of taste sensitivity. It also should be considered that different levels of regulation exist between transcript and protein product, including stability of the mRNA, the translation efficiency of the transcript and degradation of the protein, such that there is not always a direct link between mRNAs levels and their functional products, that is, their encoded proteins. Previous studies have indeed shown that adult offspring of dams fed a low protein diet exhibited decreased expression of μ -opioid receptor mRNA but the enhanced density of μ -opioid receptor binding sites [13,25]. Further studies using complementary methods, such as the 2-bottle preference test, are necessary to determine the direct consequences of the changes in the expression of taste receptor and taste signalling pathways induced by maternal malnutrition on the taste sensitivity of the offspring and to establish a mechanistic link between these changes and their associated impaired feeding pattern.

Given that animals were food-deprived for over a period of 6 h before the test meal, it could be argued that under our experimental conditions the incentive to eat is essentially driven by hunger and that there is a minimal influence on the taste system. This is however unlikely since previous studies have shown that overnight or a 24 h fasting do not alter the preference for the consumption of fatty or sugary food [33,34]. Likewise, the involvement of post-ingestive factors in the feeding pattern presented by the animals can be excluded.

Despite considerable evidence in the literature that taste preferences begin to be established during in utero development and early childhood [5,35,36], very few studies have attempted to determine the consequences of maternal undernutrition or maternal obesity on the mechanisms underpinning the detection and signalling of taste stimuli. To our knowledge, there are only two studies in which alterations in the gustatory system have been assessed in offspring following maternal undernutrition or maternal obesity. In the first of these studies, Salas et al. showed that offspring of calorie-restricted rats exhibit impaired morphology of the circumvallate papillae together with a reduced number of taste buds [37]. In the second study, Song et al. observed that maternal consumption of a high-fat diet increased the expression of both T1R1 and T1R3 receptors while reducing gustducin mRNA levels in the lingual epithelium of the offspring [38]. These changes were detected at weaning and were still present at three months of age but did not lead to preferential

consumption of a sucrose solution. Otherwise, neither the expression of PLC- β 2 nor that of TRMP5 was affected by maternal obesity. In contrast to this report, here it was observed that maternal calorie-restriction results in the upregulation of the fatty acid receptor CD36 and of all genes encoding sweet detection and signalling proteins with the exception of the T1R3 receptor gene.

In addition to being expressed in taste papillae, sweet taste receptors are present in various tissues involved in the regulation of energy homeostasis such as the pancreas, liver, adipose tissue, brain and intestine (see [39] for review). Although the physiological function of these extra-oral taste receptors remains largely undetermined, the emerging picture is that they play a pivotal role in glucose sensing and glucose homeostasis. Thus, activation of intestinal sweet taste receptors stimulates glucose absorption via the release of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) from enteroendocrine cells [40] and the upregulation of the sodium-glucose cotransporter 1 (SGLT1) in enterocytes [41]. In the pancreas, sweet taste receptors stimulate insulin secretion [42] and their activation in the arcuate nucleus of the hypothalamus results in the increased firing of glucose-excitatory neurons [17]. The characteristic of these neurones is that they become activated by high concentrations of glucose. Enhanced expression of taste receptors in the hypothalamus of malnourished animals could therefore lead to a low activation threshold of glucose-excitatory neurons and, consequently, to a potentiation of the effects of glucose. Brain injection of glucose or sucrose, an artificial sweetener whose sweetening effects are linked exclusively to its interaction with sweet taste receptors, results in decreased feeding [17]. The same has been shown upon stimulation of hypothalamic CD36 receptors by fatty acids [43]. It is thus paradoxical that malnourished animals show both increased food consumption and a higher density of hypothalamic sweet and fat taste receptors. However, sweet and fat taste receptors are also expressed in the paraventricular nucleus of the hypothalamus and it has been reported that the injection of glucose into this hypothalamic nucleus has an inhibitory effect on food intake [44]. Hence, in the absence of an integrative understanding of the function of brain taste receptors, it is not possible to draw definite conclusions about the behavioural consequences of a change in hypothalamic taste receptor expression levels.

Male and female rats reacted differently to maternal undernutrition, both in terms of food preferences and gene expression. In particular, there were no differences in sweet, fat or total food intake between control and

malnourished female rats despite the fact that the latter displayed higher expression levels of sweet and fat taste receptors in taste buds than undernourished males. Sex differences in food preferences in response to maternal undernutrition have also been documented by other studies. For example, using a very similar developmental programming model than the one used in this report, Alves et al. observed that male, but not female, undernourished rats consumed a higher amount of sweet food than non-undernourished animals [14], whereas Bellinger et al. showed that female, but not male, offspring of protein-restricted dams consumed significantly less fat when exposed to a self-selection feeding trial than their control counterparts [7]. Female rats exhibit reduced food intake during the proestrus phase of their ovarian cycle resulting from a rise in oestradiol secretion [45,46]. It could, therefore, be inferred that the lack of effect of maternal undernutrition on food preference in female rats is the consequence of the influence of female gonadal hormones on food intake and not of a sex-dependent difference in metabolic programming. However, the proestrus period in rats is 12-4 h long so that the effects of oestradiol on food intake are not maintained throughout the 4-5 days of their ovarian cycle. Furthermore, under our experimental conditions, food consumption remained stable from day 2 to day 5 of the test in both female and male rats and both male and female animals showed the same inter-individual variation in food intake across the tests. This latter result is identical to that reported recently by Smarr et al. [47]. The present observations add therefore to the existing evidence showing that metabolic programming, as many physiological functions and pathological alterations, differ between males and females and highlight the need to consider the sex as a biological variable in studies on the impact of the maternal nutritional status on offspring's health. Identifying the mechanisms underlying the differences in metabolic programming between sexes is a major challenge.

The upregulation of taste receptors and transducing pathways induced by maternal malnutrition expands on previous observations indicating that impaired nutrient sensing is a key mechanism of metabolic programming. Namely, decreased or increased activation of, respectively, mTOR and AMPK kinases were documented in adult offspring of dams fed a low protein diet during gestation and lactation [48,49]. The mTOR signalling pathway is under-stimulated in low energy conditions while, on the contrary, AMPK is over-stimulated. On the other hand, fasting entrains an upregulation of hypothalamic sweet taste receptors [50]. These observations suggest that adaptive responses of

nutrient-sensing pathways to energy shortfalls during in utero and neonatal development persist into adulthood in the absence of any nutritional deficiency.

In conclusion, maternal undernutrition induces long-lasting sex-specific changes in food preferences and gene expression in taste papillae, hypothalamus and brain reward regions. The gene expression changes in malnourished males are in favour of increased feeding and are in line with their preference for consuming sugary and fatty food.

Acknowledgements

Sandra Olvera Hernández was supported by the programme 'Food for Tomorrow' of the French Region Pays de la Loire and the European Regional Development Fund (FEDER). Luis Reyes Castro received support from the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreenSkills fellowship. Amran Daher Abdi is the recipient of a doctoral fellowship from the Ministry of Research and Innovation of the Government of the Republic of Djibouti. Carla Mezo González is supported by an international PhD fellowship from the Mexican National Council of Science and Technology (CONACYT).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the French National Research Agency (ANR, grant ANR-16-CE21-0007-01) and CONACYT (award number 2015-16-273510).

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ARTICLE.

Maternal DHA Supplementation during Pregnancy and Lactation in the Rat Protects the Offspring against High- Calorie Diet-Induced Hepatic Steatosis.

Daher-Abdi, A.; Olvera Hernández, S.; Reyes Castro, L.A.; Mezo-González, C.E.; Croyal, M.; García-Santillán, J.A.; Ouguerram, K.; Zambrano, E.; Bolaños-Jiménez, F. Maternal DHA Supplementation during Pregnancy and Lactation in the Rat Protects the Offspring against High-Calorie Diet-Induced Hepatic Steatosis. *Nutrients* 2021, 13, 3075.

Article

Maternal DHA Supplementation during Pregnancy and Lactation in the Rat Protects the Offspring against High-Calorie Diet-Induced Hepatic Steatosis

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Citation: Daher-Abdi, A.; Olvera Hernández, S.; Reyes Castro, L.A.; Mezo-González, C.E.; Croyal, M.; García-Santillán, J.A.; Ouguerram, K.; Zambrano, E.; Bolaños-Jiménez, F. Maternal DHA Supplementation during Pregnancy and Lactation in the Rat Protects the Offspring against High-Calorie Diet-Induced Hepatic Steatosis. *Nutrients* **2021**, *13*, 3075. <https://doi.org/10.3390/nu13093075>

Academic Editor: Carlo Agostoni

Received: 6 August 2021

Accepted: 30 August 2021

Published: 31 August 2021

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Abstract: Maternal supplementation during pregnancy with docosahexaenoic acid (DHA) is internationally recommended to avoid postpartum maternal depression in the mother and improve cognitive and neurological outcomes in the offspring. This study was aimed at determining whether this nutritional intervention, in the rat, protects the offspring against the development of obesity and its associated metabolic disorders. Pregnant Wistar rats received an extract of fish oil enriched in DHA or saline (SAL) as placebo by mouth from the beginning of gestation to the end of lactation. At weaning, pups were fed standard chow or a free-choice, high-fat, high-sugar (fc-HFHS) diet. Compared to animals fed standard chow, rats exposed to the fc-HFHS diet exhibited increased body weight, liver weight, body fat and leptin in serum independently of saline or DHA maternal supplementation. Nevertheless, maternal DHA supplementation prevented both the glucose intolerance and the rise in serum insulin resulting from consumption of the fc-HFHS diet. In addition, animals from the DHA-fc-HFHS diet group showed decreased hepatic triglyceride accumulation compared to SAL-fc-HFHS rats. The beneficial effects on glucose homeostasis declined with age in male rats. Yet, the preventive action against hepatic steatosis was still present in 6-month-old animals of both sexes and was associated with decreased hepatic expression of lipogenic genes. The results of the present work show that maternal DHA supplementation during pregnancy programs a healthy phenotype into the offspring that was protective against the deleterious effects of an obesogenic diet.

Keywords: developmental programming; DHA; obesity; hepatic steatosis

1. Introduction

It has well established that the nutritional status of the mother during gestation and lactation is a key determinant factor in the long-term health of the offspring. Epidemiological observations in humans show that children of obese mothers or those born to mothers who suffered from nutritional deficiencies during pregnancy are at greater risk of developing cognitive deficits and metabolic disorders than children of adequately nourished pregnant mothers [1–4]. These epidemiological observations have been corroborated by the results

of numerous experimental studies in various animal species and conceptualized under the hypothesis of the Developmental Origins of Health and Disease (DOHaD), also known as metabolic programming or nutritional programming [5–7]. According to this hypothesis, an imbalanced nutritional environment during in utero development and/or neonatal life sensitizes offspring to developing metabolic and mental diseases via epigenetic mechanisms that translate the effects of early nutritional events, leading to long-term changes in behavior and energy homeostasis. However, just as an unhealthy maternal diet can lead to adverse health consequences in the offspring, maternal supplementation with specific nutrients during pregnancy can potentially reverse and/or protect against the detrimental effects of metabolic programming [8,9]. In this respect, maternal supplementation with docosahexaenoic acid (DHA) is a common clinical practice.

DHA is a polyunsaturated fatty acid that is found in large quantities in fish oil and seaweed. It is essential for the optimal development of the brain. However, it is only produced in marginal quantities by the body—hence, the recommendation to supplement the diet of pregnant women with DHA [10]. This nutritional intervention is supposed to reduce the risk of cerebral palsy, avoid premature birth and postpartum maternal depression, and increase birth weight [10–13]. Maternal DHA supplementation, however, has been extended beyond its use in preventing developmental defects. Consumption of this fatty acid is also believed to improve the cognitive development of the child. Substantial evidence indicates that children of mothers who received DHA during pregnancy exhibit better learning and cognitive indices than children of placebo-supplemented mothers [14,15]. Nevertheless, no benefits on offspring's neurodevelopment of maternal DHA supplementation have also been documented [16].

Clinical studies have also shown that supplementing the diet with DHA and/or eicosapentaenoic acid (EPA) reduces body fat [17,18], plasma triglycerides [19] and fatty acid accretion in liver [20,21]. These effects would be mediated by increased hepatic fatty oxidation and plasma lipoprotein lipase activity associated with reduced hepatic lipogenesis and very-low-density lipoprotein (VLDL) production [22–25]. Recently, it was proposed that the production of N-acyl taurines could also be involved [26]. Lower body fat accretion and reduced dyslipidemia have also been observed in obese mice and rats fed a high-fat diet supplemented with DHA/EPA [27,28]. On the basis of these findings, we hypothesized that maternal DHA supplementation could not only improve a child's cognitive development but also promote a healthy metabolic state that would exert a protective effect against the deleterious effects of an obesogenic diet. Therefore, this study aimed to evaluate (in rat offspring) the potential protective effects of maternal DHA supplementation during pregnancy and lactation against the metabolic alterations induced by a high-calorie diet and to uncover the involved molecular mechanisms.

2. Materials and Methods

2.1. Animals and Nutritional Interventions

The workflow of the study is illustrated in Figure 1. Founder (F0) female and male Wistar rats of three months of age were obtained from Janvier Labs (Le Genest Saint Isle, France) and maintained under an inverted 12 h/12 h dark/light cycle (lights off at 7:00 AM) at 22 ± 1 °C with food and water ad libitum. After an adaptation period of ten days, females in natural proestrus were allowed to copulate with a male until at least two ejaculations were displayed. From the day after mating until the end of lactation, pregnant rats were housed individually and supplemented by mouth every day with an extract of fish oil containing 77 mg/g of EPA and 521 mg/g of DHA in the form of triglycerides (Omegavie® DHA 60 TG Qualitysilver®, Polaris, Quimper, France). The DHA formulation was contained within capsules (intended for human use) made of fish gelatin, glycerol and water which were protected from heat and light. The extract of fish oil was withdrawn by puncturing the capsule with a 1 mL syringe just before oral administration. DHA and EPA were thus protected from oxidation throughout storage and administration. The purity of the formulation was confirmed by mass spectrometric

quantification of the concentration of DHA and EPA as described in Section 2.4. The amount of delivered oil was adjusted to provide pregnant rats a daily dose of 175 mg of DHA per Kg. This corresponded, in average, to 0.85% of their daily caloric intake during the gestation period and to 0.41% of their daily caloric intake during lactation. One day after delivery, litter size was adjusted to eight pups per litter maintaining a 1:1 male to female ratio. At weaning (21 days), pups were divided into two groups to be fed either standard chow (A04, SAFE, Augy, France, controls) or a free-choice high-fat high-sugar diet (fc-HFHS) in addition to standard chow and tap water (obese), as described by La Fleur et al. [29], with minor modifications. The fc-HFHS diet consisted of powdered standard chow mixed with pig fat in the proportion of 30 g of fat per 70 g of food and a 30% sugar solution prepared with sweetened condensed milk. Four experimental groups were thus constituted: offspring of saline-supplemented dams fed standard chow after weaning (SAL-STD); offspring of saline-supplemented dams exposed to fcHFHS diet (SAL-fcHFHS diet); offspring of DHA-supplemented dams fed standard chow (DHA-STD); offspring of DHA-supplemented dams exposed to the fcHFHS diet (DHA-fcHFHS diet). The detailed composition of the standard chow can be found on the website: https://safe-lab.com/safe-wAssets/docs/product-data-sheets/diets/safe_a04_ds.pdf (accessed 29 August 2021). Its energy value was 3.15 kcal/g, while that of the standard chow mixed with pork fat was 5.10 kcal/g. The caloric value of the sweetened water solution was 1.75 kcal/mL. A total of 16 dams (8 supplemented with saline and 8 supplemented with DHA) were used. Two pups were selected at random from each litter to yield a final number of 16 saline- and 16 DHA-supplemented pups. Care was taken to split the two pups of the same dam into different experimental groups (fed standard chow or exposed to fcHFHS diet), such that the presented results corresponded to the combined data from eight animals born to eight different dams. Pups were housed four per cage until they reached 300 g of body weight and two per cage thereafter.

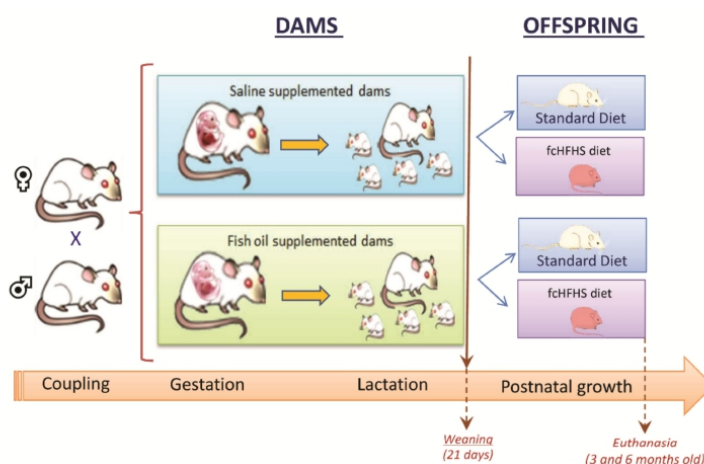


Figure 1. General scheme of the nutritional intervention.

2.2. Measurement of Body Weight and Food Intake

The weight of each dam was recorded every day throughout gestation and lactation to adjust the amount of DHA administered in relation to their body weight. Likewise, to determine the caloric contribution of DHA administration to daily energy consumption, the daily food consumption of the dams was determined by measuring the difference between the amount of pellet-food provided at the onset of their dark cycle and the amount of food remaining 24 h later. Care was taken to check through the sawdust in each cage to collect any spilled pellet of food.

2.3. Collection of Biological Samples and Euthanasia

Blood samples were collected by lateral tail vein puncture from dams on day 20 of gestation and at weaning, from supernumerary pups euthanized at the time of litter size adjustment, and from offspring at weaning and euthanasia. Sampling of the mothers took place before the oral administration of DHA. In a first series of experiments, male animals of 3 months of age were used. Thereafter, the same types of analyses were carried out in 6-month-old male and female rats. In both cases, animals were sacrificed by cervical dislocation under isoflurane anesthesia at the beginning of the dark phase of their light/dark cycle. The liver was carefully dissected, immediately frozen in liquid nitrogen and kept at -80°C until analysis. Mediastinal and abdominal (omental, perirenal, retroperitoneal, epididymal, periovarian, perivesical, and parametrial) fat depots were dissected, weighed and summed to provide a measure of body fat.

2.4. Quantification of DHA and EPA

DHA and EPA concentrations in serum and in Omegavie[®] Qualitysilver capsules were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All solvents used were LC-MS grade and were purchased from Biosolve (Valkenswaard, Netherlands). Standard compounds were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). A pool of reference standard solutions was prepared and serially diluted in ethanol to obtain seven standard solutions ranging from 0.05 to 80 $\mu\text{mol/L}$. Exogenous internal standard (2H5-DHA, 50 μL diluted at 20 $\mu\text{mol/L}$ in ethanol) was added to 100 μL of standard solutions and serum samples. Then, 850 μL of a methanol/chloroform mixture (2:1; *v:v*) were added and samples were centrifuged for 10 min at $10,000\times g$ (4°C). Supernatants were transferred to vials and dried under a gentle stream of nitrogen. Dried samples were finally resuspended with 100 μL of an isopropanol/acetonitrile/water mixture (30:65:5; *v:v:v*). LC-MS/MS analyses were performed on a Xevo[®] TQD mass spectrometer with an electrospray interface and an Acquity H-Class[®] UPLCTM device (Waters Corporation, Milford, MA, USA). Samples (5 μL) were injected onto a CSH-C18 column (1.7 μm , 2.1×100 mm, Waters Corporation) held at 60°C . Compounds were separated using a linear gradient of mobile phase B (90:10; *v:v*; isopropanol:acetonitrile containing 0.1% formic acid and 10 mmol/L ammonium acetate) in mobile phase A (60:40; *v:v*; acetonitrile:water; 10 mmol/L ammonium acetate, 0.1% formic acid) at a flow rate of 400 $\mu\text{L}/\text{min}$. Mobile phase B was linearly increased from 40% to 95% for 8 min, kept constant for 1 min, returned to the initial condition over 1 min, and then kept constant for 1.5 min before the next injection. Targeted compounds were detected by the mass spectrometer with the electrospray interface operating in the negative ion mode capillary voltage, 2.5 kV; desolvation gas (N_2) flow and temperature, 1000 L/h and 450°C ; source temperature, 150°C . The multiple reaction monitoring mode was applied for MS/MS detection with the following *m/z* transitions: EPA, 301.2 \rightarrow 257.2; DHA, 327.2 \rightarrow 283.4; and 2H5-DHA, 332.2 \rightarrow 288.3. Cone and collision energy were set at 35 V and 15 eV, respectively. Chromatographic peak area ratios between EPA or DHA and 2H5-DHA constituted the detector responses. Standard solutions were used to plot calibration curves for quantification. The linearity was expressed by the mean R^2 which was greater than 0.999 for both compounds (linear regression, $1/\times$ weighting, origin excluded).

2.5. Serum Metabolite Determinations

Animals were submitted one week before sacrifice to an oral glucose tolerance test after a fast of 8 h initiated at the beginning of the dark phase of their light/dark cycle. A drop of blood was taken immediately before administration of glucose and 20, 40, 80, and 120 min after by severing the tip of the tail. Blood glucose was determined with a blood glucose monitor (Accu-Check[®] Active, Roche Diagnostics, Vercors, Meylan, France). To determine the glucose disappearance rate (kG), the area under the curve (AUC) was calculated using the value before the administration of glucose as base line. Serum from blood collected at the time of sacrifice was assayed for insulin and leptin using assay kits

from Millipore (Billerica, MA, USA). Enzymatic diagnostic kits from BioMérieux (Craponne, France), were used to quantify the serum levels of triglycerides, glucose and cholesterol.

2.6. Quantification of Liver Triglycerides and Cholesterol

200 mg of liver were homogenized in 4 mL of a chloroform/methanol (2:1) solution. After centrifugation of the extract at 4 °C and 2500× g during 15 min, the supernatant was collected and evaporated to dryness. The residue was subsequently reconstituted in 300 µL of a solution of isopropyl alcohol containing 10% Triton X and centrifuged at 5000× g for 15 min. The resulting supernatant was used for the determination of triglycerides and cholesterol content using enzymatic diagnostic kits from BioMérieux (Craponne, France).

2.7. Real-Time Quantitative RT-PCR Experiments

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with DNase/RNase-free for 30 min at 37 °C. Afterwards, 1.5 µg of purified RNA was reversed transcribed using Superscript II RNaseH-Reverse-Transcriptase (Invitrogen) in a total volume of 20 µL, and the resulting cDNA was diluted 40-fold in DNase and RNase free water. Thereafter, 5 µL of each cDNA diluted sample was used as template for PCR amplification with 18S or β2-microglobulin as housekeeping genes and SYBR Green (Biorad, France) as fluorogenic intercalating dye. PCR was performed with the iCycler iQ detection system (BioRad Laboratories, Hercules, CA, USA), using the following parameters: an initial denaturation step of 5 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, and 30 s at 60 °C. Primers used for the amplifications are presented in Table 1.

Table 1. Sequences of primers used for real time RT-PCR analyses.

Gene Name	Symbol	Forward Primer	Reverse Primer
Carbohydrate response element binding protein	ChREBP	GTACTGTCCCTGCCTGCTCTC	CCCTCTGTGACTGCCCTTGTG
Fatty acid synthase	FASN	CGCCGTGGTGCTGGAGATTG	CTTGCCGAGGTTGGTGAGGAAG
Stearoyl-coenzyme A desaturase	SCD	GGAGCCACAGGACTTACAAGG	CGCACAAGCAGCCAACCC
Glycerol-3-phosphate acyltransferase, mitochondrial	GPAM	TGGAGTGTGGCAAGAGCGTTATC	TTCGGCAGCAGCAGCATCAGG
Diacylglycerol O-acyltransferase 2	DGAT2	CCTCATCGCTGCCTACTCC	TGAGCCAGGTGACAGAGAAG
Peroxisome proliferator-activated receptor delta	PPARγ	GGAATTAGATGACAGTACTTGGC	GGAGCACCTTGGCGAACAG
Carnitine palmitoyltransferase 1A	CPT1a	TGCCTGCCAGTTCCATTAAGC	GTCTCACTCCTTGGCCAACAG
Peroxisome proliferator-activated receptor gamma, co-activator 1alpha	PGC-1α	ACACCGCACACATCGCAATTC	TTCGTCCTCTTGGAGCCTTTCG
Medium chain acyl coenzyme A dehydrogenase	MCAD	GAGGCTAGAAGGTCTGAGAAGTG	TCTGCTGCTCCGTCAACTGG
Hydroxyacyl-Coenzyme A dehydrogenase	HADH	CTCCATGTCTCTCTTCTCTCTGC	CAGCCC GCCCGGATGAC
18S ribosomal RNA	18S	GATGCGGCGGCGTTAATCC	CTCCTGGTGGTGCCCTTCC
Beta-2 microglobulin	β2M	GATGGCTCGCTCGGTGAC	CGTAGCAGTTGAGGAAGTTGG

2.8. Data Analysis

The relative expression levels of the mRNAs in the different samples were calculated using the 2ΔΔCT method. GraphPad Prism 9 Software (GraphPad, Inc., San Diego, CA, USA) was used to perform all statistical analyses. Data were first checked for normality using the Shapiro–Wilk normality test. Statistical differences between obese and control offspring born to saline-supplemented dams were evaluated by unpaired Student’s *t*-test. The differences in the effects of maternal DHA supplementation, or between males and females, were determined by two-way ANOVA followed by Sidak’s multiple comparison test using maternal supplementation (saline or DHA), postnatal diet (chow or FcHFHS

diet), or sex as factors. Experimental results were expressed as mean \pm S.E.M. Statistical significance was set at $p < 0.05$.

3. Results

3.1. DHA Supplementation Does Not Affect Maternal Body Weight Gain or Food Intake during Pregnancy nor the Offspring's Growth Profile

Supplementation with fish oil extract increased the maternal blood levels of DHA in relation to saline-supplemented dams from 30 μM to 55 μM at the end of the gestation period and from 16 μM to 26 μM at weaning (Figure 2A,B). Serum EPA levels increased only during the gestation period, from 0.71 μM to 1.56 μM (Figure 2A). DHA-supplemented dams showed the same pattern of weight gain and food intake during gestation and lactation as their saline-supplemented counterparts (Figure 2C,D).

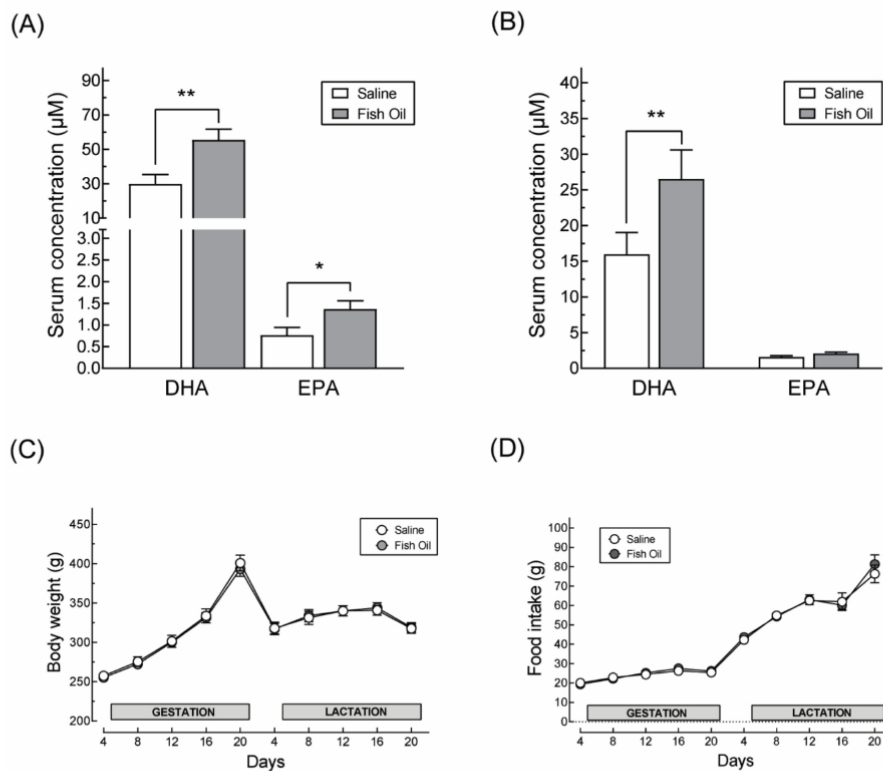


Figure 2. Effects of fish oil extract supplementation on maternal serum DHA and EPA at gestation day 20 (A) and at weaning (B). Note that the increased circulating levels of DHA and EPA has no impact on the body weight (C) and food consumption pattern (D) of the dams. * $p < 0.05$; ** $p < 0.01$ as determined by Student's *t*-test.

It is well established that the concentrations of fatty acids, including DHA, increase during pregnancy independently of the diet and decrease postpartum in both humans [30,31] and rats [32,33]. Moreover, although the levels of DHA in the offspring after birth are determined by the DHA supplied by the mother via the breast milk, this provision ceases between the 16th and 17th day of life when the offspring start to ingest solid food and, as a result, their diet becomes independent of that of their mother. In agreement with these observations, the offspring of mothers supplemented with fish oil extract showed an increase in the serum concentration of DHA and EPA only at birth (Figure 3A,B). Otherwise,

their growth profile was identical to that of the animals from saline-supplemented dams (Figure 3C).

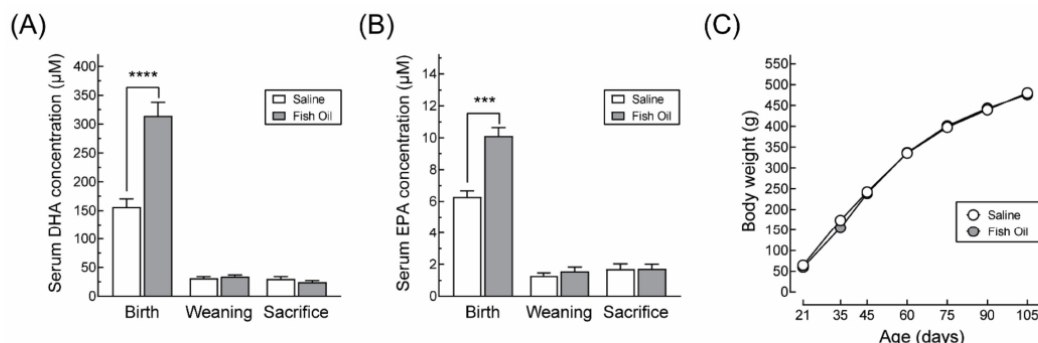


Figure 3. DHA (A) and EPA (B) levels in serum from offspring born to dams supplemented during gestation and lactation with saline or with a fish oil extract. At birth, offspring from supplemented dams exhibited increased circulating levels of DHA and EPA but showed no differences in body weight gain during lactation (C). *** $p < 0.001$; **** $p < 0.0001$ (Student’s *t*-test).

3.2. Impact of Maternal DHA Supplementation on Male Offspring at Three Months

3.2.1. Anthropometric Characteristics and Serum Metabolite Profile

To assess the potential protective effects of maternal DHA supplementation against obesity-induced metabolic alterations in the offspring, the progeny from saline- or DHA-supplemented dams were subdivided into two groups at weaning to be fed standard chow or to be exposed to a diet allowing them to freely choose between standard chow or food high in sugar and fat (FchFHFS Diet). This experimental protocol mimics the conditions of free choice feeding in humans and results in hyperglycemia, hyperinsulinemia and glucose intolerance [31]. 3-month-old male rats exposed to the FchFHFS diet gained more body weight and accumulated more body fat than rats fed standard chow, regardless of whether their mothers were supplemented with saline or DHA (Table 2). Similarly, animals born to dams supplemented with DHA or with saline who were exposed to the FchSHF diet exhibited elevated circulating levels of leptin and triglycerides compared to animals fed standard chow (Table 2). These results indicated that maternal DHA supplementation had no protective effects on the increased body weight, fat accretion and hyperlipidemia associated with obesity. In contrast, circulating insulin levels and glucose intolerance were reduced in obese animals born to DHA-supplemented dams in comparison to the SAL-fchFHFS diet group (Figure 4A,B). Otherwise, no differences in blood glucose and cholesterol levels were observed between control and obese animals.

Table 2. Anthropometric and metabolic characteristics of 3-month-old male rats born to saline or DHA-supplemented dams.

	Supplemented Saline		Supplemented DHA	
	Standard Diet	FchFHFS Diet	Standard Diet	FchFHFS Diet
Body weight (g)	510 ± 12	600 ± 16 ****	477 ± 10	599 ± 15 ****
Adipose Index	4.40 ± 0.20	7.36 ± 0.31 ****	4.76 ± 0.23	6.66 ± 0.32 ***
Leptin (ng/mL)	12.43 ± 0.83	33.10 ± 4.31 ****	13.72 ± 2.29	34.58 ± 5.44 **
Triglycerides (mg/dL)	95.48 ± 10.45	157.49 ± 15.70 **	91.27 ± 5.11	171.58 ± 28.29 *
Cholesterol (mg/dL)	67.54 ± 3.54	77.24 ± 2.96	64.72 ± 4.64	72.38 ± 4.32
Glucose (mg/dL)	189.54 ± 5.43	206.66 ± 6.27	197.20 ± 8.26	204.16 ± 9.50

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to animals fed standard diet of the same experimental group (saline or DHA supplemented). All statistical comparisons were done by two-way ANOVA using maternal supplementation and postnatal diet as factors followed by Šidák’s multiple comparisons test.

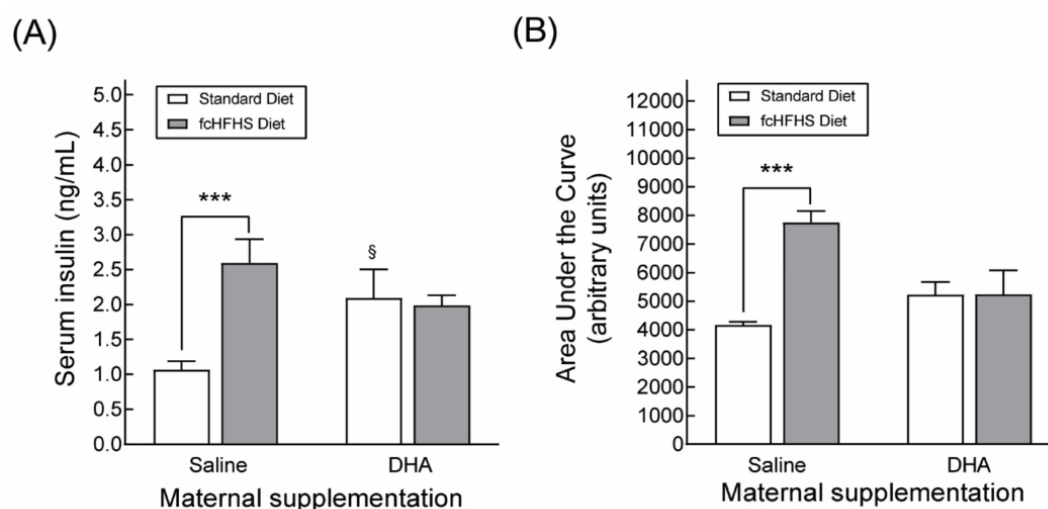


Figure 4. DHA maternal supplementation during gestation and lactation protects the offspring against insulinemia (A) and glucose intolerance (B) induced by the consumption of a high-calorie diet. Data correspond to the serum levels of insulin under ad libitum feeding conditions. The areas under the curve derive from an oral glucose tolerance test performed in 3-month-old male rats after a fasting period of 8 h. *** $p < 0.001$ (Student's *t*-test); § $p < 0.05$ compared to rats fed standard chow born to saline-supplemented dams as determined by two-way ANOVA using maternal supplementation and postnatal diet as factors.

3.2.2. Hepatic Lipid Accumulation

It has been shown that DHA supplementation in parallel with high-calorie feeding protects against the development of obesity-associated liver steatosis [34,35]. Therefore, in order to further investigate the beneficial effects of maternal DHA supplementation, we assessed the accumulation of triglycerides and cholesterol in liver samples from our four experimental groups. Rats on the FcHFHS diet born to saline-supplemented dams displayed a higher concentration of triglycerides in the liver compared to the SAL-STD group, but there were no differences in hepatic triglycerides' concentration between animals of the DHA-STD and DHA-fCHFHS diet groups (Figure 5A). In addition, obese animals born to saline-supplemented dams showed increased concentrations of cholesterol in the liver compared to rats of the SAL-STD diet group (Figure 5B). However, no significant differences in hepatic cholesterol were observed between obese and non-obese animals born to DHA-supplemented dams.

3.2.3. Hepatic Gene Expression

In order to determine the mechanisms underpinning the beneficial effects of DHA supplementation, the hepatic expression levels of genes involved in glucose and lipid metabolism were examined. The results of these analyses showed that the protective effects of maternal DHA supplementation against hepatic steatosis were associated with decreased expression of mRNAs encoding for genes that positively regulate de novo lipogenesis and triglyceride synthesis (Figure 6A). Similarly, hepatic expression of genes promoting β -oxidation was downregulated by maternal DHA supplementation (Figure 6B).

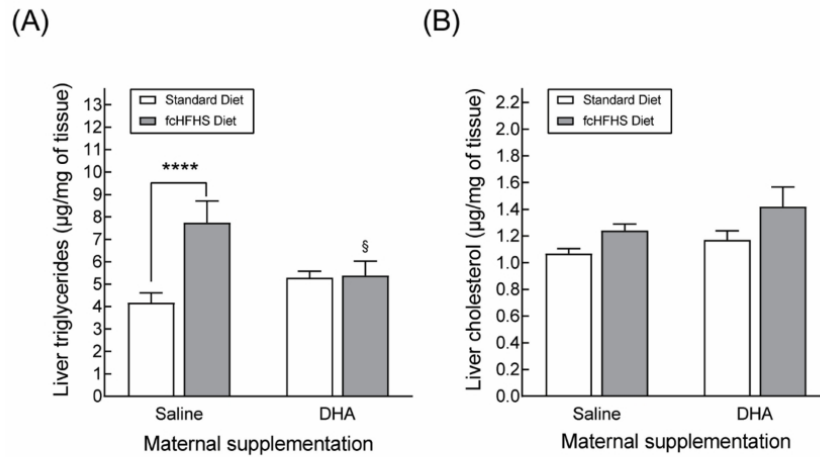


Figure 5. Protective effects of maternal DHA supplementation against obesity-induced hepatic steatosis in the offspring. Data correspond to the concentrations of triglycerides (A) and cholesterol (B) determined in liver samples from 3-month-old male rats born to saline- or DHA-supplemented dams. The offspring were fed standard chow or rendered obese by exposure to a fchFHFS diet from weaning. **** $p < 0.0001$ (Student's t -test); § $p < 0.05$ compared to rats born to saline-supplemented dams exposed to the fchFHFS diet after weaning (two-way ANOVA using maternal supplementation and postnatal diet as factors).

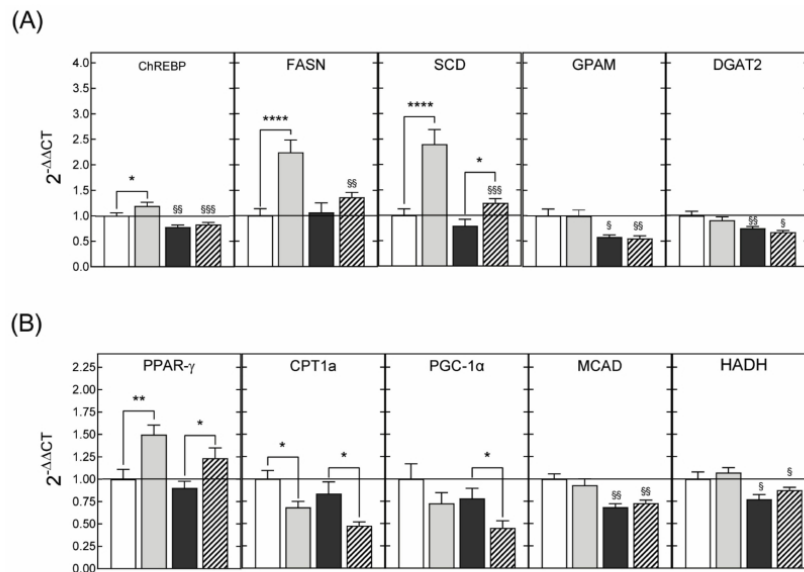


Figure 6. Expression levels of mRNAs encoding proteins involved in de novo lipogenesis (A) or fatty acid oxidation (B) in liver samples from 3-month-old male rats born to dams supplemented with saline or with DHA during gestation and lactation. The offspring were fed standard chow or rendered obese by exposure to a fchFHFS diet from weaning. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression in animals of the SAL-STD group (white bars) as a calibrator. Grey bars, SAL-fchFHFS group; black bars, DHA-STD group; stripped bars, DHA-fchFHFS diet group. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ (Student's t -test); § $p < 0.05$ §§ $p < 0.01$; §§§ $p < 0.001$ compared to animals born to saline supplemented dams that were exposed to the same diet (standard or fchFHFS diet), after weaning (two-way ANOVA with maternal supplementation and postnatal diet as factors).

3.3. Impact of Maternal DHA Supplementation on the Offspring at Six Months

The deleterious effects of metabolic programming often worsen with age and might be sex-dependent. With the aim of determining the persistence of the protective effects of DHA and potential sex differences in response to maternal DHA supplementation, a new series of experiments was conducted in which 6-month-old male and female animals born to saline- or DHA-supplemented dams were examined.

3.3.1. Anthropometric Characteristics and Serum Metabolite Profile

As expected, 6-month-old male animals exposed to the fcHFHS diet since weaning, showed an aggravation of their metabolic state compared to 3-month-old male animals exposed to the same diet. This was reflected in greater increases in body weight (+24%), body fat (+9%) and liver triglycerides (+316%) and cholesterol (+299%) in response to the fcHFHS diet (Table 3). Female rats exposed to the fcHFHS diet also showed significant metabolic alterations, some of which exceeded those observed in obese male rats in severity. This was particularly notable with respect to serum triglyceride levels (Table 3).

Table 3. Anthropometric and metabolic characteristics of 6-month-old male and female rats born to saline- or DHA-supplemented dams.

MALES				
	Supplemented Saline		Supplemented DHA	
	Standard Diet	fcHFHS Diet	Standard Diet	FcHFHS Diet
Body weight (g)	633 ± 19	754 ± 24 ***	630 ± 20	770 ± 24 ***
Adipose Index	6.51 ± 0.43	9.79 ± 0.37 ****	7.05 ± 0.11	10.72 ± 0.85 ***
Leptin (ng/mL)	14.99 ± 1.76	26.04 ± 1.91 **	15.69 ± 1.37	30.27 ± 3.55 ***
Triglycerides (mg/dL)	101.44 ± 10.96	151.09 ± 20.08 *	65.61 ± 7.49	119.45 ± 18.16 *
Cholesterol (mg/dL)	73.62 ± 5.54	74.83 ± 2.90	79.80 ± 6.11	72.94 ± 2.85
Glucose (mg/dL)	155.24 ± 9.04	177.42 ± 22.61	175.40 ± 27.71	209.04 ± 14.87
Insulin (ng/mL)	1.43 ± 0.18	2.99 ± 0.67 *	1.26 ± 0.22	4.08 ± 0.92 **
FEMALES				
	Supplemented Saline		Supplemented DHA	
	Standard Diet	fcHFHS Diet	Standard Diet	FcHFHS Diet
Body weight (g)	349 ± 80	451 ± 19 ****	357 ± 11	407 ± 26 ****
Adipose Index	7.33 ± 0.56	22.70 ± 0.42 ****	7.67 ± 0.42	10.00 ± 1.59 ***
Leptin (ng/mL)	6.53 ± 0.74	22.70 ± 3.04 ***	6.81 ± 1.05	16.13 ± 3.10 *
Triglycerides (mg/dL)	125.17 ± 13.27	339.99 ± 98.61 *	140.59 ± 20.99	285.48 ± 36.99 *
Cholesterol (mg/dL)	61.13 ± 3.91	85.11 ± 5.24	84.13 ± 4.10 §§	96.36 ± 3.83
Glucose (mg/dL)	171.65 ± 4.17	182.70 ± 10.07	183.89 ± 11.55	182.30 ± 13.42
Insulin (ng/mL)	0.93 ± 0.18	3.40 ± 0.76 **	1.45 ± 0.25	2.50 ± 0.57

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to animals fed standard diet of the same sex and the same experimental group (supplemented saline or DHA, Student's *t*-test). §§ $p < 0.01$ compared to the supplemented saline standard diet group (two-way ANOVA using maternal supplementation and postnatal diet as factors followed by Šídák's multiple comparisons test). Data correspond to six animals per group.

While male animals from both DHA-supplemented and non-supplemented dams showed glucose intolerance (Figure 7A,C), only obese female rats from saline-supplemented dams displayed impaired glucose tolerance (Figure 7B,D). In other words, maternal DHA supplementation protected female—but not male—offspring against high-calorie diet-induced glucose intolerance.

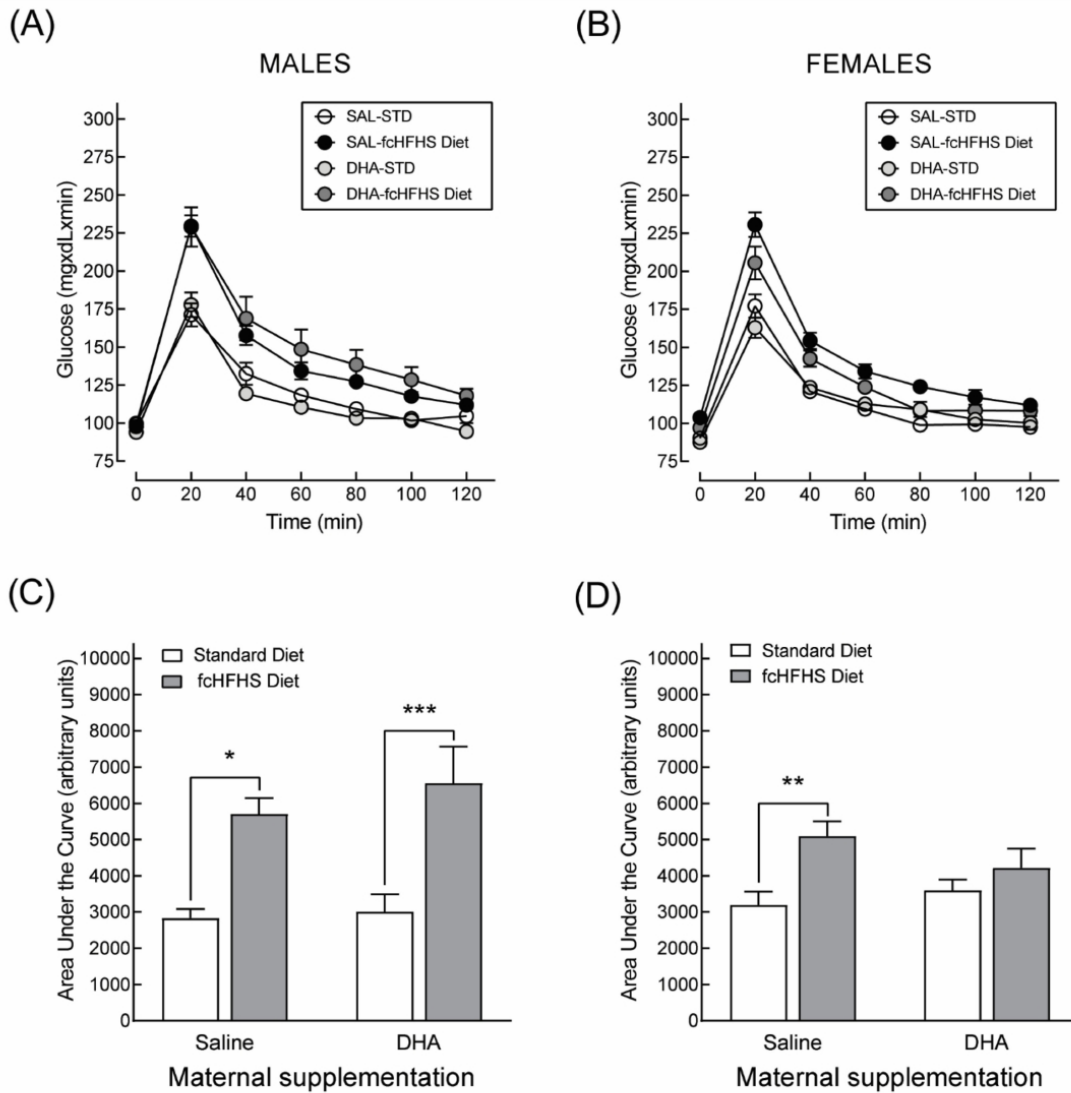


Figure 7. Long-term protective effects of maternal supplementation with DHA during gestation and lactation against high-calorie diet induced glucose intolerance. Data correspond to the curves (A,B) and the areas under the curves (C,D) derived from an oral glucose tolerance test performed in 6-month-old male and female rats born to saline or DHA supplemented dams. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; (Student's *t*-test).

3.3.2. Hepatic Liver Accumulation

Maternal DHA supplementation also protected both male and female 6-month-old animals from the accumulation of hepatic triglycerides induced by the consumption of the fcHFHS diet (Figure 8A,B). Nevertheless, sex differences were observed in liver cholesterol, both in response to maternal DHA supplementation and in response to the fcHFHS diet. Namely, obese male animals showed an increase in hepatic cholesterol levels that was partially ameliorated by maternal DHA supplementation (Figure 8C).

In contrast, control and obese female rats born to saline-supplemented dams exhibited the same accumulation of cholesterol (Figure 8D). Surprisingly, obese female rats from DHA-supplemented dams showed a greater concentration of hepatic cholesterol than their non-obese, DHA-supplemented counterparts. However, these levels were still lower than those of female rats from the SAL-STD and SAL-fcHFHS diet groups (Figure 8D).

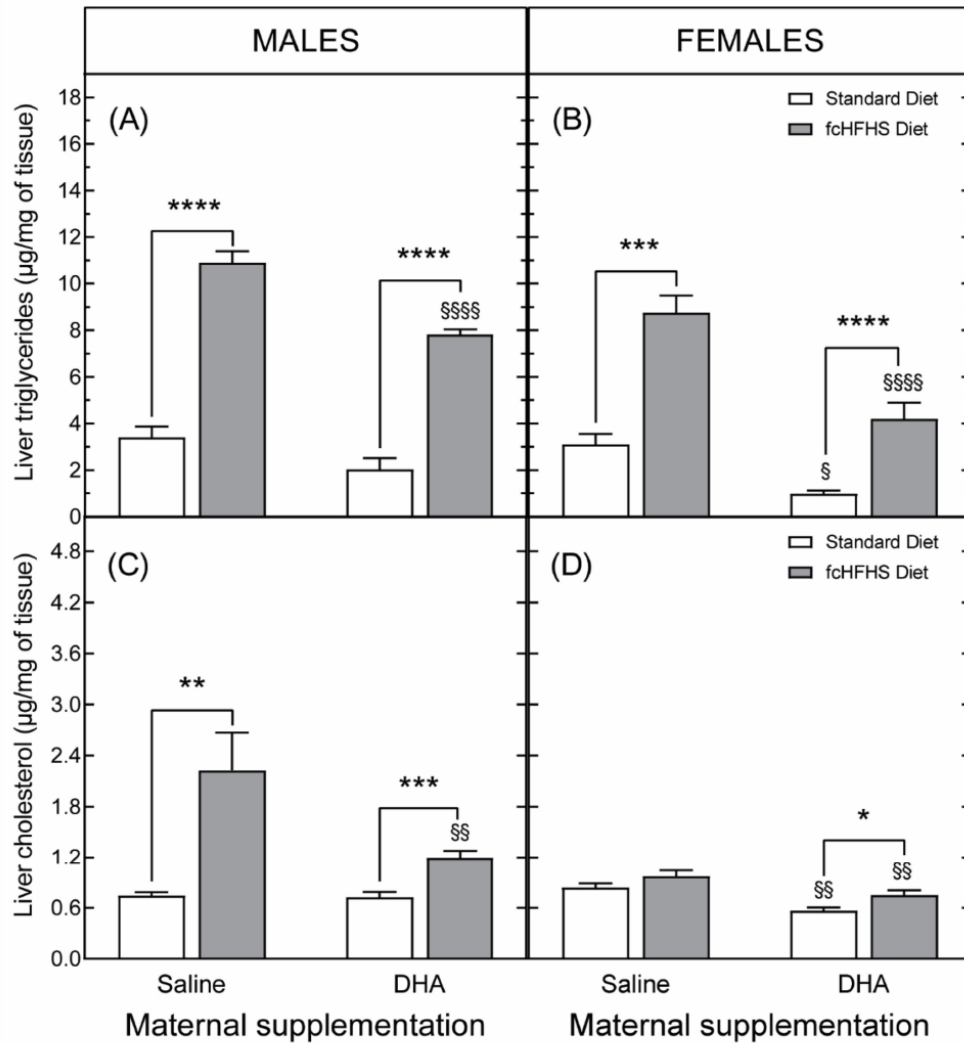


Figure 8. Long-term protective effects of DHA maternal supplementation during gestation and lactation against hepatic steatosis in the offspring. Data correspond to the concentrations of triglycerides (A,B) and cholesterol (C,D), determined in livers from 6-month-old male and female rats born to saline- or DHA-supplemented dams. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Student's *t*-test); § $p < 0.05$; §§ $p < 0.01$; §§§§ $p < 0.0001$ compared to rats born to saline-supplemented dams exposed to the same diet (standard chow or fcHFHS diet) after weaning (two-way ANOVA).

3.3.3. Hepatic Gene Expression

Finally, in agreement with the results obtained in 3-month-old animals, there was a reduction in the hepatic expression levels of genes regulating beta oxidation in both males (Figure 9) and females (Figure 10).

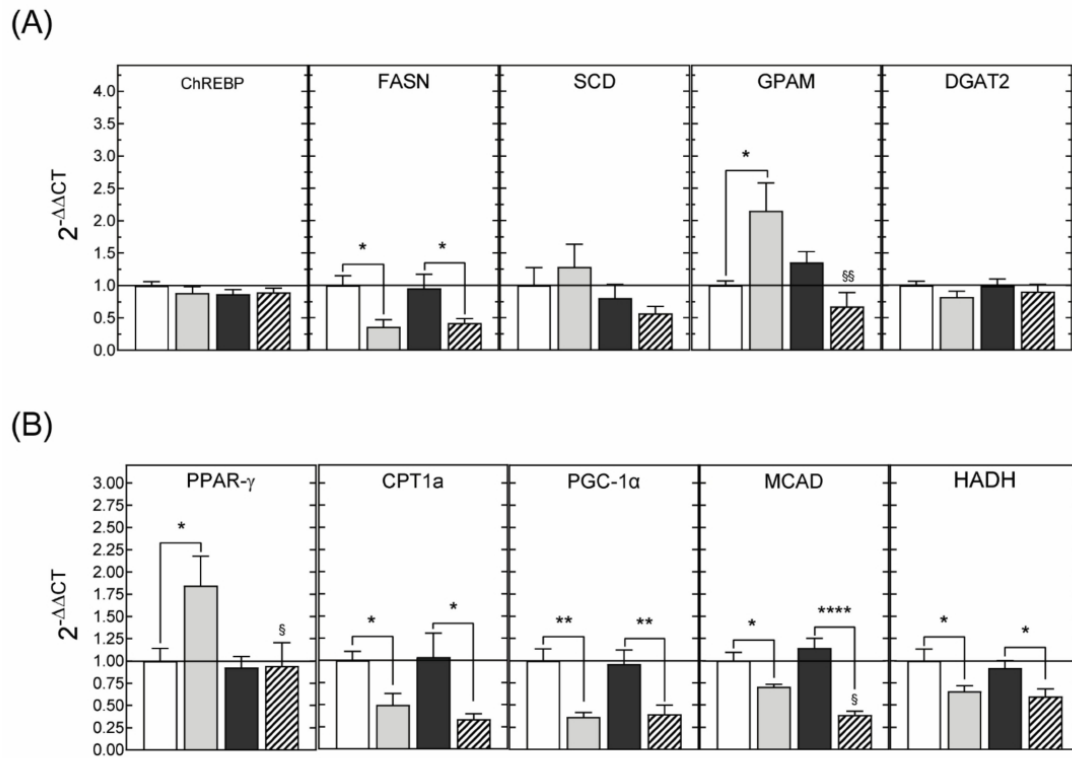


Figure 9. Expression levels of mRNAs encoding proteins involved in de novo lipogenesis (A) or fatty acid oxidation (B) in liver samples from 6-month-old male rats born to dams supplemented with saline or with DHA during gestation and lactation. The offspring were fed standard chow or rendered obese by exposure to a fCHFHS diet from weaning. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression in animals of the SAL-STD group (white bars) as a calibrator. Grey bars, SAL-fCHFHS group; black bars, DHA-STD group; stripped bars, DHA-fCHFHS diet group. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ (Student's *t*-test); § $p < 0.05$ §§ $p < 0.01$ compared to animals born to saline supplemented dams that were exposed to the same diet (standard or fCHFHS diet), after weaning (two-way ANOVA with maternal supplementation and postnatal diet as factors).

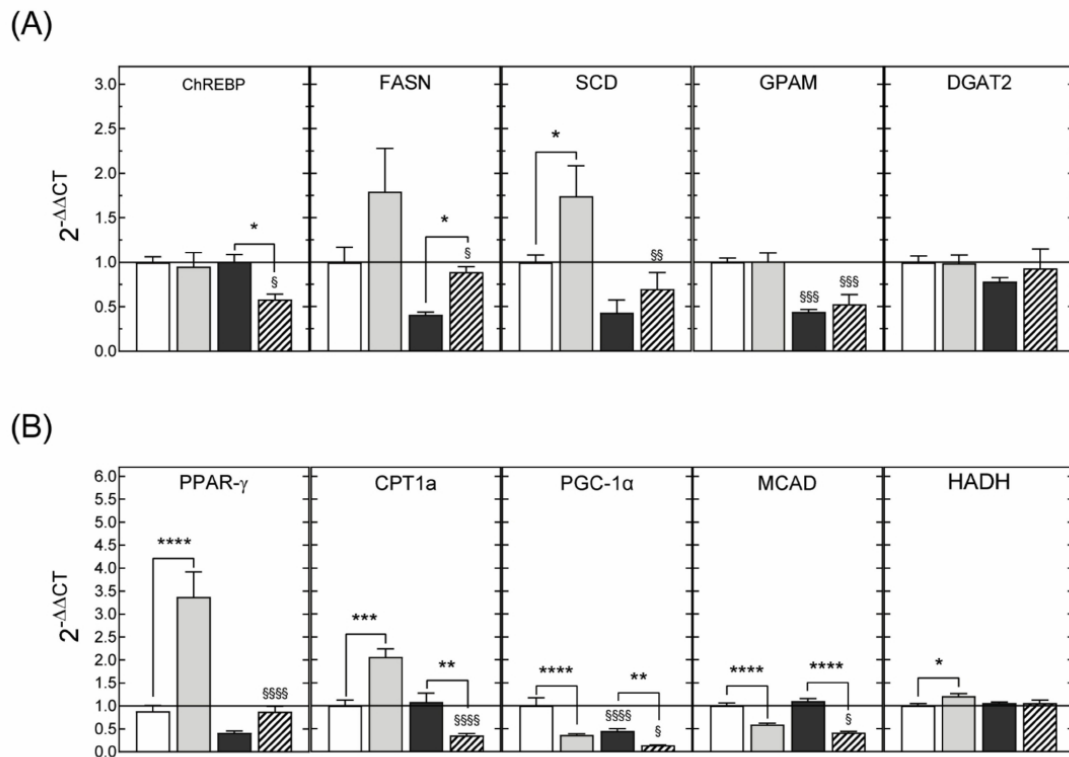


Figure 10. Expression levels of mRNAs encoding proteins involved in de novo lipogenesis (A) or fatty acid oxidation (B) in liver samples from 6-month-old female rats born to dams supplemented with saline or with DHA during gestation and lactation. The offspring were fed standard chow or rendered obese by exposure to a fcHFHS diet from weaning. Variations in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method using the expression in animals of the SAL-STD group (white bars) as a calibrator. Grey bars, SAL-fcHFHS group; black bars, DHA-STD group; stripped bars, DHA-fcHFHS diet group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Student's *t*-test); ^s $p < 0.05$ ^{ss} $p < 0.01$; ^{sss} $p < 0.001$; ^{sssss} $p < 0.0001$ compared to animals born to saline supplemented dams that were exposed to the same diet (standard or fcHFHS diet), after weaning (two-way ANOVA with maternal supplementation and postnatal diet as factors).

4. Discussion

The prevalence of obesity is increasing throughout the world, making this disorder a major public health concern. Although obesity is eventually the result of an imbalance between energy consumption and energy expenditure, numerous epidemiological and experimental studies have shown that the nutritional environment during gestation and during the very first stages of postnatal life plays a key role in determining susceptibility to obesity, as well as to developing metabolic pathologies in adulthood. The aim of this study was to investigate to what extent maternal DHA supplementation during gestation and lactation in the rat protects the offspring against metabolic disorders induced by a high-calorie diet. We focused on DHA because supplementation with this polyunsaturated fatty acid is a common clinical practice used for pregnant women [10,12,13]. Furthermore, it has been shown that the consumption of polyunsaturated fatty acids (PUFAs) reduces body weight [17,18], decreases blood triglyceride levels [36,37], improves carbohydrate homeostasis [38,39], and limits increases in body fat and the development of hepatic steatosis when consuming a high fat diet [20,21]. Based on these observations, we hypothesized

that maternal DHA supplementation could positively program the metabolism of the offspring, allowing them to better cope with an obesogenic diet.

Evaluation of the anthropometric characteristics and blood metabolic profile of animals exposed to the fCHFHSD diet from weaning showed that maternal DHA supplementation did not reduce weight gain, fat mass accumulation or the increase in circulating triglyceride levels induced by consumption of a hypercaloric diet. In contrast, obese rats from DHA-supplemented dams showed lower levels of triglycerides and cholesterol in the liver and better glucose tolerance than their saline-supplemented counterparts. These results show that maternal DHA supplementation induces physiological changes in the offspring that are protective against the development of hepatic steatosis and glucose intolerance induced by high-calorie feeding.

Our observations on the lack of effect of maternal DHA supplementation on body weight, body fat, and blood triglycerides in the offspring were consistent with the results of previous studies in humans [40–42] and rats [43] showing that the intake of omega-3 PUFAs during the perinatal period had no influence on these parameters. It should be noted, however, that some human studies have documented an inverse relationship between maternal circulating levels of n-3 PUFAs and both body and abdominal fat mass in the offspring [44,45]. These divergent results may be explained in part by differences in the methods of n-3 long chain (LC)-PUFAs supplementation, variability in the techniques used to document body measurements, and the age at which these measurements were made. Additionally, the existence of genetic polymorphisms in the fatty acid desaturase (FADS) gene that catalyze the biosynthesis of unsaturated fatty acids (HUFAs) from PUFAs could be a factor [46].

Animals of the SAL-fCHFHSD diet group, had a clear obese phenotype and high serum insulin levels but displayed identical blood glucose concentrations to those of the SAL-STD group both under ad libitum feeding conditions and after fasting. A lack of correlation between circulating glucose levels and the increase in body fat induced by the consumption of a high-fat or high-sugar diet has been reported by other authors [47,48]. It has been suggested that this dissociation could be explained by differences in the composition of the diet or by the level of propagation of insulin resistance, i.e., only in the liver or in the liver and at the peripheral level (skeletal muscle). Alternatively, in our experiments, the obesity-associated hyperglycemia could be masked by circadian variations in blood glucose levels that are at their peak during the early hours of the dark cycle [49], i.e., the time at which the animals were sacrificed. Additional studies are needed to determine which of these two mechanisms underpin our results.

The protective effects of maternal DHA supplementation against glucose intolerance in obese animals was accompanied by a decrease in serum insulin levels, suggesting that maternal DHA supplementation might protect the offspring from developing glucose intolerance by increasing insulin sensitivity. It is also worth noting that non-obese animals from DHA-supplemented dams displayed elevated insulin levels compared to non-obese animals from saline-supplemented dams. The enhanced release of insulin under acute exposure to DHA is well documented [50,51], and is generally accepted as the principal mechanism of the beneficial effects of DHA on insulin resistance. Our results therefore indicated that the protective effects of maternal DHA supplementation against the fCHFHSD diet-induced glucose intolerance might also be underpinned by a DHA insulinotropic action.

Feeding a high-calorie diet leads to the development of hepatic steatosis. The accumulation of lipids, mainly in the form of triglycerides, which characterizes this pathological condition, is the consequence of the alteration of one or more liver metabolic processes including excessive lipid entry from the diet, increased de novo fatty acid synthesis, impaired lipid export into the bloodstream, and reduced metabolism through β -oxidation [52,53]. It is generally agreed that the protective effects of the consumption of DHA against the development of hepatic steatosis result mainly from the inhibition of lipogenesis combined with an increase in β -oxidation [25,54,55]. To determine whether the reduction of hepatic steatosis in animals of the DHA-fCHFHSD diet group could be explained by the same mecha-

nisms, we assessed the hepatic expression of several genes involved in the regulation of these metabolic pathways using real time PCR. The results of these experiments showed that maternal DHA supplementation blocked the fCHFHs diet-induced increase in the expression of lipogenesis-promoting genes and reduced the levels of mRNAs-encoding genes that positively regulate triglyceride synthesis in both obese and non-obese 3-month-old animals. Therefore, the same mechanisms, operating against hepatic steatosis after the dietary consumption of DHA, seem to be activated in adult offspring rats born to DHA-supplemented dams, despite the fact that they displayed identical circulating DHA levels to animals from non-supplemented dams.

However, in contrast to reports in the literature indicating that omega-3 fatty acids promote lipid oxidation [25,55], the RT-PCR analyses that we carried out showed that maternal DHA supplementation resulted in reduced hepatic expression of genes involved in fatty acid oxidation. Several explanations can be put forward to account for these paradoxical observations. First, conflicting results exist about the impact of hepatic steatosis on the expression of genes regulating β -oxidation. For example, a decrease in CPT1a expression, coupled with enhanced long-chain acyl-CoA dehydrogenase (LCAD) and HADH mRNAs levels, was detected in liver biopsies from patients with non-alcoholic fatty liver disease [56]. Inhibition of CPT1a expression is indicative of decreased β -oxidation, whereas the increased expression of LCAD and HADH suggested that lipid oxidation in these patients was enhanced. These contradictory results were consistent with other studies showing enhanced [57,58], decreased [59], or unchanged [60] fatty acid oxidation in the liver of patients with hepatic steatosis. Second, whether increased lipid oxidation in hepatic steatosis has beneficial effects at all has been challenged [61,62]. In fact, fatty liver has been associated with impaired mitochondrial activity, such that enhanced β -oxidation can lead to excessive production of reactive oxygen species (ROS) and the activation of fatty acid oxidation in cytochromes and peroxisomes and, therefore, to hepatic damage by oxidative stress and inflammation. Third, a comparative study of the β -oxidation capacity of DHA and EPA in the liver showed that EPA is much more effective in increasing mitochondrial fatty acid oxidation than DHA [63]. Since the fish oil-extract we used contains 7 times less EPA than DHA, our results could be explained by the low proportion of EPA in relation to DHA. Fourth, fatty acids derived from triglyceride lipolysis are converted to acetyl-CoA whose transformation by carboxylation to malonyl-CoA is the committed step in the *de novo* fatty acid synthesis. Yet, rats from DHA-supplemented dams also exhibited a reduced expression of the GPAM and DGAT2 genes whose enzymatic products catalyze triglyceride synthesis. Therefore, low levels of expression of genes regulating fatty acid oxidation could be a consequence of a reduction in the amount of substrate required to initiate β -oxidation. Finally, our results are consistent with other studies showing that EPA and DHA reduced HFD-induced hepatic steatosis without affecting the expression of genes involved in β -oxidation [64].

The deleterious effects of obesity increase with age [65,66] and differ by gender [67–69]. With the aim of determining the impact of these parameters on the protective effect of maternal DHA supplementation, in a second series of experiments, the metabolic characteristics of six-month-old male and female animals born to DHA-supplemented dams and subjected to the fCHFHs diet were analyzed. The results of these studies showed that the protective effects of maternal DHA supplementation against hepatic steatosis were common to both sexes. However, only female rats from DHA-supplemented dams were protected against the development of glucose intolerance associated with the exposure to the fCHFHs diet. This latter result could be interpreted as being evidence of sexual dimorphism in the effects of DHA, as reported in previous studies [70,71]. However, females are more insulin sensitive and glucose tolerant than males [72,73]. Thus, the lack of glucose intolerance in female rats in the DHA-fCHFHs diet group may also be linked to intrinsic metabolic differences between males and females. In support of this interpretation, exposure to the fCHFHs diet did not result in increased hepatic cholesterol levels in female rats from saline-supplemented dams, whereas in male animals exposed to the same

nutritional manipulations, hepatic cholesterol concentrations were significantly increased. Regarding the impact of aging, the results obtained in male animals indicated that the protective effects of maternal DHA supplementation against hepatic steatosis persisted with age, despite the greater accumulation of triglycerides and cholesterol in the liver in 6-month-old obese animals compared to 3-month-old rats of the same experimental group.

Notwithstanding the numerous human studies involving maternal DHA supplementation during pregnancy, very few animal studies have sought to determine the biochemical and molecular mechanisms underlying the putative beneficial effects of this nutritional intervention—particularly with regard to the metabolism of the offspring. To our knowledge, there have only been two studies in which the metabolic phenotype of offspring exposed in utero to DHA was investigated. In these studies, carried out in hamsters, it was shown that maternal consumption of an EPA/DHA-enriched diet during gestation and lactation protected the offspring against increases in plasma glucose levels, hepatic triglyceride secretion and lipemia induced by feeding a high-fat diet via the enhancement of tricarboxylic acid cycle efficiency [74,75]. However, in these studies, DHA was provided via the diet, in contrast to most clinical nutritional interventions, in which DHA is provided orally in well-defined amounts. The results presented herein thus extend and deepen the limited knowledge on the consequences of maternal DHA supplementation on the metabolism of the offspring by showing, on the one hand, the protective effects of this nutritional intervention against the development of glucose intolerance and hepatic steatosis associated with obesity and, on the other hand, the existence of sex-related differences in the effects of early life exposure to DHA.

It is remarkable that the inhibition of lipogenesis underlying the protective effects of DHA against fatty liver in obese individuals—as described in intervention studies in human and rodents [25,76,77]—also acts to prevent the development of high-calorie diet-induced hepatic steatosis when DHA supplementation is provided during development via the mother as shown in the present study. This lends our model and results a translational research value and raises the question of the mechanisms by which temporary exposure to DHA during embryogenesis and neonatal life downregulates the expression of lipogenic genes in the long term. The establishment of a particular epigenetic profile following the in utero/neonatal exposure to DHA could be one of these mechanisms. Actually, maternal DHA supplementation during the last trimester of pregnancy has been shown to increase methylation levels at the differentially methylated region (DMR) of insulin growth factor 2 (IGF2) in the offspring's cord blood mononuclear cells [78]. Likewise, lower methylation levels of differentially methylated regions located in genes involved in the regulation of lipid exchange, plasma membrane function, food intake, immune function, and neurodevelopment were detected in blood spots collected at birth from babies born to mothers supplemented with DHA from 20 weeks of gestation to delivery [79]. Interestingly, some of these changes were sex-dependent and persisted until 5 years of age. However, other studies failed to detect differences in DNA methylation levels in CD4⁺ T-cells isolated from the cord blood of babies born to mothers that received DHA from 20 weeks of gestation until delivery [80]. In rodents, maternal DHA supplementation has been shown to restore intrauterine growth restriction-induced decreased levels of histone H4K20 methylation at the PPAR γ promoter gene in the lung to normal values [81]. As an initial approach to determine if the protective effects of maternal DHA supplementation against the development of hepatic steatosis could involve a modification of the epigenome, we used western blot to examine two post-translational histone modifications related to transcriptional repression (H3K9me3 and H3K27me3) and two histone modifications related to transcriptional activation (H3K4me3 and H3K14me3). No differences in these epigenetic marks were detected between the different groups (unpublished observations). However, these negative results did not exclude the involvement of other epigenetic mechanisms (DNA methylation, miRNAs expression) in the protective effects of DHA.

5. Conclusions

In summary, the present study provided compelling evidence to show that maternal DHA supplementation in rats during pregnancy protected the offspring against glucose intolerance and hepatic steatosis induced by the consumption of a hypercaloric diet. The beneficial effects on glucose homeostasis declined with age in a sex-dependent manner. However, the preventive action against hepatic steatosis persisted at least until 6 months of age and was associated with decreased hepatic expression of lipogenic genes. These results indicate that maternal DHA supplementation during pregnancy can program a healthy metabolic state in the offspring which protects against the deleterious effects of an obesogenic diet. The involvement of epigenetic mechanisms in the beneficial programming action of DHA deserves further investigation.

Author Contributions: F.B.-J. and E.Z. conceived the study and designed the experiments. A.D.-A., S.O.H., L.A.R.C., C.E.M.-G. and J.A.G.-S. performed the experiments. M.C. performed the mass spectrometry analysis. A.D.-A., S.O.H., L.A.R.C., C.E.M.-G., M.C. analyzed the data. K.O. contributed with conceptual ideas. F.B.-J. wrote the original draft. F.B.-J. and E.Z. acquired the funding. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the French National Research Agency (ANR, grant ANR-16-CE21-0007-01) and the Mexican Consejo Nacional de Ciencia y Tecnología (CONACYT, award number 2015-16-273510).

Institutional Review Board Statement: All experiments were performed following the European Union Directive on the protection of animals used for scientific purposes (2010/63/EU) and according to the guidelines of the Declaration of Helsinki in an animal facility agreed by the French Veterinary Department (register number A44276), and were approved by the Ethical Committee for Animal Experimentation of the Pays de la Loire (authorization number 2017012013318453/APAFIS 8215).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author on reasonable request.

Acknowledgments: Amran Daher Abdi is the recipient of a doctoral fellowship from the Ministry of Research and Innovation of the Government of the Republic of Djibouti. Sandra Olvera Hernández was supported by the program “Food for Tomorrow” of the French Region Pays de la Loire and the European Regional Development Fund (FEDER). Luis Reyes Castro received support from the EU in the framework of the Marie-Curie FP7 COFUND People Programme, through the award of an AgreeSkills fellowship. Carla Mezo González and Juan Antonio García-Santillán are supported by an international PhD fellowship from CONACYT. Authors are grateful to Blandine Castellano, Isabelle Grit and Agnès David for technical help.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Titre: Étude de l'implication du métabolisme cérébral du tryptophane dans les déficits de mémorisation induits par l'obésité.

Mots clés : Tryptophane, mémoire, obésité, programmation métabolique, œstrogènes.

Résumé : L'obésité a des effets délétères sur la cognition qui peuvent être transmis à la descendance via un processus de programmation métabolique. Dans ce travail de thèse, nous avons analysé le rôle du métabolisme du tryptophane (TRP) par la voie de la kynurénine (KP), dans les déficits de mémorisation et d'apprentissage induits par l'obésité. Notre hypothèse de travail étant que l'inflammation liée à l'état d'obésité augmente le taux de kynurénine (KYN), d'acide kynurénique (AK) et d'acide quinolinique (AQ) dans le cerveau et que ceci conduit à une dérégulation de la Plasticité Synaptique médiée par le Glutamate (PSmG) et, donc, à des altérations dans le processus de construction de la mémoire. Les résultats générés montrent que, chez le rat, l'obésité résultant de la consommation d'un aliment hypercalorique provoque des déficits d'apprentissage aussi bien chez les animaux mâles que chez les animaux

fémmes. Cette altération cognitive est associée à des modifications du métabolisme cérébral du TRP qui varient en fonction du sexe. Ainsi, tandis que les rats mâles obèses ont présenté des niveaux élevés d'AQ dans l'hippocampe, chez les rats femelles l'obésité a induit, au contraire, une réduction en fonction du cycle œstral de la concentration hippocampique de TRP et de ses catabolites. Enfin, l'obésité de la mère ou du père, induit chez la progéniture des déficits de mémorisation associés à une production excessive de AQ et de AK ainsi qu'à des altérations de l'expression des récepteurs NMDA du glutamate, des gènes impliqués dans le métabolisme du TRP et de marqueurs PSmG. En conclusion, l'obésité induit des déficits de mémorisation associés à des modifications du métabolisme cérébrale du TRP. Ces altérations cognitives et neurochimiques sont transmises à la progéniture.

Title: Study of the involvement of brain tryptophan metabolism in obesity-induced memory deficits.

Key words: Tryptophan, memory, obesity, metabolic programming, estrogen.

Abstract: Obesity has deleterious effects on cognition that can be transmitted to offspring via a metabolic programming process. In this thesis, we analyzed the role of tryptophan (TRP) metabolism via the kynurenine (KP) pathway in obesity-induced memory and learning deficits. Our working hypothesis was that obesity-related inflammation increases the brain levels of kynurenine (KYN), kynurenic acid (KA) and quinolinic acid (QA) and that this leads to a deregulation of Glutamate-mediated Synaptic Plasticity (GmSP) and, therefore, to impairments in the construction of the memory process. The results generated show that, in rats, obesity arising from the consumption of a high-calorie diet results in learning deficits in both male and female animals.

This cognitive impairment is associated with sex-dependent changes in brain TRP metabolism. Thus, while obese male rats showed high levels of QA in the hippocampus, in female rats, obesity induced, on the contrary, an estrous cycle-dependent reduction in the hippocampal concentration of TRP and its catabolites. Finally, maternal or paternal obesity induced memory deficits in the offspring that were associated with excessive production of QA and KA as well as with alterations in the expression of NMDA glutamate receptors, of genes involved in TRP metabolism and of GmSP markers. In conclusion, obesity induces learning deficits associated with changes in brain TRP metabolism. These cognitive and neurochemical alterations are transmitted to the offspring.