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TESIS

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ÍNDICE

Resumen

Abstract

1. **Introducción**
   - **1.1 Obesidad y estilo de vida moderno**
     - **1.1.1 Adolescentes y desarrollo de jet lag social**
     - **1.1.2 Sobreconsumo de alimentos por disrupción del sueño**
2. **Intensión de esta tesis**
3. **Antecedentes**
   - **3.1 Modelos animales de sobreconsumo de alimentos y desarrollo de síndrome metabólico**
     - **3.1.1 Modelos animales de privación de sueño y su efecto sobre la ingesta de alimentos**
     - **3.1.2 Efectos de la disrupción del sueño y el sistema límbico**
   - **3.2 Mecanismos cerebrales para la ingesta de alimentos**
     - **3.2.1 Diferencias del tipo de dietas ricas en azúcar o grasa, (absorción y procesamiento metabólico y su efecto en el cerebro)**
   - **3.3 Conductas perecidas a la adicción por alimentos palatables**
     - **3.3.1 Conductas asociadas a las adicciones y su impacto sobre el sistema límbico**
     - **3.3.2 Abstinencia de alimentos apetitosos**
4. **Planteamiento general de la tesis**
   - **4.1 Hipótesis generales**
   - **4.2 Objetivos generales**
5. **Publicación #1**
   - **5.1 Planteamiento del problema**
   - **5.2 Hipótesis**
   - **5.3 Objetivo general**
     - **5.3.1 Objetivos particulares**

6. Publicación #2
    6.1 Planteamiento del problema
    6.2 Hipótesis
    6.3 Objetivo general
        6.3.1 Objetivos particulares
7. Publicación #3
    7.1 Planteamiento del problema
    7.2 Hipótesis
    7.3 Objetivo general
        7.3.1 Objetivos particulares
8. Avances de la publicación #4
    8.1 Planteamiento del problema
    8.2 Hipótesis
    8.3 Objetivo general
        8.3.1 Objetivos particulares
    8.4 Material y método
        8.4.1 Animales y condiciones generales
        8.4.2 Diseño experimental
        8.4.3 Obtención de tejidos y procesamiento de cerebros
        8.4.4 Parámetros de síndrome metabólico
    8.5 Resultados
9. Discusión
10. Conclusiones
11. Referencias
12. Anexos

RESUMEN

El estilo de vida moderno promueve una diferencia en la hora del despertar y el tiempo de dormir, provocando una condición denominada "jet-lag social". La disrupción del sueño promueve la ingesta de alimentos altamente calóricos, que a largo plazo, puede conducir al sobrepeso, la obesidad y síndrome metabólico. La ingesta de alimentos palatables o sabrosos, provoca una alimentación hedónica y no homeostática, que contribuye al desarrollo de la obesidad. El acceso restringido a alimentos sabrosos es un fuerte estímulo para desencadenar conductas asociadas a la adicción como la conducta tipo atracón, la anticipación, conductas de esfuerzo y síntomas parecidos a la abstinencia. Dichas conductas están asociadas a la activación y a cambios plásticos en áreas corticolímbicas. Los alimentos palatables o sabrosos contienen principalmente una mezcla de grasa y azúcar, por lo tanto, la contribución de cada macronutriente para cada una de las conductas y los cambios neuronales no es clara. Con el fin de simular la situación humana, estandarizamos un modelo experimental de jet lag social, con el que asociamos el retraso de sueño con el consumo de una dieta de cafetería, de dieta alta en azúcar o dieta alta en grasa. Así mismo analizamos el desarrollo de conductas asociadas a la adicción, la obesidad, el síndrome metabólico y a cambios en la activación de áreas corticolímbicas así como de indicadores de plasticidad en las mismas. En este trabajo demostramos que el jet lag social y el consumo excesivo de dieta de cafetería, maximiza el desarrollo de la obesidad y del cumplimiento de varios criterios del síndrome metabólico. También se demostró que una dieta alta en grasa es un estímulo más fuerte que una dieta alta en azúcar para promover el desarrollo de conductas similares a la adicción y para provocar cambios plásticos en el sistema corticolímbico. Nuestros hallazgos muestran que el modelo de retraso de sueño es un factor de riesgo para desarrollar conductas parecidas a la adicción y, cuando los sujetos tienen disponible una dieta palatable alta en grasa aumenta la ocurrencia de alteraciones metabólicas y se observa activación diferencial de poblaciones neuronales especificas del hipotálamo lateral, así como una mayor activación en las regiones corticolímbicas.

ABSTRACT

Modern lifestyle promotes a difference between waking time and sleeping time in weekdays and in weekends, causing a condition called "social jet-lag". Sleep disruption promotes the intake of highly caloric food, which in the long term leads to overweight, obesity and metabolic syndrome. Ingestion of palatable food causes a hedonic and non-homeostatic ingestion that contributes to the development of obesity. Restricted access to palatable foods is a strong stimulus to trigger behaviors associated with addiction such as binge eating, anticipation, stress and withdrawal-like symptoms. These behaviors are associated with activation and plastic changes in corticolimbic areas. Palatable foods contain mainly a mixture of fat and sugar, however, the contribution of each macronutrient to each of the behaviors and neuronal changes is not clear. In order to simulate the human condition, we standardized an experimental model of social jet lag, in order to associate sleep delay with the consumption of cafeteria diet, high-sugar diet or high-fat diet; with the development of behaviors associated with addiction, obesity, metabolic syndrome and changes in the activation of corticolimbic areas and indicators of plasticity within them. This research developed and validated an experimental model of social jet lag that, when combined with excessive consumption of cafeteria diet, maximizes the development of obesity and more criteria of metabolic syndrome criteria. We also describe that a high-fat diet is a stronger stimulus than a high-sugar diet to promote the development of behaviors similar to addiction and to cause plastic changes in the corticolimbic system. Proving that sleep delay is a risk factor for developing addiction-like behaviors. Moreover we found that the combination with high fat diet, the occurrence of metabolic alterations is observed and differential activation of specific neuronal populations of the lateral hypothalamus, as well as greater activation in corticolimbic regions.

1. INTRODUCCION

1.1 Obesidad y estilo de vida moderno

La obesidad es una enfermedad crónica, de origen multifactorial, en la que participan principalmente aspectos de tipo genético, ambiental, social, psicológico y de estilo de vida. La causa principal de la obesidad es por la pérdida de equilibrio en el balance energético ocasionada por el aumento en la ingestión de calorías y por una disminución en el gasto energético [1]. Se estima que un cuarto de la población mundial adolescente padece sobrepeso u obesidad [2]. La Encuesta Nacional de Salud y Nutrición de México 2016 reportó una prevalencia del 36.3% de sobrepeso y obesidad en los adolescentes [3].

Las consecuencias de quienes padecen obesidad son diversas, entre las que se encuentran mayor propensión a padecer hipertensión arterial, resistencia a la insulina , hiperlipidemia, diabetes mellitus tipo 2 y síndrome metabólico [4–6]. El síndrome metabólico se caracteriza por la presencia de al menos 3 de los siguientes signos: hipertensión arterial, glucosa elevada, triglicéridos altos, niveles bajos de HDL y obesidad abdominal. En México se puede observar una elevada prevalencia de diabetes mellitus tipo 2 y de hipertensión en poblaciones de diferentes edades y sexo [3].

Dentro del estilo de vida actual, como un factor importante que propicia la obesidad, se incluye el sedentarismo, definido como el número de horas que se pasa frente a una computadora, televisión, celular, etc. [7]; así como el consumo de alimentos altos en grasa y carbohidratos, que son hábitos cada día más comunes en países desarrollados y en desarrollo [8]. Aunado al aumento en la obesidad, el uso de la tecnología ha llevado a estar expuestos a aparatos que emiten luz (computadoras o teléfonos celulares) en horas en las que se debería de estar dormido, provocando un desvelo o retraso de sueño, as alteraciones en el ciclo sueño y vigilia. Los anteriores se suman a la lista de factores que contribuyen al desarrollo de la obesidad.

Así mismo, los trabajadores nocturnos se encuentran entre las poblaciones que se exponen continuamente a la alteración de su ciclo de sueño y vigilia, lo cual se ha asociado al consumo de comida altamente palatable e hipercalórica (comida alta en azúcar y/o alta en grasa) durante el trabajo y se exponen a la luz por la noche [9–12]. En años recientes se ha reportado que otra población vulnerable a este problema son los adolescentes, que se desvelan (estudiando o solo navegando por la red), y su ciclo sueño y vigilia se ve importantemente afectado tanto en calidad como cantidad. Ellos también tienden a ingerir comidas hipercalóricas, por lo que son altamente susceptibles a desarrollar enfermedades asociadas a la obesidad.

1.1.1 Adolescentes y desarrollo de jet lag social

Dentro del estilo de vida adolescente se encuentran actividades recurrentes como asistir a fiestas/reuniones, uso de computadoras, ver televisión hasta tarde por la noche, inactividad física, disminución en la cantidad de sueño, etc. [13]. También hay una preferencia por consumir alimentos industrializados ricos en carbohidratos y grasas saturadas [14]. En particular, la adolescencia es un período crucial para el desarrollo de malos hábitos en los estilos de vida, que muchas veces continúan durante la edad adulta y representan un riesgo de padecer enfermedades crónicas como la obesidad [15].

En los últimos 40 años, las horas de sueño entre los adolescentes han disminuido notablemente [16], de acuerdo a las cifras obtenidas, sólo el 33% de esta población duerme las nueve horas recomendadas [17]. La disminución del sueño, ya sea total o parcial, tiene consecuencias adversas a corto y largo plazo sobre la salud [18]. Entre ellas, causa elevación de la presión arterial, sobrepeso y obesidad [19–24], lo cual puede llevar al desarrollo de enfermedades como diabetes mellitus tipo 2 [13].

Las investigaciones clínicas en ambientes controlados han demostrado que adultos con solo 4 horas de sueño por 6 días, presentan indicadores metabólicos de resistencia a la insulina, además de un apetito alterado y dirigido al consumo de

alimentos altos en calorías, aunque también mostraron que estos mismos individuos expuestos a un proceso controlado de 8 horas de sueño restaurador durante seis días, recuperaron los valores metabólicos establecidos para un individuos saludable [25]. Otros estudios señalan que son necesarias de 1-2 noches completas de sueño para la recuperación cognitiva del sueño ya sea por fragmentación, privación total o privación parcial en un periodo de hasta 90 horas de privación de sueño [26].

Investigaciones recientes han mostrado que durante los días entre semana ir al trabajo o escuela, implican levantarse temprano, esto aunado al desvelo por mantenerse despierto durante la noche disminuye la duración del sueño [27]. Cuando se presentan grandes diferencias entre los horarios de sueño de fin de semana y los días laborales se induce jet lag social [28]. Los estudios en humanos han mostrado que el jet lag social disminuye las horas de sueño durante la noche y modifica la función metabólica, la ingesta de alimentos y el peso corporal [29,30].

La población más afectada por el jet lag social son los individuos con cronotipo vespertino extremo, quienes prefieren tener actividades nocturnas y por lo tanto duermen tarde, desvelándose y levantándose muy temprano para cumplir con horarios estrictos por el trabajo o la escuela [31]. Adán y cols. (1994) observaron diferencias en el consumo de sustancias psicoactivas legales (nicotina, alcohol y cafeína) entre tipologías circadianas, siendo los sujetos vespertinos quienes consumen más de todas ellas, particularmente en la población adolescente [32,33]; mientras que la tipología circadiana matutina (duermen y se levantan temprano) parece ser un factor protector en el inicio y mantenimiento de consumo de drogas.

Estudios realizados en adolescentes han mostrado que la alteración del sueño también está asociada con menos reactividad de los sistemas cerebrales asociados a la recompensa, sugiriendo de esta forma que se necesitan recompensas más “excitantes” para generar el mismo nivel de activación neural [34], como los alimentos palatables (altos en grasa y azúcar). Se ha descrito que individuos

jóvenes forzados a reducir sus horas de sueño, muestran preferencia por alimentos dulces y ricos en calorías [23].

1.1.2 Sobreconsumo de alimentos por disrupción del sueño

El sobreconsumo de alimentos parece ser la explicación más plausible de por qué quienes duermen menos tienden a ganar más peso. Se ha reportado que la falta de sueño afecta la secreción de algunas hormonas que regulan el apetito, por ejemplo, la falta de sueño provoca un decremento en los niveles de leptina (saciedad), incrementa los niveles de grelina (hambre), altera la homeostasis de la glucosa y altera el sistema de orexinas [4]. También la reducción de las horas de sueño puede contribuir a la ganancia de peso y a la obesidad incrementando el tiempo destinado a comer por la noche.

En investigaciones recientes se ha demostrado, que individuos privados de sueño tienden a ingerir más calorías, independientemente de los cambios en los niveles de leptina y grelina, lo cual sugiere que esto depende también de mediadores hedónicos por el alimento (además de los homeostáticos) [35]. La dieta de cafetería es altamente palatable, rica en grasa y/o azúcares refinadas, por lo que se ha comprobado que este tipo de alimentos promueven un mayor consumo en volumen y menos saciedad postprandial. Por su parte, el sabor de los alimentos contribuye de forma importante con el consumo excesivo, la ganancia de peso y a largo plazo con la obesidad y las enfermedades que ésta acarrea [36]. French y cols. en 2001 encontraron que el 75% de los adolescentes estadounidenses entre las edades de 11 y 18 años comen en restaurantes de comida rápida por lo menos una vez a la semana; mientras que Cimadon y cols. en 2010 indican que el 70% de los estudiantes brasileños entre 9 y 18 años de edad, consumen comida rápida cuatro veces o más por semana [37].

2. INTENCIÓN DE ESTA TESIS

Esta tesis tiene como objetivo principal, desarrollar un modelo de jet lag social en ratas Wistar que nos permita evaluar la contribución del retraso de sueño en el

sobreconsumo de alimentos palatables o sabrosos. Así mismo, se pretende evaluar conductas parecidas a la adicción, como la conducta tipo atracón, anticipación, esfuerzo y abstinencia. También se pretende determinar síndrome metabólico y cambios en sistema nervioso central, específicamente en áreas hipotalámicas y del sistema de recompensa, todo asociado a l consumo de dietas palatables y al retraso de sueño.

3. ANTECEDENTES

3.1 Modelos animales de sobreconsumo de alimentos y desarrollo de síndrome metabólico

Para entender mejor los mecanismos que asocian la homeostasis alterada con la obesidad y el sobreconsumo de alimentos en humanos, se han desarrollado modelos experimentales con ratas, expuestas a dietas con alto contenido en grasa y carbohidratos [38,39]. Tradicionalmente estos modelos consisten en dar acceso a dietas ricas en carbohidratos o en grasa (por ejemplo 60% del contenido de la dieta) y comparar sus efectos con animales control expuestos a dietas balanceadas. También existen modelos experimentales en que se exponen las ratas a una dieta de cafetería en donde los animales tienen mayor disponibilidad y variedad de alimentos palatables, enriquecidos en contenido energético y que los humanos también consumimos [14,40]. En las dietas de cafetería se incluyen galletas, postres, leche condensada, salchichas y todo tipo de comida comercial rica en grasa y/o azúcares.

Similar a los humanos, el síndrome metabólico en animales se caracteriza por la presencia de al menos 3 de los siguientes signos: hipertensión arterial, glucosa elevada, triglicéridos altos, niveles bajos de HDL y obesidad [41]. Los modelos experimentales en donde se exponen a roedores a una dieta de cafetería reportan mayor ingesta de alimento, índices elevados en glucosa y colesterol [14], aumento de peso y de grasa abdominal [42] así como disminución en la concentración de grelina [43]. Hasta el momento la dieta de cafetería se considera el mejor estímulo

para ocasionar síndrome metabólico en modelos animales.

Otros trabajos en donde utilizan dieta de cafetería reportan que las ratas al ser expuestas a este tipo de dietas de 90 a 120 días aumentan de peso e incrementan sus niveles de glucosa y exhiben signos de intolerancia a la glucosa. También desarrollan conductas hiperfágicas similares a los episodios de *binge* (atracón) que se observan en el consumo de las drogas de abuso [44,45].

3.1.1 Modelos animales de privación de sueño y su efecto sobre la ingesta de alimentos

Existen diversas manipulaciones que intentan reproducir condiciones de privación de sueño, entre los más empleados son la plataforma invertida [46,47] y protocolos de actividad forzada [48–50]. Los estudios realizados con protocolos de actividad forzada muestran una reducción en el peso [51–53] y también se reporta un desbalance metabólico ocasionado por la disrupción ocasionada por los protocolos de actividad forzada [48,49,54]. Estos datos sugieren que la actividad combinada con la alimentación durante las horas de sueño alteran las funciones metabólicas del organismo [48].

En protocolos donde se pretende privar selectivamente de sueño REM, se ha reportado en la mayoría de los estudios una disminución del peso corporal debido al gasto energético [55,56], pero también se ha reportado un aumento en el consumo de alimentos [57,58], esto podría deberse al incremento en el gasto de energía debido al estrés y a que se mantiene el estado de vigilia por un periodo más largo de tiempo. Se ha sugerido que el sistema motivacional también juega un papel importante en dicho fenómeno, de tal forma que la regulación de la ingestión de alimentos está influenciada por dos sistemas diferentes: el homeostático y el motivacional [36].

3.1.2 Efectos de la disrupción del sueño y el sistema límbico

La privación de sueño tiene un impacto importante en la señalización dopaminérgica, por ejemplo una mayor producción de dopamina a nivel de estriado.

Esto se ha demostrado en un estudio donde después de 10 días de privación de sueño REM se ve un incremento en metabolitos asociados a la dopamina y acetilcolina en el estriado [59]. Otro estudio muestra un aumento en los niveles de metabolitos de dopamina después de tan solo 96 horas de privación de sueño REM [60]. La liberación de dopamina se ha asociado con el consumo de alimentos y la motivación por obtenerlo. En este sentido, se ha demostrado que la privación de sueño reduce el esfuerzo de los animales por obtener reforzadores (medido por "break point"), y si se administra anfetamina (agonista dopaminérgico), específicamente en el núcleo accumbens, se puede restaurar el esfuerzo y la motivación de los animales para obtener el reforzador [46], lo que indica que la privación de sueño afecta la señalización de dopaminérgica y en consecuencia la motivación por obtener cierto tipo de alimentos.

3.2 Mecanismos cerebrales para la ingesta de alimentos

Los circuitos relacionados con el consumo de alimentos están regulados por señales periféricas que son sensadas por el hipotálamo. Sabemos que el aporte energético obtenido de los alimentos consumidos puede ser transformado en reserva calórica y almacenarse en el tejido adiposo en forma de triglicéridos o en tejido muscular y en forma de glucógeno en el tejido hepático, como fuente de energía de consumo inmediato o bien en forma de proteínas las cuales serán usadas como fuente de energía sólo en casos severos de desnutrición [61].

Las señales que llevan información sobre el estado de la grasa corporal a áreas del cerebro se originan principalmente en el tejido adiposo, como en el caso de la leptina y desde el páncreas, como el caso de la insulina. Estos factores circulan en proporción a la masa grasa corporal y son referidos como "señales de adiposidad" [61]

El área cerebral encargada de procesar las señales de homeostasis del organismo, es el hipotálamo, siendo el principal centro regulador de la ingestión de alimento. Las estructuras anatómicas más importantes en el sistema de regulación de

alimentación son los núcleos ventromedial, dorsomedial, paraventricular, hipotálamo lateral y núcleo arqueado, donde se encuentran receptores de señales periféricas como los de leptina, teniendo una señal para el control de la ingestión de alimentos [62], aunque también se reportan receptores de leptina en el circuito de recompensa, principalmente el Área Tegmental Ventral, regulando la producción de dopamina [36].

El núcleo ventromedial y el hipotálamo lateral se han descrito como centros de la saciedad y el hambre respectivamente. Estos núcleos tienen interacciones con otras áreas implicadas con el comportamiento de la ingestión en el mismo hipotálamo y fuera de él, como el sistema límbico [63,64]. El núcleo dorsomedial media tanto la estimulación como la inhibición de la alimentación y depende, de manera específica, de la leptina. El núcleo paraventricular está implicado en la integración de la información de señales orexigénicas, estimulantes, así como la interacción entre neurotransmisores y neuromoduladores inhibidores del apetito [65]. Otro núcleo hipotalámico involucrado en la regulación de la ingesta de alimentos es el núcleo arqueado, que está ubicado en la base del hipotálamo medial y se asocia con la producción de neuropéptido Y (NPY) y pro-opiomelanocortinas (POMC), que ejercen efectos opuestos sobre la ingestión de alimentos. El NPY estimula la ingestión de alimentos y la POMC la inhibe [66,67].

La leptina regula la liberación de NPY y de POMC. En la vía del NPY la leptina suprime la expresión y liberación del NPY, que resulta en una disminución del consumo de alimentos y un aumento de la actividad metabólica [67,68]. Por el contrario la leptina activa a las células productoras de POMC estimulando su liberación.

Una de las hormonas peptídicas más importantes en la regulación de la ingesta de alimentos son las orexinas y se producen por el hipotálamo en dos estructuras: el área perifornical y el hipotálamo lateral. El área perifornical se ha asociado con funciones como el alertamiento y vigilia. Por su parte, el hipotálamo lateral tiene

funciones de relevo para poder registrar el valor reforzante de los alimentos que consumimos; con ello podemos decir que las orexinas tienen diferentes funciones en el encéfalo dependiendo del lugar en donde sean producidas y el lugar de acción de las mismas [69].

Las orexinas son hormonas neuropéptidas que se han encontrado en el núcleo dorsomedial del hipotálamo, en el área perifornical y en el hipotálamo lateral. El sistema orexigénico cuenta con dos ligando: OrxA y OrxB, el primero es selectivo a orexinas tipo A, mientras que el segundo no es selectivo. Ambos receptores son metabotrópicos y pueden ser encontrados en diversas partes del encéfalo, particularmente aquellas asociadas con estados de alertamiento, vigilia, ingestión de alimentos e incluso en algunas áreas relacionadas con la recompensa a drogas de abuso y adicciones [70].

La capacidad de respuesta de las neuronas productoras de orexinas tanto a glucosa como a leptina, entre otras señales metabólicas periféricas, sugiere que estas podrían actuar como un sensor del estado metabólico de los animales [71]. Dichas células proyectan a las células dopaminérgicas del mesencéfalo, constituyendo una conexión que podría ser importante para la activación emocional, así como para las respuestas de recompensa y motivación para la búsqueda de alimentos [71].

El sistema orexigénico tiene efectos sobre el ciclo sueño y vigilia, pues se ha reportado que la privación de sueño aumenta la actividad del sistema orexigénico, lo que podría promover un mayor tono del sistema nervioso simpático aumentando la actividad de grupos neuronales estimuladores del apetito (mediados por neuropéptido Y) en el núcleo arqueado hipotalámico [72]. Por lo tanto, una menor cantidad de sueño podría resultar en aumento de la actividad del sistema orexigénico hipotalámico y cambios en la organización de los estados de sueño. Además, una menor cantidad de sueño resulta en mayor somnolencia y sensación de fatiga diurna y menor gasto energético. El conjunto de estos cambios podría contribuir a mayor ganancia de peso y riesgo de obesidad [72].

Se sabe que el sistema hipotalámico orexigénico está posicionado de tal forma que puede interactuar con la vía mesolímbica, se han descrito proyecciones del hipotálamo lateral localizadas en VTA [73,74] y las terminales orexigénicas, contactan con células positivas a hidroxilasa de tirosina [75]. Incluso se ha demostrado que la administración intra-VTA de orexinas tipo A, resulta en un aumento de Fos en neuronas dopaminérgicas localizadas en la porción caudo-medial del VTA [73], además se ha descrito un aumento de la concentración de dopamina en la región shell del núcleo accumbens, sin encontrar cambios en la región core [76]. También se ha observado un aumento de dopamina en la corteza prefrontal medial. Inclusive se ha involucrado a las orexinas en la potenciación de los receptores NMDA en el sistema de recompensa, indicando un papel en la plasticidad neuronal a largo plazo [77].

De esta forma se puede entender al sistema orexigénico como multifuncional, ya que ejerce efectos sobre diversas conductas: sueño, alertamiento, ingestión de alimentos y sobre los mecanismos involucrados en los efectos reforzantes del alimento palatable.

3.2.1 Diferencias del tipo de dietas ricas en azúcar o grasa, (absorción y procesamiento metabólico y su efecto en el cerebro)

La obtención de energía que los organismos necesitan, es dada por el consumo de los principales macronutrientes: carbohidratos, proteínas y lípidos. De acuerdo al requerimiento energético, los organismos tienen la capacidad de detectar cada uno de estos componentes; principalmente a nivel oral. Los alimentos altos en azúcar o grasa son más atractivos porque rápidamente los podemos convertir a energía y cumplir los requerimientos energéticos [78]. Incluso algunos autores los han propuesto como “agentes adictivos“ por estimular áreas del cerebro asociadas al valor hedónico de los estímulos [79–82].

Las diferencias entre la detección de alimentos altos en azúcar o altos en grasa, empieza por el sentido del gusto, pues se tienen receptores específicos para poder detectar esas diferencias y comunicarlas al sistema nervioso central. El receptor al sabor dulce está formado por el heterodímero T1R2/T1R3, el cual está acoplado a una proteína G; y se encuentra en la lengua y el paladar [83]. Al ser activado en la cavidad oral, este receptor dispara una señal nerviosa, que es enviada a través de los pares craneales VII (nervio facial/información de la parte anterior de la lengua) y IX (nervio glosofaríngeo/información de la parte posterior de la lengua y el paladar) hasta la parte rostral del núcleo del tracto solitario [84,85], que comunica con el núcleo parabranquial [86,87] y haciendo un relevo talámico, llega a la corteza gustativa primaria (ínsula y opérculo frontal), la cual envía la información para ser integrada a la corteza gustatoria secundaria (corteza orbitofrontal) [88]. El núcleo del tracto solitario y la corteza gustativa primaria también proyectan a núcleos hipotalámicos (información homeostática) y al núcleo accumbens (información de relevancia del estímulo) [89,90]; creando información de lo que el organismo está probando en ese momento y enviando retroalimentación al PBN [88]. El neurotransmisor principal que recibe el núcleo accumbens, y por el cual se ha asociado a la motivación por los alimentos es la dopamina.

En cuanto a los alimentos altos en grasa, se ha demostrado que la lengua produce una lipasa lingual que rompe los triglicéridos en ácidos grasos [91–93], los cuales son detectados por los receptores CD36, DRK5, GPR 40-43MY y GPR120 y estos activan los mismos nervios gustativos que el azúcar (VII y IX) y siguen la misma vía hacia el núcleo del tracto solitario, activando las subsecuentes estructuras, para identificar el sabor ingerido [94,95].

Una vez que los alimentos son ingeridos, el resto del tracto gastrointestinal (faringe, esófago, estómago, intestino delgado e intestino grueso) también tiene forma de detectar y procesar los diferentes macronutrientes que consumimos. Por un lado, los alimentos altos en azúcar siguen activando los receptores del sabor que se encuentran a lo largo de todo el tracto gastrointestinal, estos receptores se

encuentran co-expresados con otro tipo de receptores como el de la leptina, que regula la sensibilidad a lo dulce [96]. En el intestino, este receptor de leptina se co-expresa con el receptor GLP-1, mediando su sensibilidad [97]. Cuando estos receptores son activados en el intestino delgado, se observa una mayor expresión de GLUT2 para promover la absorción del azúcar por los tejidos [98,99]. En el sistema mesentérico, hay receptores que detectan el azúcar llamados SGLT1 y SGLT3, los cuales al ser activados promueven la liberación de GLP1 y 2 [100–102]. Se piensa que la percepción post-prandial de una recompensa desempeña un papel importante en la modulación de los hábitos alimenticios [103].

Cuando en el intestino se activan los receptores que detectan el azúcar, libera diferentes hormonas que envían una señal de saciedad al torrente sanguíneo, y esto es detectado por el cerebro. Experimentos en los cuales inyectaban glucosa intragástrica pudieron medir mayores niveles de glucosa en plasma, insulina, leptina, GLP-1, PYY y menores niveles de grelina [104–107]. Así mismo, infusiones de glucosa en el duodeno activan zonas cefálicas específicas, como la corteza prefrontal, somatosensorial primaria, área piriforme, corteza orbitofrontal, núcleo caudado y putámen. Mientras que glucosa administrada en la vena porta activa solo la corteza insular, somatosensorial primaria y prefrontal [108]. Roedores mutantes que no pueden producir GLP-1 reducen su sensibilidad a sabores dulces [109]. A nivel central se ha demostrado que el azúcar puede alterar la permeabilidad de la barrera hematoencefálica [110], atravesarla y ser utilizada como fuente de energía [105].

En el tracto gastrointestinal, también se expresan receptores a ácidos grasos (GPR120, 40, 19) y al ser activados, se produce la liberación al torrente sanguíneo CCK, GLP-1 y PYY. También suprime la secreción de grelina [111]. Así mismo, el nervio vago también responde a estos cambios y los comunica al núcleo del tracto solitario, siguiendo la misma vía que el azúcar (descrita anteriormente). Wang y cols. demostraron que al inyectar GLP-1 directamente en el núcleo del tracto solitario, se

reduce el consumo de dieta alta en grasa, suprimiendo la señal dopaminérgica [112].

Las hormonas liberadas por los órganos digestivos, como ya mencionamos, además de ser inducidas por el azúcar, también son inducidas por la presencia de alimentos altos en grasa, así como a la presencia de ácidos graos, los cuales aumentan incluso cuando la grasa corporal de un individuo es alta y disminuyen cuando la grasa corporal es baja, en consecuencia, estos pueden modular el apetito [113,114].

A nivel sistémico, también se liberan ácidos grasos, los cuales pueden ser transportados dentro del cerebro y cruzar la barrera hematoencefálica [115,116]. Al entrar, el cerebro está equipado para detectar los niveles de triglicéridos que hay en la circulación, por medio de la lipasa LPL, la cual se expresa en la mayoría de los núcleos cerebrales; y se sabe que es necesaria para la regulación metabólica y energética de los individuos [117,118], en ratones deficientes de esta proteína en el sistema nervioso central, se produce altos niveles de triglicéridos en sangre y mayor ganancia de peso [119], como lo haría el consumo de dieta alta en grasa.

En contraste con los modelos con azúcar, un importante factor de confusión es que las dietas altas en grasas llevan al aumento de peso y adiposidad. Por lo tanto, en algunos casos es difícil determinar qué cambios neuronales de activación o inactivación de algunas áreas son sólo una consecuencia del aumento de la adiposidad y el peso, y cuáles están específicamente asociados con la ingesta de dietas ricas en grasas [120]. De esta manera se sabe que tanto grasa y azúcar estimulan diversos receptores, para estimular la señalización hormonal en diferentes órganos, la cual va a señalizar al cerebro mediante activación neuronal directa del órgano o mediante las hormonas en el torrente sanguíneo (Figura 1; Modificado de [121] and [66]).

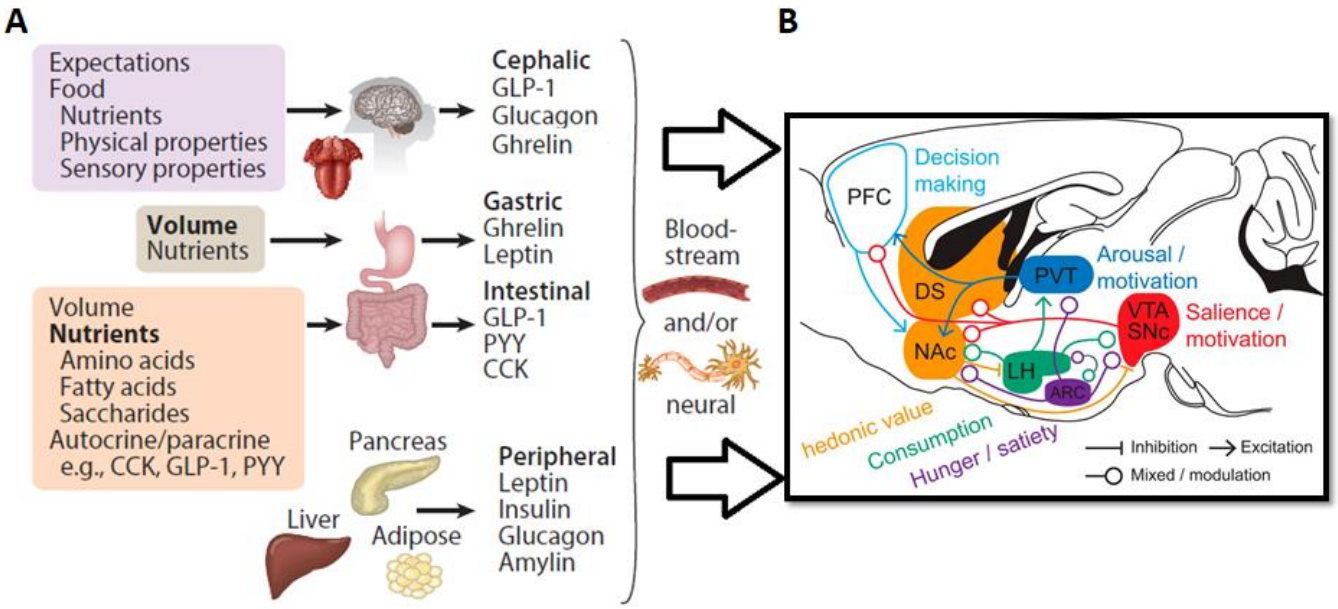

Figura 1. A) Señales endocrinas, derivadas en gran medida del intestino y transmitidas neuronalmente o mediante la circulación al cerebro, que influyen en la ingesta durante la ingesta de alimentos, determinado por el tamaño de las porciones, así como por la cantidad de los macronutrientes ingeridos. B) La detección de los macronutrientes y las hormonas en el torrente sanguíneo activan los principales nódulos neuronales que controlan la ingesta de alimentos. Modificado de [121] and [66].

Los protocolos a largo plazo con dietas altas en azúcar y altas en grasa varía de acuerdo a su tiempo de administración (semanas). Por ejemplo, la forma de administración (solido o líquido), si es restringido (unas horas al día) o *ad-libitum* y su composición (%) de macronutrientes. Las mediciones que se tienen de registro cerebral o metabólico también varían: lo hacen justo después de consumir el alimento o incluso en abstinencia (dejando a los animales algunos días sin la dieta), esto puede generar variaciones en los resultados, haciendo parecer que no son comparables; pero todo depende de las manipulaciones de cada experimento.

Lo que queda claro en la mayoría de los protocolos es que la exposición repetida a alimentos con alto contenido de grasa y/o azúcar da como resultado un consumo compulsivo de alimentos, un control deficiente de la saciedad e incrementa el

condicionamiento de estímulos alimentarios [122]. Dado lo anterior, se ha propuesto que la ingesta de alimentos está fuertemente regulada por señales hedónicas, que a menudo pueden anular las vías homeostáticas durante períodos de abundancia relativa de energía al aumentar el deseo de consumir alimentos palatables [123]

3.3 Conductas parecidas a la adicción por alimentos palatables

Los alimentos dulces, al igual que los altos en grasa han mostrado ser especialmente palatable para los seres humanos y para los roedores. Ratas que consumen una dieta alta en azúcares presentan conducta parecidas a la adicción. Estas conductas se caracterizan por no tener control en la ingestión de los alimentos (atracón), búsqueda excesiva e ingestión a pesar de cualquier efecto adverso, así como presentar conductas de esfuerzo para obtenerlo [124–129]. Así mismo, al dejar a los animales sin la dieta palatable por periodos de hasta una semana también produce signos de abstinencia y ansiedad [130,131]. En ratas expuestas a dietas altas en grasa se observan respuestas parecidas, aunque generalmente se trata de dietas mixtas en donde la grasa se acompaña de componentes dulces [126].

Una de las diferencias más importantes de los alimentos altos en azúcar y altos en grasa, son el número de kilocalorías que cada uno aporta. Un estudio reciente evaluó si las modificaciones neuronales observadas después del consumo crónico de alimentos palatables se correlacionaban con el valor hedónico de los alimentos, o con sus contenidos calóricos. Para ello, entrenaron a los ratones a presionar palancas para obtener recompensas de alimentos que fueran alimento normal (65% carbohidratos, de los cuales, 3% son de azúcar), hipercalórico (60% grasa) o saborizante isocalórico (66% carbohidratos, de los cuales 50% son de azúcar). Además, evaluaron la persistencia de la búsqueda de alimentos en los tres después de una restricción alimentaria (al 90% por 10 días). Los datos muestran que los ratones entrenados para obtener alimentos palatable isocalóricos (azúcar) mostraron mayor persistencia presionando la palanca que los otros dos grupos.

Además, ante ensayos donde la presión de la palanca no fue recompensada, el palanqueo de los animales que habían recibido el alimento isocalórico fue mayor, lo que sugiere que esta dieta promueve un comportamiento impulsivo [132].

Notablemente, Lenoir y cols. (2007) demostraron que la recompensa por azúcar es tan potente como la de la cocaína, él observo que la mayoría de las ratas prefería una recompensa dulce a una recompensa de cocaína y sólo un pequeño porcentaje de ratas comenzó a preferir cocaína, estos resultados después de una larga historia de autoadministración de cocaína [133]. Lo anterior sugiere que las propiedades reforzantes del azúcar son muy poderosas como para competir por la administración de cocaína. Esto nos muestra que este tipo de alimentos impacta en zonas cerebrales asociadas al reforzamiento, como lo hacen las drogas, de ello se encarga el sistema límbico.

3.3.1 Conductas asociadas a las adicciones y su impacto sobre el sistema límbico

La alimentación es influida no solo por mecanismos homeostáticos, sino también por el valor hedónico del alimento, el cual puede motivar el consumo y la búsqueda del mismo [134,135] por lo tanto, el consumo excesivo de alimento palatable provoca un proceso similar al que se observa en ciertos tipos de adicción, como respuestas neuroadaptativas en el circuito de recompensa del cerebro. Se propone que las drogas de abuso activan los mecanismos hedónicos que son activados por la ingestión de comida, por lo tanto, la obesidad y la drogadicción podrían depender del mismo sustrato neuronal [136]. De esta forma, investigaciones proponen que el sobre consumo de alimentos ricos en grasas y azúcares podría estar relacionado con un déficit en el circuito de recompensa del cerebro [136].

Para entender mejor los mecanismos que asocian la adicción al alimento con la obesidad en humanos, se han desarrollado modelos experimentales con ratas, expuestas a dietas con alto contenido en grasa y carbohidratos [13,136]. Tradicionalmente estos modelos consisten en administrar este tipo de dietas y

comparar sus efectos conductuales con animales control expuestos a dietas balanceadas.

Se han descrito diversas áreas cerebrales, además del hipotálamo, involucradas en la regulación de la ingestión de alimentos y que se encuentran en el llamado circuito de recompensa (involucrado en el consumo de drogas de abuso). Entre dichas regiones se encuentran la corteza gustatoria primaria y secundaria que envían sus eferencias al núcleo accumbens, el cual a su vez es influenciado por entradas de información dopaminérgicas provenientes del sistema mesolímbico y nigroestriatal [137]. Estos circuitos estriatales participan en los procesos de ingestión de comida palatable así como en el consumo de drogas de abuso [138].

Una de las estructuras cerebrales más importantes que da el valor reforzante a los diferentes estímulos que nos rodean es el núcleo accumbens, el cual está dividido anatómica y neuroquímicamente: la parte core y la parte shell. La parte shell presenta conexiones de carácter límbico y recibe entradas glutamatérgicas de hipocampo y la amígdala centromedial, así como aferencias dopaminérgicas de VTA. La parte core es una extensión de estriado dorsal y se relaciona con funciones motoras; recibe aferencias glutamatérgicas de corteza motora y aferencias dopaminérgicas de la sustancia negra; envía sus proyecciones gabaérgicas al pálido ventral, señal que forma parte de los bucles motores cortico-estrado-pálido-talámicos de carácter extrapiramidal [139].

Geiger y cols. (2009) encontraron que después de exponer a ratas durante un periodo de 15 semanas a dieta de cafetería los niveles de dopamina extracelular en el núcleo accumbens se encontraban disminuidos, lo cual podría llevar a un sobreconsumo de alimento y a su vez a mayor ganancia de peso para compensar la reducción de dopamina. Se encontró también que en el núcleo accumbens y el estriado dorsal la transmisión dopaminérgica se encontraba deprimida y que el hecho de comer dieta de cafetería restablecía dichos déficits temporalmente [140].

Con base en lo anterior, ahora sabemos que el sistema mesolímbico juega un papel muy importante al determinar la preferencia por un cierto tipo de dieta.

Similarmente, en roedores expuestos durante 15 semanas a una dieta alta en grasas, presentaban sobreconsumo de esta dieta y niveles disminuidos de dopamina en el núcleo accumbens determinados por HPLC. Además encontraron que al exponer nuevamente el alimento estándar a animales obesos la liberación de dopamina era menor, lo cual lleva a las ratas obesas a consumir preferentemente la dieta alta en grasas, normalizando de esta forma los niveles de dopamina [140]. Es por esto que el sistema mesolímbico puede jugar un papel muy importante al determinar la preferencia por un cierto tipo de dieta.

Si los patrones de ingestión de dietas altas en grasa y/o sacarosa siguen la estructura de la administración de drogas de abuso, se esperaría encontrar cambios similares a nivel del sistema nervioso central. Un cambio que se ha descrito en la literatura para el consumo de diversas drogas de abuso es el de la expresión de ΔFosB en áreas como el núcleo accumbens [141]; además de la expresión de esta proteína por la administración de drogas. También se ha visto la expresión diferencial con la exposición a reforzadores naturales como el ejercicio en rueda y la administración de dietas altas en grasa y sacarosa [142–144].

Específicamente, después de la administración aguda de drogas de abuso, las proteínas de la familia fos se inducen rápidamente en regiones específicas del encéfalo, estas respuestas se ven específicamente en el núcleo accumbens y el estriado ventral. El patrón de expresión de las proteínas de la familia fos correlacionan con el patrón de tolerancia en la adicción, es decir, se reduce su inducción en comparación con la primera exposición a la sustancia [141].

La proteína ΔFosB persiste en las neuronas por lo menos varias semanas después de la última exposición a las drogas de abuso, de esta forma se podría decir que ΔFosB ayuda a iniciar y mantener el estado adictivo [145] actuando como factor de

transcripción de genes encargados de la densidad dendrítica (caldmodulina dependiente de quinasa 5 Cdk5) y del crecimiento de espinas dendríticas (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas NFkB) [146–148].

Además, los ratones que sobreexpresan ΔFosB mostraron actividad locomotora mayor en respuesta a la administración aguda y crónica de cocaína. Así mismo, en paradigmas de condicionamiento de preferencia de lugar se observó una mayor sensibilidad a los efectos reforzantes de cocaína y morfina en estos ratones [149].

Así como otras drogas de abuso generan incremento en la expresión de ΔFosB, ciertos reforzadores naturales pueden generar incrementos similares. Esto podría deberse a que el consumo de drogas y los reforzadores naturales actúan sobre el mismo sistema en el sistema nervioso central [150]. Incluso se ha encontrado que la actividad sexual causa sensibilidad cruzada a otras drogas de abuso como las anfetaminas [129].

La actividad sexual genera plasticidad similar a la que se encuentra en el consumo de psicoestimulantes; incremento de densidad dendrítica, alteraciones en el tráfico de receptores glutamatérgicos y disminución en la fuerza sináptica en la corteza prefrontal (CPF) de proyecciones del núcleo accumbens shell [151].

La inducción de ΔFosB por reforzadores naturales es esencial para que se pueda observar la sensibilidad cruzada a psicoestimulantes, potencialmente vía espinogénesis en el núcleo accumbens durante un periodo de abstinencia. ΔFosB podría fungir como factor de transcripción para activar diversos genes involucrados en densidad sináptica y la fuerza de las sinapsis en núcleo accumbens [151], lo cual está asociado a la adicción.

En ratones que sobreexpresaban ΔFosB se observó un déficit en la producción de dopamina en células del mesencéfalo que proyectan aferencias al estriado; de esta

forma se ha inferido que el aumento de ΔFosB lleva a un incremento del valor motivacional de la comida, por lo que el consumo de alimentos con alto contenido energético restablecería los niveles de dopamina [152].

3.3.2 Abstinencia de alimentos apetitosos

El efecto adictivo de las dietas en el cerebro, tras un protocolo crónico, puede verse afectado por el tipo de protocolo o los componentes de las dietas, pero para evaluar si realmente cumple con todos los parámetros de adicción, signos conductuales y cambios cerebrales se han evaluado tras un periodo de abstinencia, pues estos efectos se ven potenciados en dicho periodo. Uno de los fármacos más usados es la naltrexona (antagonista opioide), la cual precipita los signos de abstinencia ante un protocolo con alimento palatable, por ejemplo, se ha demostrado que este fármaco altera de forma dosis-dependiente el condicionamiento de preferencia de lugar a sacarosa [153]. Otro fármaco opiáceo para precipitar los signos de abstinencia, es la naloxona [154]; la cual provoca castañeo de dientes, temblores de patas delanteras, sacudidas de cabeza y mayor ansiedad. Todos estos comportamientos aparecen después de una exposición a sacarosa al 10% o 30% por 3 semanas y un periodo de abstinencia de 36 horas más naloxona. Entre los efectos a nivel cerebral se ha observado una disminución en la liberación de dopamina, aumentando la liberación de acetilcolina y reduciendo la densidad de expresión de los RD2 en el núcleo accumbens [155–157].

En otro protocolo, donde los animales fueron expuestos a un alimento alto en azúcar, estos desarrollaron rápidamente un condicionamiento de preferencia de lugar. Cuando se trató de extinguir la conducta, con choques eléctricos en el mismo lugar donde antes recibían la recompensa, estos animales fueron más resistentes a aprender que ahora era un lugar aversivo [158]. Lo anterior sugiere que ante la abstinencia o el retiro de este reforzador, en estos animales fortalecen el aprendizaje del lugar en donde se obtenía el alimento.

Los síntomas de abstinencia inducidos por la retirada de sacarosa (10%) se han medido incluso 2 meses después de la última exposición, mediante un protocolo de condicionamiento, donde las ratas siguen palanqueando ante el estímulo condicionado [159] y donde se propone que el enriquecimiento ambiental (poniendo a 4 animales en la misma jaula con objetos novedosos), atenúa esta respuesta exacerbada de la búsqueda de sacarosa después de la abstinencia [160].

En cuanto a protocolos donde se evalúa la abstinencia a alimentos altos en grasa Teegarden y Bale (2007) mantuvieron a ratones bajo un paradigma de preferencia de alimentos altos en grasa o altos en carbohidratos y después los dejaron en un periodo de abstinencia. Encontraron que los animales que preferían la dieta alta en grasa se arriesgaban a salir a un ambiente aversivo (luz) para obtener una pequeña porción del reforzador, más que los animales que preferían la dieta alta en carbohidratos o los animales control [142]. En este trabajo se propone que la dieta alta en grasa, puede funcionar como reductor de estrés o ansiedad.

También se ha reportado que la exposición crónica a una dieta alta en grasa, disminuye la sensibilidad al estímulo estresante, mientras que la retirada (o abstinencia) de dicha dieta, la intensifica [127,152]. En muchos protocolos se menciona que los animales que consumen grasa, presentan los mismos cambios neuronales que presentan los animales que comen azúcar [161], pero los síntomas de abstinencia asociados a los provocados por opiáceos (como se ve en los animales que consumen azúcar) no son observados en animales que consumieron dieta alta en grasa [124,162,163].

4. PLANTEAMIENTO GENERAL DE LA TESIS

Se sabe que las alteraciones en el ciclo sueño y vigilia, produce cambios en el consumo de alimento, particularmente puede inducir el sobreconsumo de alimento palatable; mismo que normalmente tiene alto contenido de azúcar y/o grasa. Cada macronutriente puede afectar el metabolismo y producir obesidad y; además de impactar en el sistema homeostático central, también puede impactar a nivel hedónico y producir conductas asociadas a la adicción con marcadores específicos de activación neuronal (c-Fos) y de plasticidad neuronal (ΔFosB).

4.1 HIPÓTESIS GENERALES

- Los animales sometidos a un protocolo de jet lag social tendrán un mayor consumo de alimento palatable, desarrollaran obesidad y síndrome metabólico.

- Los animales con acceso restringido a alimento palatable, preferirán la dieta alta en grasa que la dieta alta en azúcar, desarrollando en mayor magnitud, conductas asociadas a la adicción, así como mayores niveles de los marcadores neuronales asociados a la adicción (c-Fos y ΔFosB).

- La exposición simultánea al protocolo de jet lag social y la exposición a dieta alta en grasa o azúcar, estos animales tendrán un mayor número de conductas asociadas a la adicción, como la conducta tipo atracón, la anticipación, el esfuerzo y la abstinencia, así como mayores niveles de los marcadores neuronales asociados a la adicción (c-Fos y ΔFosB)..

- Los animales sometidos al protocolo de jet lag social, tendrán un mayor consumo de dieta alta en grasa y presentarán mayor activación de áreas límbicas e hipotalámicas, así como una mayor activación de neuronas MCHérgicas y orexigénicas.

4.2 OBJETIVOS GENERALES

- Desarrollar un modelo de jet lag social en ratas Wistar, mediante la exposición a una rueda de actividad forzada en las primeras 4 horas del ciclo de descanso de la rata.

- Exponer a ratas Wistar al protocolo de jet lag social y combinarlo con dieta de cafetería para determinar si se producen cambios en el consumo de alimento y en parametros metabólicos asociados.

- Determinar qué porcentaje de dieta alta en grasa o dieta alta en azúcar es sobreconsumida por las ratas.

- Exponer crónicamente a una dieta alta en grasa o dieta alta en azúcar para evaluar si se expresan conductas asociadas a la adicción, así como la activación del sistema límbico (c-Fos y ΔFosB).

- Exponer a ratas Wistar a un modelo de jet lag social y exponer a una dieta alta en grasa o dieta alta en azúcar, para determinar la contribución de los diferentes macronutrientes en el desarrollo de a) conductas asociadas a la adicción, b) cambios asociados en el sistema límbico, c) alteraciones metabólicas y d) cambios en el sistema homeostático

5. PUBLICACION #1

5.1 PLANTEAMIENTO DEL PROBLEMA

Dado que el jet lag social es un problema recientemente descubierto en humanos, las consecuencias y riesgos de este problema aún no han sido esclarecidos del todo. No existe un modelo animal que asemeje este problema donde se puedan investigar las consecuencias a corto y largo plazo.

5.2 HIPÓTESIS

1. Las ratas expuestas a actividad forzada de lunes a viernes, desarrollaran jet lag social, teniendo un retraso de fase comparando en días entre semana y fines de semana.
2. Las ratas sometidas a jet lag social cumplirán por lo menos 3 parámetros de síndrome metabólico.
3. Las ratas expuestas a jet lag social consumirán una mayor cantidad de alimento al estar expuestos a la dieta de cafetería en comparación con ratas no expuestas a jet lag social.
4. Las ratas expuestas a jet lag social y a dieta de cafetería cumplirán con más de 3 parámetros de síndrome metabólico.

5.3 OBJETIVO GENERAL

- Desarrollar un modelo animal en rata Wistar de jet lag social.
- Evaluar síndrome metabólico en ratas expuestas a jet lag social.

5.4 OBJETIVOS PARTICULARES

1. Establecer un modelo de jet lag social mediante la exposición a actividad forzada de lunes a viernes y sin manipulación los fines de semana, midiendo las acrofases entre semana y fines de semana.
2. Medir parámetros de síndrome metabólico en ratas expuestas a jet lag social.
3. Exponer a dieta de cafetería a ratas en un protocolo de jet lag social.
4. Medir parámetros de síndrome metabólico en ratas expuestas a jet lag social y a dieta de cafetería.

5.5

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Social jet-lag potentiates obesity and metabolic syndrome when combined with cafeteria diet in rats

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ABSTRACT

Background/Objectives. Modern lifestyle promotes shifted sleep onset and shifted wake up time between weekdays and weekends, producing a condition termed "social-jet lag." Disrupted sleep promotes increased appetite for carbohydrate and fat-rich food, which in long term leads to overweight, obesity and metabolic syndrome. In order to mimic the human situation we produced an experimental model of social-jet lag (Sj-l). With this model, we explored the link between shifted sleep time with consumption of a cafeteria diet (CafD) and the development of obesity and metabolic syndrome.

Subjects/Methods. The first experiment was designed to create and confirm the model of Sj-l. Rats (n = 8–10/group) were exposed to a shifted sleep time protocol achieved by placing the rats in slow rotating wheels from Monday to Friday during the first 4 h of the light period, while on weekends they were left undisturbed. The second experiment (n = 8–12/group) explored the combined effect of Sj-l with the opportunity to ingest CafD. All protocols lasted 12 weeks. We evaluated the development of overweight and indicators of metabolic syndrome. The statistical significance for all variables was set at $P < 0.05$.

Results. Sj-l alone did not affect body weight gain but induced significant changes in cholesterol in metabolic variables representing a risk factor for metabolic syndrome. Daily restricted access to CafD in the day or night induced glucose intolerance and only CafD during the day led to overweight. Sj-l combined with CafD induced overconsumption of the diet, potentiated body weight gain (16%) and promoted 5 of the criteria for metabolic syndrome including high insulin and dislipidemia.

Conclusion. Present data provide an experimental model of social-jet lag that combined with overconsumption of CafD, and maximized the development of obesity and metabolic syndrome. Importantly, access to CafD during the night did not lead to overweight nor metabolic syndrome.

Abbreviations: BMI, body mass index; C, control group; CafD, cafeteria diet; CAF-day, group exposed to CafD during the day; CAF-night, group exposed to CafD during the night; GTT, glucose tolerance test; MetS, metabolic syndrome; s.e.m., standard error of the mean; Sj-l, social jetlag; SJL, social jetlag group; SJL + CAF, group exposed to the protocol of Sj-l and CafD; TAG, triglycerides; WN + CAF, group exposed to slow rotating wheels and CafD in the night; W-night, group exposed to slow rotating wheels during the night.

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1. Introduction

Modern life style promotes a discrepancy of sleep–wake times between workdays and weekends. During weekdays, people need to wake up early due to work or study duties, this is combined with opportunities for leisure during the night, leading to reduced sleep time. During weekends, individuals can sleep in, however social night activities also lead to shifted sleep onset. The difference in wake up time and sleep duration between weekdays and weekends creates a discrepancy between work and free days, between social and biological time, described as "social jetlag" (Sj-l) [1]. An epidemiological study indicates that around 30% in the human population suffers from 2 h or more Sj-l [2]. This condition affects also meal schedules, promoting snacking of high caloric food and is suggested to be a risk factor to develop metabolic alterations, obesity and metabolic syndrome (MetS) [2,3].

Metabolic syndrome comprises a spectrum of conditions that predisposes an individual to medical complications including cardiovascular disease and diabetes type 2, [4,5]. The criteria to diagnose MetS depend on the consulted source [6], however there is agreement that MetS is diagnosed when an individual presents at least 3 of the following criteria: abdominal obesity, hyperglycemia, glucose intolerance, hypertension, hypertriglyceridemia, dyslipidemia, and insulin resistance [3,7–9]. In animal models MetS is induced with the use of high sucrose diet alone or combined with a high fat diet or the use of cafeteria diet (CafD); the criteria used to define MetS are similar to those in humans [10–12].

Sleep disturbance is a main factor contributing to the pathogenesis of MetS [13]. Clinical studies report that short sleepers, including shift workers develop increased appetite for food rich in fat and carbohydrates [14–17] which includes consumption and snacking of CafD [18,19]. Experimental studies indicate that rodents exposed to a CafD develop overweight, increased adiposity, hepatic steatosis, and indicators of MetS [11,20,21]. Moreover, high caloric or high fat diets disrupt circadian rhythms [22,23]. The reciprocal interaction between circadian regulation and metabolism is illustrated with experimental models mimicking disturbed sleep/wake patterns as observed in shiftwork and jet-lag, providing evidence that circadian disruption favors development of overweight and metabolic disease [24–26].

Due to the growing incidence of obesity associated with circadian disruption, the aims of this study were to develop an experimental model for Sj-l in rats by shifting sleep onset between weekdays and weekends and to explore the link of this condition with overweight and MetS. In a first experiment, the influence of Sj-l on rats was examined on activity rhythms, body weight and indicators of MetS. Behavioral recordings confirmed a 2 h shift in general activity acrophase between weekdays and weekends, however subjects did not meet the criteria for MetS. In a second experiment, Sj-l was combined with the opportunity to consume CafD; this combination promoted overconsumption of CafD and potentiated development of obesity and criteria for MetS.

2. Methods and Procedures

2.1. Animals and Housing

Young male Wistar rats weighing 120–140 g were used. Rats were housed in individual acrylic cages (45 cm × 30 cm × 20 cm) placed on tilt sensors, in soundproof ventilated lockers with a 12:12 h light/dark (LD) cycle (lights on at 08.00 h), controlled temperature (22 ± 1 °C), circulating air and free access to water and chow food (Rodent Laboratory Chow 5001, Purina, Minnetonka, MN). Rats exposed to experimental manipulations during the night were acclimated to an inverted light–dark cycle (lights on at 20:00 h) for 2 weeks before starting baseline. Experiments were approved (Project 015-2012) by the committee for ethical evaluation at the Facultad de Medicina UNAM; they conform to international guidelines for the ethical use of animals and were aimed at minimizing the number of animals used and their suffering.

2.2. Experimental Design

2.2.1. Experiment 1. The experimental model of social jet-lag

The first experiment was aimed to develop a rodent model of Sj-l by exposing rats to shifting sleep onset between weekdays and weekends. Rats were randomly assigned to one of 3 groups (n = 8–10 rats/group): (1) Control (C) undisturbed rats; (2) social jet-lag (SJL), rats were located in slow rotating wheels during the first 4 h of the day; and (3) wheel night (W-night), rats were located in slow rotating wheels during the first 4 h of the night. Manipulations were performed from Monday to Friday and during the weekends, all rats remained undisturbed.

2.2.2. Experiment 2. Social Jet-Lag Combined with Access to Cafeteria Diet

This experiment explored the combined effects of Sj-l with the opportunity to eat CafD during the hours of sleep delay. Rats were randomly assigned to one of 4 groups (n = 8–12 animals/group): (1) Sj-l + CafD; rats (SJL + CAF): rats were exposed to the protocol of SJL and CafD was available inside the wheels; (2) only CafD during the first 4 h of the light phase (CAF-day); (2) wheel in the night + CafD during the first 4 h of the night (WN + CAF); and (4) only CafD during the night (CAF-night). Manipulations were performed from Monday to Friday for all the groups.

Rats exposed to the wheels and/or CafD during the night started after 2 weeks adaptation to an inverted light–dark cycle followed by one-week baseline.

Experimental conditions lasted 12 weeks. We evaluated general activity patterns, weight gain and criteria for metabolic syndrome. Blood samples and euthanasia were performed in the first 4 h of the light phase.

2.3. Social Jet-Lag Protocol

During weekdays sleep onset was delayed for 4 h by placing rats at 08:00 am (time of lights on), in slow rotating wheels (33 cm diameter × 33 cm long with four concentric subdivisions), which allow to house four rats individually. Wheels

rotate slowly (1 revolution in 3 min) forcing rats to stay awake. In the wheels, rats can sit, groom, lie down and eat and drink freely [more details in Salgado-Delgado et al. [27,28]]. After 4 h in the wheels (08:00–12:00), rats were returned to their home-cages and remained undisturbed until next day. The same procedure was performed for groups W-night and WN + CAF, which were exposed to the rotating wheels at night onset, which corresponds to the start of normal activity phase. This protocol was performed from Monday to Friday, while during Saturday and Sundays all rats remained in their home cages. Groups SJL + CAF and WN + CAF had access to CafD during the 4 h in the wheel, while SJL and W-night groups had regular chow pellets as food in the wheel.

2.4. Body Weight and Diets

Rats were weighed once a week and body weight gain was calculated for the first 10 weeks. Due to the glucose tolerance test, for which the body weight was affected, weeks 11 and 12 were not considered. All groups had free access to regular chow, which contains 4.07 kcal/g (see Table 1). CafD included palatable food items of varied composition, appearances and texture [29]; this diet is rich in fat and carbohydrates [11]. For the present study, diet was organized in two menus, which were alternated daily (see Table 1). Both menus were accompanied with 12% sugar water; rats had also access to tap water. Consumption of regular chow and CafD was assessed once per week, by weighing the ingested food for 24 h. Consumed calories are reported as a mean ± s.e.m. (standard error of the mean) for weeks 9 and 10.

2.5. Activity Recording and Analysis

General activity in the home-cage was monitored with tilt sensors placed under individual cages (Omnialva SA de CV, México). Behavioral events were collected with a digitized system and automatically stored every minute in a PC for further analysis with the program SPAD9 (Omnialva México) [27]). Double-plotted actograms were obtained by collecting the sum of activity for 15 min intervals. Mean daily activity (± s.e.m.) was obtained for weeks 8–10 of the protocol and is represented as 24 h daily curves. The same data were processed with the cosinor analysis in order to confirm amplitude and acrophase (peak activity values for 24 h) for each data series (SPAD9, Omnialva México). Data for weekdays were analyzed separate from weekends. The cosinor analysis uses the least square method to fit a sine wave to a time series (in this case to 24 h). The formula used was: $Y(t) = M + A\cos(2\pi t/\tau + \varphi) + e(t)$; where Y = collected data; M = mesor; A = amplitude; φ = acrophase; T = period; and e = error at each time.

2.6. Metabolic Syndrome Indicators and Hormonal Determinations

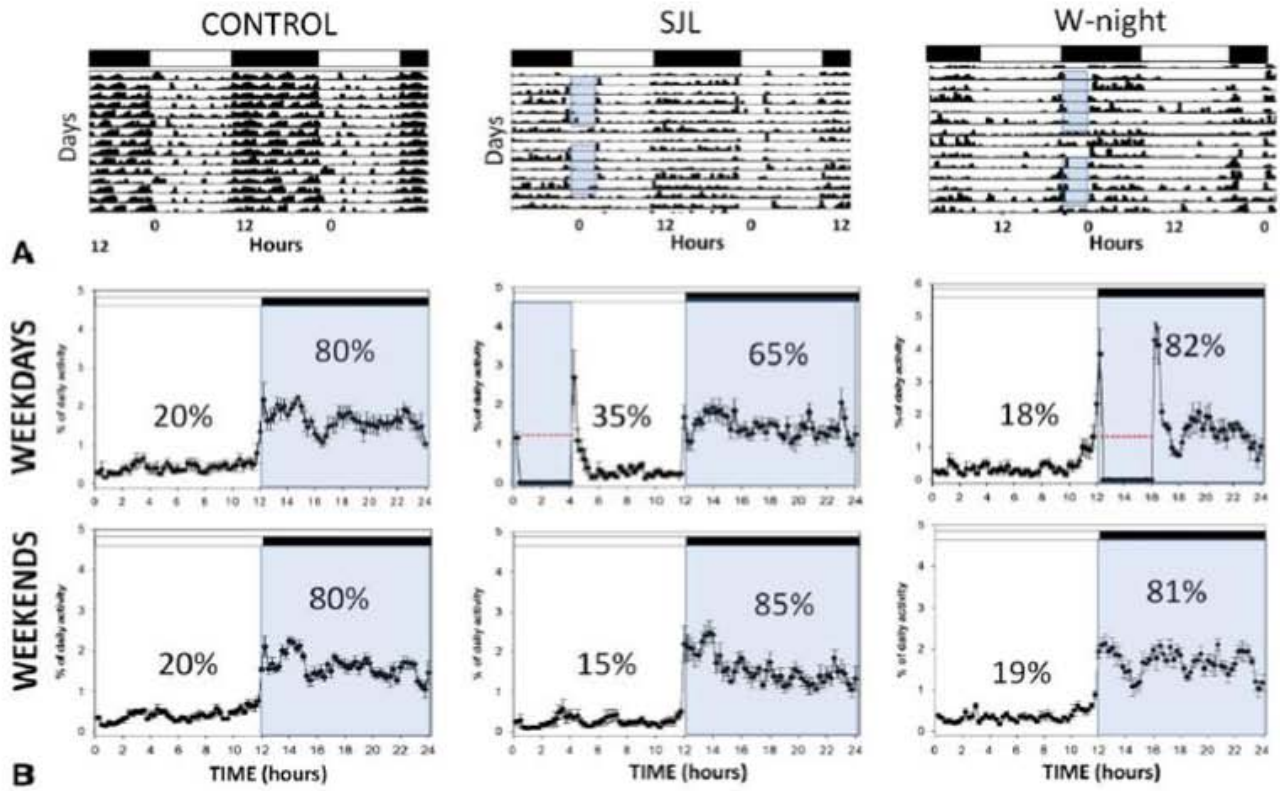
Based on the criteria described for rat models of metabolic syndrome [30], here metabolic syndrome was assumed when animals meet 3 or more of the following criteria: hyperglycemia (>100 mg/dl in fasting), in a glucose tolerance test (GTT) the last point of the curve (after 120 min) should be statistically higher than the basal levels; hyperinsulinemia (>6 ng/dL), obesity (body weight above 10%) and increased abdominal fat (more than 3% of total body weight), high cholesterol (>175 mg/dl), and hypertriglyceridemia (>150 mg/dl).

After 11 weeks in the corresponding protocol an intraperitoneal GTT was performed. Rats were fasted 16 h overnight and starting at 08:00 am, basal glucose levels were determined *via* a small incision in the tip of the tail followed by administration of a glucose solution (1 g glucose/kg body weight in 0.9% saline solution) in the abdominal cavity (see Salgado-Delgado et al. [25]). Glucose strips Ascensia Elite (Bayer, Basel, Switzerland) and a glucometer (Elite, Bayer, Elkhart, IN) were used. Glucose levels were measured 10, 30, 60, 90 and 120 min after glucose administration. At the end of week 12, rats were euthanized with an overdose of sodium pentobarbital (Pisabental, México; 50–90 mg/kg). Triglycerides (TAG) and cholesterol were assessed from blood drops obtained from the cava vein placed on strips Accutrend GCT (Roche, Mannheim, Germany). Blood (3 mL) was obtained from the cava vein for metabolic and hormonal determinations, collected in Vacutainer tubes and centrifuged for 10 min at 3000 rpm, and the resulting serum was stored in 250 μL aliquots at −45 °C for subsequent assays. Insulin was assessed with an ELISA kit for rat/mouse (EZRL-13 K, Millipore, St. Charles, MO) and an absorbance microplate reader (ELx800, Biotek). The mesenteric (abdominal) and retroperitoneal fat pads were dissected bilaterally and weighed [31,32]. In order to ensure unbiased analysis, all samples were codified and the persons in charge of analysis of samples were blind to the treatment.

Table 1 – Nutrient proportion and caloric value of regular Chow and Cafeteria diet.

	Diet composition				
	Food	kcal/g	Fat %	Carbohydrates %	Proteins %
	Chow	4.07	13	57	30
Menu 1	Sausage	1.61	52	32	16
	Chips	5.2	48	47	5
Menu 2	Chocolate cookies	5.0	40	56	4
	Crackers	4.8	39	55	6
Drink	Sugar 12% (kcal/mL)	0.48/mL	0.0	100	0

Values are given in kcal/1 g of food.



AMPLITUDE

	Control	SJL	W- night
WEEK DAYS	0.77 ± 0.04	**0.50 ± 0.07***	0.85 ± 0.02
WEEKENDS	0.80 ± 0.05	0.83 ± 0.08	0.89 ± 0.03

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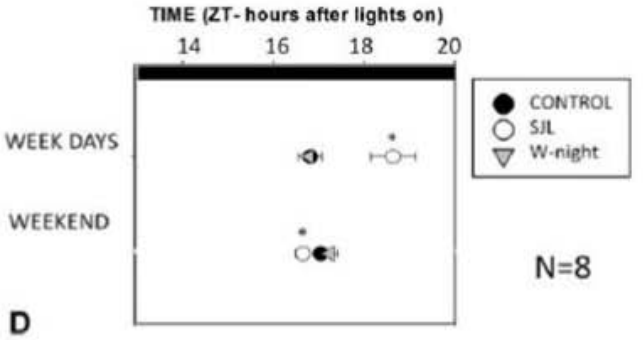


Fig. 1 – Chronic shifted sleep onset induced disruption of daily temporal patterns of activity and confirmed a condition of social jet-lag. (A) Representative examples of weeks 8 and 9 of automatic collection of general activity (actogram) are shown. Horizontal white and black bars indicate day and night phases. Gray rectangles indicate the 4 h intervals when rats were placed in slow rotating wheels and therefore were taken out of their home cages. (B) Mean ± s.e.m. activity profiles for the weekdays (top) and for the weekends (bottom). (C) Mean values ± s.e.m. for amplitude obtained with the cosinor analysis during weekdays and weekends. (D) Acrophases obtained with the cosinor analysis indicated a significant shift during weekdays and weekends. Asterisks indicate statistical difference between the SJL and the other two groups ($P < 0.05$).

2.7. *Statistical Analysis*

Data are presented as mean ± standard error of the mean. In experiment 1 the proportion of day activity, acrophases, daily caloric intake, the proportion of weight gain, glucose, cholesterol, triglycerides, insulin, and abdominal fat were analyzed with a one-way ANOVA comparing the factor groups. In experiment 2 the same variables were compared with a 2 way-ANOVA for the factors time of CafD and activity in the wheels. In both experiments body weight and the GTT were analyzed with a two-way ANOVA for repeated measures for the factor time (weeks or minutes) and the factor groups. All analyses were followed by a Tukey multiple-comparisons post hoc test with α set at $P < 0.05$. Statistical analysis and graphs were elaborated with the program PRISM.6 (GraphPad Software).

3. Results

3.1. *Chronic Shifted Sleep Onset Alters Daily Activity Patterns Creating a Condition of Sj-l*

During baseline all rats exhibited daily rhythms of general activity (data not shown), with a predominant activation during the night (~80%) and less activity during the day (Fig. 1A). SJL rats exhibited in the weekdays a drop of 65% in the night activity (Fig. 1B, top), and this was not observed in the W-night group (one way-ANOVA $F_{(2,20)} = 23.14$; $P < 0.001$). Contrasting, during the weekends no change in the distribution of day–night activity was detected (Fig. 1B bottom; one way-ANOVA $F_{(2,21)} = 0.776$; P = NS).

The cosinor analysis confirmed for weekdays a decreased amplitude for the SJL ($P < 0.05$), while the control group and the W-night group obtained similar values (one way ANOVA $F_{(2,21)} = 17.33$; $P < 0.001$). During weekends, the amplitude was similar between the three groups. (Fig. 1C). The daily acrophase (peak activity values in 24 h) in weekdays was similar for control and W-night rats, while the SJL group showed a delay of ~2 h (one way ANOVA $F_{(2,17)} = 10.6$; $P = 0.003$). For the weekends the control and W-night groups maintained similar acrophases, while the SJL showed a phase advance of 0.6 h (Fig. 1D; $F_{(2,23)} = 4.203$; $P = 0.029$). The shift of acrophases from weekdays to weekends represented a difference of 2.25 h for the SJL group.

3.2. *Chronic Sj-l Does Not Induce Obesity Neither Metabolic Syndrome*

All rats showed progressive body weight gain along the 10 weeks of the protocol (Fig. 2A). The two-way ANOVA for repeated measures indicated a significant effect for the interaction of weeks and group factors ($F_{(18,162)} = 9.37$, $P < 0.0001$). Both groups exposed to 4 h in the wheel gained less weight than the control group; this was especially evident in the W-night group (SJL [−5.7%] and W-night [−33.1%] from control; Fig. 2A and B) and this difference in body weight gain was not due to a change in daily pellet consumption (Fig. 2C; $F_{(2,27)} = 1.255$, $P = 0.3011$). In the GTT (Fig. 2D) the W-night group showed glucose intolerance according to the last point of the curve (Table 2).

After 12 weeks of experimental protocol blood samples were collected and indicators for metabolic syndrome were explored (Table 2). For all measurements, only not for abdominal fat, the one way ANOVA indicated significant differences between groups (Supplementary Table 1); however, for the SJL no values reached the criteria for a MetS (Table 2) while for the W-night group only the last point in the GTT reached the level for MetS.

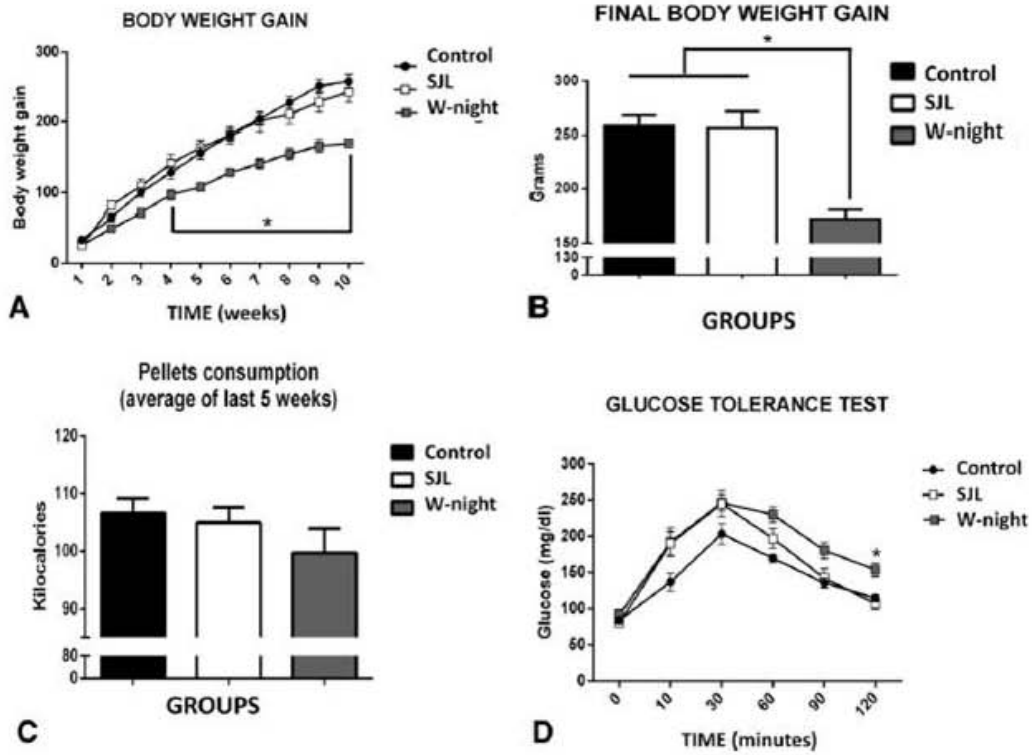


Fig. 2 – Chronic social jet-lag does induce obesity. Data are shown as mean ± s.e.m. (A) Body weight gain in control, SJL and W-night conditions along the experimental protocol. (B) After 10 weeks significant effects in body weight gain were observed among groups ($F_{(2,24)} = 11.69$, $P = 0.0003$). (C) Daily kcal intake (chow) for weeks 9 and 10 of the protocol. (D) The glucose tolerance test indicated significant difference among groups. Asterisks indicate significant difference from the control ($P < 0.05$).

Table 2 – Metabolic profile for rats exposed to daily 4 h forced activity and their controls.

METABOLIC VARIABLES	CONTROL	SJL	W-night
Fasting Glucose (mg/dL)	75.9 ± 1.2[a]	84.2 ± 3.3[ab]	85.6 ± 2.9[b]
Glucose in last point GTT (mg/dL)	114.9 ± 4.6[a]	107.1 ± 7.8[a]	153.8 ± 9.1[b]
Insulin (ng/dL)	2.0 ± 0.2[a]	4.08 ± 0.4[ab]	5.15 ± 0.7[b]
Cholesterol (mg/dL)	166.57 ± 0.9[a]	175.87 ± 2.4[b]	159.71 ± 1.9[a]
Triglycerides (mg/dL)	122.57 ± 8.4	99.55 ± 6.8	141.6 ± 8.1
Abdominal fat (%)	2.33 ± 0.2	1.85 ± 0.[a]	2.38 ± 0.2
Body weight (%from control)	257.9 ± 10.0[a]	255.6 ± 15.9[a] (-5.7%)	172.42 ± 7.9[b] (-33.1%)

Different letters indicate statistical difference between groups. Shaded cells indicate values that reached or surpassed metabolic syndrome criteria.

3.3. *Cafeteria Diet During the Rest Phase alone or Combined with Shifted Sleep Affected the Daily Patterns of Activity*

During baseline all rats exhibited daily rhythms of general activity (data not shown), with major activation during the night (80% ± 5). Actograms of 2 weeks of the experimental protocols are shown in Fig. 3A. On weekdays CAF-day rats increased the proportion of general activity during their sleep phase, and this was potentiated in SJL + CAF (Fig. 3A and B, top). In contrast, in CAF-night and WN + CAF rats the proportion of day night activity was not modified. The two-way ANOVA indicated a significant effect for the interaction of timing of CafD and activity in the wheel ($F_{(1,34)} = 8.93$; $P < 0.005$). In the weekends (Fig. 3B bottom) this proportion improved for CAF-day and SJL + CAF, however it did not reach values observed in groups receiving CafD in the night ($F_{(1,31)} = 15.07$; $P < 0.001$).

The cosinor analysis confirmed for weekdays a decreased amplitude of CAF-day and SJL + CAF groups compared with groups CAF-night and WN + CAF (two-way ANOVA interaction of timing of CafD × activity in the wheels $F_{(1,31)} = 5.25$; $P < 0.03$). During weekends this effect was diminished, however the SJL + CAF still showed lower amplitude than the other groups (Fig. 3C; two-way ANOVA interaction of timing of CafD × activity in the wheels $F_{(1,31)} = 9.77$; $P < 0.004$). In weekdays a significant delay in the daily acrophase of both groups, CAF-day and SJL + CAF, was observed ($F_{(1,34)} = 1.67.7$; $P < 0.001$), while CafD or activity in the wheels at night did not change the acrophase. During weekends, acrophases of the CAF-day and SJL + CAF groups shifted back to similar values as the night groups. The shift of acrophases from weekdays to weekends represented a difference of 2.77 h for the CAF-day group and of 3.4 h for the SJL + CAF group (Fig. 3D).

3.4. *Sj-l Combined with CafD Potentiates Obesity and Criteria for Metabolic Syndrome*

Along the 10 weeks in the experimental protocol both groups exposed to CafD during the day gained more body weight than groups ingesting CafD during the night (Fig. 4A; time × group $F_{(7,324)} = 10.67$, $P < 0.0001$), resulting in a significant body weight gain for rats ingesting CafD during the day (CAF-day [+7.8%], SJL + CAF: [+16%] from the control), while rats exposed to CafD during the night had gained significantly less (CAF-night [−17%]; WN + CAF [−24.3%] from control; Fig. 4B). The two-way ANOVA indicated a significant effect of time of CafD ($F_{(1,36)} = 81.12$, $P < 0.0001$) and the interaction time of CafD × wheels ($F_{(1,36)} = 4.4$, $P < 0.04$).

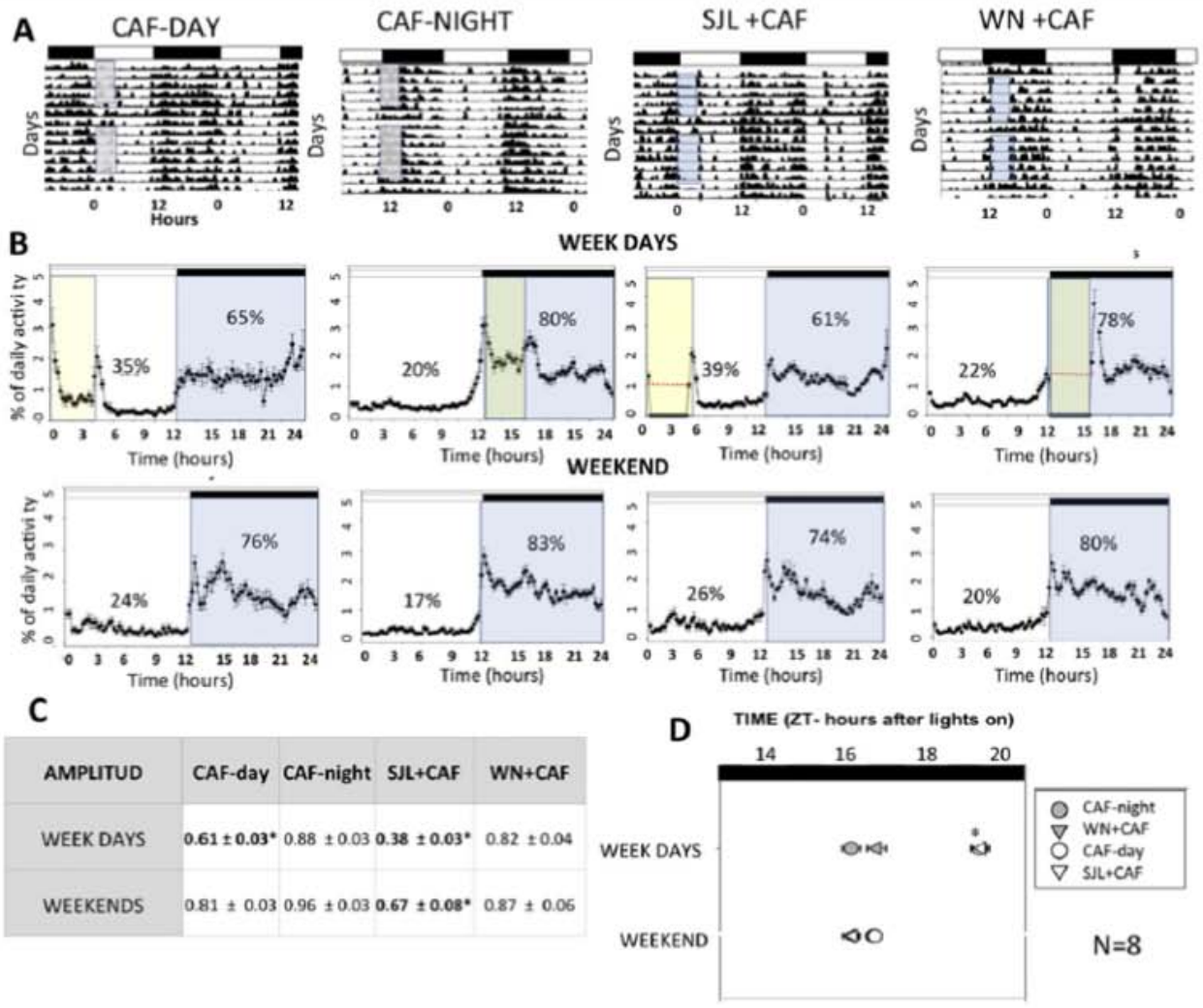
Total daily kcal intake considering Chow and CafD were similar (105–110 kcal/day) for all groups, except the SJL + CAF that consumed up to 123 kcal daily and 62% of the daily intake was provided by the CafD (Fig. 4C). The two-way ANOVA indicated a significant effect for time of CafD ($F_{(3,72)} = 3.24$, $P < 0.026$) and interaction of time of CafD × wheel ($F_{(3,72)} = 7.17$, $P < 0.0003$).

Metabolic indicators after 12 weeks of experimental protocol are shown in Table 3 and results of the two-way ANOVA for all variables are shown in Supplementary Table 2. According to the criteria set in this study for MetS, CAF-night did not develop MetS, CAF-day and WN + CAF rats reached 3 criteria, and the group SJL + CAF reached 5 criteria (Table 3), indicating that the combination of Sj-l combined with CafD has the worst outcome.

4. Discussion

Here, we used during weekdays slow rotating wheels in order to expose rats to a shifted sleep onset. Behavioral data indicate that our experimental model caused a similar condition as described for Sj-l in humans [2,33] because during the weekdays, it induced a phase delay of the acrophase and dampening of the amplitude for general activity and during the weekends the acrophase shifted back to similar values as in undisturbed rats. Sj-l caused a weekly shift of activity/sleep onset of 2 h and induced some metabolic disturbances that were statistical different from the control group, but did not reach our criteria for MetS. Groups exposed to CafD reached 2–3 criteria for MetS, excluding Caf-night; however, the combination of CafD with Sj-l delayed the weekdays' acrophase for more than 3 h, leading rats to overconsume CafD, increasing their bodyweight, and the number of criteria for MetS.

With this protocol, we mimicked the condition of going late to sleep without modifying the wake up time, not affecting the total amount of sleep and thus producing a shifted sleep/wake timing. This sleep delay promoted food



AMPLITUD	CAF-day	CAF-night	SJL+CAF	WN+CAF
WEEK DAYS	**0.61 ± 0.03***	0.88 ± 0.03	**0.38 ± 0.03***	0.82 ± 0.04
WEEKENDS	0.81 ± 0.03	0.96 ± 0.03	**0.67 ± 0.08***	0.87 ± 0.06

Fig. 3 – The 4 h access cafeteria diet at the start of the rest phase, alone or combined with SJL disrupts the daily activity patterns of activity. (A) Representative examples for week 8 and 9 of automatic collection of general activity (actogram) are shown for rats exposed to CafD during the day (CAF-day) or during the night (CAF-night) or exposed to wheels combined with CafD during the day (SJL + CAF) or during the night (WN + CAF). Horizontal white and black bars indicate day and night phases. Gray rectangles indicate the 4 h intervals when rats were placed in slow rotating wheels and received CafD; yellow rectangles indicate access to CafD. (B) Mean ± s.e.m. activity profiles for the weekdays (top) and for the weekends (bottom). (C) Amplitude obtained with the cosinor analysis indicated low amplitude for the CAF-day and SJL + CAF groups. (D) Acrophases for weekdays indicated a shift of more than 2 h between weekdays and weekends for CAF-day and SJL + CAF. Asterisks indicate statistical difference from the other groups ($P < 0.05$).

intake in the rest hours which is known to be detrimental for metabolism [34]. To our knowledge, this is the first experimental model of Sj-l proposed in rodents. It is important to note that in W-night animals this shift was not observed because they were exposed to the wheel in their normal activity phase. W-night animals gained less body weight as compared with controls and SJL group, confirming that the circadian system prepares individuals to respond efficiently and according to the day–night cycle. Previous models have tested effects of acute sleep deprivation [35,36] showing that deficient sleep is sufficient for altering metabolic balance in humans [37] and rodents [24,38–40], suggesting that shifted sleep may represent a novel risk factor for type 2 diabetes and MetS [41]. In the human population a high number of people suffer from Sj-l, however only a positive correlation between the magnitude of the lag and the severity of overweight was found when individuals had a high BMI [1,2]. Rutters et al. [42] described in healthy participants aged 18–55 years presenting Sj-l, increased levels of cortisol which is thought to predispose to visceral obesity and other metabolic diseases such as cardiovascular risk.

In our study SJL rats did not develop overweight, however were affected in their metabolic state, thus here we confirm that a chronic shift of sleep onset is sufficient to induce

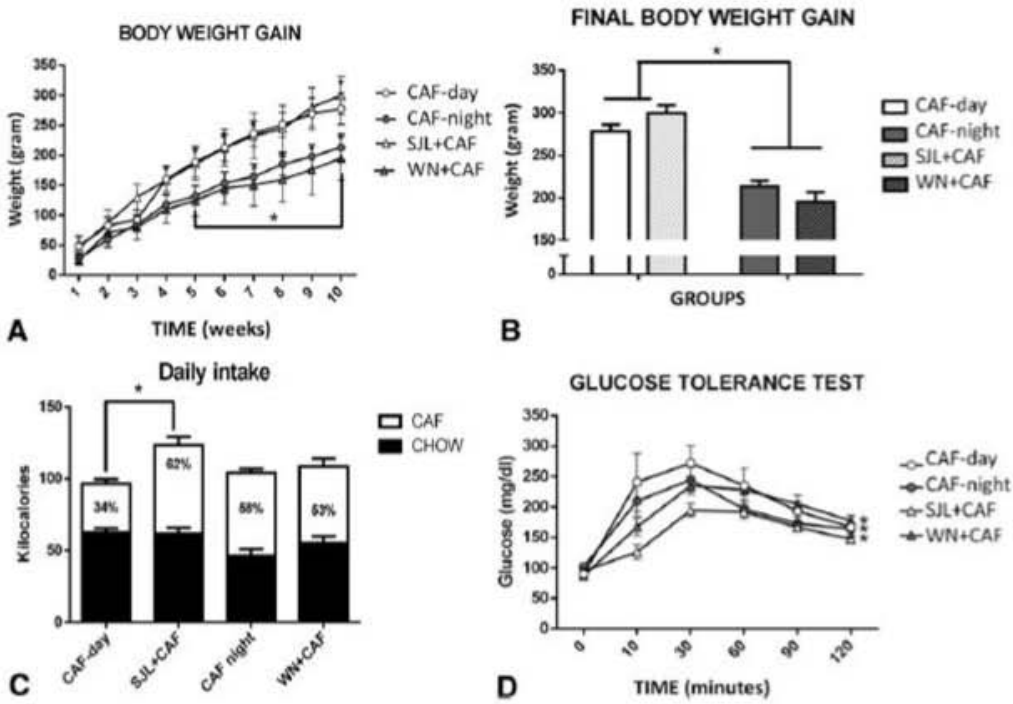


Fig. 4 – Cafeteria diet and activity in the wheel had a day–night differential outcome on body weight. Data are shown as mean ± s.e.m. (A) Body weight gain in rats ingesting CafD during the day (CAF-day), during the night (CAF-night), exposed to forced activity combined with CafD during the day (SJL + CAF) or during the night (WN + CAF) and their controls for the first 10 weeks of experimental protocol. The two-way ANOVA for repeated measures indicated a significant interaction of groups and weeks ($F_{(36,324)} = 8.57$, $P < 0.0001$). (B) After 10 weeks significant effects in body weight gain were observed among groups; the two-way ANOVA indicated a significant effect for the interaction of time and treatment ($F_{(2,54)} = 15.01$, $P < 0.0001$). (C) Daily kcal intake (chow + CafD) for weeks 9 and 10 of the protocol. The two-way ANOVA indicated a significant interaction of time and treatment on the total amount of ingested calories/day ($F_{(4,90)} = 71.38$; $P < 0.0001$). (D) The glucose tolerance test was altered in all groups ingesting CafD; the two way ANOVA indicated effects for the factor treatment ($F_{(4,35)} = 5.39$, $P < 0.0017$). Asterisks indicate significant difference from the control ($P < 0.05$).

metabolic alterations that prime individuals to develop a MetS, especially when combined with ingestion of a high caloric diet.

The use of CafD is a good model to induce obesity and MetS in rats, because it accurately reflects the variety of highly palatable, energy dense foods that are available in modern society [11,43]. Also, this diet promotes a voluntary hyperphagia resulting in rapid weight gain, inducing prediabetic parameters such as glucose and insulin intolerance [21,43]. The use of CafD is controversial [29,44]; it is argued that the choice and diversity of foods make it impossible to control the composition of the diet consumed by individual rats [44]. However, with the approach in the present study, measuring the daily consumption and calculating the intake of each component in the menus according to nutritional values provided by the manufacturer, the variations among individuals and within groups were relatively small. Here we provided CafD for only 4 h per day trying to model the snacking on energy dense foods associated with a delayed sleep pattern, while access to regular chow was *ad libitum* throughout the 24 h. This 4 h daily brief CafD exposure was sufficient to elicit MetS criteria in all groups, and when combined with Sj-l, the outcome was potentiated.

Several studies show that activity and/or food shifted to the rest phase causes circadian disruption, overweight and/or metabolic disturbance, while activity and/or food in the activity phase has a protective and positive effect on body weight and metabolism [24,25,34,45,46]. Present results agree partially with such findings; rats exposed to the CafD and/or wheels at night gained less weight than rats exposed to CafD and/or the wheels in the day. This differential effect on body weight and on metabolic balance is mediated by the circadian system; by preparing the digestive system for digestion and absorption and preparing tissues to efficiently up take energy. Present findings are in agreement with observations by other groups using timed high fat diet [47–50]. Moreover, CafD alone during the day was sufficient to induce a shift of the activity acrophase in a similar direction and magnitude as SJL in rats, supporting previous studies indicating that high/fat-high sugar diets can lead to circadian disruption [51–57].

5. Conclusion

Modern life style has created conditions to promote Sj-l, especially in young people, making them prone to shift their

Table 3 – Metabolic profile for rats exposed to daily cafeteria diet or the combination of cafeteria diet with forced activity for 4 h scheduled in the day or in the night, and their controls.

METABOLIC VARIABLES	CAF day	CAF night	SJL+CAF	WN+CAF
Fasting Glucose (mg/dL)	88.7 ± 3.8	98 ± 4.6	87.4 ± 5.7	85.2 ± 4.5
Glucose in last point of GTT (mg/dL)	166.7 ± 18.8	163.6 ± 9.1	147.3 ± 4.2	177.2 ± 8.8
Insulin (ng/dL)	7.15 ± 0.8^{ab}	4.86 ± 0.4^{a}	8.77 ± 1.2^{ab}	9.7 ± 0.6^{b}
Cholesterol (mg/dL)	168.3 ± 2.8	161.8 ± 1.5	184.4 ± 10.4	163.6 ± 5.2
Triglycerides (mg/dL)	151.07 ± 8.7^{ab}	143.5 ± 12.1^{a}	195.6 ±12.9^{b}	291.6 ± 21.0^{c}
Abdominal fat (%)	2.48 ± 0.1^{a}	2.74 ± 0.1^{ab}	3.21 ± 0.2^{b}	2.65 ± 0.1^{ab}
Final body weight gain (gr)	277.95 ± 8.3^{a} (+7.7%)	213.13 ± 7.2^{b} (-17.3%)	299.5 ± 9.9^{a} (+16.1%)	195.25 ± 11.4^{b} (-24.2%)

Different letters indicate statistical difference between groups. Shaded cells indicate values that reached or surpassed metabolic syndrome criteria.Different letters indicate statistical difference between groups. Shaded cells indicate values that reached or surpassed metabolic syndrome criteria.

sleep time and to snack during the hours of sleep delay. We have developed an experimental model of Sj-l that gave a similar outcome of shifted sleep time as observed in the human population. A strength in this study is this model with the slow rotating wheels, because in addition to keeping animals awake, it requires that they do something and so they tend to increase food intake. With this model we show that Sj-l is sufficient to cause mild metabolic changes that represent risk factors priming individuals to develop MetS. It is however necessary that Sj-l is combined with an additional factor as CafD to trigger obesity and MetS, similar to what is observed in the human population. The use of cafeteria diet has shown to be very effective to promote overweight and obesity in experimental animals; it is, however, a weakness that the control and effects of specific diet components are not possible. Therefore, further studies will need to better explore the contribution of specific diet components and especially the impact of the timing of food intake.

Based on our findings we suggest that epidemiological studies following populations at risk of circadian disruption should not only monitor the sleep delay, but also explore the timing of food ingestion as an additional risk factor for obesity and MetS.

Author Contributions

EEB: design and conduct of the study, data collection, analysis and interpretation, manuscript writing

MVR: design and conduct of the study data collection and analysis

IOR: conduct of the study, data collection and analysis

MAC: data analysis

RMB: manuscript writing

CE: design of the study, analysis and interpretation of data, manuscript writing

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Disclosure Statement

The authors declare that they have no potential conflicts of interests with any of the authors in relation to the work described.

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6. PUBLICACION #2

6.1 PLANTEAMIENTO DEL PROBLEMA

El consumo de dietas tipo cafetería es común en poblaciones humanas. En estudios experimentales con animales, se ha observado consistentemente que este paradigma alimentario induce sobreconsumo, así como problemas metabólicos. La dieta de cafetería tiene alimentos altos en azúcar y altos en grasa, por lo que no se sabe si tales consecuencias son provocadas por azúcar o grasas específicamente y en qué magnitud. Se ha propuesto que el consumo de alimentos palatables (azúcar y/o grasa) llevan al desarrollo de conductas similares a una adicción, impactando áreas asociadas a la recompensa y creando cambios plásticos en el sistema de recompensa.

6.2 HIPÓTESIS

1. Las ratas expuestas a mayores porcentajes de grasa o azúcar, tendrán un mayor sobreconsumo que aquellas que tienen un bajo porcentaje de azúcar o grasa.
2. Las ratas expuestas agudamente a dieta alta en grasa consumirán una mayor cantidad de alimento que las expuestas a dieta alta en azúcar, lo que estará asociado a una mayor expresión de c-Fos y ΔFosB en el núcleo accumbens, la corteza prefrontal y la corteza insular.
3. Las ratas expuestas crónicamente a dieta alta en grasa desarrollaran mayor conducta tipo atracón (más del doble del consumo del control), anticipación (mayor actividad locomotora una hora previa al estímulo) y mayores conductas de esfuerzo que las expuestas a dieta alta en azúcar.
4. Las ratas expuestas crónicamente a la dieta alta en grasa tendrán mayor expresión de c-Fos y ΔFosB en el núcleo accumbens, la corteza prefrontal y la corteza insular que las ratas expuestas crónicamente a dieta alta en azúcar.
5. Las ratas en abstinencia de dieta alta en grasa tendrán mayor anticipación y conductas de esfuerzo que las ratas en abstinencia de dieta alta en azúcar.

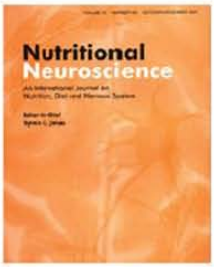
6. Las ratas en abstinencia de dieta alta en grasa tendrán mayor expresión de c-Fos y ΔFosB en el núcleo accumbens, la corteza prefrontal y la corteza insular que las ratas en abstinencia de dieta alta en azúcar

6.3 OBJETIVO GENERAL

- Comparar conductas asociadas a la adicción y cambios en el sistema de recompensa en animales con exposición aguda o crónica de dieta alta en azúcar o dieta alta en grasa.

6.4 OBJETIVOS PARTICULARES

1. Determinar el porcentaje de sobreconsumo de dietas altas en azúcar y dietas altas en grasa; calculado mediante el porcentaje en gramos que se añade al alimento estándar que normalmente ingieren las ratas.
2. Evaluar el consumo de dieta alta en azúcar o dieta alta en grasa ante una sola exposición (aguda) y evaluar los cambios producidos en el núcleo accumbens, la corteza prefrontal y la corteza insular, mediante la proteína c-Fos y la proteína ΔFosB.
3. Evaluar la conducta tipo atracón (más del doble del consumo del control), anticipación (mayor actividad locomotora una hora previa al estímulo) y conductas de esfuerzo, después del consumo crónico (4 semanas) de dieta alta en azúcar o dieta alta en grasa.
4. Evaluar cambios producidos en el núcleo accumbens, la corteza prefrontal y la corteza insular, mediante la proteína c-Fos y la proteína ΔFosB en ratas expuestas expuestas crónicamente a dietas altas en azúcar o en grasa.
5. Evaluar anticipación y conductas de esfuerzo, después de una semana de abstinencia de dieta alta en azúcar o dieta alta en grasa.
6. Evaluar los cambios producidos en el núcleo accumbens, la corteza prefrontal y la corteza insular, mediante la proteína c-Fos y la proteína ΔFosB después de una semana de retirar la dieta alta en azúcar o alta en grasa.



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Fat rather than sugar diet leads to binge-type eating, anticipation, effort behavior and activation of the corticolimbic system

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Fat rather than sugar diet leads to binge-type eating, anticipation, effort behavior and activation of the corticolimbic system

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ABSTRACT

Objectives: One factor contributing to the development of obesity is overeating palatable food. The palatability of food is driven by specific energy yielding combinations and flavor profiles that may contribute to its overconsumption. In rodents, restricted access to palatable food (PF) is a strong stimulus to trigger binge-type eating behavior (BTE), food anticipatory activity (FAA), effort behaviors and withdrawal symptoms. This is accompanied by plastic changes in corticolimbic areas associated with motivation and reward responses. Palatable food contains mainly a mixture of fat and sugar, thus, the contribution of each macronutrient for the behavioral and neuronal changes is unclear.

Methods: In this study, Wistar rats were exposed to restricted access to 50% fat rich diet (FRD) or 50% sugar rich diet (SRD) in order to compare the intensity of BTE, FAA, effort behaviors and withdrawal responses.

Results: In corticolimbic areas, c-Fos activation and ΔFosB accumulation were evaluated. After an acute exposition, rats ate more SRD than FRD, but FDR stimulated higher c-Fos. After chronic administration, the FDR group exhibited higher levels of BTE and FAA; this was associated with higher c-Fos and accumulation of ΔFosB in the corticolimbic system. Similar effects in the FRD group were observed after one week of withdrawal.

Discussion: Present data indicate that the fat rich diet is a stronger stimulus than the sugar rich diet for the development of wanting behavior for reward and the underlying plastic changes in the corticolimbic system. The differential effects may be due to the differing caloric density of the diets.

Abreviations: BTE: binge-type eating; CHOW: control group that received only chow food; CORE: accumbens core; FRD: or rich diet; ILCx: infralimbic cortex; INSCx: insular cortex; PF: palatable food; PLCx: prelimbic cortex; SHELL: accumbens shell; SRD: sugar rich diet; WD: withdrawal

KEYWORDS

ΔFosB; binge-type eating; corticolimbic system; fat rich diet; sugar rich diet; Palatable food; effort behavior; anticipatory activity

Introduction

Obesity is a worldwide risk factor for developing diabetes and cardiovascular disease. One factor contributing to the development of obesity is overeating due to compulsive behaviors to certain foods, especially high caloric palatable food (PF), which can lead to loss of control and diet-induced hyperphagia including binge eating disorder [1].

In experimental models, restricted access to PF favors the development of binge-type eating (BTE) [2], of food anticipatory activity (FAA) [3] as well as effort behaviors to obtain the diet [4]. When the diet is withdrawn, animals exhibit persistent FAA and effort behaviors [5]. Due to all these behavioral changes observed in rodents, it is suggested that PF can lead to an increased 'wanting' desire for the diet. Wanting was previously described by Berridge et al. (2009) as a component of reward and is described as 'an incentive motivation that promotes approach toward and consumption of rewarding food' [6], in this case, for the PF.

The definition of BTE is controversial. For rodent models the consensus is that animals should develop hyperphagia associated to previous caloric deprivation and/or limited access to palatable food [7]. Therefore, a criterion to measure BTE is that animals should eat at least a 2-fold increase of calorie intake, as compared to the control group during the same interval and in similar conditions [7]. Caloric deprivation or limited access to food also leads to FAA, characterized by a progressive increase of general activity, increased arousal, foraging, approaches to the food bin and food-seeking preceding access to scheduled food access [8]. The intensity of FAA can be seen as an indirect indicator of motivation for seeking food; because FAA is not observed in satiated *ad libitum* fed rodents, while FAA increases when the time

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of food access is reduced [9]. Learning, memory and decision-making are involved in the development of FAA [9], therefore, Berridge has suggested that learning is part of the food reward component [6]. Effort behaviors are usually evaluated with an operant task that determines a breakpoint to obtain the PF [10]. Effort is also observed when animals are challenged to an aversive condition, where it is required to trespass an electrified floor to obtain the diet [11]. The effort that animals are willing to exert is used as an indicator of motivation for the diet. Previous studies have suggested the use of a wire-mesh box containing the diet inside the box and to quantify behaviors directed to obtain the PF. This method entices effort behavior to obtain the food, avoids an aversive condition for animals and does not require previous operant training [12,13].

In rodents, restricted access to PF produces activation in areas of the corticolimbic system, as evaluated with the expression of the protein c-Fos, which is a marker of neural activation in response to a stimulus [14]. In animals exhibiting FAA to regular or palatable food, the c-Fos protein in brain areas involved in motivation and reward is increased [3]. Restricted access to PF also produces enforcement of synaptic connectivity in the prefrontal cortex, the nucleus accumbens, the ventral tegmental area and the insular cortex, such plastic changes are suggested to underlie the development of addiction-like behaviors [15,16]. The protein ΔFosB is a transcription factor that favors dendritic growth, and the enforcement of synaptic contacts. Therefore, the accumulation of ΔFosB in corticolimbic areas is used as an indicator of plastic changes associated with several conditions including stress, drugs and PF [17].

Experimental models with rodents have used cafeteria diet, high fat and/or high sugar diets in order to explore the mechanisms associated with PF consumption [18–20]. PF contains high proportions of sugar, fat or a combination of different percentages of both components. Previous evidence has pointed out that fat and sugar exert a differential effect on brain areas involved in homeostatic and hedonic responses [21–23]. Animal models investigating food addiction using either a sugar-model, a fat-model, or a sweet-fat model have encountered conflicting findings [24]; while sugar models have described withdrawal, associated symptoms, this is not clear in fat diet models [24]. Differences may depend on the specific properties of the diets including caloric contribution, detection, absorption and metabolism of each macronutrient [25–28]. In order to gain a better understanding, this study explored in rats the differential response in behavior and corticolimbic structures to the scheduled intermittent access to a fat rich diet (FRD) or a sugar rich diet (SRD). The intensity of BTE, FAA and effort behaviors were evaluated in rats after an acute or after a chronic exposure to FRD or SRD, as well as after one week of withdrawal (WD) from the diet. Also, the differential response in corticolimbic areas was evaluated with c-Fos and with the accumulation of ΔFosB.

Methods and procedures

Animals and housing

Male Wistar rats weighing 190–210 g were housed in individual acrylic cages (45 cm × 30 cm × 20 cm) placed on tilt sensors, in ventilated soundproof lockers with a 12:12 h light/dark cycle (lights on at 08:00 h), controlled temperature (22 ± 1°C) and free access to water and chow food (Rodent Laboratory Chow 5001, Purina, Minnetonka, MN). The committee for ethical evaluation approved experiments; they conform to international guidelines for the ethical use of animals and were aimed at minimizing the number of animals used and their suffering.

Diets

Sugar rich diet (SRD) was made by adding refined sugar to pulverized normal chow food. SRD pellets were elaborated with different proportions of sugar: 10% SRD (90 g chow + 10 g sugar), 25% SRD (75 g chow + 25 g sugar), 50% SRD (50 g chow + 50 g sugar) and 75% SRD (25 g chow + 75 g sugar). Likewise, Fat Rich Diet (FRD) was made with pulverized normal chow food plus different proportions of lard: 10% FRD (90 g chow + 10 g lard), 25% FRD (75 g chow + 25 g lard), 50% FRD (50 g chow + 50 g lard) and 75% FRD (25 g chow + 75 g lard). The kilocalories and percentage of macronutrients that each diet contributes are reported in Table 1.

Experimental design

Experiment 1. Selection of the percentage of sugar rich diet and fat rich diet

To establish which percentage of SRD and FRD was consumed in the highest amount by rats and may represent better a PF, varying % of fat or sugar were incorporated into the diets. Rats had *ad libitum* access to regular Rodent Laboratory Chow along the 24 h, while access to the PF was restricted to 1 h daily for 4 weeks. PF was placed in the feeders in the rest phase 4 h after lights on (from 12 to 13 pm). The daily consumption of the corresponding PF diet was daily recorded. Rats were randomly assigned to one of 8 groups ($n = 10$ rats/group): (1) 10% SRD; (2) 25% SRD; (3) 50% SRD; (4) 75% SRD; (5) 10% FRD; (6) 25% FRD; (7) 50% FRD and

Table 1. Percentage of macronutrients in gram and in kilocalories for Chow, sugar rich diet (SRD) and fat rich diet (FRD).

	Protein		Fat		Carbohydrate		Other elements		
	% (g)	% (kcal)	% (g)	% (kcal)	% (g)	% (kcal)	% (g)	% (kcal)	Kcal/g
CHOW	29.06	28.5	6.1	13.5	59.01	58	5.83	0	4.07
SRD (10%)	26.11	25.73	5.5	12.16	63.11	62.11	5.28	0	4.06
SRD (25%)	21.75	21.47	4.58	10.17	69.26	68.36	4.41	0	4.05
SRD (50%)	14.5	14.38	3.05	6.8	79.5	78.82	2.95	0	4.03
SRD (75%)	7.25	7.22	1.53	3.42	89.75	89.36	1.47	0	4.02
FRD (10%)	26.11	22.88	15.5	30.56	53.11	46.56	5.28	0	4.56
FRD (25%)	21.75	16.41	29.58	50.2	44.26	33.39	4.41	0	5.30
FRD (50%)	14.5	8.88	53.05	73.06	29.5	18.06	2.95	0	6.53
FRD (75%)	7.25	3.73	76.53	88.67	14.75	7.6	0.97	0	7.76

(8) 75% FRD (n = 10 per group). The diet that was eaten in the highest amount by rats during the 4th week (statistically different from other diets) was chosen for the next experiments.

Experiment 2. Acute response to the palatable diets

In order to explore the acute effects of ingesting a SRD or FRD on corticolimbic structures, rats maintained *ad libitum* with a regular chow diet received for one occasion a 50% SRD or a 50% FRD. The diet was provided for one hour in the rest phase, 4 h after lights on (from 12 to 13 pm). The diets were novel to all animals. Rats were randomly assigned to one of 3 groups: (1) Control (CHOW) undisturbed rats; (2) 50% SRD rats and (3) 50% FRD rats, (n = 10 per group). The amount of ingested food was assessed. Brains (n = 8 per group) were obtained 1.5 h after access to the diet in order to evaluate c-Fos and ΔFosB.

Experiment 3. Chronic response to the diets

This experiment was designed to explore the differential effect of a chronic restricted access to SRD or to FRD along 4 weeks. Rats were randomly assigned to one of three groups: (1) Control (CHOW) undisturbed rats; (2) Daily 1 h restricted access to 50% sugar rich diet (SRD) or (3) to 50% fat rich diet (FRD). Rats had *ad libitum* access to regular chow, while access to the PF was restricted to 1 h daily for 4 weeks. PF was placed in the feeders in the rest phase 4 h after lights on (from 12 to 13 pm). Regular food and PF consumption were evaluated daily from Monday to Friday. BTE (n = 10–18 per group) and FAA (n = 10–12 per group) were evaluated along the four weeks of exposition; while effort behavior (n = 10–12 per group) was evaluated during the fourth week of daily exposure. At the end of the 4th week brains (n = 8–10 per group) were obtained, 1.5 h after diet access in order to evaluate c-Fos and ΔFosB.

Experiment 4. Response to a withdrawal period

In order to determine the behavioral and neuronal changes due to a WD period, the same protocol for experiment 3 was performed, followed by interruption of the diets for one week. Rats were randomly assigned to one of three groups: (1) Control (CHOW); (2) 50% SRD; (3) 50% FRD. At the end of WD, rats were evaluated for FAA (n = 10 per group) and effort behaviors (n = 12–14 per group). Brains were obtained at the time when rats usually received the PF for c-Fos and ΔFosB determinations (n = 8–10 per group).

Assessment of binge-type eating, food anticipatory activity and effort behavior to obtain the diet

Binge-Type eating (BTE): Rats exposed to energy deficit or to intermittent food access develop a binge-type behavior [29,30]. Here, BTE was defined when rats ingested at least 2 fold the amount of food (in grams and kilocalories) ingested by the control Chow group during the same interval of 1 h, as described by Perello et al. [7]. During the four weeks from Monday to Friday, the consumed amount of palatable diet or Chow was weighed after the hour of SRD or FRD access.

Food Anticipatory activity: was defined as the increase of general activity in the home cage starting one hour before access to the diet (from 11 am to 12 pm). The general activity was daily recorded with an automatic monitoring system with tilt sensors that detect continuously the animal's movements during the 24 h. Activity counts were collected and automatically stored every 15 min in a PC with the program for PC SPAD9 based on Matlab (Omnialva SA de CV) [18]. The mean daily activity counts exhibited from 11 am to 12 pm during baseline was considered as 100%. The percentage of change of activity counts for the same interval along each week of access to the diet was calculated/group. In addition, in WD general activation was assessed during the hour when rats used to consume PF (12–13 pm).

Effort behavior to obtain the diet: was evaluated at the end of week 4 and of WD. A wire-mesh box (5 ×

5 × 5 cm) was placed for 5 min in each home cage as described by Blancas et al. [13]. At the end of week 4, rats were tested on two consecutive days for 5 min. The first day an empty wire-mesh box was placed in the home cage before access to PF. The following day rats were exposed to the same wire-mesh box containing their corresponding diet (CHOW, SRD or FRD). The same test was performed at the end of the WD week. Behavior was recorded with a digital camera placed in front of the home cages for later evaluation. Behavior was evaluated with an instantaneous sampling method, every 5 s the video was paused and the observer recorded the behavior occurring at that instant. Categories included as effort and interaction with the box were touching, pushing, rotating and biting, standing on and smelling the box.

Brain extraction and immunohistochemistry

Rats were anaesthetized and perfused with saline (0.9%), followed by 4% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.2), brains were removed, and cryoprotected in 30% sucrose solution. Brains were cut in sections of 40 μm with a cryostat at −18°C and organized in 4 series. One series was incubated for 72 h (4°C) in rabbit polyclonal c-Fos protein primary antibody (1:1000. SC-52-G, Santa Cruz Biotechnology) and another series was incubated in FosB/ ΔFosB protein primary antibody (1:1000 FOSB (102) SC-48, Santa Cruz Biotechnology) diluted in phosphate-buffered saline, 0.25% nutritive gelatin and 0.5% triton. Brains were processed according to the avidin biotin method [13]. Sections were mounted on gelatin-coated glasses, dehydrated in a series of alcohols, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). Sections of the Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx) were identified with the atlas of Paxinos and Watson. Three sections were chosen for each area. According to Bregma, selected sections were located in the following antero-posterior coordinates: PLCx and ILCx = +3.72, +3.00 and 2.52 mm; CORE, SHELL and INSCx = +2.16, +1.56 and + 1.08 mm. Sections were observed with an optical microscope (LEICA DM500) and microphotographs were obtained with a 20X magnification. All c-Fos and ΔFosB positive cells contained in that area (1.26 mm^2) were counted with the Image J program using the automatic threshold tool.

Statistical analysis

Data are presented as mean ± standard error of the mean. The acute consumption of each diet, and all the limbic structures evaluated with c-fos and ΔFosB were compared with one-way ANOVA. Diet consumption per week and FAA were evaluated with a two-way ANOVA for repeated measures for the factor time (days or weeks) and the factor diet. Behavior categories obtained from the wire-mesh box test were evaluated with a two-way ANOVA for the factor box (empty or full) and the factor diet. All analyses were followed by a Tukey multiple-comparisons *post hoc* test with α set at $P < 0.05$. Statistical analysis and graphs were elaborated with the program PRISM.6 (GraphPad Software).

Results

Experiment 1. Selection of the percentage of sugar rich diet and fat rich diet

Along four weeks, the highest consumption was observed for the 50% sugar rich diet and the 50% fat rich diet as compared to the other diet proportions, evaluated in grams as well as in kcal (Figure 1(A–D)). For both diets the two-way ANOVA indicated significant effects for the varying proportions in the diet ($P < 0.05$; Supplementary Table 1). In agreement with such results, for the following experiments the 50% SRD and 50% FRD were chosen.

Experiment 2. Acute response to the palatable diets

After first exposure to the diet, SRD rats consumed more grams from their diet than FRD and CHOW rats (Figure 2(A)). The one-way ANOVA indicated significant differences between groups ($P < 0.0019$; Supplementary Table 2). Kilocalories consumed by the FRD and SRD rats were significantly higher than the kilocalories consumed by the CHOW rats ($P = 0.0001$; Supplementary Table 2).

After one hour consuming SRD, a significant increase in c-Fos positive cells was observed in the PLCx, CORE and SHELL (Figure 2(D)). Ingestion of the FRD induced a significant increase in the number of c-Fos positive cells in the CORE, SHELL and INSCx (Figure 2(C)). In both regions of the nucleus accumbens, the c-Fos activation was higher in the FRD than the SRD brains. The one-way ANOVA indicated significant differences among diets in PLCx ($P = 0.031$), CORE ($P < 0.0001$), SHELL ($P < 0.0001$) and INSCx ($P = 0.011$; Supplementary Table 2). Representative microphotographs of each area with c-Fos and ΔFosB are shown in Figure 2(C).

The accumulation of ΔFosB was increased after ingestion of both diets in all evaluated areas (Figure 2(E)), with no difference between SRD and FRD. The one-way ANOVA indicated significant effects due to the

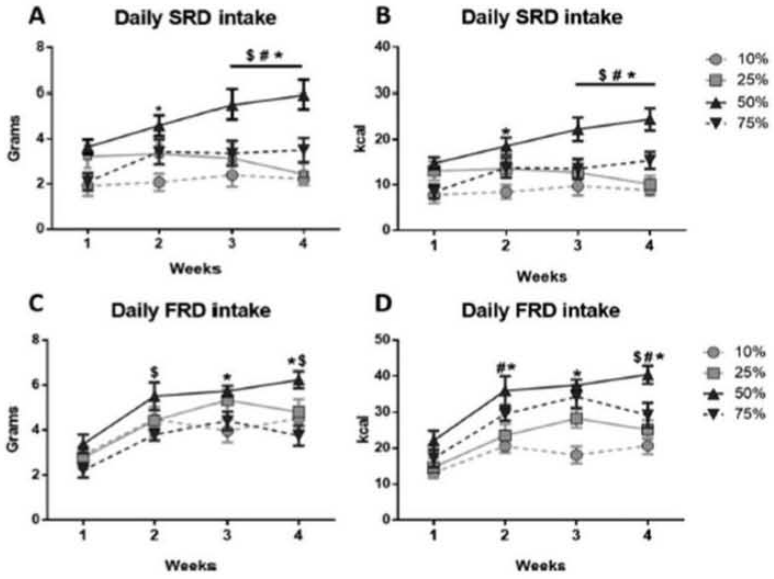

Figure 1. Chronic restricted access to different percentages of sugar rich diet (SRD) or Fat Rich Diet (FRD). Data are shown as mean ± s.e.m ($n = 10$). Mean diet consumption during 1 h of restricted access of SRD in grams (A) and in kilocalories (B); and of FRD (C and D) during 4 weeks. (*) Indicates significant difference from 10%, (#) indicates significant difference from 25% and ($) indicates significant difference from 75% ($P < 0.05$).

diets in PLCx ($P = 0.0006$), ILCx ($P < 0.0001$), CORE ($P = 0.0009$), SHELL ($P = 0.0071$) and INSCx ($P < 0.0001$; Supplementary Table 2).

Experiment 3. Chronic response to the diets

Food anticipatory activity: was evaluated for the hour preceding the restricted access to the diet (11 am–12 pm). The FRD-group exhibited FAA in all weeks (Figure 3(C)), while the SRD-group did not exhibit significant FAA. The two-way ANOVA indicated differences between diet-groups ($P < 0.0001$) and along the weeks ($P = 0.02$; Supplementary Table 1).

Effort behaviors to obtain the diet: All rats exhibited reduced effort behaviors and interaction with an empty box during the 5-minute test (Figure 3(B)). When exposed to the wire-mesh box containing the corresponding diet, the FRD and SRD rats manipulated significantly more the wire-mesh box and showed increased effort behaviors as compared to the CHOW control (Figure 3(B)). The two-way ANOVA indicated significant interaction of groups X condition of the box ($P = 0.0001$; Supplementary Table 1).

Binge type eating: During the daily hour of access to SRD and FRD rats ingested more than the double in grams and kcal from the CHOW group, indicating BTE (Figure 4(A,B)). Starting on week 2 the SRD group ate four times more grams and the FRD ate six times more grams than the CHOW group (Figure 4(A)). In addition, the FRD group ate significantly more kilocalories than SRD in spite of consuming the same amount in grams (Figure 4(B)). The two-way ANOVA indicated significant differences among groups ($P < 0.001$; Supplementary Table 1). As a consequence of SRD or FRD consumption, rats voluntarily reduced their daily 24 h chow intake (Supplementary Figure 1). This resulted in similar 24 h intake in calories for the 3 groups. Moreover, the FRD animals were heavier than SRD rats from week 3 on, however they were not different from the control group (Supplementary Figure 2).

Activation of corticolimbic structures after chronic restricted access to sugar rich or fat rich diet

After chronic restricted access to SRD similar c-Fos positive cells were observed in the limbic areas as compared with the CHOW brains. Chronic intermittent access to FRD produced increased activation in the CORE ($P = 0.0082$) and SHELL ($P = 0.0011$; Figure 4(C); Supplementary Table 2).

Chronic restricted access to SRD promoted a general increase of ΔFosB as compared to CHOW, however this was only statistically significant in the INSCx. Moreover,

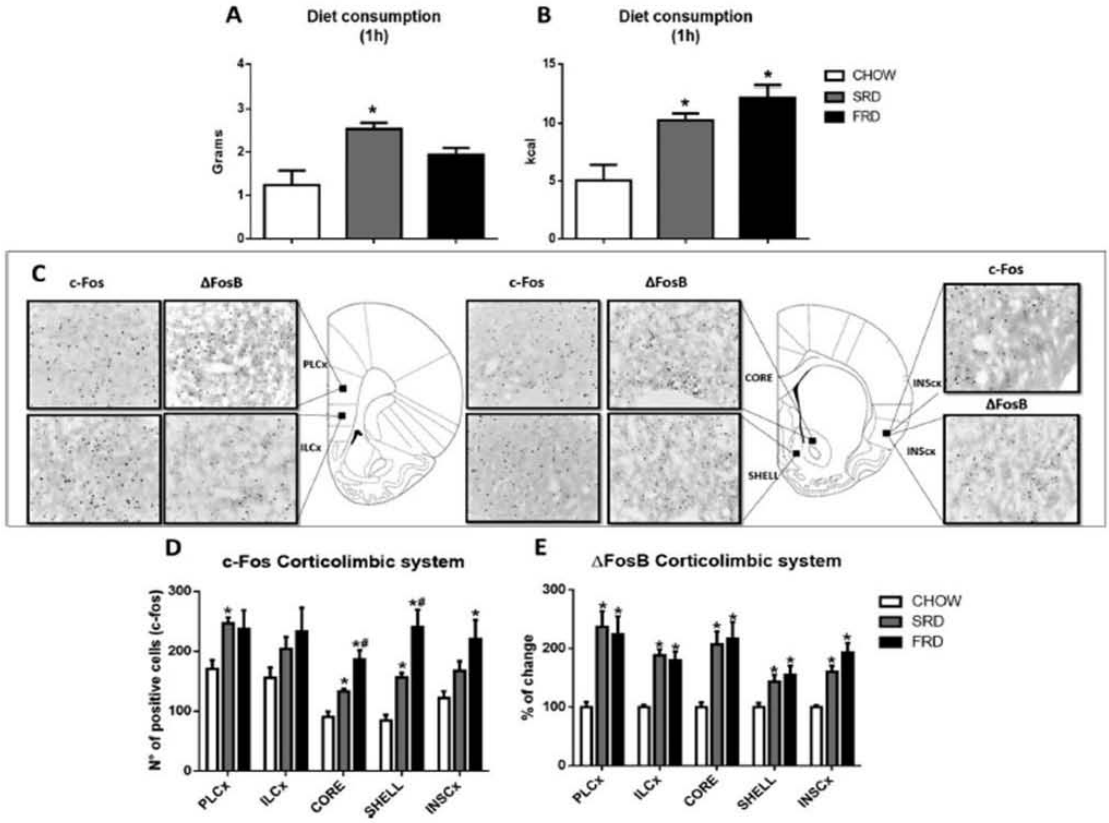

Figure 2. Acute exposure to FRD or SRD. Data are shown as mean ± s.e.m ($n = 10$). Mean consumption of sugar rich diet (SRD) or fat rich diet (HFD) in grams (A) or kilocalories (B) during 1 h of restricted access after a first exposure. C) Representative microphotographs of sampled areas of the corticolimbic system. (D) Neuronal c-Fos activation and (E) ΔFosB accumulation in corticolimbic areas after one hour of palatable food consumption. (*) indicates significant difference from control group and (#) indicates significant difference from SRD ($P < 0.05$). Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx).

chronic restricted access to FRD increased the accumulation of ΔFosB in all areas as compared with the CHOW, excepting the PLCx (Figure 4(D)), the values were however not statistically different from the SRD group. The one-way ANOVA indicated differences between groups in ILCx ($P = 0.0053$), CORE ($P = 0.028$), SHELL ($P = 0.036$) and INSCx ($P = 0.016$; Supplementary Table 2).

Experiment 4. Withdrawal effects on general activity and effort behavior

After one week of interrupting access to the diets, the FRD group and not the SRD group, exhibited increased FAA preceding and during the expected diet time (Figure 5(A,B). For the FAA the two-way ANOVA indicated significant differences for the interaction of groups X time ($P = 0003$) as well as for the activation at the expected PF time ($P = 0.0004$). Effort behaviors evaluated with the wire-mesh box were significantly higher for the FRD and SRD groups towards the full box than to the empty box and were different from the CHOW group (Figure 5(C)); the two-way ANOVA indicated statistical interaction for groups X condition ($P = 0.0006$; Supplementary Table 1).

Withdrawal effects on corticolimbic areas

After a week without the diet, the SRD group did not show a difference from the CHOW group in the number of c-Fos positive cells in corticolimbic structures, while FRD rats still exhibited increased activation in the SHELL and in the INSCx (Figure 5(F)). The one-way ANOVA indicated significant differences among groups

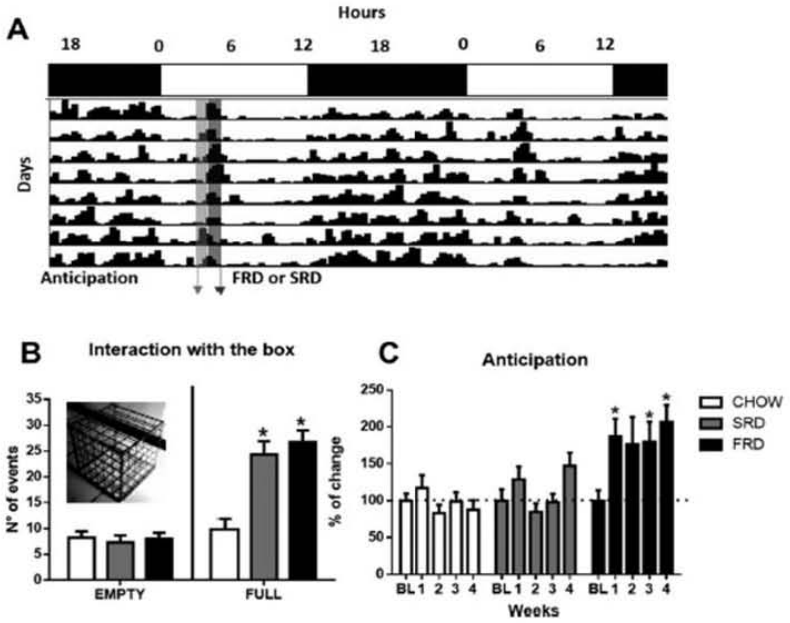

Figure 3. Anticipation and effort behaviors in rats exposed to restricted access to sugar rich diet (SRD) or fat rich diet (FRD) along 4 weeks. Data are shown as mean ± s.e.m ($n = 10–12$). (A) Representative double plotted actogram showing day-night activity counts during 8 days of restricted access to the palatable diet. Vertical grey line with arrow represents the time of access to SRD or FRD. Light grey line represents the preceding hour where anticipation was evaluated. (B) Number of events of interaction with the wire-mesh box and effort behavior to obtain the diet. (C) Percentage of change from the baseline (100%) in activity counts during the hour preceding diet access (anticipation) for the 4 experimental weeks. (*) Indicates significant difference from control group or base line ($P < 0.05$). Dotted line in (B) represents the base line (100%).

in the SHELL ($P < 0.0001$) and INSCx ($p = 0.033$) (Supplementary Table 2).

After one week of WD the SRD group exhibited high ΔFosB in the INSCx, while the FRD group exhibited high ΔFosB in all corticolimbic areas (Figure 5(G)). The one-way ANOVA showed differences between groups in all structures: PLCx ($P = 0.013$), ILCx ($P = 0.0043$), CORE ($P = 0.0007$), SHELL ($P = 0.0088$) and INSCx ($P = 0.0001$; Supplementary Table 2).

Discussion

Diets containing 50% sugar and 50% fat combined with regular chow were chosen for this study because they were consumed in a high amount surpassing more than 2 fold other proportions of the diets. This suggested that the 50% diets represented a high palatability for rats. After a first exposure, rats consumed the SRD in a higher amount in grams than the FRD, suggesting a predominating preference due to the sweet taste. However, after chronic exposure to the diets, the FRD was ingested in higher amount in grams and kcal as compared with the SRD. The FRD induced higher levels of BTE and FAA, it triggered higher c-Fos in nucleus accumbens and higher accumulation of ΔFosB in all the corticolimbic areas as compared to the SRD and CHOW. During the WD period, rats exposed to the FRD continued exhibiting high FAA, effort behavior and changes in c-Fos and ΔFosB persisted. Palatability of diets results from the combination of flavor and energy obtained from the diet 31,32. Here the FRD provided a higher proportion of calories/gram as compared with the SRD, thus the caloric density of the FRD diet attained relevance in a chronic condition and may have determined the main effects observed at the behavioral and brain level (Figure 6).

An important factor for the development of BTE, FAA and effort behaviors is the restricted access to PF. Intermittent or restricted access, create a condition where the reward is withheld and therefore stimulates the 'wanting' desire for the PF. Here we observed that the daily restriction favored the escalation of PF intake and the development of BTE. The restricted access also induced FAA and effort behavior. The relevance of the restricted access is further highlighted by previous studies showing that the intensity of FAA is negatively correlated with the duration of the restriction interval and with the caloric value of the diet [33].

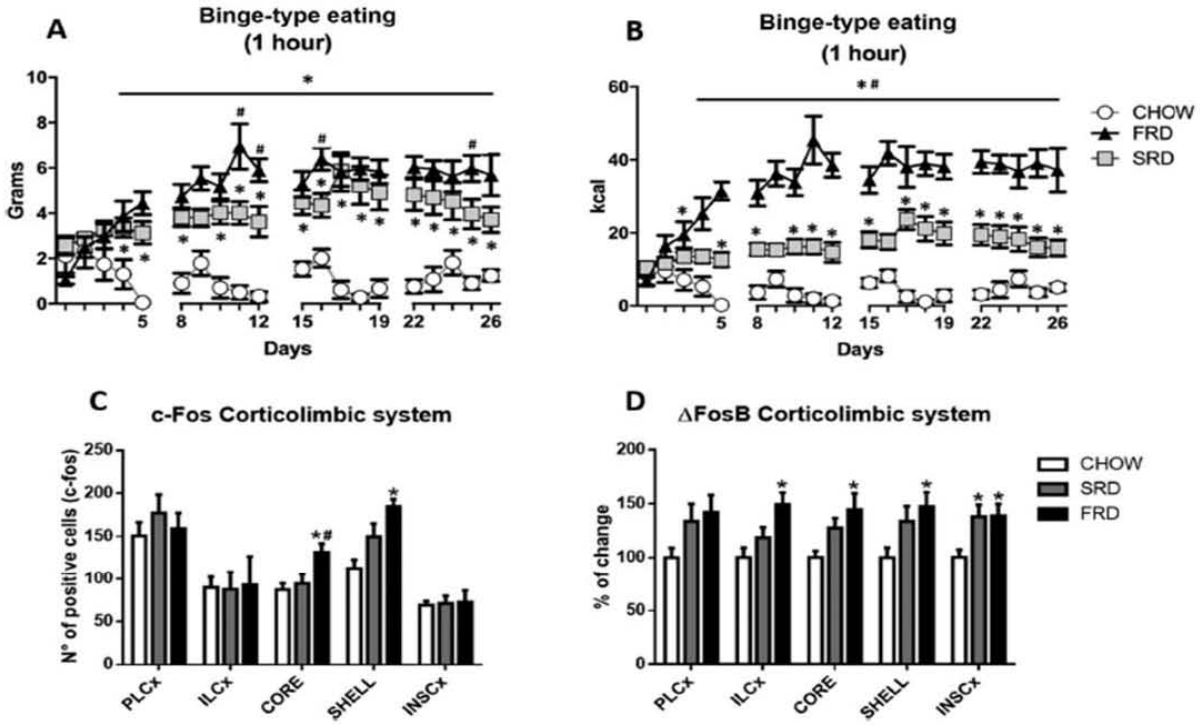

Figure 4. Binge-type eating along the 4 weeks during one hour of palatable diet access, in grams (A) and in kilocalories (B) ($n = 10–18$). (C) Neuronal c-Fos activation and (D) ΔFosB accumulation in corticolimbic areas after 4 weeks of restricted access to Sugar Rich Diet (SRD) or Fat Rich Diet (FRD; $n = 8–10$). Data are shown as mean ± s.e.m. (*) indicates significant difference from control group or base line, and (#) indicates significant difference from SRD ($P < 0.05$). Horizontal dotted line in (C) represents the base line (100%). Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx).

Both, BTE and FAA were stronger expressed in the FRD rather than the SRD group. This response may depend on the palatability, caloric contribution, absorption and metabolism of each nutrient. Along the 4 weeks, FRD rats consumed more PF in grams and kcal than the SRD rats suggesting that FRD is more attractive in taste and caloric value. Moreover, the increased c-Fos response after a first event indicated that the caloric value in the FRD might have importantly contributed to the main effects observed at the corticolimbic level. The differential response to both diets is in agreement with the study by Tenk and Felfeli who described increased BTE to high fat rather than high sugar diets after a 2 h by 3 day/week restricted access [34]. Also, animals under *ad libitum* chow developed FAA to a restricted high-fat diet [35] and to 10% sucrose solution [36]. Moreover, animals previously exposed to a high-fat diet developed anxiety during a withdrawal episode [5].

Similar effort behaviors were observed in rats exposed to restricted FRD or SRD in the wire-mesh box test after 4 weeks and during WD, suggesting that both diets increased the motivation of rats to obtain PF. A previous study described similar effects in animals exposed *ad-libitum* to a high-fat diet or high sugar diet, both developing more impulsivity in an operant test as compared with the control group [37].

After a first exposition the SRD was consumed in a higher amount in grams as compared with the FRD, however both diets triggered the activation of c-Fos in the corticolimbic system. In this first event, the FRD group showed significantly higher c-Fos activation in the nucleus accumbens as compared with the SRD rats, suggesting that the brain may respond differentially to the palatability, texture and taste as early as the first event. After chronic restricted access to the diets and during WD, a general decrease of c-Fos positive cells was observed for both diets as compared with the acute response. However, higher levels of c-Fos were still observed associated with the FRD in the 'CORE' and in the INSCx. In agreement with our study, Dela Cruz et al. [38] found higher c-Fos activation in animals fed a high-fat diet as compared with the group receiving glucose or fructose, after an acute administration. Also, previous studies that exposed animals to a gavage or *ad-*

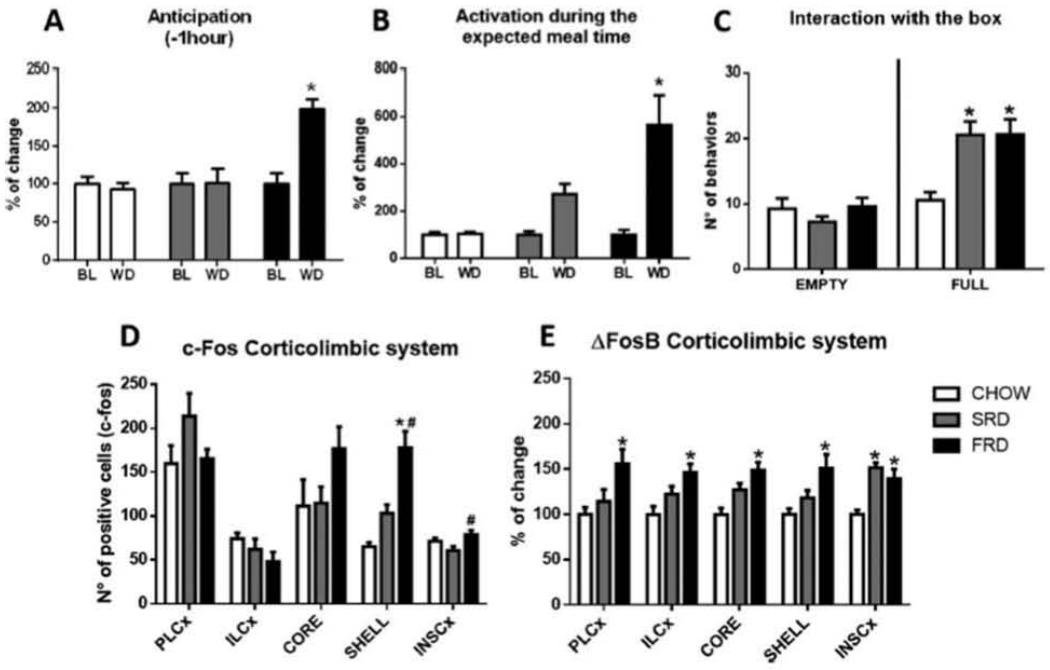

Figure 5. General activation and effort behavior for sugar rich diet (SRD) or fat rich diet (FRD) during the Withdrawal period. Data are shown as mean ± s.e.m. (A) Percentage of change from the baseline (BL = 100%) in activity counts during the hour preceding diet access (anticipation) (B) and during the hour of PF access ($n = 10$). (C) Number of active interactions with the wire-mesh box to obtain the diet ($n = 12–14$). (D) Neuronal c-Fos activation and (E) ΔFosB accumulation in corticolimbic areas after one week of Withdrawal ($n = 8–10$). (*) Indicates significant difference from control group or base line and (#) indicates significant difference from SRD ($P < 0.05$). Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx).

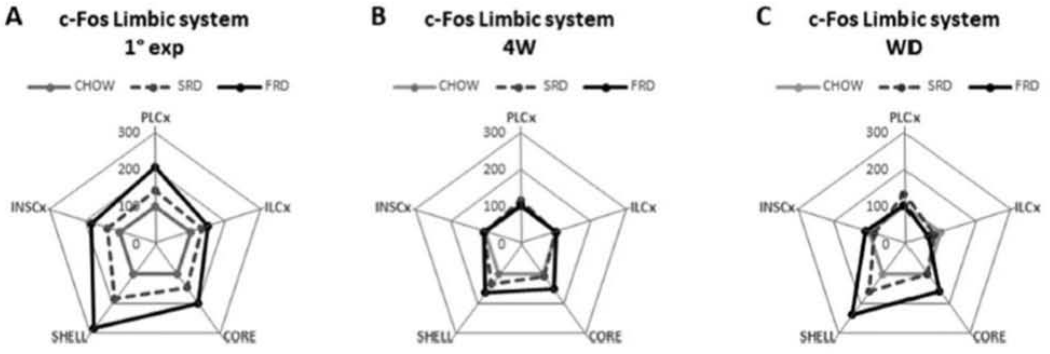

Figure 6. Percentage of change for c-Fos neuronal activation, as compared with the CHOW group (Grey continuous line; 100%). (A) Proportional change of c-Fos activation in corticolimbic areas after the first exposition to each diet, (B) after chronic exposition to each diet and (C) after one week of withdrawal. Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx).

libitum access to high-fat diet reported high expression of c-Fos in the amygdala and in the accumbens CORE as compared with administration of normal chow, ethanol, sucrose or nicotine [39,40]. Other studies have described increased c-Fos activation and dopamine release after consuming a sugar solution [14,38], however they have not compared the response with a high-fat diet.

The insular cortex is an important area related to aversive visceral feelings occurring when animals are

anticipating or craving for drugs [41]. Present results suggest that this area may be involved in the wanting behaviors including FAA for PF diet. Moreover, the low c-Fos response after chronic PF intake, as compared to the acute phase finds support in studies reporting that chronic access to PF, lead the system to react with abnormal low levels of activation [6].

After a first episode to the PF diets both groups exhibited elevated levels of ΔFosB. Importantly, after chronic intake, the accumulation of ΔFosB in corticolimbic areas persisted only in the FRD but not in the SRD, indicating the differential influence of the diets on synaptic changes in corticolimbic areas. Other studies have reported that a significant accumulation of ΔFosB can be observed after chronic *ad-libitum* consumption of high fat and/or high sugar diet [42,43] and after WD of sugar [44]. Nestler et al. [45] suggested that ΔFosB is a transcription factor that enables long-term synaptic changes underlying the development of addiction like behavior. Such changes may also favor the formation of compulsive-like eating behavior, including binge-type eating. The link of ΔFosB accumulation with addiction-like responses is supported by Kaufling, et al. [46] who demonstrated overexpression of ΔFosB after acute administration of cocaine, D-amphetamine, methylphenidate and caffeine. Altogether, data suggest that PF can change the brain by inducing ΔFosB, a factor that triggers neuronal plasticity.

Conclusion

This study indicates that a restricted access to both, a sugar rich and a fat rich diet can trigger overconsumption of PF. However, after chronic exposure to restricted access, the fat rich diet leads to increased binge-type eating and effort behaviors as compared with a high sugar diet. Also, the fat rich diet stimulated higher ΔFosB accumulation, which is a relevant transcription factor for initiating plastic changes in the corticolimbic system. This differential response in the brain (Figure 6) and behavior suggests that the caloric density provided by a fat rich diet could be a relevant factor for the development of hedonic eating, which then will lead to obesity.

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Disclosure statement

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Carolina Escobar is a Senior Professor in the Department of Anatomy, at the Faculty of Medicine, National Autonomous University in Mexico and directs a laboratory for basic research on Circadian Rhythms, Food intake and Metabolism. She studied Physiological Psychology followed by a masters and PhD in Physiological Sciences. The main contributions of her laboratory have been (1) on the behavioral, metabolic and brain mechanisms associated with food entrainment and (2) the development of experimental models of shift work in order to better understand the adverse effects of circadian disruption. Her group described mechanisms of circadian entrainment by palatable food, which now can partly explain addictive behavior. Her data on circadian disruption have provided evidence of the negative effects of food and activity during the rest phase, which has provided a new insight of the circadian system as a key process for homeostasis and for metabolic health. In this field she has published more than 90 scientific articles, 2 books and more than 25 book chapters. Since 2010 she is chief of the Research section in the Anatomy department. She is member of the Mexican Academy of Sciences and of the Mexican Academy of Medicine.

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7. PUBLICACIÓN #3

7.1 PLANTEAMIENTO DEL PROBLEMA

El protocolo experimental de jet lag social demostró que los animales expuestos a dieta de cafetería, consumían más y cumplían con más criterios de síndrome metabólico que aquellos que no estaban expuestos al jet lag social. La dieta de cafetería está compuesta por alimentos altos en azúcar y altos en grasa, por lo que no se sabe si tales consecuencias son provocadas por azúcar o grasas específicamente y en qué magnitud. Se ha propuesto que el consumo de alimentos palatables (azúcar y/o grasa) llevan al desarrollo de conductas similares a una adicción, impactando áreas asociadas a la recompensa y creando cambios plásticos.

7.2 HIPÓTESIS

1. Las ratas expuestas a un día de retraso de sueño y que consumen dieta alta en grasa consumirán más alimento que las expuestas a dieta alta en azúcar, aunado a que expresarán con mayor densidad c-Fos y ΔFosB en el núcleo accumbens, la corteza prefrontal y la corteza insular Las ratas expuestas al protocolo de jet lag social y que consumen crónicamente la dieta alta en grasa desarrollaran conducta tipo atracón (más del doble del consumo del

control), anticipación (mayor actividad locomotora una hora previa a la dieta) y mayores conductas de esfuerzo que las expuestas al protocolo de jet lag social y que consumen a dieta alta en azúcar.

2. Las ratas expuestas al protocolo crónico de jet lag social y de consumo de dieta alta en grasa tendrán mayor densidad de expresión de c-Fos y ΔFosB en el núcleo accumbens, la corteza prefrontal y la corteza insular, que las ratas expuestas al protocolo crónico de jet lag social y de consumo de dieta alta en azúcar.
3. Las ratas en abstinencia de dieta alta en grasa y sin estar en el protocolo de jet lag social tendrán mayor anticipación y conductas de esfuerzo que las ratas en abstinencia de dieta alta en azúcar.
4. Las ratas en abstinencia de dieta alta en grasa y sin estar en el protocolo de jet lag social tendrán mayor expresión de c-Fos y ΔFosB en áreas del sistema de recompensa que las ratas en abstinencia de dieta alta en azúcar

7.3 OBTETIVO GENERAL

- Evaluar los cambios en el sistema de recompensa en animales con exposición aguda o crónica de dieta alta en azúcar o dieta alta en grasa en animales expuestos al protocolo de jet lag social y comparar en abstinencia la expresión de conductas asociadas a la adicción.

7.3.1 OBJETIVOS PARTICULARES

1. Evaluar el consumo de dieta alta en azúcar o dieta alta en grasa ante una sola exposición (aguda) en animales expuestos al protocolo de jet lag social y evaluar los cambios producidos en el núcleo accumbens, la corteza prefrontal y la corteza insular, mediante las proteínas c-Fos y ΔFosB.
2. Evaluar la conducta tipo atracón, anticipación y conductas de esfuerzo, después del consumo crónico (4 semanas) de dieta alta en azúcar o dieta alta en grasa en animales expuestos al protocolo de jet lag social.
3. Evaluar cambios producidos en núcleo accumbens, la corteza prefrontal y la corteza insular, mediante la proteína c-Fos y la proteína ΔFosB en en animales

expuestos al protocolo de jet lag social y a un consumo crónico de dieta alta en azúcar o dieta alta en grasa.

4 Evaluar anticipación y conductas de esfuerzo, después de una semana de abstinencia de dieta alta en azúcar o dieta alta en grasa y sin estar en el protocolo de jet lag social.

5 Evaluar cambios producidos en núcleo accumbens, la corteza prefrontal y la corteza insular, mediante la proteína c-Fos y la proteína ΔFosB después de una semana de abstinencia de dieta alta en azúcar o dieta alta en grasa y sin estar en el protocolo de jet lag social.

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MALE WISTAR RATS EXPOSED TO CHRONIC INTERMITTENT SLEEP DELAY DEVELOP COMPULSIVE BEHAVIOR FOR HIGH FAT AND HIGH SUGAR DIETS BUT NOT FOR REGULAR CHOW

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INTRODUCTION

Modern lifestyle imposes a discrepancy between the drive to sleep and the motivation to engage in activities during the night (Wittmann *et al.*, 2006). During weekdays due to the work or school schedules, the amount and quality of sleep is reduced, while during weekends individuals try to compensate by prolonging sleep hours. This condition affects mainly the amount and quality of sleep and produces along the week a shift in the sleep-wake patterns. This also affects the adjustment of the circadian system to the external light-dark cycles, and is suggested to lead to a social jet-lag (Wittmann *et al.*, 2006). Such shifted sleep patterns are an emerging health problem and little is known about its long-term consequences. Disrupted sleep can have adverse effects on appetite and food intake, affecting metabolic function and body weight (Roenneberg *et al.*, 2007; Markwald *et al.*, 2013; Wong *et al.*, 2015). In adolescents and young adults a disrupted sleep is associated with high consumption of palatable and energetic food (Landhuis *et al.*, 2008), which represents a risk factor to develop overweight and obesity.

In animal models, sleep disruption increases food intake, however, due to the exhausting conditions and physiological stress imposed by the extended protocols (from 18- 20 h) (Barf, Van Dijk, *et al.*, 2012) this is associated with weight loss and a catabolic state (Mavanji *et al.*, 2013b; Brianza-Padilla *et al.*, 2016). Moreover, the combination of sleep deprivation combined with access to a high-fat diet or a high sugar diet has resulted in overconsumption, without increasing the body weight. Contrasting, studies using mild strategies of sleep restriction by using randomized noise, gentle handling or a reduced number of hours of sleep restriction have

observed food overconsumption and increased body weight even with a regular chow diet (Bodosi *et al.*, 2004; Husse *et al.*, 2012; Mavanji *et al.*, 2013a).

The contribution of chronic intermittent sleep delay, resembling a social jet-lag, to feeding behavior and metabolism requires more research. Especially, the possible contribution of such chronic shifted sleep as a risk factor for CFSB needs to be explored. In a previous study, we tried to mimic the condition of social jet-lag by exposing rats to a chronic protocol of intermittent sleep delay. For this purpose, from Monday to Friday, slow rotating wheels were used in order to delay 4 hours the sleep onset, while during weekends rats were left undisturbed. This protocol produced a constant shift of 2-3 hours in the activity acrophases, imposing during weekdays a conflict between the internal timing and external cycles (Espitia-Bautista *et al.*, 2017). Rats exposed to this experimental model overconsumed cafeteria diet, attained a weight gain of 16% above the controls and developed 5 out of 7 criteria for metabolic syndrome. Importantly, rats exposed to the same protocol of 4 hours in the slow rotating wheel during the active phase did not exhibit shifts in their activity acrophase, consumed less cafeteria diet, did not develop overweight and only reached 3 out of 7 criteria for metabolic syndrome. This pointed out that chronic intermittent sleep delay, but not arousal and stress produced by exposure to the wheel during the active phase, was the main factor promoting high consumption of cafeteria diet, overweight and metabolic disturbance.

Palatable food is an attractive stimulus for rodents that favors the development of compulsive food-seeking behaviors (CFSB) and produces plastic changes in the corticolimbic system. Rodents that develop CFSB exhibit high activity, seeking

behavior and craving in anticipation to the scheduled access to palatable food. They also develop binge episodes and effort behavior to obtain the food (Heyne *et al.*, 2009; Rossetti *et al.*, 2014; Burokas *et al.*, 2018). Plastic changes associated with CFSB include accumulation of ΔFosB, which is a transcription factor that triggers production of GluR2, CREB and CDk5, leading to dendritic growth in areas of the reward system (Nestler, 2008; Madsen *et al.*, 2013). Accumulation of ΔFosB also occurs after consumption of a high-sugar diet (Christiansen *et al.*, 2011), and after consumption of a high-fat diet (Teegarden, Nestler & Bale 2008). It is suggested that ΔFosB accumulation can underlie consolidation of neuronal connections required for the development for CFSB to palatable food. It is however not clear, whether the development of CFSB and overconsumption of palatable diets is mainly triggered by fat or by sugar in the diet.

In the present study, we aimed to determine in rats exposed to acute or chronic intermittent sleep delay, which element of the cafeteria diet, a high-fat or a high-sugar diet, promotes overconsumption and development of CFSB. Also, we aimed to identify whether the differential effect of the diets was associated with the activation of corticolimbic structures, as seen with c-Fos, and with accumulation of Δ-FosB.

METHODS

Animals and housing

Male Wistar rats weighing 190-210g were housed in individual acrylic cages (45cm X30cm X20cm) placed on tilt sensors, in soundproof ventilated lockers. Rats were

maintained under controlled temperature (22 ± 1ºC), free access to chow food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA.) and water, with a 12:12h light/dark (LD) cycle (lights on at 08:00 h). The committee for ethical evaluation at the Facultad de Medicina UNAM approved experiments (FM/DI/013/2018P). Experiments conform to international guidelines on the ethical use of animals; procedures were aimed at minimizing the number of animals used and their suffering.

Experimental design

Experiment 1. Acute sleep delay and chow consumption

The first experiment was aimed to test if a first event of 4 h sleep delay promotes overconsumption of chow diet and triggers activation of c-Fos and accumulation of Δ-FosB in corticolimbic areas. Rats were randomly assigned to one of 2 groups: 1. Control undisturbed rats, 2. Sleep Delay (SD); for a single occasion at the moment of lights on, rats were placed in slow rotating wheels (0.35m/ min) with chow and water access, in order to delay 4 h their sleep on set (08:00-12:00 h). After sleep delay, rats were returned to their home cages and chow consumption was measured for one hour (12:00-13:00). Rats were euthanized 30 minutes later (13:30) to obtain their brains and determine the neuronal activation in the limbic system.

Experiment 2. Chronic intermittent sleep delay and chow consumption

The second experiment was aimed to test if chronic intermittent sleep delay, as a model of social-jetlag, promotes overconsumption of chow diet and triggers activation of c-Fos and accumulation of Δ-FosB in corticolimbic areas. Rats were

randomly assigned to one of 2 groups: 1. Control undisturbed rats, 2. Chronic sleep delay (CSD) group; in order to delay 4 h their sleep on set, from Monday to Friday rats were placed in slow rotating wheels (from 08:00- 12:00) with access to chow and water inside the wheel; during weekends an undisturbed sleep onset was allowed. Chow consumption was assessed during the first hour after returning rats to their home cage along 4 weeks. At the end of the 4th week, rats were euthanized 30 min after food access (13:30) to determine the neuronal activation in the limbic system.

Experiment 3. Chronic intermittent sleep delay and ingestion of high fat or high sugar diet

This experiment explored in rats exposed to CSD the differential overconsumption of a high-sugar or a high-fat diet and the consequent response in corticolimbic areas. Rats were randomly assigned to one of 3 groups: 1.Chronic sleep delay (CSD), 2. Chronic sleep delay + high-sugar diet (CSD-S) and 3. Chronic sleep delay + high-fat diet (CSD-F).

Rats were exposed to the protocol of 4 hours sleep delay from Monday to Friday (08:00- 12:00) and had access to the corresponding diet during the following hour after the return to their home cage (12:00-13:00). Daily intake of palatable diet was assessed along 4 weeks (for one hour after the CSD protocol). During weekends rats were left undisturbed. At the end of the 4th week and in order to determine the neuronal activation in the limbic system, rats were euthanized 30 min after intake of the palatable diet (13:30).

Experiment 4. Compulsive Food-Seeking behavior (CFSB) after interrupting the diets (withdrawal)

The aim of this experiment was to determine whether the chronic intermittent sleep delay protocol combined with restricted access to palatable food (High-Fat or High-Sugar Diet) would elicit behaviors related with CFSB and whether this would be associated with activation of c-Fos and accumulation of Δ-FosB in the corticolimbic system. Therefore, rats were randomly assigned to one of 3 groups:

1.Chronic sleep delay (CSD), 2. Chronic sleep delay + high-sugar diet (CSD-S) and 3. Chronic sleep delay + high- fat diet (CSD-F). Rats were exposed to the same 4 week protocol as in experiment 3. This was followed by one week of withdrawal in which the experimental protocols were interrupted. At the end of the withdrawal week, rats were evaluated for CFSB and were euthanized 1.5 h after expected food access to determine the neuronal activation in the limbic system associated with withdrawal.

Diets

All groups received standard chow food (4.07 kcal/g). High-sugar diet was made by adding refined sugar to normal chow food in a proportion of 50% (50g chow + 50g sugar. 4.03 kcal/g). Likewise, pellets of high-fat diet were made with normal chow plus lard (50g chow + 50g lard, 6.53 kcal/g). More details of the macronutrients for each diet see Supplementary table 1.

Activity recording and analysis

General activity was recorded with an automatic monitoring system with tilt sensors, detecting animal's movements during the 24 hours. Counts were collected and stored every 15 minutes in a PC for further analysis with the program for PC SPAD9 (version 1.1.1) designed for this system and based on Matlab (Omnialva SA de CV, Mexico City, Mexico); more details see (Espitia-Bautista *et al.*, 2017). Double-plotted actograms were obtained by collecting the sum of activity for 15 min intervals. Mean daily activity (± s.e.m.) was obtained along the protocol and are represented as 24h daily curves. Data were processed with the cosinor analysis to obtain the acrophase and amplitude (peak activity values for 24h). Data for weekdays were analyzed separate from weekends.

Assessment of Compulsive Food-Seeking Behaviors

The indicators used to measure Compulsive Food-Seeking Behaviors (CSB) were

1) Binge eating, 2) Craving (food anticipatory activity at the expected meal time) and 3) Effort to obtain the diet.

Binge eating: was classified as the double or more gram ingested in one hour of restricted access, compared with the CSD group that consumed chow.

Craving : was defined as the increase of general activity in the home cage during one hour before access to the diet (ZT3-ZT4, 11:00 – 12:00h) during the expected meal time (ZT4-ZT5, 12:00 – 13:00h). The mean number of activity counts exhibited from 11:00 to 12:00 and from 12:00-13:00 hours during one week of baseline was considered as 100% for each group. The percentage of change of activity counts for the same interval in the withdrawal week is shown as craving.

Effort to obtain the diet: was evaluated at the end of the withdrawal week. Rats were exposed in two consecutive days to a wire-mesh box (5X5X5 cm) as described by Blancas et al, 2014 (Blancas *et al.*, 2014). The first day rats were exposed in their home cage to an empty wire-mesh box for 5 minutes before access to the corresponding diet. The following day the same wire-mesh box was placed in the home cage containing their corresponding diet (chow, high-sugar diet or high-fat diet). Behavior was recorded with a digital camera for later evaluation according to different categories: effort and interaction with the box, anxiety, and exploration (See Supplementary table 2). Behavior was assessed with an instantaneous sampling method (Martin & Bateson, 1993); every 5 seconds the video was paused and the observer recorded the behavior occurring at that instant. 60 samples were obtained for the 5 min recording.

Brain extraction and immunohistochemistry

At the end of each experiment, rats were euthanized one hour and a half after the chow or palatable consumption, to determine the neuronal activation in the limbic system. Brains were obtained by perfusion with saline (0.9%), followed by 4% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.2); brains were cryoprotected in 30% sucrose solution and cut with a cryostat at -18°C, sections of 40 µm and organized in series. 1 series was incubated for c-Fos for 72 h (4°C) in protein primary antibody (1:1000. SC-52-G, Santa Cruz Biotechnology) and another series in rabbit polyclonal FosB/ΔFosB protein primary antibody (1:1000. FOSB (102) SC-48, Santa Cruz Biotechnology); both diluted in phosphate-buffered saline (PBS), 0.25% nutritive gelatin and 0.5% triton (PBSGT). Brains were processed according to the

avidin biotin method (more details see (Blancas *et al.*, 2014)). Sections were mounted on gelatin-coated slides, dehydrated in a series of alcohols, cleared with xylene and coverslipped with Entellan (Merck, Darmstadt, Germany). Areas of the corticolimbic system were identified in the atlas of Paxinos and Watson (Paxinos & Watson, 1997). Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx). Three slices were chosen corresponded to the following distances from bregma: PLCx and ILCx = +3.72, +3.00 and 2.52 mm; CORE, SHELL and INSCx = +2.16, +1.56 and +1.08 mm. Microphotographs were obtained at a 20X magnification with an optical microscope (LEICA DM500), see Supplementary Figure 1 and 2. Immunopositive c-Fos and ΔFosB neurons were analyzed with the Image J program setting an automatic threshold.

Statistical analysis

Data are presented as mean ± standard error of the mean. In experiment 1 the chow consumption and mesurments in the brain were compared with a two-tailed unpaired Student´s t-Test. Acrophases, amplitude and craving were compared with a 2 way-ANOVA for the factors time in the protocol (BL, 4W) and weekdays (week, weekend). The effort to obtain the diet was compared with a two way-ANOVA for the factors box (empty or full) and the factor diet (CSD, CSD-S, CSD-F). Measurements in the brain (c-Fos and ΔFosB) in experiment 3 and 4 were compared with one-way ANOVA. Diet consumption per week (chow or palatable diet) was evaluated with a two-ways ANOVA for repeated measures for the factor time (weeks) and the factor groups. All analyses were followed by a Tukey multiple-comparisons post hoc test

with α set at P<0.05. Statistical analysis and graphs were elaborated with the program PRISM.6 (GraphPad Software).

RESULTS

Acute sleep delay decreased chow food consumption and activated the limbic system

After a first experience of sleep delay SD rats decreased their chow consumption during the first hour following the return to their home cages, as compared to the controls (two-tailed unpaired Student t-test P=0.027; Figure 1A). In the SD rats, in spite of les food ingestion, the number of positive c-Fos cells in corticolimbic areas was higher than in the control group (Figure 1B). The two-tailed unpaired Student t-test indicated significant differences between groups in the PFCx (P=0.002), PLCx (P=0.016), CORE (P=0.0004), SHELL (P=0.0007) and INSCx (P=0.0001). After this first event, the accumulation of ΔFosB was also increased in the SD group as compared with the controls (Figure 1C). The two-tailed unpaired Student t-test indicated significant differences between groups in PFCx (P<0.0001), PLCx (P<0.0001), CORE (P<0.0001), SHELL (P<0.0001) and INSCx (P=0.0002).

Chronic intermittent sleep delay decreased chow food consumption and elicited plastic changes in the limbic system

Animals exposed to chronic intermittent sleep delay during weekdays, exhibited shifts in the activity acrophases as previously reported for our experimental model of social jet-lag (Espitia-Bautista *et al.*, 2017). At base line, control and CSD animals

showed daily rhythms of general activity, with stable acrophases of a mean of 16.5 h during weekdays and weekends (Figure 2B). After 4 weeks of CSD, rats exhibited a delayed acrophase of ~ 3.3 h during weekdays as compared with the weekend and with their own base line (Figure 2C), confirming a weekly shifted activity pattern, similar to social jet-lag. The two-way ANOVA indicated significant effects in the interaction of experimental phase X weekdays vs weekends ($F_{(1,46)}$=44.48; P<0.0001) and with the baseline ($F_{(1,46)}$=72.06; P<0.0001). No difference was observed in the day-night amplitude of activity rhythms for both groups (Supplementary Figure 1A and 1B).

Along the 4 weeks of the chronic intermittent sleep delay protocol and during the first hour following sleep delay in the wheels (ZT4-ZT5), the amount of chow food consumed by the CSD was lower than the controls ($F_{(1,22)}$=39.08; P<0.0001; Figure 3A). However, the number of positive c-Fos cells in corticolimbic areas was similar for both groups (control and CSD) except for the CORE (P=0.047; Figure 3B), with higher values for the CSD group. The accumulation of ΔFosB was higher in the PLCx (P=0.01), PFCx (P=0.028) and CORE (P=0.0011) for the CSD as compared with the control group (Figure 3C).

Chronic intermittent sleep delay promotes binge eating for High-Fat Diet rather than High-Sugar Diet

Rats exposed to the chronic intermittent sleep delay protocol combined with one-hour access to high-sugar diet or high-fat diet exhibited stable acrophases during baseline with a mean peak activity of ~ 16.15 h during weekdays and weekend. After 4 weeks of the shifted sleep, the CSD-S group showed a delayed acrophase of ~2.5h

(Figure 4A) and the CSD-F group a delay of ~3.0h (Figure 4B) during weekdays as compared with the weekend and with its own base line. The two-way ANOVA indicated significant effects for the interaction experimental phases X weekdays and weekends for the CSD-S ($F_{(1,36)}$=15.01; P=0.0004) and for the CSD-F ($F_{(1,56)}$=25.27; P<0.0001). There were no differences in the amplitude of activity rhythms between groups (Supplementary Figure 1C and 1D).

Along the protocol and during the following hour after sleep delay, both groups exposed to a palatable diet, CSD-S and CSD-F, consumed more diet than the CSD that had access to chow (Figure 4C). Moreover, the CSD-F group ate more diet in gram and kilocalories (Figure 4D) than the CSD and CSD-S groups. The two-way ANOVA for repeated measures indicated a significant interaction for groups X time in weeks ($F_{(6,114)}$=5.23; P<0.0001) for the weight of ingested diet in gram and for the amount of calories ($F_{(6,114)}$=7.08; P<0.0001).

After 4 weeks of chronic intermittent sleep delay combined with restricted access to palatable diet, no difference was observed between the CSD, CSD-F and CSD-S groups in the number of positive cells to c-Fos in corticolimbic areas. For ΔFosB accumulation the one-way ANOVA indicated significant difference among groups in the SHELL ($F_{(2,24)}$=7.27; P=0.0034) and in the INSCx ($F_{(2,24)}$=4.21; P<0.027). The CSD-S group had higher accumulation in the SHELL, and the CSD -F had higher accumulation in INSCx as compared with the CSD group (Figure 5).

Compulsive Food-Seeking Behaviors during the withdrawal week in rats ingesting high fat or high sugar diets

At the end of the withdrawal week, effort behaviors were evaluated with the wire-mesh box test. The CSD group receiving chow did not develop effort behavior towards the full box as compared with the empty box, while groups exposed to palatable food showed a low interaction with an empty box and high interaction with the box containing the corresponding diet (Figure 6A). The two-way ANOVA indicated significant effects due to the interaction of experimental phases X groups ($F_{(2,27)}$=15.19; P<0.0001). During the effort test the CSD-F group performed less grooming than the two other groups, the two-way ANOVA indicated significant effects between groups ($F_{(2,27)}$=3.74; P=0.036; Figure 6B). Also, both groups exposed to palatable food displayed less cage exploration during the test with the full box. The two-way ANOVA indicated a significant effect due to the interaction experimental phase X groups ($F_{(2,27)}$=8.33; P=0.0015; Figure 6C).

During the withdrawal week, both groups consuming a palatable diet, CSD-S and CSD -F, exhibited increased general activity in anticipation to the hour of palatable food access (ZT3-ZT4; Figure 6D). They also exhibited increased activity during the hour when they were normally exposed to the diet (ZT4-ZT5; Figure 6E). The Two-way ANOVA indicated a significant effect between experimental phases (baseline vs withdrawal week) ($F_{(1,59)}$=7.96; P=0.006) for anticipation and for activation at ZT4-ZT5 ($F_{(1,55)}$=15.49; P=0.0002).

At the end of the withdrawal period, c-Fos expression was evaluated and significant effects between groups were only observed in the INSCx ($F_{(2,22)}$=20.03; P<0.0001),

with higher positive cells in both groups that ate palatable food. The CSD-F group exhibited increased accumulation of ΔFosB cells in the PLCx ($F_{(2,24)}$=5.47; P=0.011; one-way ANOVA) and both groups exposed to palatable diet had increased accumulation of ΔFosB in the CORE ($F_{(2,24)}$=17.56; P<0.0001); and SHELL ($F_{(2,24)}$=5.83; P=0.0086) as compared with the CSD group.

DISCUSSION

Acute and chronic intermittent sleep delay combined with access to regular chow did not lead to chow overconsumption, rather it produced a reduction of food intake. However, after the first sleep delay, rats already exhibited c-Fos activation and overproduction of ΔFosB in corticolimbic areas, which is suggested to favor the development of CFSB. The chronic weekly protocol of intermittent sleep delay induced shifted activity acrophases of around 3 hours on weekdays as previously described for a model of social jet-lag (Espitia-Bautista *et al.*, 2017). This condition combined with restricted access to a palatable diet favored binge eating behavior of high fat and high sugar diet. When diets were withdrawn, only rats exposed to the palatable diets, and not the CSD group, exhibited CFSB. This was observed as effort behaviors to obtain the diet and increased activation at the expected time of the diet. In the chronic stage c-Fos activity was decreased in the limbic system as compared with the acute stage, only a significant c-Fos activation remained in the and INSCx and ΔFosB accumulation in PLCx, and CORE and SHELL for the CSD-F group. Present data point out that chronic intermittent sleep delay combined with a high fat or high sugar diet, but not with regular chow, represent a risk factor for developing CFSB.

Acute and chronic intermittent sleep delay induced lower chow food ingestion. The effects of sleep deprivation on the 24h food consumption of regular chow are not clear, because they vary depending on the duration of the protocol and the strategy used to disrupt sleep (Guerrero-Vargas et al 2018). Using a rotating floor Baud *et al.* (2013) observed increased chow intake (Baud *et al.*, 2013); likewise Mavajni *et al.* (2013) using loud noise for 8 hours to disturb sleep reported increased regular food intake (Mavanji *et al.*, 2013b). However, using similar rotating drums as used in the present study Barf et al (2012) reported no effects on the 24h chow consumption (Barf, Van Dijk, *et al.*, 2012). In the present study food intake was evaluated for only one hour following sleep delay, thus the reduced chow intake may be related with the increased drive to sleep rather than eating. Contrasting, rats having access for 1 hour to palatable food following the 4h sleep delay, developed binge eating already after one week of the protocol and this was significantly amplified in CSD-F rats. Consistent with our findings, other studies reported binge behavior for sweet food (Koban et al., 2008; Hanlon et al., 2010; Martins et al., 2011) as well as for fat food (Barf, Desprez, et al., 2012; Ho et al., 2016) associated with sleep disruption.

Protocols aimed at disrupting sleep, expose rodents to stressful conditions and may stimulate a state of high arousal and activity. The differential response to over consume a palatable food vs. regular chow is also observed in animals exposed to stress, where a reduction in chow consumption (Sticht *et al.*, 2018), and a preference for palatable diets is reported (Kant & Bauman, 1993; Romaní-Pérez *et al.*, 2017; Giudetti *et al.*, 2018). It is suggested that sleep disruption as well as stress, trigger the search for a "comfort food" aimed to relieve emotional and physical discomfort

(Farooqui *et al.*, 1996; Dallman *et al.*, 2003; Pecoraro *et al.*, 2004; la Fleur, 2006). It is therefore not clear which factor associated with sleep disruption is the main cause of the hyperphagia for palatable food because also stress and forced behavioral activation may elicit the search for high caloric food. In our protocol of chronic sleep delay rats did not exhibit elevated levels of corticosterone after the exposure to the wheel (Supplementary Figure 2), suggesting low levels of stress and indicating adaptation to the chronic intermittent sleep delay condition.

The initial activation observed with c-Fos after a first exposure, was not observed after 4 weeks of regular sleep delay. In the chronic protocol, all groups exposed to sleep delay (chow or palatable food) exhibited similar elevated c-Fos activation in the corticolimbic system independent of the diet. Other studies described higher activation of c-Fos after ingestion of a high fat diet (Dela Cruz *et al.*, 2016) as well as after ingestion of a high-sugar diet (Pomonis *et al.*, 2000). In our study, the main effects are due to the first event of sleep delay in which arousal and increased activity may be associated with the levels of c-Fos.

In the withdrawal condition, the CSD rats eating regular chow did not exhibit CFSB. Only rats exposed to the high fat or high sugar diet exhibited CFSB, showing binge eating along the 4 weeks and after one week of withdrawal. Both groups exhibited effort behaviors to obtain the diet out of the wiremesh box and craving at the expected time of the diet. This indicates that sleep delay alone is not a risk factor for hyperfagia and obesity, while the combination with palatable food represents a risk for CFSB. During withdrawal, the levels of c-Fos were increased in all groups with CSD as compared with the control group, however, only both groups previously

exposed to palatable food exhibited a significant increase of c-Fos in the INSCx as compared with the CSD group receiving chow. The INSCx has been associated with aversive visceral feelings occurring when animals are expecting or craving for drugs (Cosme *et al.*, 2015; Arguello *et al.*, 2017; Fedota *et al.*, 2018) which may drive individuals to relapse. The activation of the INSCx observed in the CSD-F and CSD-S during withdrawal may reflect an aversive feeling due to the lack of the palatable food.

Present data show that after the first event of sleep delay an increase of ΔFosB accumulation in corticolimbic areas was already evident. Sleep disruption and stress, are associated with accumulation of ΔFosB, which is suggested to stimulate dendritic growth and production the GluR2 subunit in AMPA receptors aimed to initiate binge eating and CFSB for palatable food (Nestler *et al.*, 2001; Wallace *et al.*, 2008). Thus in our study the accumulation of ΔFosB may be a priming stimulus to favor binge eating of palatable food. In the long term ingestion of palatable food associated with sleep delay produced specific accumulation of ΔFosB in PLCx, CORE and SHELL, while this was not observed with chow, suggesting a main effect due to the diets. In the SHELL the accumulation was higher for the group that consumed sugar, similar to a previous report (Christiansen *et al.*, 2011). Moreover, the group consuming fat diet exhibited accumulation in the INSCx. Other studies have observed that high-fat diet causes an overproduction of ΔFosB in different areas of the reward system (Teegarden *et al.*, 2009; Sharma & Fulton, 2012; Baker & Reichelt, 2016). The accumulation of ΔFosB has been associated with the development of addiction-like behavior (Sharma & Fulton, 2012; Sharma *et al.*, 2013).

CONCLUSIONS

Young adults and adolescents are adopting shifted sleep patterns, based on sleep delay as part of their life style. Present data show the adverse effects of such sleep habits as a risk factor to develop compulsive food seeking behavior when combined with access to palatable food. We demonstrate that sleep delay can stmulate plastic changes in the brain that may prime individuals to develop binge eating of high fat or high sugar diet. The compulsive overeating and the preference for high fat or high sugar diets represent a risk for developing obesity and metabolic disease.

All together, data show that sleep delay combined with a high fat or high sugar diet and not with regular chow, represents a risk factor for developing CFSB.

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AUTHOR´S CONTRIBUTION

CE and EEB are responsible for the study concept, design and drafted the manuscript and interpretation of findings; EEB and LUR contributed to the acquisition of animal data and data analysis. All authors critically reviewed content and approved final version for publication.

DATA ACCESSIBILITY

Data and materials will be accessible in the laboratory website:

https://anatomia.facmed.unam.mx/investigacion/laboratorios/metabolismo

ABBREVIATIONS

CFSB: Compulsive food-Seeking Behavior; **CORE**: Accumbens Core; **CSD**: Chronic Sleeep Delay; **ILCx**: Infralimbic Cortex; **INSCx**: Insular Cortex; **PLCx**: Prelimbic Cortex; **SD**: Sleep Delay; **SHELL**: Accumbens Core; **CSD**: Chronic Sleep Delay; **CSD-F:** Chronic Sleep Delay +High-Fat Diet; **CSD-S**: Chronic Sleep Delay +High-Sugar Diet.

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FIGURE LEGENDS

Figure 1. (A) Chow consumption after one event of 4 hours of sleep delay (SD). (B) Neuronal Activation and (C) ΔFosB accumulation in areas of the corticolimbic system after the first hour of food consumption. Prelimbic Cortex (PLCx), Infralimbic Cortex (ILCx), Accumbens Core (CORE), Accumbens Shell (SHELL) and Insular Cortex (INSCx). Data are shown as mean ± s.e.m (n=8-10). (*) indicate significant difference from Control ($P < 0.05$).

Figure 2. (A) Actogram representing the chronic intermittent shifted sleep protocol based on Chronic Sleep Delay (CSD). Grey boxes represent the time when rats were in the slow rotating wheels. (B,C) Acrophases obtained for base line (BL) and the 4th week (4W) during weekdays (5days) and weekends (2 days). Data are shown as mean ± s.e.m (n=7-14). (*) indicates significant difference from Weekend. (#) indicates difference from Base Line (BL) ($P < 0.05$).

Figure 3. (A) Chow consumption during the first hour after 4 in the wheel (CSD) along 4 weeks. (B) Neuronal Activation and (C) ΔFosB accumulation in areas of the corticolimbic system on week 4 of the protocol. Data are shown as mean ± s.e.m (n=8-12). (*) indicate significant difference from Control ($P< 0.05$).

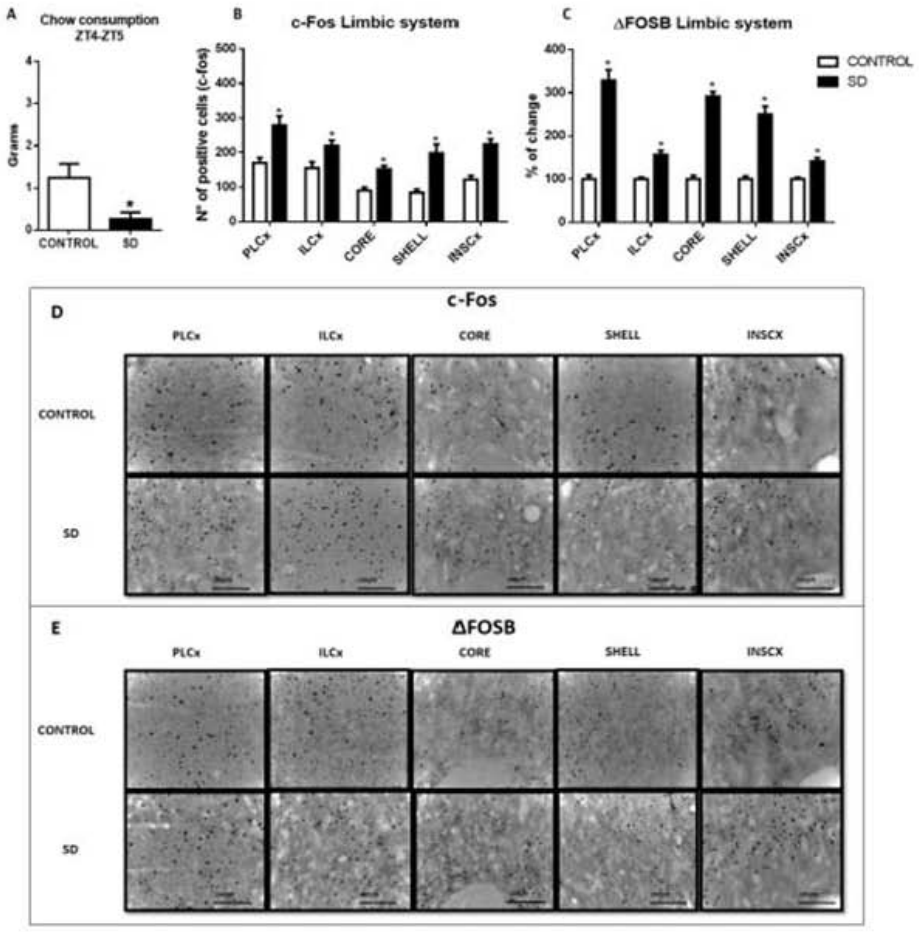
Figure 4. (A,B) Acrophases of Chronic Sleep Delay +Sugar (CSD-S) and Chronic

Sleep Delay +Fat (CSD-F) groups during base line (BL) and the 4th week (4W) of shifted sleep. (C,D) Sugar Diet or Fat Diet consumption during one hour following 4 hours of sleep delay (CSD) along 4 weeks of protocol in grams (C) and in kilocalories (D). Data are shown as mean ± s.e.m (n=10-17). (*) indicates significant difference from Weekend and CSD. (#) indicates difference from Base Line (BL). ($) indicates difference from CSD-S ($P < 0.05$).

Figure 5. (A) Neuronal Activation of areas of corticolimbic system after one hour of food consumption in the 4° week. (B) ΔFosB accumulation in areas of corticolimbic system after one hour of food consumption in the 4° week. Data are shown as mean ± s.e.m (n=8-12). (*) indicate significant difference from CSD ($P< 0.05$).

Figure 6. (A) Behaviors related with the interaction and effort to obtain the diet in the wire mesh box (n=10-14). (B) Behaviors related with grooming in presence to the wire mesh box (n=10-14). (C) Behaviors related with exploration in the home cage (n=10-14). (D) Average of general activity one hour before access to the diet (Craving-anticipation ZT3-ZT4) in Base Line (BL) and in Withdrawal (WD) (n=7-14). (E) Average of general activity at the expected time of diet consumption (Craving-anticipation ZT4-ZT5) in BL and in WD (n=7-14). (*) indicates significant difference from empty box. (#) indicates difference from base line (BL) ($P < 0.05$).

Figure 7. (A) Neuronal Activation of areas of corticolimbic system in WD. (B) ΔFosB accumulation in areas of corticolimbic system in WD (n=8-9). Data are shown as mean ± s.e.m. (*) indicates significant difference from CSD group. ($) indicates difference from CSD-S ($P < 0.05$).



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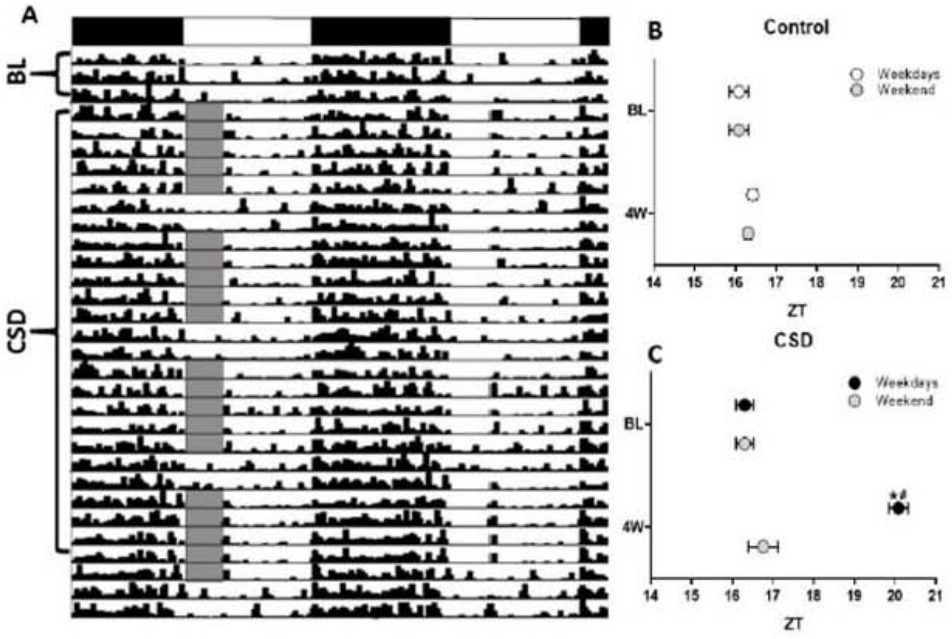

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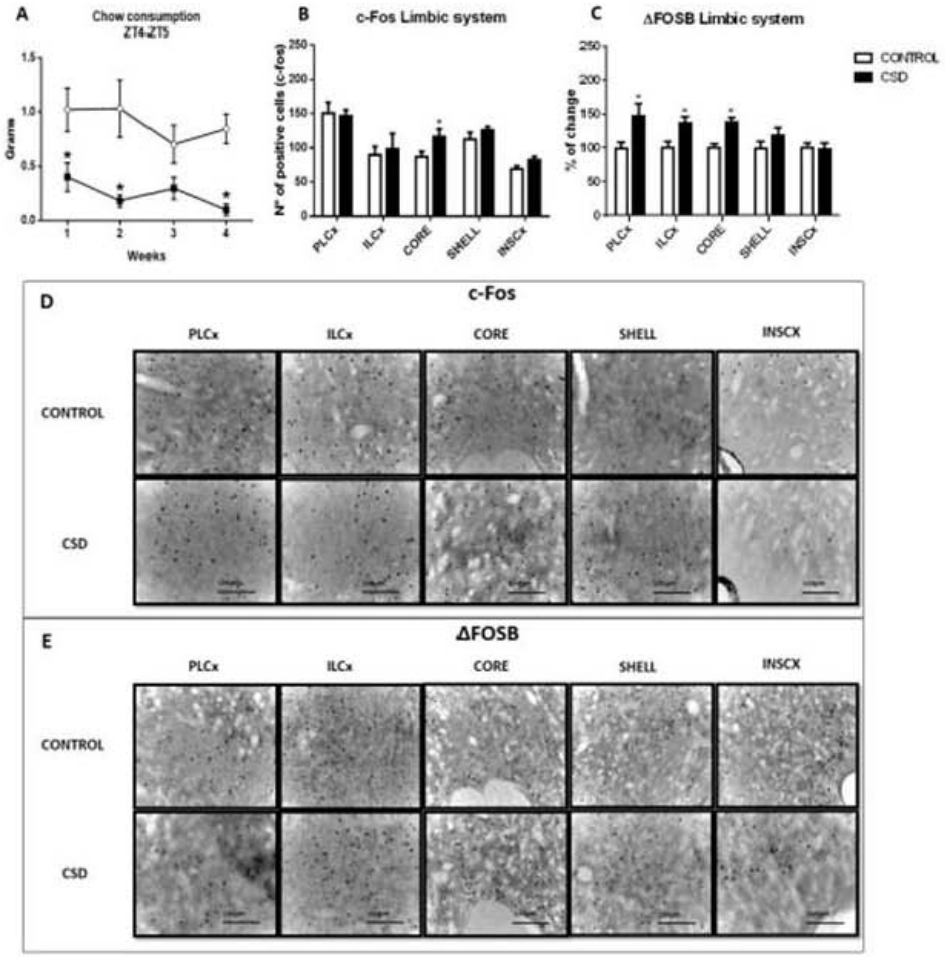

(A) Chow consumption during the first hour after 4 in the wheel (CSD) along 4 weeks. (B) Neuronal Activation and (C) ΔFosB accumulation in areas of the corticolimbic system on week 4 of the protocol. Data are shown as mean ± s.e.m (n=8-12). (*) indicate significant difference from Control ($P < 0.05$).

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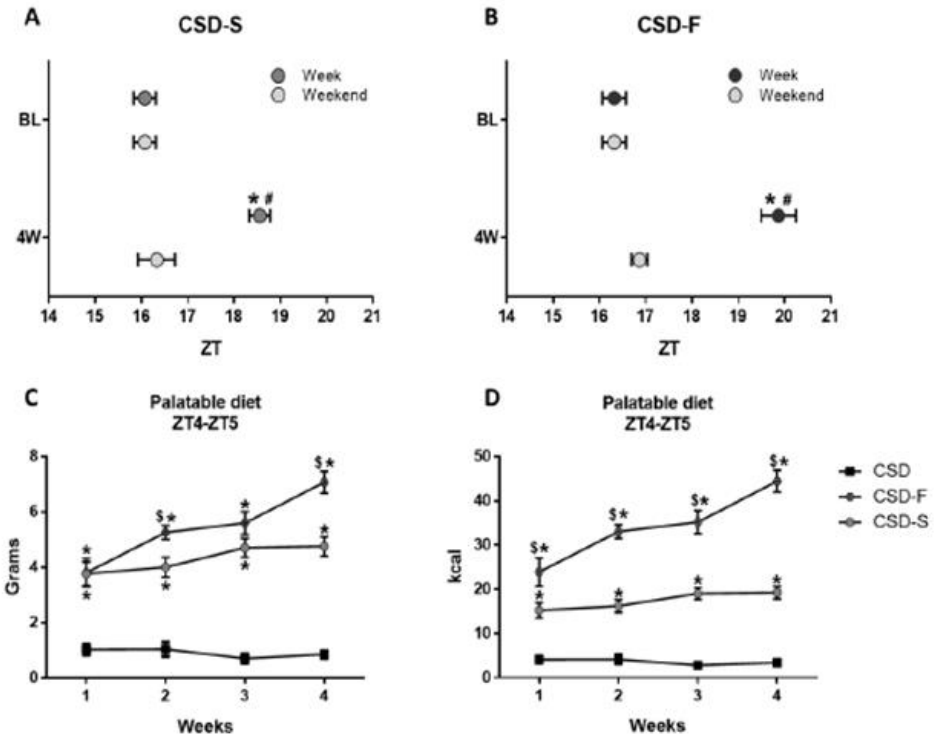

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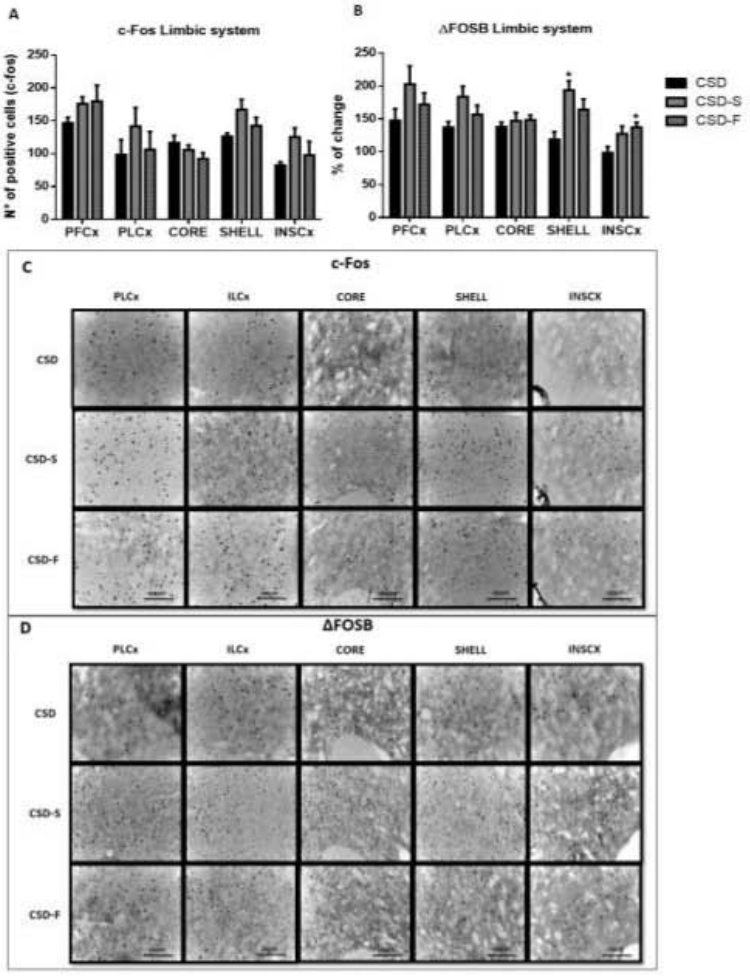

(A) Neuronal Activation of areas of corticolimbic system after one hour of food consumption in the 4° week. (B) ΔFosB accumulation in areas of corticolimbic system after one hour of food consumption in the 4° week. Data are shown as mean ± s.e.m (n=8-12). (*) indicate significant difference from CSD ($P < 0.05$).

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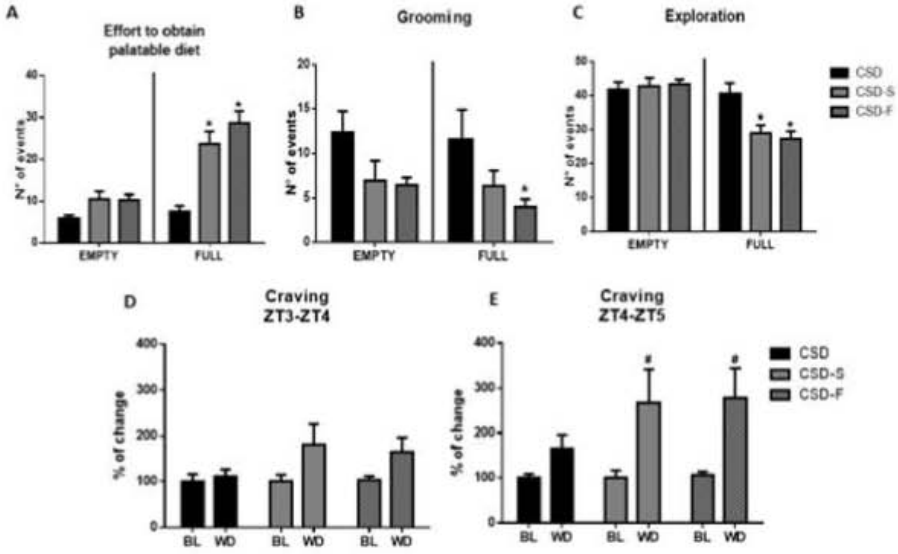

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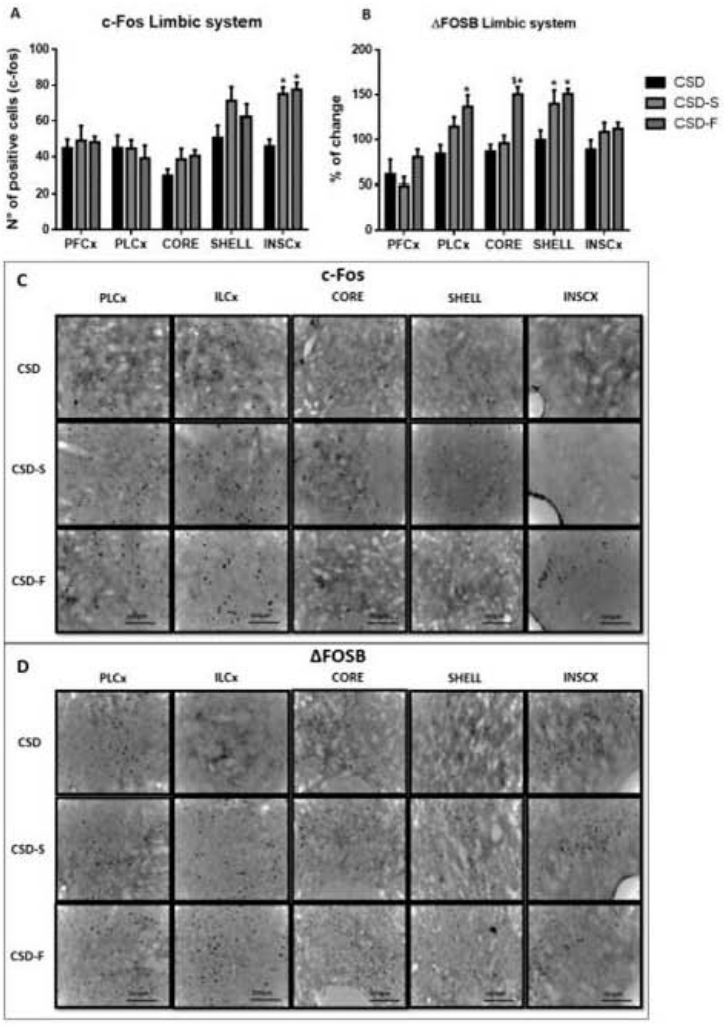

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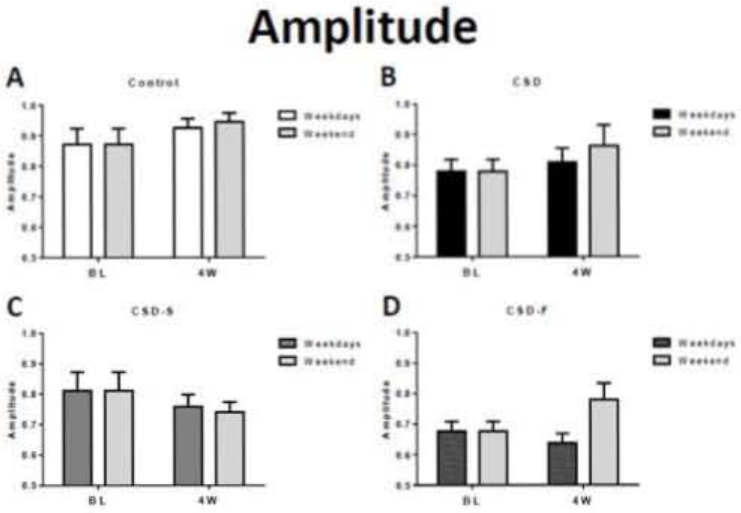
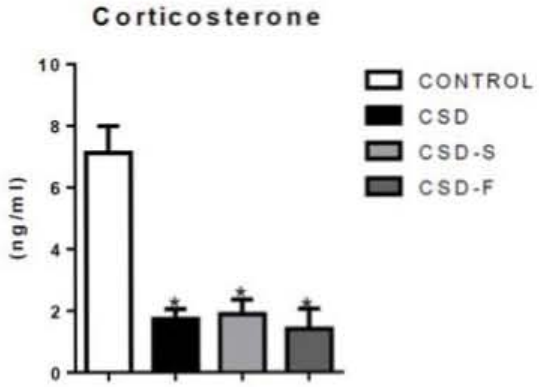


Supplementary figure 1. Amplitude for general activity rhythms for the Base Line (BL) and week 4 (4W). The amplitude was calculated for 5 weekdays and for 2 days of the weekend. Data are shown as mean ± s.e.m (n=7-14).



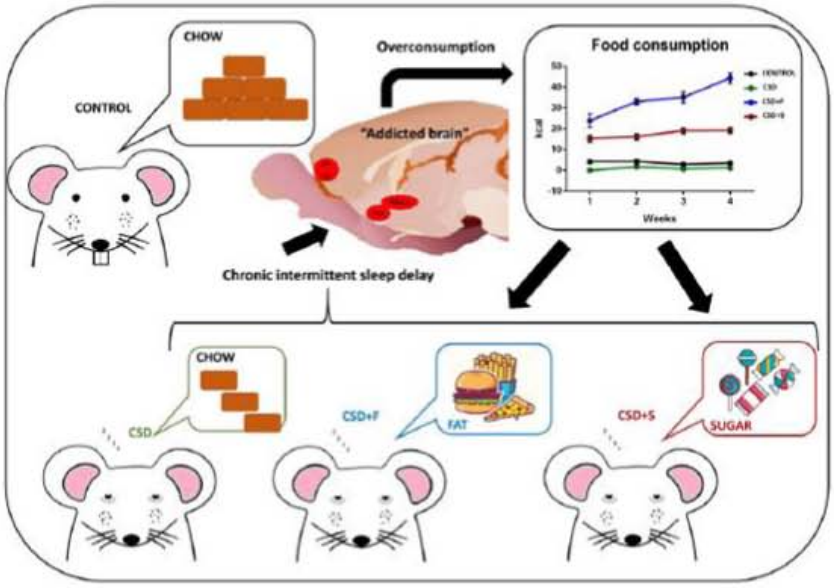
Supplementary figure 2. Levels of corticosterone after 4 weeks in the protocol of Chronic intermittent sleep delay. Blood samples were taken before perfusion in the 4° week of protocol. Data are shown as mean ± s.e.m (n=5-6). (*) indicate significant difference from Control ($P < 0.05$).

Supplementary table 1. Percentage of each macronutrient in gram and in kilocalories for the Chow, High-Sugar Diet (HSD) and High-Fat Diet (HFD).

	Protein		Fat		Carbohydrate		Other elements		
	% (g)	% (kcal)	% (g)	% (kcal)	% (g)	% (kcal)	% (g)	% (kcal)	Kcal/g
CHOW	29.06	28.5	6.1	13.5	59.01	58	5.83	0	4.07
HSD (50%)	14.5	14.38	3.05	6.8	79.5	78.82	2.95	0	4.03
HFD (50%)	14.5	8.88	53.05	73.06	29.5	18.06	2.95	0	6.53

Supplementary Table 2. Behaviors evaluated with the wire-mesh box in 5 min recorded per subject, every 5 seconds the video was paused and the observer recorded the behavior occurring in that instant and according this table. 60 behaviors were obtained for the 5 min. recording.

Clasification	Behavior
Effort and interaction with the box	Pushing the box
	Rotating the box
	Biting the box
	Going up the box
	Smelling the box
	Touching with the legs
Grooming	Grooming
	Burying in the sawdust
	Digging in the sawdust
	Burying the box in the sawdust
Exploration	Walking
	Standing in two legs
	Smelling
	Still



Graphical abstract text

Chronic intermittent sleep delay (CSD) was induced in rats by shifting sleep onset between weekdays and weekends. CSD promoted plastic changes creating an "addicted brain" that primed individuals for overconsumption of high-fat (CSD +F) or high-sugar (CSD +S). Importantly, chow was not overconsumed. At the end of the protocol, access to the diet was interrupted and rats exhibited compulsive eating behaviors, indicating that CSD is a risk factor for the development of addictive-like eating behaviors.

8. AVANCES DE LA PUBLICACION #4

8.1 PLANTEAMIENTO DEL PROBLEMA

El protocolo experimental de jet lag social mostró que los animales expuestos a dieta de cafetería, consumían una mayor cantidad de alimento y cumplían con más criterios de síndrome metabólico que aquellos que no estaban expuestos al jet lag social. La dieta de cafetería está compuesta por alimentos altos en azúcar y altos en grasa, y las publicaciones siguientes mostraron que las ratas expuestas al protocolo de jet lag social prefieren la dieta alta en grasa, desarrollan con mayor magnitud conductas asociadas a las adicciones y producen mayores cambios en el sistema de recompensa, que aquellos que están expuestos a dieta alta en azúcar. A partir de lo anterior, es posible sugerir que la preferencia por dietas altas en grasa podría estar asociada a la generación de cambios compatibles con el síndrome metabólico, además de impactar en el sistema de recompensa, también afectar el funcionamiento dl sistema regulatorio homeostático.

8.2 HIPÓTESIS

1. Las ratas expuestas a una noche de retraso de sueño y con dieta alta en grasa, tendrán peores valores metabólicos que aquellos expuestos a dieta alta en azúcar o sin retraso de sueño.
2. Las ratas expuestas a una noche de retraso de sueño y con dieta alta en grasa, tendrán mayor activación hipotalámica, de neuronas orexigénicas y de neuronas MCHérgicas que aquellos expuestos a dieta alta en azúcar o sin retraso de sueño.
3. Las ratas expuestas al protocolo de jet lag social y el consumo crónico de dieta alta en grasa, tendrán peores valores metabólicos que aquellos expuestos a dieta alta en azúcar o sin el protocolo de jet lag social.
4. Las ratas expuestas al protocolo de jet lag social y el consumo crónico de dieta alta en grasa, tendrán mayor activación hipotalámica, de neuronas orexigénicas y de neuronas MCHérgicas que aquellos expuestos a dieta alta en azúcar o sin el protocolo de jet lag social.

5. Una semana de recuperación sin dieta y sin actividad forzada, reestablecerá los patrones de alimentación, pero no el peso corporal, la grasa acumulada, la activación de áreas hipotalámicas, de neuronas orexigénicas ni de neuronas MCHérgicas.

8.3 OBJETIVO GENERAL

- Comparar variables metabólicas (altos niveles de glucosa, triglicéridos, insulina, leptina, corticosterona y una mayor cantidad de acumulación de grasa visceral) en animales con y sin exposición al protocolo de jet lag y sometidos a regímenes alimentarios agudos o crónicos de dieta alta en azúcar o grasa o dieta estándar (chow).

8.3.1 OBJETIVOS PARTICULARES

1. Evaluar, después de una noche de retraso de sueño y una sola exposición (aguda) de dieta alta en grasa o azúcar, la respuesta metabólica.
2. Evaluar activación de áreas hipotalámicas, de neuronas orexigénicas y de neuronas MCHérgicas ante una noche de retraso de sueño y de una exposición (aguda) de dieta alta en grasa o azúcar.
3. Evaluar variables metabólicas ante el protocolo de jet lag social y consumo crónico de dieta alta en grasa o azúcar.
4. Evaluar la activación de áreas hipotalámicas, de neuronas orexigénicas y de neuronas MCHérgicas ante el protocolo de jet lag social y consumo crónico de dieta alta en grasa o azúcar.
5. Evaluar si con una semana de recuperación (sin dieta y sin actividad forzada) se reestablecen los patrones de alimentación, el peso corporal, la grasa acumulada, así como la activación de áreas hipotalámicas, de neuronas orexigénicas y de neuronas MCHérgicas, es decir sí los animales se comportan como un animal control.

8.4 Material y método

8.4.1 Animales y condiciones experimentales generales

Se utilizaron ratas Wistar obtenidas del bioterio general de la Facultad de Medicina de la UNAM y se mantuvieron individualmente en cajas de acrílico (45x30x35) en lockers con flujo de aire, temperatura contantes (21-23 C°) y un ciclo luz-oscuridad controlados 12:12, donde el prendido de luz fue a las 07:00 horas (ZT0) y el apagado a las 19:00 horas (ZT12). Todos los procedimientos se realizaron siguiendo las recomendaciones de la norma Oficial Mexicana sobre "Especificaciones técnicas para la producción, cuidado y uso de animales de laboratorio" (NOM-062-ZOO-1999). Los protocolos experimentales del presente estudio fueron aprobados por el comité de ética de la Facultad de Medicina (FM/DI/013/2018). Se realizó monitoreo continuo y automatizado de conducta y se tomó el peso corporal y de la comida ingerida (chow y dieta) durante todo el protocolo. Todos los grupos tuvieron acceso libre a agua y a la dieta chow.

8.4.2 Diseño experimental

***Experimento 1*:** Para la evaluación de variables metabólicas y activación neuronal ante una exposición de retraso de sueño, aunado a la exposición a dietas altas en grasa o alta en azúcar, se obtuvieron 6 grupos:

1. **Control** (n=10): Sujetos en condiciones ad-libitum de comida y agua.
2. **Retraso de sueño (SD)** (n=10): Sujetos sometidos a ruedas de actividad forzada por las primeras 4hrs de su fase de descanso (ZT0-ZT4). Después de esas 4hrs en la rueda, los sujetos fueron devueltos a sus cajas.
3. **Azúcar (HSD)** (n=10): Sujetos con acceso a dieta alta en azúcar (50% chow + 50% azúcar) de ZT4 a ZT5.
4. **RS+Azúcar (SD+HSD)** (n=10): Sujetos sometidos a ruedas de actividad de ZT0 a ZT4, al ser regresados a sus cajas se les da acceso a dieta alta en azúcar (50% chow + 50% azúcar) de ZT4 a ZT5.
5. **Grasa (HFD)** (n=10): Sujetos con acceso a dieta alta en grasa (50% chow + 50% grasa) de ZT4 a ZT5.

6. **RS+Grasa (SD+HFD)** (n=10): Sujetos sometidos a ruedas de actividad de ZT0 a ZT4, al ser regresados a sus cajas se les da acceso a dieta alta en grasa (50% chow + 50% grasa) de ZT4 a ZT5.

Ante un solo evento de exposición a la rueda, a la dieta o ambas fueron sacrificados 90 min después del acceso a las dietas, para la extracción de sangre y perfusión de cerebros para analizar inmunohistoquímicamente con c-Fos la activación del núcleo arqueado, el hipotálamo lateral, el área perifornical, el núcleo paraventricular del tálamo, la zona inserta; además de la evaluación de la activación de la población MCHérgica y orexigénica en el hipotálamo lateral y el área perifornical.

***Experimento 2*:** Para la evaluación de variables metabólicas y activación neuronal ante el protocolo de jet lag social, aunado a la exposición a dietas altas en grasa o alta en azúcar, se obtuvieron los mismos grupos del experimento 1, solo que en vez de ser condiciones agudas, la exposición a la rueda de actividad forzada fue por 4 semanas de lunes a viernes (las siglas se sustituyen a SJL, en vez de SD), sin manipulaciones en los fines de semana, para crear el modelo de jet lag social. La administración de las dietas fueron crónicas, después del acceso a las ruedas por una hora (ZT4-ZT5). A lo largo de las semanas fue monitoreado el peso corporal, la cantidad de alimento chow y la cantidad de dietas consumidas. Los animales fueron sacrificados al final de la semana 4, 90 min después del acceso a las dietas, para la extracción de sangre y perfusión de cerebros para analizar inmunohistoquímicamente con c-Fos la activación del núcleo arqueado, el hipotálamo lateral, el área perifornical, el núcleo paraventricular del tálamo, la zona inserta; además de la evaluación de la activación de la población MCHérgica y orexigénica en el hipotálamo lateral y el área perifornical.

***Experimento 3*:** Para la evaluación de los patrones de alimentación, el peso corporal, la grasa acumulada, así como la activación del núcleo arqueado, el hipotálamo lateral, el área perifornical, el núcleo paraventricular del tálamo, la zona inserta; además de la evaluación de la activación de la población MCHérgica y

orexigénica en el hipotálamo lateral y el área perifornical, ante una semana de recuperación, después de 4 semanas en el protocolo de jet lag social y la administración de dietas, se realizó otra serie de la misma forma que el experimento 2, después de 4 semanas en el protocolo de jet lag social, se devolvieron a los animales a condiciones control por una semana, midiendo el peso corporal y el consumo de alimento. Los animales se sacrificaron al final de la semana 5 entre ZT5 y ZT6.

8.4.3 Obtención de tejidos y procesamiento de cerebros

Para la obtención sangre, grasa y cerebros, las ratas fueron sacrificadas por medio de una sobredosis de pentobarbital (0.63 grams/ kg; Pisabental, Pisa agropecuaria S.A. de CV., México). Al caer sedadas, se extrajeron 3ml de sangre por animal y fueron perfundidas a través del ventrículo izquierdo del corazón hacia la arteria aorta. Se utilizó solución salina isotónica al 0.9% seguido de paraformaldehido al 4.0% en buffer fosfato (PBS, 0.1 M, con un PH de 7.2). Se extrajeron los cerebros, las almohadillas de grasa gonadal y retroabdominal con ayuda de instrumental quirúrgico; los cerebros se post fijaron en paraformaldehido al 4.0% durante una hora. Posteriormente, los tejidos fueron almacenados en sacarosa al 30% para su crioprotección y posteriormente fueron congelados y cortados a -20°C en secciones coronales de 40µm. Las secciones de tejido fueron incubados por medio de inmunohistoquimica en anticuerpo para c-Fos (rabbit polyclonal, 1:3000; Millipore Corp., USA). Para la evaluación de la activación de neuronas orexigénicas, otra serie de tejido fue incubada con c-Fos (rabbit polyclonal, 1:3000; Millipore Corp., USA) y con ORX (goat polyclonal, 1:5000; Santacruz). Para la evaluación de la activación de neuronas MCHérgicas, otra serie de tejido fue incubada con c-Fos (rabbit polyclonal, 1:3000; Millipore Corp., USA) y con MCH (obsequiado por el Dr. Rudolf M. Buijs).

8.4.4 Parámetros de metabolismo alterado

El cumplimiento los siguientes criterios, es suficiente para ser considerado como metabolismo alterado:

	Parametros
Hiperglicemia (mg/dl. Fasting)	>100
Test de tolerancia a la glucosa (área bajo la curva) (mg/dl)/min	>150
Ganancia de peso (%)	10%
Hiperinsulinemia (ng/dl)	>6
Grasa visceral (%)	3%
Alto colesterol (mg/dl)	>175
Hipertrigliceridemia (mg/dl)	>150
Glucosa despues del consume de la dieta (mg/dl)	>180 (2 horas postprandial)
Corticosterona (ng/ml)	ZT5 (>20)
Leptina (ng/ml)	>8

8.4 Resultados

Tras una sola exposición de dieta sabrosa, pudimos observar que los animales consumían una mayor cantidad de dieta alta en azúcar que dieta alta en grasa y chow consumido por el control (Tabla 1). El AVOVA de una vía mostro significancia entre grupos ($F_{(2,27)}$=7.19; p<0.001). Pero cuando se calcularon las kilocalorías consumidas, los animales con las dietas sabrosas consumieron el mismo número de kilocalorías, que fueron mayores al control (Tabla 1). El AVOVA de una vía mostro significancia entre grupos ($F_{(2,27)}$=12.81; p<0.0001). Cuando los animales fueron expuestos a una noche de retraso de sueño mediante la rueda de actividad forzada, el ANOVA mostró que la dieta que seguía consumiéndose en mayor cantidad era la dieta alta en azúcar, seguida por la dieta alta en grasa y en menor cantidad el chow ($F_{(2,21)}$=51.05; p<0.0001). Cuando se calcularon las kilocalorías consumidas, se observó el mismo efecto que cuando sólo se administraban las

dietas solas, no hubo diferencias en el consumo de las dietas sabrosas entre si, pero ambas fueron consumidas en mayor cantidad que el chow ($F_{(2,21)}$=30.52; p<0.0001) (Tabla 1). Se realizó un ANOVA de dos vías para medir los efectos del factor dieta y el factor retraso de sueño y se encontraron diferencias significativas en la interacción de ambos factores en el consumo de alimento medido en gramos ($F_{(2,48)}$=7.78; p<0.001) y en kilocalorías ($F_{(2,48)}$=5.85; p<0.005).

Una hora después del consumo de las dietas, se tomaron muestras de sangre para establecer la reacción metabólica a las dietas; pudimos observar que ambos grupos que comieron dieta alta en azúcar (con o sin retraso de sueño) obtuvieron mayores niveles de glucosa a diferencia de los que solo comieron chow o dieta alta en grasa El ANOVA de una vía indicó diferencias en los que sólo consumían las dietas ($F_{(2,33)}$=33.72; p<0.0001) y en los grupos expuestos al retraso del sueño ($F_{(2,33)}$=7.04; p<0.0028) (Tabla 1). Cuando se realizó el ANOVA de dos vías para evaluar la significancia de los efectos del factor dieta y el factor retraso del sueño, se encontraron diferencias significativas en la interacción de ambos factores en los niveles de glucosa ($F_{(2,66)}$=4.25; p<0.018).

En cuanto al manejo de lípidos, aquellos grupos que ingirieron dieta alta en grasa mostraron altos niveles de triglicéridos en sangre, tanto los que solo consumieron la dieta ($F_{(2,21)}$=39.15; p<0.0001) como los expuestos al retraso del sueño ($F_{(2,20)}$=11.75; p<0.0004). Cuando se aplicó el ANOVA de dos vías para medir los efectos del factor dieta y el factor retraso de sueño, se observaron diferencias significativas en la interacción de ambos factores en los niveles de triglicéridos ($F_{(2,41)}$=6.78; p<0.002). En cuanto a los niveles de insulina y leptina, no se encontraron diferencias significativas entre grupos (Tabla 1). Cabe mencionar que dentro de los rangos para establecer el síndrome metabólico, en esta primera exposición a las dietas, el único parámetro rebasado fue el de triglicéridos, con aquellos animales que consumían dieta alta en grasa.

Tabla 1: Consumo de dietas (1h). Parámetros metabólicos medidos una hora después de haber consumido la dieta una sola vez. Los recuadros amarillos indican

el cumplimiento de lo definido para síndrome metabólico. Números en negritas indican diferencias significativas. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\$}$) Diferente de SD+HFD.

	CTRL	HSD	HFD	SD	SD+HSD	SD-HFD
Consumo de alimento (gramos)	1.24 **+/-** 0.33	**2.53 +/- 0.14***	1.93 **+/-** 0.16	0.26 **+/-** 0.15	**3.32 +/- 0.19^{+}**	**1.58 +/- 0.27^{+}°**
Consumo de alimento (kcal)	5.04 **+/-** 1.36	**10.27 +/- 0.57***	**12.19 +/- 1.00***	1.07 **+/-** 0.62	**13.47 +/- 0.8^{+}**	**9.9 +/- 1.72^{+}**
Glucosa después de la dieta (mg/dl)	96.36 **+/-** 2.18	**122.97 +/- 2.92***	100.38 **+/-** 2.23	101.31 **+/-** 2.38	**116.35 +/- 3.4$^{\$}$**	108.5 +/- 2.61
Hiperinsulinemia (ng/dl)	2.96 **+/-** 0.65	4.6 **+/-** 0.3	4.36 **+/-** 0.55	4.59 **+/-** 0.68	3.14 **+/-** 0.61	4.84 **+/-** 1.09
Hipertrigliceridemia (mg/dl)	96.74 **+/-** 12.61	124.75 **+/-** 10.69	**242.91 +/- 14.19***	105.27 **+/-** 12.1	109.79 **+/-** 10.74	**170.03 +/- 9.17^{+}**
Leptina (ng/ml)	3.4 **+/-** 0.33	4.11 **+/-** 0.16	2.95 **+/-** 0.47	3.99 **+/-** 0.43	3.58 **+/-** 0.38	3.74 **+/-** 0.19

Tras el consumo de las dietas en cada una de las condiciones, se evaluó la activación hipotalámica para determinar si existía alguna activación diferencial en los núcleos de interés. En los grupos que sólo consumieron la dieta, el ANOVA mostró diferencias significativas y con una mayor activación tras el consumo de una dieta alta en grasa en el área perifornical (PFA) ($F_{(2,24)}$=19.41; p<0.0001), el hipotálamo lateral (LH) ($F_{(2,27)}$=9.61; p<0.0007), el núcleo paraventricular del tálamo (PVT) ($F_{(2,24)}$=20.63; p<0.0001), la zona inserta (ZI) ($F_{(2,22)}$=4.8; p<0.018) y el núcleo arqueado (ARQ) ($F_{(2,21)}$=7.64; p<0.0032) (Fig. 1A). Cuando los animales se sometieron a la condición de retraso del sueño, la dieta alta en grasa sólo tuvo impacto en el LH ($F_{(2,27)}$=4.10; p<0.03), el PVT ($F_{(2,27)}$=6.85; p<0.0039) y la ZI

($F_{(2,24)}$=6.48; p<0.005); mientras que en los demás núcleos evaluados no se identificaron diferencias significativas (Fig. 1D). Al realizar el ANOVA de dos vías para evaluar los efectos del factor dieta y el factor retraso del sueño, sólo se encontraron diferencias significativas en la interacción en los niveles de c-Fos en el PFA ($F_{(2,45)}$=6.83; p<0.0026), el PVT ($F_{(2,48)}$=3.19; p<0.04) y el ARQ ($F_{(2,42)}$=5.36; p<0.008).

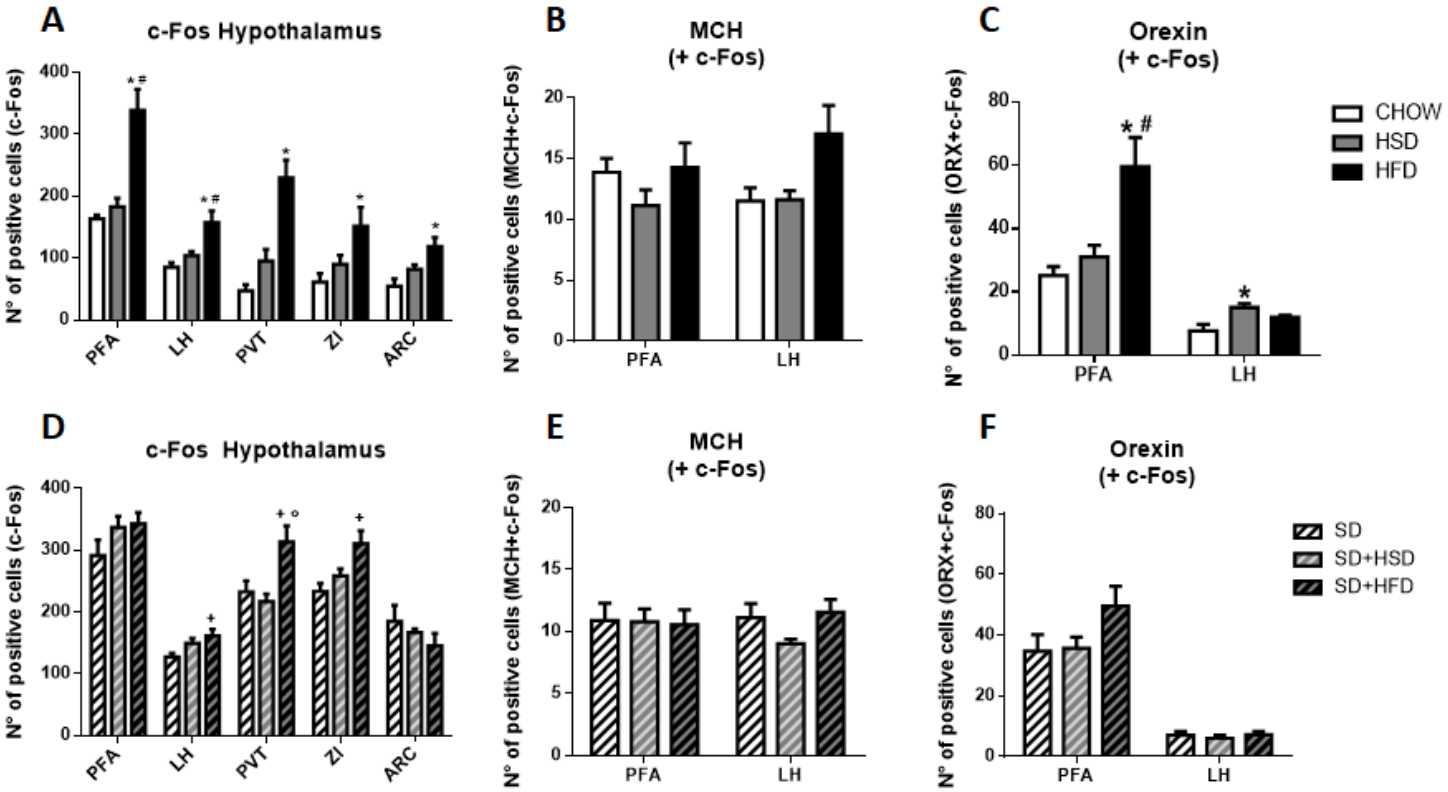


Figura 1: (A, D) Activación hipotalámica después del consumo aguda de dieta alta en azúcar (HSD o SD+HSD) o dieta alta en grasa (HFD o SD+HFD); medida en el área perifornical (PFA), el hipotálamo lateral (LH), el núcleo paraventricular del tálamo (PVT), la zona inserta (ZI) y el núcleo arqueado (ARQ). (B, E) Activación de neuronas positivas a la hormona concentradora de melanocitos (MCH) en PFA y LH. (C, F) Activación de neuronas positivas a orexinas (ORX) en PFA y LH. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\#}$) Diferente de HSD.

Cuando evaluamos la activación de los grupos neuronales específicos de MCH y ORX, observamos que en el PFA y el LH, las neuronas que producían MCH no fueron diferencialmente activadas por alguna dieta en específico (Fig. 1B). Tampoco fueron diferencialmente activadas cuando los animales estuvieron expuestos al retraso del sueño (Fig. 1E). Sin embargo, cuando se evaluó la activación de neuronas que producen ORX, pudimos observar que los animales expuestos a dieta alta en grasa producían una mayor activación en el área perifornical ($F_{(2,27)}$=4.06; p<0.032), mientras que la dieta alta en azúcar tuvo una mayor activación en el LH ($F_{(2,21)}$=9.44; p<0.0008) (Fig. 1C). Después de una noche de retraso del sueño no se encontraron diferencias significativas en este grupo neuronal en ningún grupo. (Fig. 1F). Al realizar el ANOVA de dos vías para conocer la significancia de la diferencia de los efectos del factor dieta y el factor retraso de sueño, sólo se encontraron diferencias significativas en la interacción en las neuronas productoras de orexinas activas en el LH ($F_{(2,42)}$=5.76; p<0.0061).

El siguiente experimento crónico mostró que a lo largo de las semanas, los grupos expuestos a la dieta alta en grasa, consumieron mayor cantidad de kilocalorías que aquellos que consumían la dieta alta en azúcar o chow (todos los grupos sin exposición al jet lag social). El ANOVA de medidas repetidas mostró diferencias en la interacción (dieta X tiempo) ($F_{(6,135)}$=5.06; p<0.0001) (Fig. 2A), mientras que con la exposición al protocolo de jet lag social, el ANOVA de medidas repetidas mostró diferencias en la interacción (dieta X tiempo) ($F_{(6,114)}$=7.45; p<0.0001) (Fig. 2D). Esto se corroboró promediando del consumo a lo largo de las 4 semanas de exposición, tras lo que se identificaron diferencias marcadas por el ANOVA de una vía ($F_{(2,43)}$=155.5; p<0.0001) (Tabla 2), lo mismo que en los sujetos expuestos al jet lag social ($F_{(2,36)}$=313.6; p<0.0001) (Tabla 2). También se pudo observar que los animales que consumían dieta palatable, consumían una menor cantidad de chow el resto del día en ambas condiciones, para compensar kilocalóricamente el consumo de la dieta palatable (Fig. 2B, E). El ANOVA de medidas repetidas mostro diferencias en la interacción (dieta X tiempo) ($F_{(6,99)}$=5.76; p<0.0001) en el consumo

de chow en animales que sólo consumían la dieta palatable; mientras que para los sujetos expuestos al jet lag social el ANOVA de medidas repetidas, se encontraron diferencias en la interacción (dieta X tiempo) ($F_{(6,87)}$=2.69; p<0.01). Cuando se sumaba el consumo de la dieta y el chow, solo el grupo que sólo consumía dieta alta en grasa tuvo un sobreconsumo la primera semana de exposición, mientras que las consecuentes semanas no mostraron diferencias (Fig. 2C); el ANOVA de medidas repetidas mostro diferencias en la interacción (dieta X tiempo) ($F_{(6,99)}$=2.71; p<0.01). Cuando los animales estuvieron expuestos a jet lag social, no se encontraron diferencias en el consumo kilocalórico total a lo largo de las semanas, pero el ANOVA de medidas repetidas mostro diferencias en el factor tiempo ($F_{(3,99)}$=2.71; p<0.03) (Fig. 2F).

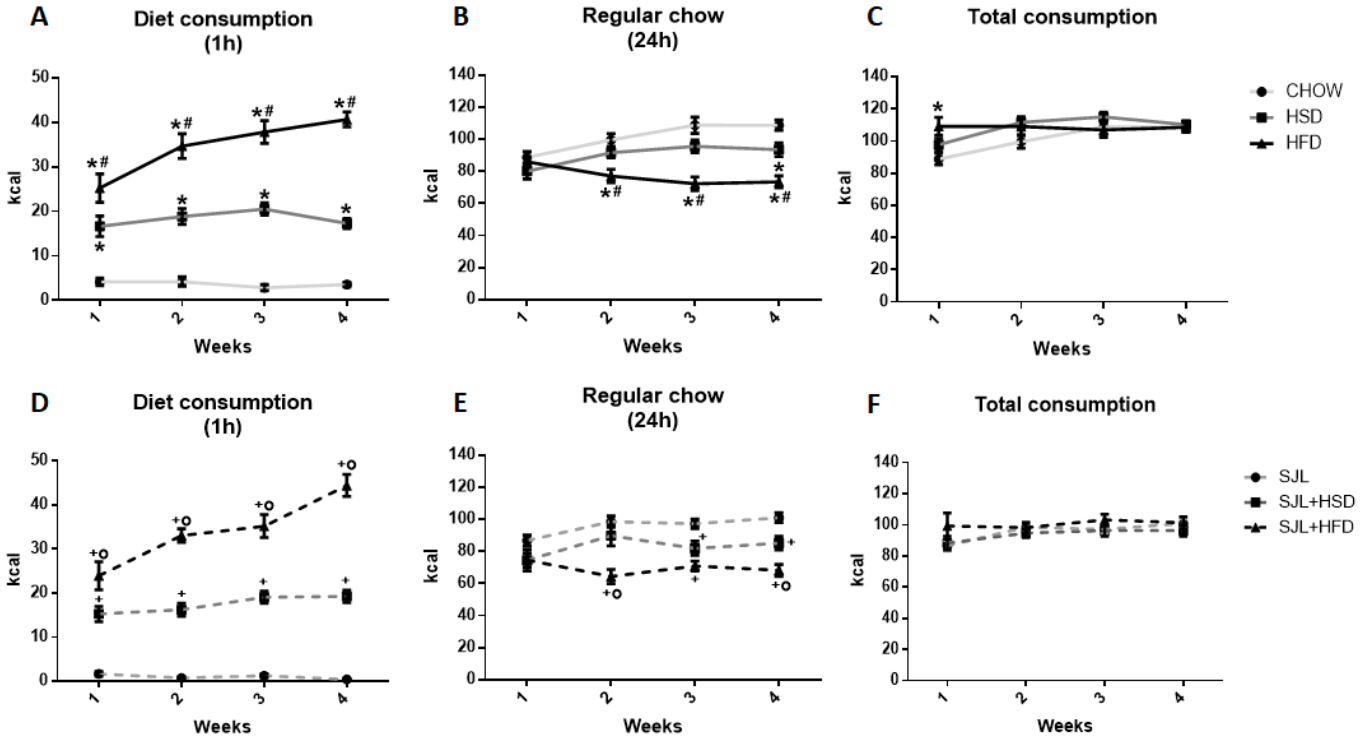


Figura 2: (A, D) Consumo kilocalórico diario de las diferentes dietas. (B, E) Consumo kilocalórico solo de chow a lo largo de 24 horas. (C, F) Suma kilocalórica del consumo de dietas y el consumo de chow. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\#}$) Diferente de HSD.

Se registró el peso de los animales a lo largo del protocolo y pudimos observar que los animales que consumían dieta alta en grasa, mostraban una mayor ganancia de peso a partir de la segunda semana de exposición a la dieta en comparación con los que solo consumían chow y dieta alta en azúcar. El ANOVA de medidas repetidas mostró diferencias en el factor tiempo ($F_{(3,99)}$=701.2; $p<0.0001$) y el factor dieta ($F_{(2,33)}$= 4.79; $p<0.01$) (Fig. 3A). En el protocolo de jet lag social, no se encontraron diferencias significativas entre grupos a lo largo de la semana, pero el ANOVA de medidas repetidas mostró diferencias en el factor tiempo ($F_{(3,87)}$=193.1; $p<0.0001$ (Fig. 3E). Al final de la exposición a dietas, el grupo que consumía dieta alta en grasa ganó más peso corporal ($F_{(2,32)}$=6.61; $p<0.003$) (Fig. 3B), mientras que no hubo diferencias en los que estuvieron expuestos a la rueda de actividad forzada (Fig. 3F).

Al final de la cuarta semana, se realizó un test de tolerancia a la glucosa y observamos que los animales que consumían dieta alta en grasa, tuvieron mayores niveles de glucosa a lo largo de la prueba en comparación con los que solo consumían chow o dieta alta en azúcar, el ANOVA de medidas repetidas mostro diferencias en la interacción (dieta X tiempo) ($F_{(8,84)}$=2.07; $p<0.05$) (Fig. 3D); al igual que los animales expuestos a la dieta alta en grasa y con jet lag social, mostrado por el ANOVA de medidas repetidas, significancia en la interacción (dieta X tiempo) ($F_{(8,88)}$=5.05; $p<0.0001$) (Fig. 3H). Al final de la prueba, se obtuvo el área bajo la curva de cada grupo, para establecer el cumplimiento de síndrome metabólico. Se observó que el grupo que consumía dieta alta en grasa sin jet lag ($F_{(2,21)}$=4.68; $p<0.02$) y con jet lag ($F_{(2,22)}$=5.33; $p<0.012$) mostró un área mayor (Tabla 2). A pesar de no ser diferentes significativamente, los grupos de dieta alta en azúcar y jet lag social, superaron los valores establecidos para el cumplimiento de síndrome metabólico.

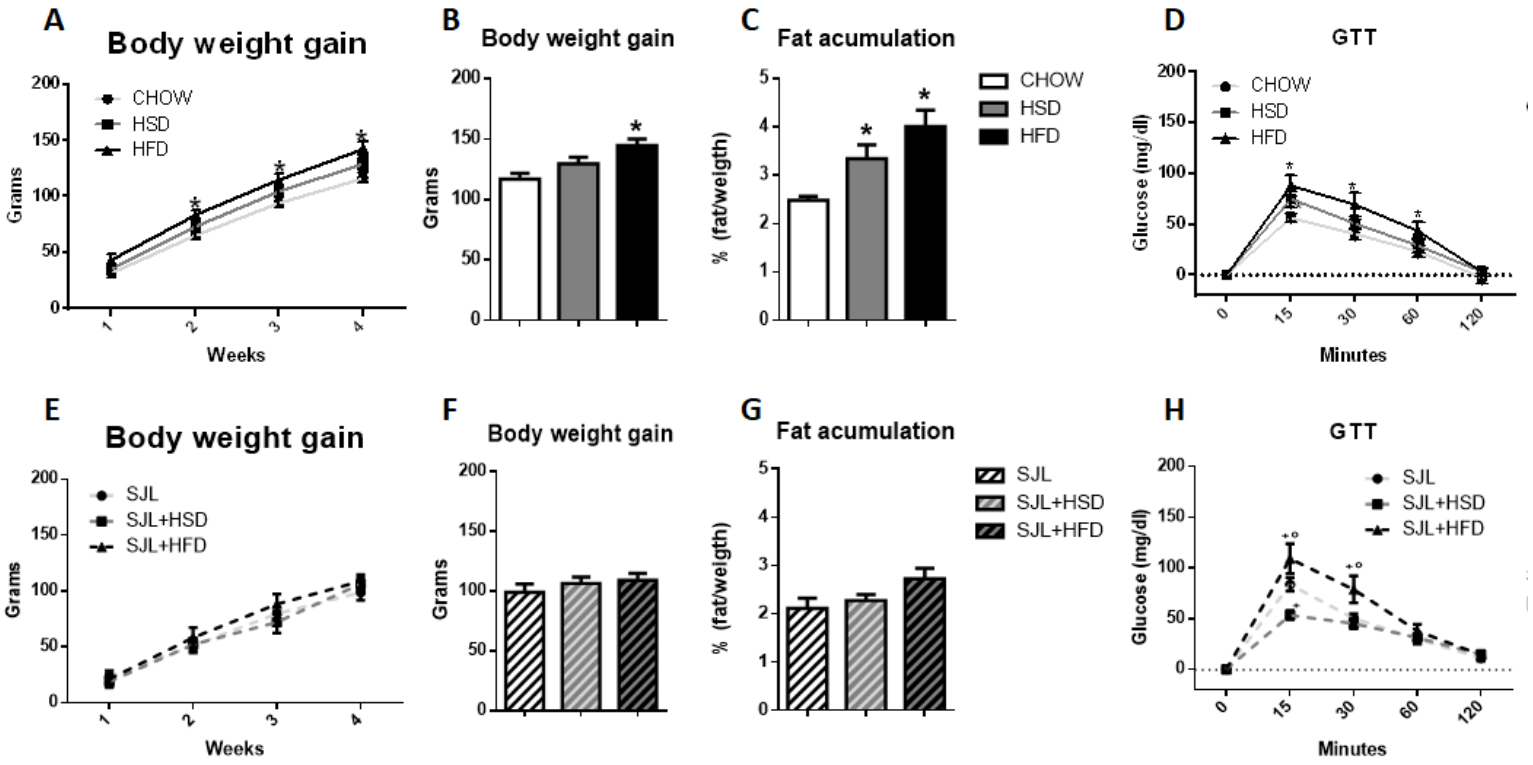


Figura 3: (A, E) Ganancia de peso a lo largo de 4 semanas de exposición a las dietas y/o el protocolo de jet lag social. (B, F) Ganancia de peso total. (C, G) Acumulación de grasa abdominal, después de 4 semanas de exposición a las dietas y/o el protocolo de jet lag social. (D, H) Test de tolerancia a la glucosa. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\#}$) Diferente de HSD.

Dentro de los parámetros para determinar si los animales desarrollaron síndrome metabólico, se obtuvo el porcentaje de peso ganado a lo largo del protocolo y el ANOVA de una vía mostró diferencias significativas en el grupo que consumía dieta alta en grasa ($F_{(2,33)}$=6.05; p<0.005), por su parte, el grupo que consumía dieta alta en azúcar ganó 10.7%, y aunque no fue estadísticamente significativa la diferencia, superó el parámetro de síndrome metabólico. Los grupos expuestos a jet lag social no mostraron diferencias significativas entre ellos, pues incluso la ganancia fue menor que el grupo control. Asociado al peso corporal, otro de los parámetros evaluados fue la acumulación de grasa y pudimos observar que tanto los animales

que comían dieta alta en azúcar o grasa, tuvieron una mayor acumulación de grasa en comparación con el control ($F_{(2,18)}$=11.85; p<0.0005) (Fig. 3C, Tabla 2) rebasando el cumplimiento del criterio para síndrome metabólico; esto no se vio en ninguno de los grupos expuestos al jet lag social (Fig. 3G, Tabla 2).

Los niveles de glucosa fueron evaluados en ayuno y después de consumir las dietas. En ayuno pudimos observar los animales que consumieron dieta alta en grasa, tuvieron mayores niveles ($F_{(2,22)}$=17.7; p<0.0001); mientras que dentro de los grupos expuestos a jet lag social, los que mostraron mayores niveles fueron los que consumían dieta alta en azúcar ($F_{(2,26)}$=9.48; p<0.0008). Pero ninguno rebasó el parámetro de síndrome metabólico. Al realizar el ANOVA de dos vías para conocer la significancia estadística de los efectos del factor dieta y el factor retraso del sueño, hubo diferencias significativas en la interacción en cuanto a los niveles de glucosa en ayuno ($F_{(2,48)}$=3.98; p<0.025). Cuando los niveles de glucosa se evaluaron después del consumo de las dietas, no hubo diferencias significativas con o sin exposición al jet lag social. En cuanto a los niveles de insulina, pudimos observar cambios significativos en aquellos animales que consumían dieta alta en grasa, tanto sin la exposición al jet lag social ($F_{(2,15)}$=7.97; p<0.004), como en aquellos que estaban expuestos al jet lag social ($F_{(2,15)}$=14.97; p<0.0003). Sin embargo, todos los grupos con o sin jet lag social, rebasaron el criterio de síndrome metabólico (Tabla 2).

Los niveles de corticosterona mostraron diferencias significativas en el grupo que consumía dieta alta en grasa ($F_{(2,10)}$=26.38; p<0.0001) cumpliendo con el parámetro para síndrome metabólico, mientras que en los animales con jet lag social no hubo diferencias significativas, sorprendentemente tuvieron valores más bajos que el control. Se realizó el ANOVA de dos vías para evaluar la significancia los efectos del factor dieta y el factor retraso del sueño, y hubo diferencias significativas en la interacción en cuanto a los niveles de corticosterona ($F_{(2,24)}$=34.37; p<0.0001).

En cuanto a los niveles de triglicéridos en sangre, los grupos expuestos a dieta alta en grasa obtuvieron niveles significativamente mayores, tanto sin exposición al jet lag social ($F_{(2,20)}$=13.7; p<0.0002), como con exposición a jet lag social ($F_{(2,21)}$=109.0; p<0.0001). A pesar de eso, el grupo expuesto solo a dieta alta en azúcar, también rebasó el criterio para el cumplimiento de síndrome metabólico (Tabla 2).También se realizó el ANOVA de dos vías para determinar los efectos del factor dieta y el factor retraso de sueño, y hubo diferencias significativas en la interacción en cuanto a los niveles de triglicéridos ($F_{(2,41)}$=6.17; p<0.004). En cuanto a los niveles de leptina, se encontraron diferencias significativas en los grupos expuestos a dieta alta en grasa, sin jet lag ($F_{(2,18)}$=7.62; p<0.004) y con jet lag ($F_{(2,17)}$=11.74; p<0.0006). Aun así, los sujetos expuestos a la dieta alta en azúcar volvieron a rebasar el criterio para el cumplimiento de síndrome metabólico (Tabla 2). También se realizó el ANOVA de dos vías para conocer los efectos del factor dieta y el factor retraso de sueño, y hubo diferencias significativas en la interacción en cuanto a los niveles de leptina ($F_{(2,35)}$=4.13; p<0.024).

Tabla 2: Parámetros metabólicos asociados al síndrome metabólico, medidos al final de la 4° semana de protocolo y justo después del consumo de las dietas. Los recuadros amarillos indican que se cumplió el criterio para síndrome metabólico. Números en negritas indican diferencias significativas. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\#}$) Diferente de HSD.

	CTRL	HSD	HFD	SJL	SJL+HSD	SJL-HFD
Dieta consumida durante 4 semanas (kcal-mean)	3.7 +/- 0.38	**17.79 +/- 1.07***	**33.44 +/- 1.41*$^{\#}$**	1.00 +/- 0.17	**16.87 +/- 0.97^{+}**	**34.74 +/- 1.22^{+}°**
Ganancia de peso (%)	0% +/- 4.16	10.7% +/- 4.61	**22.26% +/- 4.77***	-15.41% +/- 5.95	-9.03% +/- 4.65	-6.8% +/- 4.97
Hiperglicemia (mg/dl. ayuno)	81.0 +/- 1.59	79.55 +/- 2.55	**95.25 +/- 1.67*$^{\#}$**	85.0 +/- 1.49	**77.6 +/- 0.66^{+}**	**88.33 +/- 2.77°**

Test de tolerancia a la glucosa (Área bajo la curva) mg/dl)/min	123.55 +/- 9.51	157.02 +/- 13.96	**204.36 +/- 27.75***	169.43 +/- 13.03	137.25 +/- 9.54	**233.5 +/- 31.22^{+}**
Hiperinsulinemia (ng/dl)	3.05 +/- 0.43	6.04 +/- 0.01	**8.32 +/- 1.55***	6.23 +/- 0.48	7.27 +/- 0.71	**12.24 +/- 1.15^{+}°**
Grasa visceral %	2.48 +/- 0.08	**3.34 +/- 0.29***	**4.01 +/- 0.34***	2.1 +/- 0.21	2.27 +/- 0.12	2.72 +/- 0.21
Hipertrigliceridemia (mg/dl)	123.1 +/- 12.63	185.03 +/- 5.91	**316.02 +/- 42.08*$^{\#}$**	136.29 +/- 6.98	124.56 +/- 7.46	**403.96 +/- 24.14^{+}°**
Glucosa después de la dieta (mg/dl)	100.52 +/- 2.48	109.2 +/- 2.75	108.71 +/- 5.62	99.72 +/- 2.71	104.87 +/- 3.89	112.71 +/- 7.99
Corticosterona (ng/ml)	7.12 +/- 0.87	16.9 +/- 2.74	**39.3 +/- 5.33*$^{\#}$**	1.73 +/- 0.32	1.87 +/- 0.48	1.4 +/- 0.65
Leptina (ng/ml)	5.82 +/- 0.58	12.12 +/- 0.76	**15.23 +/- 2.55***	6.47 +/- 0.45	6.58 +/- 0.29	**9.18 +/- 0.5^{+}°**

Después de una exposición crónica a las dietas y/o al jet lag social, se extrajeron los cerebros para evaluar la activación que tenían después de comer la respetiva dieta. Encontramos que independientemente de la exposición al jet lag social, la activación de las áreas hipotalámicas evaluadas ante el consumo de las dietas no difirió significativamente (Fig. 4A, D). Sin embargo, cuando realizamos el ANOVA de dos vías para evaluar los efectos del factor dieta y el factor retraso de sueño, encontramos diferencias significativas en la interacción en cuanto a la activación del ARQ ($F_{(2,42)}$=3.52; p<0.038).

Cuando se evaluaron las neuronas MCHérgicas en la PFA, las dietas palatables produjeron mayor activación, que la dieta del grupo control (Fig. 4B) ($F_{(2,21)}$=6.75; p<0.005), pero no hay diferencias significativas entre los grupos expuestos a jet lag social (Fig. 4E). En cuanto a este grupo neuronal en el LH, encontramos que solo el grupo expuesto a dieta alta en azúcar tuvo mayor activación que el grupo control

($F_{(2,27)}$=5.71; p<0.0085) (Fig. 4B); mientras que en los grupos expuestos al jet lag social, tuvieron una mayor activación aquellos que consumían dieta alta en grasa ($F_{(2,21)}$=4.31; p<0.02) (Fig. 4E). También se realizó el ANOVA de dos vías para ver los efectos del factor dieta y el factor retraso de sueño, y encontramos diferencias significativas en la interacción en cuanto a las neuronas activadas, productoras de MCH en el LH ($F_{(2,48)}$=5.12; p<0.009).

Cuando las neuronas orexigénicas fueron evaluadas, pudimos observar que los animales que consumieron dieta alta en grasa tuvieron una mayor activación de este grupo neuronal, en comparación con el control y el que comía dieta alta en azúcar ($F_{(2,21)}$=4.37; p<0.02) (Fig. 4C), mientras que no hubo diferencias en los grupos sometidos al jet lag social (Fig. 4F). Tampoco se mostraron cambios en el LH con o sin jet lag social (Fig. 4C, F), aunque cuando se realizó el ANOVA de dos vías para evaluar los efectos del factor dieta y el factor retraso de sueño, encontramos diferencias significativas en la interacción en cuanto a las neuronas activadas, productoras de ORX en el LH ($F_{(2,48)}$=3.23; p<0.048).

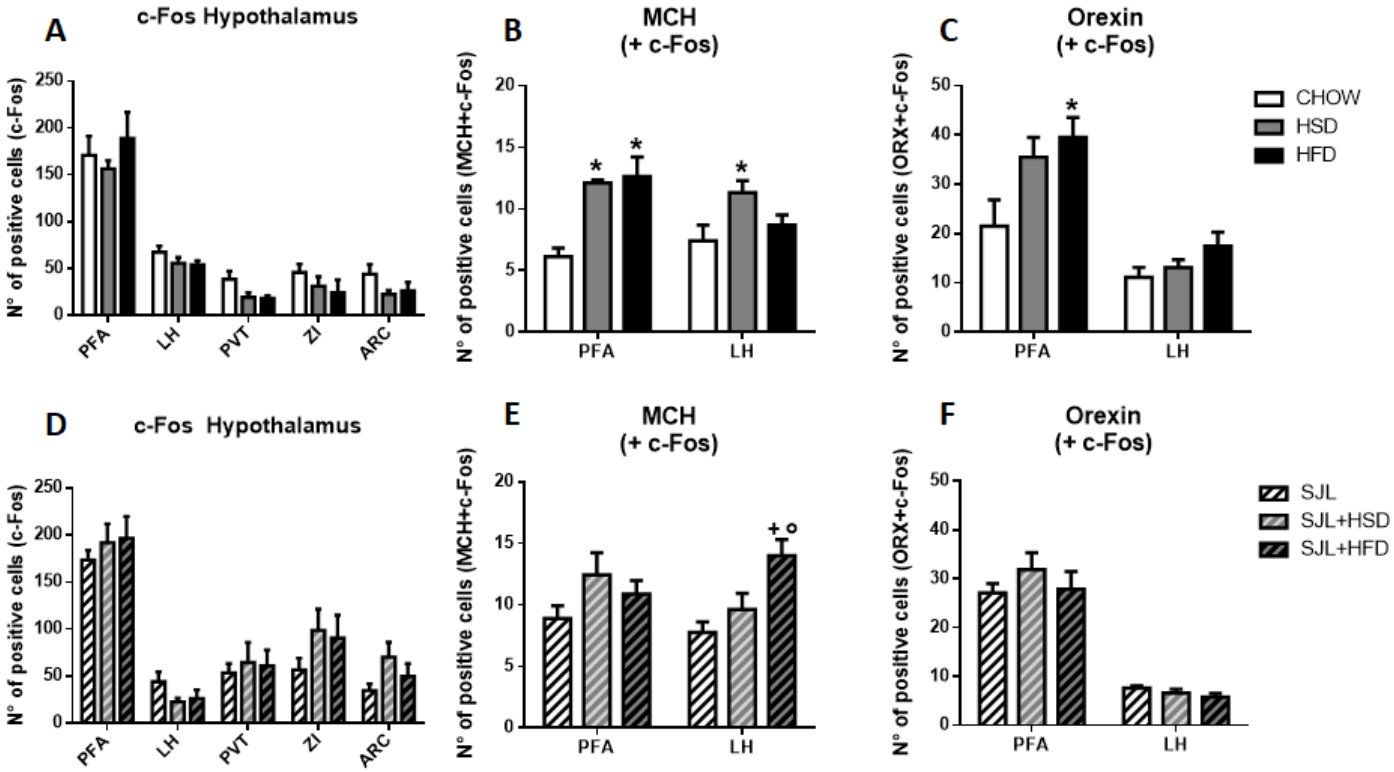


Figura 4: (A, D) Activación hipotalámica después del consumo crónico de dieta alta en azúcar (HSD o SD+HSD) o dieta alta en grasa (HFD o SD+HFD); medida en el área perifornical (PFA), el hipotálamo lateral (LH), el núcleo paraventricular del tálamo (PVT), la zona inserta (ZI) y el núcleo arqueado (ARQ). (B, E) Activación de neuronas positivas a la hormona concentradora de melanocitos (MCH) en PFA y LH. (C, F) Activación de neuronas positivas a orexinas (ORX) en PFA y LH. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD.

Al terminar las 4 semanas de exposición crónica a las dietas y/o al jet lag social, los animales no fueron manipulados por una semana, para ver algún tipo de recuperación. En cuanto a la ganancia de peso, el ANOVA de medidas repetidas mostró diferencias en la interacción (dietas X tiempo) ($F_{(8,124)}$=3.19; p<0.0025), en donde podemos ver que durante la semana 3 y 4 de las dietas, el grupo que consumía grasa tiene una mayor ganancia de peso, la cual se mantiene en la semana de recuperación (Fig. 5A). En los grupos expuestos al jet lag social, el ANOVA de medidas repetidas mostró diferencias a lo largo del tiempo ($F_{(4,132)}$=610.9; p<0.0001) y no se muestran diferencias entre los grupos, y esto se mantiene en la semana de recuperación (Fig. 5F). Cuando tomamos el valor absoluto de la ganancia de peso en la semana de recuperación, el ANOVA de una vía no mostró diferencias significativas en los grupos de dietas, ni en los grupos expuestos al jet lag social (Fig. 5B, G). La acumulación de grasa en los animales expuestos a dieta alta en grasa se mantuvo en la semana de recuperación, lo cual no sucedió para los animales con dieta alta en azúcar ($F_{(2,17)}$=15.84; p<0.0001) (Fig. 5C). Cuando evaluamos la acumulación de grasa en los grupos expuestos al jet lag social, el ANOVA de una vía mostró diferencias significativas en donde los grupos expuestos a dieta palatable acumulan más grasa que los que solo tienen jet lag social con acceso a chow ($F_{(2,17)}$=6.48; p<0.0081) (Fig. 5H), diferencias que no se mostraron a la 4° semana. Al realizar el ANOVA de dos vías para conocer la significancia de los efectos del factor dieta y el factor retraso de sueño, hubo diferencias significativas en la interacción en cuanto a la acumulación de grasa ($F_{(2,34)}$=4.85; p<0.014).

Cuando se evaluó el consumo de chow a la quinta semana, se pudo observar que los animales sin exposición al jet lag social comieron la misma cantidad de energía que las semanas anteriores, volvían a consumir la cantidad requerida en chow para mantener el equilibrio kilocalórico (Fig. 5D, E). El ANOVA de medidas repetidas mostró diferencias en el factor tiempo ($F_{(4,132)}$=7.69; p<0.0001) y en el factor dieta ($F_{(2,33)}$=10.71; p<0.0003), pero no en la interacción entre los factores. Lo mismo se vió en los grupos expuestos a jet lag social (Fig. 5I, J), donde el ANOVA de medidas repetidas mostró diferencias en la interacción de los factores (dietas X tiempo) ($F_{(8,132)}$=8.48; p<0.0001).

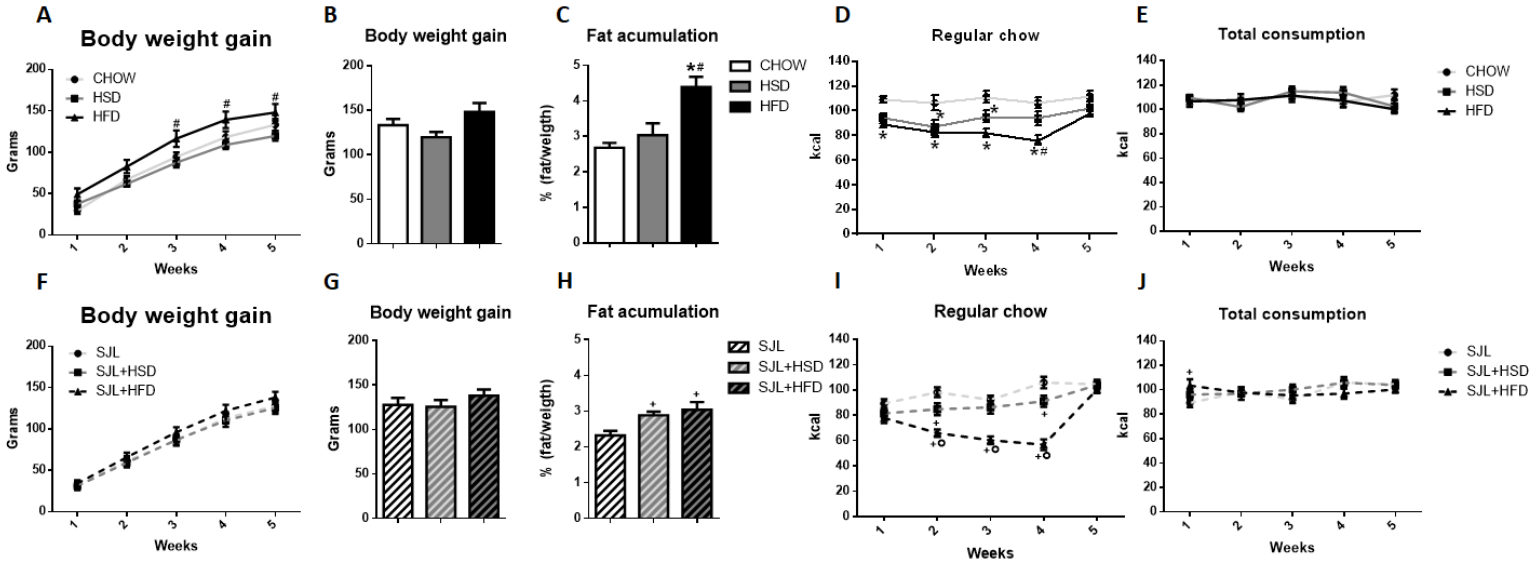


Figura 5: (A, F) Ganancia de peso a lo largo de 4 semanas de exposición y una semana de recuperación a las dietas y/o el protocolo de jet lag social. (B, G) Ganancia de peso total. (C, H) Acumulación de grasa abdominal, después de una semana de recuperación, después de una exposición crónica a las dietas y/o el protocolo de jet lag social. (D, I) Consumo kilocalórico solo de chow a lo largo de 24 horas. (E, J) Suma kilocalórica del consumo de dietas y el consumo de chow. (*) Diferente del control. ($^{+}$) Diferente de SD. (°) Diferente de SD+HSD. ($^{\#}$) Diferente de HSD.

Al analizar el cerebro después de esta semana de recuperación, en los grupos expuestos solo a las dietas pudimos observar que en el PFA los animales que consumían dieta alta en grasa mostraban menos neuronas activas que el control ($F_{(2,24)}$=4.04; p<0.03), al igual que en el PVT ($F_{(2,24)}$=3.5; p<0.04), en la ZI ($F_{(2,21)}$=6.76; p<0.005) y en el ARQ ($F_{(2,27)}$=44; p<0.02). El grupo de grasa solo tuvo una hipoactivación en la ZI en comparación con el control. Cuando se analizó la activación de estos núcleos en los grupos expuestos al jet lag social, el ANOVA de una vía mostró diferencias significativas solo en el ARQ ($F_{(2,24)}$=3.92; p<0.03), en donde en ambas áreas el grupo que consumía dieta alta en grasa mostró una mayor activación. Los demás núcleos evaluados no presentaron diferencias significativas. Al realizar el ANOVA de dos vías para evaluar los efectos del factor dieta y el factor retraso del sueño, hubo diferencias significativas en la interacción en cuanto a las neuronas activadas en el LH ($F_{(2,51)}$=3.67; p<0.032) y el ARQ ($F_{(2,51)}$=7.5; p<0.0014).

Al evaluar el grupo neuronal productor de MCH, no se encontraron diferencias significativas cuando los animales se encontraban en recuperación, con o sin el protocolo de jet lag social. Cuando se evaluó el grupo neuronal productor de ORX, el ANOVA de una vía mostró diferencias significativas en el PFA, cuando los animales tenían un previo tratamiento de jet lag social ($F_{(2,21)}$=4.69; p<0.02). Asimismo, cuando realizamos un ANOVA de dos vías para ver los efectos del factor dieta y el factor retraso del sueño, hubo diferencias significativas en la interacción en cuanto a las neuronas orexigénicas activadas en el PFA ($F_{(2,48)}$=4.68; p<0.013).

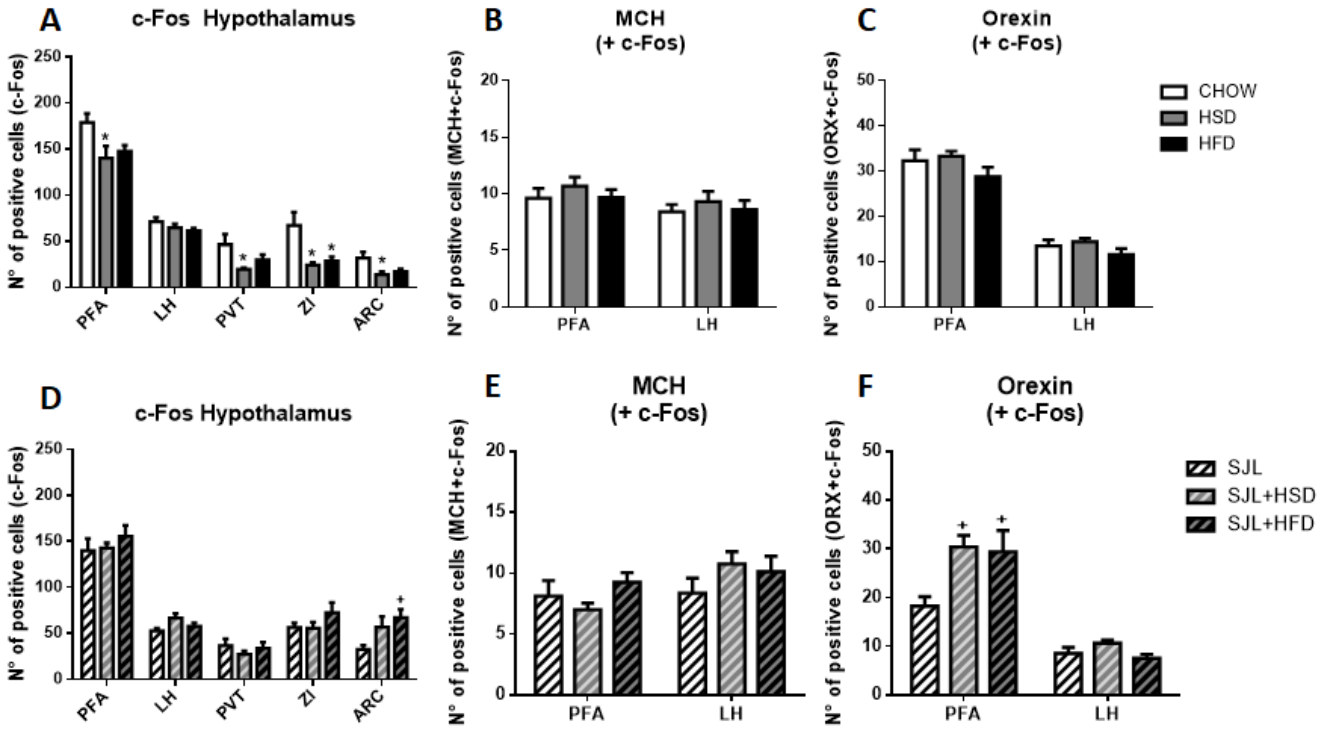
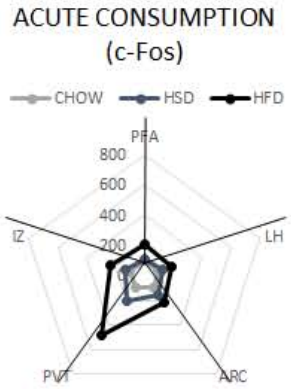
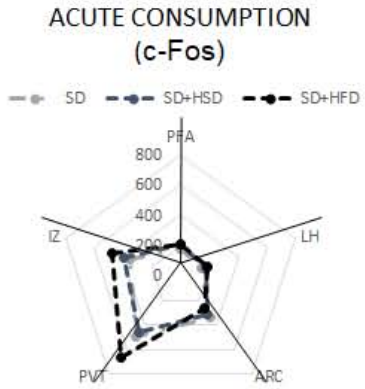


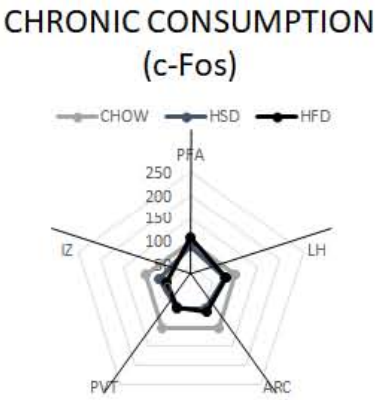
Figura 6: (A, D) Activación hipotalámica después de una semana de recuperación al consumo crónico de dieta alta en azúcar (HSD o SD+HSD) o dieta alta en grasa (HFD o SD+HFD); medida en el área perifornical (PFA), el hipotálamo lateral (LH), el núcleo paraventricular del tálamo (PVT), la zona inserta (ZI) y el núcleo arqueado (ARQ). (B, E) Activación de neuronas positivas a la hormona concentradora de melanocitos (MCH) en PFA y LH. (C, F) Activación de neuronas positivas a orexinas (ORX) en PFA y LH. (*) Diferente del control. ($^{+}$) Diferente de SD.

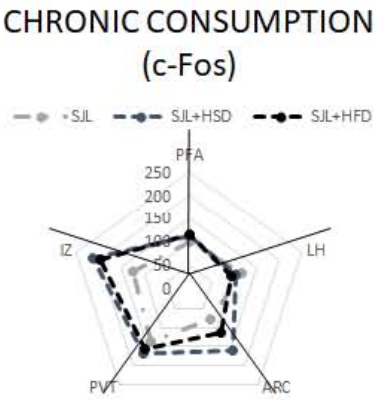
De manera gráfica, podemos comparar la activación hipotalámica ante una exposición aguda, crónica y de recuperación, en donde podemos observar que los grupos expuestos a retraso de una noche de sueño o una situación crónica de jet lag social, muestran niveles más altos, que aquellos que solo comen la dieta. Mientras que tan solo con la dieta, de manera crónica, el hipotálamo ya no es capaz de responder ante el consumo. Y en el periodo de recuperación observamos que al no consumir la dieta, hay una hipoactivación en comparación con el control,

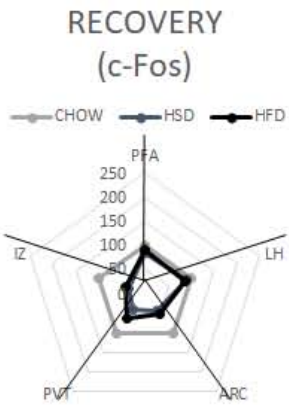
mientras que los que estaban expuestos a las ruedas de actividad forzada siguen con un nivel alto, con más notoriedad en el núcleo arqueado.











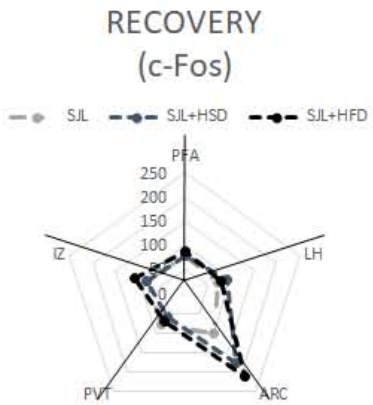


Figura 7: Activación hipotalámica después de un consumo de dieta alta en azúcar o dieta alta en grasa; con o sin el protocolo de jet la social. Fase aguda (A), fase crónica (B) y después de una semana de recuperación (C).

9. DISCUSION

La implementación de ruedas de actividad forzada para crear el modelo en rata Wistar de jet lag social resultó en lo esperado, pues se muestra una condición similar a la descrita en humanos [164], los resultado exhiben que en los días entre semana hay un retraso de fase en la acrofase en la actividad de los animales, mientras que los fines de semana la acrofase tiene valores similares a los del grupo control. El retraso de fase fue de aproximadamente 2 horas y se observaron algunas diferencias metabólicas, aunque no se alcanzaron los valores establecidos para el cumplimiento de síndrome metabólico (Articulo 1). Cuando los sujetos fueron expuestos sólo a la dieta de cafetería, los criterios de síndrome se cumplieron (2-3), pero la combinación del protocolo de jet lag social y la dieta de cafetería mostró que el retraso de fase fue de alrededor de 3 horas, además de un incremento de fase y un aumento en el cumplimiento de más criterios del síndrome metabólico (Articulo 1).

En la población humana hay una correlación positiva entre la las horas de retraso en el jet lag social y la gravedad del sobrepeso, solo cuando los individuos tenían un IMC alto [165,166]. En nuestro estudio, las ratas en el protocolo de jet lag social no desarrollaron sobrepeso, sin embargo, se vieron afectadas en su estado metabólico, por lo que confirmamos que un cambio crónico del inicio del sueño es suficiente para inducir alteraciones metabólicas, especialmente cuando se combinan con una dieta alta en calorías.

Este retraso en el sueño promovió la ingesta de alimentos en las horas de descanso, lo que se sabe que es perjudicial para el metabolismo [167]. Por lo que sabemos, este es el primer modelo experimental de jet lag social propuesto en roedores. Es importante notar que en los animales que tenían las manipulaciones en la noche

(fase de actividad de las ratas) no presentaron cambios metabólicos ni de aumento de peso, lo que sugiere que el sistema circadiano prepara a las personas para responder de manera eficiente y de acuerdo con el ciclo día-noche. Algunos modelos han probado los efectos de la privación aguda del sueño [168,169] mostrando que el sueño deficiente es suficiente para alterar el equilibrio metabólico en humanos [25] y roedores [170–173], sugiriendo que la falta sueño puede representar un nuevo factor de riesgo para el desarrollo de diabetes y de síndrome metabólico [174].

Varios estudios muestran que la actividad y/o los alimentos con acceso en la fase de descanso, causan trastornos circadianos, metabólicos y sobrepeso: mientras que la actividad y/o los alimentos en la fase de actividad, tienen un efecto protector sobre el peso corporal y el metabolismo [54,167,172,175,176]. Nuestros resultados coinciden parcialmente con dichos hallazgos, las ratas expuestas a la dieta de cafetería o a la rueda de actividad forzada de noche, ganaron menos peso que las ratas expuestas a la dieta de cafetería o a la rueda de actividad forzada de día. Este efecto diferencial sobre el peso corporal y el equilibrio metabólico está mediado por el sistema circadiano; preparando el sistema digestivo para la digestión y absorción y preparando tejidos para que consuman energía de manera eficiente. Los hallazgos concuerdan con las observaciones de otros grupos que utilizan una dieta alta en grasas programada en la fase adecuada [177–180].

El uso de la dieta de cafetería es un buen modelo para inducir obesidad y síndrome metabólico en ratas, ya que refleja con precisión la variedad de alimentos altamente palatable y densamente energéticos disponibles en la sociedad moderna [14,181]. Además, esta dieta promueve hiperfagia voluntaria que induce parámetros prediabéticos como glucosa alta e intolerancia a la insulina [181,182]. El uso de la dieta de cafetería es controversial [183,184], pues se argumenta que la elección y la diversidad de los alimentos hacen imposible controlar la composición de la dieta consumida por cada individuo [183]. En este estudio se midió cada uno de los componentes de la dieta de cafetería por individuo y se calculó el consumo

kilocalórico de acuerdo a los datos ofrecidos por cada uno de los fabricantes de los componentes. Entre sujetos no hubo diferencias significativas de consumo dentro de cada grupo.

Dada la controversia del uso de dietas de cafetería en los modelos animales, decidimos investigar por separado sus principales componentes: grasa y azúcar. Dado que ambos son sabrosos y se han utilizado más controladamente en los estudios animales, quisimos comparar los efectos que tienen ambas dietas por separado. En primera instancia quisimos saber qué porcentaje de grasa o azúcar provocaba un mayor consumo en ratas expuestas a un intervalo breve de una hora diaria. Se encontró que las dietas que contenían un 50% de azúcar (50% de azúcar + 50% de chow) y un 50% de grasa (50% de grasa+ 50% de azúcar) se consumieron en una cantidad alta que superó en más de 2 veces las dietas de 10 y 25%. Esto sugirió que las ratas podrían presentar una sobreingesta y por lo tanto las dietas al 50% representaban una alta palatabilidad. A partir de este estudio se decidió usar esas dietas a lo largo de nuestra investigación en todas las manipulaciones.

Uno de nuestros principales puntos de investigación era saber qué efectos tenían las dietas en el sistema de recompensa, por lo que tras una primera exposición mostramos que las ratas consumieron en una cantidad mayor, la dieta alta en azúcar que la dieta alta en grasa; sin embargo, ambas dietas mostraron una activación similar de c-Fos y de acumulación de ΔFosB en áreas corticolímbicas. Se sugiere esta activación aguda para iniciar cambios neuronales aumentan la vulnerabilidad al consumo excesivo de comida sabrosa o incluso drogas. Esta idea se observó en un estudio, donde roedores expuestos a dieta alta en grasas o en azúcar, aumenta la vulnerabilidad al consumo de cocaína [185]. Otro estudio mostró que una dieta alta en grasas estimuló el consumo de etanol; mientras que una dieta alta en azúcar al igual que el grupo que se le administró solución salina disminuyeron el consumo de alcohol [186]. Además, se ha mostrado que una dosis aguda intraperitoneal de lípidos estimuló la liberación de dopamina en el núcleo accumbens en comparación con una dosis de glucosa y de solución salina [187].

Otros estudios han asociado esta activación inicial con el sabor del azúcar o la grasac [89,188].

Pocos estudios han comparado azúcar y grasa y el impacto que tienen en áreas corticolímbicas. Cruz et al, (2016) después de una administración aguda, encontraron una mayor activación de c-Fos en animales alimentados con una dieta alta en grasas en comparación con el grupo que recibió glucosa o fructosa [189]. También en un estudio que expuso a los animales a una sonda de lípidos durante una semana, observó una alta expresión de c-Fos en la amígdala y en el núcleo de Accumbens en comparación con la administración de etanol, sacarosa o nicotina. [190]. Contrastando, otros estudios describieron una mayor activación de c-Fos y la liberación de dopamina después de consumir una solución de azúcar [191,192].

Dado que ambas dietas impactan el sistema de recompensa, se ha propuesto que los alimentos sabrosos pudieran generar conductas parecidas a la adicción. Dentro de los criterios propuestos para evaluar adicción a los alimentos son: la conducta tipo atracón, búsqueda, conductas de esfuerzo y abstinencia. En este estudio mostramos que los animales que comían dieta alta en grasa por un periodo largo, mostraban niveles más altos de atracón y anticipación que aquellos que comían dieta alta en azúcar; mientras que ambas dietas presentaron conductas de esfuerzo (Articulo 2).

Un factor importante para el desarrollo de la conducta tipo atracón, anticipación y conductas de esfuerzo es que los animales solo tienen un acceso restringido a las dietas sabrosas. Mientras que el acceso intermitente o restringido, aumenta la motivación y favorece el desarrollo de la conducta tipo atracón, comiendo tanto como sea posible en un corto intervalo de tiempo [193–195], el acceso *ad libitum* a la dieta sabrosa, induce obesidad y disminuye la motivación para la dieta. Del mismo modo, la activación anticipatoria a los alimentos solo se observa cuando el acceso a estos es restringido. La intensidad de la anticipación se correlaciona con la duración del intervalo de restricción [196] y con el valor calórico de la dieta.

Nuestros resultados concuerdan con el estudio realizado por Tenk y Felfeli, donde describieron un aumento de la conducta tipo atracón de dieta alta en grasa en lugar de dieta alta en azúcar después de un acceso restringido de 2 horas cada 3 días por semana [197]. También, se ha demostrado en estudios independientes, que animales alimentados con chow ad-libitum y con acceso restringido a dieta alta en grasa o una solución de sacarosa al 10%, desarrollaron anticipación [198–200]. Sin embargo, estos estudios no comparan los efectos de ambas dietas.

Las ratas expuestas a dieta alta en grasa o dieta alta en azúcar mostraron un esfuerzo similar para obtener la dieta (Articulo 2), lo que sugiere que ambas dietas aumentan la motivación de las ratas para obtener el alimento sabroso. Un estudio previo mostró que los animales expuestos a una dieta alta en grasas reduce la motivación cuando se compara con animales expuestos a una dieta alta en azúcar (ambas dietas ad-libitum), pero ambos grupos desarrollaron una mayor impulsividad en comparación con el grupo control [201]. Otros estudios que evalúan el esfuerzo, utilizan una tarea operante llamada razón progresiva, donde el objetivo es evaluar en los roedores expuestos a grasa y/o azúcar o incluso una droga de abuso, cuanto están dispuestos a palanquear por el reforzador, lo que ha mostrado que el alimento sabroso produce una mayor motivación y esfuerzo para obtener la dieta [202–204].

Después de estar una semana en abstinencia sin acceso a la dieta palatable, nuestras ratas tanto con dieta alta en grasa como las que comían dieta alta en azúcar, mostraron anticipación y conductas de esfuerzo en una misma magnitud. Otros estudios muestran que animales bajo un protocolo de abstinencia después de la exposición a una dieta alta en grasa y/o alta en azúcar desarrollan ansiedad, persistencia de actividad locomotora y conductas de esfuerzo [136,142,198,205–207]. Este estudio respalda dichos hallazgos e indica que, a largo plazo, ambas dietas pueden conducir a conductas similares a la adicción.

A pesar de una disminución general de c-Fos después de una ingesta crónica de dieta alta en grasa y durante la abstinencia, las ratas que consumían dieta alta en

grasa, mantuvieron una activación en el núcleo accumbens core y en la corteza insular. La corteza insular es un área importante relacionada con las sensaciones viscerales aversivas que ocurren cuando los animales están en craving o en abstinencia por drogas de abuso. La baja respuesta de c-Fos aquí reportada después de la ingesta crónica de las dietas, encuentra apoyo en los estudios que informan que el consumo crónico de estos alimentos, hace que el sistema reaccione con niveles anormales de activación bajos [208,209]. Es importante notar que en algunos estudios, la dieta alta en azúcar se ha combinado con drogas para observar mejor el efecto de abstinencia [157,192].

Se sugiere que ΔFosB se acumula gradualmente después del consumo de alcohol o drogas para estimular la plasticidad neuronal que subyace a los cambios a largo plazo [210]. Estos efectos se han descrito principalmente en el núcleo accumbens, en el área tegmental ventral, la amígdala, el hipocampo y en la corteza prefrontal [211,212]. Los datos actuales indican que desde de la primera exposición, ambas dietas indujeron la sobreexpresión de ΔFosB. Esto concuerda con el estudio de Kaufling, et al. (2010) donde demostraron la sobreexpresión de ΔFosB después de la administración aguda de drogas como cocaína, D-anfetamina, metilfenidato y cafeína [213]. En nuestro estudio, después de la ingesta crónica, solo en la dieta alta en grasa pero no el grupo con dieta alta en azúcar, persistió la acumulación de ΔFosB en áreas corticolímbicas. Otros estudios han informado que después de un consumo crónico de grasa y/o azúcar, también se produce una acumulación significativa de ΔFosB [142,214–216]; esto también se ha visto en periodos de abstinencia de azúcar [143] o grasa [142]. Todos los datos en conjunto sugieren que la dieta sabrosa puede cambiar el cerebro induciendo plasticidad neuronal y que, principalmente, una dieta alta en grasa puede favorecer el desarrollo de conductas asociadas a la adicción.

Ahora que comprobamos que la dieta alta en grasa produce más conductas asociadas a la adición, así como mayor sobreconsumo, mayor activación del sistema límbico y mayor plasticidad; nuestra siguiente pregunta fue si el protocolo

de jet-lag inducía una diferencia en el consumo de dieta alta en grasa y dieta alta en azúcar. Por lo que mostramos que una sola noche de retraso de sueño, provocado por la exposición de la rueda de actividad forzada las primeras 4 horas de su fase de descanso, inducia un menor consumo de chow justo después de la exposición a la rueda (1 hora); a pesar de que consumían menos chow, el acceso a la rueda provocó una activación del sistema límbico muy importante, así como la inducción de ΔFosB, lo que nos hizo pensar que estos animales podrían desarrollar más fácilmente conductas asociadas a la adición por comida sabrosa. Al medir la activación del sistema límbico y la acumulación de ΔFosB en animales que fueron expuestos a un protocolo crónico de jet lag social, pudimos observar que seguían comiendo menos chow y que solo algunas estructuras del sistema límbico conservaron la sobreacumulación de la proteína ΔFosB. Los efectos de la falta o disrupción de sueño en el consumo de alimentos durante las 24 horas de chow no son claros, ya que varían según la duración del protocolo y la estrategia utilizada para interrumpir el sueño [56]. Por ejemplo, utilizando un piso giratorio Baud et al. (2013) observó una mayor ingesta de chow [217]; Así mismo, Mavajni et al. (2013) usó ruidos fuertes durante 8 horas para perturbar el sueño, informó un aumento en la ingesta regular de alimentos [218]. Sin embargo, en otro estudio donde se utilizaron tambores giratorios similares a los utilizados en el presente estudio, no informaron efectos sobre el consumo de comida 24 horas [55]. Es importante mencionar que en el presente estudio, la ingesta de alimentos se evaluó durante solo una hora después del retraso del sueño, por lo que la ingesta reducida de chow puede estar relacionada con el aumento de la capacidad para dormir en lugar de comer. Sin mostrar cambios en la ingesta kilocalórica a lo largo de 24 horas.

Cuando el protocolo de jet lag social se combinó con dieta alta en grasa o dieta alta en azúcar, se pudo ver que ambas dietas provocaban atracón por ambas dietas, pero el grupo expuesto a dieta alta en grasa consumió más que las de azúcar. De acuerdo con otros estudios asociados a la disrupción de sueño, se ha mostrado conducta tipo atracón de alimentos dulces [219–221] como también por comida alta en grasa [173,222].

Los protocolos dirigidos a perturbar el sueño, exponen a los roedores a condiciones estresantes y pueden estimular un estado de alta excitación y actividad. La respuesta diferencial al consumo excesivo de alimentos sabrosos versus chow regular también se observa en animales expuestos al estrés, donde se observa un menor consumo de chow [223][223,224][223,224], y se reporta una preferencia por dietas sabrosas [225–227]. Hay un estudio que muestra cómo la privación puede promover el consumo diferencial de alimentos altos en grasas o altos en carbohidratos, donde encontraron que después de 96 horas de privación de sueño REM, los animales expuestos a dieta alta en carbohidratos no presentan diferencias significativas en el consumo de dieta comparado con los animales que no están privados de sueño, pero los que estuvieron expuestos a privación de sueño y a dieta alta en grasa, si tuvieron un consumo mayor de la dieta en comparación con quienes no estaban privados de sueño [228], lo que sugiere que la privación de sueño puede impactar en la elección del tipo de alimentos que los sujetos consumen después de la privación, así como nuestros resultados sugieren.

Se sugiere que la interrupción del sueño y el estrés desencadenen la búsqueda de un alimento reconfortante destinado a aliviar el malestar físico y emocional [60,229–231]. Por lo tanto, no está claro qué factor asociado con la interrupción del sueño es la causa principal de la hiperfagia para alimentos sabrosos, ya que también el estrés y la activación forzada pueden provocar la búsqueda de alimentos con alto contenido calórico. En nuestro protocolo de jet lag social, las ratas no mostraron niveles elevados de corticosterona, medidos después de la exposición a la rueda de actividad forzada, sugiriendo bajos niveles de estrés e indica una adaptación a la condición crónica.

Después de una semana de abstinencia a las dietas evaluamos conductas asociadas a la adición, y pudimos ver que los sujetos expuestos a las dietas sabrosas persistieron en las conductas asociadas a la adicción, mientras que los sujetos que solo comieron alimento chow, no mostraron estas conductas. Ambos

grupos mostraron comportamientos de esfuerzo para obtener la dieta; mayor activación locomotora en el momento esperado de la dieta. Esto indica que el retraso del sueño por sí solo no es un factor de riesgo para el sobreconsumo y la obesidad, mientras que la combinación con alimentos sabrosos representa un riesgo para desarrollar conductas compulsivas hacia la obtención de alimentos con alto contenido kilocalórico. Durante la abstinencia, los niveles de c-Fos aumentaron en todos los grupos con jet lag social en comparación con el grupo control, sin embargo, solo ambos grupos expuestos previamente a alimentos sabrosos mostraron un aumento significativo de c-Fos en la corteza insular en comparación con el grupo de jet lag social, que solo comía alimento chow. La corteza insular se ha asociado con sensaciones viscerales aversivas que ocurren cuando los animales son dependientes a alguna droga de abuso [232–234], lo que puede llevar a los individuos a recaer. La activación de la corteza insular, observada en los grupos que consumían las dietas sabrosas, durante la retirada puede reflejar una sensación de aversión debido a la falta de estos.

Los datos en nuestro estudio muestran que después del primer evento de retraso de sueño, la acumulación de ΔFosB es evidentemente alto en áreas corticolímbicas. La interrupción del sueño y el estrés se asocian con la acumulación de ΔFosB, que se sabe que es necesario para estimular el crecimiento dendrítico y la producción de la subunidad GluR2 perteneciente a los receptores de AMPA destinados a iniciar la conducta tipo atracón y las conductas asociadas y parecidas a la adicción de alimentos sabrosos [143,210]. Por lo tanto, en nuestro estudio, la acumulación de ΔFosB puede ser un estímulo primordial para favorecer el consumo excesivo de alimentos sabrosos. En el largo plazo, la ingesta de alimentos sabrosos asociados con el retraso del sueño produjo una acumulación específica de ΔFosB en la corteza prefrontal y en el núcleo accumbens, específicamente en la región shell, la acumulación fue mayor para el grupo que consumió dieta alta en azúcar, similar a un informe reportado anteriormente [235]. Por otra parte, el grupo que consumió dieta de grasas exhibió mayor acumulación en la corteza insular. Otros estudios han observado que las dietas alta en grasas causan una sobreproducción de ΔFosB en

diferentes áreas del sistema de recompensa [214,216,236]. También se ha asociado la acumulación de ΔFosB con el desarrollo de conductas similares a las adicciones [216,237].

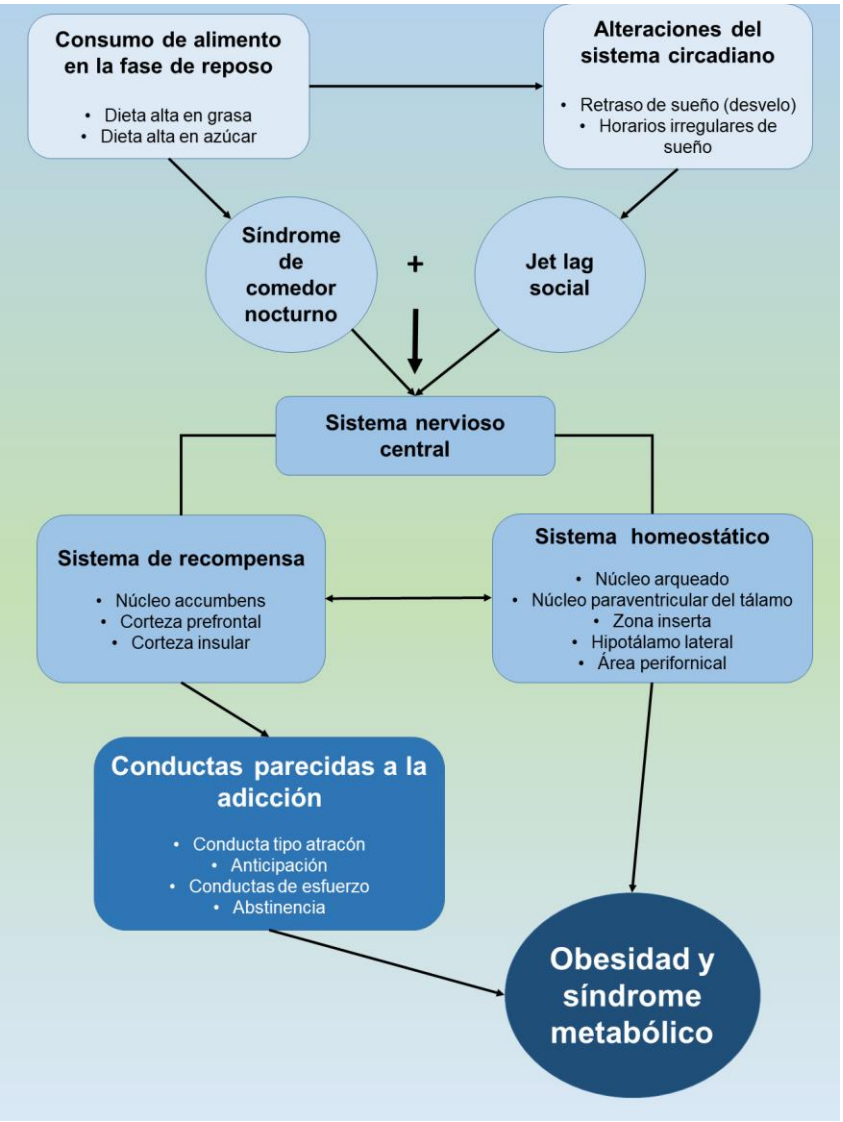
10. CONCLUSIONES

El estilo de vida moderno ha creado condiciones para promover el desarrollo del jet lag social, especialmente en los jóvenes, haciéndolos propensos a cambiar sus horas de sueño y a comer durante esas horas. Nuestro modelo experimental dio un resultado similar de tiempo de sueño cambiado, como se observa en la población humana. Una de las fortalezas de este estudio es la implementación de ruedas de actividad forzada, ya que además de mantener a los animales despiertos, pueden a su vez consumir alimentos. Con este modelo, demostramos que el jet lag social es suficiente para causar cambios metabólicos leves que representan factores de riesgo, que preparan a los individuos para desarrollar síndrome metabólico.

Este estudio demostró que principalmente una dieta rica en grasa y no una dieta rica en azúcar, desencadena cambios plásticos en el sistema corticolímbico y favorece el desarrollo de atracones, anticipación, comportamientos de esfuerzo y respuestas de abstinencia, todos ellos indicadores de comportamientos alimenticios similares a la adicción. La alta motivación para comer alimentos con alto contenido de grasa puede iniciar la pérdida del equilibrio homeostático y el desarrollo de la alimentación hedónica, lo que a su vez conducirá a la obesidad y problemas metabólicos.

También demostramos que el retraso del sueño puede estimular cambios plásticos en el cerebro, que pueden desencadenar una alimentación compulsiva preferentemente a dietas altas en grasas en lugar de dietas altas en azúcar. Sin embargo, algunas conductas asociadas a la adicción se desarrollaron en una intensidad similar para ambas dietas. La sobrealimentación compulsiva y la preferencia por dietas altas en grasa o en azúcar representan un riesgo para

desarrollar obesidad y conductas compulsivas. De manera general, proponemos un modelo mecanístico que resume la interacción de los factores explorados en este trabajo, y su contribución en el desarrollo de obesidad y el síndrome metabólico (Figura 2).



De acuerdo con nuestros hallazgos, sugerimos que los estudios epidemiológicos hechos en poblaciones, incluyan la evaluación de alteraciones circadianas, no sólo monitoreando el retraso del sueño, sino también explorando el momento de la ingesta de alimentos como un factor de riesgo adicional para la obesidad, el síndrome metabólico y conductas asociadas a la posible adicción a alimentos altos en kilocalorías.

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ANEXOS 1

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RESEARCH ARTICLE Open Access

Circadian disruption promotes tumor growth by anabolic host metabolism; experimental evidence in a rat model

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Abstract

Background: Light at night creates a conflicting signal to the biological clock and disrupts circadian physiology. In rodents, light at night increases the risk to develop mood disorders, overweight, disrupted energy metabolism, immune dysfunction and cancer. We hypothesized that constant light (LL) in rats may facilitate tumor growth via disrupted metabolism and increased inflammatory response in the host, inducing a propitious microenvironment for tumor cells.

Methods: Male Wistar rats were exposed to LL or a regular light-dark cycle (LD) for 5 weeks. Body weight gain, food consumption, triglycerides and glucose blood levels were evaluated; a glucose tolerance test was also performed. Inflammation and sickness behavior were evaluated after the administration of intravenous lipopolysaccharide. Tumors were induced by subcutaneous inoculation of glioma cells (C6). In tumor-bearing rats, the metabolic state and immune cells infiltration to the tumor was investigated by using immunohistochemistry and flow cytometry. The mRNA expression of genes involved metabolic, growth, angiogenes and inflammatory pathways was measured in the tumor microenvironment by qPCR. Tumor growth was also evaluated in animals fed with a high sugar diet.

Results: We found that LL induced overweight, high plasma triglycerides and glucose levels as well as reduced glucose clearance. In response to an LPS challenge, LL rats responded with higher pro-inflammatory cytokines and exacerbated sickness behavior. Tumor cell inoculation resulted in increased tumor volume in LL as compared with LD rats, associated with high blood glucose levels and decreased triglycerides levels in the host. More macrophages were recruited in the LL tumor and the microenvironment was characterized by upregulation of genes involved in lipogenesis (*Acaca, Fasn, and Pparγ*), glucose uptake (*Glut-1*), and tumor growth (*Vegfa, Myc, Ir*) suggesting that LL tumors rely on these processes in order to support their enhanced growth. Genes related with the inflammatory state in the tumor microenvironment were not different between LL and LD conditions. In rats fed a high caloric diet tumor growth was similar to LL conditions.

Conclusions: Data indicates that circadian disruption by LL provides a favorable condition for tumor growth by promoting an anabolic metabolism in the host.

Keywords: Light at night, Circadian disruption, Tumor development, Inflammation, Metabolism and obesity

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Background

The alternation of day-night cycles is necessary for entrainment of the master circadian clock to efficiently transmit temporal signals to the organism in order to adapt behavioral and physiological responses to the cycling conditions of the environment [1]. Modern lifestyle, night-work and leisure schedules change the sleep-wake timing and due to the extended and sometimes inverted activity, individuals are exposed to light at night creating a conflicting signal to the circadian clock and disrupting circadian regulation of physiology.

Circadian disruption increases the risk to develop disease in humans [2] and rodents [3], it also promotes an obesogenic condition, altered metabolism [4–6], immune dysfunction [7, 8] and increased the vulnerability to develop cancer [9, 10]. In rodents, light at night increases the growth rate of mammary adenocarcinomas [11], chemical induced hepatocarcinogenesis [12], and accelerates aging and tumorigenesis in young rats [13]. These studies have related the increased tumor growth to the decreased nocturnal production of melatonin and its reduced blood concentration due to light at night. However, in addition to melatonin suppression, other deleterious changes triggered by constant illumination conditions (LL), may favor the process of tumor development.

Inflammatory environments and altered immune function are recognized as carcinogenic promoters [14–16]. Tumor-secreted inflammatory mediators such as Interleukin 6 (IL-6) and Tumor necrosis factor α (TNF-α), can regulate host metabolism in multiple tissues [17, 18], suggesting a possible role of an inflammatory state in mediating tumor-induced metabolic changes in the host. We have previously demonstrated that circadian disruption induces a increased inflammatory response [8] and promotes metabolic disturbances, including, dyslipidemia, insulin insensitivity and increased adipose mass [5], all of them leading to an obesogenic environment, which is an additional factor that could provide a favorable internal environment for tumor growth [19].

Here we hypothesized that circadian disruption induced by LL will favor tumor development via altering the inflammatory response and metabolism in the host, resulting in a propitious condition for the proliferative activities required for tumor growth.

Methods

Experimental design

The aim of this study was to investigate in rats exposed to LL and their controls the metabolic and the inflammatory state in the host, and the resulting conditions of the tumor microenvironment that may favor tumor's growth. For this purpose, after 12 days of baseline, rats were randomly assigned to one of 2 groups: 1. Control LD, rats were left undisturbed in their home cages during 5 weeks and remained in 12:12 h LD; 2. Constant light (LL), rats were maintained with the lights on (200–250 lx at the level of the cage) for 5 weeks. Body weight and food intake were determined at the baseline and every week along the protocol. All animals included in the LL group were completely arrhythmic both in locomotor activity and body temperature after 5 weeks of LL exposure.

Experiment 1. Behavioral and metabolic consequences of 5 weeks in LL

A first series of LD (n = 8) and LL (n = 8) rats were used to confirm arrythmicity of general activity and core body temperature (Tb) after 5 weeks in LL conditions. Intra-abdominal temperature sensors (iButtons) were implanted before starting experiments and programmed to measured Tb during the last two days on week 5 of the protocol. A glucose tolerance test (GTT) was performed at the end of the 4th week; glucose and triglycerides (TG) in plasma were assessed at the end of the 5th week.

Experiment 2. Evaluation of the inflammatory response to LPS after 5 weeks in LL

A series of LD (n = 8) and LL (n = 8) rats were cannulated in the external jugular vein and were implanted with intra-abdominal temperature sensors (iButtons). After 1-week recovery (5 weeks in the lighting condition), rats received intravenous LPS (2 μg/kg) in the morning at ZT2 based on previous studies [8, 20] and in order to have the influence of light in both groups. Blood samples were collected from the jugular cannula and TNF-α was determined. In order to measure sickness behavior, food and fluid ingestion, body weight and temperature response were monitored before, following, 24 h and 48 h post the LPS administration. After this experiment, rats were euthanized and temperature sensors were collected for temperature analysis.

Experiment 3. Evaluation of tumor development, tumor microenvironment and the influence of the tumor on the host metabolism

In another series of LD (n = 8) and LL (n = 8) rats, at the end of the 5th week, rats were subcutaneously inoculated with C6 tumor cells and 13 days later, rats were euthanized and tumors as well as blood were collected for further metabolic analysis of the host and tumor. Another series of LD (n = 8) and LL (n = 8) rats were subcutaneously inoculated with tumor cells and after 9 days a GTT was performed. All animals remained in their lighting schedules i.e., LD or LL until the end of the experiments.

Animals and general housing conditions

Adult male Wistar rats weighing 190 to 200 g at the beginning of the experiments were obtained from the animal facility of the Faculty of Medicine of the Universidad Nacional Autónoma de México (UNAM). Animals were housed in individual cages placed in isolated lockers with controlled lighting conditions located in a soundproof monitoring room maintained at a controlled temperature of 22 ± 1 °C and with continuous air flow. All rats were given free access to food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA) and water. For a baseline all rats were under a 12:12 h light-dark cycle (LD), lights-on at 7:00, defined as Zeitgeber time 0 (ZT0) and lights off at 19:00 h (ZT12).

Automatic monitoring of general activity

General activity was automatically monitored daily with tilt sensors placed under the individual cages. Behavioral events were collected with a digital system (Omnialva SA de CV, México) and automatically stored every minute in a PC for further analysis. Analysis was performed with the program for PC SPAD9 designed for this system and based on Matlab. Double plotted actograms were constructed for each animal representing the number of activity counts every 15 min and periodicity with a χ^2 periodogram for the last 14 days of the experimental protocol.

Intra-jugular cannula insertion and intra-abdominal temperature sensors implantation

All surgeries were performed as previously described [20] using aseptic procedures.

For temperature recordings, the iButtons were programmed to collect core temperature data every 60 min and implanted in the rat peritoneum. For experiment 1, recordings started on week 5; for experiment 2, data were collected starting 2 days before LPS administration and continued until sacrifice. Temperature recordings were collected according to geographical time and the subjetive day-night phases for LL rats were selected based on the 12 h day and 12 h night of LD animals.

Blood sample collection TNF-α and metabolic determinations

Blood samples (250ul) drawn from the intrajugular catheter were collected in Microvette®/500 tubes (Sarsted, Nümbrecht Germany) before LPS (0 min) and post-infusion times 40, 80, 120 and 180 min. Samples were centrifuged and plasma TNF-α levels were determined by ELISA according to the manufacturer's recommendations (Invitrogen #KRC3011). Glucose and TG plasma levels were determined with enzymatic methods (ELITech Clinical Systems, France). Blood samples were taken from tail puncture between ZT2-ZT3 under ad libitum conditions.

Glucose tolerance test

During week 4, the GTT was performed after 16 h of overnight fasting. A basal blood sample was obtained at ZT0 (7:00 h), and an intraperitoneal injection of 1 g of glucose/kg in saline solution was immediately given. After glucose administration, subsequent blood samples were collected from tail puncture (15, 30, 60 and 120 min respectively). Glucose level was determined with a blood glucose monitor (Glucose meter, Accu-Chek active. Roche).

Inflammatory response

Inflammation was induced by a single intravenous (iv) injection of LPS (2μg/kg lipopolysaccharide from *Escherichia coli* serotype 0127:B8, Sigma-Aldrich, St. Louis, MO).

Tumor xenografts

The glioma C6 cell line has shown to be a convenient model to assess factors influencing tumor proliferation. This C6 cell line has a similar growth rate in the brain and in the subcutaneous region, it is already visible on day 5 and it starts decreasing on day 15, providing a 10 day window for observations and manipulations. Moreover the histological characteristics are similar for C6 cells implanted in the brain and subcutaneously, showing high nuclear cell ratio, mitosis and pseudopalisading with small populations of GFAP positive cells [21]. Therefore subcutaneous implantation has the advantage that it can be measured with a caliper, and can be easily monitored externally without killing rats on different days. For this study the glioma C6 cell line was kindly provided by Dra. Patricia García López from the Instituto Nacional de Cancerología México and was obtained from ATCC® CCL-107™ (Rockville, Maryland, USA). This cell line was cloned from a rat glial tumor induced by N-nitrosomethylurea [22]. The cell culture was maintained as a monolayer in RPMI-1640 medium supplemented with 5% fetal bovine serum and incubated at 37 °C in a 5% CO_2 atmosphere at high humidity. The C6 cell line was tested negative for *Mycoplasma*.

Rats were subcutaneously inoculated with 5×10^6 C6-cells in the back right flank; tumor size was assessed every 2 days from day 7 to day 13. The volume of C6 tumors reaches a maximum on day 15 in intact rats, after which the tumor reabsorbs [21]. Tumor volume was determined with a caliper using the following relation: $V = \pi/6 \times (\text{large diameter} \times [\text{short diameter}]^2)$.

Tumor macrophages immunohistochemistry and cell count

Tumors were fixed in 4% paraformaldehyde (ph 7.2) for 24 h at 4 °C, and cryo-protected in 30% sucrose 1 mM PB (ph 7.2) for 3 to 4 days. Tumors were frozen and cut in 20 μm coronal sections at −20 °C. Free-floating tumor

sections were incubated for 24 h under constant shaking at 4 °C with rabbit anti F4/80 antibody (1:2000; Santa Cruz) and were processed according to the avidin-biotin peroxidase method [20]. Immunoreactivity to F4/80 was quantified in six representative sections using a light microscope (Leica ICC50HD) and captured with a 40× ocular. Immunoreactive-positive areas were counted using computerized image analysis system (Image J, 1.42q, National Institutes of Health Bethesda, MD) using 12 squares grid over the tumor picture. Positive staining grids (inflammatory loci) were counted by free hand.

Tumor q-PCR

Total RNA from tumors was harvested using Trizol reagent (life technologies). RNA was reverse transcribed to generate cDNA using SuperScript III first-stand synthesis super mix (Invitrogen). Specific primer sets (Additional file 1: Table S1) and Kapa Sybr Master Mix (Kapa biosystems) were used for qPCR. Data were collected using a A Prism 7000 real-time PCR system (Life Technologies), samples were run in duplicate. Relative quantification studies were performed with the collected data using the Prism 7000 System SDS software 1.3 (Life Technologies) and the relative expression ratio (R) of a target gene was calculated based on Efficiency and the CP deviation of an unknown sample versus a control, and expressed in comparison to the reference genes [23] hypoxanthine phosphoribosyltransferase (HPRT) and TATA box binding protein (TbP).

Flow cytometry

Tumor dissociation was performed as previously described [24]. Cells were washed twice by PBS and counted in a Neubauer chamber; cell viability was evaluated using Trypan Blue dye exclusion. Immunofluorescence staining was carried out by antibodies to rat lymphocyte markers (Additional file 1: Table S2). In brief, 2 X 10^5 cells were suspended in PBS containing 0.2% bovine serum albumin and 0.2% sodium azide, and incubated with fluorescent antibodies for 30 min at 8°C. After washing, 10,000 cells were analyzed on a MACSQuant flow cytometer (Miltenyi Biotech, Germany). First, acquired cells were gated by their physical properties (forward and side scatter); immediately, a second gate was done based on CD45 expression and forward scatter, from which was drawn a histogram to analyze CD43, CD3, CD4, CD8, CD45R and CD161 expression.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Weight gain, tumor volume, core temperature, food and water intake, ad libitum-fasted glucose, glucose levels for the GTT and TNF-α plasma levels were compared with a two-way ANOVA for repeated measures for two factors (Condition LL or LD x time). Mean day-night temperature was compared with a two-way ANOVA. ANOVA's were followed by Bonferroni's post-hoc test for multiple comparisons. An unpaired one-tail Student T test was used to analyze food ingestion, serum TG, AUC, CD43 cells and genes measured in the tumor. Mann-Whitney test was used to analyze F4/80-IR positive grids.

All data and the Area Under the Curve (AUC) for plasma glucose were analyzed by using GraphPad Prism (version 6.03; Graph Pad Software, Inc.). Statistical significance was set at $\alpha = 0.05$.

Results

Constant light induced loss of circadian rhythms in general activity and core body temperature

Control rats in LD exhibited a clear day-night general activity alternation, characterized by high activity levels during the night (Fig. 1a). In contrast, LL induced a progressively loss of general activity rhythmicity until no clear day–night difference was observed (Fig. 1b). The periodogram corresponding to the last 14 days of experiment confirmed circadian rhythmicity in general activity for all rats in LD (Fig. 1c) and loss of circadian rhythmicity for all LL rats (Fig. 1d).

Circadian rhythms in core body temperature (Tb) were also monitored during the last 2 days of the lighting protocol. LD rats showed a clear day-night Tb rhythm characterized by low temperature levels during the day and high levels during the night (Fig. 1e) while LL rats showed constant temperature values along the subjective day-night 24 h period. Interestingly, the mean daily temperature of LL rats was higher than the mean of the day values of LD rats (Fig. 1d; $p < 0.05$).

Constant light modified metabolism in the host

After 5 weeks, LL animals had gained more weight than the control LD rats; this reached significant difference from LD animals on weeks 4 and 5 of the protocol (Fig. 2a; $p < 0.01$). The increased body weight gain observed in LL rats, was not due to a difference in food consumption (Fig. 2b).

Plasma TG levels were higher in LL as compared with LD rats (Fig. 2c; $p < 0.01$); similarly, glucose plasma levels were significantly higher in LL rats as compared with LD rats both under fasted (before GTT) and ad libitum conditions (Fig. 2d; $p < 0.01$). In addition, LL rats showed an impaired glucose clearance, as demonstrated with the GTT (Fig. 2e-d; $p < 0.05$).

Constant light increases the inflammatory response to LPS

Basal TNF-α plasma levels, measured at time 0 were very low or undetectable in both LD and LL rats. LPS administration triggered a significant increase of TNF-α

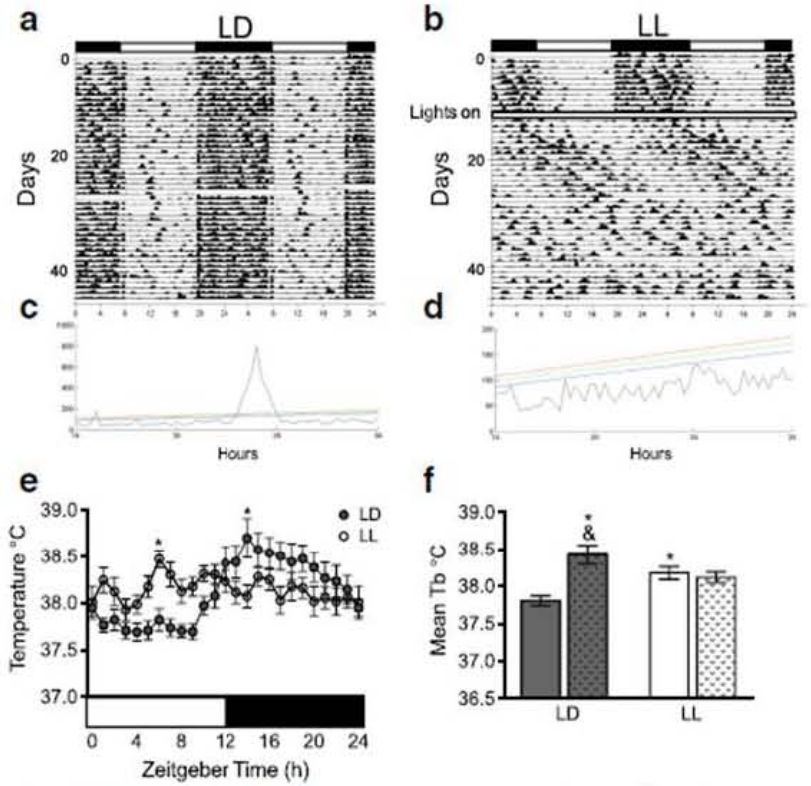

Fig. 1 Constant light for 5 weeks disrupts circadian rhythms in general activity and core body temperature (Tb). (**a**) Representative double-plotted actograms from LD and (**b**) LL rats respectively. Black and white horizontal bars on top of the actograms represent night and day. The change in the lighting condition after 12 days of baseline (12:12 h LD cycle) is indicated with the legend "lights on" and the large white bar representing the constant light condition. (**c**) The χ^2 periodogram test for the last 14 days demonstrates a 24 h rhythm for LD and (**d**) the absence of a circadian rhythm for LL rats. (**e**) Mean temperature values from the last 2 days of the lighting schedule for LD (grey circles) and LL (white circles) rats along 24 h. (**f**) Mean day-night temperature values for LD (grey bars) and LL (white bars), stripped bars in each group represent the night. Data are the mean ± SEM ($n = 8$/group). For E the repeated-measures two-way ANOVA, indicated significant interaction of the lighting condition versus time $p < 0.0001$. For F the two-way ANOVA indicated significant interaction of the lighting condition versus time $p < 0.0001$. The Bonferroni test indicated statistical difference LL from LD *$p < 0.05$ for F and indicated & $p < 0.001$ between day and night in the LD group

plasma levels in both groups, reaching the highest levels after 40 and 80 min, LL rats reached significantly higher TNF-α plasma levels as compared with LD rats (Fig. 3a; $p < 0.05$).

After the LPS challenge, analysis of Tb indicated that both groups exhibited an initial increase in Tb with a first peak 1 h after LPS administration and a second peak 5–6 h later, the mean temperature of the day in LL rats was significantly higher as compared to LD rats (Fig. 3b; $p < 0.05$) and the Tb difference between day and night in LD rats was dampened. 24 h post LPS injection Tb of both groups returned to pre injection levels (Fig. 3c). Both groups reduced food consumption on the day of LPS administration, this was more severe in LL rats, which consumed 41.66 ± 2.76% less food as compared to the 12.59 ± 2.41% reduction observed in LD rats (Fig. 3d; $p < 0.05$); water intake was also reduced in both groups (Fig. 3e). 24 h after LPS administration both groups increased food and water intake; nevertheless 48 h post LPS, LL rats were still consuming significantly less food than the LD group (Fig. 3d; $p < 0.05$).

This initial food and water reduction impacted on body weight for both groups on the day of LPS administration; however, there was no difference in the weight loss between groups (19 ± 2.67 g in LD and 12.83 ± 2.46 g in LL rats). Animals had not recovered body weight 48 h post LPS. Altogether these results indicate that LL aggravates the sickness response, especially cytokine production and food consumption.

Inoculated tumor cells grow more in LL rats

Inoculated tumor cells formed bigger tumors in LL rats, that were significantly different from LD tumors on days 11 and 13 (Fig. 4a; $p < 0.05$). At the end of the

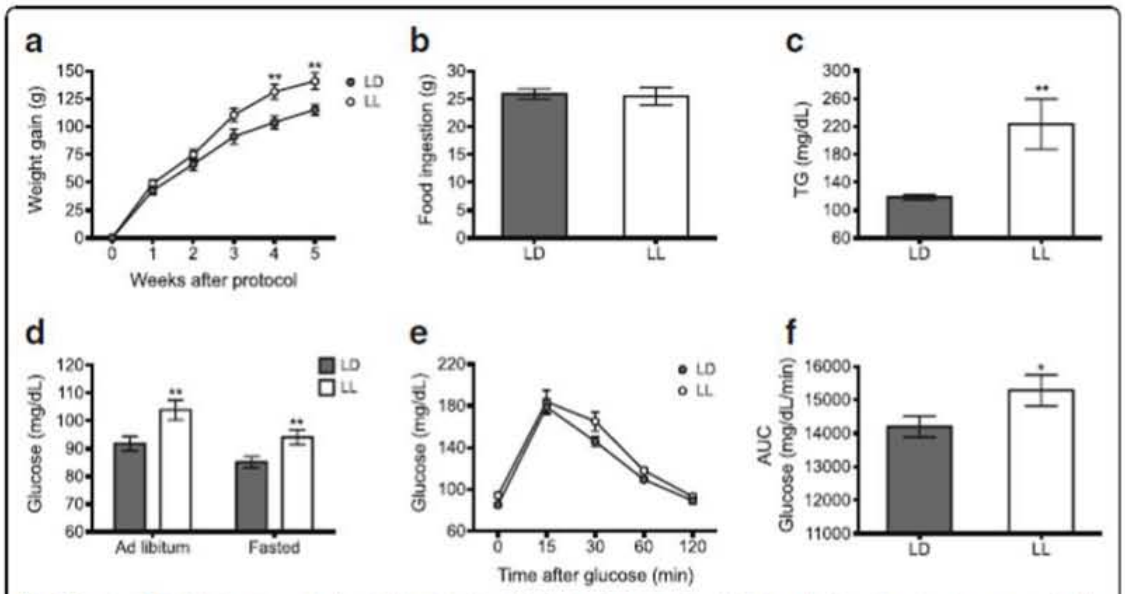

Fig. 2 Constant light (LL) disrupts metabolism. (**a**) LL rats (white circles) gained more body weight along the 5-week protocol as compared with LD rats (grey circles). The repeated-measures two-way ANOVA indicated significant interaction of the lighting condition versus time, $p = 0.0011$. The Bonferroni test $^{**}p < 0.01$ indicated statistical difference from LD. (**b**) Food ingestion assessed for 24 h during week 5 indicated no differences between groups. Data are expressed as mean ± SEM (n = 7–8/group). (**c**) Basal plasma TG and (**d**) glucose levels under ad libitum and fasted conditions were significantly increased in LL rats. Data are the mean ± SEM (n = 12/group), $^{**}p < 0.01$ indicates statistical difference from LD; unpaired t test. (**e**) Glucose tolerance test (GTT, 0–120 min) and (**f**) area under the curve (AUC) following i.p. administration of 1 g of glucose/kg. Data are the means ± SEM (n = 14–15/group). $^{*}p < 0.05$ indicates statistical difference from LD; unpaired t test

experiment, isolated tumors from LL were also significantly heavier than LD tumors (Fig. 4b-c; $p < 0.5$). Together these findings suggest that LL induces a suitable environment for tumor growth.

Tumor development changed the metabolic profile in the host

Tumor development affected the body weight between LL and LD groups (Fig. 2a). Before tumor inoculation LL rats were heavier than LD rats, 4 days after inoculation differences disappeared between groups and by the end of the experiment (13 days after) LD rats had gained more weight as compared to LL rats (Fig. 5a; $p < 0.05$), suggesting that in LL rats the increased tumor growth resulted in a higher metabolic demand.

Tumor development also decreased food ingestion in both groups as compared to their own basal levels (Fig. 5b; $p < 0.05$) without difference between groups. TG levels in LL tumor-bearing rats diminished 26% as compared to their previous condition. In contrast, TG levels in LD tumor-bearing rats increased 10% as compared to their previous condition; thus TG levels were not different between LL and LD tumor-bearing rats (Fig. 5c). The presence of the tumor induced an increase of glucose levels in both groups. LL animals increased 55.01% ± 6, while LD rats increased 45.92% ± 8 from their basal glucose levels. In addition, tumor development also increased fasting blood glucose levels in LL rats as compared to LD (Fig. 5d; $p < 0.05$); nevertheless glucose clearance was not different between LL and LD tumor-bearing rats on day 9 after tumor cells inoculation as demonstrated with the GTT.

In order to test whether the initial metabolic condition induced by LL may be the promoting factor for tumor growth, a different group of rats in LD condition, was exposed to a 1 h daily access to high sugar diet for 4 weeks (Additional file 2). The high sugar diet induced increased body weight and similar metabolic disturbances as observed in LL rats (Additional file 3: A-D). A sugar diet favored the growth of bigger tumors as compared to rats consuming a chow diet (Additional file 3: E; $p < 0.01$), reaching similar size as tumors in the LL rats at day 13.

Tumors from LL rats recruit more macrophages

Because the infiltration of immune cells is an important event that correlates with tumor growth or elimination (depending on the infiltrating immune cell type), we investigated the inflammatory condition in the tumor. Tumor infiltration of T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$), NK cells ($CD161^+$) and B cells (CD45R) was not different between LD and LL (Additional file 4: A-D). However,

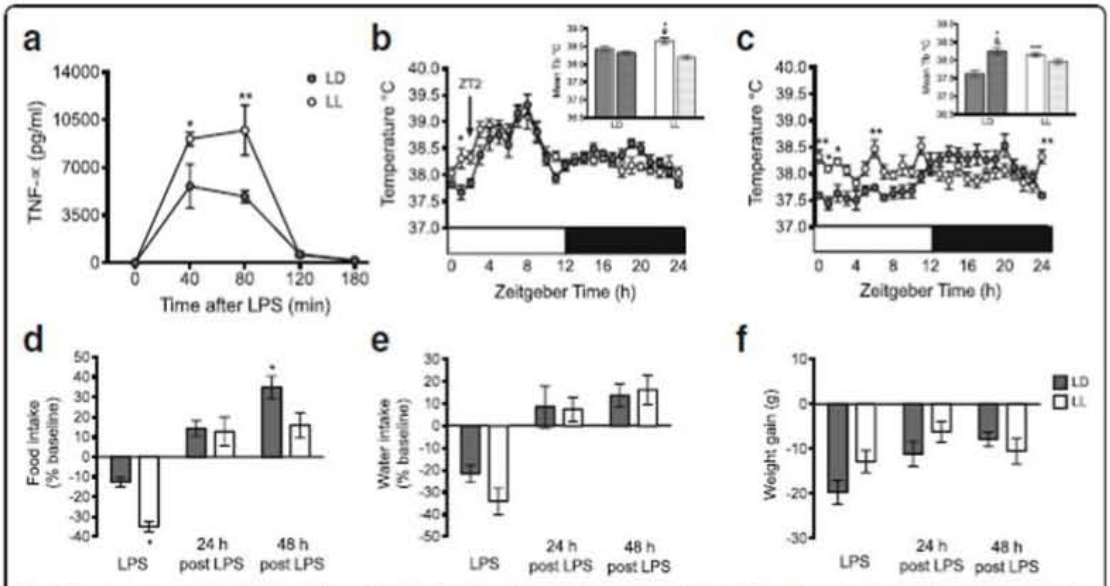

Fig. 3 LL rats produced more TNF-α and showed increased sickness behavior in response to LPS. (**a**) TNF-α plasma levels after the administration of 2μg/kg of LPS. The repeated-measures two-way ANOVA indicated a significant effect due to the interaction condition versus time, $p = 0.0065$. The Bonferroni test $^{**}p < 0.01$ indicated statistical difference from LD. (**b**) Thermoregulatory response to LPS. The arrow represents LPS administration at ZT2 for both groups. The mean day-night core body temperature (Tb) is shown in the box, for LD (grey bars) and LL (white bars), stripped bars in each group represent the night. The repeated-measures two-way ANOVA indicated a significant effect due to light condition $p = 0.0107$; and time $p = 0.0011$. The Bonferroni test $p < 0.05$ indicated statistical difference from LD and # between LL. (**c**) Thermoregulatory response 24 h post LPS. The mean day-night core body temperature (Tb) is shown in the box. The subjective day-night phases for LL rats were selected based on the 12 h day and 12 h night of LD animals. The repeated-measures two-way ANOVA indicated effects due to the interaction condition versus time, $p = 0.0011$; the Bonferroni test indicated $^{*}p < 0.05$ statistical difference from LD and & between LD. (**d**) Food intake, (**e**) Water intake and (**f**) Weight gain during the day of LPS administration, 24 and 48 h post LPS in LD and LL rats. Values are expressed as a percentage of the baseline value established prior to LPS administration. For d, the repeated-measures two-way ANOVA indicated effects due to lighting condition $p = 0.0107$ and time $p = 0.0011$. Data are the mean ± SEM ($n = 6$/group). $^{*}p < 0.05$ indicates statistical difference from LD; with Bonferroni test

tumors of LL rats tend to recruited more monocytes ($CD43^{+}$, Fig. 6a). Because monocytes are the precursors of macrophages, we evaluated the presence of macrophages inside the tumors, using the F4/80 marker. The inmmunohistochemical staining indicated that tumors from LL rats recruited more macrophages as compared to LD rats (Fig. 6b-c; $p < 0.05$). The increased number of tumor macrophages in LL did not result in increased levels of circulating TNF-α. Undetectable TNF-α plasma levels were measured on both LD and LL rats (Data not shown).

The LL tumor microenvironment is characterized by an altered metabolic profile

In order to identify the factors in the tumor microenvironment that may favor its growth, a set of genes related with metabolism, cytokines, growth and angiogenesis

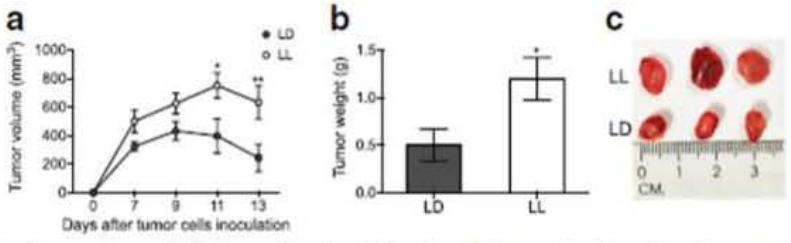

Fig. 4 Constant light enhances tumor growth. (**a**) Tumor volume along 13 days after subcutaneous C6 cells inoculation. The repeated-measures two-way ANOVA indicated a significant interaction of condition versus time, $p = 0.0240$. The Bonferroni test indicated $^{*}p < 0.05$, $^{*}p < 0.01$ statistical difference from LD. (**b**) Tumor weight at day 13. $^{*}p < 0.05$ indicates statistical difference from LD; unpaired t test. (**c**) Representative pictures from LD and LL tumors. Data are the mean ± SEM ($n = 7$/group)

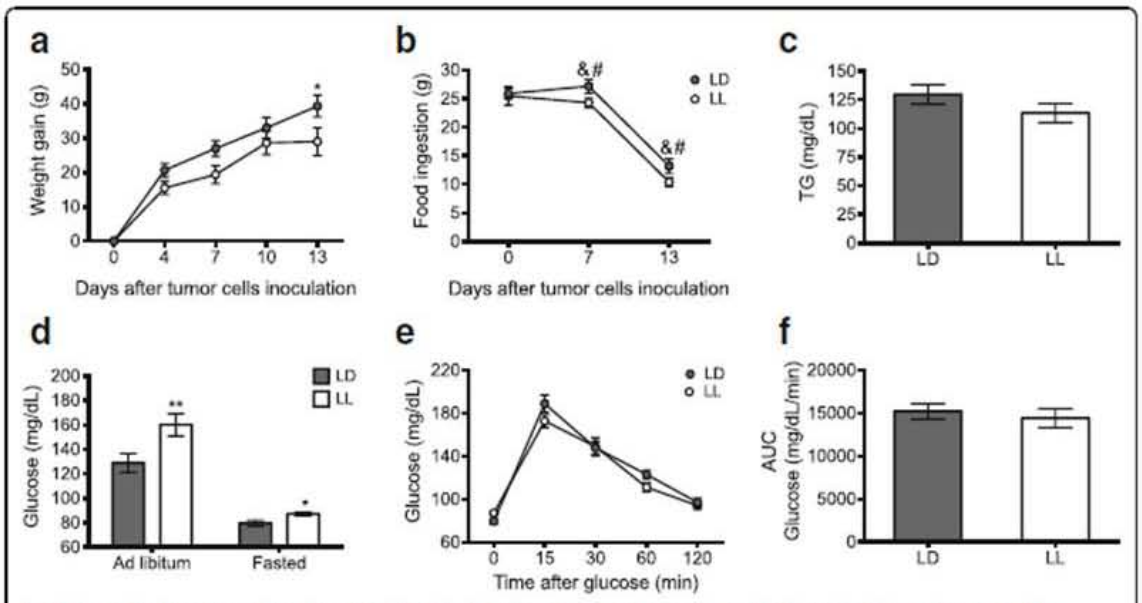

Fig. 5 Tumor development modifies the metabolic profile of the host. (**a**) Accumulative gain weight along the 13 days after tumor cells inoculation (**b**) Food ingestion before, on day 7 and day 13 after tumor cells inoculation. Data are the mean ± SEM (n = 7/group). For A and B, the repeated-measures two-way ANOVA indicated a significant effect of time, $p < 0.0001$. Bonferroni test indicated *$p < 0.01$ statistical difference from LD, & between LD and # between LL. (**c**) TG and (**d**) glucose levels under ad libitum and fasted conditions in LD and LL tumor-bearing rats (13 days) D. Unpaired t test **$p > 0.001$ indicated statistical difference from LD. Data are the mean ± SEM (n = 7/group). (**e**) Glucose tolerance test (0–120 min) and (**F**) AUC following i.p. administration of 1 g glucose/kg. Data are the mean ± SEM (n = 8/group)

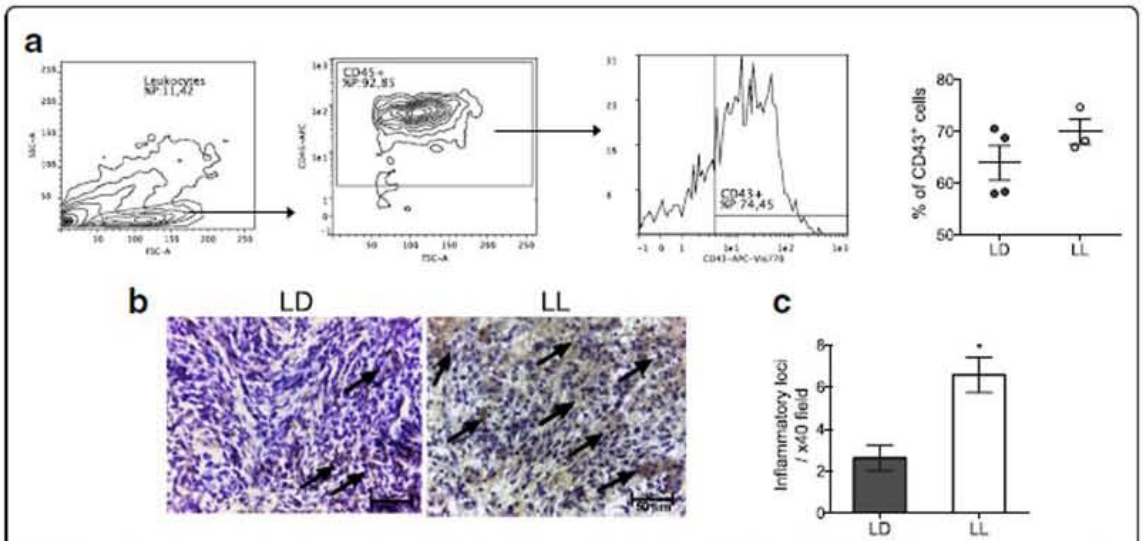

Fig. 6 Tumors from LL rats recruit more macrophages. (**a**) Gating strategy employed to identify CD43$^+$ cells in LD and LL tumors and percentage of CD43$^+$ cells (n = 3–4/group). Analysis was done on whole lysates from tumors removed from LD and LL rats on day 13. (**b**) Immunohistochemistry staining of LD and LL tumors against an anti-macrophage antibody F4/80. Arrows indicate inflammatory loci. (**c**) Quantification of inflammatory loci in the grid area. Data are the mean ± SEM (n = 5–6/group) *p = 0.0173 indicates statistical difference from LD; Mann-Whitney test

pathways were evaluated in tumors obtained from LD and LL 13 days after inoculation.

Genes involved in lipogenesis Acetyl-CoA carboxylase alpha (*Acaca*), Fatty acid synthase.

(*Fasn*) and Peroxisome proliferator activated receptor gamma (*Pparγ*) were highly expressed in the tumors of LL rats as compared to LD tumors (Fig. 7a; $p < 0.5$), suggesting an up regulation of lipid production in tumors of LL animals in order to support their growth. Contrasting, Sterol regulatory element binding transcription factor 1 (*Srebp-1*) a transcriptional activator of the genes involved in lipogenesis was decreased in the tumors of LL rats ($p < 0.05$). The expression of genes related to lipid oxidation Carnitine palmitoyltransferase IA (*Cpt1a*), Acyl-CoA dehydrogenase (*Acads*), Hydroxyacyl-CoA dehydrogenase (*Hadha)* and Peroxisome proliferator-activated receptor alpha (*Ppar*) was not different between LD and LL tumors (Fig. 7b).

From the genes involved in glycolysis, the expression of the Glucose transporter 1 (*Glut1*) was increased in LL as compared to LD tumors (Fig. 7c; $p < 0.05$) suggesting a higher glucose uptake. The expression profile of other glycolytic genes Hexokinase II (*HkII*), Pyruvate kinase muscle isozyme M2 (*Pkm2*) and Lactate dehydrogenase (*Ldh*) was not different between the two groups (Fig. 7c). In line with the increased lipogenesis and glucose transport, tumors from LL rats expressed high levels of the oncogene *Myc*, the Insulin transporter (*Ir*) and the pro-angiogenic gene *Vegf-α*(Fig. 7d and e; $p < 0.05$). In contrast, no differences between tumors in the two groups were found in the expression of the Hypoxia-inducible factor 1-alpha (*Hif1-α*) and the measured cytokines Transforming growth factor beta (*Tfgα*), Interleukin 10 (*Il10*), Interleukin 6 (*IL6*), Interleukin 1 beta (*Il1α*) and *Tnfα* (Fig. 7f).

Discussion

Light at night is a modern life style problem, especially for individuals living in big cities; it affects night workers as well as young people and children that are exposed to artificial light for extended hours of the night. The effects of light at night on human and rodent health have been the focus of several studies reporting a loss of body homeostasis, body weight gain, depression and increased tumor development [11, 25, 26]; however, the mechanisms involved in this process are not well established.

This study demonstrates that light at night disrupts the host's metabolism as well as the inflammatory response creating an obesogenic environment, favorable for tumor growth. Tumors induced in LL rats showed an increased number of macrophages, expressed high mRNA levels of key enzymes involved in lipogenesis as well as in the uptake of glucose; this was associated with increased mRNA levels of markers of tumor development.

LL disrupts metabolism

The control of cellular metabolism is essential for cell survival, and the role of aberrant cellular metabolism in cancer

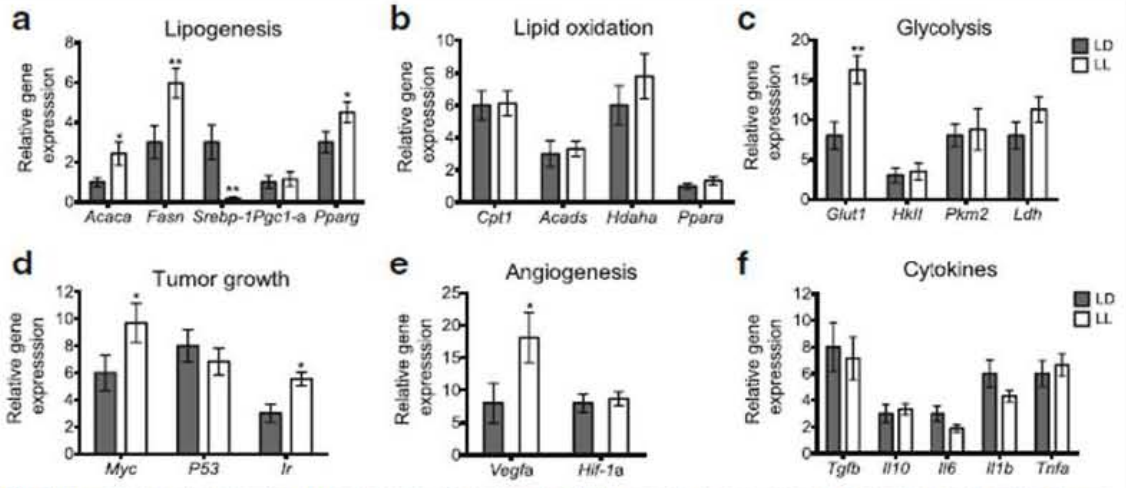

Fig. 7 In tumors from rats exposed to LL the expression of genes involved in lipogenesis, glucose uptake, angiogenesis and cell proliferation was increased. (**a**) Relative expression of genes (*Acaca, Fasn, Srebp-1 and Ppary*) involved in lipogenesis; (**b**) Relative expression of genes involved in lipid oxidation (*Cpt1, Acads, Hadha and Ppara*); (**c**) glycolysis (*Glut-1, HkII, Pkm2, Ldh*); (**d**) Tumor growth (*Myc, P53, Ir*); (**e**) angiogenesis (*Vegfa, Hif1-α*) and (**F**) cytokines (*Tfgα, Il10, IL6, Il1a and Tnfa*). All genes were measured using qRT-PCR analysis. Gene expression in tumors was normalized to the expression of Hprt and Tbp as endogenous controls. Data are plotted as the mean ± SEM. ($n = 7–10$/group). *$p < 0.05$, **$p < 0.01$ indicates statistical difference from LD; unpaired t test

is becoming evident. In humans, over weight and an anabolic metabolism are associated with cancer development [19, 27]. Several systemic and metabolic alterations that accompany obesity, such as insulin resistance, hyperglycemia, fat accumulation, low-grade systemic inflammation and immune deregulation, also correlate with cancer development [28]. Present data are in agreement with this approach, since the increased tumor growth was associated with increased body weight gain, induced dyslipidemia, high glucose levels and altered glucose clearance in LL animals. Similar metabolic changes and tumor growth were observed after a high sugar diet, confirming that increase tumor development profits from the host's metabolism shifted to an obesogenic condition. Indeed, high glucose levels are associated with poor survival in patients with glioblastoma [29, 30], the same kind of tumors induced in the present study after the inoculation of C6 cells in the rat [31]. Importantly, fasting regimens, which are associated with decreased levels of glucose and insulin, delayed the progression of cancer, have cancer preventive effects and increase the efficacy of chemotherapy agents [32–34].

It is well described that tumor cells "reprogram" the host's metabolism in order to survive and proliferate under conditions that otherwise would arrest or kill normal cells [35]. We report that after 13 days of tumor induction, in both groups plasma glucose levels were increased while TG levels decreased in LL animals, correlating with increased tumor growth and suggesting TG uptake.

Increased expression of glucose transporter 1 in LL tumors

Tumor cells take up nutrients such as glucose, lipids and aminoacids to fuel their metabolic pathways [36]. Glucose metabolism in cancer cells is known to be elevated due to altered membrane transport, that leads to increased intracellular glucose levels. Glucose is used by tumors to generate energy mainly through aerobic glycolysis (increased conversion of glucose to lactic acid to produce ATP) [37]. The main product lactate, is associated with increased tumor angiogenesis, heightened metastasis, and can also induce a pro-inflammatory state in the tumor microenvironment [38]. In line with this, tumors isolated from LL rats exhibited increased mRNA levels of the glucose transporter 1 (*Glut-1*), which promotes glucose import into the cytoplasm. Besides a primary substrate for ATP generation, glucose is a carbon source for the biosynthesis of other macromolecules; hence a critical nutrient for fast proliferating cells [39]. Contrasting, we did not find significant differences in the expression of key enzymes involved in the aerobic glycolytic pathway such as *HKII, Pkm2* and *Ldh*. Thus differences in enzymatic activity may be present since previous findings relate the growth of C6 tumor cells to the high expression of *Glut-1* coupled to glucose metabolism [40]. This is supported by observations in which glucose was withdrawn from culture medium inducing apoptosis in glioblastoma cell lines [41].

In this study the glucose tolerance test suggests insulin resistance induced by LL, which is in agreement with others findings [4], the increased *Ir* mRNA levels observed in LL tumors coupled with the increased insulin levels in LL observed by others, offer another possible pathway (46) by which tumor growth can be stimulated under the metabolic conditions of LL. This possible mechanism is further supported by the increased mRNA levels of the transcription factor *Myc* in LL tumors. Interestingly, besides regulating the transcription of genes involved in cell growth, cell proliferation, cell cycle, protein biosynthesis and apoptosis (under nutrient or growth factor deprivation conditions) [42], other genes targeted by the transcription factor *Myc* include key genes involved in glucose metabolism such as *Glut-1* [43], lipid metabolism and angiogenesis [44]. The increased expression of *Glut-1* probably promoting increased glucose influx to the LL tumor cells, together with the up-regulation of *Myc*, may favor glucose metabolism and the supply of acetyl-coA used as a substrate for lipid biosynthesis and for other nuclear processes.

High lipid synthesis in LL tumors

Alterations in lipid metabolic pathways are another well-recognized metabolic adaptation that enables tumors to take up exogenous lipids or up-regulate endogenous synthesis (50,51). Decreased circulating TG levels in LL tumor bearing rats, suggest tumor lipid uptake, which is supported by the observed up-regulation of *Vegf*-α known target of *Ppar*-γ which was also up-regulated in LL tumors and it is known to be activated by fatty acids in the tumor microenvironment [45]. Moreover, present data suggest that LL tumors have increased lipid synthesis because they expressed high mRNA levels of all the key enzymes involved in lipid synthesis such as *Acaca* that generates malonyl-CoA from actetyl-Coa, *Fasn*, which catalyzes fatty acid chain elongation and *Pparγ*, a transcription factor that regulates the expression of genes involved in lipid metabolism as well as tumorogenesis [46]. Strikingly, LL tumors expressed decreased mRNA levels of *Srepb-1* (a transcription factor that regulates the activation of genes involved in fatty acid synthesis), which suggest the role of other regulatory mechanisms for the increased expression of lipogenic genes in LL tumors. The increased fatty acids synthesis observed in LL tumors may favor energy production, cell signaling and tumor growth by inducing membrane synthesis, angiogenesis, migration and immunosuppression [46].

Constant light disrupts the inflammatory response to LPS

Undetectable TNF-α plasma levels were measured in LL rats before the LPS challenge, indicating that LL increases the sensitivity to an immune challenge without changing the inflammatory state of the host at least in the circulation, as observed in other circadian desynchronization protocols such as experimental shift-work and jet lag in rodents [8, 47]. However, LL aggravated certain components of the sickness response such as cytokine production, and food consumption after LPS administration, which is in agreement with other studies [7]. Constant light also decreases the amplitude of the diurnal rhythmicity of leukocyte counts as well as the number and cytotoxicity of splenic NK cells in rats [48, 49]. Moreover, rats exposed to LL produce fewer antibodies in response to a T-cell dependent antigen [50]. Altogether these results also indicate that LL affects the function of the immune system in a way that may favor the development of disease and tumor growth.

The inflammatory microenvironment of LL tumors

The exacerbated inflammatory response observed in LL animals suggested a deregulated inflammatory response affecting the tumor microenvironment. Our analysis confirmed that LL tumors recruited more macrophages as compared to LD tumors favoring tumor growth. Macrophages are the major immune cell population recruited in gliomas [51] and support tumor progression, angiogenesis, metastasis and immunosuppression [52]. In this sense, increased number of TAMs observed in LL tumors may have contributed to the observed tumor growth via the production of soluble factors such as VEGF a well-recognized angiogenic promoter. Here we show that the highly macrophage infiltrating LL tumors expressed increased pro-angiogenic factor *Vegf-a* mRNA levels, which regulates blood vessel formation but also exert mitogenic actions that may contribute to the enhance tumor growth observed in LL rats. Importantly, targeted deletion of TAMs in glioma xenografts promotes tumor regression [53].

Angiogenesis is an essential mechanism for tumor growth and maintenance, which may occur in response to environmental cues such as hypoxia stabilizing the transcription factor *Hif-1α*, that in turn activates the expression of angiogenic genes like *Vegf-a*. Levels of the *Hif-1α* mRNA were not different between LL and LD tumors, which can be explained by its relatively short-lived mRNA [54], or the oscillating O2 tumor levels (over the course of hours and days), which induce periodic fluctuations of tumor *Hif-1α* expression [55].

Conclusions

The obesogenic metabolism observed in LL hosts associated to an altered immune response may have favored a propitious internal tumor environment. We have demonstrated that tumors from LL rats up-regulate key enzymes involved in glucose uptake and lipogenesis, which correlates with increased expression of tumor growth markers. Of clinical relevance is the fact that circadian disruption by LL exposure induces several metabolic features that are also observed in Type II diabetes mellitus patients or with metabolic syndrome; conditions that also are associated with increased cancer incidence.

Light at night suppresses melatonin in both, diurnal (humans) and nocturnal subjects [56–58] and has shown to exert adverse effects in diurnal species in a similar way as in nocturnal rodents [59, 60]. In this regard light at night is an environmental risk factor that appears to favor conditions for tumor growth, similar to obesity and diabetes. Present data highlight the importance of developing strategies to prevent circadian disruption and raise the need to continue exploring the link between circadian regulation and health problems including cancer.

Limitations of our study

The tumor cell line used in this study does not enable us to follow tumor development at latter survival times because for this type of cells the host immune system induces tumor involution. However, this cell line allowed us to study tumor development in rats with an intact immune system and the interaction with the host's homeostatic conditions. Although we induced the tumor by inoculating tumor cells, present data suggest that the metabolic condition observed in LL rats per se may promote spontaneous tumor formation at later stages as has been recently demonstrated in a model of circadian desynchronization by chronic jet lag exposure [61]. More studies are necessary to corroborate this.

Additional files

Additional file 1: Table S1. and **Table S2.** (PDF 102 kb)

Additional file 2: Supplementary methods. (PDF 67 kb)

Additional file 3: High sugar diet (HS) induces a suitable metabolic environment for tumor growth. (A) HS rats (white circles) gained more body weight along the 4-week protocol as compared with chow diet rats (grey circles). Data are the mean ± SEM (n = 7/group). The repeated-measures two-way ANOVA indicated significant effects for condition versus time, interaction $p = 0.0014$. The Bonferroni test ***$p < 0.001$ indicated statistical difference from chow diet. (B) HS rats ingest more Kcal in 24 h. Data are the mean ± SEM (n = 7/group). ** $p < 0.01$ indicates statistical difference from chow diet; unpaired t test. (C) Basal plasma triglycerides (TG) and glucose levels (D) under ad libitum conditions were significantly increased in HS rats. Data are the mean ± SEM (n = 6–7/group), **$p < 0.01$, ***$p < 0.001$ indicates statistical difference from chow diet; unpaired t test. (E) Glucose tolerance test (GTT, 0–120 min) following i.p. administration of 1 g of glucose/kg. Values are expressed as mean ± SEM (n = 7/group). The repeated-measures two-way ANOVA indicated significant effects for condition versus time interaction $p = 0.016$. The Bonferroni test **$p < 0.01$ indicated statistical difference from chow diet. (F) Tumor volume along 13 days after

subcutaneous C6 cells inoculation. The repeated-measures two-way ANOVA indicated a significant interaction for condition versus time, $p = 0.0032$. Data are expressed as mean ± SEM (n = 4-7/group). The Bonferroni test indicated $^{**}p < 0.01$ statistical difference from chow diet. (PDF 328 kb)

Additional file 4: Tumors from LL and LD rats similar percentages of immune cells. (A) Percentage of $CD8^+$, (B) $CD4^+$, (C) $CD45R^+$ and (D) $CD161^+$ cells. Data are expressed as the mean ± SEM (n = 3–4/group). Analysis was done on whole lysates from tumors removed from LD and LL rats on day 13. (PDF 23 kb)

Abbreviations

Acaca: Acetyl-CoA carboxylase alpha; *Acads*: Acyl-CoA dehydrogenase; *Cpt1a*: Carnitine palmitoyltransferase IA; *Fasn*: Fatty acid synthase; *Glut1*: Glucose transporter 1; GTT: Glucose tolerance test; *Hadha*: Hydroxyacyl-CoA dehydrogenase; *Hif1-α*: Hypoxia-inducible factor 1-alpha; *Hkll*: Hexokinase II; *Il10*: Interleukin 10; *Il1a*: Interleukin 1 beta; *Il6*: Interleukin 6; *Ir*: Insulin transporter; *iv*: Intravenous; LD: Light-dark cycle; *Ldh*: Lactate dehydrogenase; LL: Constant illumination conditions; LPS: Lipopolysaccharide; *Pkm2*: Pyruvate kinase muscle isozyme M2; *Ppara*: Peroxisome proliferator-activated receptor alpha; *Ppary*: Peroxisome proliferator activated receptor gamma; *Srebp-1*: Sterol regulatory element binding transcription factor 1; Tb: Core body temperature; *Tfga*: Transforming growth factor beta; TG: Triglycerides; TNF-α: Tumor necrosis factor α; *Vegfa*: Vascular endothelial growth factor alpha; ZT: Zeitgeber time

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Authors' contributions

N.N.G-V. and C.E. designed and conceived the research; N.N.G-V., R.N-F., M.A.G-R, M.C.B., E.F-B, A.L.-B, and C.C-M conducted experiments. N.N.G-V., M.A.G-R., M.C.B., A.L-B., C.E., R.L. and R.M.B analyzed data. N.N.G-V. and C.E., wrote the paper. N.N.G-V., RN-F, M.A.G-R, M.C.B, E.F-B, A.L.-B, R.L., R.M.B and C.E. reviewed and edited the manuscript. C.E. is the guarantor of this work and, as such, had full access to all data in the study. All authors read and approved the final manuscript.

Ethics approval

Experimental procedures used in this study were approved by the committee for ethical evaluation at the Faculty of Medicine, UNAM (019/2015), in strict accordance with international guidelines for animal handling. All efforts were made to minimize the number of animals and their suffering.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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ANEXOS 2

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Shift-work: is time of eating determining metabolic health? Evidence from animal models

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The circadian disruption in shift-workers is suggested to be a risk factor to develop overweight and metabolic dysfunction. The conflicting time signals given by shifted activity, shifted food intake and exposure to light at night occurring in the shift-worker are proposed to be the cause for the loss of internal synchrony and the consequent adverse effects on body weight and metabolism. Because food elicited signals have proven to be potent entraining signals for peripheral oscillations, here we review the findings from experimental models of shift-work and verify whether they provide evidence about the causal association between shifted feeding schedules, circadian disruption and altered metabolism. We found mainly four experimental models that mimic the conditions of shift-work: protocols of forced sleep deprivation, of forced activity during the normal rest phase, exposure to light at night and shifted food timing. A big variability in the intensity and duration of the protocols was observed, which led to a diversity of effects. A common result was the disruption of temporal patterns of activity; however, not all studies explored the temporal patterns of food intake. According to studies that evaluate time of food intake as an experimental model of shift-work and studies that evaluate shifted food consumption, time of food intake may be a determining factor for the loss of balance at the circadian and metabolic level.

Circadian disruption: Shift-work: Scheduled feeding: Light at night: Obesity: Metabolic syndrome

Shift-work and night work require individuals to labour outside conventional daytime hours, imposing shifted activity and sleep schedules[1]. It is estimated that worldwide about 20 % of individuals participate in some form of shift-work. Besides shift- and night workers, another 25 % of young adults worldwide are exposed voluntarily to shifted sleep-activity habits[2], mainly due to the modern life style that promotes social or leisure activities at normal rest times. Individuals engaged in nocturnal leisure activities shift their sleep-activity patterns differentially between weekdays and weekends, leading them to similar conditions as shift-workers. This shifted sleep timing is now referred as social jet-lag[3]. Both social jet-lag and shift-work share similar features because in both conditions individuals are awake and active outside conventional daytime hours; they suffer from altered sleep-activity habits, shifted eating patterns and are exposed to light at night[4,5]. Such conditions cause a conflict with the internal biological clock and promote circadian disruption triggering loss of homeostasis[6].

While we know little about the short- or long-term consequences of social jet-lag, a vast number of studies provide information about the short- and long-term effects of night and shift-work. Shift-workers are identified as a population with higher risk to develop adverse health effects including myocardial infarction, ischemic stroke and CVD[7,8]. Shift-workers complain of disturbed sleep and excessive fatigue[1,9], which have adverse

Abbreviation: LL, constant light intensity throughout 24 h; SCN, suprachiasmatic nucleus.

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consequences on their work performance, leading to high levels of stress[10,11] to a reduced reaction time and sleepiness-related accidents[12,13]. In the long term, shift-work is associated with psychiatric disorders, depression and substance abuse[14–16].

Metabolically, shift-work is associated with a higher propensity to develop overweight or obesity[17,18] and it is a risk factor for metabolic syndrome[17,19], insulin resistance[20], dyslipidaemia and type 2 diabetes[21,22]. Due to the worldwide increasing incidence of obesity and metabolic disease, attention has focused on individuals at risk, especially shift-workers and groups exposed to disrupted sleep-activity patterns. In regard to social jet-lag, studies are needed to confirm this association; however, in individuals presenting overweight, a higher BMI was associated with a higher number of shifted hours between weekdays and weekends[2]. Clinical and experimental studies have indicated that shift-work and other conditions that cause circadian disruption prime individuals for a higher vulnerability to lose metabolic balance and obesity[18,23,24].

Circadian rhythms are relevant in order to adjust the intensity and efficiency of the organism's response to the daily challenges required by the day–night cycles. Disrupted circadian rhythms will result in a time-deficient response that in the long term will lead to loss of homeostasis and disease. Circadian rhythms are driven by the circadian system, which is a complex internal timing system constituted by a biological clock, the suprachiasmatic nucleus (SCN), and by peripheral oscillators[25]. The circadian system fluctuates, synchronised to the external light–dark cycle, which is the main environmental time reference; however, other inputs, relevant for the group or the individual's survival, can also provide time information, including the internal energetic state and food availability.

At the cellular level, clock mechanisms are driven by transcription–translation feedback loops of several interacting genes better known as clock genes[26]. Clock genes impose a temporal order to the transcription of other genes necessary for metabolic functions in the cells. The SCN coordinates such rhythms by means of hormonal and autonomic mechanisms, allowing in this way the time signal to reach cells that are not directly exposed to light[27]. However, other internal stimuli that provide time information to the cells are elicited by feeding cycles that induce metabolic rhythms. Peripheral organs respond to the changing levels of glucose, insulin, temperature and corticosterone, in different ways, which depend on their function and involvement in metabolic balance[26]. Therefore, time of food intake has shown to be a powerful signal for the circadian system, driving brain and peripheral oscillators as well as behaviour[28]. When time of food does not coincide with the normal sleep–activity cycle, driven by the biological clock, food creates an internal conflict with temporal signals driven by the SCN for the regulation of metabolic efficiency favouring weight gain, obesity and metabolic syndrome[29–31].

Several studies demonstrate that shift-workers develop shifted food intake patterns, with increased consumption towards late at night[32,33], moreover during their shifts they show a preference for high-energetic and high-fat food[34,35] creating a shifted time pattern of energy signals to the cells, organs and components of the circadian system. Thus, it is possible that the shifted mealtime may trigger an internal conflict promoting internal desynchrony, leading to a deficient temporal response by organs involved in digestion and metabolic balance, and to a loss of homeostasis. Food intake currently of the normal activity phase may be the solution to counteract the adverse physiological consequences frequently observed in human shift-workers. Founded on this assumption, studies based on chrono-nutrition suggest implementing time-organised eating schedules for shift- and night workers in such a way that food does not represent a conflicting temporal signal with the normal light–dark cycle. More information is necessary in order to dissect the contribution of the time of eating for circadian disruption and to verify if restricted feeding schedules can be a possible intervention for individuals at risk of circadian disruption.

Experimental models in rodents have been used to better understand how shift-work impacts the circadian system, and to uncover factors associated with circadian disruption that exert adverse effects on behaviour and metabolic efficiency. Therefore, experimental protocols have implemented conditions of shifted timing of sleep, shifted timing of activity or shifted timing of food intake, and the exposure to light at night[36]. The advantage of experimental models is that variables can be better controlled and causal relations can be determined. A limitation is that mainly rats or mice, which are nocturnal animals, have been used for these models.

In this review, we have searched in the experimental protocols modelling shift-work whether the time of food intake could be the cause of circadian disruption and metabolic disease.

Data bases used for this review were Google scholar and PubMed, and keywords used for the bibliographic search were (shift work circadian disruption metabolism rat mice obesity) including 'sleep deprivation' and not review = 271 articles; (shift work circadian disruption metabolism rat mice obesity) including 'forced activity' and not review = 37 articles; (shift work circadian disruption metabolism rat mice obesity) including 'light at night' and not review = 131 articles; (shift work circadian disruption metabolism rat mice obesity) including 'restricted feeding' and not review = 262 articles.

Studies published in languages different from English were not considered. Reviews were discarded. Citations referring to human studies were discarded; only studies using repeated manipulations (usually more than 3 d) as a model of shift-work were included, leaving out numerous studies that explore acute effects (manipulations for a single occasion). For models of experimental shift-work, we identified the following protocols: shifted timing of sleep, shifted timing of activity or shifted timing of food intake, as well as the exposure to light at night. From these models, only studies describing circadian disruption and/or overweight and/or metabolic dysfunction were analysed, discarding studies that

explored other physiological systems or other mechanisms. Models using shifted light–dark cycles that resemble a jet-lag condition were not included as well as models using knock out or GM mice. For the condition of shifted food intake, a big spectrum of diets and timing schedules were observed ranging from 2 to 16 h food access[29], which led to diverse metabolic outcomes. In this analysis, we have included studies using 8–16 h food access with a regular chow or a high-fat, high energetic diet, because shorter access to food promotes a hypoenergetic condition leading animals to lose weight. Considering all criteria, a total of fifty-four studies were included in this review.

Experimental models of shift- and night work: what do they indicate?

Experimental models of chronic sleep disruption

The most well-known feature of shift-work in human subjects is the disruption of the normal sleep-activity patterns; therefore, animal models aimed at mimicking shift-work have used protocols in rodents to chronically reduce or shift the sleep timing. Table 1 summarises studies that explored the consequences of chronic sleep disruption, and the effects on the circadian system, on body weight and/or metabolic function. Studies causing chronic sleep disruption vary in their strategies and in the time employed to produce a chronic sleep deprivation, some (ten studies) reduce total sleep, others inhibit rapid eye movement sleep (seven studies) or induce sleep fragmentation (two studies). In general, all strategies led to a redistribution of sleep–wake phase[37–40], suggesting a circadian disturbance. However, the majority of such studies have not assessed circadian rhythms.

Due to sleep deprivation, food ingestion was decreased (seven out of fifteen studies), or not changed (four out of fifteen), while in some studies (four studies) this behaviour was not monitored (Table 1). In the majority of the studies, a decrease in body weight gain was observed, which is suggested to be the result of increased energy expenditure[40] due to the exhausting conditions and physiological stress imposed by the extended protocols (from 18 to 20 h) of sleep deprivation[40–47]. Studies observing a reduced body weight reported metabolic changes indicating a catabolic state or fasting like state, with reduced levels of glucose, low TAG, low cholesterol, low leptin levels, and in some studies accompanied by high levels of ghrelin and corticosterone[38,40–50]. Thus, an anabolic state was associated with sleep deprivation.

Importantly, studies that have implemented a milder strategy of sleep restriction by using randomised loud noise or reducing the period and hours of sleep restriction observed increased body weight using a regular diet[39,45]. Using gentle handling for 6 h at the start of the rest phase resulted in disrupted circadian rhythmicity of clock and metabolic genes in the liver[51], altered glucose and TAG blood levels, as well as modifications in adipocytes transcription profile[52]. While some studies did not find a significant effect on body weight, they observed glucose intolerance, insulin insensitivity[41,42,53,54] and increased circulating insulin[44]. An important feature of protocols using milder strategies for sleep deprivation is that animals were able to maintain a normal feeding rate. An example is the study by Caron and Stephenson[45] in which, by using milder sleep deprivation and giving rats the opportunity to have short recovery sleep bouts, this reduced the sleep debt and improved temperature regulation, food intake and metabolic efficiency.

The relevance of the time of food intake on body weight and the metabolic outcome was assessed in five studies[23,38,43,51,52] combining sleep deprivation with daytime feeding, where food consumption was shifted to the hours that animals were kept awake. The effects of shifted food intake are not consistent; it did not increase body weight using a regular diet[38,51]; in two studies it resulted in decreased bodyweight[43,52], and in one study it promoted overweight when combined with a highly palatable energy-dense diet, better known as cafeteria diet[23]. Overweight remained during the recovery period after chronic rapid eye movement sleep deprivation combined with high-fat diet[44]. Metabolic effects were also worsened when combining cafeteria diet with sleep deprivation leading to a metabolic syndrome[23,53], and in aged mice, the combination of a high-fat diet with sleep deprivation led to damage to the pancreas[55].

All together, chronic disrupted sleep causes metabolic alterations favouring in some cases a metabolic syndrome and in others reflecting a fasted state, even when body weight is reduced or not affected. Only a few studies using milder strategies for sleep disruption report increased and shifted food intake towards the forced hours of wake time and this was associated with indicators of metabolic syndrome.

Experimental models of forced activity

Animal models that shift activity to the resting phase are scarce (Table 2). A main difficulty in defining such models is that some protocols that shift activity coincide with manipulations used for sleep deprivation.

The strategies used to keep animals awake and active mainly consist of motorised wheels that vary in their construction. Similar to the studies for sleep deprivation, we found a variety of studies requiring from the animals different intensities of activity and effort (Table 2), which is reflected by the number of revolutions per minute, by the frequency of locomotor adjustments or the number of responses of the animal. The majority of studies required from the animals a strong effort during their forced activity schedules, keeping continuous alertness and emitting effortful movements that mimic more of an exercise routine[56]. Some schedules represented a stressful condition[57] or even drove animals to exhaustion and to a torpor state due to the negative metabolic state driven by the exhausting protocol[58]. Models of forced activity induced disrupted circadian rhythms mainly by shifting activity towards the rest phase.

Body weight was decreased (five out of eight studies)[56–60], in two studies body weight was mildly (7 %) increased[61,62] and one study did not assess it[63].

Table 1. Experimental models of chronic sleep disruption

Protocol				Findings				
Photoperiod	Feeding schedules	Diet	Duration (d)	Circadian disruption	Body weight	Metabolic findings	Effects on food intake	Reference
12/12 LD	*Ad libitum*	Standard chow	21	NE	↓	Reduced leptin and leptin receptor, deficient glucose clearance, increased TNF-α and IL-6, macrophage infiltration in white adipose tissue, reduced retroperitoneal fat	↓	Venancio & Suchecki[41]
12/12 LD	*Ad libitum*	Standard chow	4	NE	↓	Decreased leptin and insulin levels, no change in glucose, increased leptin receptors in the hypothalamus	↑ Food intake in the light phase	Moraes *et al.*[42]
12/12 LD	*Ad libitum*	Standard chow	4	NE	↓ Adipose tissue	Decreased glucose, TAG, leptin and VLDL	NE	Rosa Neto *et al.*[47]
12/12 LD	*Ad libitum*	Standard chow, HFD or liquid diet	4	NE	↓	Fasting like metabolic profile: increased ketone bodies, reduced liver glycogen and TAG	↓	Martins *et al.*[48]
12/12 LD	*Ad libitum*	Standard chow	90	NE	↓	Glucose intolerance with a GTT and insulin insensitivity	NE	Xu *et al.*[47]
12/12 LD	*Ad libitum*	Standard chow	7	NE	↓	Low glucose levels and TAG levels, low insulin and leptin, increased ghrelin and corticosterone. Effects proportional to the duration of sleep deprivation	↓	Brianza-Padilla *et al.*[51]
12/12 LD	*Ad libitum*	Standard chow followed by HFD during sleep recovery	21	NE	↓	Increased insulin, resistin and inflammation indicators	=	de Oliveira *et al.*[44]
12/12 LD	*Ad libitum*	Standard chow and HFD	9	NE	↓ With Chow diet and body weight loss was ameliorated with HFD	High-fat feeding in combination with sleep disruption impairs glucose tolerance	↓ With regular chow ↑ When exposed to HFD	Ho *et al.*[53]
12/12 LD	*Ad libitum*	Standard chow	14	NO The daily peak of corticosterone remained in the same phase	=	Glucose intolerance with a GTT	↑	Baud *et al.*[54]
12/12 LD	*Ad libitum*	Standard chow	7	NE	NE	High glucose levels and low insulin with low insulin sensitivity, UPR protein in endocrine pancreatic cells	NE	Naidoo *et al.*[55]
12/12 LD	*Ad libitum*	45 % Fat diet	8	NE Microsleeps during sleep deprivation	↓	Decreased glucose, insulin and leptin levels, increased energy expenditure	=	Barf *et al.*[43]

Proceedings of the Nutrition Society

12/12 LD	*Ad libitum*	Standard chow	60	NO Overall diurnal pattern of sleep was not affected. Total wake time correlated with total cell loss in the VLPO	↓	40 % sleep reduction reduced glucose TAG, cholesterol, C-reactive protein, increased ghrelin and lower leptin	=	Vetrivelan *et al.*[48]
12/12 LD	*Ad libitum*	Standard chow	9	YES Increased wake time in the light phase and more sleep in the night, decreased SWS and REM time, more phase transitions	↑	NE	↑	Mavanji *et al.*[39]
12/12 LD	*Ad libitum*	Standard chow	4	YES Pronounced redistribution of sleep/wake cycle, with sporadic microsleep episodes during the activity phase	NE	NE	NE	Gronli *et al.*[37]
12/12 LD	*Ad libitum*	Standard chow	5	YES Compensatory redistribution of sleep stages, activity increased 11 % during hours of sleep restriction	NE	Ghrelin and corticosterone increased during sleep deprivation, leptin levels were not changed	↓	Bodosi *et al.*[38]
12/12 LD	*Ad libitum*	Standard chow	10	NE	↓	Metabolic rate and body temperature related to the rate of accumulation of sleep deprivation	=	Caron and Stephenson[45]
12/12 LD	*Ad libitum*	Standard chow	5	YES Redistribution of activity levels in the 24 h cycle, low activity in the last half of the night	↓	Leptin 3-fold higher, lipid and carbohydrate metabolism transcripts overexpressed in white adipose tissue, indicating increased lipogenesis	↓ During the hours of sleep restriction. Total daily food =	Husse *et al.*[50]
12/12 LD with high light intensity 500 lux during the day	*Ad libitum*	Standard chow	10	YES Shifted corticosterone peak, gradual decline of nocturnal activity. In DD no change in daily activity patterns or in SCN clock genes	=	Loss of rhythmicity in genes involved in carbohydrate metabolism in the liver	↑ Food intake during the light phase	Barclay *et al.*[51]
12/12 LD	*Ad libitum*	Standard chow and cafeteria diet	70	YES Shifted acrophases and low amplitude of general activity	↓	Sleep restriction combined with cafeteria diet promoted criteria for metabolic syndrome	↓With regular chow ↑With cafeteria diet	Espitia-Bautista *et al.*[23]

DD, constant dark; GTT, glucose tolerance test; HFD, high-fat diet; LD, light–dark; NE, not explored; REM, rapid eye movement; SCN, suprachiasmatic nucleus; UPR, unfolded protein response; VLPO, ventrolateral preoptic nucleus; =, similar to controls; ↑ increased; ↓decreased.

Table 2. Experimental models of forced activity

Protocol							Findings			
Forced activity model	Animal model	Photoperiod	Feeding schedules	Diet	Duration (d)	Circadian disruption	Body weight	Metabolic findings	Effects on food intake	Reference
Timed forced activity with automatic slow rotating (0.3 rpm) wheels 8 h/5 d/ week	Male Wistar rats	12/12 LD	*Ad libitum*	Standard chow	35	YES Nocturnal activity decreased, temperature and metabolic rhythms shifted to the workhours	↑ 7 %	Dyslipidaemia, steatosis, glucose intolerance	Shifted feeding patterns to the light phase	Salgado-Delgado *et al.*[60,61]
Timed forced activity with automatic slow rotating (3 rpm) wheels 8 h	Male Wistar rats	12/12 LD	*Ad libitum*	Standard chow	4	NO Core body patterns not changed, feeding patterns shifted	↓	In the liver disturbed genes encoding for insulin sensitivity and lipid metabolism	Shifted feeding patterns to the light phase	Marti *et al.*[59]
Floor rotates bidirectionally at varying speed. 8 h/5 d/week	Male Wistar rats	12/12 LD	*Ad libitum*	Standard chow	35	NO Core body patterns not changed, daily activity patterns not changed, nocturnal activity decreased	↓	NE	NE	Leenaars *et al.*[57]
Access to activity wheel + food only in the day or only in the night	Male Long Evans rats	12/12 LD	12 h restricted food access	Standard chow	22	YES Nocturnal activity decreased, altered temperature rhythms shifted to the work hours	↓	NE	=	Murphy *et al.*[60]
Shifted LD cycles combined with 12 h exercise in a running wheel	Male F344 rats	12/12 LD and shifted	*Ad libitum*	Standard chow	91	YES The combination of LD shifts + wheel running altered temperature rhythms	↓	NE	Exercised rats ate more	Tsai & Tsai[56]
Motorised wheels 10 h in the light phase	Male Syrian hamsters	14/10 LD	*Ad libitum*	Standard chow	21	YES Reduced daily running with 6 lux light, shifted activity to the light phase	NE	NE	NE	Dallman & Mrosovsky[63]
'Work for food' protocol increasing the number of revolution for a reward pellet	Male CBA mice	12/12 LD	*Ad libitum*	Standard chow	40 (approximately)	YES Shifted activity towards the day	↓	Decreased temperature, torpor	↓ Food intake	Hut *et al.*[58]

LD, light–dark; NE, not explored; rpm, revolutions per minute; =, similar to controls; ↑ increased; ↓decreased.

Table 3. Experimental models for light at night

Protocol						Findings				
Shift-work Model	Animal model	Photoperiod	Feeding schedules	Diet	Duration (d)	Circadian disruption	Body weight	Metabolic findings	Effects on food intake	Reference
Constant light	Male Swiss-Webster mice	L/L constant light (150 lx)	*Ad libitum*	Standard chow	56	YES Locomotor activity arrhythmicity, blunted corticosterone levels	↑	Weight gain, increased epididymal fat deposition, glucose intolerance	↑ During the subjective day	Fonken *et al.*[69]
Constant light	Male C57Bl/6 J mice	L/L constant light (180 lx)	*Ad libitum*	Standard chow	30	YES Locomotor activity arrhythmicity. Decreased SCN neuronal activity (electrophysiological recordings). Blunted corticosterone levels	↑	Weight gain, glucose intolerance. More energy intake during the subjective day period, RER during the subjective day period. Decreased total energy expenditure levels. Abolishment of circadian variation in insulin sensitivity	NE	Coomans *et al.*[68]
Constant light	Male C57BL/6 J mice	L/L constant light (about 85 μW/cm^2)	*Ad libitum*	Standard chow	35	YES Loss of circadian rhythm in behavioural activity	=	Increased body fat mass, reduced uptake of TAG-derived fatty acids and glucose by BAT, decreased activity of brown adipocytes	NE	Kooijman *et al.*[67]
Constant light	Male Sprague-Dawley rats	L/L constant light (>100 lx)	*Ad libitum*	Standard chow	70	YES Locomotor activity arrhythmicity	=	Loss of diurnal variations in insulin plasma concentrations, damped Per-1 amplitude in isolated pancreatic islets from LL rats, disrupted islet architecture, increased B-cell apoptosis	NE	Qian *et al.*[64]
Constant light	Male Sprague-Dawley rats	L/L constant light (300 lx)	*Ad libitum*	Standard chow	42	YES Low melatonin concentrations and loss of metabolic rhythms	=	Constantly elevated plasma fatty acid levels	=	Dauchy *et al.*[71]
Constant light	Diabetic prone HIP rats	L/L constant light (>100 lx)	*Ad libitum*	Standard chow	70	YES Locomotor activity arrhythmicity. Disrupted melatonin rhythms	=	Increased glucose levels, decreased glucose and arginine stimulated insulin secretion.	NE	Gale *et al.*[70]
Constant light	Male Long Evans rats	L/L constant light (450 lx)	*Ad libitum*	Standard chow	17	AMBIGOUS Free-running locomotor activity but low melatonin concentrations during the night	NE	More positive fed efficiency value and increased visceral adiposity	↓	Wideman & Murphy[72]

Interestingly, only two groups have explored the metabolic outcome of shifted forced activity using a mild strategy to enforce activity. Both groups report shifted feeding patterns towards the rest phase and a resulting disrupted metabolism[59,61,62]. The study by Marti *et al.*[59] imposed this protocol for only 4 d and could already observe in the liver a disturbed pattern of genes encoding insulin sensitivity and lipid metabolism. A series of studies by our group, using slow rotating wheels, reported that activity during the resting phase for 4 weeks induced rats to gain more bodyweight and to develop abdominal obesity accompanied by liver steatosis and glucose intolerance[61,62].

With this protocol, rats developed disrupted circadian rhythms characterised by a shift in core body temperature, in general activity and serum TAG, a loss of the rhythm in glucose, in the rhythm in clock genes in the liver and no change in corticosterone rhythm.

Importantly, forced activity in the slow rotating wheel induced a shift in the timing of food consumption to the light phase, suggesting this as a possible factor inducing circadian disruption and loss of metabolic balance[64]. To confirm this association, rats were prevented from ingesting food during the forced activity hours and only had access to food during the night (which is the normal activity phase for rats). This procedure prevented circadian disruption and the adverse metabolic effects observed in rats exposed to the working schedule. Moreover, daytime food access alone recapitulated the effects of this working schedule on metabolism[61,64]. A possible effect of food intake in other protocols of forced activity in the rest phase was not assessed.

All together, most of the experimental models using forced activity in the rest phase have not provided evidence that this factor may cause circadian disruption and metabolic dysfunction. This is probably due to the exhausting protocols used to induce activity. Similar as observed with the sleep deprivation studies, milder protocols favouring rats to eat during the rest phase induced increased body weight and adverse metabolic changes in the direction of metabolic syndrome, pointing out shifted food intake as an important risk factor for circadian and metabolic disruption.

Experimental models for light at night

In rodents, light exposure at night has been used as a strategy to mimic one of the most disrupting and common conditions experienced by human shift-workers. Light is a signal that immediately activates the SCN; however, for nocturnal rodents, light is a rest signal, and for human subjects, light is associated with activity. Table 3 summarises studies that explored the metabolic consequences of continuous light exposure in mice and rats either implementing constant light intensity throughout 24 h (LL) or alternating bright light during the day with dim light exposure at night (L/DL).

From studies involving LL, seven out of eight reported clear circadian disruption based on arrhythmic locomotor activity patterns, on low SCN neuronal activation or disturbed corticosterone and melatonin rhythms[65–71]. From the other two studies, we may assume that the cir cadian system was also affected because they report low melatonin levels, which is also an indicator of disruptio at the level of the biological clock[71,72]. To note is tha two studies using alternating bright light during the da with dim light at night did not induce circadian arrythmi city at least in general activity and corticosteron levels[65,73], and a third study did not explore it[74].

The effects of LL on body weight are inconsistent: i two studies body weight was increased[65,66], in four stud ies animals remained similar to controls[67,68,70,71] and i two studies body weight gain is not reported[69,72] Interestingly, metabolic dysfunction was consistentl reported in all studies independently of the body weigh outcome[65–72]. Rodents in LL developed increased glu cose levels, glucose intolerance, decreased insulin sensi tivity, increased fat mass deposition, elevated plasm fatty acids, decreased activity of brown adipocytes higher RER during the subjective day and decrease energy expenditure[65–71]. Adverse effects of LL are als described in organs involved in energy balance. Qia *et al.*[68] reported disrupted pancreatic islet architectur as well as increased apoptosis due to LL. Loss of circa dian rhythmicity in clock genes in the liver and th colon were also reported while rhythmicity in the duode num was preserved[69,72].

In the three studies alternating bright light with din light at night, consistent increased body weight gai was observed, together with higher RER, glucose intoler ance, increased insulin levels during the light phase an decreased energy expenditure[65,74,75], similar as observe in LL.

Interestingly only two of the cited studies using LL (Table 3) assessed the 24 h pattern of food consumption. Polidarova *et al.*[69] reported loss of circadian rhythms in feeding behaviour, Wideman and Murphy[72] indicated that rats in LL consumed less food throughout the 24 h cycle, however attained a positive feed efficiency value (g body weight change/g food intake), which suggests that this condition may favour body weight gain. Contrasting, only the study by Fonken *et al.*[65] reported that mice exposed to alternating bright light during the day with dim light at night shifted their feeding patterns and consumed a higher amount of food in the day.

Altogether, studies using constant light report a consistent disruptive effect of light on metabolism leading to increased adiposity and disruptive glucose balance. Because few studies assessed patterns of food ingestion, the contribution of food timing to metabolic dysfunction is not clear. Nevertheless, eating while the nocturnal animal is exposed to light suggests a circadian conflict, which requires further studies.

Experimental models of shifted food timing

Restricting food access to the rest phase has been used in rodents as a strategy to reproduce the shifted feeding schedule of human shift-workers. Studies that explored the metabolic consequences of shifted feeding schedules are summarised in Table 4. The majority of the studies used a protocol of restricting food access to 12 h during

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Constant light	Male Wistar rats	L/L constant light (50-300 lx)	*Ad libitum*	Not mention	30	YES Locomotor activity arrhythmicity.	NE	NE		Loss of circadian rhythm in feeding behaviour	Polidarova *et al.*[69]
Dim light at night	Female Swiss Webster rats	L/DL 16-8 (150 lx/14 h-5 lx 8 h)	Ad libitum	Standard chow	56	NE	↑	Weight gain		↓	Aubrecht *et al.*[74]
Dim light at night	Male Swiss-Webster mice	L/DL 14-10 (150 lx/14 h-5 lx 10 h)	*Ad libitum*	Standard chow	14	NO Dim light did not cause arrhythmic activity	↑	Less O_2 consumption, higher RER values, decreased energy expenditure		NE	Bomiger *et al.*[75]
Dim light at night	Male Swiss-Webster mice	L/DL 16-8 (150 lx/16 h-5 lx 8 h)	*Ad libitum*	Standard chow	56	NO Typical circadian rhythm in locomotor activity and corticosterone levels	↑	Glucose intolerance, increased insulin levels during the light phase		↑During the day (55-5 %)	Fonken *et al.*[85]

BAT; brown adipose tissue; HIP, human islet amyloid polypeptide; L/DL, light/dim light; L/L, light/light; lx, lux; NE, not explored; SCN, suprachiasmatic nucleus; =, similar to controls; ↑ increased; ↓ decreased.

the day, which is the rest phase, and compared this with food access for 12 h during the night. Diets mainly consist of standard chow; however, some studies also have used high-fat diet or high-fat and high-sucrose diets. There are also some studies using shortened food access for 4 or 5 h during the day; however, we have not included them in this review because this daily brief access to food can lead to food entrainment, resets circadian metabolism and induce energy restriction resembling a fasted day[29].

From the studies examined here, twelve out of fourteen reported circadian disturbances, while the other two studies did not explore this feature[76, 77]. Disrupted circadian rhythms were observed in the fluctuations of clock genes in the liver, muscle and heart[61,78–83]. Studies also report loss of temperature rhythms, shifted locomotor activity towards the rest phase, shifted glucose, TAG, leptin and ghrelin rhythms and decreased leptin immunoreactivity rhythms in the organum vasculosum of the lamina terminals[61,64,78–86]. In the study of Ramirez-Plascencia *et al.*[87], authors described shifted or blunted activity rhythms of orexin, melanin-concentrating hormone and α-melanocortin-stimulating hormone neurons in the hypothalamus. Therefore, it is a consistent finding that shifted food access to the rest phase affects brain and peripheral clocks involved in metabolic regulation. Such findings emphasise the importance of food as a powerful circadian synchroniser.

The effect of shifting food to the light period on body weight gain is quite clear. In ten studies using 12 h day feeding, animals gained significantly more body weight[61,64,76–78,81–84,87]; in four studies, animals remained similar to controls[79,80,85,86]. In two studies, where authors did not find differences in body weight gain[79,80], this was associated with decreased food consumption. In the case of the studies by Shamsi *et al.*[85] (8 or 16 h day feeding) and Rocha *et al.*[86], animals' body weight remained similar to controls; however, animals developed metabolic alterations (Table 4).

Among rodents that gained weight, metabolic dysfunction was reported, mainly fat mass accumulation, dyslipidaemia, high glucose levels and glucose intolerance, decreased insulin sensitivity, lower RER, decreased energy expenditure and disrupted circadian rhythms of metabolic genes in the liver and muscle[61,76–78,82–84,87].

All together, studies using restricted food access to the rest phase reported circadian disruption in organs related with metabolic function. This loss of temporal order among organs regulating metabolism may be the cause of a disturbed metabolism that can have potential health consequences, such as metabolic syndrome, obesity and diabetes[6,88].

What do experimental models indicate about the association between circadian disruption, overweight and metabolic disturbance?

All models described here induce circadian disruption and affect the metabolic state in one or another direction (Table 5), indicating a clear association between

Table 4. Experimental models of shifted food timing

PROTOCOL						FINDINGS				
Shift-work model	Animal model	Photoperiod	Feeding schedules	Diet	Duration (d)	Circadian disruption	Body weight	Metabolic findings	Effects on food intake	Reference
Shifted feeding	Male C57BL/6 J mice	12/12 LD	Day (12 h) or Night (12 h)	HFD	42	NE	↑	Slightly increased in fat mass accumulation	NE	Arble *et al.*[77]
Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or night (12 h)	Standard chow	28	YES Loss of temperature rhythms, disrupted rhythms of clock genes in the liver	↑	Dyslipidaemia. Increased fat mass accumulation. High glucose levels and glucose intolerance	=	Salgado-Delgado *et al.*[81,84]
Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or *ad libitum*	Standard chow	21	YES Shifted activity towards the light phase. Decreased nocturnal activity	↑ Only in 24 months old rats	NE	↓ In young rats ↑ In old rats	Reddy & Jagota[56]
Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or night (12 h)	Standard chow	21	YES Shifted activity of ORX neurons in the LH-PeF. Blunted daily rhythm of MCH neuron activation. Loss of circadian activity of α-MSH neurons in the arcuate nucleus	↑	NE	↓	Ramirez-Plascencia *et al.*[85]
Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or night (12 h)	Standard chow, fat/sugar diet	31	NE	↑ Only with high-sugar diet in the day	Hyperphagia, lower RER in the light period. Decreased food efficiency, increased fat pads	↑ Consumption of energy during the light period	Oosterman *et al.*[73]
Shifted feeding	Male wild-type mice (on FVB/N background)	12/12 LD	Day (12 h) or night (12 h)	Standard chow	9	YES Disrupted rhythms in clock and metabolic genes expression in the liver, epididymal fat, muscle and heart	↑	Food consumption during the day induced: lower energy expenditure, higher RER	↑ Food intake at the beginning of the day	Bray *et al.*[76]
Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or night (12 h)	Standard chow	56	YES Disrupted circadian rhythms in leptin levels. Altered rhythms of clock genes in the liver	=	Increased perirenal fat mass deposition. Increased feed efficiency. Increased RER	↓	Opperhuizen *et al.*[78]

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Shifted feeding	Male Wistar rats	12/12 LD	Day (12 h) or *ad libitum*	Standard chow or HFD	21	YES Disrupted rhythms in insulin, leptin and NEFA levels. Disrupted circadian rhythms of clock genes in the liver and muscle. Decreased nocturnal activity and oxygen consumption	=	Increased day corticosterone. Shifted RER profile to the day. Increased glycogen in the muscle	↓ In food intake (3–5 d)	Reznick *et al.*[83]
Shifted feeding	Male C57BL/6 J mice	12/12 LD	Day (12 h) or night (12 h)	Standard chow	15, 30 and 90	YES Disrupted clock genes rhythms in the liver and pancreas	↑	Increased glucose, free fatty acids levels and corticosterone in the circulation. Increased peritoneal fat mass deposition. Glucose intolerance. Decreased insulin levels. Increased triacylglycerols and cholesterol in the liver	NE	Mukherji *et al.*[81,82]
Shifted feeding	Male C57BL/6 mice	8/16 LD 16/8 LD	Day (16 h) or day (8 h)	Standard chow	56	YES Altered rhythms of clock and metabolic genes in the liver	=	Increased insulin levels. Decreased TAG. Increased glucose tolerance. Decreased liver weight	NE	Shamsi *et al.*[80]
Shifted feeding	Male C57BL/6 J mice	12/12 LD	Day (8 h) night (8 h)	High-fat and high-sucrose diet	7	YES Disrupted circadian rhythms of metabolic genes in the liver. Shifted rhythms of ghrelin, insulin and leptin and reduced nocturnal activity	↑	Hyperphagia. Physical inactivity. Increased adiposity, insulin, leptin, total cholesterol and ghrelin levels. Increased hepatic lipids	↑	Yasumoto *et al.*[83]
Shifted feeding	Male Wistar rats	12/12 LD	Day 80 % of their total food intake Night 20 %	Standard chow	60	YES Shifted daily feeding and glucose patterns	=	Glucose intolerance, fat mass accumulation, accumulation of fat in the liver	↑ hyperprotein and hyperglucidic diet = total 24 h intake	Rodha *et al.*[86]

HFD, high-fat diet; LH-PeF, lateral hypothalamus-perifornical area; LD, light–dark; MCH, melanin-concentrating hormone; α-MSH, α-melanocyte-stimulating hormone; NE, not explored; ORX, orexin; OVLT, organum vasculosum lamina terminalis; ↑ increased; ↓ decreased.

conditions that affect daily cycles and the development of an adverse metabolic condition. In some studies due to the lack of circadian assessment or metabolic follow-up, this association is not always evident. However, protocols using light exposure at night or shifted food to the rest phase highlight the adverse effects of food intake at the wrong time or light at night on metabolic health. Both factors are present in the modern life style and affect the shift-worker.

Metabolic effects largely depended on the specific manipulations by each model, i.e. duration, intensity of activity, feeding schedule and type of diet. Regarding sleep deprivation and forced activity models, the majority of the studies report decreased body weight gain associated with a catabolic metabolism which, as previously discussed, may be related to the physical requirements and stressful conditions of the protocols. Importantly, studies using milder protocols for sleep disruption or for enforced activity are scarce, however report metabolic changes that suggest the development of metabolic syndrome[51,52,61,64]. Contrasting, light exposure at night has proved to be an effective and consistent model to mimic the metabolic alterations observed in human shift-workers. Most of the studies using light at night reported increased body weight gain and all of them described alterations reflecting a dyslipidaemia and criteria for a metabolic syndrome. Likewise models using shifted food to the rest phase found consistent metabolic dysfunction and showed to be effective to produce body weight gain, in spite of a few reports that observed no difference from the control.

The relevance of shifted food intake as a cause of circadian and metabolic disorder in shift-work model

When looking at all models of shift-work, it is clear that many studies did not explore the possible role of the time of food intake as a risk factor for circadian disruption and metabolic alterations (Table 5). Evidently, the models implementing shifted food access to the rest phase have paid attention to this factor and reported consistently changes in the metabolic state towards dyslipidaemia and glucose intolerance. Other models that observed shifted food consumption to the normal rest hours have provided strong evidence about this association. In the studies by Salgado-Delgado *et al.*[61,64], animals shifted their food intake to the rest phase and this had an adverse metabolic outcome. When animals were not allowed to eat at the wrong phase, the adverse metabolic effects were prevented, pointing out the relevance of food timing as a main risk factor for metabolic problems as observed in human shift-workers. One of the studies using dim light at night also observed a shift in meal patterns, and this shift was associated with overweight and metabolic changes[65].

Food has proved to be a powerful entraining signal for the circadian system. Metabolic signals elicited by food intake impact organs at the cellular level and provide timing to cellular processes and genes involved in glucose and lipid metabolism[28]. Under shift-work conditions several external and internal timing signals are shifted due to abnormal exposure to activity, to light and to food, resulting in an internal conflict with time signals from the SCN transmitted to organs and cells via the autonomic nervous system. This affects differentially organs and regulatory genes depending on their dependence on metabolism, endocrine or autonomic signals[28]. The loss of an internal temporal order among different regulatory systems leads to incorrect or deficient adaptive responses to external demands, which can be the cause of a loss of homeostasis and a higher propensity to disease[25].

Main internal signals that influence metabolic function

Food intake as a time signal for the circadian system

Studies implementing shifted feeding schedules towards the rest phase described shifted rhythms of clock and metabolic genes in organs involved in metabolic balance, especially in the liver[61,79,82,83,85], in the muscle and adipose tissue[78,80]. As we have mentioned earlier, clock genes impose a temporal order to the transcription of other genes necessary for metabolic functions in the cells.

In the study by Salgado-Delgado *et al.*[64], where forced activity in slow rotating wheels induced a shifted food intake towards the day, also shifted and blunted clock genes in the liver were reported, suggesting a circadian disruption at the cellular level. Such studies indicate the relevant effect of the time of food intake as a potent disrupting factor, when time of food does not coincide with the light–dark cycle. The disruption of clock gene expression in the liver is associated with disturbed liver metabolism and development of liver steatosis (see for review[89]).

The circadian conflict at the cellular level is suggested to occur between the shifted food-related signals (glucose, insulin) and the biological clock transmitting light–dark information to peripheral organs and cells[27]. Melatonin and corticosterone are the main hormonal pathways used by the biological clock to transmit time information of the light–dark cycle to peripheral organs[90,91] and may be the source of conflict with the food-entrained rhythms.

Corticosterone

Corticosterone is proposed as an internal timing signal for a variety of organs[92] and reaches peak levels at the beginning of the active phase, which in rodents corresponds to the beginning of the night. In the liver[93,94], corticosterone influences gluconeogenesis as demonstrated *in vivo* while *in vitro* exerts synchronizing effects on fibroblasts[95] and adipose tissue[96].

In rats, sleep deprivation induced increased levels of corticosterone during the protocol[38], mild forced activity, as well as exposure to food restriction to the resting phase, induced a peak of corticosterone at the beginning of the schedule in addition to the normal peak at the beginning of the night[38,64,80]. Similarly, animals exposed to LL exhibit increased levels of corticosterone

Table 5. Proportion of studies providing evidence for the relationship between circadian disruptions and adverse metabolic function

Shift-work model	Animal model	Circadian disruption	Body weight	Metabolic findings	Effects on food intake
Sleep deprivation (19 studies)	63·2 % Male rats 31·5 % Male mice 5·2 % Female rats	YES 31·6 % NO 10·5 % NE 57·9 %	= 10·5 % ↓ 68·4 % NE 15·8 %	31 % Metabolic syndrome 37 % Catabolic state 16 % No effects 16 % NE	↑ 26·3 % = 21·1 % ↓ 31·5 % NE 21·2 %
Forced activity (8 studies)	75 % Male rats 12·5 % Male mice 12·5 % Male hamsters	YES 75 % NO 25 %	↑ 25 % NE 14·3 % ↓ 62·7 %	37·5 % Metabolic syndrome 12·5 % Catabolic state 50 % NE	↑ 12·5 % = 12·5 % ↓ 12·5 % NE 25 % Shifted to the light phase 37·5 %
Light exposure at night (11 studies)	45·4 % Male rats 45·4 % Male mice 9·1 % Female rats	YES 63·6 % NO 18·1 % NE 9·1 % AMBIGUOUS 9·1 %	↑ 45·4 % = 36·4 % NE 18·2 %	100 % Metabolic syndrome	↑ 18·2 % = 9·1 % ↓ 18·2 % NE 45·4 % 9·1 % No loss of rhythm in feeding behaviour
Shifted feeding (14 studies)	42·8 % Male mice 57·2 % Male rats	YES 85·7 % NE 14·3 %	↑ 71·6 % = 21·4 %	85·7 % Metabolic syndrome 14·3 % NE	↑ 35·7 % = 14·3 % ↓ 21·4 % NE 28·5 %

Metabolic syndrome = overweight or obesity, increased adipose tissue, glucose intolerance, increased TAG, increased cholesterol; NE, not explored; =, similar to controls; ↑ increased; ↓ decreased.

along the 24 h period with a loss of the circadian rhythmicity[65,66]. Such high levels of glucocorticoids affect glucose homeostasis and promote gluconeogenesis in the liver[97]. It is also known that high doses of glucocorticoids result in increased weight gain, glucose intolerance and high insulin and TAG levels[98]. Elevated corticosteroid levels and dampened or disrupted glucocorticoid rhythmicity have been reported in obese adults and in genetically obese Zucker rats and db/db mice[92]. Moreover, the shifted timing of corticosterone release may function as an altered time signal and exert a disruptive effect on the circadian system.

Melatonin

It is suggested that the SCN uses the nocturnal melatonin secretion to distribute circadian signals within the brain or the periphery, to organs and cells possessing melatonin receptors[91]. In rodents, daily administration of melatonin entrains activity rhythms in free-running rats[99–101], and some studies suggest that melatonin can entrain adipocytes[102] and protein synthesis in hepatocytes[103]. In three of the experimental models of light at night, altered timing and/or levels of melatonin were observed[70–72].

It is well described that low light intensities at night are sufficient to inhibit melatonin[71]; however, this hormone under the dim light at night schedule has not been evaluated. Likewise in animal protocols that restrict food access to the day, melatonin has not been evaluated. Importantly, the three studies involving LL that reported low melatonin levels associated the hormone levels with metabolic alterations[70–72].

Melatonin in addition to being an important regulator of circadian rhythms is also involved in the regulation of glucose metabolism[104]. The relevance of melatonin for metabolic balance is further demonstrated under conditions of low hormone concentrations such as ageing, in which, melatonin supplementation effectively decreased fat mass accumulation, body weight gain and restored insulin and leptin levels[105,106]. In diabetic rats and mice, melatonin treatment also improved glucose and TAG levels, diminished body weight and insulin levels[107,108]. Based on this evidence, we suggest that low melatonin levels resulting from circadian disruption may promote the metabolic dysfunction; however, more data from the experimental models will be necessary to support this association.

Main contributions of experimental models, limitations and perspectives

The analysis of the four experimental models for shift-work indicates that the strategies and variables assessed by different groups are diverse with respect to intensity, time of exposure and variables used to assess circadian and/or metabolic state, leading in some studies to contrasting or inconclusive findings. It is important to indicate that studies here reported have included a significant number of subjects in their experimental designs, which permits to draw conclusions; however, the variability in the use of factors has led to different outcomes. Moreover, not all studies performed a circadian screening, not all determined the temporal order of food intake and only a few studies have explored the association

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between the time of food intake as the cause of circadian and metabolic disruption. Importantly, studies that explored this relationship report that the time of food intake is indeed essential for the development of metabolic disturbances.

A growing body of epidemiological evidence in human populations indicates that short sleep is a risk factor for the development of obesity and metabolic disturbance. Animal studies exploring the effects of restricted sleep indeed observed a reduction in insulin sensitivity and changed levels of hormones involved in appetite and neuroendocrine regulation, such as ghrelin, leptin and insulin[109,110]. However, experimental models using sleep deprivation have not provided conclusive effects of the contribution of shifted feeding schedules because very few have explored the possibility of a shifted food intake. Moreover, models for sleep deprivation or forced activity require more uniformity, using mild protocols in order to provide conclusive results about metabolic mechanisms.

As a model of shift-work, light at night exposure has proved to be an effective and a consistent model to mimic the metabolic alterations observed in human shift-workers, highlighting the importance of being exposed to dark nights in order to promote metabolic health. The fact that light at night will activate neurons in the SCN that are normally inactive and inhibit melatonin secretion when melatonin is normally high indicates that light at night is a strong circadian disruptive signal. In such conditions, scheduled food has shown to ameliorate metabolic conditions and to exert strong entraining signal for metabolic genes in the liver[111]. More evidence is needed to determine the role of food timing under constant light. The majority of studies exploring experimental shift-work have been performed in nocturnal animals, which is a limitation when translating their findings to the diurnal human species. As indicated earlier, melatonin is involved in the regulation of glucose metabolism, and in nocturnal rodents, melatonin treatment improved the metabolic state in obese mice[108] suggesting that low melatonin levels resulting from circadian disruption may be partly the cause of metabolic dysfunction. In nocturnal rodents, melatonin release coincides with their active phase and with the time of food intake, while in human subjects, melatonin release coincides with the rest and sleep phase and not with the moments of maximal digestion and food absorption. This important difference requires a better understanding for the role that melatonin could play linking circadian disruption and metabolism. In a similar way, light at night has shown to be a disruptive signal for the circadian system, and while nocturnal rodents are normally active at night, human subjects sleep, thus the disrupting effect on sleep-activity patterns is inverted. Therefore, a next necessary step for the experimental models of shift-work is to use diurnal species in order to confirm such adverse effects and to better translate experimental results to the problem of the human shift-worker.

Interestingly, the majority of studies here reported have used male animals and only two studies were found that explored the response in females[42,74]. In the study by Xu *et al.*[42], authors do not discuss a possible difference in their outcomes associated with the sex of the animals. In this regard, Aubrecht *et al.*[74] discuss their previous work using male animals and stated that dim light affects in a similar way body mass and likely metabolic function in both males and females. Other studies testing a high-fat diet in male and female rodents indicate a differential influence associated with sex in rats[112,113], mice and hamsters[114,115]. This is partly explained by a differential response to metabolic signals in brain areas involved with metabolic regulation[116]. Since women are also exposed to shift-work, further studies exploring the effects of circadian disruption and shifted food timing in female rodents are necessary.

We conclude that so far experimental evidence confirms the association of circadian disruption with metabolic alterations, hereby some models indicate that the shifted time of food intake may be a determining factor for the loss of internal synchrony because of the differential response of individual organs to internal entraining signals. Thus, the adverse consequences of shift-work on metabolism may be explained by a loss of coordinated rhythmicity among different organs due to shifted food elicited signals, low melatonin and shifted or increased corticosterone levels[117].

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Conflicts of Interest

None.

Authorship

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