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HYPOADENOSINERGIC PATHOLOGIES IN A RAT MODEL OF THE WILLIS-EKBOM DISEASE

Dissertação no âmbito do Mestrado em Bioquímica orientada pelo Professor Doutor Attila Köfalvi e pelo Professor Doutor Ângelo Tomé e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Outubro 2020

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Dissertation presented to the Faculty of Science and Technology of the University of Coimbra. The work was performed in Neuromodulation and Metabolism Lab at CNC - Center for Neuroscience and Cell Biology,University of Coimbra, under the supervision of Doctor Attila Köfalvi and co-supervision of Doctor Ângelo Tomé.

2020

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Acknowledgements/Agradecimentos

O primeiro agradecimento terá de ser ao meu orientador, Dr. Attila Köfalvi, por todas as oportunidades, seja de realizar esta tese, como pelo trabalho desenvolvido desde a rotação laboratorial, por todos os ensinamentos, todo o apoio, carinho, ajuda e tremenda confiança que demonstrou ao longo deste ano. À Dra. Samira Ferreira, por todos os conhecimentos que me passou, por toda a paciência, ajuda e confiança que demonstrou desde início. Ao Professor Ângelo por ser meu orientador interno e por estar sempre disponível para ajudar. Ao Dr. Henrique Silva pela passagem de conhecimento de eletrofisiologia. Ao Dr. Rodrigo Cunha, por me ter recebido sempre bem. Aos restantes membros do grupo de Neuromodulação do CNC que, de alguma forma me ajudaram no dia-a-dia em laboratório.

O segundo agradecimento, aos meus pais, por "abrirem os cordões à bolsa" e tornarem isto possível, por todo o apoio e esforços que fizeram pela "filhota". Obrigada mãe e avó Béu por me encherem a barriga de comidinha de tacho! Às minhas tias e avó, Ana, Cidália e Luísa por todo apoio, seja do trabalho, como das notas extra que dão à vossa "netinha" e até pelo grande interesse de querer saber dos falecidos. À minha peludinha, a estrelinha mais brilhante.

O terceiro, ao que Coimbra me deu de melhor:

Às minhas tábuas de cinco e muitos mais anos. As que foram para o norte, Marta Lourenço e Raquel Pinto, mas que estiveram sempre à distância de um ícone de chat e de um bom chá. À Marina Barbosa, pelas pipocas covid-free e por me encher de bolos e chá durante este ano. Amiga que alimenta e apresenta gatas, é amiga para a vida! Por último, à Margarida Pereira, a amiga de todas as horas, a parceira de mestrado e o meu maior pilar nele, por toda a paciência que teve (mútuo!), por todo o apoio e companheirismo. Por todas as aventuras e momentos associados, todas as neuras e trabalhos na esplanada de casa, por todos os prints e viagens planeadas. No fim disto e, se o covid deixar, vamos a todas, amiga!

À minha madrinha de praxe e sempre amiga, Susana Costa, por estar lá, literalmente, desde o primeiro dia, por todos os apontamentos que contribuíram para estar aqui hoje e todas as "desbundas" e momentos passados juntas. Ao padrinho, Mário Coutinho, dealer de memes, companheiro de jantares e cafés. À minha pinipom, Sofia Ruivo. Obrigada pessoas, por todo o apoio, confiança e por todos os abracinhos. Somos uma "famelga" muito desnaturada, mas linda!

Por último, à Beatriz Rito, colega de licenciatura e amiga furada de mestrado, por tudo o que deixámos para o último dia, por todos os "cafés de boa língua" e toda a comida partilhada virtualmente. À Marta Barão, por acompanhar um certo grupo de "desbundeiras", por todo o

apoio e amizade desde sempre. À Bárbara Rodrigues, por ser a melhor e mais fiel “homie” da vivenda do Montarroio, pela amizade e confiança de sempre.

A todos os outros que Bioquímica e Coimbra deram a conhecer e que de alguma forma contribuíram para estes anos.

O último agradecimento, aos de Almeirim. Às amigas dos cafés e encontros muito ocasionais, Marta e Debbie. À Ana Carolina, por todos os convites para ir a Viseu (a culpa é desta menina, mas prometo que vou!), apoio, amizade e por estar sempre à distância de uma mensagem. Aos Almeirinenses Alentejanos que Coimbra conhece, Jaime, José e Catarina, pelos jantares, chats errados e todaaas as boleias.

Ao Covid-19 por me atrasar a entregar o próximo best-seller de cabeceira.

Obrigada a todos! O que vem a seguir, ninguém sabe. Mas agora? O que importa é que:

A vossa Tilde é MESTRE!

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Abbreviations List

2-AG - 2-arachidonylglycerol

A_1R - A_1 adenosine receptors

$A_{2A}R$ - A_{2A} adenosine receptors

ACh- Acetylcholine

ADA - Adenosine deaminase aminopropanesulfonic acid

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP - Adenosine triphosphate

BCA – Bicinchoninic acid

BID - Brain Iron Deficiency

BSA – Bovine serum albumin

CB_1R- Cannabinoid receptor type 1

CB_2R- Cannabinoid receptor type 2

CNS - Central nervous system

CTRL – Control

D_1R - type 1 dopamine receptors

D_2R - type 2 dopamine receptors

DAT - Dopamine transporter

DOR - δ -opioid receptors

EDTA – Ethylenediaminetetracetic acid disodium salt dihydrate

ENTs - Equilibrative nucleoside transporters

EPM – Elevated plus maze

FSC – Forward Scatter

GPCR – G proteins coupled receptors

GPi - Internal segment of Globus Pallidus

HBM – HEPES buffered medium

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IV – Intravenous

KOR - κ -opioid receptors

mGluRs - Metabotropic receptors

MOR - μ-opioid receptors

MSN - Medium spiny neurons

nAChRs - Nicotinic acetylcholine receptors

NIDA - National Institute on Drug Abuse

NMDA - N-methyl D-aspartate

OF – Open field

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

PLMS - Periodic leg movement during sleep

RLS - Restless leg syndrome

RT – Room temperature

SEM – Standard error of the mean

SNc - substantia nigra pars compacta

SNr - substantia nigra pars reticulata

SSC – Side Scatter

STN - Subthalamic nucleus

TFR – Transferrin receptor

TH - Tyrosine hydroxylase

VAChT- Vesicular acetylcholine transporter

VGluT – Vesicular glutamate transporters

WED – Willis-Ekbom Disease

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Abstract

Willis-Ekbom's disease, also known as restless leg syndrome, is a neurological disease characterized by an unsettling need of the patients to move their legs and sometimes arms, even when they are at rest. Nocturnal aggravation of symptoms strongly impar sleep quality. It is believed that at the neurophysiological level, there is a hyperarousal of the nervous system, which is associated with a hyperexcitability of the corticostriatal glutamatergic terminals in the striatum. Iron deficiency is as a major comorbidity for WED has been recognized. Iron deficiency may be restricted to the nervous tissue, which is called brain iron deficiency (BID). Both iron deficiency (and consequent anemia) and WED are more prevalent in women, which underlines the need to compare the effect of iron deficiency on the striatum of laboratory rodents of both genders.

The striatum is the main input structure for excitatory stimuli from the basal ganglia and is involved in both motor and cognitive processes. Glutamatergic corticostriatal neurotransmission is modulated by several neurotransmitters such as GABA and neuromodulators including dopamine, adenosine, opioids, acetylcholine and endocannabinoids. Impaired neuromodulation at corticostriatal synapses can lead to a variety of neurological diseases. Many studies point to the existence of changes in the adenosinergic system in patients and models of BID. Recently, a hypoadenosinergic state was described by our collaborators, caused by an increase in the density of type A_{2A} adenosine receptors and a decrease in A_1 receptor density, or both.
Thus, here I studied the possible presynaptic changes in striatal glutamatergic as well as cholinergic nerve terminals, isolated from young adult rats, after completing a four-week diet with iron poor chow (BID rats), or normal chow (controls). Both males and females were studied in order to purported gender-specific phenotypes.

First, the animals were subjected to behavioral tests to evaluate locomotor activity, anxiety and memory. Subsequently, the animals were euthanized, and their brains were collected to prepare purified striatal synaptosomes. My aims were to evaluate changes in the density of glutamatergic and cholinergic nerve terminals endowed with adenosine receptors, as well as μ-opioid receptors (MOR) – since MOR agonists are efficacious in controlling patients' sensorimotor symptoms, and finally, cannabinoid CB_1 receptors, because it is capable of modulating the functioning of both adenosine receptors and MOR via heteromerization.

Since BID was induced on the 21st postnatal day, that is, after that the brain development is largely completed, overt behavioral changes in locomotion, habituation and anxiety-like behaviour of BID rats were not observed in the open field and elevated-plus maze tests. Spatial

working memory in the Y-maze spontaneous alternation test also remained intact in the BID rats.

However, the technique “flow synaptometry” revealed strong tendencies towards a reduction in the density of MOR-positive terminals and an increase in A_{2A}R-positive terminals. Altogether, for the first time an imbalance has been documented in inhibitory and facilitatory neuromodulation at the synaptic level in individually identified nerve terminals, which will certainly contribute to understanding of the pathomechanism and the adequate pharmacotherapy of WED.

Resumo

A doença de Willis-Ekbom, também conhecida como síndrome da perna inquieta, é uma doença neurológica caracterizada pelos seus sintomas. Os portadores experienciam uma inquietante necessidade de estar em movimento, mesmo quando se encontram em repouso sendo que, há um agravamento dos sintomas no período noturno. Outro aspeto clínico é o experienciamento de pequenos episódios de despertares durante o sono, que normalmente precedem o início do movimento das pernas. Dado o estado de excitamento, acredita-se que a nível neurofisiológico exista um hiper-excitamento do sistema nervoso, mais concretamente, associado a uma hiperexcitabilidade dos terminais glutamatérgicos no estriado. A condição mais bem estudada e mais associada a WED é a deficiência de ferro que, pode não ser evidente sistemicamente e ocorrer localmente, como no cérebro. Quer a deficiência de ferro (e a anemia consequente) quer a WED são mais prevalentes em mulheres, que sublinha a necessidade de comparar o efeito de deficiência de ferro no estriado de roedores laboratoriais de ambos os géneros.

O estriado é a principal estrutura de entrada de estímulos excitatórios dos gânglios da base e encontra-se envolvido tanto em processos motores como cognitivos. A neurotransmissão de glutamato é modulada por vários neurotransmissores como o GABA e neuromoduladores como a dopamina, adenosina, opioides, acetilcolina e endocanabinóides. A existência de uma perturbação nestes sistemas de modulação pode levar a uma variedade de doenças neurológicas. Muitos estudos apontam para a existência de alterações a nível do sistema adenosinérgico em pacientes e modelos de BID. Recentemente foi descrito, pelos nossos colaboradores, um estado hipoadenosinérgico causada por um aumento na densidade dos recetores de adenosina do tipo A_{2A} e/ou uma diminuição dos recetores A_1.

Assim, recorrendo a um modelo animal (foram alvo de estudo ambos machos e fêmeas, de modo a avaliar se existe um fenótipo específico entre géneros) sujeito a uma deficiência de ferro através da comida. Primeiramente, os animais foram sujeitos a testes comportamentais para avaliar a atividade locomotora, ansiedade e memória. Mais tarde, o cérebro foi recolhido e realizou-se a purificação de sinaptossomas do estriado para realizar o objetivo desta tese: avaliar alterações de densidade dos recetores de adenosina, tal como a percentagem de co-localização entre ambos, em terminais gutamatérgicos cortico-estriatais e tálamo-estriatais e, em terminais colinérgicos,

recorrendo à técnica de “sinaptometria” de fluxo. Para além destes, também foram avaliadas alterações dos recetores μ-opióides (MOR), uma vez que agonistas do MOR são eficazes no controlo de sintomas sensorimotores dos pacientes. e canabinóide do tipo 1 (CB1R), o recetor que é capaz de modular o funcionamento tal dos receptores adenosinérgicos como do MOR via heteromerização, tal como a sua percentagem de co-localização.

Uma vez que a deficiência de ferro cerebral (DFC) foi induzido no dia 21 pós-natal, ou seja, depois de maioritariamente concluir o desenvolvimento cerebral, já não foram observadas alterações na locomoção e na habituação dos animais com BID no teste “open field”, tal como no comportamento ansioso realizado no “elevated-plus maze”.

No entanto, a técnica da sinaptometria de fluxo revelou fortes tendências para uma redução na densidade dos terminais positivos para MOR e um aumento nos terminais com o recetor facilitatório A_{2A}.

Tudo junto, observou-se pela primeira vez um desbalanço no controlo inibitório e facilitatório no nível sináptico em terminais nervosos individualmente identificados, que certamente contribuirá para o entendimento sobre o patomecanismo e a farmacoterapia adequada da WED.

1. Introduction

1.1 Restless Legs Syndrome

Restless legs syndrome (RLS) was first described by an English Physicist in 1685, Sir Thomas Willis, after observing that his patients had trouble sleeping due to uncontrolled movements of the arms and legs. Later, in 1945, a Swedish neurologist, Karl Axel Ekbom, described the same symptoms, but since he was unaware that it also affects the upper limbs, he named this condition "restless legs syndrome" (Coccagna et al., 2004). Nevertheless, Willis-Ekbom Disease (WED) is the preferred term now in modern neurology, and not only for honoring of the two scientists, but also because the patients' arms may be also affected. Hereafter, I will use the term WED in place of RLS in my Thesis.

WED is being widely studied in order to find a treatment to improve the quality of life of the patients. WED is a neurological disorder of the sensory-motor system and is associated with sleep disturbances. WED affects approximately 10% of the adult population with higher prevalence in women and the elderly but, can also affect men and at any age (Ohayon et al., 2012).

WED is characterized by the feeling of restlessness and an urgent need for movement, especially during the nocturnal period or during the rest when the aggravation of symptoms is observed. In addition to the feeling of restlessness, sometimes it can be painful and only movement or physical activity provides relief. One of the clinical phenomena is the so-called periodic leg movement during sleep (PLMS), described as the repetition of short duration episodes of repetitive movements during short time intervals. Moreover, another clinical aspect is the presence of small episodes of awakening during sleep or hyperarousal, which usually precedes the beginning of the movement of the legs, being related to, but not caused by PLMS. This state of excitement leads to the disease being classified as a sleep disorder since patients have reduced hours of sleep (less than 6 hours) without daytime sleepiness. Also, at the neurophysiologic level, WED is associated with a hyperexcitability of central nervous system (CNS) (Ferré et al., 2017; Venkateshiah & Ioachimescu, 2015).

Currently, no diagnostic method allows direct detection of the disease, rather, differential diagnosis is done based on the symptoms of the patient and his medical history (Ohayon et al., 2012).

1.1.1 Pathomechanisms of WED

The cause of WED is mostly unknown, however, the disease may have genetic roots with 50-60% heredity, thus being considered a multifactorial complex disease with genetic and non-genetic factors (Allen, 2015).

Currently, several genes have been associated with WED, and those genes have multiple roles in neurogenesis, neuronal differentiation and synaptogenesis, namely, MEIS1, BTBD9, PTPRD, MAP2K5, SKOR1, and TOX3. Interestingly, MEIS1 polymorphism is considered the most relevant risk factor, since it seems to play an important role in the motor component and, a lower expression of this gene is observed in WED patients. Also, heredity studies suggest an autosomal dominant transmission with high penetrance (Dhawan et al., 2006; Ferré, García-Borreguero, et al., 2019).

Nevertheless, in the absence of a clear genetic cause, WED is often sporadic and secondary to health conditions including end-stage renal disease, pregnancy and iron deficiency (Ferré, García-Borreguero, et al., 2019). This project will focus on the influence of iron deficiency, since recent studies blame brain iron deficiency for sporadic WED, even in the absence of systemic iron deficiency and anemia (Allen, 2015).

1.2 Brain Iron Deficiency (BID)

The chemical element iron is important for the normal performance of the CNS since it is involved in energy metabolism, neurotransmitter synthesis, myelin formation, and brain development among others. Furthermore, it presents a heterogeneous distribution in the brain, exhibiting greater concentrations in the basal ganglia and deep nuclei of the cerebellum (Mills et al., 2010). At the global level, anemia and iron deficiency are of major nutritional concerns as they may cause severe physiological and cognitive disturbances.

Since the first studies, Ekbom observed that most of the WED patients had iron deficiency and that iron intravenous replacement successfully treated their condition. Thus, WED is more prevalent (about ~30%) in patients with changes in iron homeostasis, however, not all WED patients have a systemic iron deficiency, but an exclusive deficiency in brain tissue (Allen, 2015; Connor et al., 2011; Earley et al., 2014).

The brain obtains iron from the blood stream via transport through the endothelial cells of the blood-brain barrier. This transport involves various proteins including ferritin, which is downregulated in WED, as well as the natural resistance-associated macrophage protein 2, ferroportin, transferrin and its receptor, which are upregulated in (Connor et al., 2011). BID can be safely documented based on increased levels of transferrin and transferrin receptors; and by the decrease in ferritin, the protein responsible for its storage, in the brain tissue and the cerebrospinal fluid of iron deficient patients and also in animal models (Connor et al., 2011; Ferré, García-Borreguero, et al., 2019)

1.2.1 Animal Models

It is possible to create laboratory rodent models of BID, which have face, predictive and construct validity for WED, including changes in dopaminergic system and the hyperarousal (Ferré, García-Borreguero, et al., 2019; Quiroz et al., 2010).

One way to induce BID in pups is through a low iron diet, which can be administered in dams, from pregnancy until mid-lactation or, just when lactation begins. In the first case, pups will acquire a mild iron deficiency, as only a few brain structures will show a decrease in iron content. On the other hand, in the second case, the offspring will acquire a severe decrease in the brain iron content. The great disadvantage of these two models is a remarkable phenotype in the offsprings. This is because the induction of iron deficiency so early in life is inevitably associated with alterations in the development of the brain that largely completes around the postnatal day 12 (Beard et al., 2006).

An alternative rat model was previously established by our NIDA collaborators. In detail, Sprague-Dawley pups of both sexes receive iron deficient chow for 4 weeks, starting on the day of the weaning, which is in our case was the arbitrarily chosen day 21 of post-natal life. Since by post-natal day 21, the rats already have a fairly developed

brain, BID does to trigger a marked phenotype in this model (Quiroz et al., 2010), which is our interest when considering the principles of the 3Rs.

1.2.2 Association of BID with Other Diseases

According to Rudy et al (2017), rodents exposed to iron deficiency postnatally have increased susceptibility to seizures. Epilepsy is a neurological disease that affects mental and physical functions. It is mostly characterized by episodes of short- or long-lasting seizures, depending on the severity of the disease (Rudy & Mayer-Proschel, 2017)

When seizure is experienced, several changes occur to the CNS, especially to the hippocampus and the cerebral cortex, such as an increase in adenosine extracellular levels secondary to A_1R downregulation and $A_{2A}R$ upregulation, which consequently lead to increased release of glutamate (Canas et al., 2018). This can be considered, similarly to WED, a hyperarousal condition, as they share putative disturbances in inhibitory control of glutamatergic terminals, but in different brain structures. Thus, given these data, it is hypothesized that BID may also affect hippocampal excitability, and may also be related to pathological mechanisms of other neurological diseases such as migraine (Eidlitz-Markus et al., 2017; Pamuk et al., 2016). Therefore, it is not surprising that several anticonvulsants appear to be effective in the pharmacotherapy of WED (See 1.6).

These observations together with the principle of "Reduction" of the 3Rs' prompted me to assess the possible inhibitory/excitatory imbalance in the density of GABA, and glutamatergic nerve terminals and some of their neuromodulator receptors in the cortex and the hippocampi of the same rats I have been using for the execution of my Master project. However, those experiments are beyond the focus of this Thesis, and will not be presented here.

1.3 Treatments for WED

Currently there are several palliative treatments for idiopathic WED to provide a higher quality of life for the patients. The existing treatments for idiopathic WED include L-DOPA and dopamine receptor agonists, α2δ ligands, anticonvulsants, opioids

and benzodiazepines. However, these medicines do not cure the pathomechanisms of the disease, and their prolonged intake may lead to unwanted side effects, tolerance and loss of effectiveness, and in turn, the exacerbation of symptom severity (Ferré, García-Borreguero, et al., 2019; Garcia-Borreguero et al., 2018).

When WED appears to be secondary to a primary condition, the specific treatment of that condition – such as the administration of iron, folic acid and vitamins in anemia and pregnancy or kidney transplant in renal end-stage diseases – usually improves or abolishes WED symptoms.

1.3.1 Dopamine Receptor Agonists

Dopaminergic signaling is altered in patients with WED and consequently, dopamine receptor agonists (ropinirole, pramipexole, and lately rotigotine) and the dopamine precursor, levodopa are efficacious treatments (at least in the first periods). D_2-like agonists were approved at an early stage of the disease, and – in the short term – they did not cause significant side effects. Nevertheless, on the long run, these drugs cause an increase in the severity and intensity of the symptoms experienced by WED patients, which is thought to be caused by increased D_1 dopamine receptor activity (Trenkwalder et al., 2013).

1.3.2 α2δ Ligands

An alternative therapeutic strategy is to target glutamatergic neurotransmission. The administration of α2δ ligands is currently the most recommended treatment for early stages of the disease. These drugs bind to the α2δ subunits of certain voltage-gated Ca^{2+} channels present in the glutamatergic terminals, causing a reduction in Ca^{2+} entry and consequently, decreasing the release of excitatory neurotransmitters. The exact mechanism by which these ligands are effective in the treatment of WED is not yet known, therefore remain an empirically validated choice. The best-known ligands are gabapentin, pregabalin and gabapentin-enacarbil (Ferré, Quiroz, et al., 2019; Yepes et al., 2017). Several studies have shown that these medicines are effective to counteract arousals and motor symptoms in the short and long term, however, pregabalin is not approved as a treatment. In addition, (Garcia-Borreguero et al., 2013) showed in a

clinical trial in patients that pregabalin is less effective in treating motor symptoms than pramipexole (Allen et al., 2014).

1.3.3 Iron

The administration of oral or intravenous (IV) iron is one of the treatments of WED, given that iron insufficiency is one of the most well-established secondary causes of the disease. Iron administration is recommended for patients with low serum ferritin levels (<50 µg/mL). IV treatment is more effective and faster in replacing iron levels, since it does not pass through the gastrointestinal tract like oral medication and, thus, is not subject to the limiting processes until it is absorbed. Also, the efficacy of oral formulations are limited by uncontrolled and slow absoption – especially in patients with gastrointestinal pathologies –, and they can cause secondary conditions such as gastritis, diarrhea and nausea (Ferré et al., 2017).

One of the main problems associated with the administration of iron via IV are allergic reactions caused by some formulations. In addition, the absorption of iron in the brain is higher during the nighttime, so it is necessary that the formulations have longer lifespan, for the iron be released during that period. Currently, there are several iron formulations, with short lifetime (iron sucrose and ferric gluconate) and with longer life times (LMW-iron dextran, ferric carboxymaltose, ferumoxytol, and iron isomaltoside), however none has the perfect pharmacokinetic properties for replenishing brain iron levels (Allen & Earley, 2007; Auerbach & Macdougall, 2017).

1.3.4 Opioids

Anecdotally, the first medical use of opioids in WED dates to the 17th Century (Walters, 2013). It is even more intriguing that the µ-opioid receptor knockout mouse displays hyperexcitability, iron deficience and anemia, dopaminergic dysfunction as well as impaired circadian rythm (Lyu et al., 2020), which are comorbities frequently associated with WED. This relationship between the µ-opioid signaling (see below) and WED symptoms is further supported by postmortem studies in which patients showed a deficit of β-endorphin and Met-enkephalin in thalamus (Sun et al., 2011; Trenkwalder et al., 2017). Therefore, it is not surprising that the first clinical trial with oxycodone in severe refractory WED cases was successful (Trenkwalder et al., 2013), thus providing the proof-of-concept for the level-2 use of slowly releasing oxycodone/naloxone in the

treatment of WED. Notwithstanding, opioid treatment is still much discussed by the medical community because of its side effects and the high probability of addiction.

1.4 Basal Ganglia in the Crosshair of WED

The main components of the basal ganglia are the striatum, globus pallidus, ventral pallidum, substantia nigra and the subthalamic nucleus. Most of the iron in the brain is stored in the substantia nigra pars compacta (SN) and basal ganglia, the latter reaching levels equivalent to the liver (Singh et al., 2014).

The whole central nervous system is affected by iron deficiency and therefore, by a set of pathologies, including the hyperexcitability of glutamatergic terminals. Nevertheless, the best documented manifestation of the symptoms involves the hyperactivity of cortico-striatal input terminals. It is clear therefore why the basal ganglia is in our focus when studying the effects of BID. Basal ganglia contains many subsets of intertwined neuronal circuits involved in motor control, habit formation, cognitive processes, emotional responses among others, which I am going to summarize below.

1.4.1 The Dorsal Striatum and its Connections

The pair of striata is the only GABAergic projecting nuclei of the brain. It is the main relay station for excitatory inputs from frontocortical and limbic regions (coming from the cortex that are VGLUT1-positive and from the thalamus which are VGLUT2-positive) and dopaminergic projections from the substantia nigra pars compacta (SNc) of the basal ganglia. This structure is divided into 1) dorsal striatum, composed by caudate nucleus and putamen, and has a major role in motor control, and by 2) ventral striatum or nucleus accumbens in rats, mostly responsible for motivation, aversion, reward, and in a broader sense, reinforcement learning. The dorsal striatum is further subdivided, according to its cortical afferents, into dorsomedial which receives inputs from association cortices, and dorsolateral which receives inputs from the sensorimotor cortex (Bamford et al., 2018; Shipp, 2017).

The principal cell types of the striatum are projection neurons called GABAergic medium spiny neurons (MSN). A low percentage of "true" GABAergic interneurons subserving local loops are also present, together with a minor number of cholinergic interneurons (Zhou et al., 2002). The MSNs projections give rise to two pathways with opposite effects: the caudate and putamen projections to the internal segment of globus pallidus (GPi) (entopeduncular nucleus in rodents) and substantia nigra pars reticulata (SNr) are part of the direct pathway and express type 1 dopamine receptors (D_1R), substance P and the opioid dynorphyn. This pathway has the function of freeing upper motor neurons from tonic inhibition. On the other hand, the indirect pathway, which plays a role in modulating the disinhibitory actions of the direct pathway, MSNs express type 2 dopamine receptors (D_2R), A_{2A} adenosine receptors ($A_{2A}R$) and enkephalin. Indirect MSNs projects to the external segment of globus pallidus (GPe) which, in turn, projects to the SNr and GPi, these being the largest output sources from the basal ganglia. Consequently, the subthalamic nucleus (STN) cells become more active due to the inhibition of GPe and, excite the GPi cells, increasing the inhibitory outflow of basal ganglia and thus inhibiting movement (Bamford et al., 2018; Brimblecombe & Cragg, 2017; Shipp, 2017).

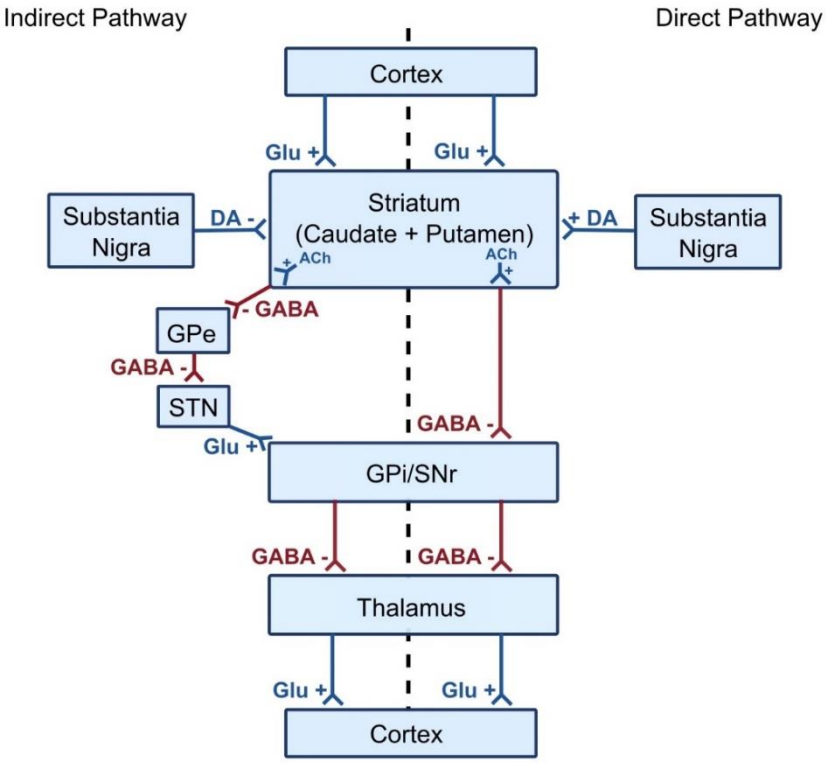

Figure 1. Schematic Representation of the Basal Ganglia Circuitry. Figure taken from https://www.neurovascularmedicine.com/basalgangliastrokes.php.

1.4.2 *Glutamate*

Glutamate is the principal excitatory neurotransmitter responsible for the information flow in the nervous system. The glutamate-glutamin cycle also plays an essential role in brain energy metabolism (Dienel, 2019).

By activating ionotropic and metabotropic receptors, glutamate acts as a fast and slow modulator of neuronal and glial excitability. Both chronic and acute dysregulation of glutamatergic neurotransmission are associated with serious neurological disorders. The most well-known acute examples are excitotoxicity leading to neuronal apoptosis and cortical spreading depression, a pathophysiological substrate of migraines. Chronic dysbalances of glutamatergic neurotransmission are detected in most if not all neurological and psychiatric disorders (Jewett & Thapa, 2020).

Without the glutamatergic corticostriatal afferents, the basal ganglia would be idle. Therefore, anything that alters corticostriatal synaptic transmission will profoundly affect our behavior (Bamford et al., 2018; Brimblecombe & Cragg, 2017). Consequently, movement disorders including WED are strongly associated with impaired synaptic plasticity at corticostriatal synapses (Ferré, García-Borreguero, et al., 2019; Garcia-Borreguero & Cano-Pumarega, 2017),

1.4.2.1 Glutamate receptors

The ionotropic glutamate receptors, N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors are considered to be fast-acting receptors since they can produce a fast signal transmission. AMPAR are responsible for rapid neurotransmission while NMDAR mediates longer and slower responses and, also, regulate dopamine release since they are present in dopaminergic terminals (Marti et al., 2002; Nankai et al., 1998). In the basal ganglia, mainly in the striatum, they are present mostly post-synaptically in glutamatergic neurons and are not expressed in GABAergic neurons.

On the other hand, metabotropic receptors (mGluRs) are slow acting as they are receptors coupled to G proteins (GPCRs) that activate a second messenger system to modulate ion channel function. There are three divisions for metabotropic receptors, which may belong to group I ($mGluR_{1,5}$), II ($mGluR_{2,3}$) or III ($mGluR_{4-8}$), and what differentiates them are their functions and structure. Group I mGluRs are mostly post-synaptic, coupled to Gq proteins and, are associated with the production of

endocannabinoids whereas the remaining groups, II and III, are mostly presynaptic and regulators of synaptic transmission which, when activated, inhibit the release of glutamate (Galvan et al., 2006; Zhang & Sulzer, 2003).

Glutamatergic neurotransmission is regulated, in addition to mGluRs, by other neurotransmitters – the far most known example is GABA – and by a tremendous variety of neuromodulators, such as dopamine, adenosine, opioids and endocannabinoids (see below).

1.4.3 *Dopamine*

Dopamine is a monoamine neuromodulator that belongs to the catecholamine subgroup, and is a major indirect modulator of glutamatergic neurotransmission, especially in the striatum. Somewhat surprisingly, the direct presynaptic effects of dopamine on glutamate release is not fully elucidated (Bamford et al., 2018).

In dopaminergic neurons of the substantia nigra pars compacta and ventral tegmental area, dopamine is synthesized from the amino acid tyrosine which undergoes hydroxylation by the enzyme tyrosine hydroxylase (TH) to form 3,4-dihydroxyphenylalanine (L-DOPA), the main precursor, which undergoes decarboxylation to form dopamine. In the synapse, it is eliminated predominantly via the dopamine transporter (DAT), but it is also a substrate of other monoamine transporters. Once taken up, it is metabolized by monoamine oxidases and catechol-*O*-methyltransferase. The highest content of dopamine in the brain is found in the striatum and its action strongly depends on whether it activates inhibitory D_1-like or facilitatory D_2-like receptors and the cellular and subcellular location of the respective receptors (Gerfen & Surmeier, 2011; Sulzer et al., 2016).

Dopamine receptors are exclusively metabotropic of the GPCR family, which are divided into two groups depending on the type of G protein they activate: D_1-like ones that include D_1 and D_5 receptors and D_2-type that include D_2, D_3 and D_4 receptors. Also, all dopamine receptor subtypes are expressed in the central nervous system. In the striatum, D_1Rs, which are expressed in MSNs of the direct pathway, are excitatory receptors since they are coupled to G_s proteins and are exclusively postsynaptic. On the other hand, the receptors found in the MSNs of the indirect pathway, the D_2Rs, have an inhibitory role since they are G_i-coupled receptors and are both pre and postsynaptic (Smith & Kieval, 2000; Sulzer et al., 2016).

The presynaptic presence of dopamine D_2 and D_4 receptors at the corticostriatal glutamatergic terminals, in heteromeric form, play an important role in the modulation of the release of glutamate. The D_4 receptor, depending on its polymorphic variations, has the ability to modify the constitutive activity of D_2R in the heteromer, *i.e*, the D_2 receptor has a gain in function and is able to inhibit the release of glutamate through the corticostriatal terminals (Bonaventura et al., 2017). Impaired dopaminergic neuromodulation (too high or too low dopamine levels) is associated with several psychiatric and neurological disorders, such as Parkinson's disease, schizophrenia, depression, hyperactivity, among others. A post-mortem study showed that WED patients have changes in dopaminergic and glutamatergic neurotransmission, which have been associated with motor symptoms and sleep disturbances (Connor et al., 2009).

On the other hand, in animals subject to an iron-deficient diet, none motor symptoms are observed, however, they present identical dopaminergic alterations as humans: 1) increased tyrosine hydroxylase activity in the striatum and SN; 2) a decrease in D_2R density in the striatum, indicating the existence of a pre-synaptic hyperdopaminergic state. This state can be explained by the hypersensitivity of the glutamatergic terminals for the release of glutamate, also suggesting the existence of a presynaptic hyperglutamatergic state, since the release of glutamate controls the release of dopamine (Ferré, García-Borreguero, et al., 2019; Jiménez-Jiménez et al., 2015). The hypersensitivity of glutamatergic terminals was confirmed by an optogenetic-microdialysis study in rodents with BID that also showed the efficacy of dopamine agonists in blocking the glutamate release (Yepes et al., 2017).

What leads to impaired dopamine signaling in the striatum besides neurodegeneration is only partially understood, but an impaired cholinergic control of the dopaminergic afferents may be one such factor (Abudukeyoumu et al., 2019).

1.4.4 *Acetylcholine*

Acetylcholine (ACh) is also a major modulator of bodily physiology – the best-known examples are the neuromuscular junction and the autonomic nervous system. In the CNS, ACh is believed to have important roles in neuromodulation, cognition and memory, associative learning, behavioral flexibility, arousal, motivation and reward

(Ahmed et al., 2019). Impaired ACh signaling in the CNS is implicated in the ethyology of neuropsychiatric disorders such as Alzheimer's disease and addiction (Soreq, 2015).

ACh is synthesized by diverse groups of neurons in the mammalian brain. The most well-known groups are the pontomesencephalotegmental group of nuclei, the septal nuclei, and the basal nucleus of Meynert. Nevertheless, these are projection neurons innervating other brain areas, whereas the only nucleus of the brain which contains GABAergic projections, *i.e.* the striatum has its own intrinsic cholinergic (Ahmed et al., 2019; Zhou et al., 2002). Although only a few percent in the total number of striatal neurons, these giant cells embroil the whole striata with extremely dense varicose ramifications, which are vesicular ACh transporter (VAChT-positive). This transporter ensures the refilling of cholinergic synaptic vesicles with ACh. Once released, the effect of ACh is rapidly terminated by extracellular cholinesterases, and the end product choline is taken up by choline transporters to support ACh synthesis (Picciotto et al., 2012).

Unlike dopamine, ACh has both metabotropic and ionotropic receptors. Metabotropic or muscarinic ACh receptors (mAChRs) are classified as inhibitory ($G_{i/o}$-coupled) such as the M_2 and M_4 receptors, while the $G_{q/11}$-coupled M_1, M_3 and M_5 receptors are facilitatory (Brown, 2019)They are commonly activated by muscarine. In contrast to the five well-known mAChRs, nicotinic ACh receptors come in an enormous diversity, due to the possibility that the nine central α subunits ($\alpha_{2\text{-}10}$) and the three central β subunits ($\beta_{2\text{-}4}$) can form homo- or heteropentameric ligand-gated ion channels. These receptors all have different pharmacology (different sensitivity to ACh and nicotine).

It is well-known that mAChRs and nAChRs are located virtually everywhere in the brain, both pre- and post-synaptically in neurons, and they can influence fast synaptic transmission (Brown, 2019; Wittenberg et al., 2020). In the striatum, cholinergic interneurons receive a large number of glutamatergic, GABAergic, cholinergic and dopaminergic inputs. In turn, they innervate reciprocally their afferents and the dendrites of the MSN. In summary they can modulate corticostriatal neurotransmission both pre- and post-synaptically, both via muscarinic and nicotinic ACh receptors (Abudukeyoumu et al., 2019). Thanked to its central hub role in organizing striatal neuromodulation, several procholinergic therapies have been proposed in sever polytreatment-resistant WED (Jiménez-Jiménez et al., 2015), but still

very little is known about the changes and the therapeutic potential of the striatal cholinergic system in WED. One leading hypothesis is that μ-opioid receptors in striatal cholinergic terminals may underlie the beneficial effects of low doses of opioids in WED.

1.4.5 *Opioids*

Opioids are intensively studied peptide neuromodulators thanked to their role in analgesia and in the infamous pandemy of opioid addiction. Opioids have profound – and often lethal – effects in humans, due to the widespread expression of their receptors inside and outside the CNS. The three best studied opioid receptors are the μ- (MOR), the δ- (DOR) and the κ-opioid receptor (KOR). These categories can be further sub-classified by function or structure. The number of additional receptor subtypes is contentious, but some sources report σ, ε, ζ and nociceptive receptor that belong to this class (Feng et al., 2012).

Besides morphine, our body synthesizes three large pro-compounds: proenkephalin, prodynorphin, and pro-opiomelanocortin, and the tetrapeptides, endomorphines. The pro-compounds can further decompose to small fragments, oligomers, which are still active, including, enkephalins, dynorphins and endorphins, which bind to opioid receptors according to their selectivity. In the striatum, the DOR/MOR agonist, enkefalin is produced by the D_2R-positive MSN, while the preferentially KOR but also MOR and DOR agonist, dynorphin is produced by the D_1R-positive neurons of the direct pathway. MOR is present in a variety of pre- and post-synaptic element in the striatum, including glutamatergic afferents, and cholinergic and GABAergic nerve terminals, respectively. MOR is a favorite target of recent studies, since it plays a major role in drug addiction, neuropsychiatric disorders and pain management (Gibula-Tarlowska & Kotlinska, 2020; Prager & Plotkin, 2019; Sandor et al., 1992; Whalley, 2016).

MOR activation stimulates inhibitory $G_{i/o\alpha}$ proteins, leading to the inhibition of adenyl cyclase and, consequently, of cAMP formation. On the other hand, the activation of the $G_{i/o\beta\gamma}$ subunits, the suppression of voltage-gated calcium currents and the activation of inwardly rectifying potassium currents, cause hyperpolarization. The presynaptic consequence of all these is the decreased release of neurotransmitters, such

as glutamate, and may thus be beneficial in the hyperglutamatergic state in WED. Indeed, MOR activation appears to be a successful therapeutic strategy in otherwise therapy-resistant WED (de Oliveira et al., 2016; Trenkwalder et al., 2013, 2017). Indeed, a recent study revealed that the MOR knockout mice have disturbances in cerebral iron homeostasis and exhibit an RLS-phenotype (Lyu et al., 2020).

1.4.6 *Adenosine*

Recently, evidence has emerged from the laboratory of our collaborators on the present study that there is a change in the adenosinergic system in BID that disrupts the adenosine-dopamine-glutamate balance (Ferré, Quiroz, et al., 2018; Ferré, 2019; Quiroz et al., 2010, 2016), consistent with the fact that the brain regions most affected by lack of iron are those that, under normal conditions, are rich in adenosine receptors. These changes may also be an explanation for the sleep disorders characteristic of the pathology.

Adenosine is a key neuromodulator and allostatic regulator in the brain (Rodrigo A. Cunha, 2019). It is a major modulator of glutamatergic transmission and also shapes the activity of the dopaminergic system by pre- and post-synaptic mechanisms (Borycz et al., 2007). For instance, the release of corticostriatal glutamate is strictly controlled by presynaptic adenosine receptors (Ferré, Quiroz, et al., 2019; Köfalvi et al., 2020)

Adenosine can be synthesized within cells via 5′-nucleotidases enzymes, depending on the breakdown of adenosine triphosphate (ATP) in adenosine di- and monophosphate (ADP / AMP) and finally, in adenosine. Once formed, within the cell it can also undergo the action of adenosine deaminase (ADA) to form inosine or the action of adenosine kinase to form AMP. The release of adenosine by equilibrative nucleoside transporters (ENTs) due to neuronal stimuli, raises the levels of extracellular adenosine, which can also be generated in the extracellular space through the catabolism of ATP and other purines, via ectonucleotidases CD39 and CD73. ATP can be release with e.g. glutamate and acetylcholine, by the reversed transport from astrocytes, by lysosomal exocytosis and via hemichannels. Obviously, astrocytes can also eliminate extracellular adenosine via the nucleoside transporter ENT. Hence, adenosine is a sign of increased glutamatergic neurotransmission (Cellai et al., 2018).

When accumulated in the extracellular space after prolonged wakefulness, adenosine may induce sleepiness mainly activating the corticobasal and thalamic A_1Rs. With the activation of A_1R, the accumulation of adenosine leads to inhibition of the cells of origin in the corticopetal basal forebrain system and the prefrontal corticofugal neurons that innervate the cells of origin of the routine ascending arousal systems, increasing sleepiness. Hence, the general adenosine receptor antagonist, caffeine is considered beneficial in brain disorders, and its analogues may be useful as an adjunct therapy (Cunha, 2019; Ferré, Quiroz, et al., 2018). Therefore, a decrease in the density of functional A_1R and an increase in extracellular adenosine contributes to the hyperexcitability of the corticostriatal terminals (Ferré, Quiroz, et al., 2018, 2019).

There are 4 types of G protein-coupled adenosine receptors, the A_1, A_{2A}, A_{2B} and A_3 receptors, however, only the A_1R and A_{2A}R receptors are documented as major neuromodulators in the brain (Cunha, 2001).

The A_1R are found in greater density in the brain, both in neurons and glial cells, particularly in structures such as the cortex, cerebellum and hippocampus. Although at lower levels than the structures mentioned above, in the striatum they are both pre and postsynaptic in glutamatergic and dopaminergic terminals. It is the receptor that has higher affinity for adenosine (70 nM), and upon activation, it signalizes via inhibitory $G_{i/o}$ proteins, being important in the regulation of synaptic plasticity and release of neurotransmitters (Dunwiddie & Masino, 2001; Van Dort et al., 2009).

On the other hand, the A_{2A}R are found in greater numbers in the basal ganglia, especially in the striatum, mostly in GABAergic striatopallidal MSN, cholinergic interneurons, glutamatergic corticostriatal terminals and in lowest number in glial cells, olfactory tubercle, hippocampus and cortex. They have a lower affinity for adenosine (150 nM) (Dunwiddie & Masino, 2001) and are excitatory GPCR, as they are coupled to G_s outside the striatum and G_{olf} in the MSN protein.

1.4.6.1 Adenosine Receptor Heteromers

It is more the rule than the exception that metabotropic (and even, ionotropic) receptors form heteromultimers, mosaics with one another. These novels signaling entities function as precomputational units in neurons, that is, instead of a neuron summarizing the activity of various individual inhibitory and excitatory receptors which would constantly tug on the excitability of the membranes, causing serious energy leak,

these receptors form one signaling unit which computes the overall outcome of the contrasting inhibitory and exctitatory inputs. For instance, A_{2A}Rs form heteromers with the A_1Rs in corticostriatal terminals (Ferré et al., 2011; Ferré & Ciruela, 2019). These heteromers signalize via inhibitory A_1Rs at low (ambient) adenosine levels. If the firing of the nerve terminal is not salient, then ambient adenosine levels would silence these terminals (Figure 2). But when the corticostriatal afferent fires salient information, enough ATP is co-released with glutamate to reach the critical synaptic levels to activate presynaptic A_{2A}Rs. In turn, A_{2A}Rs in the heterotetramer would silence their co-partner A_1Rs, and thus the receptor complex would couple to cAMP generation, exerting a feed-forward facilitation on glutamate release, hence rescuing salient information flow.

Presynaptically, the A_{2A}R also forms heterotetramers with the cannabinoid CB_1 receptors (CB_1Rs) (Figure 2). Post-synaptically, in the dendrites of the MSN of the indirect pathway, A_{2A}Rs also form heterotetramers with dopamine D_2 receptors and $mGluR_5$ (Figure 2) (Ferré et al., 2011; Ferré & Ciruela, 2019). To understand this complex picture, we must discuss in a nutshell at last the final effector of neuromodulation, the endocannabinoid system.

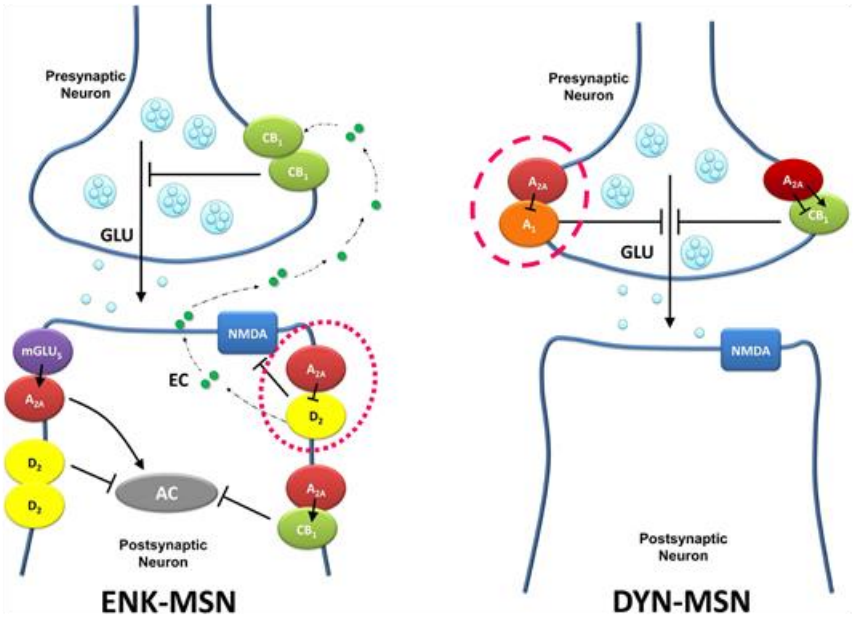

Figure 2. Pre- and postsynaptic orchestration of adenosine A_{2A} receptor heteromers at corticostriatal synapses. Modified from Ferré et al., 2011.

1.4.7 The Endocannabinoid System

A brief and overly simplified depiction of the endocannabinoid system would include the G protein-coupled cannabinoid CB_1 and CB_2 receptors (CB_1Rs and CB_2Rs), two major lipid-derivative endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG) and their turnover enzymes (Di Marzo, 2018; Solymosi & Kofalvi, 2017). A large variety of developing and mature neuronal and glial cells express one or both cannabinoid receptors (Jordan & Xi, 2019; Katona & Freund, 2012; Robin et al., 2018). The CB_1R is thought to be responsible for the psychoactive effects of marihuana's Δ^9-tetrahydrocannabinol, although the CB_2R has also been recognized in brain (patho)physiology (Jordan & Xi, 2019; Solymosi & Kofalvi, 2017). Both cannabinoid receptors have pivotal roles in the development, signaling, metabolism and protection of brain cells. This means that persistent alterations in endocannabinoid turnover could severely affect these versatile roles of the CB_1R and the CB_2R (Alpár et al., 2016; Fernández-Ruiz et al., 2015; Katona, 2015; Rodrigues et al., 2020).

Below, I present the spatiotemporal and molecular orchestration of A_{2A}R-CB_1R interaction at the corticostriatal synapse, which also serves to highlight the complexity of endocannabinoid function in the regulation of network activity. Presynaptically, half of the corticostriatal nerve terminals is equipped with CB_1Rs, and the half of these CB_1R-positive terminals is also endowed by A_{2A}R as our group has shown before with the technique "flow synaptometry" (Ferreira et al., 2015), which has been a major workhorse technique in my Master Thesis work. Presynaptic CB_1Rs also inhibit ATP release from depolarized striatal synaptosomes (Ferreira et al., 2015), suggesting that CB_1Rs could control the generation of synaptic adenosine, too.

Post-synaptic A_{2A}Rs are highly coexpressed with both D_2Rs and $mGluR_5$, which co-localize with diacylglycerol lipase α (DAGLα) in the dendritic spines of the MSNs (Ferré et al., 2011; Uchigashima et al., 2007). The activation of $G_{q/11}$ protein-coupled metabotropic receptors such as the $mGluR_5$ stimulates DAGLα-mediated 2-AG release (Figure 2) (Solymosi & Kofalvi, 2017). $mGluR_5$ is typically activated by glutamate spill-over as a result of high-frequency corticostriatal discharge (Zhang & Sulzer, 2003), which leads to 2-AG release in the synapse. D_2Rs have been also reported in MSN dendritic spines to prolong $mGluR_5$-induced transient 2-AG release (Figure 2) (Kreitzer & Malenka, 2008). Post-synaptic A_{2A}Rs are negatively coupled to both $mGluR_5$ and D_2Rs (Ferré et al., 2011; Ferré & Ciruela, 2019) (Figure 2), thus A_{2A}R activation by

phasic adenosine levels inhibits post-synaptic metabotropic receptor-induced endocannabinoid formation.

In conclusion, if certain corticostriatal terminals reach sufficient firing frequency, the consequent retrograde endocannabinoid signaling will no longer affect glutamate release via presynaptic CB_1Rs and, simultaneously high adenosine levels will allow the A_{2A}R to overrule the A_1R-mediated (F Ciruela et al., 2006; Rodrigo A Cunha, 2008) and the CB_1R-mediated inhibition of corticostriatal glutamate release. Although it is beyond our focus, it is noteworthy to mention that pre- and post-synaptically, various subtypes of adenosine, cannabinoid, dopamine and opioid receptors form heteromultimers in the striatum, thus contributing to a seemingly enormous complexity of neuromodulation.

1.4.8 BID-induced alterations in the adenosinergic system

In the striatum of Sprague-Dawley rats with severe BID, a reduced density of A_1Rs and an increased density of A_{2A}Rs was observed, with a consequent physiological alterations including hyperexcitability of corticostriatal nerve terminals (Quiroz et al., 2010, 2016). This is at least in part in concert with a previous finding in both human neuroblastoma culture and the mouse C57bl/6 BID model where iron deficiency caused an upregulation of A_{2A}R expression (Gulyani et al., 2009). Our collaborators, leaders of the field termed this condition a “hypoadenosinergic state”, responsible for the hypersensitivity of the corticostriatal terminals in that sense that adenosine in general should reduce corticostriatal (Ferré, Quiroz, et al., 2019).

The best way to test this hypothesis would be the direct administration of an A_1R agonists to WED patients, however, they are not FDA approved due to the side effects they cause (e.g bradycardia, atrio-ventricular blocks). Thus, an alternate target is the nucleoside transporters ENT1, a bidirectional equilibrative carrier driven by chemical gradients present in the astrocytic processes. The use of inhibitors such as dipyridamole may be a possible therapy for WED, since they cause an increase in extracellular adenosine levels, leading to an inhibition of glutamate release in the corticostriatal terminals.

Dipyridamole in a human clinical carried out in 30 WED patients, greatly improved both sensory and motor symptoms, as well as sleep (Garcia-Borreguero et al., 2018). This provides evidence that hypoadenosinergic mechanisms play a central role in

WED. Thus, all the evidence described above leads to the conclusion that hypoadenosinergic neurotransmission associated with the downregulation of A_1Rs is directly or indirectly involved in WED symptoms (Ferré, Quiroz, et al., 2019).

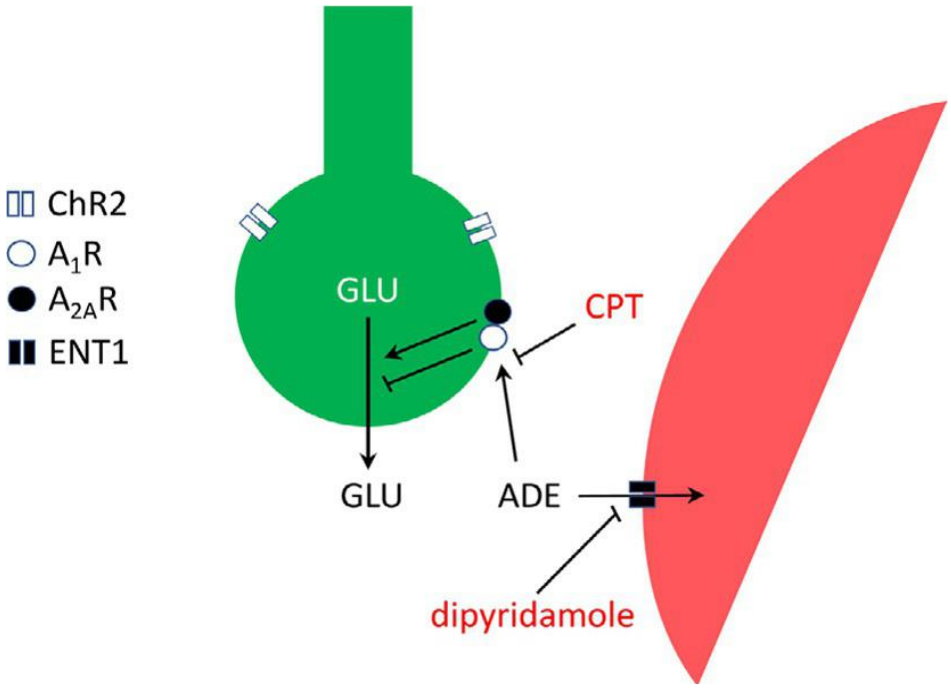

Figure 3. Scheme representing the A1R-mediated modulation glutamate release by corticostriatal terminals (green). A_1Rs are activated by elevated extracellular levels of adenosine, as a result of the inhibition of adenosine uptake into astrocytes (red) by dipyridamole inhibition. CPT, 8-cyclopentyltheophylline, an A_1R antagonist (Ferré, Quiroz, et al., 2019).

1.5. Summary

This Introduction served evidence for BID being sufficient to cause sporadic RLS/WED in humans and for that animal models of BID are appropriate to study cellular and molecular changes associated with WED.

We identified signaling systems – glutamate, dopamine and acetylcholine – in the striatum that are believed to be severely affected by putative changes in presynaptic density of adenosine A_1 and A_{2A} receptors, μ-opioid and cannabinoid CB_1 receptors upon BID. My Master Thesis Project aimed at documenting these putative changes in the respective nerve terminals. Additionally, I also tested for the first time if there is any gender difference at the levels of behavior and the neurochemical markers in the BID model.

2. Rationale and Aims

As documented in the Introduction, recent preclinical and clinical studies indicate that adenosinergic dysbalance may be present in WED and in BID models, and provides an attractive therapeutic target.

1) Nevertheless, the exact mechanism by which these alterations happen remains to be defined. The existence of a disturbance in the adenosinergic system, a hypothetical hypoadenosinergic state caused by either $A_{2A}R$ upregulation or by A_1R downregulation or both, is the first hypothesis of my MSc work to address.

2) Additionally, the well-known therapeutic efficacy of opioids in WED leads to a second working hypothesis to test, namely I would like to explore if changes in MOR receptor densities and distribution are present in the striatum of the BID model.

3) Finally, as discussed in the Introduction, WED is more prevalent in women, perhaps due to their higher probability of suffering from iron deficiency. Thus, my third working hypothesis is the existence of a gender-specific phenotype in the BID model.

To achieve my goals,

- I will characterize our newly set up model of BID in Sprague-Dawley rats (the strain used by our collaborators at NIDA-IMR) by documenting severe anemia, changes in body weight and an increased density of transferrin receptors in their striata;

- I will assess and compare basic behavioral paradigms between treatments and genders;

- And finally, I will measure the density and the frequency of colocalization among the most relevant presynaptic markers. We selected the following combinations as our FACSCalibur at CNC only allows observing three channels: A_1R and $A_{2A}R$ as well as CB_1R and MOR in $VGluT_1$-positive corticostriatal, $VGluT_2$-positive thalamostriatal glutamatergic terminals and VAChT-positive cholinergic terminals in the striatum of brain iron deficient rats with those of the control rats.

3. Materials and Methods

3.1 Animals

The present project, approved with the ORBEA 257, is a collaboration with National Institute of Drug Abuse (NIDA) that counts as the continuation of a previous project (PO# HHSN271201700216P), regularized in ORBEA 167. All experiments were performed in accordance with the local animal welfare committee (Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal), European Union guidelines and the Federation of Laboratory Animal Science Associations (FELASA).

Animals were kept according to strict standards of health and biosafety. They were under a controlled cycle of light (12h day/night cycle), humidity (45-65%) and ad libitum access to food and water. For the execution of this study, a colony of Sprague-Dawley was established to avoid the transport of pregnant females or litters from Charles-River (Barcelona, Spain), where they were purchased. The selection of the lineage of animals is based on previous studies who used the same model, that support the present one, all carried out by Dr. Sergi Ferré.

For all litters, weaning was carried out arbitrarily on the 21st postnatal day. The dams received control diet (see below) starting from the 14th postnatal day to habituate their pups to the special diet. On the day of the weaning, the litter was randomly stratified into four groups, according to sex and the diet they would receive: male and female control group – food with adjusted iron content (containing 48 mg/kg Fe(II)SO_4, code: TD.80396, ssniff - Spezialdiäten GmbH); and male and female treatment group – iron deficient food (6-8 mg/kg Fe(II)SO_4, TD.80396). Each group of animals received the respective diets for four weeks which is the necessary length of this procedure to obtain reliable BID in the groups fed with the iron deficient chow (Quiroz et al., 2010).

After the completion of the feeding protocol, the rats were weighed (Figure 4A), then deeply anesthetized by isoflurane vapor (no reaction to tail pinch while still breathing) and euthanized via a stainless-steel guillotine, for tissue collection. In addition, blood was collected in tubes with and without 0.5 M K_2EDTA, in order to perform supplementary hematological analysis by “Beatriz Godinho, Análises Clínicas”. Complete blood count was performed and serum iron was determined for each rat, which is summarized in Figure 4B-D. Although, serum ferritin levels were also requested, the laboratory used the wrong antibody that did not recognize the rat ferritin protein.

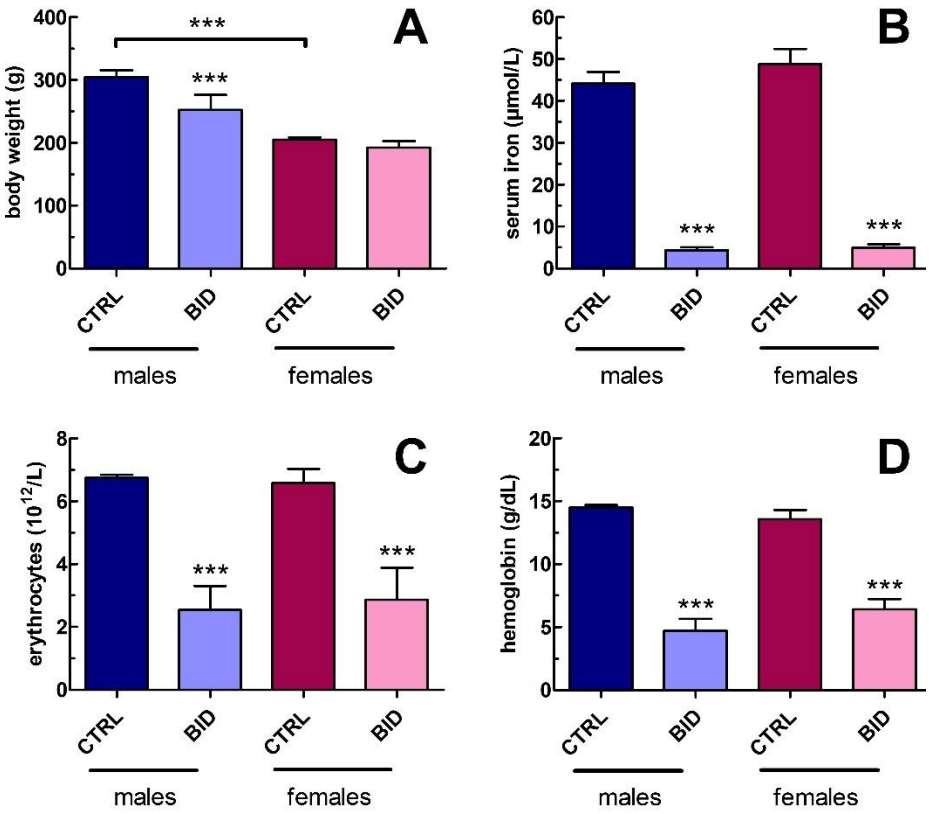

Figure 4. Body weight, serum iron, erythrocyte count and hemoglobin levels in CTRL and BID animals after the 4-week feeding regimen. Sprague-Dawley littermates were weaned on postnatal day 21 and randomly grouped into 2×2 groups based on gender, then immediately subjected to special rat chow containing 48 mg/kg (CTRL diet) or ~7 mg/kg $Fe(II)SO_4$ (BID diet) for four weeks. Upon completion of the feeding regimen, the rats were weighed. Panel A shows a somewhat surprising finding that only the male rats exhibited significant growth retardation after being fed with iron poor chow for 4 weeks. Male BID rats weighed 17.2% less ($n = 7$; $P < 0.0001$ by 2-way-ANOVA followed by Tukey's multiple comparison's *post-hoc* test) than their control fed littermates. Such difference was not observed with the female rats. Female rats grew slower at this age compared to their male littermates ($n = 7$ and 8, $P < 0.0001$). However, 4 weeks on iron poor chow did not significantly affect their growth curve (-5.6% body weight vs. control female rats, $n = 8$, $P > 0.05$). Thus, this is an intriguing finding suggesting that iron is either necessary for the faster growth or it is required for the growth of highly blood-dependent peripheral tissues such as the skeletal muscle. Additional anatomical studies are required to assess the gender-specific development of major organs and tissues in this BID model. Bars represent mean + S.E.M. of $n = 7$ and 8 independent observations.

3.2 Behavioral tests

The behavior tests were designed according to previous protocols already established in our laboratory (Leffa et al., 2018). All the tests, performed in the order described below, were done during the light phase of the circadian cycle (9 AM to 5 PM) in the last two days of the diet (days 27 and 28). All the animals were transported from the animal house to a room adjacent to the test room, where they were left for at least one hour for acclimatization. In order to minimize the interferences in the animal's behavior, between each test, the apparatus was cleaned with a 70% ethanol to eliminate olfactory clues. Tested animals waited in holding cages and returned to their original cages only after all the cage mates were tested.

The data were collected and analyzed with the help of the ANYmaze® video tracking software (Stoelting, US).

Day 1

Open Field (OF) test

The open field test is used to assess the locomotor activity of animals. Because rodents tend to avoid open areas, which they consider threatening, the relative exploration of the center of the maze is used as an index of anxiety-like behavior (Belzung & Griebel, 2001; Walsh & Cummins, 1976).

The animals were placed in the center of an open field arena (width × length × wall height: 50 cm x 50 cm x 1 m), with defined peripheral and central (36% of total area) zones. They were left to explore for 30 min, in order to also assess within session habituation.

- **Locomotion:** Locomotion was assessed by measuring the total distance travelled by each animal.
- **Anxiety-like behavior:** Percentage of time spent, and percentage distance travelled in the center of the OF in the first 5 minutes of the test was used as an index of anxiety like behavior. The more anxious the animals are, the less time they spend in the center of the OF, which is an open and therefore threatening area (Belzung & Griebel, 2001).
- **Short-term (within session) habituation:** Habituation describes the progressive decrease of a behavioral response upon repeated exposure to non-threatening sensory stimulus (Typlt et al., 2013). It is considered a basic form of learning, allowing to filter irrelevant from relevant stimuli (Poon & Young, 2006). This is believed to be necessary for other forms of learning (Rankin et al., 2009). Disruption of habituation, which is for

instance found in patients with schizophrenia and autism spectrum disorder, strongly correlates with cognitive impairment (Ludewig et al., 2003; Perry et al., 2007).

To evaluate habituation, rats were left to explore the OF arena for 30 minutes, time during which they should progressively decrease locomotion as they become habituated to the environment.

Elevated plus maze (EPM) test

EPM is one of the most used tests to assess anxiety-like behavior in rodents. It is based on their innate curiosity to explore new environments and their fear of open spaces (Pellow et al., 1985). The EPM consists of four arms, elevated from the ground: two open arms, perceived as threatening and two closed arms, perceived as safe. The basic principle of this test is that anxious will explore less the threatening open arms (Pellow et al., 1985).

The cross-shaped apparatus we used, consisted of four arms of the same size (54 cm × 10 cm), with the two closed arms enclosed by 40-cm-high walls. The apparatus was raised 50 cm above the floor. Rats were placed at the center of the four arms, facing the open arm opposite to the experimenter. Animal behavior was recorded for 5 min. Entries were counted whenever all the four paws of the animal crossed into one of the arms. Anxiety-like behavior was measured as a lower percentage of open arm entries and lower percentage of time spent in open arms (Pellow et al., 1985).

Day 2

Y-maze spontaneous alternation test

Spontaneous alternation in a Y-maze is a test that is commonly used to assess spatial working memory. Working memory is a representation of an object, stimulus, or spatial location that is used within a testing session to guide behavior (Dudchenko, 2004). The test is based on the natural tendency of animals to choose a different arm than the one previously chosen (Dudchenko, 2004).

The test was carried out in a Y-shaped Plexiglas apparatus (width × length × wall height: 10 cm x 35 cm x 25 cm) with 3 arms, separated by equal angles. The equipment had defined visual cues on the walls at the end of each arm to help with geographical orientation of the animals. Rats were placed at the end of the start arm, facing the wall, and were allowed to freely explore the maze for 5 min. Since the animal's natural tendency is to explore a new arm instead of re-entering in the arm that they were previously, the entry sequences in each arm

were recorded in order to calculate the percentage of correct alternations for every 3 entries. 3 sequential entries in three different arms is a correct alternation. The percentage of spontaneous alternation was calculated as the percentage of alternations of the total possible number of alternations (total number of arm entries − 2) (Augusto et al., 2013).

3.3 Flow Synaptometry

Techniques such as binding assay and Western blotting allow us to evaluate the global changes in the density of receptors in a brain area, however, they cannot specifically tell in which cell types or subsynaptic compartment the changes occur (*e.g.* in corticostriatal glutamatergic terminals, or rather in cholinergic terminals). Therefore, to achieve the main objective of this work, a different and more sensitive technique has been developed and optimized at our lab termed as "flow synaptometry" (Ferreira et al., 2015). Coincidentally, other groups also have developed similar procedures with the idea of achieving a robust proteomic and statistical analysis of a pool of isolated nerve terminals (Biesemann et al., 2014; Hobson & Sims, 2019).

The flow synaptometry technique has the same principles as flow cytometry, however, instead of cells, synaptosomes are analyzed. It is both a quantitative and qualitative technique, often used to evaluate the morphology, size and properties of single particles as they move, one by one in a fluid flow. Flow cytometers detect particles inside the light beams by axial frontal and lateral sensors that, as the synaptosome passes through the laser, the light is spread in all directions, thus allowing the analysis and differentiation of synaptosomes. The light scattered in the frontal direction is called forward scatter (FSC) gives information about the particle size while the lateral scattering of the light (or side scatter, SSC), is proportional to the complexity and granularity of the particle. In addition, the biological samples (in our case, the synaptosomes) are to be labeled with antibodies linked to fluorochromes, thus allowing to study changes in the density of markers and receptors.

3.4 Synaptosomal Preparation

3.4.1 Preparation of S1 fraction

Synaptosomal preparation was be carried out according to the protocol described by Dunkley et al, with some changes. The brains were quickly collected in an ice-cold solution of 320 mM sucrose, 1 mM Ethylenediaminetetracetic acid disodium salt dihydrate (EDTA) and 5 mM Tris, pH 7.4. The pair of striata were isolated and homogenized, using a Potter Teflon, in 6

mL of buffer. The final preparation was divided into 3 Eppendorf tubes of 2 mL each, and centrifuged at 4000 g, 5 min. The first supernatants (S1) of the first two low-speed centrifugation will be layered on top of the discontinuous Percoll gradient for purification. The P1 fractions were snap-frozen for Western blotting analysis of transferrin receptor (TFR).

3.4.2 Discontinuous Percoll gradient

Percoll gradients were prepared following the protocol described by (Dunkley et al., 2008). The solutions for the Percoll gradients were made by diluting concentrated Percoll with a solution of 320 mM sucrose, 1 mM EDTA and 5 mM Tris, pH 7.4, and prepared according to the data in table 1.

Table 1. Preparation of Percoll gradient solutions.

% Percoll (vol/vol)	Buffer (mL)	Percoll (mL)
3%	24.25	0.75
10%	22.5	2.5
15%	21.25	3.75
23%	19.25	5.75

Subsequently, the solutions were placed in 15 mL tubes, using a peristaltic pump, in order to form gradients and, at the top, about 2 mL of the above S1 synaptosomal fractions was layered gently. The gradients were centrifuged at 25000 g for 11 min at 4ºC. In order to avoid a sudden stop that could stir up the gradient, we brake the centrifuge at the slowest deceleration rate. The purified synaptosomes were removed between the 15% and 23% layers and subsequently diluted to 15 mL with HEPES buffered medium (HBM) with the following constitution: 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Again, the samples were centrifuged at 22000 g for 11 min at 4ºC and returned to slow the brake from maximum rate to 0g as the pellet obtained here moves freely in the bottom of the tube. The pellet was collected, diluted in 2 mL of HBM and centrifuged at 11000 rpm for 11 min at 4ºC. The final pellet was frozen at -80ºC until use.

3.5 Immunolabeling and Flow Synaptometry Analysis

The pellets obtained after purification in gradients were fixed in 1 mL of 0.25% paraformaldehyde in phosphate buffered saline (PBS; 135 mM NaCl, 1.3 mM KCl, 3.2 mM NaH_2PO_4, 0.5 mM KH_2PO_4 and 10 mM EDTA) for 1 h at 4ºC. After fixation, they were centrifuged at 5000 rpm for 3 minutes at 4ºC. Then, in order to permeabilize the synaptosomal membrane for marking intracellular antigens, the pellet was incubated with PBS with 0.2% Tween 20 for 15 min at 37ºC and then centrifuged at 5000 rpm, and subsequently, 0.5 mL of PBS was added to wash for 3 min at 4ºC. Finally, the resulting pellet was resuspended and diluted in PBS (150-200 µL - volume dependent on the amount of material).

For immunolabeling, 5 µL of resuspended synaptosomes were incubated with 100 µL of primary and secondary antibodies, described in Table 2, both diluted in PBS with 2% normal goat serum, for 30 min at 4ºC. The secondary antibodies were conjugated with fluorescein isothiocianate (FITC) or cyanine 3 (Cy3) or cyanine 5 (Cy5). After each incubation, 3 washes were carried out, 3 min each, with PBS 0.2% Tween 20, at 5000 rpm at 4ºC. All the primary antibodies have been titrated to obtain concentration-saturation isotherms to determine lowest antibody concentration that yields a signal close to saturation. $A_{2A}R$ and CB_1R antibodies were validated in the CB_1R KO and the $A_{2A}R$ KO mice before by us (Ferreira et al., 2015), while several others were validated in synaptosomes obtained in control Wistar rats, to determine the most appropriate dilutions/protocols to carry the colocalization studies. Furthermore, negative controls were also carried out, containing only synaptosomes and secondary antibody in order to quantify non-specific labeling.

Labeled samples were then resuspended in PBS and were analyzed in the FACSCalibur flow cytometer (Becton, Dickinson and Company, USA – 4 channels). The right dilution for each sample was adjusted to work within a count of 300-400 events per second. Approx. 30,000 events were collected for analysis.

In order to evaluate the purity of the synaptosomal preparations obtained through discontinuous Percoll gradients, several flow synaptometry assays were made with synaptosomes labelled with synaptic markers and, this process was carried out in both purified synaptosomes and in the crude P2 fraction as a comparison (Data not shown).

Table 2. List of antibodies used for flow cytometry.

Antibody	Supplier	Host	Type	Dilution
Synaptophysin	Synaptic Systems	Rabbit	Monoclonal	1:300
VGlut1	Synaptic Systems	Mouse	Monoclonal	1:100
VGlut2	Abcam	Mouse	Monoclonal	1:10000
VAchT	Novus Biologicals	Mouse	Monoclonal	1:300
CB_1R	Frontiers/Nittobo	Rabbit	Polyclonal	1:3000
	Frontiers/Nittobo	Guinea-pig	Polyclonal	1:300
A_1R	Invitrogen	Rabbit	Polyclonal	1:300
MOR	Frontiers/Nittobo	Rabbit	Polyclonal	1:100
$A_{2A}R$	Frontiers/Nittobo	Guinea-pig	Polyclonal	1:30
Anti-mouse Cy3	Jackson Immunoresearch	Goat	IgG	1:200
Anti-rabbit FITC	Jackson Immunoresearch	Goat	IgG	1:200
Anti-guinea pig Cy5	Abcam	Goat	IgG	1:200

3.5.1 Data analysis

Data analysis was performed using BD Cell Quest Pro software. For a single labeling, the data were plotted in single-parameter fluorescence histograms and, from these graphs, the percentage of positive particles in the samples was calculated. The negative and positively labeled regions (M1 and M2 regions, respectively) were calculated for each negative control, with the aid of the overlay tool (Figure 5B and 5C), where the intersection of the two histograms represents the beginning of the population of positive synaptosomes. The specific labeling of each sample is calculated by subtracting the percentage of labeling the sample with that of the respective controls and with the percentage of PBS debris. For double and triple labeling, a dual-parameter dot plot was used in order to analyze the percent of co-localization through the upper right quadrant (Figure 5D)

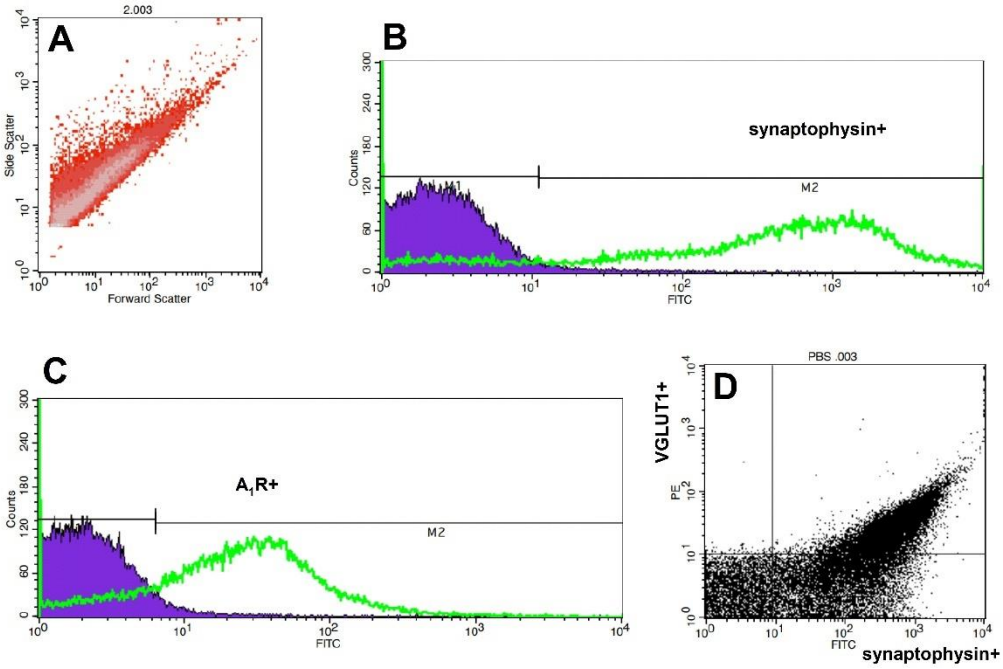

Figure 5. Flow cytometry data analysis. A) Example of dot-plot showing synaptosomes. The parameters are Forward Scatter (FSC - x axis) that gives information about the particle size and Side Scatter (SSC - y axis) that is proportional to the complexity and granularity of the particle. B) and C) Single-parameter fluorescence histograms for synaptosomes labeled with synaptophysin and A_1R (represented in green), respectively, and synaptosomes only incubated with secondary antibodies (showed in purple). D) Dual-parameter dot-plot with synaptosomes labeled with synaptophysin and VGluT1.

4. Results

4.1 Effect of BID in locomotor activity

First, I evaluated if animals with BID exhibit alterations on locomotor activity. As shown in Figure 6A, there was no difference in the total distance traveled between CTRL and BID in either gender. Nevertheless, a tendency could be observed for females to travel a greater distance within the open field than males, independently of the treatment. Additionally, Figure 6B illustrates that initial locomotor activity was higher, for both genders and treatments, but as the test progressed, the animals gradually slowed down and their activity stabilized at lower levels, suggesting locomotor habituation. Nevertheless, there was a significant effect of time ($P < 0.0001$) in the distance traveled for the animals, but no treatment effect in both genders.

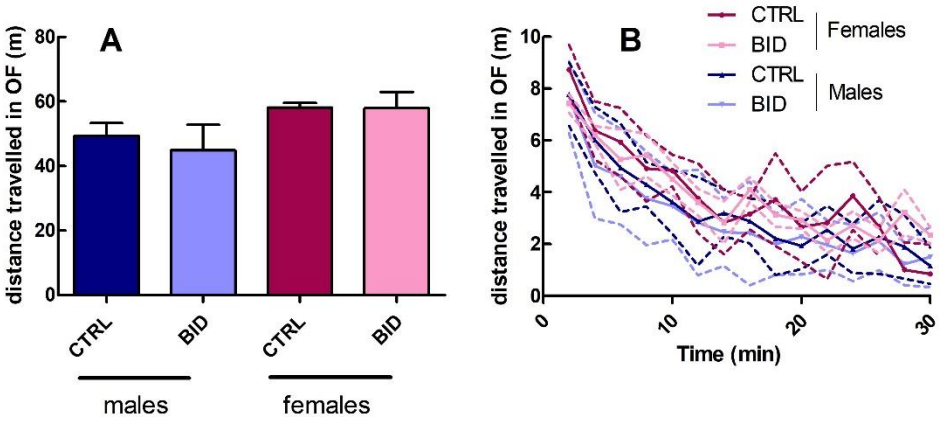

Figure 6. Effect of BID in locomotor activity of animals. A) Total distance travelled in meters for CTRL and BID animals of both genders. B) Open field (OF) habituation of exploratory behavior during the 30 min test. Dotted lines represent SEM. All results are shown as mean + SEM, n = 5-(Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

4.2 Effect of BID on Anxiety-like Behavior

OF test can also serve to evaluate anxiety-like behaviour. As shown in Figure 7A and B, there was no significant treatment effect beyond tendency on anxiety-like behavior in either gender since they travelled similar distances and also spent similar amounts of time in the center of OF.

In the EPM test, males with BID showed a tendency to spend more time in the open arms and, also, to show more entries in open arms than the control group. This behavior was virtually reversed in the females, since the control group tended to spend more time in open

arms (Figure 7C and D). Thus, this suggests an interaction between treatment and gender that was almost statistically significant (p = 0.07). Moreover, there was a tendency for gender effect on anxious behavior (% time in open arms: p = 0.06; % entries in open arms: p = 0.09).

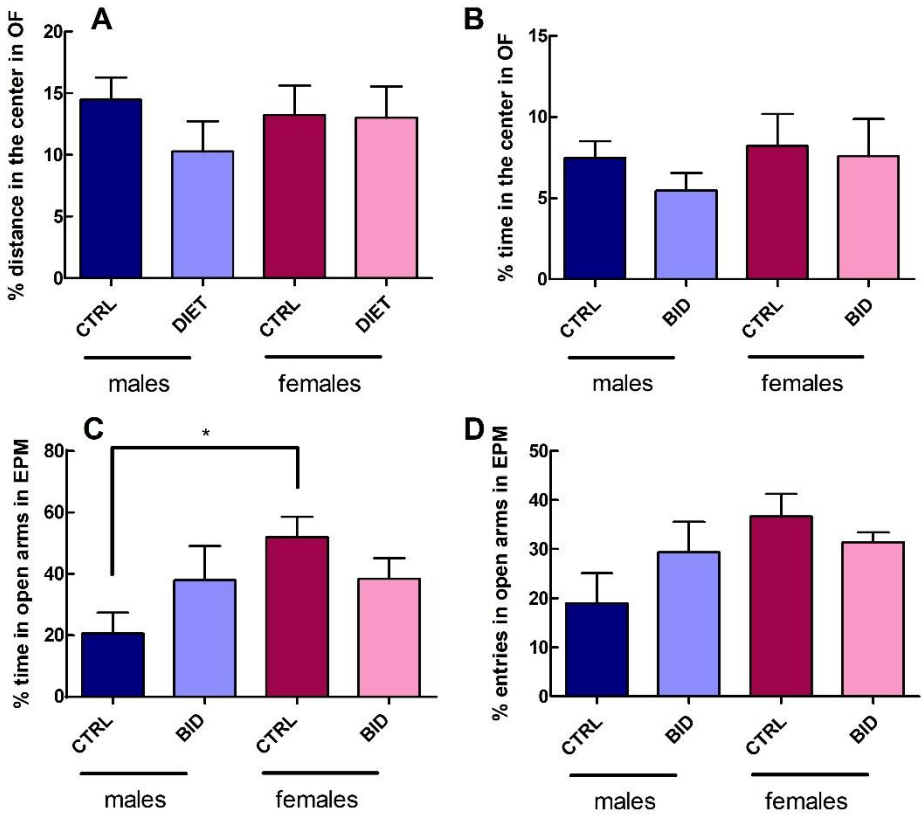

Figure 7. Anxiety-like Behavior in the OF and EPM tests. A) Percentage of distance that animals travelled in the center of OF. B) and C) Percentage of time that the rats spent in the center of OF and in the open arms of the EPM, respectively. D) Percentage of animal entries in the open arms of EPM. All results are shown as mean + SEM, n = 5-7. *P < 0.05 as compared between genders and respective controls (Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

4.3 Effect of BID on Spatial Working Memory

The alternation behavior was assessed in the Y maze test. Figure 8 documents that there was no significant difference in spontaneous alternation and number total of entries in the Y maze's arms, respectively, between control and BID groups. Also, Two-way ANOVA analysis with Tukey's multiple comparisons *post-hoc* test revealed no statistical significance for treatment (P = 0.37) or gender (P = 0.27) effects in spatial working memory and, respectively).

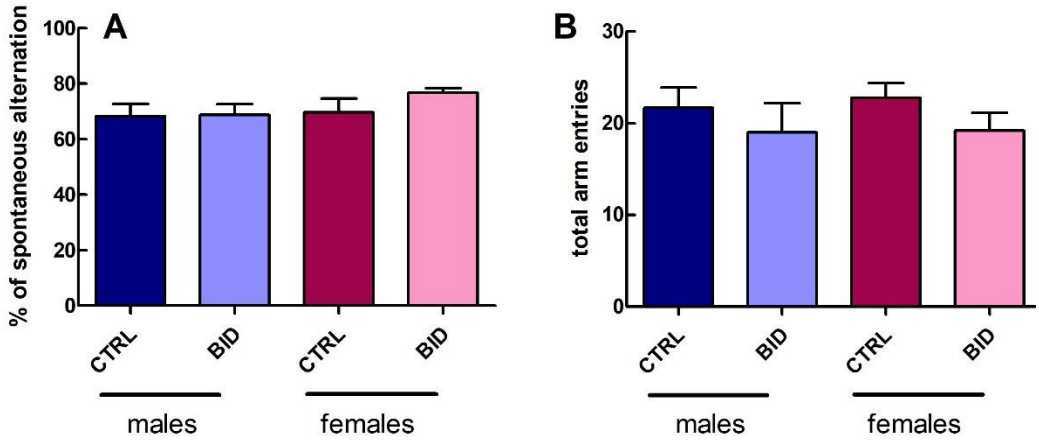

Figure 8. Spatial working memory in the Y-maze Spontaneous Alternation test. A) Percentage of Spontaneous Alternation in the Y-maze. B) Total number of entries in maze's arms. All results are shown as mean + SEM, n = 5-7 (Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

4.4 Presynaptic density of A_1R, $A_{2A}R$, CB_1R and MOR in striatal nerve terminals

The density of receptors was evaluated using double and triple labeling of purified synaptosomes and the analysis was made using dual dot-plots. Please kindly note that data presented in this version of the thesis contains mean ± SEM of only 3 or 4 pairs out of the 7 pairs of male and 8 pairs of female rats used for our study. Also, this study originally aimed at testing the density of neuromodulator receptors in dopaminergic terminals, too, but the DAT antibody from Sigma failed to stain the synaptosomes up to the dilution of 1:100.

As shown in Figure 9A, approximately 80% of the particles were positive for synaptophysin, independently of gender and treatment. As expected, the frequency of corticostriatal (positive for VGLUT1), thalamostriatal (positive for VGLUT2) and cholinergic terminals (positive for VAChT) among all synaptophysin- (SYN-) positive terminals was not affected by either gender or treatment (Figure 9B-D). As an important quality control, the ratio between the VGLUT1+ and VGLUT2+ terminals amounted to ~48%/~12%, *i.e.* 4:1, which is exactly the same as what was described previously in a milestone electronmicroscopy study, that is, 78% of all striatal glutamatergic terminals is VGLUT1-positive (Raju & Smith, 2005).

Likewise, there was no significant changes in the presynaptic density of A_1R and $A_{2A}R$. However, there was a tendency for presynaptic A_1Rs to decrease in BID males (Figure 9E) while presynaptic $A_{2A}Rs$ levels tended to increase in BID males (Figure 9F). Similarly, presynaptic CB_1R density tendentiously increased in the BID males (Figure 9G) while MOR density appears to decrease in both genders when subjected to a low iron diet. Hopefully, upon

completion of the samples from all the 30 animals, the results will be sufficiently clear to draw solid conclusions.

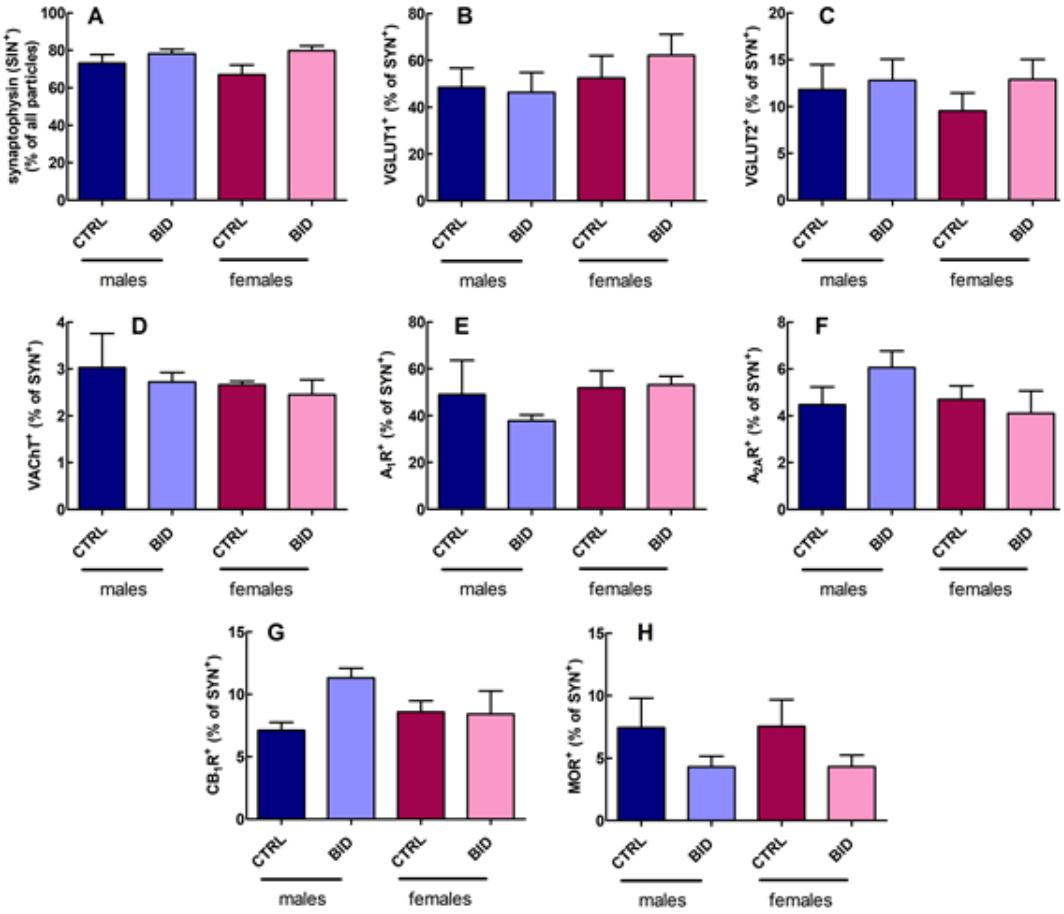

Figure 9. Effect of iron deficiency in density of A_1R, $A_{2A}R$, MOR and CB_1R. A) to H) Average percentage of particles positive for synaptophysin (aka, synaptosomes), and the percentage of VGLUT1-, VGLUT2-, VAChT-, A_1R-, $A_{2A}R$-, CB_1R- and MOR-positive particles among all synaptophysin-positive particles , in both male and female CTRL and BID animals, respectively. All results are shown as mean ± SEM, n = 3-4 (Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

4.5 Density of A_1R, $A_{2A}R$, CB_1R and MOR in Corticostriatal Terminals

Next, I evaluated each receptor in all three nerve terminal types separately, in triple labeling, that is anti-A_1R with anti-$A_{2A}R$ as well as anti-CB_1R together with anti-MOR in all three nerve terminals. These 6 triplets of antibody combinations were carefully selected with the help of our collaborator, Dr. Sergi Ferré, who is the pioneering neurologist in WED research at NIDA, Baltimore. Although at this stage, only 3 pairs of females and 4 pairs of males have been processed, there are already clear tendencies – or the lack of thereof for certain receptors at a given nerve terminal, as one can see in the following results.

Figure 10 documents that in corticostriatal (*i.e.* VGLUT1$^+$) terminals, none of the above 4 receptors seem to be affected by BID in the male animals, which is somewhat surprising. However, there is a greater chance for both the $A_{2A}R$ (Figure 10B) and the MOR (Figure 10E) to be downregulated by BID in the females, which already points toward a gender-specific effect of BID. When analyzing the percentage of synapses bearing both A_1R and $A_{2A}R$, it becomes immediately clear that all $A_{2A}R^+$ terminals are also A_1R^+, and BID does not affect the frequency of such terminals, at least in the males. In contrast, apparently around 60% of all MOR and CB_1R immunoreactivity colocalize in a subset of nerve terminals. While there is absolutely no change in this number in the males, BID tends to halve the number of CB_1R and MOR double$^+$ terminals in females.

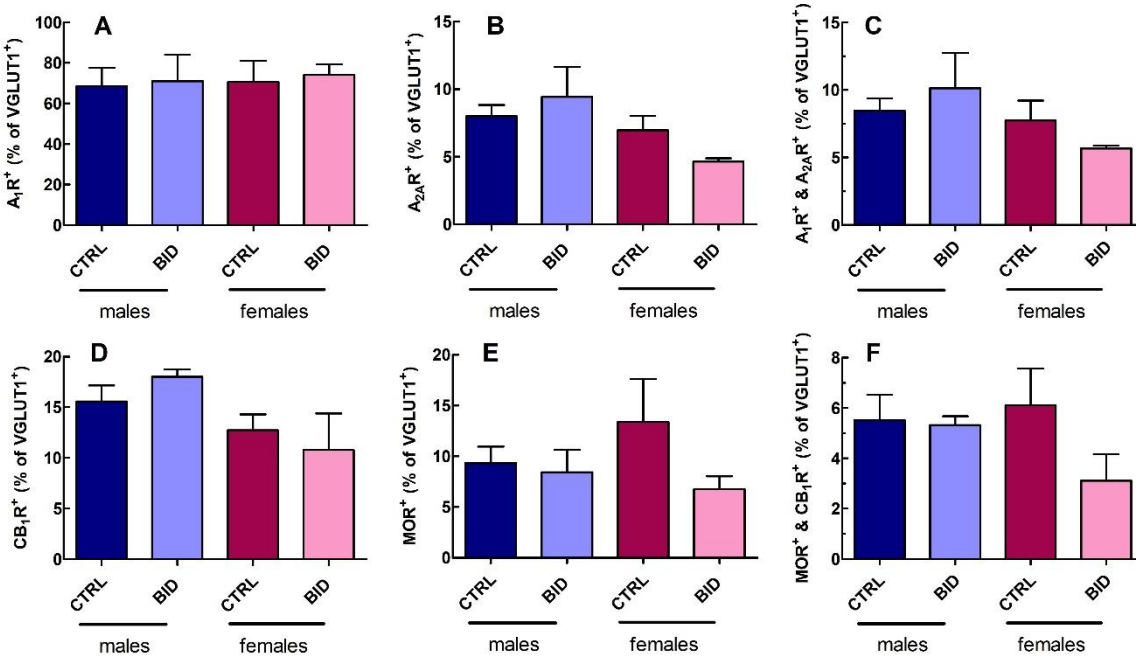

Figure 10. Effect of iron deficiency in density of receptors in corticostriatal terminals. Percentage of VGLUT1$^+$ terminals expressing A) A_1R, B) $A_{2A}R$ or C) both; or D) CB_1R, E) MOR or F) both in both genders. All results are shown as mean + SEM, n = 3-4 (Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

4.6 Density of A_1R, $A_{2A}R$, CB_1R and MOR in Thalamostriatal Terminals

Thalamostriatal afferents are important modulators of corticostriatal inputs. These VGLUT2$^+$ terminals amount to one fifth of all glutamatergic terminals in the striatum (Raju & Smith, 2005), and of course, may be differently affected by BID. Indeed, as Figure 11 illustrates, the amount of $A_{2A}R^+$ terminals was approximately the double that was found in VGLUT1$^+$ terminals Figures 10B vs. 11B), while the proportion of A_1R^+ terminals was similar in both terminal types (~70%; Figures 10A vs. 11A). Once again, all $A_{2A}R^+$ terminals were also A_1R^+ (Figure 11C).

In contrast, a substantially greater, 20-25% of thalamic inputs were endowed by either the CB_1R (Figure 11D) and the MOR (Figure 11E), and once again, half of this immunoreactivity was found in the very same terminals (Figure 11F). BID apparently had no appreciable effect on the adenosine receptors and the CB_1R, but it reduced thalamostriatal MOR expression in both genders, which is expected to reach statistical significance with increasing the sample number. Apparently, mostly the non-CB_1R bearing MOR$^+$ terminals were affected by BID (Figure 11E vs. F).

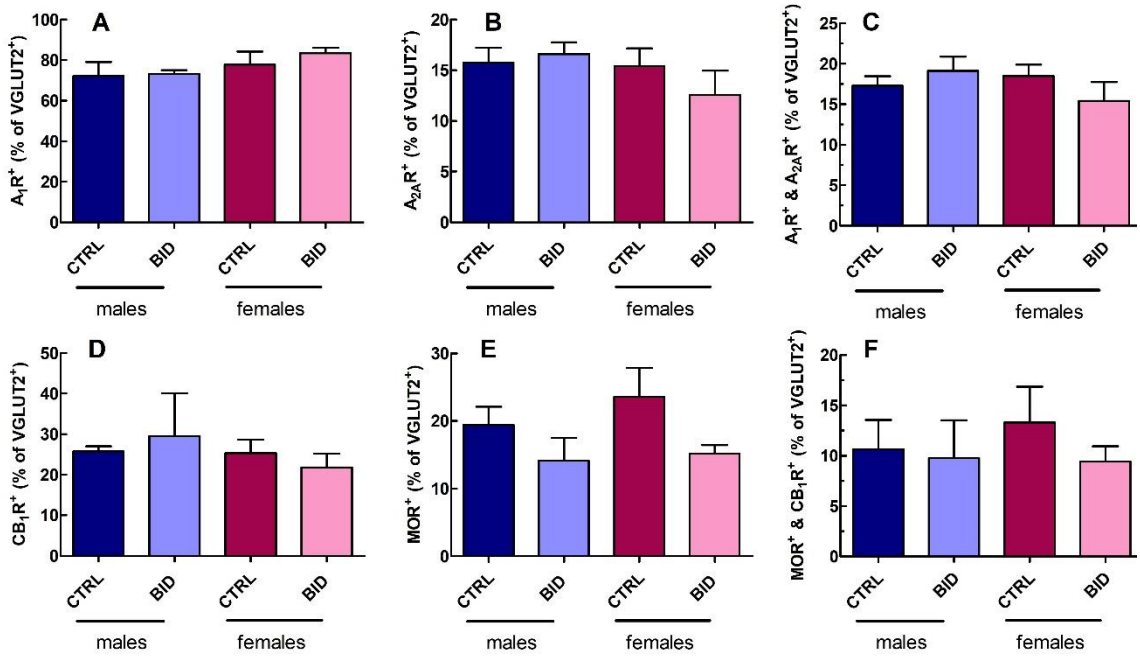

Figure 11. Effect of iron deficiency in density of receptors in thalamostriatal terminals. Percentage of VGLUT2$^+$ terminals expressing A) A_1R, B) $A_{2A}R$ or C) both; or D) CB_1R, E) MOR or F) both in both genders. All results are shown as mean + SEM, n = 3-4.

4.7 Density of A_1R, $A_{2A}R$, CB_1R and MOR in Cholinergic Nerve Terminals

Figure 12 presents that the frequency of A_1R^+ cholinergic terminals was ~70%, while only ~25% of cholinergic terminals bears the $A_{2A}R$, and again, all $A_{2A}R^+$ terminals were also A_1R^+ (Figure 12A-C). There was a minor and opposing tendency for change in $A_{2A}R$ expression in male and female BID rats (Figure 12B).

One third of cholinergic terminals appeared to be positive for CB_1R, while 50% of them were endowed with the MOR. In contrast to the glutamatergic terminals, all CB_1R^+ terminals were also MOR^+. Finally, BID does not appear to significantly affect the density of these two receptors in cholinergic terminals of either gender.

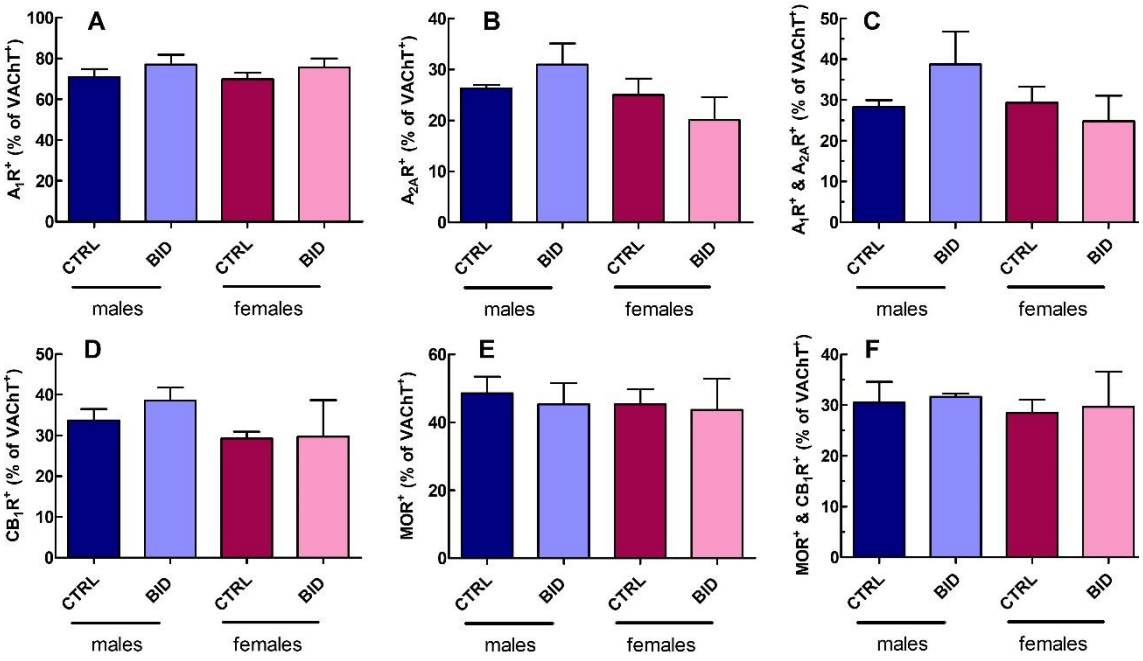

Figure 12. Effect of iron deficiency in density of receptors in cholinergic terminals. Percentage of $VAChT^+$ terminals expressing A) A_1R, B) $A_{2A}R$ or C) both; or D) CB_1R, E) MOR or F) both in both genders. All results are shown as mean + SEM, n = 3-4 (Two-way ANOVA with Tukey's multiple comparisons *post-hoc* test).

5. Discussion

5.1 Brain Iron Deficiency

Iron is a double-edged micronutrient, because both its deficiency and its excess accelerate the onset of serious chronic disorders, including neurological conditions. Besides storing in the hemoglobin and the liver, our brain, especially the basal ganglia accumulates iron at high quantities (Grubić Kezele & Ćurko-Cofek, 2020; Singh et al., 2014). There is a growing body of evidence supporting gender-specific iron homeostasis (Grubić Kezele & Ćurko-Cofek, 2020). This is intriguing and alarming in the same time, because neurological symptoms which are often more prevalent in women, including WED and migraine, and which may be linked to gender-specifically impaired iron homeostasis, are often studied in male rodents, in order to avoid the estrus cycle as a confounding factor.

Nevertheless, animal models of BID are also excellent RLS/WED models and have been employed for decades. It is also important which aspect of BID one wants to study, because early childhood iron deficiency may lead to severe developmental deficits. Therefore, an effort has been made to assess the impact of the severity and timing of nutritional iron deficiency on developing rats, starting from the in-utero life until the post-weaning (Beard et al., 2006; Erikson et al., 1997; Piñero et al., 2000). Based on these, we chose a reliable model with little impact on brain development and behavior.

The animals after the four-week diet showed great differences. We found an intriguing gender-specific effect for anemia on body weight – males rather than females showed a mild but significant growth retardation. Besides, blood test confirmed severe anemia. Nevertheless, these alterations were seemingly not associated with overt behavioral phenotype.

5.2 Behavioral Assessment of our BID Model

WED is a chronic sensorimotor disorder, characterized by the feeling of restlessness and an urgent need for movement, especially during the nocturnal period or during the rest when the aggravation of symptoms is observed. Besides motor symptoms, common comorbidities of WED in humans include insomnia, anxiety, and depression (Earley & Silber, 2010). Association with impaired cognitive and executive functioning has also been reported (Earley & Silber, 2010; Fulda et al., 2011; Jung, 2015).

To better characterize our model, we performed basic behavioral characterization of male and female rats with and without BID. Of note, these experiments were not controlled for the estrus cycle of the female adolescent rats. On the other hand, the experiments were carried

out daytime, *i.e.* the diurnal cycle corresponding to the resting period for rats, since in humans WED symptoms are exacerbated during nighttime.

In the open field test, we found no changes in locomotor activity and no changes in habituation between the control and the BID groups, suggesting that exploratory behavior and sensory filtering are not affected in rats with BID. However, exploratory activity recorded for 90 minutes has been previously found to be lower in the same model, but only in males (Quiroz et al., 2010). This difference could be due to the duration of the test, which for us was 30 minutes, compared to their 90 minutes. We also need to increase the number of animals to consolidate our conclusions.

For emotional behavior, we evaluated exploratory activity in the center of the open field and in the open arms of the elevated plus maze. With limited number of animals, we could find only tendency for decreased anxiety-like behavior in the open field for male BID rats and increased anxiety-like behavior for female BID rats. However, baseline anxiety-like behavior in the EPM was different in males and females, regardless of diet. This is a well-known phenomenon: Scholl et al. (2019) have thoroughly documented that female rats spend significantly more time in open areas than did male rats in the EPM rather than in the open field paradigm; regardless of estrous cycle stage.

Nevertheless, our EPM results in males are in contrast to a previous report by Li and colleagues (2011) using the same animal model we used, as those authors reported an increased anxiety-like behavior, given by the rats' lower exploration of the open arms of the EPM. Higher anxiety levels have been reported in WED patients, too (Earley & Silber, 2010), but given the higher tendency for these patients be women, gender is a confounding factor. Therefore, more animals should be tested, as well as other behavior tests are necessary to detect subtle changes, such as sucrose preference test to evaluate anhedonia, light-dark box to evaluate anxiety-like behavior and fear conditioning to evaluate emotional processing. From those experiments it would be possible to tell whether BID rats show symptoms consistent with anxiety- and depressive-like states.

As a first assessment of cognitive function, we assessed working memory in the Y-maze spontaneous alternation test. We found no differences between control and BID rats. It is worth noting that it has been previously reported that WED patients show deficits in working memory (for review see Jung, 2015). Therefore, it would be interesting to test the animals in more sensitive working memory tests, such as the radial arm maze or the working memory version of the Morris water maze. Since deficits in behavior flexibility has also been reported for (Fulda et al., 2011; Jung, 2015), it will also be important to test behavior flexibility, for instance reversal learning in the Morris water maze.

5.3 Flow Synaptometry Analysis Suggests Presynaptic Changes in BID

I achieved one of the main goal of the thesis: I set up optimal parameters for the double and triple detection of presynaptic markers in a recent technique what we call "flow synaptometry". I got reliable ratios for VGLUT1 and VGLUT2 labeling among synaptophysin$^+$ terminals, even after only processing 4 pairs of male and 3 pairs of female rats. The percentage of cholinergic terminals was relatively low in comparison with glutamatergic terminals, and this was also expected, although quantitatively never before documented. Since the vast majority of striatal terminals are glutamatergic, the remaining 10-20% of terminals are expected to be GABAergic and dopaminergic, and only a minor fraction remains for cholinergic, noradrenergic and serotonergic terminals.

In our model, BID did not affect the density of glutamatergic and cholinergic innervations in the striatum. This is an important feature of our model, because we chose the post-weaning period to induce BID, in order to avoid gross neurodevelopmental effects. Nevertheless, as Fig. 9E and F demonstrates, there was already a clear inhibitory-excitatory dysbalance in the adenosinergic signaling, since the overall A_1R density tends to decrease while $A_{2A}R$ density increases among the nerve terminals, at least in the male BID rats. Also, there was a clear tendency for the increase in CB_1R^+ terminals in the male rats. These altogether underlie the importance of testing laboratory animals from both genders. Finally, the frequency of MOR$^+$ terminals visibly decreased in both genders and it will eventually reach statistical significance. If so, it could be suggestive of an opioid deficiency at least in the striatum. This could in part explain the increased pain sensation in WED and the need for external opioid supplementation, which is one of the most successful strategies for these patients (de Oliveira et al., 2016; Trenkwalder et al., 2017).

In spite the overall tendencies for these neuromodulator receptors, the low sample number so far has not allowed me to ascribe gross changes in one particular nerve terminal type. It is also possible that some of the gross changes would appear in those terminal types we have not investigated yet, *i.e.* in GABAergic and dopaminergic synaptosomes. Unfortunately, our effort to label dopaminergic synaptosomes with anti-DAT for flow synaptometry has not been successful so far.

5.3.1 Corticostriatal Terminals

A_1R, $A_{2A}R$, CB_1R and MOR are well documented functional presynaptic receptors in corticostriatal, *i.e.* VGLUT1$^+$ terminals (Ciruela et al., 2006; Ferré et al., 2018; Köfalvi et al., 2020), and among them, only the $A_{2A}R$ is facilitatory. Interestingly, the earlier electron microscopy study by Ciruela and colleaues (2006) indeed confirmed that virtually all

glutamatergic (VGLUT1$^+$ and VGLUT2$^+$)-positive terminals that are endowed with the $A_{2A}R$ is also A_1R^+, thus corroborating our findings.

In contrast to what is expected at least in human patients, based on previous studies (Ferré et al., 2019), we found so far, no tendency for a decrease in the number of A_1R^+ terminals, especially not in corticostriatal afferents. Albeit it seems a bit unexpected, it can be simply the limitation of our technique. Let's imagine that A_1R density is reduced in a single corticostriatal terminal by 90% from 100 individual A_1R proteins down to 10 receptor proteins due to BID, this nerve terminal will be still counted as an A_1R^+ terminal, even though, A_1R function would be strongly compromised in this terminal. Therefore, flow synaptometry is not expected to detect functional deficits, but only an overall frequency change of the receptor bearing terminals in the total pool.

Additionally, knowing that A_1R is subject to heteromerization with $A_{2A}Rs$, it equally possible that an increase in $A_{2A}R$ density negatively affects A_1R functioning via excessive heteromerization (Ferré et al., 2011; Ferré and Ciruela, 2019). Finally, I observed an overall increase in CB_1R density in male BID subjects. Since CB_1R $G_{i/o}$ signaling competes with A_1R signaling in most if not all nerve terminals where the two are present (Solymosi & Kofalvi, 2017)

Finally, corticostriatal terminals isolated from three female BID rats showed a strong ~50% reduction in CB_1R-MOR double positive terminals. We cannot ignore here the fact that there is a strong correlation between the opioid and endocannabinoid systems (Solymosi & Kofalvi, 2017). As an example, opioids and cannabimimetics exhibit similar pharmacological effects, including analgesia, hypotension, hypothermia, motor impairment and sedation. Some of these converging effects are in fact due to converging signaling of the CB_1R and the MOR, taken that the CB_1R-MOR heteromer is one more example of GPCR heteromerization (Rios et al., 2006). The interaction between the CB_1R and the MOR is so strong that the the shell of the nucleus accumbens of the CB_1R global KO mice exhibits reduced number of MOR$^+$ MSN dendrites and a loss of MOR$^+$ corticoaccumbal glutamatergic afferents, nevertheless, the density of MOR$^+$ staining in each remaining afferents increased substantially (Lane et al., 2010). Therefore, it is possible that the two receptors help each other's trafficking and also serve as a trophic factor.

5.3.2 Thalamostriatal Terminals

These afferents are originated from the parafascicular complex of the thalamus and are important modulators of the corticostriatal information flow. As above discussed, the presence of A_1R-$A_{2A}R$ heteromers in both cortico- and thalamostriatal afferents are well documented (Ciruela et al., 2006; Ferré and Ciruela, 2019). Furthermore, MORs at striatal VGLUT2$^+$

terminals modulate reward (Reeves et al., 2020). As for the CB_1R, there is compelling evidence for the significantly lower CB_1R density in thalamostriatal than in corticostriatal terminals (Uchigashima et al., 2007; Wu et al., 2015). This is perhaps not reflected in the frequency of CB_1R^+ terminals among each glutamatergic cell type, but rather, in the amount of receptor packed in both terminal types. Hence, it is clear that the CB_1R does not participate in thalamostriatal synaptic plasticity (Wu et al., 2015), and therefore may have other roles including metabolic control or axon guidance (Solymosi & Kofalvi, 2017).

In our BID model, we found a tendentious reduction in MOR^+ terminals among all $VGLUT2^+$ terminals, but it is important to wait with the interpretation of this until gathering conclusive data.

5.3.3 Cholinergic Terminals

Cholinergic ($VAChT^+$) terminals modulate myriad aspects of striatal circuit function (including cellular excitability, synaptic transmission and plasticity, dopamine release, and circuit responses to salient cues) (Abudukeyoumu et al., 2019; Prager & Plotkin, 2019). Among many other functions, an interesting one is that ACh, via post-synaptic M_1R activation, triggers the release of the endocannabinoid, 2-AG in MSN dendrites, which in turn can reduce presynaptic GABA release onto MSN dendrites (Uchigashima et al., 2007).

Cholinergic terminals are also subject of modulation by several converging neuromodulator inputs. For instance, adenosine via presynaptic A_1R inhibits while via presynaptic $A_{2A}R$ stimulates acetylcholine release in the striatum.

As for opioids, clear evidence indicates that both MOR mRNA and MOR protein are present in cholinergic interneurons in the limbic/prefrontal territory but not by those in the sensorimotor territory of the dorsal striatum. Activation of these MORs by endogenously released enkephalin reduces ACh release (Jabourian et al., 2005).

Whether endocannabinoids can modulate intrastriatal ACh release it is contentious, but the general consensus is that these striatal giant cholinergic interneurons are devoid of CB_1R expression (Solymosi & Kofalvi, 2017; Uchigashima et al., 2007). Actually, these cholinergic interneurons are the only known cholinergic neurons which are devoid of CB_1R expression, and both A. Köfalvi and others have shown that the most efficacious CB_1R agonist, WIN55212-2 fails to affect striatal acetylcholine release. In contrast, extrastriatal ACh sources are subject to presynaptic modulation by CB_1R (for review see Solymosi & Köfalvi, 2017).

Therefore, it came as a surprise that I found that 30% of striatal cholinergic terminals were CB_1R^+. The only resolution of this conundrum could be the assumption that the striatum receives previously overlooked external cholinergic afferents too, from the midbrain, where cholinergic cells do express the CB_1R. And to our greatest surprise, this seems to be the case! It

has been very recently published in Nature Communications that the midbrain, a previously unknown source of acetylcholine in the striatum, is a major contributor to cholinergic transmission in the striatal complex. Dauton and colleagues (2020) found that neurons of the pedunculopontine and laterodorsal tegmental nuclei synapse with striatal cholinergic interneurons and give rise to excitatory responses. Furthermore, they produce uniform inhibition of spiny projection neurons.

5.3 Concluding Remarks and Future Aims

In conclusion, even with a low number of animals assayed with flow synaptometry, we can detect changes in presynaptic neuromodulator receptors which will surely enrich our knowledge about how iron modulates brain functioning and how the negative impacts of BID can be efficiently combated with pharmacotherapy.

This project was conceived in the realm of an ongoing collaboration between our group and the group of Dr. Sergi Ferré (Molecular Targets and Medications Discovery Branch, Integrative Neurobiology Section, NIDA). This collaboration has yielded several coauthorsips on milestone papers for the two groups (Borycz et al., 2007; Köfalvi et al., 2020). Dr. Ferré's group have pioneered the field of BID research in RLS/WED and reached the conclusion that adenosiergic dysfunction takes center stage and serves as a possible therapeutic target (Garcia-Borreguero 2018; Ferré et al., 2019). As his group still lacked a refined target to mass evaluate presynaptic changes in various nerve terminal types, my Master Thesis project served a good alternative solution to this challenge. My project is only finished when I complete the desired sample number for the here presented experiments, because we must investigate two more major nerve terminal types, the GABAergic and dopaminergic terminals in the BID animals. Furthermore, I have prepared synaptosomes from both the hippocampus and the frontal cortex of the 30 rats involved so far in this project with the aim of looking outside the striatum to seek for aberrant changes in BID. My group became interested in this model as it can subserve different additional animal models including migraine and epilepsy (Pamuk et al., 2016; Rudy & Mayer-Proschel, 2017), both of which are more prevalent in women, and which are dependent on arousal in cortical and hippocampal synaptic transmission, attributable to impaired neuromodulation and energy metabolism.

Another technique not immediately available for Dr. Ferré's group is electrophysiology. Since I have got acquainted with this technique during my project, I would like to expand our horizon on this collaboration and investigate the possible dysfunctions present in striatal circuitries. In detail, I would like to explore how corticostriatal synaptic plasticity is affected in the male and female BID animals. Finally, I would like to further assess our BID rats in refined behavioral tests to tease out possible (and gender-specific) differences.

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