UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

"Neural mechanisms mediating locomotor performance during forced wheel running in adolescent rats. Stress responses and role of the dopaminergic system"

Mecanismos neurales que median el rendimiento motor en rueda forzada en ratas adolescentes. Respuestas de estrés y rol del sistema dopaminérgico.

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Memoria presentada para optar al grado de Doctor con Mención Internacional por:

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José Luis Ferrán Bertone

Luis Puelles López

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A mi padre

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PREFACE

This doctoral thesis is presented as a compendium of publications and it applies for the International Doctorate mention in accordance with the rules for the regulation of official doctoral studies of the University of Murcia (RD-99/2011 and R-310/2015) and with the approval of the thesis supervisors, the Academic Commission of The Doctoral Program in Signals Integration and Modulation in Biomedicine and the General Committee for Doctoral Studies. This thesis is composed of three research studies published in international journals indexed in *Journal Citation Reports* (JCR). Additionally, this document provides a general introduction, which presents the studies and justifies the scientific unity of the thesis (section 1) and an overall summary of the aims of the research and the final conclusions, which unify the partial results presented in each one of the published studies (sections 2 and 4).

Resumen extendido en castellano

De acuerdo con el reglamento por el que se regulan las enseñanzas oficiales de doctorado de la Universidad de Murcia se presenta en este anexo un resumen extendido traducido al castellano de la tesis doctoral:

Esta tesis doctoral es presentada como compendio de publicaciones y opta a la mención de Doctorado Internacional de acuerdo con el reglamento por el que se regulan las enseñanzas oficiales de doctorado de la Universidad de Murcia (RD-99/2011 y R-310/2015) y con la aprobación de los directores de tesis, la Comisión Académica del Programa de Doctorado en Integración y Modulación de Señales en Biomedicina y la Comisión General de Doctorado. Esta tesis está compuesta de tres trabajos de investigación publicados en revistas internacionales indizadas en *Journal Citation Reports* (JCR) (Toval et al., 2017; Toval et al., 2020; Toval et al., 2021). Todos los trabajos aquí presentados configuran una unidad científica en el área de la neurobiología del ejercicio. Además, este documento de tesis ofrece una introducción general en la que se presentan los trabajos y se justifica la unidad científica de la tesis (sección 1) y un resumen general de los objetivos de investigación y las conclusiones finales, que unifican los resultados parciales presentados en cada uno de los trabajos publicados (secciones 2 y 4).

La práctica regular de actividad física reduce el riesgo de desarrollar enfermedades crónicas, como la diabetes tipo 2, la obesidad, algunos tipos de cáncer, y enfermedades cardiovascular (Dishman et al., 2012; Lavie et al., 2015; Ortega et al., 2016; Pedersen et al., 2016; Ruegsegger and Booth, 2017; Warburton and Bredin, 2017; WHO, 2018; Bull et al., 2020). Además, el ejercicio físico se asocia con la prevención de enfermedades mentales como la depresión, la ansiedad, el Alzheimer o el Parkinson, así como con el retraso en la aparición y desarrollo de su sintomatología (Blumenthal et al., 1999; Dishman et al., 2012; Cooney et al., 2014; Ruegsegger and Booth, 2017; Warburton and Bredin, 2017). La actividad física es un potente estimulante de la

plasticidad cerebral y tiene un impacto positivo en una amplia variedad de funciones motoras y cognitivas (Dishman et al., 2006; Foley and Fleshner, 2008; Dayan and Cohen, 2011; Erickson et al., 2012; Fernandes et al., 2017; El-Sayes et al., 2019; Bliss et al., 2020). De hecho, la capacidad física es un importante predictor de la mortalidad, morbilidad y el estado de salud (Bedford et al., 1979; Goldstein, 1990; Myers et al., 2002; ACSM, 2018). Por todo esto, la investigación en la actividad física ha experimentado un crecimiento significativo durante las últimas décadas. Sin embargo, los mecanismos neurobiológicos por los que ejercicio produce todos estos efectos aún se desconocen, y esto es en parte debido a la falta de uniformidad y parametrización en los modelos de ejercicio físico empleados en investigación (Erickson et al., 2012; Toval et al., 2017). La intensidad, el volumen o la frecuencia desarrollados en cada modalidad de ejercicio producen adaptaciones fisiológicas específicas. Por este motivo, para asegurar la fiabilidad y reproducibilidad de los diseños experimentales, es necesario que todos los animales sean expuestos a las mismas condiciones (Leasure and Jones, 2008; Toval et al., 2017; Garrigos et al., 2021).

Los modelos de roedor empleados para determinar los mecanismos neurobiológicos involucrados en el control neural del movimiento así como las adaptaciones fisiológicas producidas por la actividad física, pueden llevarse a cabo con el uso de paradigmas voluntarios o forzados. La modalidad más utilizada en experimentación con roedores es la rueda voluntaria (de Visser et al., 2005; Toval et al., 2017). Sin embargo, una de las principales dificultades de la rueda voluntaria es que la intensidad y el volumen de carrera son diferentes entre sujetos del mismo grupo experimental. Estas diferencias individuales pueden evitarse con la utilización de modalidades forzadas, como la cinta de correr o la rueda a motor, que permiten exponer a todos los animales a la misma carga de entrenamiento. Sin embargo, correr en una cinta o en una rueda motorizada puede suponer un desafío para los roedores, que normalmente alcanzan niveles de rendimiento menores o incluso rechazan correr (Kregel et al., 2006; Toval et al., 2017). El objetivo a largo plazo de nuestro grupo de

investigación es comprender y desenmarañar los mecanismos neurobiológicos que producen y modulan respuestas motoras y metabólicas en relación a la actividad física. Por esta razón, el primer paso necesario es disponer de un modelo de ejercicio forzado en roedores que sea fiable y reproducible.

Por ello, en el **estudio 1** (Toval et al., 2017) nos propusimos desarrollar y evaluar una fase de habituación al ejercicio en rueda forzada para mejorar el rendimiento motor de ratas adolescentes. Con este propósito, se desarrolló un protocolo de habituación de 8 días de duración basado en un aumento progresivo de la velocidad y el tiempo de carrera. Una vez concluido el protocolo, se evaluó su efecto sobre el rendimiento motor mediante un test de ejercicio incremental. Nuestro estudio determinó que ocho días de habituación mejoraron significativamente el rendimiento motor durante el test incremental en ratas Sprague-Dawley. Además, estos datos implican que la implementación de una fase inicial de habituación es clave para lograr respuestas motoras satisfactorias (100% de los roedores) durante programas de entrenamiento más exigentes. Según la literatura publicada, esta mejora en el rendimiento motor observada después de un corto periodo de habituación no puede ser atribuida cambios adaptativos en los sistemas musculares, cardiovasculares o respiratorios (Booth and Holloszy, 1977; Terjung and Hood, 1986; Toval et al., 2017). Por el contrario, el sistema nervioso central parece tener un rol esencial en la regulación de la respuesta motora observada. El modelo desarrollado en el estudio 1 sienta las bases para la exploración de los mecanismos neurales que modulan la capacidad física. En particular, el rol de las respuestas de estrés y el sistema dopaminérgico han sido analizados en los estudios 2 y 3 de la presente tesis doctoral.

El ejercicio agudo es considerado una condición de estrés, ya que desencadena una serie de respuestas fisiológicas que facilitan la utilización de energía. La intensidad de carrera ha sido descrita como un factor clave en la regulación de las respuestas de estrés y, de acuerdo con la literatura, son necesarias altas velocidades de carrera para inducir aumentos en los niveles de las hormonas de estrés que

desencadenen cambios en el lactato y la glucosa, biomarcadores de estrés (Raastad et al., 2000; Saito and Soya, 2004; Soya et al., 2007; Rezaei et al., 2017). Algunos autores sugieren que el ejercicio forzado produce además un estrés adicional derivado de la coerción de los animales, e inespecífico del estímulo físico del ejercicio (Moraska et al., 2000; Dishman et al., 2006; Yanagita et al., 2007; Lin et al., 2012; Morgan et al., 2015). Estas respuestas de estrés podrían actuar tanto reduciendo como potenciando las funciones motoras y, en este último caso, podrían ser el origen de la mejora motora observada después de la habituación (Toval et al., 2017; Toval et al., 2020). Por este motivo, en el **estudio 2** (Toval et al., 2020) nos propusimos determinar si los biomarcadores plasmáticos de estrés lactato y glucosa varían durante el protocolo habituación al ejercicio en rueda forzada y durante el test de carrera incremental, comparándolo con ratas no habituadas. Además, se evaluaron los cambios transcriptómicos en la expresión de ARN mensajero de Avp y Crh en el hipotálamo después de la habituación, mediante hibridación in situ y qPCR. Los resultados obtenidos mostraron que los biomarcadores de estrés plasmáticos e hipotalámicos permanecieron invariables durante el protocolo de habituación. Según estos datos, las respuestas de estrés no parecen estar involucradas en la mejora del rendimiento motor observada después del programa de habituación. Curiosamente, después del test incremental las ratas no habituadas mostraron niveles de lactato y glucosa significativamente más elevados que las ratas habituadas, lo que implica que la implementación de una fase adaptativa previa a programas de ejercicio forzado podría minimizar las respuestas de estrés no específicas del ejercicio.

Los mecanismos que regulan las respuestas motoras observadas durante la carrera forzada parecen depender por tanto del sistema nervioso central. En particular, el sistema dopaminérgico es un candidato importante en la modulación de estos comportamientos. En el **estudio 3** (Toval et al., 2021) nos propusimos averiguar cuáles son los mecanismos neurales involucrados en la capacidad física durante la adolescencia. La adolescencia es un periodo de crecimiento y maduración

caracterizado por una serie de cambios comportamentales y biológicos claves para preparar al individuo para su supervivencia durante la vida adulta (Spear, 2000; Caballero et al., 2016; Caballero and Tseng, 2016). Mejorar la capacidad física durante este sensible período ha mostrado tener un impacto positivo en diversas funciones motoras y cognitivas (Hopkins et al., 2011; Bergeron et al., 2015; Lloyd et al., 2015; Belcher et al., 2020). Por lo tanto, entender los mecanismos neurales que contribuyen a potenciar el rendimiento físico durante la adolescencia podrá contribuir a: 1) lograr diseños de actividades y tareas físicas más eficientes basados en los mecanismos neurobiológicos precisos que regulan estas respuestas funcionales, 2) adaptar los programas de entrenamiento a la etapa madurativa específica de acuerdo a criterios biológicos bien fundados. En conjunto, contribuirá a promover un desarrollo físico y neural saludable en jóvenes deportistas

La dopamina central tiene un importante rol en el desarrollo y maduración de circuitos neurales asociados con el aprendizaje motor y cognitivo durante la adolescencia, mediante la activación de los receptores dopaminérgicos D1 y D2 (Wickens et al., 2003; Willuhn and Steiner, 2008; Crisp et al., 2012; Tritsch and Sabatini, 2012; Caballero et al., 2016; Caballero and Tseng, 2016; Dwyer and Leslie, 2016; Steiner and Tseng, 2016). Por ello, el objetivo del estudio 3 ha sido determinar si los receptores dopaminérgicos D1 y D2 contribuyen a la modulación de la capacidad física durante la adolescencia y si esta modulación tiene lugar en el estriado, núcleo principal de los ganglios de la base, asociados al control motor, y una de las regiones cerebrales con mayor expresión de receptores dopaminérgicos. Los resultados obtenidos han mostrado que la dopamina está involucrada en la regulación del rendimiento motor a través de la acción de los receptores D1 estriatales y D2 extra-estriatales.

De forma resumida y tomado en conjunto, los estudios 1 y 2 proporcionan un novedoso modelo en roedores para el estudio de la neurobiología del ejercicio, en el que es posible aplicar las mismas cargas de entrenamiento a todos los animales, logrando niveles altos de rendimiento y evitando respuestas de estrés no específicas

del ejercicio. Y en el estudio 3 se exploran los mecanismos neurales asociados a la capacidad física durante la adolescencia, que es dependiente de dopamina y está ligada a la activación de los receptores D1 estriatales y D2 extraestriatales.

Esta tesis doctoral está constituida por los siguientes trabajos de investigación publicados:

1. Habituation Training Improves Locomotor Performance in a Forced Running Wheel System in Rats
    - Autores: A. Toval, R. Baños, E. De la Cruz, N. Morales-Delgado, J. Pallarés, A. Ayad, K.Y. Tseng and J.L. Ferran
    - Revista científica: Frontiers in behavioral neuroscience
    - Fecha de publicación: Marzo del 2017
    - Volumen: 11
    - Página: 42
    - Factor de impacto (JCR): 3.33 (2017)
    - Categoría de la revista: Behavioral Neurosciences (neurociencia del comportamiento
    - Cuartil: Q1
    - DOI: 10.3389/fnbeh.2017.00042
    - Disponible online en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5340750/

2. Hypothalamic Crh/Avp, Plasmatic Glucose and Lactate Remain Unchanged During Habituation to Forced Exercise
    - Autores: A. Toval, F. Vicente-Conesa, P. Martínez-Ortega, Y. Kutsenko, N. Morales-Delgado, D. Garrigos, A. Alonso, B. Ribeiro Do Couto, M. Popović and J.L. Ferran
    - Revista científica: Frontiers in Physiology

- Fecha de publicación: Mayo del 2020
- Volumen: 11
- Página: 410
- Factor de impacto (JCR): 3.37 (2019)
- Categoría de la revista: Physiology (fisiología)
- Cuartil: Q1
- DOI: 0.3389/fphys.2020.00410
- Disponible online en: https://pubmed.ncbi.nlm.nih.gov/32499715/

3. Dopaminergic modulation of forced-running performance in adolescent rats: role of striatal D1 and extrastriatal D2 dopamine receptors
    - Autores: A. Toval, D. Garrigos, Y. Kutsenko, M. Popović, B. Ribeiro Do-Couto, N. Morales-Delgado, K.Y. Tseng, J.L. Ferran
    - Revista científica: Molecular Neurobiology
    - Fecha de publicación: January, 2021 (online version)
    - Factor de impacto (JCR): 4.5 (2019)
    - Categoría de la revista: Neurosciences (neurociencias)
    - Cuartil: Q1
    - DOI: 10.1007/s12035-020-02252-2
    - Disponible online en: https://link.springer.com/article/10.1007%2Fs12035-020-02252-2

INDEX

INTRODUCTION

1.1. PRESENTATION AND SCIENTIFIC UNIT

This doctoral thesis is presented as a compendium of publications and it applies for the International Doctorate mention. This thesis is composed of three research studies (Toval et al., 2017; Toval et al., 2020; Toval et al., 2021) published in international journals indexed in *Journal Citation Reports* (JCR). All the articles configure a scientific unity in the field of the neurobiology of exercise.

Regular practice of physical activity reduces the risk of developing chronic diseases, such as type 2 diabetes, obesity, several types of cancer and cardiovascular diseases (Dishman et al., 2012; Lavie et al., 2015; Ortega et al., 2016; Pedersen et al., 2016; Ruegsegger and Booth, 2017; Warburton and Bredin, 2017; WHO, 2018; Bull et al., 2020). Furthermore, exercise has been strongly associated with the prevention and slower progression of mental illness, including depression, anxiety, Parkinson or Alzheimer disease (Blumenthal et al., 1999; Dishman et al., 2012; Cooney et al., 2014; Ruegsegger and Booth, 2017; Warburton and Bredin, 2017). Physical activity is a powerful enhancer of brain plasticity and has a positive impact in a broad set of cognitive and motor functions (Dishman et al., 2006; Foley and Fleshner, 2008; Dayan and Cohen, 2011; Erickson et al., 2012; Fernandes et al., 2017; El-Sayes et al., 2019; Bliss et al., 2020). Indeed, exercise capacity and physical fitness are important predictors of mortality, morbidity and health status (Bedford et al., 1979; Goldstein, 1990; Myers et al., 2002; ACSM, 2018). For all this, the research on physical activity has experienced a significant growth during the last decades. However, the neurobiological mechanisms by which exercise produces health benefits remain to be elucidated, and this is partly due to the lack of uniformity and parametrization in the experimental models of exercise (Erickson et al., 2012; Toval et al., 2017). The intensity, volume or frequency developed in each modality of exercise produce specific physiological adaptations. Thus, to ensure the reliability and repeatability of the experimental designs, all the animals need to be exposed to same conditions (Leasure and Jones, 2008; Toval et al., 2017; Garrigos et al., 2021).

Rodent models employed to determine the neurobiological mechanisms involved in the neural control of movement and the physiological adaptations produced by physical activity, can be developed using voluntary or forced paradigms. The most used modality in rodents is the voluntary running wheel (de Visser et al., 2005; Toval et al., 2017). However, one of the main difficulties of the voluntary wheel is that intensity and volume of running varies from subjects of the same experimental group. These individual differences can be avoided using forced modalities such as treadmill and forced running wheel, which allow similar training loads for all the animals. Nevertheless, running in a treadmill or motorized wheel can be challenging for the rodents, which typically achieve low levels of performance or even reject running (Kregel et al., 2006; Toval et al., 2017). The long-term goal of our research group is to unravel and understand the neurobiological mechanisms producing and modulating motor and metabolic responses in relation to physical activity. For this reason, it is first necessary to have a reproducible and reliable rodent model of forced exercise.

Thus, in the **study 1**, we aimed to develop and evaluate a phase of habituation to exercise in forced running wheel in order to improve the locomotor performance of young rats. For this purpose, we have developed an 8-days habituation protocol based on a progressive increase of the speed and time of running. After the protocol, we evaluated its effect in the locomotor performance by an incremental exercise test. Our study determined that eight days of habituation significantly improved the locomotor performance of Sprague-Dawley rats during the incremental test. Also, our data reveal that the implementation of the habituation phase is a key component to achieve successful locomotor responses (100% of the rodents) during longer and more demanding training programs. According to the published research studies, the improved locomotor performance observed after a short period of habituation cannot be attributed to adaptive changes of the muscular, cardiovascular or respiratory systems (Booth and Holloszy, 1977; Terjung and Hood, 1986; Toval et al., 2017). By

contrast, the central nervous system and the stress circuits might be playing a key role in the regulation of the observed motor response. The model developed in study 1 lays the foundations for the exploration of the neural mechanisms modulating exercise capacity. Specifically, the role of stress responses and the dopaminergic system have been analyzed in the study 2 and 3 of the present doctoral thesis.

Acute exercise is considered a stressor since it promotes a series of physiological responses to facilitate energy utilization. Intensity of running has been described as a key factor regulating the stress response; and according to literature, high speeds are required to increase stress hormones inducing plasmatic changes in the stress biomarkers lactate and glucose (Raastad et al., 2000; Saito and Soya, 2004; Soya et al., 2007; Rezaei et al., 2017). Some authors suggest that forced exercise models produce an additional stress component, derived from coercion of the animals and not related to the physical stimulus of exercise (Moraska et al., 2000; Dishman et al., 2006; Yanagita et al., 2007; Lin et al., 2012; Morgan et al., 2015). These observed stress responses might act either diminishing or enhancing motor functions, and in the latter condition could be the source of the observed improvement of locomotor performance after habituation (Toval et al., 2017; Toval et al., 2020). Thus, in **study 2**, we aimed to determine whether plasmatic stress biomarkers, lactate and glucose, vary during the exercise habituation protocol in forced running wheel and during the incremental running test compared with non-habituated rats. Furthermore, we assessed chronic transcriptomic changes in hypothalamic *Crh* and *Avp* mRNA expression after habituation, by in situ hybridization and qPCR. The results obtained in study 2 showed that plasmatic and hypothalamic stress biomarkers remain unchanged during the habituation protocol. According to these results, stress responses do not appear to be involved in the improved locomotor performance observed after the habituation program. Interestingly, non-habituated rats showed significantly higher levels of plasmatic lactate and glucose during the incremental test, which implies that the

implementation of an adaptive phase prior to forced exercise programs might minimize non-specific stress responses.

Thus, the mechanisms regulating the observed locomotor responses during forced running appears to be dependent on the central nervous system. In particular the dopaminergic system is an important candidate to modulate these motor behaviors. In the **study 3** we aimed to find out the neural mechanisms involved in exercise capacity during adolescence. Adolescence is a period of development characterized by key behavioral and biological changes that prepare the individual for adulthood survival (Spear, 2000; Caballero et al., 2016; Caballero and Tseng, 2016). It is known that improving exercise capacity during this sensitive period impacts positively on cognitive and motor functions (Hopkins et al., 2011; Bergeron et al., 2015; Lloyd et al., 2015; Belcher et al., 2020). Therefore, understanding the neural mechanisms that contribute to enhance physical performance during adolescence will contribute to: 1) achieve more efficient designs of exercise tasks based on the neurobiological mechanism that regulates these functional responses and 2) adapt the training programs to the specific maturational stage of life according to well-founded biological criteria. Altogether it will contribute to promote a healthy physical and neural development of youth athletes.

The central dopamine system is known to play a key role in the development and maturation of neural circuits associated with cognitive and motor learning behaviors during adolescence through the activation of D1 and D2 receptors (Wickens et al., 2003; Willuhn and Steiner, 2008; Crisp et al., 2012; Tritsch and Sabatini, 2012; Caballero et al., 2016; Caballero and Tseng, 2016; Dwyer and Leslie, 2016; Steiner and Tseng, 2016). Thus, the aim of the study 3 was to determine whether the D1 and D2 receptors contribute to modulate exercise capacity during adolescence and whether this modulation occurs through the striatum, which is the main input structure of the basal ganglia circuitry involved in motor control, and one of the brain regions with the highest expression of dopamine receptors. The results showed that the dopamine

system is involved in the regulation of locomotor performance through a recruitment of striatal D1 and extrastriatal D2 receptor signaling.

Collectively, **study 1 and 2** provide a novel rodent model of analysis of the neurobiology of exercise in which similar training loads can be applied to all the animals, achieving successful levels of motor performance and avoiding non-specific of exercise stress responses. Thus, guaranteeing the reproducibility and reliability of the experimental procedures. And **study 3** explores the neural mechanisms associated to exercise capacity during adolescence, which is dopamine-dependent and mechanistically linked to the activation of striatal D1 and extra-striatal D2 receptors.

The present doctoral thesis is constituted by the following published articles:

1. Habituation Training Improves Locomotor Performance in a Forced Running Wheel System in Rats
    - Authors: A. Toval, R. Baños, E. De la Cruz, N. Morales-Delgado, J. Pallarés, A. Ayad, K.Y. Tseng and J.L. Ferran
    - Journal: Frontiers in behavioral neuroscience
    - Date of publication: March, 2017
    - Volume: 11
    - Page: 42
    - Impact factor (JCR): 3.33 (2017)
    - Journal Category: Behavioral Neurosciences
    - Quartile: Q1
    - DOI: 10.3389/fnbeh.2017.00042
    - Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5340750/

2. Hypothalamic Crh/Avp, Plasmatic Glucose and Lactate Remain Unchanged During Habituation to Forced Exercise
    - Authors: A. Toval, F. Vicente-Conesa, P. Martínez-Ortega, Y. Kutsenko, N. Morales-Delgado, D. Garrigos, A. Alonso, B. Ribeiro Do Couto, M. Popović and J.L. Ferran
    - Journal: Frontiers in Physiology
    - Date of publication: May, 2020
    - Volume: 11
    - Page: 410
    - Impact factor (JCR): 3.37 (2019)
    - Journal Category: Physiology
    - Quartile: Q1
    - DOI: 0.3389/fphys.2020.00410
    - Available online at: https://pubmed.ncbi.nlm.nih.gov/32499715/

3. Dopaminergic modulation of forced-running performance in adolescent rats: role of striatal D1 and extrastriatal D2 dopamine receptors
    - Authors: A. Toval, D. Garrigos, Y. Kutsenko, M. Popović, B. Ribeiro Do-Couto, N. Morales-Delgado, K.Y. Tseng, J.L. Ferran
    - Journal: Molecular Neurobiology
    - Date of publication: January, 2021 (online version)
    - Impact factor (JCR): 4.5 (2019)
    - Journal Category: Neurosciences
    - Quartile: Q1
    - DOI: 10.1007/s12035-020-02252-2
    - Available online at: https://link.springer.com/article/10.1007%2Fs12035-020-02252-2

1.2. THEORETICAL CONCEPTUALIZATION

1.2.1. Physical activity, brain function and adolescence

The terms "Physical activity", "exercise", and "sport" are closely related, however, they describe different concepts. "Physical activity" is defined as any form of body movement performed by skeletal muscles that results in an increase of energy expenditure, while the term "exercise" refers to a subcategory of physical activity that is planned, structured, repetitive and with a purpose. Finally, the term "sport" also involves physical activity, skill and/or hand-eye coordination but it is subject to specific rules (Caspersen et al., 1985; WHO, 2018). In this thesis we will also refer to "active people" as individuals who achieve at least the minimum amount of physical activity recommended by the World Health Organization (WHO) (Bull et al., 2020), which is over 150 minutes of moderate-intensity of physical activity per week for adults, and more than 60 minutes of moderate to vigorous-intensity physical activity per day for adolescents. Global estimates indicate that 27.5% of adults and 81% of adolescent population are not active enough (Bull et al., 2020).

The lack of physical activity is one of the leading risk factors for death worldwide according to the WHO (WHO, 2018). Insufficient physical activity increases the risk of developing non-communicable diseases such as obesity, osteoporosis, type 2 diabetes or heart disease (Dishman et al., 2012; Ruegsegger and Booth, 2017; Warburton and Bredin, 2017; Piercy et al., 2018; WHO, 2018). Moreover, physical activity reduces the risk of developing several types of cancer, as well as the risk of disease recurrence (Pedersen et al., 2016). Interestingly, recent studies support that the practice of any type of regular physical activity has a beneficial impact on the human immune response to infectious diseases. In this way, physical activity acts as a protective factor and contributes to the improvement of the prognosis against COVID-19 (Burtscher et al., 2020; Casas-Rojo et al., 2020; Chastin et al., 2020; da Silveira et al., 2020). Besides the increment of morbidity and premature mortality, the

current pandemic of physical inactivity has a significant economic cost to health-care systems. Recent studies have estimated that the cost of physical inactivity is approximately INT $ 53.8 billion worldwide per year (Ding et al., 2016). On the other hand, it is estimated that increasing the levels of physical activity of the population to at least the lower threshold of the 2020 WHO´s recommendations, will contribute INT $ 8.6 trillion to the global economy cumulatively by 2050 (Hafner et al., 2020).

In addition to the well-known positive impact of physical activity in cardiovascular health and fitness, growing evidence has shown strong correlations between physical activity and mental health, improving brain function and preventing mental disorders such as depression (Blumenthal et al., 1999; Cooney et al., 2014), Parkinson (Smith and Zigmond, 2003) or Alzheimer disease (Santos-Lozano et al., 2016; Stephen et al., 2017; Valenzuela et al., 2020). Indeed, physical activity is a powerful enhancer of brain plasticity and has a positive impact on a wide range of cognitive and motor functions, for example enhancing neurogenesis, synaptogenesis, brain angiogenesis, neural repair, and minimizing oxidative stress (Dishman et al., 2006; Foley and Fleshner, 2008; Dayan and Cohen, 2011; Erickson et al., 2012; Fernandes et al., 2017; El-Sayes et al., 2019; Bliss et al., 2020) (Figure 1).

Of special interest is the effect of physical activity during adolescence. The adolescent period is characterized by key behavioral and biological changes that prepares the individual for adulthood survival (Spear, 2000; Hopkins et al., 2011; Bergeron et al., 2015; Lloyd et al., 2015; Caballero et al., 2016; Caballero and Tseng, 2016; Belcher et al., 2020). Due to the enhanced brain plasticity and cognitive flexibility, adolescence is often considered as a period of both opportunity and vulnerability (Belcher et al., 2020). The practice of physical activity during this sensitive period has been strongly correlated with an enhanced cognitive and physical development and with a healthier adult life (Bergeron et al., 2015; Lloyd et al., 2015; Bull et al., 2020). A decrease in the levels of regular physical activity is considered one of the main causal factors of obesity during the adolescence, which also imply a higher

risk of becoming obese adults (Hills et al., 2011). Therefore, exercise programs must be adapted to the biological maturity of this sensitive period in order to promote a healthy development and to enhance the potential talent of youth athletes (Bergeron et al., 2015; Lloyd et al., 2015).

Figure 1. Benefits of physical activity highlighting those on the nervous system.

For all these reasons, research in physical activity has experienced a significant growth in recent decades (Figure 2). However, most of the research that relates physical activity and brain function has focused on descriptive facts, while few studies have explored neurobiological mechanisms. How physical activity modulates the brain and how the brain modulates physical activity remains poorly understood (Erickson et al., 2012; El-Sayes et al., 2019).

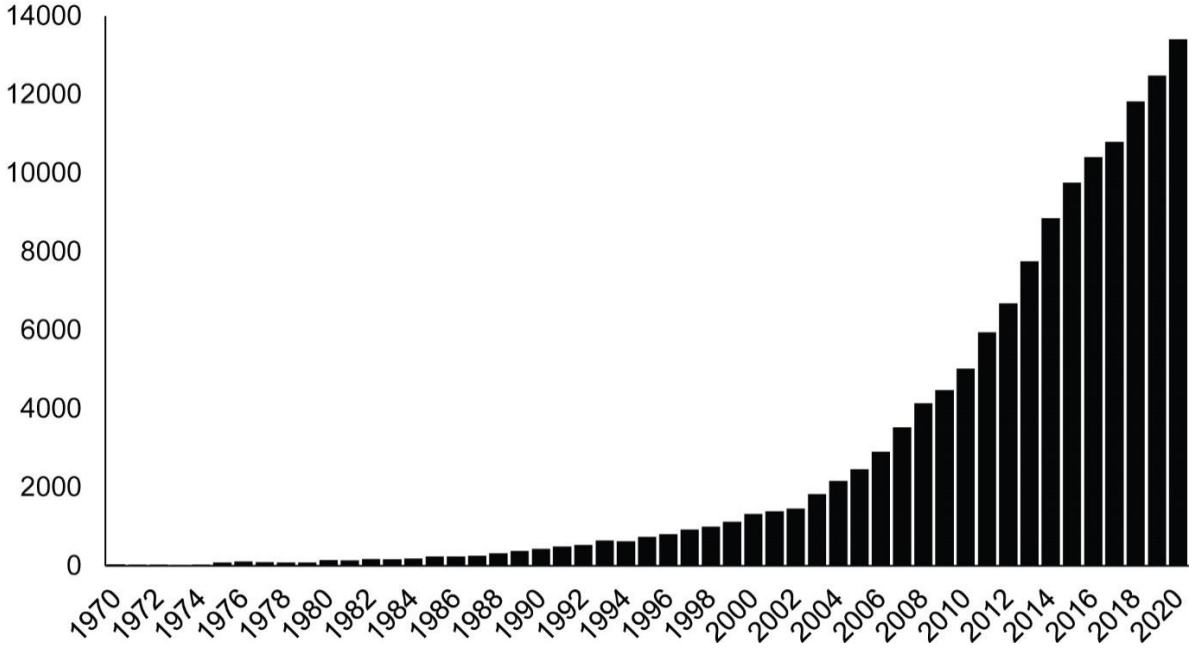


Figure 2. Number of papers published in PubMed per year from the key word "physical activity" from 1970 to 2020.

1.2.2. Animal models of exercise

Most of the research that studies the biological mechanisms underlying physical activity is carried out in animal models, as it requires invasive procedures or involves lifelong studies, which is not feasible with human subjects (Kregel et al., 2006). Historically, rodents have been the most widely used animal species in biomedical research, due to their anatomical, physiological, and genetic homologies with humans added to their small size, short life cycle, ease of maintenance, and

availability of abundant genetic resources (Bryda, 2013). However, studies evaluating the impact of physical activity in rodents are controversial due to the lack of uniformity and parameterization in the experimental exercise protocols used (Toval et al., 2017; Garrigos et al., 2021). Variables such as the type of exercise (e.g. voluntary vs forced), the duration (acute vs chronic), the intensity (e.g. speed of running), or the frequency of the training will produce different physiological adaptations. Therefore, researchers must take all of these factors into account when selecting and implementing the most appropriate animal model, to minimize undesirable effects and to ensure the reproducibility of the experiments.

Exercise in rodents can be classified in different modalities, based on the type of exercise (e.g. swimming or running in a treadmill or wheel), the will to run of the animals (forced or voluntary), the intensity (lineal, ramp or interval training), the duration, the metabolic response (aerobic or anaerobic), or the physical capacity developed during the activity (e.g. endurance, resistance, speed, coordination…). In order to study the neural mechanisms involved in each modality, the parameters of the exercise stimulus need to be accurately controlled (e.g. ensuring that the same training load is applied to all the animals) (Kregel et al., 2006; Leasure and Jones, 2008; Garrigos et al., 2021). One of the most frequently used classifications group rodent modalities of exercise into two main paradigms: voluntary and forced. The study by Leasure and Jones (2008) comparing the effects of exercise in voluntary and forced conditions showed that both types of exercise produce different physiological and behavioral responses. The voluntary running wheel is the most widely used modality in exercise research. Rodents have *ad libitum* access to the running wheels, requiring minimal intervention from the researchers. However, the stimulus of physical activity, such as the running intensity and volume, differs significantly from subject to subject due to the "voluntary" nature of the model. These individual differences can be avoided by using forced running wheel or treadmill modalities, as speed and running

time can be controlled and manipulated ensuring that the same training load is applied to all subjects.

Among the main modalities of forced exercise in rodents are the treadmill and the forced running wheel. The treadmill has been extensively used over the past decades (Kregel et al., 2006), as the total amount of external work performed by the animals can be easily calculated and manipulated according to the specific interests of the research questions. Nevertheless, running on a treadmill can be challenging for rodents and requires the application of an aversive stimulus (e.g. electric shock or high-pressure blast of air) to encourage the animals to run. This stressful component can interfere with the responses and adaptations produced by exercise. On the other hand, forced running wheel system is a novel modality of forced exercise. Although the current number of reports using the forced wheel is much lower than the treadmill, it is a booming model during the last two decades (Garrigos et al., 2021). This system includes most of the advantages of the forced models but, unlike the treadmill, it does not require the application of any aversive stimulus. Although forced models guarantee the reproducibility of the applied training load and seem to be the most suitable to study the effects of exercise, two major disadvantages of their use have been reported: 1) irregular motor performance and 2) non-specific stress responses (Toval et al., 2017; Toval et al., 2020). In fact, 10% of animals refuse to run during forced exercise protocols (Koch and Britton, 2001; Kregel et al., 2006), and runners rarely reach the speeds observed during voluntary running (Narath et al., 2001; Leasure and Jones, 2008).

In STUDY 1 of the present thesis (Toval et al., 2017) we aimed to develop and optimize a reproducible and reliable rodent model of exercise using forced running wheel in order to achieve successful levels of locomotor response, in terms of intensity and volume of running, and to reduce the number of rats rejecting running. For that purpose, we developed and implemented an 8-days habituation protocol, with a gradual increase of speed and time of running. After the protocol, locomotor

performance was assessed using an incremental exercise test to exhaustion. Our data showed that habituation significantly improved motor performance during the incremental test, getting a 100% success rate response. The observed motor improvement after habituation was again corroborated in STUDY 2 (Toval et al., 2020) and STUDY 3 (Toval et al., 2021).

Figure 3. Adolescent rats running in the forced wheel system during an exercise session of the habituation period. All the sessions were performed during the active period of the rats (at Zeitgeber time 14 and 20, which is equivalent to 2 and 8 hours after lights were off, in a 12-h light/12-h dark cycle).

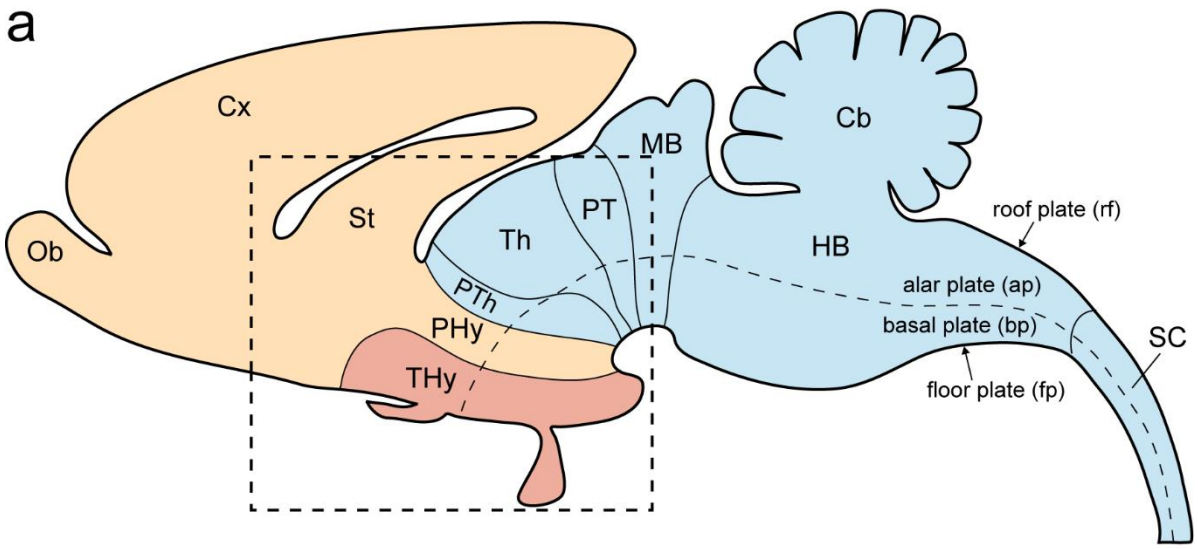
1.2.3. Stress responses of exercise

Survival of all living organisms depends on maintaining a complex dynamic and harmonious balance, which is constantly challenged by disturbing forces termed "stressors". This biological process of self-regulation to maintain equilibrium is known as homeostasis (Chrousos and Gold, 1992; Chrousos, 2009).

The term "stress" is often used with different meanings. For the present thesis we will use the standard definition of stress as a state of disharmony or disruption of the body´s homeostasis, caused by a real or perceived threat or challenge (Chrousos

and Gold, 1992; Chrousos, 2009). When emotional or physical stressors appear, the body prepares to respond to the challenge or threat by activating various physiological and behavioral adaptive responses, mediated by the stress system, which include the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the activation of the sympathetic adrenomedullar (SAM) axis. These central responses from HPA and SAM axes trigger the production of glucocorticoids and the catecholamines norepinephrine and epinephrine, respectively (Chrousos, 2009; Godoy et al., 2018). Acute exercise is considered a stress condition since it initiates a coordinate series of physiological responses, which include the stimulation of the HPA and SAM axes, inducing metabolic and endocrine changes to maintain homeostasis under the new physiological conditions (Raastad et al., 2000; Leal-Cerro et al., 2003; Morgan et al., 2015; Soria et al., 2015; Chen et al., 2017). Both the HPA and SAM axes initiate their response in the paraventricular nucleus (PVN) of the hypothalamus.

According to the prosomeric model, the hypothalamic region is part of the two most rostral prosomeres: peduncular and terminal, caudally and rostrally respectively (Puelles et al., 2012; Ferran et al., 2015c; Puelles and Rubenstein, 2015; Diaz and Puelles, 2020), (Figure 4). Each prosomere is a rostrocaudal partition of the neural tube arranged in floor, basal, alar and roof dorsoventral domains (Puelles, 2013, 2019). The hypothalamic region, according to the updated prosomeric model, can be identified in the entire basal region of both peduncular and terminal prosomeres, and as part of the most ventral domains of the alar plate identified as paraventricular and subparaventricular domains (Puelles et al., 2012; Ferran et al., 2015c; Puelles and Rubenstein, 2015; Diaz and Puelles, 2020; Puelles et al., 2020). The PVN is localized in the paraventricular domain (alar region) of the peduncular hypothalamic prosomere (Morales-Delgado et al., 2011; Morales-Delgado et al., 2014; Ferran et al., 2015c) (Figure 4).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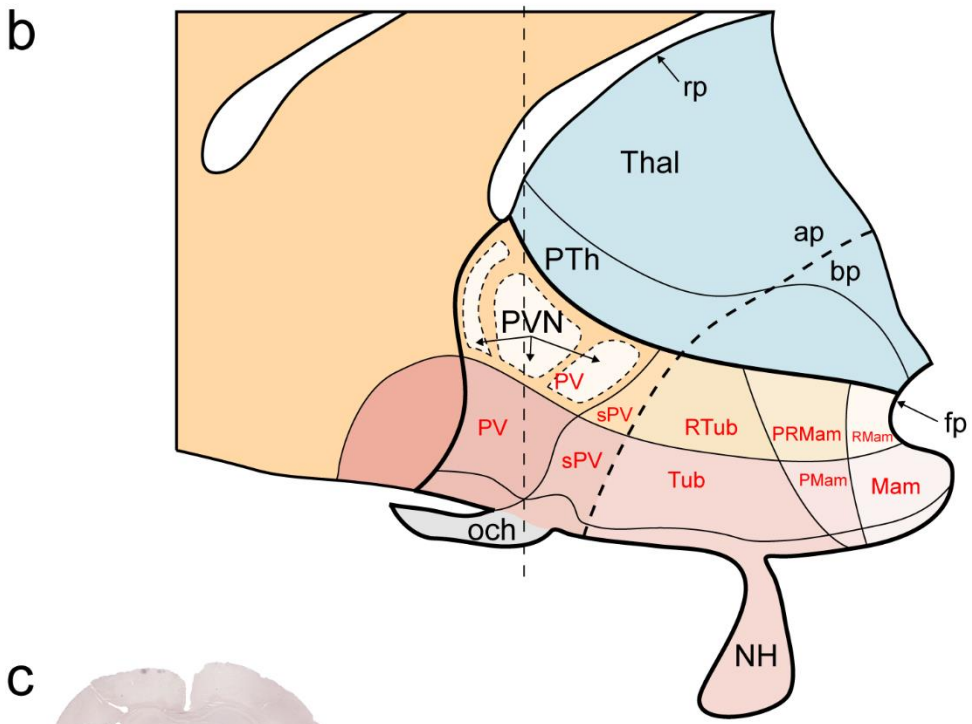
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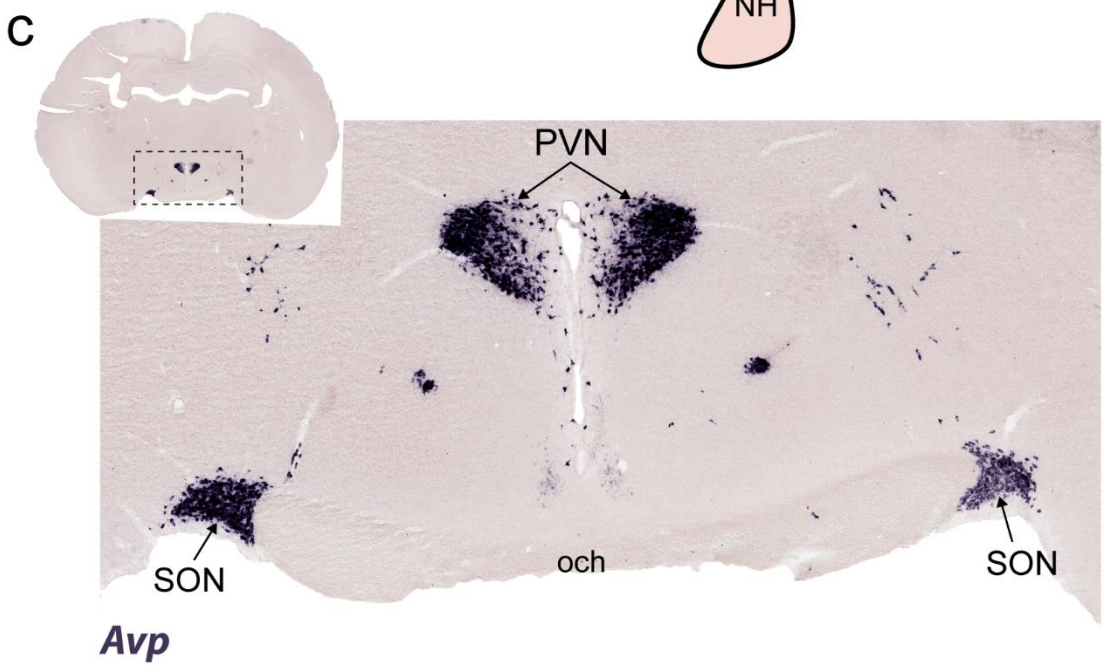
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Figure 4. a) Schematic representation of the rat brain illustrating hypothalamic position and main subdivisions according to the prosomeric model. Spinal cord (SC), hindbrain (HB), cerebellum (Cb), midbrain (MB) and diencephalon (pretectum, PT, Thalamus, Th, and prethalamus, Pth) are in blue, and the hypothalamo-telencephalic prosomeres (peduncular, and terminal) in orange and red, respectively. Peduncular (PHy) and terminal (Thy) hypothalamus can be identified as part of these prosomeres. The longitudinal alar/basal boundary is showed as a dashed black line and the roof (rp), alar (ap), basal (bp) and floor (fp) plates are indicated. b) Schematic representation of the main hypothalamic areas distributed across the anteroposterior and dorsoventral dimensions. The hypothalamic area is subdivided rostrocaudally into the prosomeres terminal (Thy) and peduncular (PHy) (orange and red colors, respectively). The alar hypothalamus is subdivided dorsoventrally into paraventricular (PV) and subparaventricular (SV) regions; and the basal hypothalamus is subdivided dorsoventrally in both the peduncular and terminal prosomeres into the tuberal and retrotuberal (Tub/Rtub), perimamilar and periretromamilar (PMam/PRMam), and mamilar and retromamilar areas (Mam/RMam). The terminal prosomere include rostrally the acroterminal domains. The paraventricular nucleus (PVN) is located in the paraventricular area of the peduncular hypothalamic prosomere (PHy), and is constituted by three partitions (dorsal, central and ventral). Adapted from Ferran et al. (2015), and Puelles and Rubenstein (2015). c) In situ hybridization of a coronal section showing Avp mRNA expression (dark blue), identifying the magnocellular neurons of the paraventricular nucleus (PVN), and the supraoptic nucleus (SON). In situ hybridization procedures were developed according to Ferran et al. (2015a, b). St, striatum, Cx, cortex, Ob, olfactory bulb. och, optic chiasma, NH, neurohypophysis.

The PVN is composed of parvocellular neurons (from latin "parvus", meaning small) expressing corticotropin-releasing hormone (CRH) and magnocellular neurons (from latin "magnus" which means great) expressing vasopressin (AVP) and oxytocin (OXT) (Herman et al., 2002; Aguilera and Liu, 2012; Morales-Delgado et al., 2014). CRH parvocellular neurons induces adrenocorticotropic hormone (ACTH) in the pituitary gland, leading to the production and release of glucocorticoids through the cortex of the adrenal gland (Figure 5). On the other hand, AVP magnocellular neurons stimulate the sympathetic-adrenal and catecholaminergic responses (Chrousos, 2009; Geerling et al., 2010; Hernández et al., 2016; Godoy et al., 2018). These changes promote energy utilization preparing the organism for the fight-or-flight response by increasing the heart rate, blood pressure or plasmatic glucose and lactate

concentrations (Tan et al., 1992; Moraska et al., 2000; Chennaoui et al., 2002; Andersen et al., 2004; Kawashima et al., 2004; Soya et al., 2007; Yanagita et al., 2007; Chrousos, 2009; Chen et al., 2017; Godoy et al., 2018).

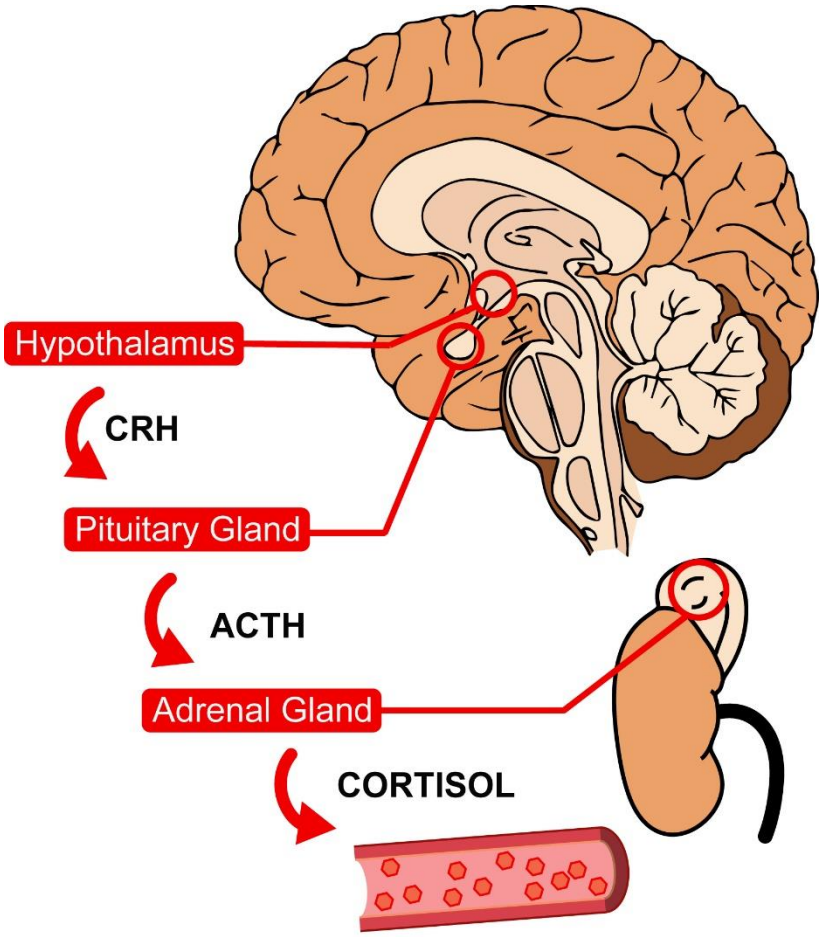


Figure 5. Diagram representing the hypothalamic-pituitary-adrenal axis (HPA)

Physical exercise has been shown to induce most of the mentioned changes to promote energy utilization. For example, it has been found a higher number of C-FOS/CRH positive cells in the PVN after forced running (Timofeeva et al., 2003; Yanagita et al., 2007). Also, plasmatic glucose and lactate levels have been considered useful markers of stress response after high intensity running (Raastad et al., 2000; Garcia-Alvarez et al., 2014; Rezaei et al., 2017). The blood lactate curve and lactate thresholds (LTs) are widely used for the diagnosis of endurance capacity and for

prescription of intensity in exercise programs. When the lactate threshold is reached during high intensity running in treadmill or motorized wheel, a quick gain of lactate, but also glucose and ACTH is observed (Timofeeva et al., 2003; Soya et al., 2007; Rezaei et al., 2017). Activation of the HPA axis triggers the release of glucocorticoids, which play an important role in the regulation of glucose homeostasis. Glucocorticoids stimulate gluconeogenesis in the liver, while reducing glucose uptake and utilization by antagonizing insulin response in the skeletal muscle. Glucocorticoids also regulate glycogen metabolism, increasing glycogen storage in the liver, promoting glycogenolysis in the skeletal muscles, and modulating insulin and glucagon secretion from the pancreas. Therefore, a raise of glucocorticoids finally leads to hyperglycemia. This effect of glucocorticoids regulating glucose homeostasis aims to preserve plasmatic glucose for the brain during stressful situations, promoting the availability of vital substrates to potentiate brain function (Charmandari et al., 2005; Kuo et al., 2015).

Fluctuations of these mentioned physiological responses depend on the different types and intensities of exercise. Actually, forced exercise models in rodents have been described to cause higher and prolonged stress responses compared with voluntary paradigms (Yanagita et al., 2007; Hayes et al., 2008; Griesbach et al., 2012). It is frequently assumed that forced exercise models induce a non-specific emotional stress, which can be added to the physical stress arisen from high intensities of exercise. This additional source of stress may mask the proper effects derived from the physical activity (Lin et al., 2012; Morgan et al., 2015).

Therefore, once resolved the difficulties with irregular motor responses in STUDY 1 (Toval et al., 2017), in STUDY 2 (Toval et al., 2020) we aimed to determine whether the exercise habituation protocol in forced running wheel alters plasmatic and hypothalamic stress biomarkers. Our data show that plasmatic concentrations of glucose and lactate do not present any changes throughout each session of habituation, with a tendency to decrease at the end of the protocol. Also, hypothalamic

Crh and *Avp* gene expression were not chronically activated by the habituation program, assessed by in situ hybridization and qPCR. Furthermore, rats without previous contact with the wheel showed significantly higher plasmatic levels of glucose and lactate after an incremental running test, suggesting that the implementation of an adaptive phase prior to forced exercise programs might avoid non-specific stress responses.

1.2.4. Dopamine system and motor control

Dopamine (DA) is the major catecholaminergic neurotransmitter in the central nervous system and plays an important role modulating a wide variety of cognitive and motor functions including learning, memory, motivation or locomotor activity (Wickens et al., 2003; Wise, 2004; Foley and Fleshner, 2008; Salamone and Correa, 2012; Steiner and Tseng, 2016; Salamone et al., 2018; Klein et al., 2019).

Dopaminergic neurons are distributed throughout different brain areas, from the neuronal groups A8 to A16, following the nomenclature of the classic study of (Dahlstroem and Fuxe, 1964), which is still the model of reference to identify these neuronal groups (Björklund and Dunnett, 2007). The major source of dopamine in the mammalian brain is located in the ventral tegmental area (VTA, A10) and the substantia nigra pars compacta (SNc, A9) (Figure 6 and 8). Classic studies identify these cell groups as mesencephalic dopaminergic neurons; however, Smits et al. (2006) described their origin from the tegmental area of the diencephalon and the mesencephalon, and thus referring to these groups of dopaminergic neurons as meso-diencephalic. These meso-diencephalic dopaminergic neurons contain approximately 90% of the total number of brain dopaminergic cells (Chinta and Andersen, 2005; Björklund and Dunnett, 2007) (Figure 6).

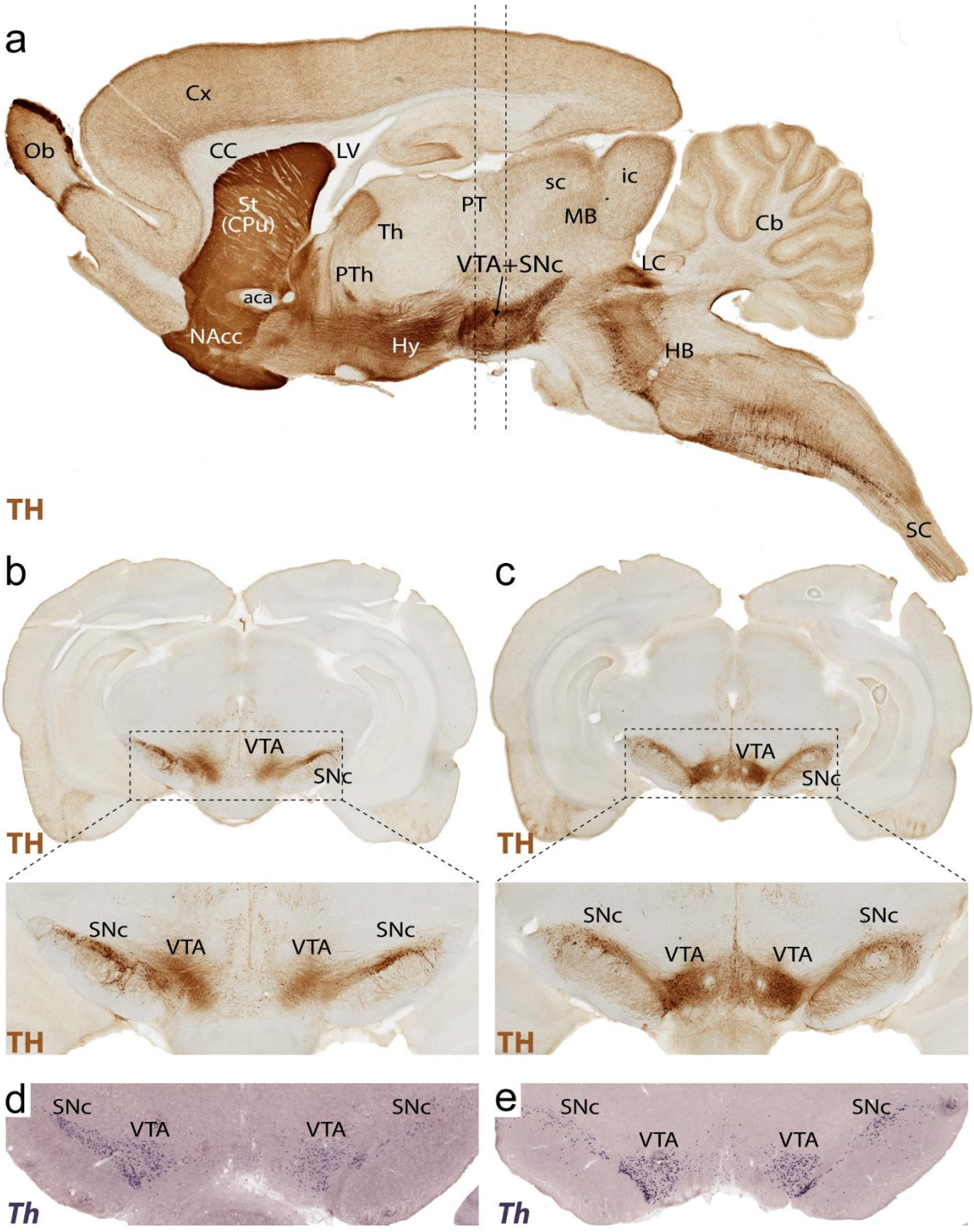


Figure 6. a) Rat brain sagittal section of the tyrosine hydroxylase immunohistochemistry that helps to identify some of the main dopaminergic cell groups and their axonal projections. Projections from the meso-diencephalic dopaminergic neurons (VTA and SNc) to the striatum (CPu and NAcc) can be observed. Increased expression of TH can be also observed from the noradrenergic neurons in the locus coeruleus (LC). b and c) Tyrosine hydroxylase immunohistochemistry identifying dopaminergic neurons in selected rostral and caudal

coronal sections from the VTA and SNc. d and e) Tyrosine Hydroxylase mRNA expression identifying dopaminergic neurons in selected rostral and caudal coronal sections from the VTA and SNc. TH-positive nuclei belonging to dopaminergic neurons can be identified in dark blue. In situ hybridization and immunohistochemistry procedures were developed according to Ferran et al. (2015a, b). SC, spinal cord; HB, hindbrain; Cb, cerebellum; MB, midbrain; PT, pretectum; Th, Thalamus; PTh, prethalamus; Hy, hypothalamus; aca, anterior part of the anterior commissure; CPu, caudate putamen; NAcc, nucleus accumbens; CC, corpus callosum; Cx, cortex, LV, lateral ventricule; and Ob, olfactory bulb.

Dopamine is synthesized from the amino acid tyrosine, which is produced in the liver from phenylalanine through the action of phenylalanine hydroxylase and taken up into the dopaminergic neurons of the brain, where it is converted into dopamine through a series of reactions (Figure 7). Tyrosine hydroxylase (TH) hydroxylates tyrosine to L-DOPA. TH is the first and the rate-limiting enzyme of catecholamines biosynthesis (including dopamine, epinephrine and norepinephrine), therefore, TH modulation is an important process by which optimal levels of catecholamines are maintained. Once formed, L-DOPA is then rapidly converted to dopamine by aromatic amino acid decarboxylase (Dunkley et al., 2004; Daubner et al., 2011; Ayano, 2016) (Figure 7). Dopamine synthesis occurs in the cytoplasm and most of the times it is transported from the cytosol by a vesicular monoamine transporter (VMAT2) into synaptic vesicles where it is stored until release by exocytosis into the synaptic cleft (Südhof, 1995). Once released, dopamine can elicit a physiological response in postsynaptic or nearby cells. Typically, neurotransmission occurs quickly and the dopamine molecules are rapidly 1) metabolized by enzymes (Kanner and Schuldiner, 1987), 2) taken back up by the dopamine transporter of the presynaptic neuron (Iversen, 1971), or 3) bound to postsynaptic neurons or target cells' receptors (Garris et al., 1994; Klein et al., 2019) (Figure 7).

Dopamine elicits excitatory and inhibitory effects on the targeted neurons depending on which receptor is being activated. Dopamine receptors belong to the family of G-protein coupled receptors (GPCR), thus all of them are metabotropic and lead to the formation of second messengers which stimulate specific signaling

pathways. Five different types of dopaminergic receptors have been described, which can be grouped into two major families: D1-like (comprising D1R and D5R) and D2-like (comprising D2R, D3R, and D4R). D1-like receptors family are located in postsynaptic cells and have an excitatory effect, whereas D2-like receptors can be localized both post and pre-synaptically (autoreceptors) and exerts an inhibitory effect (Schwartz et al., 1992; Kumar and Patel, 2007; Beaulieu and Gainetdinov, 2011) (Figure 7).

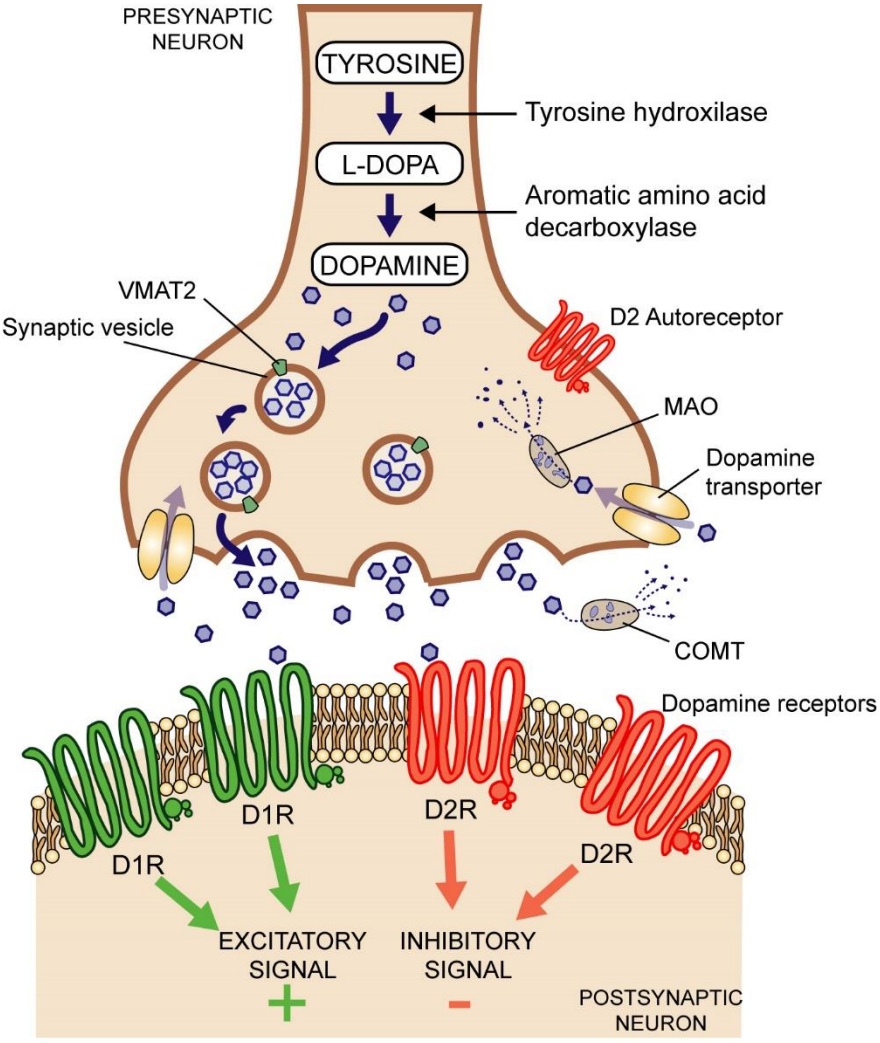


Figure 7. The biosynthetic pathway of dopamine and liberation process

Dopaminergic neurons send their projection to different brain nuclei, originating different dopaminergic systems or pathways. Among the main pathways of dopamine are 1) the nigrostriatal pathway, from SNc to the striatum, 2) the mesocortical and mesolimbic pathways, sourced in the VTA and projecting to the cortex and accumbens, respectively, and 3) the tuberoinfundibular pathway, from the arcuate nucleus in the hypothalamus (tuberal region) targeting to the median eminence (infundibular region) (Cooper et al., 2003). Furthermore, it has been recently described a new parallel descending pathway from SNc (A9) and from A13 to the mesencephalic locomotor region (MLR) which might be also involved in the control of movement (Sharma et al., 2018) (Figure 8).

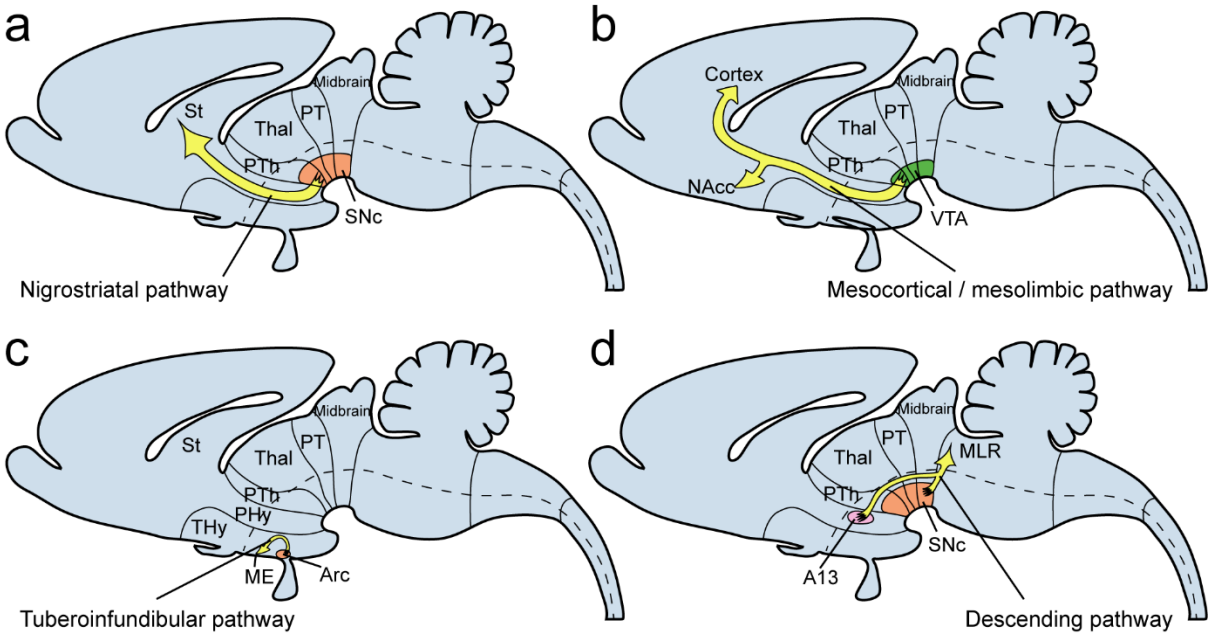


Figure 8. Visual representation of the main dopaminergic pathways: a) nigrostriatal, b) mesocortical and mesolimbic, c) tuberoinfundibular and d) descending pathway. St, striatum; PT, pretectum; Thal, Thalamus; PTh, prethalamus; PHy, peduncular hypothalamus; THy, terminal hypothalamus; SNc, substantia nigra pars compacta; VTA, ventral tegmental area; NAcc: nucleus accumbens; Arc, arcuate nucleus; ME, median eminence.

Among the main targets of the dopamine is the striatum, which is the main input structure of the basal ganglia circuitry and one of the brain regions with the highest expression of dopamine receptors. Basal ganglia nuclei are composed by the striatum, including caudate-putamen and nucleus accumbens, the internal and external segment of the globus pallidus, the subthalamic nucleus and the substantia nigra, and are associated with a variety of functions, but are best-known for their role in motor control (Kreitzer and Malenka, 2008; Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012; Steiner and Tseng, 2016). Striatum receives excitatory inputs from the cortex and thalamus and it is the origin of the direct and indirect pathways, two parallel cortico-basal ganglia-thalamo-cortical loops that control movement in opposing ways (Figure 9). Dopamine differentially modulates direct and indirect pathways activity binding to D1 (direct pathway) and D2 (indirect pathway) receptors of the striatal output neurons (medium spiny neurons, MSNs). Direct pathway facilitates initiation and execution of movement projecting from striatum to substantia nigra pars reticulata (SNr) and the internal portion of globus pallidus (GPi). On the other hand, indirect pathway sends their primary striatal projections to the external portion of globus pallidus (GPe) leading to an inhibition of the movement (Tritsch and Sabatini, 2012; Steiner and Tseng, 2016) (Figure 9).

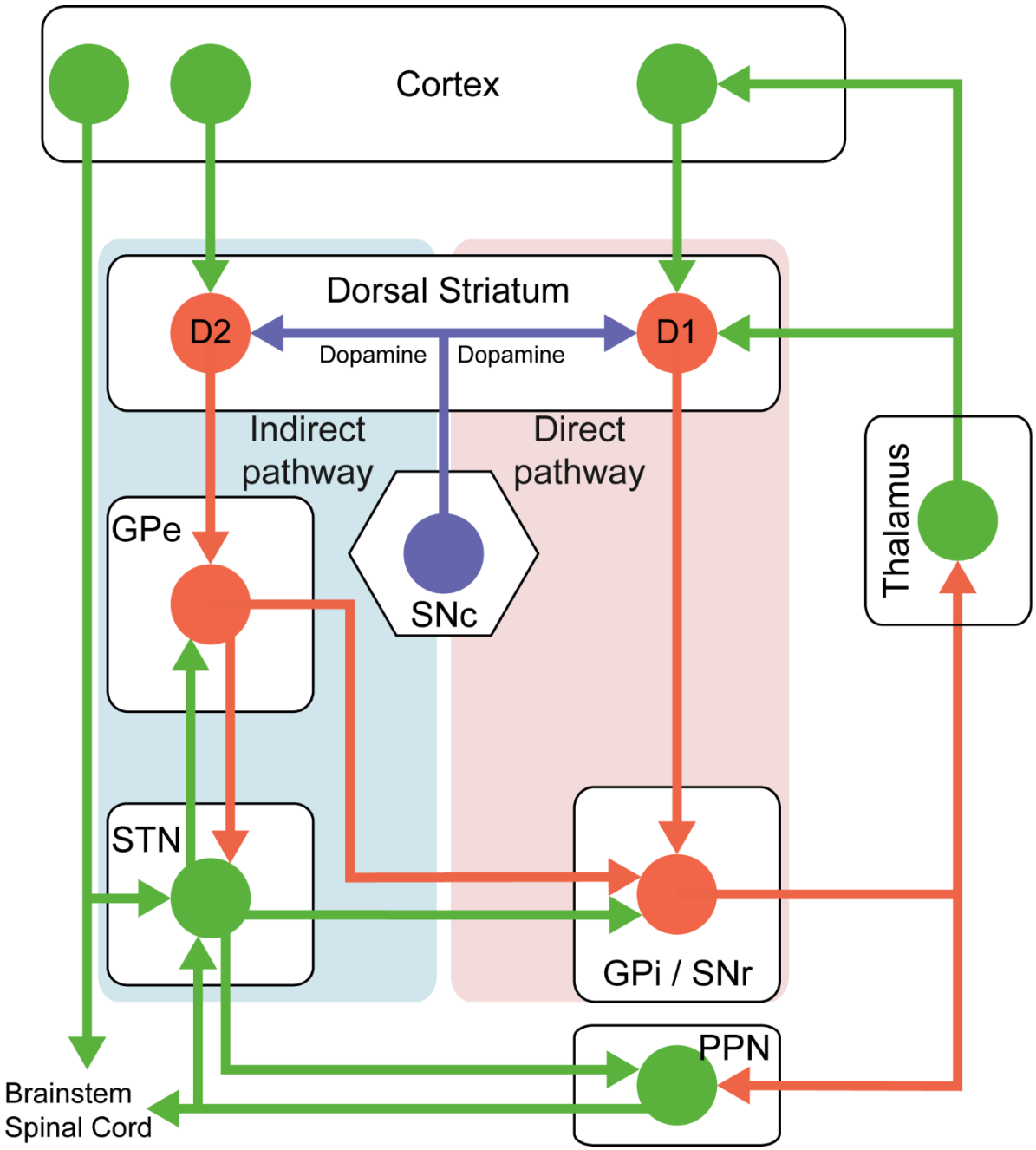


Figure 9. Diagram of motor control circuitry. Green arrows indicate excitatory (glutamatergic) connections; red arrows represent inhibitory (γ-aminobutyric acid [GABA]-ergic) connections. Dark blue arrows indicate dopaminergic connections, which act as excitatory when targeting D1-containg neurons and as inhibitory when targeting D2-containg neurons. GPe and GPi, external and internal segments of the globus pallidus; STN, subthalamic nucleus; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; PPN, pedunculopontine nucleus (Adapted from DeLong and Wichmann (2007); and Flores-Barrera, personal communication, May 2019).

In STUDY 3 of the present thesis we aimed to explore the neural mechanisms involved in exercise capacity during adolescence. The striatum has been identified as a major site of activity-dependent synaptic plasticity, and among all the neuromodulators regulating circuit function and plasticity in the mammalian brain, dopamine (DA) stands out as one of the most behaviorally powerful (Kreitzer and Malenka, 2008; Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012). The central dopamine is well known to play a key role in the development and maturation of neural circuits associated with cognitive and motor learning during adolescence through the activation of D1 and D2 receptors (Wickens et al., 2003; Willuhn and Steiner, 2008; Crisp et al., 2012; Tritsch and Sabatini, 2012; Caballero et al., 2016; Caballero and Tseng, 2016; Dwyer and Leslie, 2016; Steiner and Tseng, 2016). Furthermore, genetic and pharmacological studies have observed increments in dopamine content and release in response to physical activity (Fisher et al., 2004; Petzinger et al., 2007; Foley and Fleshner, 2008; Beeler et al., 2010; Petzinger et al., 2015) whereas reduction in dopamine function could limit the activation of motor circuits and negatively impact the exercise capacity (Foley and Fleshner, 2008; Balthazar et al., 2010; Cordeiro et al., 2017). Therefore, the central dopamine system and the dopaminergic modulation of striatum are postulated as candidates to play an important role in the regulation of locomotor performance during adolescence.

Thus, once the rodent model of exercise was optimized in STUDY 1 and 2, the aim of the STUDY 3 was to determine whether the dopaminergic system contributes to modulate exercise capacity during adolescence through the activation of D1 or D2 receptors, and whether this modulation occurs through the striatum. To test this, adolescent rats were exposed to an 8-days habituation period in the running wheel before evaluating their locomotor performance in an incremental exercise test. During the test dopamine receptors were pharmacologically blocked by systemic and intrastriatal administration of D1 and D2 dopamine receptors antagonists. Selective blockade of striatal dopamine receptors was achieved implanting intracerebral

cannulas in the dorsal striatum of the adolescent rats by stereotaxic surgery (Figure 10, see STUDY 3 for further methodological details). Our results showed that the level of locomotor response to incremental loads of forced running in adolescent rats is dopamine dependent and mechanistically linked to the activation of D1 and D2 receptors. It was also observed that striatal blockade of D1, but not D2 receptors, reduced the response during the incremental test, which implies that a recruitment of striatal D1 and extrastriatal D2 receptor signaling is needed to sustain proper levels of locomotor performance during forced running.

Taken together, the results from STUDY 3 indicate that coordinated modulation of striatal and extra-striatal neural circuits' activity by dopamine could play a major role in adjusting the level of locomotor response during forced running. Our results also suggest that the dopaminergic system might be recruited during the habituation phase, potentiating corticostriatal transmission and plasticity and leading to an enhancement of the motor coordination and the response to incremental loads of running. Further studies are warranted to unravel the neural interactions associated with exercise capacity, including the involvement of other motor-related neurotransmitter systems and brain structures.

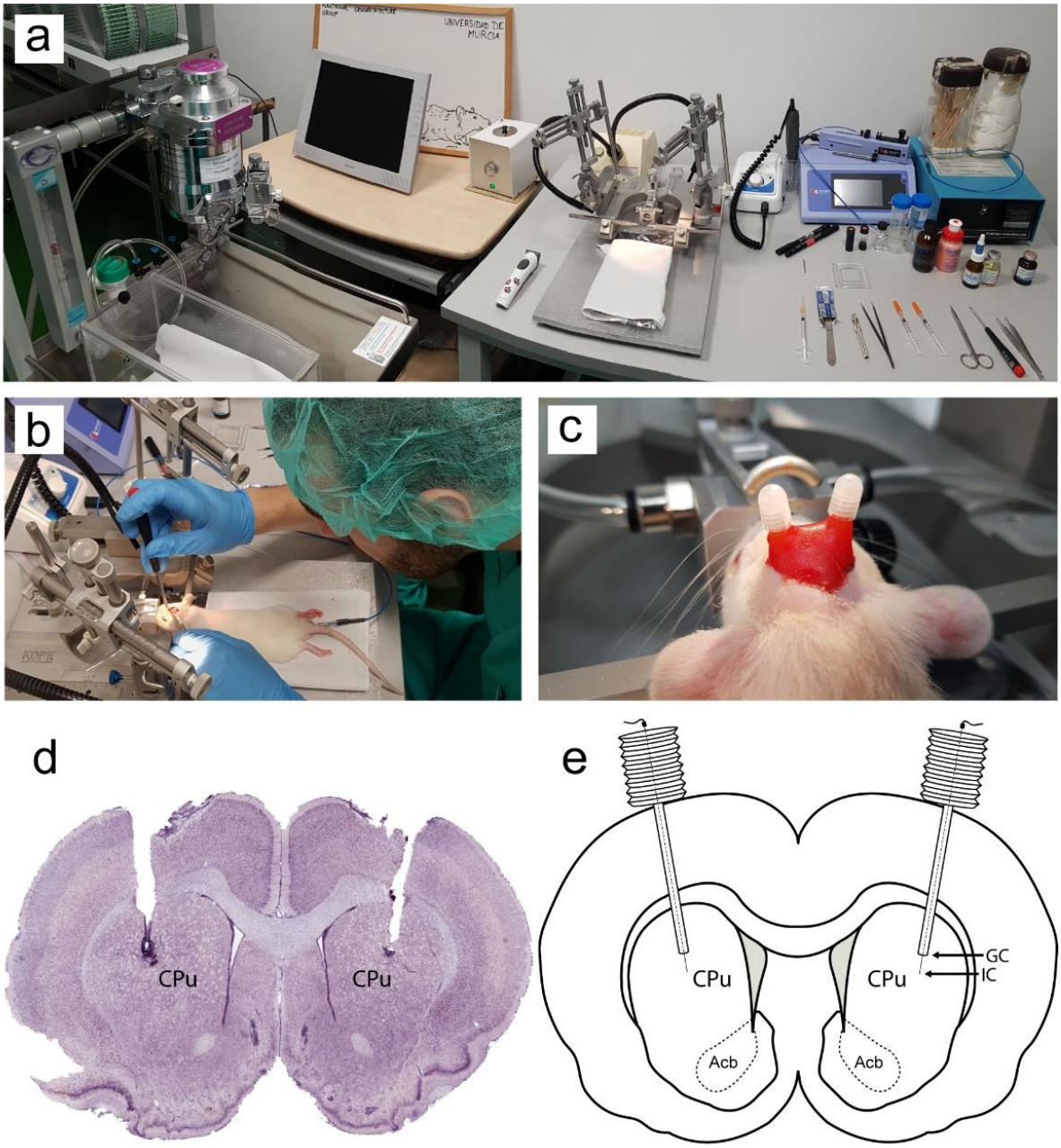


Figure 10. Photograph of a) the stereotaxic equipment b) the stereotaxic surgery procedure, and c) Sprague-Dawley rat just after implantation of intracerebral cannulas; d) Example of Nissl-stained coronal section showing the site of injection (CPu, caudate-putamen, striatum) and e) schematic diagram of coronal section showing the location of the implanted guide cannula (GC) and the infusion cannula (IC).

AIMS AND OBJECTIVES

GENERAL AIM:

Develop an exercise model in rodents to determine the neurobiological mechanisms underlying physical activity

STUDY 1:

AIM:

1. Develop and optimize a reproducible and reliable rodent model of exercise in forced running wheel.

OBJECTIVES:

1.1. Achieve successful locomotor responses, in terms of intensity and volume of running, by adolescent rats in forced wheel system.

1.2. Reduce the number of rats rejecting running in forced running wheel.

1.3. Determine the effect of a habituation protocol, with gradual increase of the training load, in the locomotor performance assessed by a maximal incremental test.

1.4. Determine long-term effects of the habituation protocol (1, 3, 31 and 33 days) on the locomotor performance during an incremental running test in forced wheel.

STUDY 2:

AIM:

2. Determine whether the exercise habituation protocol in forced running wheel alters plasmatic and hypothalamic stress biomarkers.

OBJECTIVES:

2.1. Determine whether plasmatic lactate is increased during the habituation sessions.

2.2. Determine whether plasmatic glucose is increased during the habituation sessions.

2.3. Determine changes in plasmatic lactate and glucose after an incremental exercise test comparing habituated and non-habituated (wheel and cage control) rats, and their locomotor responses during the test

2.4. Determine chronic transcriptomic changes after habituation in Crh and Avp mRNA expression in the hypothalamus.

STUDY 3:

AIM:

3. Determine whether the dopaminergic system modulates exercise capacity during adolescence.

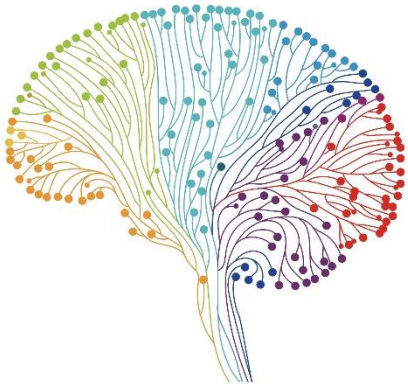
OBJECTIVES

3.1. Determine the effect of systemic administration of dopamine D1 (SCH23390) and D2 (raclopride) receptor antagonists on the level of locomotor performance of adolescent rats.

3.2. Determine the effect of intrastriatal administration of dopamine D1 (SCH23390) and D2 (raclopride) receptor antagonists on the level of locomotor performance of adolescent rats.

PUBLICATIONS

STUDY 1



Habituation Training Improves Locomotor Performance in a Forced Running Wheel System in Rats

A. Toval, R. Baños, E. De la Cruz, N. Morales-Delgado, J. Pallarés, A. Ayad, K.Y. Tseng & J.L. Ferran

Journal: Frontiers in behavioral neuroscience

Doi: 10.3389/fnbeh.2017.00042

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5340750/

Abstract: Increasing evidence supports that physical activity promotes mental health; and regular exercise may confer positive effects in neurological disorders. There is growing number of reports that requires the analysis of the impact of physical activity in animal models. Exercise in rodents can be performed under voluntary or forced conditions. The former presents the disadvantage that the volume and intensity of exercise varies from subject to subject. On the other hand, a major challenge of the forced training protocol is the low level of performance typically achieved within a given session. Thus, the aim of the present study was to evaluate the effectiveness of gradual increasing of the volume and intensity (training habituation protocol) to improve the locomotor performance in a forced running-wheel system in rats. Sprague-Dawley rats were randomly assigned to either a group that received an exercise training habituation protocol, or a control group. The locomotor performance during forced running was assessed by an incremental exercise test. The experimental results reveal that the total running time and the distance covered by habituated rats was significantly higher than in control ones. We conclude that the exercise habituation protocol improves the locomotor performance in forced running wheel.

STUDY 2

Hypothalamic Crh/Avp, Plasmatic Glucose and Lactate Remain Unchanged During Habituation to Forced Exercise

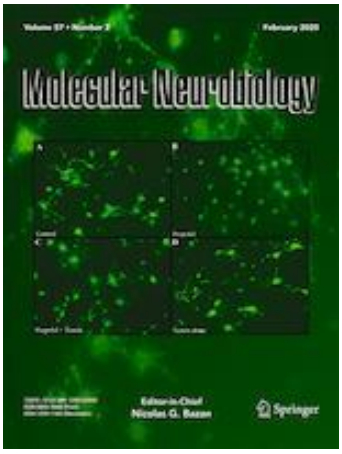
A. Toval, F. Vicente-Conesa, P. Martínez-Ortega, Y. Kutsenko, N. Morales-Delgado, D. Garrigos, A. Alonso, B. Ribeiro Do Couto, M. Popović & J.L. Ferran

Journal: Frontiers in Physiology

Doi: 0.3389/fphys.2020.00410 https://pubmed.ncbi.nlm.nih.gov/32499715/

Abstract: It has been demonstrated that physical activity contributes to a healthier life. However, there is a knowledge gap regarding the neural mechanisms producing these effects. One of the keystones to deal with this problem is to use training programs with equal loads of physical activity. However, irregular motor and stress responses have been found in murine exercise models. Habituation to forced exercise facilitates a complete response to a training program in all rodents, reaching the same load of physical activity among animals. Here, it was evaluated if glucose and lactate - which are stress biomarkers - are increased during the habituation to exercise. Sprague-Dawley rats received an 8-days habituation protocol with progressive increments of time and speed of running. Then, experimental and control (non-habituated) rats were subjected to an incremental test. Blood samples were obtained to determine plasmatic glucose and lactate levels before, immediately after and 30 min after each session of training. Crh and Avp mRNA expression was determined by two-step qPCR. Our results revealed that glucose and lactate levels are not increased during the habituation period and tend to decrease toward the end of the protocol. Also, Crh and Avp were not chronically activated by the habituation program. Lactate and glucose, determined after the incremental test, were higher in control rats without previous contact with the wheel, compared with habituated and wheel control rats. These results suggest that the implementation of an adaptive phase prior to forced exercise programs might avoid non-specific stress responses.

STUDY 3



Dopaminergic Modulation of Forced-Running Performance in Adolescent Rats: Role of Striatal D1 and Extrastriatal D2 Dopamine Receptors

A. Toval, D. Garrigos, Y. Kutsenko, M. Popović, B. Ribeiro Do-Couto, N. Morales-Delgado, K.Y. Tseng, J.L. Ferran

Journal: Molecular Neurobiology

Doi: 10.1007/s12035-020-02252-2

https://link.springer.com/article/10.1007%2Fs12035-020-02252-2

Abstract: Improving exercise capacity during adolescence impacts positively on cognitive and motor functions. However, the neural mechanisms contributing to enhance physical performance during this sensitive period remain poorly understood. Such knowledge could help to optimize exercise programs and promote a healthy physical and cognitive development in youth athletes. The central dopamine system is of great interest because of its role in regulating motor behavior through the activation of D1 and D2 receptors. Thus, the aim of the present study is to determine whether D1 or D2 receptor signaling contributes to modulate the exercise capacity during adolescence and if this modulation takes place through the striatum. To test this, we used a rodent model of forced running wheel that we implemented recently to assess the exercise capacity. Briefly, rats were exposed to an 8-day period of habituation in the running wheel before assessing their locomotor performance in response to an incremental exercise test, in which the speed was gradually increased until exhaustion. We found that systemic administration of D1-like (SCH23390) and/or D2-like (raclopride) receptor antagonists prior to the incremental test reduced the duration of forced running in a dose-dependent manner. Similarly, locomotor activity in the open field was decreased by the dopamine antagonists. Interestingly, this was not the case following intrastriatal infusion of an effective dose of SCH23390, which decreased motor performance during the incremental test without disrupting the behavioral response in the open field. Surprisingly, intrastriatal delivery of raclopride failed to impact the duration of forced running. Altogether, these results indicate that the level of locomotor response to incremental loads of forced running in adolescent rats is dopamine dependent and mechanistically linked to the activation of striatal D1 and extra-striatal D2 receptors.

CONCLUSIONS

CONCLUSIONS FROM STUDY 1:

1.1. All the rats reached similar intensities and volume of running during all sessions of the habituation protocol. Thus, the forced running wheel system allows all animals to be exposed to the same training load (intensity and volume), guaranteeing experimental reproducibility.

1.2. 100% of the habituated rats achieve a regular running pattern after habituation.

1.3. A progressive phase of habituation to exercise significantly improves motor performance during an incremental running test. All the habituated rats covered higher distances and reached higher speeds than non-habituated rats during the test.

1.4. The locomotor improvement achieved after habituation is transient as the running speed and time decreases progressively over time within a period of 30 days.

CONCLUSIONS FROM STUDY 2:

2.1. The plasmatic stress biomarker lactate remain unchanged during the habituation sessions, therefore discarding a stress response linked to this biomarker during the habituation program.

2.2. The plasmatic stress biomarker glucose remain unchanged during the habituation sessions, therefore discarding a stress response linked to this biomarker during the habituation program.

2.3. Habituated rats spent a longer time in the incremental test than non-habituated rats (cage and wheel control), but the total running time of the wheel control group was significantly higher than the cage control group. Despite this, habituated and wheel control rats showed lower increases of both plasmatic lactate and glucose concentrations than the cage control group after the incremental running, which

implies that a progressive exposure to the wheels minimize non-specific stress reactions in forced models.

2.4. Hypothalamic *Crh* and *Avp* mRNA expression remained unchanged after the habituation protocol, suggesting that habituation is not producing chronic activation of the hypothalamic-pituitary-adrenal axis or the sympathetic adrenomedullar axis, main source of stress responses.

CONCLUSIONS FROM STUDY 3:

3.1. Systemic blockade of both, D1 and D1 dopamine receptors, reduced the response during the incremental test, which implies that the level of locomotor response to incremental loads of forced running in adolescent rats is dopamine-dependent and mechanistically linked to the activation of D1 and D2 receptors

3.2. Striatal blockade of D1, but not D2 receptors, reduced the response during the incremental test which implies a recruitment of striatal D1 and extra-striatal D2 receptor signaling to sustain proper level of locomotor performance during forced running in adolescent rats.

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APPENDIXES

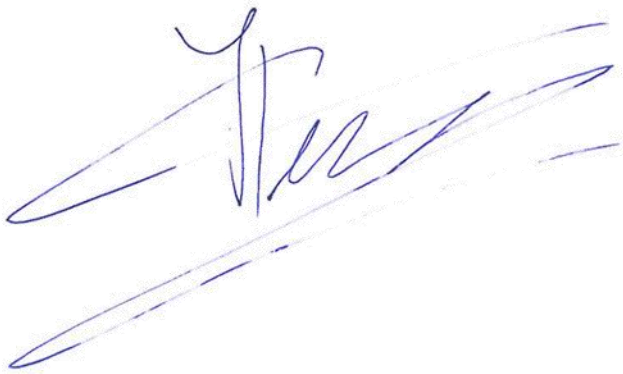
APPENDIX 1: Supervisors Approval

UNIVERSIDAD DE
MURCIA

D. José Luis Ferrán Bertone, Profesor Titular de Universidad del Área de Anatomía y Embriología Humana en el Departamento de Anatomía Humana y Psicobiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Neural mechanisms mediating locomotor performance during forced wheel running in adolescent rats. Stress responses and role of the dopaminergic system / Mecanismos neurales que median el rendimiento motor en rueda forzada en ratas adolescentes. Respuestas de estrés y rol del sistema dopaminérgico", realizada por D. José Ángel Toval Sánchez, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 8 de Febrero de 2021



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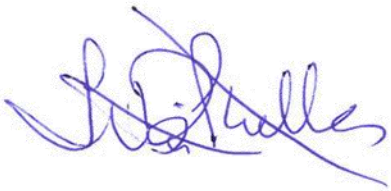
Appendix 1: Supervisors approval

UNIVERSIDAD DE
MURCIA

D. Luis Puelles López, Catedrático de Universidad del Área de Anatomía y Embriología Humana en el Departamento de Anatomía Humana y Psicobiología, AUTORIZA:

La presentación de la Tesis Doctoral titulada "Neural mechanisms mediating locomotor performance during forced wheel running in adolescent rats. Stress responses and role of the dopaminergic system / Mecanismos neurales que median el rendimiento motor en rueda forzada en ratas adolescentes. Respuestas de estrés y rol del sistema dopaminérgico", realizada por D. José Ángel Toval Sánchez, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

En Murcia, a 8 de Febrero de 2021



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APPENDIX 2: Research stays

UNIVERSIDAD DE MURCIA | ESCUELA INTERNACIONAL DE DOCTORADO

MARIA DOLORES HIDALGO MONTESINOS, SECRETARIA DE LA ESCUELA INTERNACIONAL DE DOCTORADO DE LA UNIVERSIDAD DE MURCIA Y CON EL VISTO BUENO DEL VICERRECTOR DE INVESTIGACIÓN Y TRANSFERENCIA

HACE CONSTAR:

Que **D. José Ángel Toval Sánchez**, con nº D.N.I: 48655369L, estudiante del programa de Doctorado en INTEGRACIÓN Y MODULACIÓN DE SEÑALES EN BIOMEDICINA por la Universidad de Murcia, durante su formación doctoral y como consta en su expediente **ha acreditado las siguientes estancias:**

(1) Laboratorio de Conducta Animal y Neurociencia (LCAyN), Departamento de Psicología Experimental de la Facultad de Psicología de la Universidad de Sevilla. Duración de la estancia: desde el 25 Junio al 31 de Julio de 2017. Investigador receptor: Dr. Manuel Portabella García. Objetivo de la estancia: Aprendizaje de metodología y aplicaciones de las cirugías estereotáxicas.

(2) Laboratory of Molecular Neurobiology, University of Warsaw, Polonia. Duración de la estancia: desde el 1 de Enero al 15 de Febrero de 2018. Investigador receptor: Dra Marta B Wisniewska. Objetivo de la estancia: Aprendizaje de los fundamentos y producción de AAVs y optogenética.

(3) Department of Anatomy and Cell Biology, University of Illinois at Chicago. Duración de la estancia: desde 1 de Abril hasta 1 de Junio 2019. Investigador receptor: Dr. Kuei Tseng. Objetivo de la estancia: Aprendizaje de las técnicas experimentales: canulación cerebral mediante cirugías estereotáxicas y principios de la electrofisiología invivo

UNIVERSIDAD DE MURCIA | ESCUELA INTERNACIONAL DE DOCTORADO

Y para que conste y surta los efectos oportunos donde proceda, se expide y firma la presente en

Murcia, 26 de noviembre de 2019

Firmado con certificado electrónico reconocido.
La información sobre el firmante, la fecha de firma y el código de verificación del documento se encuentran disponibles en los márgenes izquierdo e inferior

Laboratorio de Conducta Animal
y Neurociencia (LCAyN)
Departamento de Psicología Experimental
Facultad de Psicología

31 de julio de 2017

Manuel Portavella García, Profesor Titular de Universidad del Área de Psicobiología adscrito al Departamento de Psicología Experimental, en la Facultad de Psicología de la Universidad de Sevilla INFORMA que:

D. José Ángel Toval Sánchez, con DNI: 48655369-L, estudiante de Doctorado, que desarrolla su trabajo actualmente en el Departamento de Anatomía Humana y Psicobiología de la Universidad de Murcia, ha realizado una estancia de investigación en el Laboratorio de Conducta Animal y Neurociencia (LCAyN), de la Facultad de Psicología de la Universidad de Sevilla, durante el periodo de un mes comprendido entre el 25 de junio al 31 de julio de 2017.

Durante dicho periodo, se formó tanto en el manejo de la cirugía estereotáxica para la realización de microinyeccion, estimulación, lesión cerebral y electrofisiología de registro de campo eléctrico y microdiálisis en roedores, como en técnicas de registro conductual y condicionamiento. Tales como: condicionamiento operante en cajas de Skinner, aversión condicionada al sabor e inhibición latente, y tests de ansiedad y actividad en campo abierto y termografía *in vivo*.

En Sevilla a 31 de Julio de 2017

Fdo: Dr. Manuel Portavella García

Profesor Titular de Universidad
Responsable del Bienestar Animal del LCAyN
Departamento de Psicología Experimental
Universidad de Sevilla
c/Camilo José Cela s/n
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Warsaw, 17/02/2018

CERTIFICATE OF SCIENTIFIC VISIT

To whom it may concern:

This letter is to confirm that Mr. José Ángel Toval Sanchez (ID number: 48655369L) stayed in my laboratory 6 weeks (from January 1, 2018 to February 15, 2018), under my supervision at the Laboratory of Molecular Neurobiology, University of Warsaw. This scientific exchange is a part of his doctoral studies. During this Mr. Toval was trained in optogenetics and production of adeno-associated viruses. He also prepared samples that are to be used in Spain in experiment related to his doctoral project.

Yours faithfully,

M. Wisniewska

THE
UNIVERSITY OF
ILLINOIS
COLLEGE OF
MEDICINE
CHICAGO PEORIA ROCKFORD URBANA

DEPARTMENT OF ANATOMY
AND CELL BIOLOGY
CHICAGO

Kuei Y. Tseng, MD & PhD
Professor
tsengky@uic.edu

May 31st, 2019

To whom it may concern,

This letter is to certify José Ángel Toval Sanchez (ID number: 48655369L) received research training for a period of 2 months (from April 1, 2019 to June 1, 2019) in my laboratory at the University of Illinois at Chicago (UIC) – College of Medicine as a visiting scholar. The scientific training Ángel received in my laboratory is part of his PhD/doctoral studies. During his visit, Ángel gained proficiency on experimental design and implementation of electrophysiological recordings in vivo, stereotaxic surgeries, cannulation and infusion of agonists and antagonists into the brain including the delivery of DREADD.

Should you need further information, do not hesitate to contact me through any of the means below.

Sincerely yours,

Kuei Y Tseng, MD & PhD
Professor, Department of Anatomy and Cell Biology
Director of Graduate Studies, Neurobiology Research Concentration of GEMS
College of Medicine, University of Illinois at Chicago
Chicago, IL 60612
Email: tsengky@uic.edu

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APPENDIX 3: Contribution to the published studies

UNIVERSIDAD DE MURCIA

INFORME JUSTIFICATIVO SOBRE LA CONTRIBUCIÓN DEL DOCTORANDO A LAS PUBLICACIONES INTEGRADAS EN LA TESIS DOCTORAL

D. José Angel Toval Sánchez, a efectos de obtención de la autorización de la Comisión General de Doctorado para la presentación de la Tesis Doctoral "Neural mechanisms mediating locomotor performance during forced wheel running in adolescent rats. Stress responses and role of the dopaminergic system", justifica su contribución a las publicaciones que la integran, en los aspectos que se detallan a continuación, con el aval de sus directores, D. José Luis Ferran Bertone y D. Luis Puelles López.

Artículo 1:

Toval A, Baños R, De la Cruz E, Morales-Delgado N, Pallares JG, Ayad A, Tseng KY, Ferran JL. 2017. Habituation Training Improves Locomotor Performance in a Forced Running Wheel System in Rats. Front Behav Neurosci 11:42. doi: 10.3389/fnbeh.2017.00042

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

- Contextualización teórica, análisis del estado de la cuestión y desarrollo de experimentos piloto
- Conceptualización y diseño de los experimentos
- Coordinación y realización de los experimentos (entrenamientos de los roedores, manutención y cuidado de los animales, test de rendimiento motor y toma de datos)
- Análisis e interpretación de los resultados y discusión
- Redacción del manuscrito

Artículo 2:

Toval A, Vicente-Conesa F, Martínez-Ortega P, Kutsenko Y, Morales-Delgado N, Garrigos D, Alonso A, Do Couto BR, Popović M, Ferran JL. 2020. Hypothalamic Crh/Avp, plasmatic glucose and lactate remain unchanged during habituation to forced exercise. Frontiers in Physiology 11. doi: 10.3389/fphys.2020.00410

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

- Contextualización teórica, análisis del estado de la cuestión y desarrollo de experimentos piloto
- Conceptualización y diseño de los experimentos

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- Realización de los experimentos (entrenamientos de los roedores, manutención y cuidado de los animales, extracción de sangre y medición de glucosa y lactato, extracción de cerebros y procesamiento mediante hibridación *insitu* y qPCR)
- Análisis e interpretación de los resultados y discusión
- Redacción del manuscrito

Artículo 3:

Toval A, Garrigos D, Kutsenko Y, Popović M, Do-Couto BR, Morales-Delgado N, Tseng KY, Ferran JL. 2021. Dopaminergic Modulation of Forced Running Performance in Adolescent Rats: Role of Striatal D1 and Extra-striatal D2 Dopamine Receptors. Molecular Neurobiology. doi: 10.1007/s12035-020-02252-2

El doctorando, y primer autor de esta publicación, ha contribuido de forma relevante en:

- Contextualización teórica, análisis del estado de la cuestión y desarrollo de experimentos piloto
- Conceptualización y diseño de los experimentos
- Aprendizaje y puesta a punto de las técnicas experimentales utilizadas (modelo roedor de actividad física, cirugía estereotáxica y administración de fármacos de forma sistémica e intracerebral)
- Realización de los experimentos (entrenamientos de los roedores, manutención y cuidado de los animales, administración de fármacos, canulaciones cerebrales mediante cirugías estereotáxicas, test de rendimiento motor en rueda forzada y test de actividad ambulatoria en campo abierto -open field-, extracción y procesamiento de cerebros)
- Análisis e interpretación de los resultados y discusión
- Redacción del manuscrito

Fdo: el doctorando
José Angel Toval Sánchez

Con el visto bueno de los directores:

V.° B.°: José Luis Ferrán Bertone

V.° B.° : Luis Puelles López

APPENDIX 4: Favorable report of the Ethics Committee

UNIVERSIDAD DE MURCIA | Vicerrectorado de Investigación e Internacionalización

CEEA Comité Ético de Experimentación Animal

CAMPUS MARE NOSTRUM

Código CEEA: 693/2020	INFORME DE COMITÉ ÉTICO

INFORME DEL COMITÉ ÉTICO DE EXPERIMENTACIÓN ANIMAL

DATOS DEL CENTRO

Nombre: **CEEA Universidad de Murcia**

Número de Registro del Centro: REGA **ES300305440012**

Título de la Tesis Doctoral: *"Mecanismos neurales que median el rendimiento motor en rueda forzada en ratas adolescentes. Circuitos de estrés y rol del sistema dopaminérgico"*

Doctorando: D. José Ángel Toval Sánchez
Director de la Tesis Doctoral: D. José Luis Ferrán Bertone

Título del Proyecto en el que se enmarca la Tesis Doctoral: *"Neural bases of resistance to fatigue and reduction of adipose tissue in an exercise forced program"* y *"Determinación de los blancos dopaminérgicos y de los niveles de carga de entrenamiento en la respuesta a la habituación en rueda forzada en rata"*
Investigador responsable: D. José Luis Ferrán Bertone

Aspectos que han sido considerados para su evaluación:

- Capacitación del personal investigador
- Idoneidad del procedimiento en relación a los objetivos del estudio.
- Metodología empleada
- Posibilidad de conseguir conclusiones válidas con el menor nº posible de animales
- Consideraciones de métodos alternativos
- Idoneidad de las especies seleccionadas
- Supervisión, Criterios de Punto Final y Finalización del Procedimiento

Una vez evaluado el procedimiento antes mencionado, atendiendo a los puntos indicados y de conformidad con lo acordado el día cuatro de febrero de dos mil veintiuno[1], el Comité Ético de Experimentación Animal de la Universidad de Murcia, INFORMA **FAVORABLEMENTE** sobre la realización de dicho procedimiento.

D.ª Carmen Lagares Martínez — Secretaria CEEA

D. ª María Senena Corbalán García — Presidenta CEEA

[1] A los efectos de lo establecido en el art. 19.5 de la Ley 40/2015 de 1 de octubre de Régimen Jurídico del Sector Público (B.O.E. 02-10), se advierte que el acta de la sesión citada está pendiente de aprobación