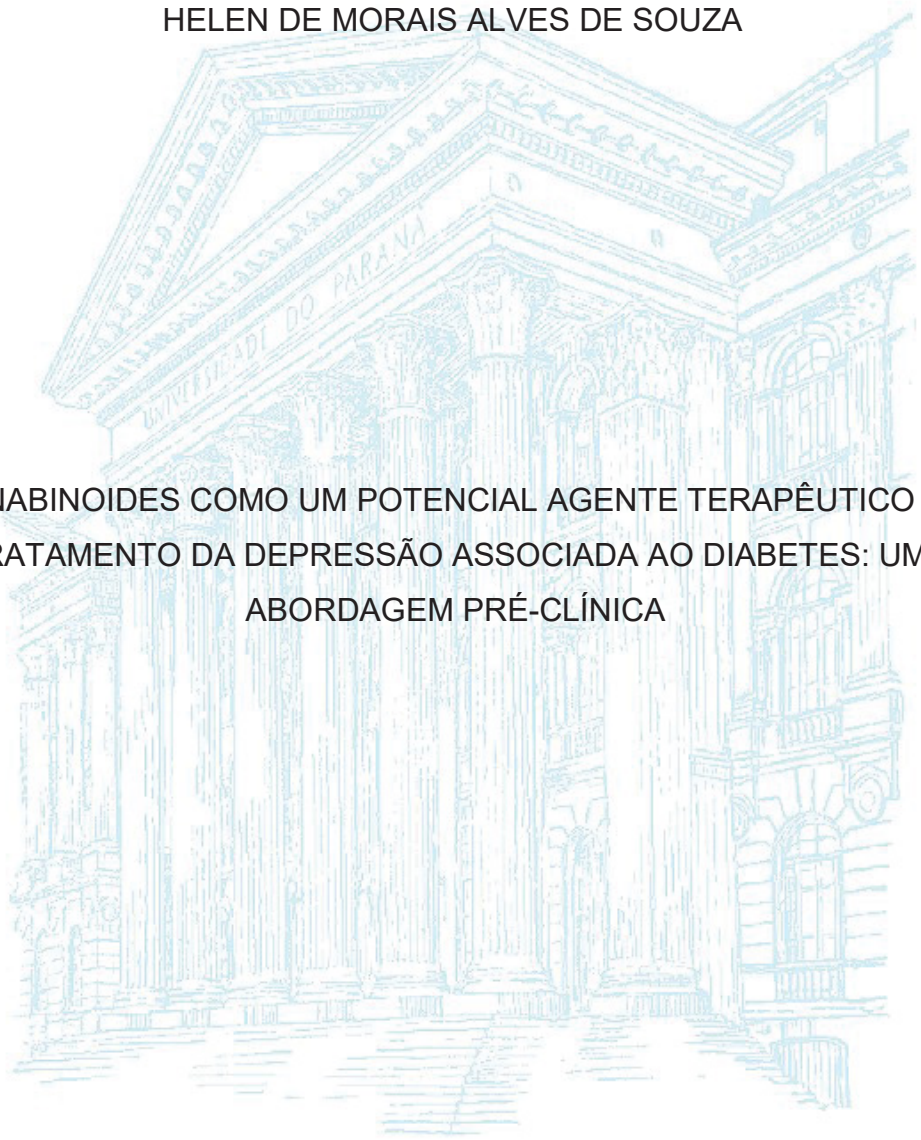
UNIVERSIDADE FEDERAL DO PARANÁ

HELEN DE MORAIS ALVES DE SOUZA



CANABINOIDES COMO UM POTENCIAL AGENTE TERAPÊUTICO NO TRATAMENTO DA DEPRESSÃO ASSOCIADA AO DIABETES: UMA ABORDAGEM PRÉ-CLÍNICA

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, setor Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Farmacologia

Orientadora: Profª Drª Janaína Menezes Zanoveli

Co-orientadora: Profª Drª Joice Maria da Cunha

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **HELEN DE MORAIS ALVES DE SOUZA** intitulada: **Canabinóides como um potencial agente terapêutico no tratamento da depressão associada ao diabetes: uma abordagem pré-clínica** , após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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Curitiba, 27 de Abril de 2018.

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Dedico este trabalho aos meus familiares, em especial ao meu filho Otávio, meu esposo Eduardo, meu pai Jair, minha mãe Helena e a minha irmã Hely. A vocês, os meus mais belos sentimentos.

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“Deixe algum sinal de alegria onde passes.”

Francisco Cândido Xavier

NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo, como artigos científicos, sendo um publicado e um submetido para publicação, de acordo com as normas do Programa de Pós-Graduação em Farmacologia, do Setor de Ciências Biológicas da Universidade Federal do Paraná. A tese consta de uma revisão bibliográfica, hipótese e objetivos gerais do trabalho e dois artigos científicos com os experimentos realizados, resultados e discussão, além das considerações finais.

RESUMO

A depressão associada ao diabetes tem sido descrita como uma comorbidade altamente debilitante. Devido aos seus mecanismos complexos e multifatoriais, o tratamento da depressão associada ao diabetes representa um desafio clínico. Dessa forma, o sistema endocanabinoide (SEC) tem sido apontado como um sistema promissor, envolvido na neurobiologia de muitas doenças, incluindo depressão e diabetes. Assim, objetivamos investigar o papel do SEC na fisiopatologia da depressão associada ao diabetes. Para isso, ratos Wistar machos diabéticos (DBT) e normoglicêmicos (NGL) foram tratados com antagonistas canabinoide CB1 ou CB2, seguidos de anandamida (AEA) e então submetidos ao teste de natação forçada modificado (TNFm). Parâmetros de estresse oxidativo, expressão do receptor CB1 e níveis de serotonina (5-HT) e noradrenalina (NA) no hipocampo e no córtex pré-frontal também foram realizados. Observou-se que os animais DBT apresentaram um comportamento do tipo depressivo mais pronunciado e aumento da expressão do receptor CB1 no hipocampo. O tratamento com AEA induziu uma melhora significativa no comportamento do tipo depressivo, que foi revertido pelo antagonista CB1, sem afetar a hiperglicemia ou o ganho de peso. A AEA também foi capaz de restaurar a expressão elevada CB1 e também elevar o nível reduzido de 5-HT no hipocampo de animais DBT. Além disso, o AEA restaurou os níveis elevados de NA no córtex pré-frontal e induziu um efeito neuroprotetor ao restaurar a glutationa reduzida e aumentar os níveis de lipoperoxidação, juntamente com a redução da atividade da superóxido dismutase observada no hipocampo e no córtex pré-frontal. Também foi de interesse desse trabalho, avaliar o potencial efeito antidepressivo do tratamento agudo ou subcrônico com o canabidiol (CBD) em ratos diabéticos usando o TNFm. Para melhor compreender a funcionalidade do sistema endocanabinoide em animais DBT, também avaliamos o efeito do URB597, um inibidor da hidrolase de amida de ácido graxo. Quatro semanas após o tratamento com estreptozotocina ou tampão citrato, animais DBT receberam uma injeção intraperitoneal aguda de CBD, 1 hora antes do TNFm, ou URB597, 2 horas antes do TNFm. Em outro grupo experimental, os animais foram tratados subcrônicamente com CBD, 24, 5 e 1 hora antes da TNFm ou URB597, 24, 5 e 2 horas antes do TNFm. O grupo de NGL foi tratado de forma aguda com CBD ou URB597. O tratamento agudo com CBD ou URB induziu um do tipo antidepressivo em ratos NGL, mas não em ratos DBT. No entanto, o tratamento subcrônico com CBD, mas não com o URB597, induziu um discreto efeito antidepressivo em animais DBT. Nem o peso corporal, nem os níveis de glicose sanguinea foram alterados pelos tratamentos. Considerando a importância do sistema endocanabinoide para os mecanismo envolvidos na depressão associada ao diabetes, consideramos importante aprofundar os estudos envolvendo o sistema endocanabinoide, particularmente em animais DBT.

Palavras chaves: Diabetes. Depressão. Estresse oxidativo. Sistema endocanabinoide. Anandamida. Canabidiol. Serotonina.

ABSTRACT

Depression associated with diabetes has been described as a highly debilitating comorbidity. Due to its complex and multifactorial mechanisms, the treatment of depression associated with diabetes represents a clinical challenge. Thus, the endocannabinoid system (ECs) has been singled out as a promising system involved in the neurobiology of many diseases, including depression and diabetes. Thus, we aimed to investigate the role of ECS in the pathophysiology of depression associated with diabetes. For this, male Wistar rats diabetic (DBT) and normoglycemic (NGL) were treated with cannabinoid antagonists CB1 or CB2, followed by anandamide (AEA) and then submitted to the modified forced swimming test (mFST). Parameters of oxidative stress, CB1 receptor expression and levels of serotonin (5-HT) and noradrenaline (NA) in the hippocampus and prefrontal cortex were also performed. It was observed that DBT animals presented more pronounced depressive behavior and increased CB1 receptor expression in the hippocampus. Treatment with AEA induced a significant improvement in the behavior of the depressive type, which was reversed by the CB1 antagonist, without affecting hyperglycemia or weight gain. AEA was also able to restore high CB1 expression and also raise the reduced level of 5-HT in the hippocampus of DBT animals. In addition, AEA restored elevated NA levels in the prefrontal cortex and induced a neuroprotective effect by restoring reduced reduced glutathione and increasing lipoperoxidation levels, along with a reduction in superoxide dismutase activity observed in the hippocampus and prefrontal cortex. It was also of interest in this study to evaluate the potential antidepressant effect of acute or sub-chronic treatment with cannabidiol (CBD) in diabetic rats using mFST. To better understand the functionality of the endocannabinoid system in diabetic animals, we also evaluated the effect of URB597, a fatty acid amide hydrolase inhibitor. Four weeks after treatment with streptozotocin or citrate buffer, acute DBT animals received an intraperitoneal injection of CBD 1 hour before mFST or URB597 2 hours before mFST. In another experimental group, the animals were treated sub-chronically with CBD, 24, 5 and 1 hour before to mFST or URB597 24, 5 and 2 hours before to mFST. The NGL group was treated acutely with CBD or URB597. The NGL group was treated acutely with CBD or URB597. Acute treatment with CBD or URB induced a similar effect to antidepressant in NGL rats, but not in DBT rats. However, sub-chronic treatment with CBD, but not with URB597, induced a mild antidepressant effect in DBT animals. Neither body weight nor blood glucose levels were altered by treatments. Considering the importance of the ECS for the mechanisms involved in depression associated with diabetes, we consider it important to deepen studies involving the ECS, particularly in DBT animals.

Keywords: Diabetes. Depression. Oxidative Stress. Endocannabinoid System. Anandamide. Cannabidiol. Serotonin.

LISTA DE ABREVIATURAS

AEA – Anandamida

AM251 – Antagonista do Receptor CB1

AM630 – Antagonista do Receptor CB2

ANOVA – Análise de Variância

ATP – Adenosina Trifosfato

CBD – Canabidiol

CAT – Catalase

CB1 – Receptor CB1

CB2 – Receptor CB2

PFC – Córtex Pré-Frontal

DBT – Diabético

DM – *Diabetes Mellitus*

DM1 – *Diabetes Mellitus* tipo 1

DM2 – *Diabetes Mellitus* tipo 2

DNA – Ácido Desoxirribonucleico

ERN – Espécie Reativa de Nitrogênio

ERO – Espécie Reativa de Oxigênio

FST – Teste de Natação Forçada

mFST – Teste de Natação Forçada Modificada

GLUT2 – Transportador de Glicose tipo 2

GPX – Glutationa Peroxidase

GSH – Glutationa Reduzida

GSSG – Glutationa Oxidada

HIP – Hipocampo

HHA – Hipotálamo-Hipófise-Adrenal

HU210 – Agonista do Receptor CB1

IMI – Imipramina

I.P. – Intraperitonial

LPO – Peroxidação Lipídica

LOOH – Hidroperoxido de Lipídio

MGL – Monoacilglicerol Lipase

NA – Noradrenalina

NGL – Normoglicêmico

SBD – Sociedade Brasileira de Diabetes

SOD - Superóxido Dismutase

STZ – Estreptozotocina

TNF – Teste de Natação Forçada

URB597 – Inibidor da Amida Hidrolase de Ácido Graxo

2AG – 2-araquidonoilglicerol

5-HT – Serotonina

SUMÁRIO

1 INTRODUÇÃO

1.1 *DIABETES MELLITUS*: EPIDEMIOLOGIA, CLASSIFICAÇÃO E COMPLICAÇÕES

O D*iabetes* M*ellitus* (DM) é uma disfunção metabólica caracterizada por hiperglicemia persistente (KLEINBERGER e POLLIN, 2015; DE OLIVEIRA *et al*., 2017; BHARTI *et al*., 2018) resultante de uma falha na secreção de insulina, na ação da insulina ou de ambas (BROWNLEE, 2005; XIANG *et al*., 2010; ALMEIDA *et al*., 2018). Em 2017 foi estimado que o número de pessoas no mundo vivendo com a doença era de 425 milhões, destes uma em cada duas pessoas não são diagnosticadas. Em 2045 o número de diabéticos poderá chegar a 629 milhões de pessoas e em relação à faixa etária estima-se que em 2045, 438 milhões de pessoas terão diabetes entre 20 a 64 anos e 191 milhões de pessoas entre 65 a 79 anos. Com relação ao custo financeiro, foram gastos em 2017 cerca de 727 bilhões de dólares no tratamento dessa doença (INTERNATIONAL DIABETES FEDERATION, 2017). O Brasil é o quarto país a nível mundial com maior prevalência de DM na população adulta, que está em torno de 12,5 milhões pessoas (INTERNATIONAL DIABETES FEDERATION, 2017).

O DM é classificado em três tipos principais: DM tipo 1 (DM1), DM tipo 2 (DM2) e DM gestacional. De acordo com a *INTERNATIONAL DIABETES FEDERATION* (IDF, 2017), o DM1 representa cerca de 5 a 10 % de todos os casos de DM (IDF, 2015, 2017; SKYLER *et al*., 2017). Já o DM2 atinge cerca de 95% dos pacientes diabéticos e geralmente acomete pacientes com idade superior a 40 anos, porém o número de crianças e jovens com DM2 também vem crescendo ao longo dos últimos anos (WILSON, 2013; DIRETRIZES DA SOCIEDADE BRASILERIA DE DIABETES 2015-2016; SKYLER *et al*., 2017).

De particular interesse para o presente estudo é importante mencionar que o DM1 pode acometer pessoas em qualquer faixa etária, mas geralmente ocorre em crianças e jovens (BARAT *et al*., 2008; ATKINSON *et al*., 2014). De acordo com a IDF (2017), existem aproximadamente 542 mil crianças de 0 a 14 anos de idade que possuem DM1. Estudos mostram que a incidência e prevalência do DM1 variam substancialmente. DM1 é mais comum na Finlândia (>60 casos para

cada 100.000 pessoas a cada ano) e na Sardenha (por volta de 40 casos para cada 100.000 pessoas a cada ano). Curiosamente e de maneira diferente, o DM1 não é comum na China, Índia e Venezuela (por volta de 0-1 casos para cada 100.000 pessoas a cada ano) (ATKINSON *et al*., 2014). Outra observação importante é que a incidência de DM1 não tem ocorrido igualmente quando se avalia o fator idade. Os mecanismos relacionados com incidência geográfica e com o aumento das taxas da incidência de DM1 ainda são desconhecidos; porém, fatores ambientais parecem contribuir, tais como condições de higiene, viroses e também uma relação com a microbiota intestinal, além de fatores genéticos (ATKINSON *et al*., 2014).

Informações sobre a epidemiologia do DM1 especificamente em adultos são muito reduzidas. Em muitos estudos epidemiológicos, pacientes adultos que apresentam hiperglicemia acima dos valores de referências são considerados como simplesmente diabéticos, sem levar em conta as diferenças do DM1 versus DM2 (ATKINSON *et al*., 2014; FAZELI *et al*., 2017; BODEN, 2018), indicando que até 50% dos casos reais de DM1 sejam diagnosticados erroneamente como DM2, significando que o número de casos de DM1 é amplamente subestimado (ATKINSON *et al*., 2014; DIAZ-VALENCIA *et al*., 2015).

Em relação à doença em si, o DM1 resulta da destruição autoimune das células β do pâncreas. Uma pequena porcentagem de pacientes afetados é classificada como tipo 1B, sem evidência de autoimunidade, e a patogênese nesses casos é considerada idiopática (ATKINSON *et al*., 2014; PASCHOU *et al*., 2018). Quando ocorre destruição das células β pancreáticas, ocorre também uma deficiência da produção de insulina (IQBAL *et al*., 2018; SUBRAMANIAN *et al*., 2018). Esta deficiência resulta de um processo complexo em que fatores genéticos e ambientais conduzem a uma resposta autoimune levando à destruição das células β pancreática secretora de insulina (ATKINSON *et al*., 2014; PASCHOU *et al*., 2018). O DM1 geralmente segue um curso clínico agudo, com pacientes apresentando poliúria, polidipsia e perda de peso, além da hiperglicemia (FAZELI *et al*., 2017; IQBAL *et al*., 2018). O tratamento do DM1 é baseado na substituição de insulina inexistente ou deficiente (IQBAL *et al*., 2018; SUBRAMANIAN *et al*., 2018).

O diabetes *mellitus* é confirmado quando a glicose sérica (jejum) é igual ou superior a 126 mg/dL em caso de pequenas elevações da glicemia, ou se a glicemia após sobrecarga oral, que é realizado com 75 gramas de glicose, varia acima de 200 mg/dL no teste de tolerância a glicose. O diagnóstico deve ser confirmado pela repetição do teste em outro dia, tais situações ocorrendo ao menos em dois eventos distintos. Outro parâmetro importante a ser avaliado é a hemoglobina glicada (HbA1) que é utilizada como parâmetro de referência para avaliar o grau de hiperglicemia crônica, sendo a doença identificada quando o valor for superior a 6,5% (LEONG E WHEELER, 2018; SIEGEL *et al*., 2018).

Sabe-se que há muitas complicações do diabetes, resultante do aumento prolongado de açúcar no sangue, e estas podem ser macro e microvasculares, como a aterosclerose, doenças coronarianas, trombose, doença vascular periférica, retinopatia, nefropatia e neuropatia; além de algumas comorbidades associadas que prejudicam muito a qualidade de vida, como por exemplo, a neuropatia diabética e transtornos psiquiátricos como a depressão (MELENDEZ-RAMIREZ *et al*., 2010; SCHREIBER *et al*. 2015; DE MORAIS *et al*., 2014, 2016; DA SILVA DIAS *et al*., 2016), sendo a depressão associada ao DM1 o principal foco de interesse do nosso trabalho.

1.2 DEPRESSÃO ASSOCIADA AO DM1: ESTUDOS CLÍNICOS E PRÉ-CLÍNICOS

Estudos mostram uma alta incidência de depressão em pacientes diabéticos (LUSTMAN *et al*., 1992; ANDERSON *et al*., 2011; SANTOS *et al*., 2013; MOULTON *et al*., 2015; ZANOVELI *et al*., 2016; RÉUS *et al*., 2017; POUWER, 2017; ALOHA *et al*., 2018; BODEN, 2018). Em relação especificamente ao DM1, alguns estudos apontam um aumento da incidência de DM1 em pacientes depressivos (POUWER, 2017; KAMPLING *et al*., 2017). Estudo conduzido por ROY e LLOYD (2012) apontou que a incidência é três vezes maior de depressão em pacientes com DM1 e duas vezes maior em pessoas com DM2 quando comparados com indivíduos não diabéticos.

A depressão é considerada como a segunda principal causa de incapacidade em indivíduos entre 15 e 44 anos e está associada a alterações em diversos sistemas, tais como, alterações cognitivas, emocionais, alterações nos

sistemas psicomotores, neuroendócrinos e neuroquímicos (LEE *et al*., 2010; RÉUS *et al*., 2017). Esta doença está entre as 20 mais comuns, independentemente da região mundial avaliada (SEVILLA-GONZÁLEZ *et al*., 2018). Para que a depressão seja diagnosticada, segundo critérios do Manual de Diagnóstico e Estatística das Doenças Mentais (DSM-V, 2013), é necessário que no mínimo cinco dos sintomas abaixo estejam presentes durante um período de duas semanas, sendo pelo menos um deles o humor deprimido ou a perda do interesse ou prazer. Os sintomas são: humor deprimido, acentuada diminuição do interesse ou prazer, perda ou ganho de peso corporal, insônia ou hipersonia, agitação ou retardo psicomotor, fadiga ou perda de energia, sentimento de inutilidade ou culpa excessiva, redução da capacidade de pensar ou concentrar, pensamentos recorrentes de morte, além de idealização suicida.

De acordo com estudos clínicos, pacientes com depressão apresentam uma diminuição na neurotransmissão monoaminérgica, baixos níveis de fator neurotrófico derivado do encéfalo (*brain-derived neurotrophic factor*, BDNF), além de apresentar uma desregulação do eixo hipotálamo-hipófise-adrenal (LEE *et al*., 2010; ZEUGMANN *et al*., 2010; MAES *et al*., 2011; BUTTENSCHON *et al*., 2017; RÉUS *et al*., 2017; JESULOLA *et al*., 2018). Também foram encontradas alterações em encéfalos de pacientes depressivos *pós mortem* e estas incluem alterações anatômicas corticais e subcorticais, como uma menor densidade neuronal no cortéx pré-fontal (SIBILLE e FRENCH, 2013) e um volume hipocampal reduzido nesses pacientes (MALYKHIN e COUPLAND, 2015), além de alterações de certos genes que aumentam o risco de desenvolvimento da depressão (WRAY *et al*., 2012).

Alguns estudos apontam que uma das possíveis causas para o desenvolvimento da depressão em pacientes com DM1 seja decorrente inicialmente de uma mudança no estilo de vida, isto é, esses indivíduos precisam se adaptar ao um novo estilo de vida que inclui práticas regulares de exercícios físicos, privação de comer alimentos ricos em gorduras e açucares que são vistos como alimentos prazerosos, além do tratamento crônico medicamentoso de reposição de insulina e de todas as comorbidades associadas. Todas essas mudanças trazem ao paciente um grande desconforto, além do aumento dos gastos financeiros e aumento da frequência de hospitalizações, acarretando

muitas vezes em uma baixa adesão ao tratamento. Consequentemente haverá uma piora do controle glicêmico, que tem sido associada com alterações neurobiológicas que podem predispor a depressão (WREDLING *et. al*., 1992; MOULTON *et al*., 2015; ZANOVELI *et al*., 2016; BELVEDERI *et al*., 2017; SEVILLA *et al*., 2018). Neste aspecto, recentemente o estudo de SCHMITT e colaboradores (2017) mostrou em um estudo conduzido com 181 participantes uma associação entre glicemia e sintomas depressivos, ou seja, uma redução dos sintomas depressivos estaria relacionada com uma melhora do controle glicêmico.

Em relação à fisiopatologia da depressão associada ao DM, há uma discussão sobre uma possível relação bidirecional entre essas doenças (BELLUSH *et al*., 1991; CHAMPANERI *et al*., 2010; RENN *et al*., 2011; GRAGNOLI *et al*., 2014; MOULTON *et al*., 2015; PETRAK *et al*., 2015; ZANOVELI *et al*., 2016; GILSANZ *et al*., 2018), não sendo ainda esclarecido o mecanismo fisiopatológico que associa a depressão com o DM. É proposto que processos fisiológicos alterados decorrentes da hiperglicemia, tais como elevada oxidação da glicose, deficiência ou sensibilidade alterada da ação da insulina e respostas neuroadaptativas podem predispor a depressão (GUPTA *et al*., 2014; DE MORAIS *et al*., 2014; DA SILVA DIAS *et al*., 2016). Por outro lado, sabe-se também que a depressão é um fator de risco crucial para induzir uma piora no controle da glicemia, uma vez que a depressão está associada com a hiperatividade do eixo Hipotálamo-Hipófise-Adrenal (HHA), elevando os níveis de cortisol em humanos e corticosterona em ratos (CAMERON *et al*., 1984; BELLUSH *et al*., 1991; CHAMPANERI *et al*., 2010). Nesse sentido, estudos clínicos e pré-clínicos apontam um nível aumentado de cortisol em humanos e corticosterona em animais deprimidos, sugerindo uma maior desregulação do eixo HHA (YOUNG e ALTEMUS, 2004; VIAU *et al*., 2005; CHOPRA *et al*., 2009). Sendo o cortisol um hormônio com ação contra-regulatória, a exposição prolongada a esse hormônio induz adiposidade visceral, resistência à insulina, dislipidemia entre outros precursores metabólicos relacionados também com o diabetes (BELLUSH *et al*., 1991; JURUENA *et al*., 2003; CHAMPANERI *et al*., 2010). Independente da ordem com que esses eventos aconteçam, é sabido que a depressão leva a sérios danos a saúde e a qualidade de vida do paciente,

aumentando a morbidade e mortalidade (ALONSO *et al*., 2011; RÉUS *et al*., 2017; SCHIMITT *et al*., 2017).

Interessante notar que vários estudos pré-clinicos também associam a depressão ao diabetes, ou seja, mostram um comportamento do tipo depressivo mais pronunciado tanto em ratos quanto em camundongos com diabetes induzido quimicamente por uma única injeção de estreptozotocina (STZ). Esses animais apresentam um tempo de imobilidade maior (quando avaliados no teste de natação forçada e no teste de suspensão pela cauda), quando comparados com animais normoglicêmicos (GOMEZ e BARROS, 2000; HO *et al*., 2012; DE MORAIS *et al*., 2014, 2016; GUPTA *et al*., 2014; DA SILVA DIAS *et al*., 2016; REDIVO *et al*., 2016; REBAI *et al*., 2017).

A STZ é um antibiótico de natureza glicosamina-nitrosuréia com propriedades tóxicas e que foi inicialmente isolada e caracterizada como um antimicrobiano de largo espectro a partir de colônias de *Streptomyces achromogenes (*XIANG *et al*., 2010; SZKUDELSKI, 2001; DELFINO *et al*., 2002). A indução deste estado diabético experimental é devido a sua capacidade de destruição das células β pancreáticas levando a diminuição da secreção de insulina (SZKUDELSKI, 2001; SKZUDELSKI *et al*., 2013). A forma pela qual a STZ danifica as células produtoras de insulina se deve a semelhança da molécula de STZ com a molécula de glicose o que permite que a mesma seja internalizada via transportadores de glicose do tipo GLUT2, expressos na superfície das células β pancreáticas (KARUNANAYAKE *et al*., 1974; SZKUDELSKI, 2001). A ação tóxica intracelular da STZ se dá pelo aumento dos níveis de espécies reativas de oxigênio molecular ocasionando alquilações de bases nitrogenadas que compõem o ácido desoxirribonucleico (DNA) celular (ELSNER *et al*., 2000; SZKUDELSKI, 2001; LENZEN, 2008), as quais, quando reparadas, causam alterações no metabolismo de células β por acarretarem diminuição celular de nicotinamida adenina dinucleotídeo (NAD) e consequentemente de adenosina trifosfato (ATP). Assim, este esgotamento da energia celular resulta, leva à necrose das células β pancreáticas (SANDLER e SWENNE, 1983; DELFINO *et al*., 2002; LENZEN, 2008). Vale ressaltar que duas horas após a injeção de STZ, a hiperglicemia é observada juntamente com a queda de insulina no sangue. Cerca de seis horas depois, ocorre a hipoglicemia com altos níveis de insulina no sangue. Após esse

período, a hiperglicemia se desenvolve e níveis de insulina no sangue diminuem (WEST *et al*. 1996; SZKUDELSKI *et al*., 2001).

Interessante que o uso da administração da STZ como modelo de diabetes do tipo 1 se justifica pela alta capacidade de mimetizar quadros patológicos observados em pacientes diabéticos como poliúria, polifagia e hiperglicemia (GOYARY e SHARMA, 2010). Além disso, foi demonstrado que a STZ não ultrapassa a barreira hematoencefálica e possui uma rápida taxa de excreção (KARUNANAYAKE *et al*., 1974; HIRANO *et al*., 2006), indicando que o efeito do tipo depressivo verificado nos animais DBT não é devido a ação da STZ *per si*. Utilizando esse modelo animal de DM1, dados do nosso laboratório e de vários outros grupos mostram que animais diabéticos apresentam uma série de alterações no encéfalo, tais como diminuição nos níveis de fator neurotrófico derivado do encéfalo no córtex pré-frontal e hipocampo (REDIVO *et al*., 2016; ABDELWAHED *et al*., 2018), hiperativação do eixo hipotálamo-pituitária-adrenal (JOSEPH e GOLDEN, 2017). alterações no sistema imune, como um aumento de citocinas pró-inflamatórias no hipocampo (DA SILVA DIAS *et al*., 2016), deficiência de neurotransmissores como a noradrenalina e serotonina em diferentes áreas encefálicas envolvidas no comportamento emocional (BELLUSHI *et al*., 1991; NESTLER *et al*., 2006; HO *et al*., 2012; GUPTA *et al*., 2014; ZANOVELI *et al*., 2016; DA SILVA DIAS *et al*., 2016) e alterações na plasticidade sináptica neuronal (GISPEN *et al*., 2000; MOCKING *et al*., 2013; PRABHAKAR *et al*., 2015; ASWAR *et al*., 2017). Além dessas alterações, um prejuízo significativo de mecanismos de defesas antioxidantes, além de um aumento do estresse oxidativo tem sido demonstrado em animais diabéticos, tanto perifericamente como no sistema nervoso central (VALKO *et al*., 2007; GIACCO e BROWNLEE, 2010; WAYHS *et al*., 2014; DE MORAIS *et al*., 2014, 2016; INAM *et al*., 2018).

Em relação ao envolvimento da serotonina, um neurotransmissor importante na mediação de respostas emocionais e que parece estar envolvido na associação diabetes/depressão, sabe-se que os neurônios serotoninérgicos originam-se nos núcleos da rafe, a partir do qual eles apresentam tanto projeções ascendentes quanto descendentes (BILLARD *et al*., 2014). O receptor 5-HT_{1A} é um dos receptores de serotonina melhor caracterizado; além disso, está

relacionado com a fisiopatologia da depressão (ARANGO *et al*., 2001; SAVITZ *et al*., 2009; MILLER *et al*., 2013). Estudos demonstram que a depressão está associada a uma redução na responsividade desses receptores 5-HT_{1A} (SARGENT *et al*., 2000). Mais ainda, um polimorfismo no gene que codifica estes receptores foi associado a um aumento na vulnerabilidade à depressão maior (HENSLER, 2002; LEMONDE *et al*., 2003). Corroborando tais estudos, foi demonstrada, em pacientes depressivos que cometeram suicídio, uma densidade reduzida de receptores 5-HT_{1A} em análises de áreas como o hipocampo e córtex pré-frontal desses indivíduos (CHEETHAM *et al*., 1990; ARANGO *et al*., 2001; PRABHAKAR *et al*., 2015).

Interessante que estudos conduzidos utilizando técnicas *in vivo* mostram que animais com o diabetes induzido pela STZ apresentam baixos níveis de serotonina em todo o conteúdo do cérebro (GUPTA *et al*., 2014). Outros estudos sugerem que as alterações na concentração extracelular de serotonina parecem ser dependentes da área encefálica nesses animais. Por exemplo, foi observada uma redução neste conteúdo no hipocampo e hipotálamo (SHIMIZU, 1991; YAMATO *et al*., 2004; DA SILVA DIAS *et al*., 2016), mas nenhuma alteração no estriado (BRODERICK e JACOBY, 1989). O mecanismo da depressão relacionada ao diabetes, como citado anteriormente, ainda não está totalmente esclarecido, porém um outro mecanismo possível pode ser as alterações enzimáticas no cérebro, incluindo diminuição dos níveis de fração livre de L-triptofano e a inibição da enzima triptofano-5-hidroxilase 2, que é responsável pela conversão da fração livre da L-triptofano para serotonina (HERRERA *et al*., 2005). Foi confirmado que o diabetes perturba o equilíbrio da fração livre da L-triptofano com outros aminoácidos neutros que desempenham um papel importante na determinação da quantidade dessa fração livre disponível para conversão em serotonina, levando finalmente à supressão da síntese de serotonina (MANJARREZ-GUTIÉRREZ *et al*., 1999; HERRERA *et al*., 2005). Outros achados sugerem alterações nos receptores serotonina. Assim, evidencias apontam que os receptores 5-HT_{1A} estão diminuídos no diabetes em áreas como o hipocampo (PRABHAKAR *et al*., 2015) e o efeito de fármacos antidepressivos está reduzido em animais com diabetes induzida por STZ, ressaltando o prejuízo

na neurotransmissão serotoninérgica também em animais diabéticos (SHIMIZU, 1991; YAMATO *et al*., 2004).

Além da desregulação dos sistemas de neurotransmissores, como o serotonérgico, que parece ser comum no diabetes e na depressão, cabe ressaltar os danos nos tecidos e sistemas decorrentes da hiperglicemia, levando a um prejuízo de mecanismos de defesas antioxidantes, além de um aumento do estresse oxidativo (VALKO *et al*., 2007; GIACCO e BROWNLEE, 2010; WAYHS *et al*., 2014; DE MORAIS *et al*., 2014, 2016; INAM *et al*., 2018). Os antioxidantes podem ser definidos como qualquer molécula que diminui ou inibe um processo de oxidação. Assim, um antioxidante protege estruturas celulares ou biomoléculas contra os efeitos deletérios de substancias que promovem a oxidação (HALLIWELL e GUTTERIDGE, 2007; INAM *et al*., 2018). Esses agentes antioxidantes podem ser classificados em enzimáticos, dentre os quais estão a enzima superóxido dismutase e catalase, e não enzimáticos, como a glutationa reduzida (GSH) (VALKO *et al*., 2007).

A superóxido dismutase é uma enzima antioxidante que catalisa a dismutação do ânion superóxido a peróxido de hidrogênio envolvendo processo sucessivo de oxidação e redução, tornando o produto menos reativo que o anterior, sendo fundamental para a prevenção da toxicidade induzida pelas espécies reativas (FRIDOVICH, 1997; STOCKER e KEANEY, 2004). Já a catalase, apresenta a função de promover a decomposição do produto da ação da superóxido dismutase, que é um composto tóxico e reativo denominado peróxido de hidrogênio. Essa enzima catalisa a reação entre duas moléculas de peróxido de hidrogênio, resultando na formação de água e oxigênio molecular (FRIDOVICH, 1997; HALLIWELL e GUTTERIDGE, 2007).

Em relação aos antioxidantes não enzimáticos, como por exemplo, a glutationa (L-γ-glutamil-L-cistenilglicina), ela está presente no organismo em duas formas: reduzida (GSH) e a forma oxidada (GSSG). A GSH é um tiol não proteico constituído de glutamato, cisteína e glicina que participa direta ou indiretamente de diversos processos celulares, tais como a síntese de DNA, proteínas e também da modulação da função proteica (STOCKER e KEANEY, 2004; DRINGEN *et al*., 2005). A GSH pode servir de substrato para a ação da enzima glutationa peroxidase (GPx) agindo na detoxificação de peróxidos orgânicos e de

hidrogênio. Por servir como cofator para diversas enzimas a GSH representa um dos principais compostos endógenos que combatem as espécies reativas (FRIDOVICH, 1997; STOCKER e KEANEY, 2004; MAHER, 2005). Em condições fisiológicas basais existe um balanço controlado entre moléculas pró-oxidante e as moléculas antioxidantes, sendo que um desequilíbrio entre essas moléculas é denominado estresse oxidativo (HALLIWELL; GUTTERIDGE, 2007; ROHENKOHL *et al*., 2011).

Interessante que esse desequilíbrio induz uma elevação da concentração intracelular de moléculas altamente reativas que provocam danos à estrutura das células como danos a estrutura proteica, quebra no DNA e também danos a membrana celular, como a peroxidação lipídica (LPO) (SIES, 1997; VALKO *et al*., 2007).

A LPO pode ser definida como uma cascata de eventos bioquímicos que provoca a oxidação de lipídios poliinsaturados de membranas celulares (HALLIWELL e GUTTERIDGE, 1999; DAL-PIZZOL *et al*., 2000). As principais consequências da LPO são as alterações na estrutura e na permeabilidade da membrana, podendo levar à destruição de sua estrutura celular, alteração dos mecanismos de troca de metabólitos e, promovendo também liberação do conteúdo das organelas e formação de produtos citotóxicos o que numa condição extrema pode levar à morte celular (HALLIWELL e GUTTERIDGE, 1999; DAL-PIZZOL *et al*., 2000).

Diante disso, é importante ressaltar que o encéfalo é particularmente propenso aos danos ocasionados por espécies reativas de nitrogênio e oxigênio, pois é um tecido altamente rico em ácidos graxos poliinsaturados oxidáveis e pobre em defesas antioxidantes (VALKO *et al*., 2007; WANG e MICHAELIS, 2010; INNOS *et al*., 2013, DE MORAIS *et al*., 2014; TABATABAEI *et al*., 2017). Assim, dados do nosso laboratório (DE MORAIS *et al*., 2014) e de outros laboratórios (WAYHS *et al*., 2013; 2014) sugerem que o estresse oxidativo possui uma participação na fisiopatologia da depressão associada ao diabetes, uma vez que nesses estudos os animais diabéticos apresentaram um comportamento do tipo depressivo mais pronunciado que pode estar relacionado com um aumento na atividade da superóxido dismutase e da catalase (DE MORAIS *et al*., 2014), além de um aumento na peroxidação lipídica no córtex pré-frontal e no hipocampo

enquanto que os níveis de GSH encontram-se reduzidos nessas áreas (WAYHS *et al*., 2013, 2014; DE MORAIS *et al*., 2014; REBAI *et al*., 2017).

Cabe mencionar que os mecanismos fisiopatológicos que associam a depressão ao diabetes ou vice-versa e que levam aos danos no sistema nervoso central vão muito além dessas alterações supracitadas. Nesse sentindo, é crescente o interesse em aprofundar os estudos acerca da fisiopatologia da depressão diabética. Mais ainda, de buscar conhecer um novo agente terapêutico que seja capaz de aliviar a depressão e melhorar os parâmetros associados ao diabetes. Na atualidade, os antidepressivos são o tratamento de primeira escolha para a depressão associada ou não ao diabetes.

Tem sido descrito em muito estudos que os antidepressivos podem ou não alterar a glicemia (ERENMEMISOGLU *et al*., 1999; HENNINGS *et al*., 2012; NICOLAU *et al*., 2013; ZUCCOLI *et al*., 2013; BYSTRITSKY *et al*., 2014; PRABHAKAR *et al*., 2015; FATHALLAH *et al*., 2015). Por exemplo, tem sido relatado que alguns antidepressivos tricíclicos podem causar hiperglicemia e podem ser potencialmente perigosos em pacientes diabéticos (HENNINGS *et al*., 2012; PRABHAKAR *et al*., 2015). Também foi demonstrado que pacientes com depressão associada ao diabetes tratados com citalopram apresentaram uma melhora significativa no escore de depressão; no entanto, não foram encontradas diferenças no controle glicêmico avaliado pela HbA1c (NICOLAU *et al*., 2013). PAILE-HYVÄRINEN e colegas (2007) trataram os pacientes que apresentavam essa comorbidade com paroxetina e placebo e verificaram que não houve diferença estatisticamente significativa entre os grupos tratamento (paroxetina) e placebo, nem para o controle glicêmico nem para o escore de depressão (PAILE-HYVÄRINEN *et al*., 2007).

Diante do exposto, torna-se evidente a necessidade de estudos que busquem esclarecer todas essas alterações observadas na depressão diabética. Mais ainda, de descobrir novos tratamentos cujo efeito antidepressivo apareça rapidamente após o início do tratamento, com menos efeitos colaterais e/ou riscos para o paciente diabético, além de promover uma melhora nos parâmetros relacionados com o diabetes em si. Assim, na atualidade grande interesse tem sido voltado para o sistema canabinoide, por este apresentar um papel modulador em diferenças condições associadas ou não a doenças.

1.3 CANABINOIDES

A *Cannabis sativa*, conhecida popularmente como maconha, foi uma das primeiras plantas cultivadas pelo ser humano, sendo seus derivados conhecidos há muitos anos por suas propriedades medicinais e pelo seu uso recreativo (RUSSO, 2007; COSTA *et al*., 2011; MALDONADO *et al*., 2011; DESAI *et al*., 2017). Suas folhas secretam uma resina que contém compostos ativos chamados de canabinoides, sendo que os principais compostos isolados desta planta são o canabidiol (CBD) e o Δ9-tetrahidrocanabinol (Δ9-THC). O CBD diferentemente do Δ9-tetrahidrocanabinol (Δ9-THC), é desprovido de efeitos psicoativos (MECHOULAM *et al*., 1998; MECHOULAM and HANUS 2002; PERTWEE et al., 2005; MALDONADO 2011).

Em 1964, os pesquisadores GAONI e MECHOULAN identificaram o composto psicoativo presente na *cannabis*, o Δ9-THC. De lá até a atualidade muitos estudos foram e estão sendo conduzidos para um maior entendimento do sistema endocanabinoide (SEC) (WALKER e HUANG, 2002).

O SEC é um sistema complexo composto por receptores endógenos, sendo o receptor do tipo CB1 o primeiro a ser descrito por MATSUDA e colaboradores (1990). Dois anos depois, em 1992, seu primeiro ligante endógeno foi caracterizado, sendo denominado de N-araquidoniletanolamida (anandamida-AEA) (DEVANE *et al*., 1992). Esse sistema também é composto pelo receptor do tipo CB2 e pelos ligantes endógenos 2-araquidoniletanolamida (2-AG), o O-araquidonoiletanolamina (virodamine) e o N-araquidonoildopamina (SUGIURA *et al*., 1995; WALKER *et al*., 2002). Os receptores canabinoide são acoplados à proteína Gi/o que quando ativada inibe a atividade da enzima adenilato ciclase, ativa canais de potássio e inibe canais de cálcio voltagem dependente (HOWLETT e MUKHOPADHYAY, 2000; PIOMELLI, 2003; MENDIGUREN *et al*., 2018). Os receptores são localizados predominantemente sobre terminais axônicos pré-sinápticos e são capazes também de regular o influxo de cálcio causando, portanto, uma redução na liberação de neurotransmissores (FREUND *et al*., 2003; SCHLICKER e KATHMANN, 2001). Dentre os ligantes endógenos, os mais expressivos e estudados são as moléculas lipofílicas, a AEA e o 2-AG

(SUGIURA *et al*., 1995; DEVANE *et al*., 1992). Esses compostos são formados principalmente a partir da hidrólise de ácidos graxos poliinsaturados de cadeia longa, especificamente o ácido araquidônico. A AEA e o 2-AG são formados por vias dependentes de fosfolipídios, sendo sintetizados respectivamente, pelas enzimas N-acilfosfatidil etanolamida-fosfolipase seletiva (NAPE-PLD) e a diacilglicerol lipase seletiva (DAG Lipase) (BIZOGNO *et al.*, 2002; LIU *et al*., 2006). O 2-AG é um agonista total em ambos os receptores CB1 e CB2, mas apresenta menos afinidade do que a AEA para ambos os receptores. Por outro lado, a AEA é um agonista parcial em ambos os receptores CB1 e CB2, e exibe uma maior afinidade para os receptores CB1 (PERTWEE E ROSS 2002). A AEA é metabolizada e hidrolisada, através de um sistema proteico de transporte que promove sua receptação, pela ação da enzima FAAH (amida hidrolase de ácidos graxos) e o 2-AG pela ação da enzima MGL (monoacilglicerol lipase) (BIZOGNO *et al*., 2002; KUNOS *et al.*, 2008).

Ambas as moléculas agem como neurotransmissores atípicos e são formados pós-sinapticamente “sob demanda” por ação excitatória. Consequentemente, os compostos endógenos são liberados na fenda sináptica onde atuam de um modo retrógrado, ou seja, atuam em receptores pré-sinápticos localizados em neurônios de diferentes neurotransmissores (FREUND *et al*., 2003; HILL e GORZALKA 2005; MENDIGUREN *et al*., 2018). Dessa maneira, atuam exercendo ações modulatórias amplas mediando diferentes comportamentos, tais como modulação da dor, memória, ansiedade e depressão (SCHREIBER *et al*., 2012; HILL *et al*., 2005; CAMPOS *et al*., 2012; FERREIRA-VIEIRA *et al*., 2014, DE MORAIS *et al*., 2016; CAMPOS *et al*., 2017).

O SEC tem sido apontado como um sistema promissor para o tratamento de diversas doenças, dentre estas a depressão (GOBBI *et al*., 2005; HILL *et al*., 2009; CAMPOS *et al*., 2012; ZHOU *et al*., 2017) e o diabetes (para uma revisão ver BOOZ *et al*., 2011; HORVÁTH *et al*., 2012; FREITAS *et al*., 2017). Estudos demostram que a administração do URB597 que inibe a amida hidrolase de ácido graxo (FAAH), enzima que degrada a AEA, provoca efeito do tipo antidepressivo em animais submetidos ao teste de suspensão pela cauda e ao teste de natação forçada (GOBBI *et al*., 2005). Interessante que esses mesmos autores observaram que o URB597 aumentou a atividade de disparo de neurônios

serotoninérgicos no núcleo dorsal da rafe e de neurônios noradrenérgicos no locus ceruleus. Esta interação entre o SEC e o sistema serotoninérgico tem sido observada em vários estudos (MORALES e BÄCKMAN, 2002; ZANELATI *et al*., 2010; SOARES *et al*., 2010; MCLAUGHLIN *et al*., 2012; SARTIM *et al*., 2016; DE MORAIS *et al*., 2016). Assim, tem sido demonstrado que o URB597 possui potencial analgésico, ansiolítico e antidepressivo (HOLT *et al*., 2005; PATEL e HILLARD, 2006; BORTOLATO *et al*., 2007) e parece não produzir catalepsia, hipotermia e também não alterar as respostas hipofágicas que são respostas típicas apresentadas por agonistas canabinoides (FEGLEY *et al*., 2005).

Tendo em vista a importância do SEC na modulação/mediação de respostas comportamentais associadas com as emoções, recentemente vem ganhando grande destaque o canabidiol (CBD), composto não psicotomimético mais abundante presente na planta *Cannabis sativa,* como um agente farmacológico de grande potencial no tratamento de diversas psicopatologias ou condições, tais como ansiedade, depressão, ataque epiléptico, esquizofrenia, dor crônica, dentre outras (IZZO *et al*., 2009; RÉUS *et al*., 2011; DE MELLO SCHIER *et al*., 2014). De interesse para o presente estudo, estudos mostram um potencial do CBD em induzir efeitos benéficos em relação ao estado diabético *per se*, reduzindo a incidência de DM1 em camundongos e a inflamação pancreática precoce nesse tipo de diabetes, podendo também retardar os danos causados pelo diabetes nas células β pancreaticas (WEISS *et al*., 2006; DI MARZO *et al*., 2011; LEHMANN *et al*., 2016). Estudo conduzido por EL-REMESSY e colaboradores (2006) mostrou que o tratamento com CBD em animais diabéticos manteve a integridade da barreira hemato-retinal, reduziu significativamente o número de células apoptóticas evitando a permeabilidade vascular e a morte neuronal, além de bloquear os aumentos no estresse oxidativo reduzindo a peroxidação lipídica, diminuição dos níveis de fator de necrose tumoral-α, fator de crescimento endotelial vascular e molécula de adesão intercelular-1 e também atuou reduzindo as citocinas pró-inflamatórias.

2 HIPÓTESE

Uma desregulação no sistema canabinoide participa do comportamento do tipo depressivo dos animais diabéticos.

3 OBJETIVOS

- Avaliar o efeito do tratamento com anandamida sobre respostas comportamentais relacionadas com a depressão em animais diabéticos.
- Avaliar o envolvimento do receptores canabinoides CB1 e CB2 de animais diabéticos sobre o efeito comportamental induzido pela anandamida.
- Avaliar o efeito do tratamento com anandamida sobre a expressão do receptor CB1 no hipocampo e córtex pré-frontal de ratos diabéticos.
- Avaliar parâmetros de estresse oxidativo no hipocampo e córtex pré-frontal de ratos diabéticos.
- Avaliar o conteúdo extracelular de serotonina e noradrenalina no hipocampo e córtex pré-frontal de ratos diabéticos.
- Avaliar o efeito do tratamento agudo com CBD sobre respostas comportamentais relacionadas com a depressão em animais diabéticos.
- Avaliar o efeito do tratamento subcrônico com o CBD sobre respostas comportamentais relacionadas com a depressão em animais diabéticos.
- Avaliar a funcionalidade do sistema endocanabinoide em animais diabéticos após tratamento agudo com o inibidor da enzima que degrada a anandamida, URB597, sobre respostas comportamentais relacionadas com a depressão em animais diabéticos.
- Avaliar a funcionalidade do sistema endocanabinoide em animais diabéticos após tratamento subcrônico com o inibidor da enzima que degrada a anandamida, URB597, sobre respostas comportamentais relacionadas com a depressão em animais diabéticos.

4 PRIMEIRO ARTIGO CIENTÍFICO: ANANDAMIDE REVERSES DEPRESSIVE-LIKE BEHAVIOR, NEUROCHEMICAL ABNORMALITIES AND OXIDATIVE-STRESS PARAMETERS IN STREPTOZOTOCIN-DIABETIC RATS: ROLE OF CB1 RECEPTORS

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Anandamide reverses depressive-like behavior, neurochemical abnormalities and oxidative-stress parameters in streptozotocin-diabetic rats: role of CB1 receptors.

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4.1 ABSTRACT

The pathophysiology associated with increased prevalence of depression in diabetics is not completely understood, although studies have pointed the endocannabinoid system as a possible target. Then, we aimed to investigate the role of this system in the pathophysiology of depression associated with diabetes. For this, diabetic (DBT) male Wistar rats were intraperitoneally treated with cannabinoid CB1 (AM251, 1 mg/kg) or CB2 (AM639, 1 mg/kg) receptor antagonists followed by anandamide (AEA, 0.005 mg/kg) and then submitted to the forced swimming test (FST). Oxidative stress parameters, CB1 receptor expression and serotonin (5-HT) and noradrenaline levels in the hippocampus (HIP) and prefrontal cortex (PFC) were also performed. It was observed that DBT animals presented a more pronounced depressive-like behavior and increase of CB1 receptor expression in the HIP. AEA treatment induced a significant improvement in the depressive-like behavior, which was reversed by the CB1 antagonist AM251, without affecting the hyperglycemia or weight gain. AEA was also able to restore the elevated CB1 expression and also to elevate the reduced level of 5-HT in the HIP from DBT animals. In addition, AEA restored the elevated noradrenaline levels in the PFC and induced a neuroprotective effect by restoring the decreased reduced glutathione and increased lipid hydroperoxides levels along with the decreased superoxide dismutase activity observed in HIP or PFC. Together, our data suggest that in depression associated with diabetes, the endocannabinoid anandamide has a potential to induce neuroadaptative changes able to improve the depressive-like response by its action as a CB1 receptor agonist.

Keywords: Diabetes; Depression; Oxidative stress; CB1 receptor; serotonin; noradrenaline.

4.2 INTRODUCTION

Diabetes *mellitus* (DM) is a chronic metabolic disorder characterized by hyperglycemia resulting from failure in insulin secretion and/or in its action (Brownlee, 2005; Whays et al., 2010; Zanovelli et al., 2015). It is known that the damage caused by hyperglycemia includes dysfunction and failure of various organs being the brain one of the most affected organs (Sevick et al., 2007; Moulton et al., 2015; Zanoveli et al., 2015), which can explain the higher incidence of depression among diabetic (DBT) patients (Anderson et al., 2001, Smith et al., 2013; Nicolau et al., 2013). Depression has been related to a general worsening in the health of DBT patients, increasing the morbidity and mortality (Nefs et al., 2016). It has been proposed that this comorbidity may be the result of changing in lifestyle (dietary restriction, chronic daily treatment, increased in financial expenses and in frequency of hospitalization) and/or of physiological changing due to the diabetic condition (Wredling et al., 1992; Zanoveli et al., 2015).

Many proposals have been made to try to understand the pathophysiological relationship between depression and diabetes. For example, it has been reported in animal models of diabetes several brain alterations, such as disturbed neurotransmission (Bellush et al., 1991; Gupta et al., 2014; Prabhakar et al., 2015), impaired synaptic plasticity, reduction of adult neurogenesis in the dentate gyrus, increased oxidative stress in brain areas related to depression such as hippocampus (HIP) and prefrontal cortex (PFC) (Beauquis et al., 2010; Whays et al., 2010; Naudi et al., 2012; De Morais et al., 2014). Indeed, the cause of depression associated with diabetes is still not conclusive, leading to the proposal of ineffective treatments. To worsen, the antidepressant chronic treatment may induce significant effects over diabetic condition, including disturbed patient's blood glucose levels (Zanoveli et al., 2015). In that sense, it is important to note that in recent years a system has gained attention, the endocannabinoid system, which plays a regulatory role in several brain functions and seems to be a potential therapeutic target for the diabetes (Di Marzo et al., 2011) and depression treatments (Hill and Gorzalka, 2005; Mclaughlin et al., 2007; Booz et al., 2011; Micale et al., 2013, Gatta-Cherifi and Cota, 2015, Micale et al., 2015).

The endocannabinoid system includes the endogenous ligand araquidoniletanolamide (anandamide; AEA) that acts as atypical neurotransmitter being formed postsynaptically on demand by excitatory action. Consequently, this endogenous compound is released into the synaptic cleft acting in a retrograde manner by activating CB1 or CB2 receptors located pre-synaptically and thus inhibiting the release of different neurotransmitters, such as noradrenaline (NA) and serotonin (5-HT) (Devane et al., 1992; Fonseca et al., 2013). Therefore, the endocannabinoid system seems to act protecting the brain of being overwhelmed by excessive excitatory or inhibitory activity. The endocannabinoid system dysfunctions, such as extreme excitation or inhibition, may occur and lead to neuropsychological states, such as mania or hyperexcitability, at one extreme and depression, anhedonia and apathy on the other. Thus, a decrease of the endocannabinoid system activity could explain the anhedonia, anxiety, and also a decrease in the serotonergic activity that often accompanies depression (Wilson et al., 2001; Ashton and Moore, 2011).

Besides a possible role of the endocannabinoid system in the pathophysiology of neuropathologies commonly reported in DBT patients, this system seems to be particularly involved in the diabetic pathogenesis itself. In this sense, abnormalities in the endocannabinoid system have been reported in both patients and animal models of type 1 and type 2 diabetes (for review see Di Marzo et al., 2011). Interestingly, patients with type 2 diabetes exhibit increased activity of the endocannabinoid system in visceral fat and increased plasma concentrations of AEA and 2-arachidonoylglycerol (2-AG; Matias et al., 2006). Besides, studies show that monoacylglycerol lipase (MGL) activity is increased in adipocytes from animal models of diabetes (Cable et al., 2014). Considering the type 1 diabetes, current research showed that endocannabinoids have immunosuppressive properties, such as leukocyte proliferation inhibition, reduction of pro-inflammatory cytokines and T cells apoptosis induction (for review see Katchan et al., 2016). Given the role of the endocannabinoid system also in glucose homeostasis, in the food intake and energy balance (for review see Cristino et al., 2014), cannabinoid receptor antagonists, mainly CB1 receptor antagonist, have been shown to be effective in weight and hyperinsulinemia reduction (Matias et al., 2006), in beta cells proliferation (Kim et al., 2011) and dyslipidaemia and blood pressure

reduction (for review see Scheen and Paquot, 2009) while CB2 receptor agonist seems to be a potential therapeutic alternative to diabetic nephropathy (Zoja et al., 2016).

It is known that brain areas such as HIP and PFC, two brain areas extremely involved in the neurobiology of depression, together with the amygdala and ventral striatum present high densities of CB1 receptors (Herkenham, 1991, Mato and Pazos, 2004; Kano et al., 2009). Interestingly, it has been reported reciprocal neural connections between HIP and PFC (Duman and Monteggia, 2006). Thus, it is not surprising that the CB1 receptor activation in both the structures, PFC or dentate gyrus region of the HIP, induces antidepressant-like response when an animal is exposed to the forced swim test (FST) (Bambico et al., 2007; McLaughlin et al., 2007; McLaughlin and Gobbi, 2012).

Considering the exposed above, as well as the lack of studies addressing the role of the endocannabinoid system on depression associated with diabetes, in this study, we primarily aimed to evaluate the effect of the AEA treatment on the depressive-like behavior in streptozotocin-induced DBT rats and the role of the CB1 or CB2 receptors in its mechanism of action. Additionally, it was evaluated the effect of the AEA treatment over the HIP and PFC CB1 receptor expression, oxidative stress parameters and the 5-HT and NA e contents.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Animals

Male *Wistar* rats (180-220 g) provided by the Federal University of Paraná were assigned four per cage (41 × 32 × 16.5 cm) and received food and water *ad libitum and* maintained in a temperature-controlled room (22 ± 2 °C) under 12/12-h light/dark cycle (lights on at 7 a.m.). The experiments were carried out according to Brazilian Society of Neuroscience and Behavior guidelines for care and use of Laboratory Animals and all efforts were made to minimize the number of animals as well as the animal suffering. All experimental procedures were approved by the local Ethics Committee for Research on Animals UFPR (CEUA/BIO-UFPR; #749).

4.3.2 Drugs

The following drugs were used: Streptozotocin (STZ; 60 mg/kg, i.p., Santa Cruz Biotechnology Inc., USA), sodium citrate (Merck S.A., Brazil), CB1/CB2 cannabinoid agonist N-arachidonoyl ethanolamide (anandamide, 0.001, 0.005, 0.01 or 0.05 mg/kg; i.p., Cayman Chemical, Ann Arbor, MI, USA); CB1 receptor antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide trifluoroacetate (AM251, 1 mg/kg, i.p., Cayman Chemical, Ann Arbor, MI, USA) and CB2 receptor antagonist 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone (AM630, 1 mg/kg, i.p., Cayman Chemical, Ann Arbor, MI, USA). STZ was dissolved in citrate buffer (10 mM, pH 4.5); anandamide (AEA) was freshly diluted with 0.9% NaCl. AM251 or AM630 were dissolved in a 1:1:18 solution of dimethyl sulfoxide/ Tween 80/ 0.9% sterile saline. Imipramine (IMI; 15 mg/kg, i.p., Novartis Pharmaceutical Industry, Brazil) was dissolved in saline. All doses were based on previous studies and prior experiments conducted in our laboratory (De Morais et al., 2014; Hill and Gorzalka, 2005).

4.3.3 Diabetes Induction

Experimental diabetes was induced following an overnight fast by a single intraperitoneal (i.p.) injection of STZ at a dose of 60 mg/kg freshly dissolved in citrate buffer (10 mM, pH 4.5). Normoglycemic animals received a single intraperitoneal (i.p.) injection of the vehicle, citrate buffer (10 mM, pH 4.5). Blood glucose levels were measured 72 h after STZ or vehicle administration by a strip operated reflectance meter in a blood sample obtained by tail prick and again at the end of all behavioral tests. Animals with fasting blood glucose levels ≥ 250 mg/dL were considered diabetic and maintained in the study (De Morais et al., 2014). All animals were observed daily and weighed throughout the experiment.

4.3.4 Open-field test

This test was conducted as described previously (De Morais et al., 2014). Briefly, all the animals were placed in the center of a wooden rectangular open field (40 cm × 50 cm × 63 cm) with a floor divided into 6 rectangular units. The number

of squares crossed with all four paws in an interval of 5 min was observed and quantified as a parameter of locomotor activity.

4.3.5 Forced swimming test (FST)

All animals were submitted to FST as described by Porsolt (Porsolt et al., 1978) with minor modifications. The test was conducted in two sessions. The animals underwent a pre-test session, in which they were placed individually to swim in a tank (30 cm × 40 cm height, containing 25 cm of water at 24 ± 1 °C) for a period of 15 min. Twenty four hours later, animals were submitted to a 5 min session of forced swim (test). During this session, the total time of immobility (except the small movements necessary to float) was evaluated. Moreover, the frequency of active behaviors, i.e. climbing and swimming, and the passive behavior of immobility was also quantified over 5-s intervals during the test session (300 seconds). Climbing is defined as upward directed movements of the forepaws usually along the side of the swim chamber and swimming behavior is defined as movement (usually horizontal) throughout the tank which includes crossing across quadrants of the cylinder (Cryan et al., 2005). After each session (pre-test and test session), the animals were removed and allowed to dry in a separate cage before being returned to their home cages.

4.3.6 Analysis of the cb1 receptor expression by western blotting

Sample processing to Western blotting was performed as previously described (Gomes et al., 2010) with some modifications. HIP and PFC were removed and homogenized separately in a solution containing protease inhibitors (T-Per Tissue protein extraction reagent, Thermo Scientific, USA; sodium orthovanadate, Sigma Aldrich, USA; phenylmethanesulfonyl fluoride, Sigma Aldrich, USA and protease inhibitor cocktail, Sigma Aldrich, USA). Samples were sonicated and the homogenates were centrifuged at 15,000 rpm for 30 min at 4ºC. After determining the protein concentrations of membrane supernatants through Nanodrop (Thermo Scientific, USA), equal amounts of protein (50 µg) from each sample were transferred to a nitrocellulose membrane, which was subsequently stained with

Ponceau S solution (Sigma Aldrich, USA) and blocked with milk 5% in Tris-buffered saline containing 0.2% of Tween 20 (polyoxyethylene sorbitan monooleate, Sigma Aldrich, USA). The samples were then probed with an antibody against CB1 (1:1000, Cayman) and with a secondary goat anti-rabbit IGG (1:5000; Cell). The immunoreactivity was detected by chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Scientific, USA), and the bands were quantified by optical density using Image Lab software (USA).

4.3.7 Noradrenaline and serotonin quantification by high-performance liquid chromatography (HPLC)

For neurotransmitter analysis, HPLC system was equipped with a reverse-phase column (Hypurity Elite C18, 250 mm x 4.6 mm, 5 µm and 100-Å pore diameter particle size; Hypersil, Cheshire, UK), coupled with electrochemical detection. Collected samples of the HIP and PFC were homogenized in 0.2 M perchloric acid containing dihydroxy-benzylamine (DHBA), centrifuged at 15,000 rpm for 20 min at 6ºC and stored at -70°C for 15 days, and 50 µL was injected into the HPLC-EC system. The HPLC system consisted of a Shimadzu LC-10 AD chromatograph, with a CBM-10A communication bus module, an on-line DGU-14A degassing unit, and an L-ECD-6A electrochemical detector with a glassy-carbon electrode and an LC-10 AD pump. The potential was set at 850 mV (versus an Ag/AgCl reference electrode). The mobile phase containing 150 mM chloroacetic acid, 120 mM NaOH, 0.67 mM EDTA, 0.86 mM sodium octylsulfate, 3.5% acetonitrile, and 2.6% tetrahydrofuran, adjusted to pH 3.0, was filtered and pumped through the system at a flow rate of 1.2 mL/min. For quantification of neurotransmitter levels, peak areas were compared to standard curves (Carvalho et al., 2012).

4.3.8 Indirect analysis of oxidative stress parameters

For analysis of oxidative stress markers in the PFC and HIP, tissues were homogenized in 200 mM of potassium phosphate buffer (pH 6.5) and then used to determine the reduced glutathione (GSH) and lipid hydroperoxides (LOOH). The

supernatant was used for the determination of superoxide dismutase (SOD) activity.

The content of LOOH, a highly reactive product of lipid peroxidation, was determined by the Ferrous Oxidation-Xylenol Orange (FOX2) method as previously described (Jiang et al., 1992). For this analysis 90% methanol was added to the homogenate, sonicated and centrifuged at 9000 × g for 20 min at 4◦C. The resulting supernatant was mixed with FOX2 reagent and led to incubate for 30 min at room temperature. The absorbance was determined at 560 nm and the results were expressed as mmol/mg of tissue.

For determination of GSH levels, aliquots of tissue homogenate from the PFC and HIP were mixed with12.5% trichloroacetic acid, vortexed for 10 min and centrifuged for 15 min at 900 × g (Sedlak et al., 1968). The supernatant was then mixed with TRIS buffer (0.4 M, pH 8.9) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 M). For posterior analysis, the absorbance was measured by spectrophotometry at 415 nm with a microplate reader. Results were compared to a standard curve of GSH (0.375–3 µg) and expressed as µg/g of tissue.

For the SOD activity analysis, a method that determines its capacity to inhibit pyrogallol autoxidation was used (Gao et al., 1998). For this, pyrogallol (1 mM) was added to the buffer solution (200 mM Tris HCl–EDTA, pH 8.5) and to supernatant aliquots, and then agitated in the vortex for 1 min. Afterwards, a period of incubation of 20 min occurs for the reaction to happen, which is later finished by adding 1NHCl and centrifuged for 4 min at 18,700 × g. The absorbance was then measured at 405 nm and the amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The results were expressed as U/mg of protein.

4.3.9 Data analysis

The Kolmogorov–Smirnov and Levene tests were initially employed to ensure that the data satisfied the criteria for carrying out one-way analysis of variance (ANOVA). When criteria were satisfied, the results were reported as the mean ± Standard Error of Mean (SEM). The data between DBT and NGL were

analyzed by the Student *t* test, while the treatment effect between DBT groups was evaluated using also Student *t* test or ANOVA. When appropriated, Newman–Keuls *post-hoc* tests were applied. Differences were considered statistically significant when $p \leq 0.05$.

4.4 EXPERIMENTAL DESIGN

4.4.1 Experiment 1 – Effect of the treatment with anandamide (dose-response curve) in diabetic rats submitted to the open field and forced swimming tests.

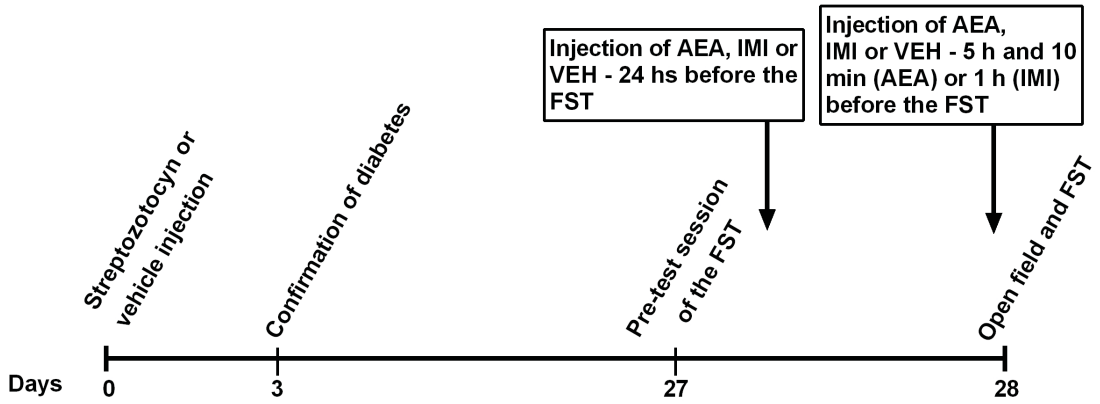

Figure 1 – Timeline of the Experiment 1. Diabetic rats induced by streptozotocin (STZ) were randomly treated with anandamide (AEA; 0.001, 0.005, 0.01 or 0.05 mg/kg; i.p.), Imipramine (IMI, 15 mg/kg; i.p.) or its respective vehicle (VEH). The pre-test session of the forced swim test (FST) was conducted on the 27 th day after the treatment with VEH (citrate buffer – normoglycemic rats) or induction of diabetes with STZ. The open field and FST were conducted in the 28th day. All treatments were performed in 3 applications (i.p.): immediately after the pre-test (24h before FST), 5h and 10 min (AEA) or 1h (IMI) before the FST. After evaluation of the behavioural parameters elicited by each dose of AEA, the best dose was chosen for further experimentation.

4.4.2 Experiment 2 – Effect of the previous treatment with AM251 (a CB1 receptor antagonist; 1 mg/kg, i.p.) or AM630 (a CB2 receptor antagonist; 1 mg/kg, i.p.) in diabetic animals treated with anandamide (0.005 mg/kg) and submitted to the open field and forced swimming tests.

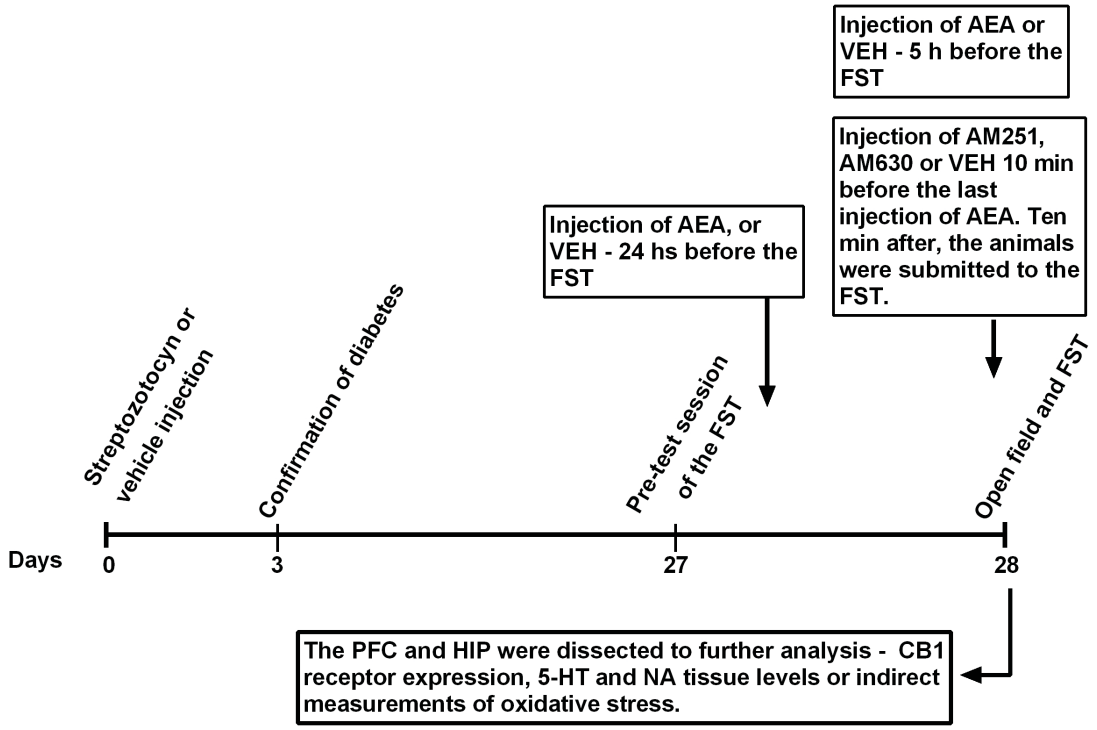

Figure 2 - Timeline of the Experiment 2. The CB1 receptor antagonist AM251 (1 mg/kg; i.p.) or CB2 receptor antagonist AM630 (1 mg/kg; i.p.) were injected 10 minutes before the last AEA injection (0.005, i.p.). Ten minutes later, rats were evaluated in the open-field test followed by forced swim test (FST). Immediately after the last determination of glycaemia, the animals were euthanized by decapitation and the prefrontal cortex and hippocampus were dissected for further analysis (western blot - CB1 receptor expression; HPLC - serotonin and noradrenaline tissue levels or indirect measurements of oxidative stress - GSH and LPO levels; SOD activity).

4.5 RESULTS

4.5.1 Effect of AEA in diabetic rats submitted to the forced swimming test

As shown in Figure 3, Student *t* test showed difference between NGL and DBT rats (t=3.864; df=14; p≤0.05). Also, one way ANOVA showed difference between experimental DBT groups [F(5,42)=19.08; p≤0.05]. The *post-hoc* test of Newman-Keuls showed that DBT animals treated AEA at the dose of 0.005 mg/kg was able to significantly decrease immobility time when compared to control DBT group (p≤0.05) indicating an antidepressant-like effect. Thus, the dose of 0.005 mg/kg was chosen for further experiments. All AEA doses were also tested in NGL rats and one-way ANOVA did not show significant difference between these NGL groups [F(4,36)=2,20; p≤0.05; data not shown]. As expected, post-hoc test of Newman-Keuls showed a significant antidepressant-like effect in DBT animals treated with IMI when compared to control DBT group (p≤0.05).

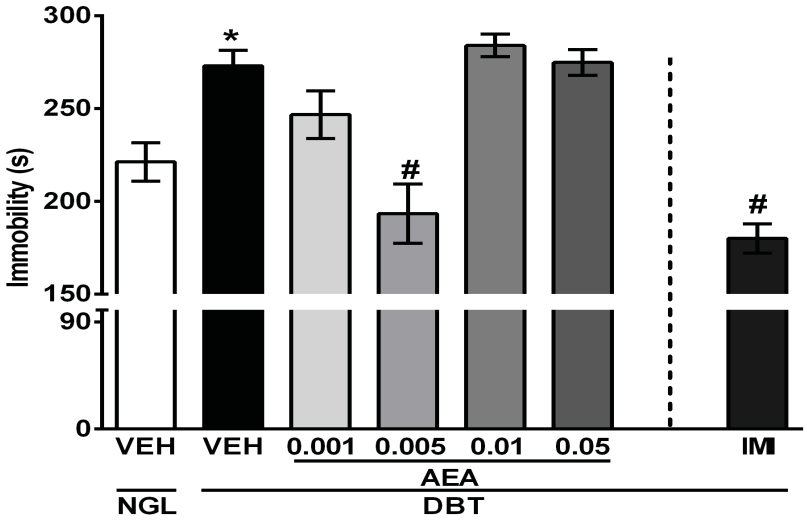

Figure 3. Effect of treatment with anandamide (AEA; 0, 0.001, 0.005, 0.01 or 0.1 mg/kg; i.p.) or Imipramine (IMI, 15 mg/kg; i.p.) in diabetic (DBT) animals on the immobility time evaluated during the Forced swimming test. The values are expressed as the mean ± SEM of 7-9 animals/experimental group. * indicates p≤0.05 compared to normoglycemic (NGL) animals treated with vehicle (VEH) and # indicates p≤0.05 compared to DBT animals treated with VEH.

4.5.2 Role of CB1 or CB2 receptors on the antidepressant-like effect of AEA in diabetic animals

Student *t* test showed difference between NGL and DBT on mean counts of immobility (t=5.054; df=13; p≤0.05), climbing (t=3.719; df=13; p≤0.05) and swimming, (t=3.365; df=13; p≤0.05) as shown in Figure 4. One-way ANOVA also showed a significant effect of the experimental groups among DBT animals on mean counts of immobility [F(5,42)=5.1; p≤0.05], climbing [F(5,42)=4.8; p≤0.05] and swimming [F(5,42)=3.6; p≤0.05], as shown in Figure 4. The *post-hoc* test of Newman-Keuls showed that AEA at the dose of 0.005 mg/kg decreased immobility and increased climbing and swimming mean counts in these DBT animals (p≤0.05). The *post-hoc* analysis also showed that the antidepressant-like effect of the AEA was prevented by pretreatment with CB1 receptor antagonist AM251 (p≤0.05), but not by CB2 receptor antagonist AM630 (p>0.05).

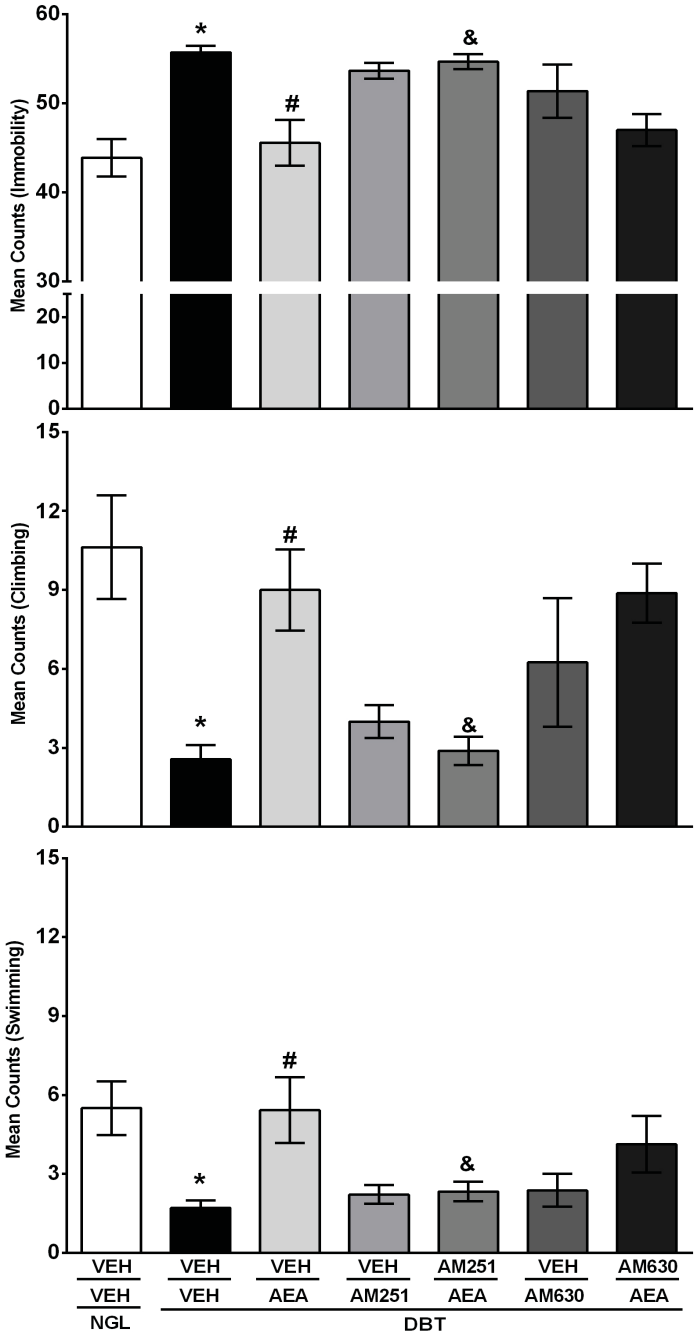

Figure 4. Effect of the previous treatment with CB1 receptor antagonist AM251 or CB2 antagonist AM630 on immobility, climbing and swimming mean counts in diabetic (DBT) animals treated with anandamide (AEA) or vehicle (VEH). The values are expressed as the mean ± SEM of 9-10 animals/experimental group. * indicates p≤0.05 compared to normoglycemic (NGL) animals treated with VEH; # indicates p≤0.05 compared to DBT animals treated with VEH and & indicates p≤0.05 when compared to DBT animals treated with VEH/AEA.

4.5.3 Effect of the condition (normoglycemic or diabetic) and/or treatments on blood glucose levels, weight gain and locomotor activity

As shown in the table 1, regarding to glycaemia and weight gain analysis, the Student *t* test showed difference among NGL and DBT groups (t=14.90; df=14; p≤0.05; t=4.10; df=14; p≤0.05), respectively. Moreover, the one-way ANOVA did not show a difference between DBT experimental groups [Glycaemia: F(6,52)=0.076; p>0.05; Weight Gain: F(6,52)=0.06; p>0.05]. As for the number of crossings, Student *t* test did not show a difference between DBT and NGL animals (t=0.99; df=18; p>0.05). Also, any difference of treatments employed was shown by one-way ANOVA among the DBT animals [F(6,54)=1.77; p>0.05].

Table 1- Effect of condition (normoglycemic-NGL or diabetic-DBT) and/or treatment (anandamide-AEA – 0.005 mg/kg; AM630 – 1 mg/kg; AM251 – 1 mg/kg; imipramine-IMI – 15 mg/kg; or vehicle-VEH) on glycaemia, weight gain and number of crossings evaluated in the open field test.

Condition/Treatment	Glycaemia (mg/dL)	Weight Gain (g)	N.of Crossings
NGL (VEH/VEH)	105 ± 2	127.5 ± 8.2	53 ± 0.9
DBT (VEH/VEH)	460 ± 23*	63 ± 13*	50 ± 2
DBT (AEA/VEH)	460 ± 27	62 ± 9	46 ± 3
DBT (AM 251/VEH)	476 ± 18	64 ± 11	40 ± 2
DBT (AM 251/AEA)	475 ± 25	66 ± 11	40 ± 2
DBT (AM 630/VEH)	474 ± 32	63 ± 7	43 ± 3
DBT (AM 630/AEA)	471 ± 27	69 ± 7	43 ± 1
DBT (IMI)	467 ± 19	67 ± 9	28 ± 3*#

Results are expressed as mean + SEM; n = 6–9, *p<0.05 when compared to NGL (VEH/VEH); #p< 0.05 when compared to DBT (VEH/VEH).

4.5.4 Effect of AEA treatment over CB1 receptor expression in the hippocampus and prefrontal cortex from diabetic animals

As shown in the Figure 5, Student t test showed a difference between NGL and DBT animals on CB1 receptor expression in the HIP ($t=2.86$; $df=4$; $p\leq0.05$) and also a difference between the vehicle or AEA-treated DBT rats ($t=3.17$; $df=4$; $p\leq0.05$; Figure 5A). Regarding the CB1 receptor expression in the PFC, the Student t test showed no difference between NGL and DBT groups ($t=1.87$; $df=4$; $p>0.05$; Figure 5B) neither between vehicle or AEA-treated DBT animals ($t=0.63$; $df=4$; $p>0.05$; Figure 5B).

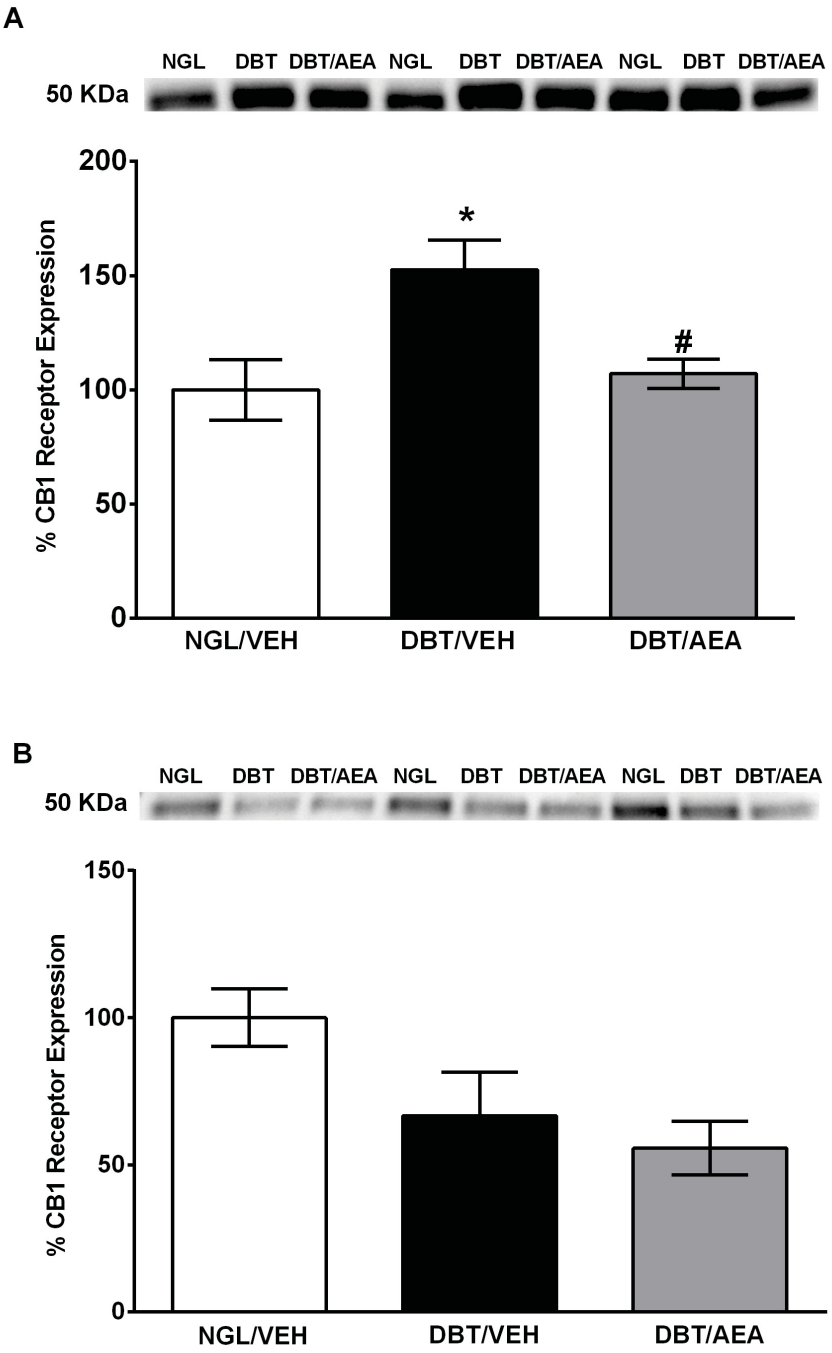

Figure 5. Effect of anandamide (AEA) treatment on CB1 receptor expression in hippocampus (panel A) and prefrontal cortex (panel B) of diabetic (DBT) animals. The values are expressed as the mean ± SEM of samples obtained from 3 animals per experimental group. * indicates $p \leq 0.05$ compared with normoglycemic (NGL) animals treated with vehicle (VEH); # indicates $p \leq 0.05$, compared with DBT treated with VEH.

4.5.5 Effect of AEA on noradrenaline and serotonin content in the hippocampus and prefrontal cortex from in diabetic animals

As shown in Figure 6, Student *t* test showed a difference between NGL and DBT animals when noradrenaline content was quantified [HIP: t=3.150; df=13; p≤0.05; PFC: t=2.11; df=12; p≤0.05] and also when 5-HT content was investigated [HIP: t=3.37; df=13; p≤0.05; PFC: t=2.59; df=13; p≤0.05]. Moreover, Student *t* test showed that the decrease of 5-HT content in the HIP from DBT rats was significantly reversed by AEA treatment (t=4.93; df=13; p≤0.05). However, this treatment was not able to reverse the decrease in hippocampal NA levels from DBT rats (t=1.37; df=13; p>0.05; Figure 6A). At the PFC, while the NA content was increased in DBT animals (p≤0.05), the 5-HT content in significantly reduced (p≤0.05) when compared to the NGL. At the PFC from DBT rats, the AEA treatment significantly reverved the increased NA content (t=2.55; df=12; p≤0.05) but not the reduced 5-HT (t=0.27; df=13; p>0.05; Figure 6B).

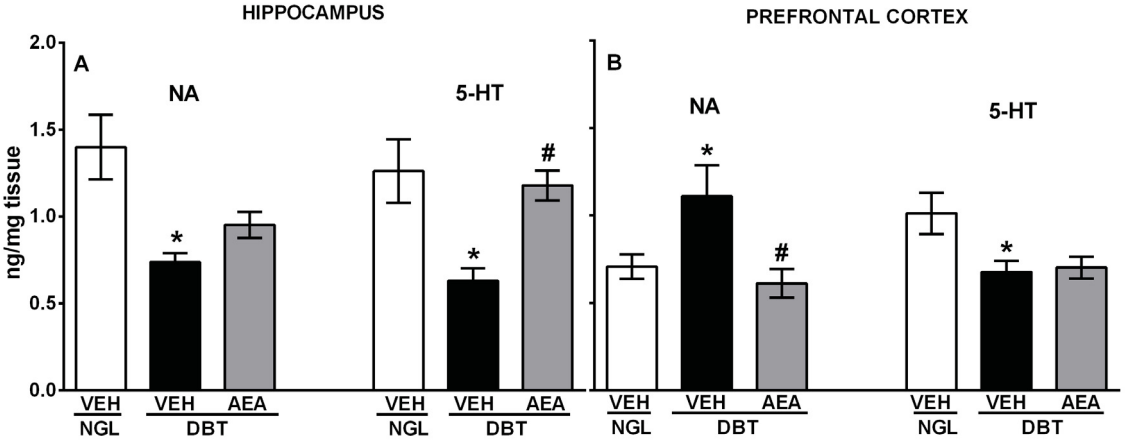

Figure 6. Effect of anandamide (AEA) treatment on noradrenaline (NA) or serotonin (5-HT) levels in the hippocampus and prefrontal cortex of diabetic (DBT) animals. The values are expressed as the mean ± SEM of 7-8 animals/experimental group. * indicates p≤0.05 when compared to normoglycemic (NGL) animals treated with vehicle (VEH); # indicates p≤0.05 when compared to DBT animals treated with VEH.

4.5.6 Effect of the AEA on oxidative stress parameters in the hippocampus and prefrontal cortex from diabetic animals

As shown in the Figure 7A and 7B, Student *t* test showed a significant decrease in the GSH levels in tissues from DBT animals when compared to NGL ones [HIP: t=3.35; df=13; p≤0.05; PFC: t=5.60; df=12; p≤0.05] which is restored by AEA treatment [HIP: t=5.39; df=12; p≤0.05; PFC: t=6.49; df=11; p≤0.05].

Student *t* test also showed a significant increase in LOOH levels in tissues from DBT animals when compared to NGL ones (Figure 7, panels C and D) [HIP: t=3.24; df=13; p≤0.05; PFC: t=2.81; df=14; p≤0.05] which is significantly reduced by AEA treatment [HIP: t=2.76; df=12; p≤0.05; PFC: t=2.57; df=14; p≤0.05].

Analysis by Student *t* test also showed a significant increase in the SOD activity in tissues from DBT animals when compared to NGL ones (Figure 7, panels E and F) [HIP: t=5,72; df=14; p≤0.05; PFC: t=2,51; df=15; p≤0.05] which is significantly restored by AEA treatment [HIP: t=6.65; df=11; p≤0.05; PFC: t=4.83; df=15; p≤0.05].

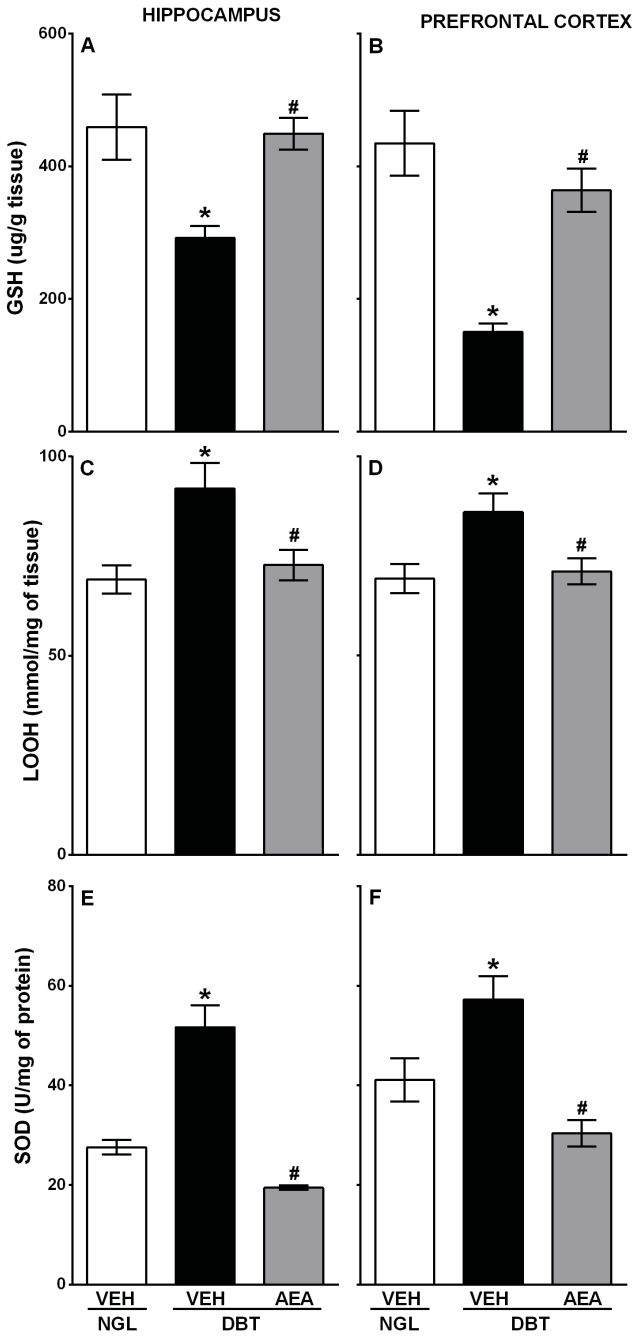

Figure 7. Effect of anandamide (AEA) treatment in diabetic (DBT) animals on reduced glutathione (GSH) levels (panels A and B); lipid hydroperoxides (LOOH) content (panels C and D) and superoxide dismutase (SOD) activity (panels E and F) in the hippocampus and prefrontal cortex of DBT animals. The values are expressed as the mean ± SEM of 6-8 animals/experimental group. * indicates p≤0.05 when compared to normoglycemic (NGL) animals treated with vehicle (VEH); # indicates p≤0.05 when compared to DBT animals treated with VEH.

4.6 DISCUSSION

The central finding of this study is that the treatment with AEA induced an antidepressant-like effect in DBT rats and this effect seems to be associated to a reestablishment of 5-HT levels in the HIP and the NA levels in the PFC. Moreover, AEA treatment improved the altered indirect parameters of oxidative stress in the HIP and PFC from these DBT animals, indicating a neuroprotective effect. Once the elevated CB1 receptor density in the HIP was restored by AEA treatment as well as the CB1 receptor antagonist blocked the antidepressant-like effect of AEA, our hypothesis is that the CB1 receptors play the main role in AEA effects.

The more pronounced depressive-like behavior in DBT animals observed in the present study has already been observed in previous studies from our group (De Morais et al., 2014; Da Silva Dias et al., 2015) and also by others (Gomez and Barros, 2000; Ho et al., 2012; Gupta et al., 2014). Here, it is important to point out that despite the FST receive criticism regarding its use to study the depressive-like behavior, according to these studies, the FST seems to be able to identify pro-depressant effects (see Slattery and Cryan, 2012). Thus, despite the weakness of face validity, such evidence shows that the FST can be an appropriate tool for preclinical mood disorder research.

This study, to our knowledge, is the first to demonstrate an antidepressant-like effect of AEA in DBT animals. Thus, as observed with the tricyclic antidepressant IMI, used in the present study as a positive control of the antidepressant-like behavior, the effective dose of AEA (0.005 mg/kg) was able to decrease the immobility time of DBT animals evaluated in the FST (Figure 3) and also to induce the reduction in the immobility mean count and the increase in the mean counts of active behaviors of climbing and swimming (Figure 4). In this dose regimen, the AEA treatment was not able to alter the hyperglycemia, the locomotor activity and the reduced weight gain of DBT animals (Table 1). Differently and as previously observed (De Morais et al., 2014), IMI treatment reduced the locomotor activity in the open field test. However, this impairment did not prejudice its antidepressant-like effect characterized by reducing the immobility time during the FST (Table 1). It is important to point out that a previous study from our laboratory have already

observed that the same IMI dose regimen induced a significant reduction of total immobility time in DBT and non-DBT rats during the FST (de Morais et al., 2014).

In the present study, we did not observe the antidepressant-like effect of AEA in NGL animals (at all tested doses; from 0.01 to 0.05 mg/kg; please see description in the 4.5.1 item). However, data in the literature have shown that the depressive-like behavior in normoglycemic has been primarily inhibited by fatty acid amide hydrolase inhibitors (Hill and Gorzalka, 2005; Kruk et al., 2015) and/or CB1 receptor activation (Rutkowska and Jachimczuk, 2004; Hill and Gorzalka, 2005; Mclaughlin et al., 2007). Considering the effect of AEA itself, a recent work of Sartim et al. (2016) showed that the microinjection of AEA directly in the infralimbic subregion of prefrontal cortex induced a significant decrease in the immobility time in rats submitted to the FST. Besides, the intracerebroventricular infusion of AEA was also able to reduce the total immobility time in the normoglycemic mice, as proposed by Umathe et al. (2011). Then, the antidepressive-like effect of AEA in normoglycemic rats appears to be preferentially related to its central action.

In order to study the mechanisms involved in the antidepressant-like effect of AEA in DBT animals, our data showed that the pretreatment with the CB1 receptor antagonist AM251, but not with CB2 receptor antagonist AM630, induced an impairment in the antidepressant-like effect induced by AEA, suggesting the CB1 receptor is primarily the target of the AEA. In this same direction, studies conducted in non-DBT animals have shown that the pharmacological stimulation of the CB1 receptor causes antidepressant-like effects in animals subjected to FST (Hill and Gorzalka, 2005; Gobbi et al., 2005; Kruk et al., 2015). The HIP seems to be a key brain area involved in the antidepressant effect of the endocannabinoid system since the bilateral administration of the CB1 receptor agonist HU-210 into the dorsal HIP induced a significant antidepressant-like effect in rats submitted to FST (McLaughlin et al., 2007). Furthermore, the HIP appears to be one of the brain areas of higher expression of CB1 receptors (Irving et al., 2002; Hill et al., 2008). Interestingly our data showed an increased CB1 receptor expression in HIP from the DBT animals when compared to normoglycemic group, been this increased CB1 receptor expression normalized after AEA treatment (Figure 5A). According to our data, Duarte and coworkers (2007) also observed an

increase in the CB1 receptor density in HIP of DBT rats, which was associate to the an adaptive response in an attempt to counterbalance the reduced endocannabinoid communication due to postsynaptic calcium depreciation in these animals (Duarte et al., 2007).

Many evidence have pointed out that the endocannabinoid system may induce its beneficial effects on behavior related to depression by acting on the serotoninergic and noradrenergic systems (Zanelati et al., 2010; Morales and Bäckman, 2002; Gobbi et al, 2005; Reyes et al., 2012). Thus, McLaughlin and Gobbi (2012) observed that direct injection of AEA hydrolysis inhibitor URB597 into the PFC induced an increase in the firing of serotoninergic neurons of dorsal raphe nucleus suggesting that the endocannabinoid signaling in the PFC can modulate stress-related behaviors, as active responses, through regulation of 5-HT neurotransmission. It is important to highlight that these studies were conducted in non-DBT animals and differently from our study, the AEA was injected directly into the PFC. Beside the PFC, the HIP is also appointed as a possible target brain area in which an interaction between these systems clearly occurs (Morales and Bäckman, 2002; Zanelati et al., 2010). In relation to noradrenergic neurotransmission, a study conducted by Reyes et al (2012) demonstrated that the pretreatment with the CB1 receptor agonist WIN 55,212-2 into the PFC decreased the noradrenergic transmission induced by stress, suggesting that a relationship between the endocannabinoid system and noradrenergic system fundamentally influence the modulation of the stress-related behavior (Hill and Gorzalka, 2004; Gobbi et al, 2005; Reyes et al., 2012). In agreement, our data showed a reestablishment of noradrenergic transmission in PFC from DBT animals submitted to FST (Figure 6B), which could be due to the fact that there are CB1 receptors located in noradrenergic axon terminals in this region (Oropeza et al., 2007).

Importantly, a possible dysregulation in the noradrenergic and serotonergic neurotransmission has been related to depression associated with diabetes (Bellush et al., 1991; Gupta et al., 2014; Prabhakar et al., 2015; da Silva Dias, 2015), explaining the use of antidepressants which inhibit the reuptake of 5-HT and/or NA as the first-line drugs in the treatment of depression associated with

diabetes (de Long et al., 2015). Accordingly, we also aimed to study the effect of AEA treatment over the 5-HT and NA tissue levels in the HIP and PFC from DBT animals. Our data clearly showed that when compared to NGL animals, DBT animals presented reduced 5-HT levels in the HIP and PFC, a reduction in the NA levels in the HIP and an increase of this neurotransmitter in the PFC (Figure 6). These data are corroborated by the fact that the DBT animals presented a decrease in climbing frequency (which is related to a decrease in the NA neurotransmission), and a decrease in the swimming frequency (which is related to the decrease in the 5-HT neurotransmission; Figure 4; Detke et al, 1995; Cryan et al, 2002, 2005). The treatment with AEA was able to restore the reduced 5-HT levels in the HIP which may explain the AEA effect over the swimming counts and also the elevated NA levels in the PFC which seems to be related to AEA effect over the climbing counts in the FST. Interestingly, Prabhakar and collaborators (2015) suggested that the serotonergic system is a multifactorial target involved in the pathophysiology of depression associated with diabetes since they observed that DBT animals showed an increase in the activity of the monoamine oxidase enzyme and consequently an elevation in the 5-HT metabolism, leading to a low level of 5-HT in brain areas (Gupta et al., 2014; Prabhakar et al., 2015). It is important to point out that regarding to 5-HT, our data indicate a possible interaction between the endocannabinoid system and 5-HT in the HIP from DBT animals in view of the fact that AEA treatment restored the reduced CB1 receptor expression in this brain area as well as reestablished the reduced 5-HT levels. However, since AEA also restored the increased NA levels in PFC to the control (NGL) levels, we cannot rule out that NA in PFC also plays an important role in mediating the depression associated with diabetes. Altogether, it seems that the 5-HT in the HIP and NA in the PFC from DBT animals have a singular role in the mediation of behavior related to the depression.

The endocannabinoid system has also been related to the neuroprotective effects through an antioxidant activity (Booz et al., 2011). Confirming this observation made in normoglycemic animals, we demonstrated that AEA treatment induced a beneficial effect on the increased oxidative stress in the HIP and PFC from DBT animals (Figure 7; de Morais et al., 2014). More specifically, we observed that AEA treatment significantly reduced the increased GSH and LOOH levels, and

increased the reduced activity of SOD in the HIP and PFC from DBT animals (Figure 7), suggesting that endocannabinoid system could exert an antioxidant or neuroprotective effect on DBT individuals. According to our data, Dagon et al. (2007) observed that the treatment with the CB1 receptor agonist HU-210 reduced the brain levels of lipid peroxidation, correcting the cognitive impairment in DBT mice.

Taken all together, depression associated with diabetes is a complex comorbidity in which the patophysiological mechanism is still far from be clarified. It seems to involve various biological systems such as the endocannabinoid, serotonergic and noradrenergic. Thus, our findings indicate that the antidepressant-like effect induced by AEA in DBT animals may be related to its neuroprotective action associated with a functional improvement of endocannabinoid system with consequent reestablishment of the hippocampal 5-HT and cortical NA levels to the basal levels. Although further studies are required to better understand the neurobiology of depression associated with diabetes, the present study points out the endocannabinoid system as a potential therapeutic target for the treatment of this comorbidity.

4.7 CONFLICTS OF INTEREST

The authors do not have any conflict of interest in the conduct and reporting of this research.

4.8 ACKNOWLEDGMENTS

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5 SEGUNDO ARTIGO CIENTÍFICO: SUB-CHRONIC TREATMENT WITH CANNABIDIOL BUT NOT WITH URB597 INDUCED A MILD ANTIDEPRESSANT-LIKE EFFECT IN DIABETIC RATS

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Sub-chronic treatment with cannabidiol but not with URB597 induced a mild antidepressant-like effect in diabetic rats

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5.1 ABSTRACT

Depression associated with diabetes has been described as a highly debilitating comorbidity. Due to its complex and multifactorial mechanisms, the treatment of depression associated with diabetes represents a clinical challenge. Cannabidiol (CBD), the non-psychotomimetic compound derived from *Cannabis sativa*, has been pointed out as a promising compound for the treatment of several psychiatric disorders. Here, we evaluated the potential antidepressant-like effect of acute or sub-chronic treatment with CBD in diabetic rats using the modified forced swimming test (mFST). Also, to better understand the functionality of the endocannabinoid system in diabetic animals we also evaluated the effect of URB597, a fatty acid amide hydrolase inhibitor. Four weeks after the treatment with streptozotocin (60 mg/kg; i.p.; diabetic group-DBT) or citrate buffer (i.p.; normoglycemic group-NGL), DBT animals received an acute intraperitoneal injection of CBD (0, 0.3, 3, 10, 30 or 60 mg/Kg), 1 hour before the mFST, or URB597 (0, 0.1, 0.3 or 1 mg/Kg) 2 hours before the mFST. In another set of experiments, animals were sub-chronically treated with CBD (0, 0.3, 3, 30 or 60 mg/Kg i.p.), 24, 5 and 1 hour before the mFST or URB597 (0, 0.1, 0.3 or 1 mg/Kg i.p.) 24, 5 and 2 hours before the mFST. The NGL group was acutely treated with CBD (0, 30 mg/kg i.p.) or URB597 (0, 0.3 mg/kg; i.p.). Acute treatment with either CBD or URB induced an antidepressant-like effect in NGL rats, but not in DBT rats. However, sub-chronic treatment with CBD (only at a dose of 30 mg/kg), but not with URB597, induced a mild antidepressant-like effect in DBT animals. Neither body weight nor blood glucose levels were altered by treatments. Considering the importance of the endocannabinoid system to the mechanism of action of many antidepressant drugs, the mild antidepressant-like effect of the sub-chronic treatment with CBD, but not with URB597 does not invalidate the importance of deepening the studies involving the endocannabinoid system particularly in DBT animals.

Keywords: Cannabidiol, URB597, Diabetes, Depression, Streptozotocin

5.2 INTRODUCTION

Diabetes mellitus (DM) is a highly prevalent chronic metabolic disorder that is characterized by hyperglycemia due to a relative or absolute deficiency of insulin action or secretion [1–3]. According to the International Diabetes Federation [4], the number of diabetic patients is more than 425 million people worldwide, a number that can reach 642 million in 2040. However, considering that one of two people with DM has not yet been diagnosed, these numbers are still underestimated [4].

It is known that chronic hyperglycemia leads to micro and macrovascular complications, such as nephropathies, retinopathies, heart diseases and also dementia and depression [2,3,5–8]. Clinically, the high prevalence of depression in diabetic patients is well known, as well as an increased risk of patients with DM to develop depression [9–14]. Evidence suggests that this comorbidity may be the result of lifestyle changes such as stress, a non-rigid glycemic control, dietary restriction and/or physiological changes due to the diabetic condition itself [2,3,15].

Although antidepressants in combination with hypoglycemic drugs are the treatment of choice for the depression associated with diabetes, this condition is badly controlled by these drugs with only few of the patients achieving true recovery or remission [2,16]. Moreover, antidepressants should be cautiously prescribed for diabetic patients as some medications may directly influence glycemic control or interact with hypoglycemic drugs [17–19]. Thus, there is a clear demand to explore novel therapeutic compounds for the management of this important clinical condition whose the therapeutic effect appears quickly after the start of treatment, with fewer side effects and/or fewer risks for the patient and with a higher rate of adhesion and effectiveness.

In view of this, loads of evidence point out to compounds like cannabidiol (CBD), the most abundant non-psychotomimetic compound present in the *Cannabis sativa*. CBD has recently gained prominence as a therapeutic tool for a wide range of disorders, such as anxiety, epileptic seizure, schizophrenia, chronic pain and also depression [20–24]. Interestingly, studies in humans have shown that CBD is well tolerated and does not appear to induce obvious adverse effects,

even after prolonged treatment [25]. Of particular interest, preclinical studies conducted in non-diabetic animals show that CBD induces an antidepressant-like effect [21,26] in a similar manner to the classical antidepressant imipramine after acute or prolonged treatment [22,26]. In addition, other beneficial properties of CBD have been documented, such as neuroprotective, antioxidant and anti-inflammatory [27–29].

Regarding to diabetes, studies show that CBD may have beneficial effects in relation to the diabetic state *per se*, reducing the incidence of type 1 diabetes in mice and the early pancreatic inflammation in this type of diabetes [30–32]. Furthermore, CBD reduces the neurotoxicity, inflammation, and blood-retinal barrier breakdown in streptozotocin-induced diabetic animal [33,34]. Using this animal model of diabetes, our group recently showed that acute treatment with CBD (at doses of 3 mg/Kg; i.p.) exerted a significant reduction of mechanical allodynia, suggesting that CBD may be effective in the treatment of painful diabetic neuropathy (Jesus et al., submitted data).

Thus, the present study aimed to evaluate the effect of acute or sub-chronic treatment with CBD in depressive-like behaviors in streptozotocin-induced diabetic rats using the modified forced swimming test. Moreover, given the fact that the endocannabinoid effects are tightly regulated by their degradation rate by fatty acid amide hydrolase (FAAH) and that the endocannabinoid system seems to be unregulated in diabetic animals [8,35], it was also evaluated the effects of URB597, a FAAH inhibitor that elevates the brain anandamide levels in rodents [36,37].

5.3 MATERIAL AND METHODS

5.3.1 Animals

Adult male Wistar rats (180–220 g), provided by the Federal University of Paraná, were housed in plastic cages (41× 32 × 16.5 cm) with food and water available *ad libitum* and maintained in a temperature-controlled room (22 + 2◦C) under 12h/12h light/dark cycle (lights on at 7:00 a.m.). All the protocols were performed in accordance with the ethical guidelines of Brazilian legislation on animal welfare and previously approved by the Federal University of Parana

Institutional Committee for the Ethical Use of Animals (CEUA/BIO-UFPR; authorization #749). All efforts were made to minimize the number and suffering of the animals used.

5.3.2 Drugs and treatment

The following drugs were used: streptozotocin (STZ; Santa Cruz Biotechnology Inc., USA), sodium citrate (Merck S.A., Brazil), cyclohexylcarbamic acid 3-carbamoylbiphenyl-3-yl ester (URB597; 0.1; 0.3 or 1 mg/Kg; i.p.; Sigma Aldrich, USA), cannabidiol (CBD; 0.3, 3, 10, 30 or 60 mg/Kg, i.p.; 99.6% pure was kindly supplied by BSPG-Pharm, Sandwich, United Kingdom). STZ was dissolved in citrate buffer (10 mM, pH 4.5). URB597 was dissolved in 2–3 drops of ethanol and diluted as required in a 1% aqueous solution Tween 80. CBD was freshly diluted in 2%Tween 80 and saline [8,21,38,39].

5.3.3 Diabetes induction

Type-1 experimental diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/Kg) freshly dissolved in citrate buffer (10 mM, pH 4.5) in overnight fasten rats. Hyperglycemia was confirmed 72 h after STZ administration by applying a small volume of peripheral blood collected from the tail on test strips impregnated with glucose oxidase (Accu-Check ActiveTM, Roche) and confirmed again at ending of the behavioural tests. Animals with fasting blood glucose levels ≥250 mg/dL were considered diabetic and maintained in the experimental groups [8].

5.3.4 Modified forced swim test

The potential antidepressant-like effect of CBD or URB597 was investigated using the modified forced swim test (mFST) as described initially by Porsolt et al. [40] and modified by Detke et al. [41]. The test was conducted in two sessions. First, in the pre-test session rats were placed individually to swim in a tank (30 cm × 40 cm height, containing 30 cm of water at 22 + 1°C) for 15 min. Twenty four hours later, animals were submitted to a 5 min session of forced swim (test) and the session was filmed for later analysis. Every 5 seconds of interval of the test session, the predominant behavior was evaluated: (1) immobility (except the small

movements required to float), (2) swimming (movements through the plastic cylinder) and (3) climbing (movement with the front legs in the cylinder wall in an attempt to leave it). After each session (pre-test and test session), the animals were removed and allowed to dry in a separate cage before being returned to their home cages and the tank was cleaned.

5.3.5 Open-field test

The open field test (OFT) was conducted as described previously [8]. Briefly, all the animals were placed in the center of a wooden rectangular open field (40 X 50 X 63 cm) with a floor divided into 9 rectangular units. The number of squares crossed with all four paws in an interval of 5 min was observed and quantified as a parameter of general motor activity.

5.3.6 Experimental design

In the first set of experiments, four weeks after the treatment with streptozotocin (60 mg/kg; i.p.), diabetic (DBT) animals received an acute injection of CBD (0, 0.3, 3, 10, 30 or 60 mg/Kg i.p.). Normoglycemic animals (NGL; treated with citrate buffer, vehicle of STZ) received an acute injection of CBD (only at a dose of 30 mg/Kg i.p.) 1 hour before the OFT followed by the mFST. Independent groups of DBT animals received an acute injection of URB597 (0, 0.1, 0.3 or 1 mg/Kg; i.p.) and NGL animals received an acute injection of URB597 (0.3 mg/Kg; i.p.) 2 hours before the OFT followed by the mFST. The injection of CBD or URB597 in NGL was made as a positive control of the antidepressant-like effect of these drugs as observed previously [21,42,43].

The second set of experiments aimed to evaluate the effect of subchronic treatment with CBD or URB597. Thus, DBT animals were submitted to the regimen of 3 intraperitoneal injections of CBD (0, 0.3, 3, 30 or 60 mg/Kg), 24, 5 and 1 hour before the OFT followed by the mFST or URB597 (0, 0.1, 0.3 or 1 mg/Kg) 24, 5 and 2 hours before the OFT followed the mFST.

In all set of experiments, animals had their body weight and blood glucose checked weekly.

5.3.7 Statistical analysis

The Kolmogorov-Smirnov and Levene tests were initially employed to ensure that the data satisfied the criteria for carrying out parametric tests. When criteria were satisfied, the results were reported as the mean + Standard Error of Mean (SEM). The data were analyzed by one-way ANOVA with groups as a single independent factor. When appropriated, Newman–Keuls tests were used for *post hoc* analyses. Differences were considered statistically significant when $p<0.05$.

5.4 RESULTS

5.4.1 Acute treatment with cannabidiol did not change the depressive-like behavior in diabetic animals

As showed in Fig.1 (panels A, B and C), the one-way ANOVA revealed that the different groups were able to change the frequencies of immobility [$F(7,55)=20.15$; $p<0.05$], swimming [$F(7,55)=12.69$; $p<0.05$] and climbing [$F(7,55)=11.84$; $p<0.05$]. Newman-Keuls *post hoc* test showed that DBT animals presented an increase of the immobility frequency, while the frequencies of swimming and climbing was reduced ($p<0.05$) when compared with NGL animals. Interestingly, significant difference was observed between vehicle-treated NGL animals when compared with NGL animals treated with CBD (30 mg/kg; $p<0.05$), showing decreased frequency of immobility and increased frequencies of swimming and climbing ($p<0.05$). However, the *post hoc* test showed no significant difference between the DBT groups treated with vehicle or CDB ($p>0.05$).

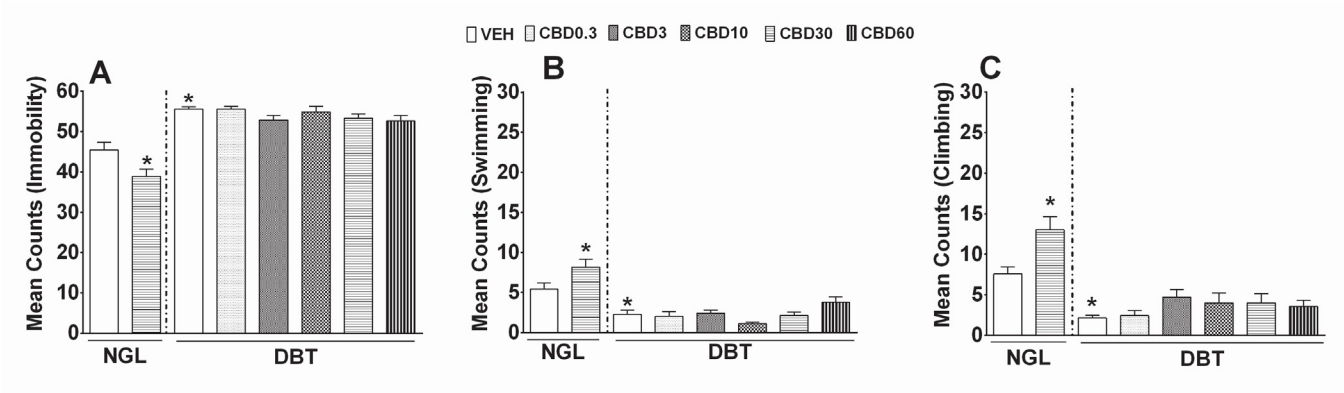

Fig 1: Effect of acute treatment with CBD (0.3, 3, 10, 30 and 60 mg/kg i.p.) or vehicle (VEH) on the frequency of immobility (figure 1A), swimming (figure 1B) and climbing (figure 1C) in NGL and DBT animals submitted to the mFST. Values were expressed as mean ± SEM (n = 7-12). * p <0.05 when compared with vehicle-treated normoglycemic (NGL/VEH) animals.

5.4.2 Cannabidiol subchronic treatment induced antidepressant-like effect in diabetic animals

One-way ANOVA (see Fig.2, panels A, B and C) revealed that the different groups were able to change the frequencies of immobility [$F(6,72)=36.63$; $p<0.05$], swimming [$F(6,72)=9.22$; $p<0.05$] and climbing [$F(6,72)=12.32$; $p<0.05$]. Newman-Keuls *post hoc* test showed that DBT animals presented increased frequency of immobility, decreased frequency of swimming and climbing ($p<0.05$) when compared with NGL animals. Also, the *post hoc* test showed that CBD treatment (only at the higher doses of 30 mg/kg) significantly decreased the frequency of immobility and increased the frequencies of swimming and climbing ($p<0.05$).

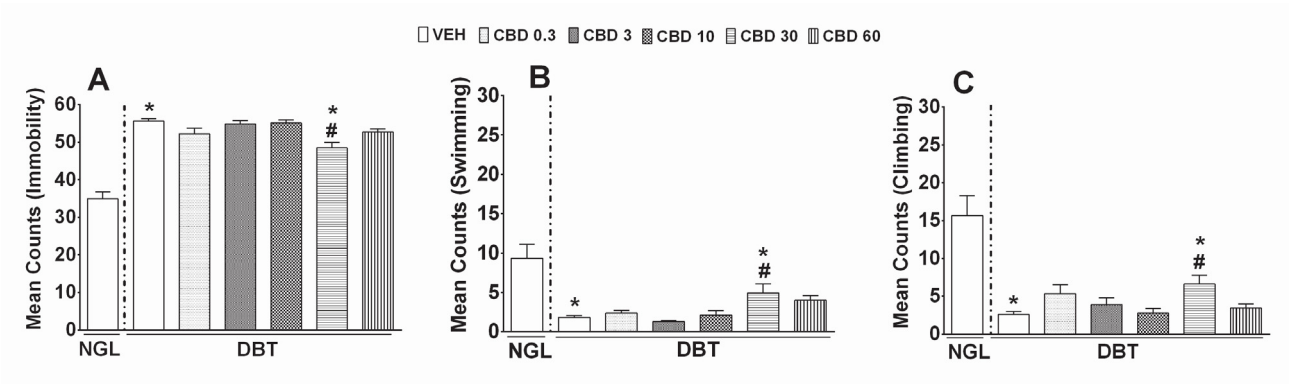

Fig 2: Effect of sub-chronic treatment with CBD (0.3, 3, 10, 30 and 60 mg/kg i.p.) or vehicle (VEH) on the frequency of immobility (figure 2A), swimming (figure 2B) and climbing (figure 2C) response in NGL and DBT animals submitted to FST. Values were expressed as mean ± SEM (n=9-13). *p <0.05 when compared with vehicle-treated normoglycemic (NGL/VEH) animals and $^{\#}$p <0.05 when compared to vehicle-treated diabetic animals (DBT / VEH).

5.4.3 Effect of condition (normoglycemic-NGL or diabetic-DBT) and/or treatment (cannabidiol-CBD; or vehicle-VEH) on glycaemia, weight gain and number of crossings evaluated in the open field test

As showed in Table 1, one-way ANOVA revealed that different groups submitted to the CBD acute treatment had altered the glycaemia [F(7,55)=92,38; p<0.05], weight gain [F(7,55)=15,92; p<0.05] and number of crossings [F(7,55) =9,805; p<0.05]. The same was observed when a CBD subchronic treatment was performed [glycaemia: F(6,72)=73,56; p<0.05; weight gain: F(6,72)=17,23; p<0.05 and number of crossings: F(6,72)=9,114; p<0.05]. Newman-Keuls *post hoc* test showed that all DBT animals were different of NGL animals, *i.e.* presented hyperglycemia (p<0.05), reduced weight gain (p<0.05) and decrease in the number of crossings in the open field (p<0.05).

Condition/AcuteTreatment	Glycaemia (mg/dL)	Weightgain (g)	No. of crossings
NGL-VEH	102±2.5	140±8	68±1
NGL-CBD (30 mg/Kg)	101±1	149±3.5	57±1
DBT-VEH	531±10*	58±3*	38±1*
DBT-CBD (0.3 mg/Kg)	510±11.5*	51±3*	35±1.5*
DBT-CBD (3 mg/Kg)	520±10*	57±2*	39±1.5*
DBT-CBD (10 mg/Kg)	506±11*	55±3*	38±2*
DBT-CBD (30 mg/Kg)	506±8*	54±4*	39±1*
DBT-CBD (60 mg/Kg)	524±8*	45±2*	30±1*
Condition/Subchronictreatment	**Glycaemia (mg/dL)**	**Weightgain (g)**	**No. of crossings**
NGL-VEH	99±1	121±3	60±2
DBT-VEH	521±5*	48±2*	34±1*
DBT-CBD (0.3 mg/Kg)	513±7*	48±2*	35±1*
DBT-CBD (3 mg/Kg)	511±6*	45±2*	34±1*
DBT-CBD (10 mg/Kg)	501±8*	44±2*	24±1*
DBT-CBD (30 mg/Kg)	490±5*	49±1*	40±1*
DBT-CBD (60 mg/Kg)	505±6*	44±2*	22±1*

Results are expressed as mean±SEM; n=7-14.*p≤0.05 when compared to NGL-VEH.

5.4.4 URB597 acute treatment did not change the depressive-like behavior in diabetic animals

As showed in Fig.3 (panels A, B and C), the one-way ANOVA revealed that the different groups were able to change the frequencies of immobility [$F(5,47)=59.98$; $p<0.05$], swimming [$F(5,47)=8.16$; $p<0.05$] and climbing [$F(5,47)=25.91$; $p<0.05$]. Newman-Keuls *post hoc* test showed that DBT animals presented increased frequency of immobility, decreased frequency of swimming and climbing ($p<0.05$) when compared with NGL animals. Interestingly, significant difference was observed between NGL animals treated with vehicle and NGL treated with URB597 (0.3 mg/Kg), *i.e.* the treated animals presented decreased frequency of immobility and increased frequencies of swimming and climbing ($p<0.05$). However, the *post hoc* test showed no significant difference between the DBT groups treated with vehicle or URB597 ($p>0.05$).

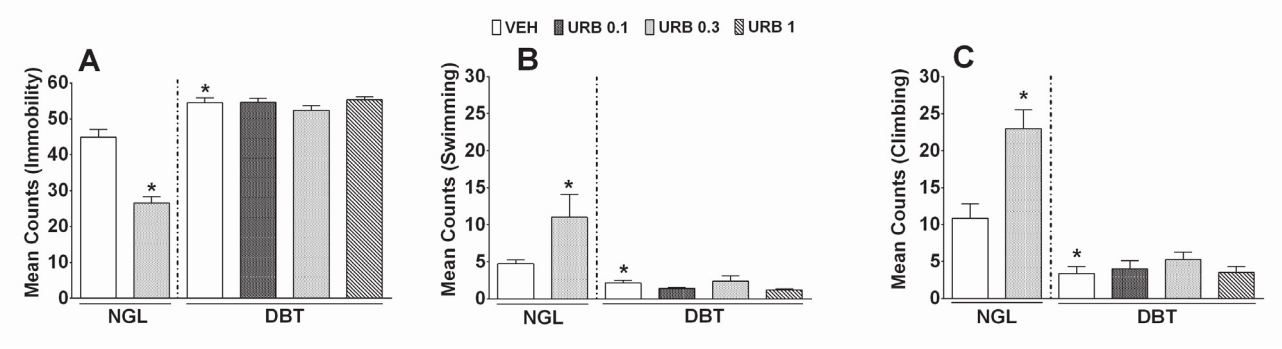

Fig 3: Effect of acute treatment with URB597 (0.1, 0.3, 1 mg/kg i.p.) or vehicle (VEH) on the frequency of immobility (figure 3A), swimming (figure 3B) and climbing (figure 3C) in NGL and DBT animals submitted to the mFST. Values were expressed as mean ± SEM (n=8-10). * $p<0.05$ when compared with vehicle-treated normoglycemic (NGL/VEH) animals.

5.4.5 URB597 subchronic treatment did not change the depressive-like behavior in diabetic animals

One-way ANOVA (Fig.4, panels A, B and C) revealed that the different groups were able to change the frequency of immobility [F4,42)=10.25; p<0.05], swimming [F4,42)=5.64; p<0.05] and climbing [F(4,42)=7.21; p<0.05]. Newman-Keuls *post hoc* test showed that DBT animals presented increased frequency of immobility, decreased frequencies of swimming and climbing (p<0.05) when compared with NGL animals. However, the *post hoc* test showed no significant difference between the DBT groups treated with vehicle or URB597 (p>0.05).

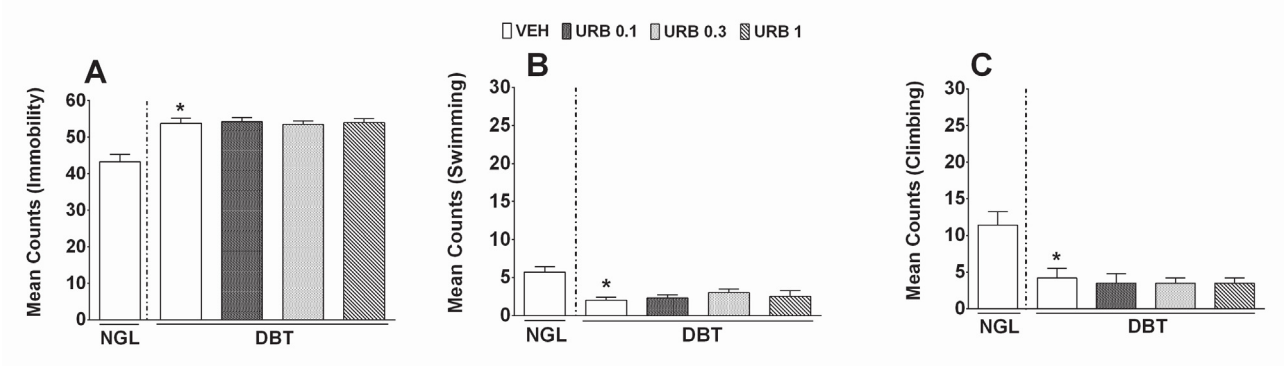

Fig 4: Effect of sub-chronic treatment with URB597 (0.1, 0.3, 1 mg/kg i.p.) or vehicle (VEH) on the frequency of immobility (figure 4A), swimming (figure 4B) and climbing (figure 4C) in NGL and DBT animals submitted to the mFST. Values were expressed as mean ± SEM (n=7-10). *p <0.05 when compared with vehicle-treated normoglycemic (NGL/VEH) animals and $^{\#}$p <0.05 when compared to vehicle-treated diabetic animals (DBT / VEH).

5.4.6 Effect of condition (normoglycemic-NGL or diabetic-DBT) and/or treatment (URB597; or vehicle-VEH) on glycaemia, weight gain and number of crossings evaluated in the open field test

As showed in Table 2, one-way ANOVA revealed that different groups submitted to the URB597 acute treatment had altered the glycemia [F(5,47)=271,2; p<0.05], weight gain [F(5,47)=25,56; p<0.05] and number of crossings [F(5,47)= 10,27;

p<0.05]. The same was observed when a subchronic treatment with URB597 was performed [glycemia: F(4,42)=49,93; p<0.05; weight gain: F(4,42)=17,98; p<0.05 and number of crossings: F(4,42)=4,695; p<0.05]. Newman-Keuls *post hoc* test showed that all DBT animals were different of NGL animals, *i.e.* presented hyperglycemia (p<0.05), reduced weight gain (p<0.05) and decrease in the number of crossings in the open field (p<0.05).

Condition/AcuteTreatment	**Glycaemia (mg/dL)**	**Weightgain (g)**	**No. of crossings**
NGL-VEH	104±1.5	116±3	50±1
NGL-URB597 (0.3 mg/Kg)	106±1	119±2.5	53±1
DBT-VEH	535±7*	50±3*	37±1*
DBT-URB597 (0.1 mg/Kg)	543±5*	49±2*	31±1*
DBT-URB597 (0.3 mg/Kg)	550±5*	55±2*	38±1*
DBT-URB597 (1 mg/Kg)	546±5*	52±1*	31±1*
Condition/Subchronictreatment	**Glycaemia (mg/dL)**	**Weightgain (g)**	**No. of crossings**
NGL-VEH	105±1	117±3	63±2
DBT-VEH	471±7*	49±2*	41±1*
DBT-URB597 (0.1 mg/Kg)	459±8*	46±2*	42±1*
DBT-URB597 (0.3 mg/Kg)	498±6*	49±2*	40±1*
DBT-URB597 (1 mg/Kg)	478±7*	48±2*	42±1*

Results are expressed as mean±SEM; n=7-10.*p≤0.05 when compared to NGL-VEH.

5.5 DISCUSSION

The main finding of this study is that, although both CBD and URB597 induced an antidepressant-like effect when administered acutely to normoglycemic (NGL) animals, these treatments were not able to induce this effect in diabetic (DBT) animals. Interestingly, even after sub-chronic treatment, URB597 did not induce an antidepressant-like effect in DBT animals. Conversely, this regimen of treatment with CBD induced a mild antidepressant-like effect in these rats. This work is, to our knowledge, the first to suggest that CBD has a favorable profile in a similar model predictive of antidepressant-like activity (3 injections; [40]) in DBT animals.

As clinically observed, DBT animals exhibit a more pronounced depressive-like behavior when compared to NGL ones [8,18,44–46] evidenced in this study by an increase in the immobility frequency and a reduction in the frequencies of climbing and swimming in the mFST [8,46]. As well demonstrated in the literature, these behaviors reflect dysregulations on the noradrenergic and serotonergic systems [8,44,46,47] which is strictly related to the neurobiology of depression [2,3,48–51].

Even if the behaviors exhibited by NGL animals in the mFST are analyzed separately, they are consistent with depressive-like behaviors, and therefore susceptible to modulation by drugs with antidepressant potential. In this study, the acute treatment with CBD (at a dose of 30 mg/Kg) induces an antidepressant-like effect in NGL animals, supported by the reduction of the frequency of immobility and an increase of swimming and climbing frequencies (Figure 1). This acute CBD antidepressant-like propriety has already been observed in both rats and mice [21,26]. Although it was not the aim of this study, the mechanisms by which the CBD exhibits this effect are multiple and complex and therefore, not fully understood. In this sense, it has been proposed that CBD may exert the antidepressant-like effect acting directly or indirectly on cannabinoid receptors CB1 and/or CB2 and also, although controversial, through a low affinity antagonistic action on both cannabinoid receptors [25,52–54]. CBD can also exert the antidepressant-like effect by increasing the endocannabinoid tone through the inhibition of fatty acid amide hydrolase (FAAH), leading to anandamide increase in the central nervous system [55]. Although there are these hypotheses, it seems to be the serotoninergic system most related to many biological effects of CBD. In

fact, the antidepressant-like effect of CBD seems to be dependent on 5-HT1A serotonergic receptors [26] and also due to increased levels of serotonin and glutamate in the prefrontal cortical area [56]. Interestingly, both antidepressant-like effect and enhanced cortical 5-HT/glutamate neurotransmission induced by CBD were prevented by blocking 5-HT1A receptor, reinforcing the involvement of this serotonergic receptor.

In contrast, in DBT animals the acute treatment with CBD (30 or even a higher dose of 60 mg/Kg) was not able to induce an antidepressant-like effect. Many hypotheses could be relevant to explain this lack of acute CBD effect. The DBT state is closely related to hyperglycemia-induced oxidative stress [8,45,57,58] decreased hippocampal cell proliferation [59], neuroinflammation [46,60], dysregulation of the Hypothalamus-Pituitary-Adrenal-axis [61,62], and also dysregulation of endocannabinoid system [8,35,63], which may account for the development of depression. A previous study from our group has already observed that the more pronounced depressive-like behaviors exhibited by DBT rats are significantly improved only after sub-chronic treatment with anandamide [8]. Interestingly, DBT animals also showed an increase in the activity of the monoamine oxidase enzyme and consequently an elevation in the 5-HT metabolism, leading to a low level of 5-HT in brain areas [44,46,49,64–66]. Considering the serotonergic system as a multifactorial target in the pathophysiology of depression associated with diabetes, a single CBD injection may not be sufficient to counterbalance the chronic consequences of the DBT state. Nonetheless, the sub-chronic treatment with CBD (only at a dose of 30 mg/Kg) was able to induce an antidepressant-like effect in DBT animals. This effect was not an expressive effect, but a discrete effect when compared with the depressive-like behavior exhibited by the NGL animals in the mFST. Curiously, the sub-chronic treatment with CBD at the highest dose (60 mg/kg) was not able to reverse the depressive-like behavior in DBT animals, in a typical U-curve pattern of cannabinoid-mediated effects. The effective sub-chronic dose of CBD (30 mg/kg i.p.) was able to reduce the frequency of immobility and increase the frequency of swimming and climbing in DBT animals (Figure 2), suggesting an improvement in the monoaminergic function.

The antidepressant-like effect of CBD does not seem to depend on the improvement of general health status or hyperglycemia since the sub-chronic treatment with CBD did not change these parameters (Table 1). This absence of effect may be due to the short time of treatment with the CBD. In that sense, it has been noticed that a more prolonged treatment with CBD has a protective effect on pancreatic cells, reducing the markers of inflammation in the microcirculation of pancreas from non-obese DBT mice [31] and the insulitis [30]. In addition, as already shown in previous studies, DBT animals present a reduction in locomotor activity (Tables 1 and 2; [8,67,68]) that was not altered by acute or sub-chronic treatment with CBD. This reduction in locomotor activity seems not to be interfering in the antidepressant-like effect of sub-chronic injection of CBD since this regimen of treatment induced not only a reduction of immobility frequency but also a significant increase on the swimming and climbing frequencies during the mFST.

Another set of experiments was designed to further explore the role of the endocannabinoid system in the maintenance of depressive-like status in DBT animals. Previous data from our group suggested that a dysregulation in this system can be related with the more pronounced depressive-like behavior observed in DBT animals [8]. In this study, it was observed that the sub-chronic treatment with anandamide (AEA) reverted the depressive-like behaviors in DBT rats, without affecting the hyperglycemia or weight gain. Moreover, AEA treatment also restored the elevated CB1 receptor expression and increased the levels of serotonin in the hippocampus of DBT rats. Similarly, Duarte and coworkers [69] had already observed an increase in CB1 receptor density in hippocampus of DBT rats, which was associated to an adaptive response in an attempt to counterbalance the reduced endocannabinoid system tone in these animals. Plenty of evidence have pointed out that the endocannabinoid system plays a regulatory role in several brain functions and seems to be a potential therapeutic target for the diabetes [70–73] or depression treatment [74–78]. A dysfunction of the endocannabinoid system, *i.e.* a reduction of the endocannabinoid tone has been associated with a homeostatic mechanism for inhibitory synapses such as the gabaergic ones [79], leading to different neuropsychological states [80,81]. Therefore, the effects of acute or sub-chronic treatment with the FAAH inhibitor

URB597 were tested. Our data showed that both acute and subchronic treatment with URB597 (0.1, 0.3 or 1 mg/Kg; i.p.) did not change the frequencies of immobility, climbing and swimming of DBT animals submitted to mFST (Figure 3 and 4). These doses and regimen of treatments were not able to influence the reduced weight gain, the hyperglycemia and the reduced locomotor activity of the DBT animals (Table 2). Nevertheless, the acute treatment with URB597 (0.3 mg/Kg, i.p.) showed an antidepressant-like effect in NGL animals submitted to mFST, evidenced by a significant decrease in the frequency of immobility and an increase in the frequencies of climbing and swimming (Figure 3), as already observed previously [42,43]. It is known that the URB597 increases the anandamide levels in the central nervous system, leading to an antidepressant-like effect [42,82,83] which is related to the modulation of serotonin and norepinephrine neurotransmission through CB1 receptor activation [42,84–86]. Corroborating the importance of the serotonergic system in the modulation of the depression and therefore in the effect of drugs with antidepressant profile, Bambico et al. observed that para-chlorophenylalanine, a serotonin synthesis inhibitor, prevented the URB597-mediated antidepressant-like effect evaluated in the FST. Moreover, a single URB597 administration was able to gradually increase the serotonin neuron firing rate in dorsal raphe nucleus, consistent with other previous findings [42,84,85,86]. Then, the absence of antidepressant-like effect after acute or subchronic treatment with URB597 in DBT animals (Figures 3 and 4) can be due to a dysregulation of both serotonergic and endocannabinoid systems observed in these animals [44,46,49,64–66]. Perhaps the reestablishment of neurotransmission is only achieved after an even longer treatment in DBT animals. In this sense, it has been demonstrated that only after a chronic treatment with URB597, rats exposed to moderate chronic stress exhibited a reduction of the depressive-like behaviors, an increase in the anandamide levels in some brain areas and also a normalization of the body weight gain [82].

Considering the importance of raising the levels of endocannabinoids in some encephalic areas related to the emotions to the mechanism of action of many antidepressant drugs (as evidenced by Smaga et al.[38]), it is plausible to hypothesize that the endocannabinoid system may serve as a target for drug design and discovery of new therapies for depression, especially depression

associated with diabetes. Even after the observation of the mild antidepressant-like effect of the sub-chronic treatment with CBD, but not with URB597, the data obtained in this study do not invalidate the importance of deepening the studies involving the endocannabinoid system particularly in DBT animals. A better understanding of the pathophysiological mechanisms that associate diabetes with depression will permit the proposition of more effective drugs, with fewer side effects and faster clinical response.

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5.7 CONFLICT OF INTEREST

JAC is co-inventor (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent “Fluorinated CBD compounds, compositions and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023” Def. US no. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to “develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson’s disease, and anxiety disorders.” JAC received travel support from and is medical advisor of BSPG-Pharm.

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6 CONSIDERAÇÕES FINAIS

Em conjunto, a depressão associada ao diabetes é uma comorbidade complexa na qual o mecanismo fisiopatológico ainda está longe de ser esclarecido. Parece envolver vários sistemas biológicos, como o endocanabinoide, serotoninérgico e noradrenérgico. Assim, nossos achados indicam que o efeito do tipo antidepressivo observado após o tratamento com anandamida em animais diabéticos pode estar relacionado a sua ação como um composto neuroprotetor associado a uma melhora funcional do sistema endocanabinoide com o consequente restabelecimento dos níveis de serotonina hipocampal e dos níveis de noradrenalina no córtex pré-frontal. Mais ainda, nossos achados indicam que o efeito do tipo antidepressivo observado após o tratamento com AEA envolve a ativação de receptor canabinoide do tipo CB1. Além disso, observamos que apesar do efeito antidepressivo discreto com o canabidiol, podemos sugerir que o sistema endocanabinoide sirva como alvo para o desenho de drogas e descoberta de novas terapias para a depressão, especialmente a depressão associada ao diabetes.

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