UNIVERSIDADE FEDERAL DO PARANÁ

YANE COSTA CHAVES

EFEITO DO CANABIDIOL SOBRE A CONSOLIDAÇÃO DA MEMORIA DE MEDO ESPECIFICA E GENERALIZADA, BEM COMO SOBRE A PERSISTENCIA DESSAS MEMORIAS: UMA ABORDAGEM PRECLINICA EM UM MODELO ANIMAL DE DIABETES *MELLITUS* TIPO-1.

CURITIBA

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Dissertação apresentada ao programa de Pós-graduação em farmacologia da Universidade Federal do Paraná como Requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Profª. Janaína Menezes Zanoveli

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ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRA EM FARMACOLOGIA

No dia nove de março de dois mil e vinte e dois às 09:00 horas, na sala PPG Farmacologia - auditório, Auditorio, foram instaladas as atividades pertinentes ao rito de defesa de dissertação da mestranda **YANE COSTA CHAVES**, intitulada**: Efeito do canabidiol sobre a consolidação da memória de medo específica e generalizada, bem como sobre a persistência dessas memórias: uma abordagem preclínica em um modelo animal de diabetes *mellitus* tipo-1.**, sob orientação da Profa. Dra. JANAÍNA MENEZES ZANOVELI. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: JANAÍNA MENEZES ZANOVELI (UNIVERSIDADE FEDERAL DO PARANÁ), SAMIA REGIANE LOURENCO JOCA (AARHUS UNIVERSITY), ROBERTO ANDREATINI (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestra está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, JANAÍNA MENEZES ZANOVELI, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 09 de Março de 2022.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **YANE COSTA CHAVES** intitulada: **Efeito do canabidiol sobre a consolidação da memória de medo específica e generalizada, bem como sobre a persistência dessas memórias: uma abordagem preclínica em um modelo animal de diabetes *mellitus* tipo-1.**, sob orientação da Profa. Dra. JANAÍNA MENEZES ZANOVELI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestra está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. Neste formato o texto apresenta uma revisão de literatura e objetivos do trabalho (em português), além do artigo científico do trabalho (em inglês) abordando os experimentos realizados, com resultados e discussão. Por fim, uma breve conclusão (em português). O artigo foi formatado conforme as normas propostas por periódicos de circulação internacional.

AGRADECIMENTOS

Em meio a maior concretude do postulado de Darwin quanto a seleção natural e ao maior esforço político e social para uma distopia, partilhei esse tempo e espaço com pessoas fundamentais para minha chegada até aqui.

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.”

Fernando Pessoa

RESUMO

Evidências indicam que muitos pacientes com diabetes *mellitus* tipo 1 (DM1) apresentam prejuízo no processamento das emoções, bem como da memória associada ao medo, demonstrado pela alta prevalência de transtornos relacionados ao estresse e/ou ansiedade. Com base na falta de tratamento eficaz dessas condições, particularmente nesses pacientes com DM1, o canabidiol (CBD), componente não psicotomimético da planta *Cannabis sativa*, tem-se destacado pelo seu potencial em melhorar diversas condições relacionadas com as emoções, bem como aspectos específicos associados ao diabetes *mellitus*. Assim, investigamos em animais com DM1 induzido experimentalmente se uma única injeção ou um tratamento mais prolongado (1 semana) com CBD (0, 10, 30 ou 60 mg/kg; ip) interferiria no processo de consolidação da memória de medo contextual (teste em contexto condicionado), bem como na sua generalização (teste em contexto neutro) e persistência (reavaliação dos animais 1 semana após). Além disso, os efeitos da injeção única de CBD sobre a consolidação da memória de medo também foram analisados através da expressão da proteína associada ao citoesqueleto regulada por atividade (Arc) no hipocampo dorsal (HD), uma importante área do cérebro relacionada à memória e processos emocionais. Também estudamos se esta injeção única com CBD interferiria em uma memória de curto prazo. A fim de avaliar se os efeitos eram exclusivamente associados ao processamento da memória de medo, após o tratamento prolongado com CBD também investigamos respostas comportamentais relacionadas com a ansiedade. Por fim, estudamos os efeitos dos tratamentos único ou contínuo com CBD em outros tipos de memórias não associadas ao medo, como memória de reconhecimento e localização de objetos, bem como memória espacial. Nossos dados demonstraram que uma única injeção de CBD foi capaz de prejudicar apenas a generalização da memória do medo, não sendo este efeito persistente quando animais foram reavaliados 1 semana após. O tratamento agudo com CBD não foi capaz de alterar a memória de medo contextual de curto prazo nesses animais com DM1 induzido em ambos os contextos. Porém, o tratamento agudo foi capaz de reduzir a expressão da Arc no HD, indicando uma redução no processamento exacerbado de consolidação dessa memória de medo nesses animais. Diferentemente do tratamento agudo, um tratamento mais prolongado com CBD prejudicou a persistência da memória do medo condicionado exacerbada e esse efeito pode estar relacionado ao

processo de memória de longo prazo. Além disso, não podemos descartar o envolvimento dos aspectos emocionais nesses processos, uma vez que esses animais tratados por mais 1 semana com CBD demonstraram um claro efeito ansiolítico. Nos testes de memória não associada ao medo (memórias de reconhecimento e localização de objetos e espacial), os animais com DM1 induzido apresentaram prejuízo no índice de discriminação e nem o tratamento agudo e o contínuo com CBD alteraram significativamente esses índices. Em conjunto, apesar da necessidade de mais estudos, nestas condições experimentais específicas, nossos dados indicam que o CBD atua de forma distinta nesses animais com DM1 induzido dependendo do tipo de memória envolvida e dos tipos de tratamentos, como a duração do tratamento e a dose empregada. Enquanto o tratamento com CBD foi capaz de induzir efeitos benéficos sobre o processamento da memória de medo ou sobre a ansiedade dos animais, os mesmos tratamentos não alteraram as memórias espacial e de reconhecimento/localização de objetos que estão prejudicadas.

Palavras-chave: estreptozotocina, canabidiol, memória de medo, diabetes, labirinto em cruz elevado.

ABSTRACT

Evidence indicates a higher prevalence of impaired fear memory processing in patients with type-1 diabetes mellitus (T1DM). Based on the lack of effective treatment of these conditions, it has been reported that cannabidiol (CBD), a non-psychotomimetic component of Cannabis sativa plant, presents a great potential to treat emotional aspects, in addition to improving several other pathological aspects associated with diabetes mellitus (DM). We investigated in induced T1DM animals whether a single injection or a more prolonged treatment (1 week) with CBD (0, 10, 30, or 60 mg/kg; i.p.) would interfere in the consolidation process of contextual fear memory, as well as in its generalization and persistence. In addition, the effects of a single CBD injection on fear memory consolidation were also analyzed through the expression of activity-regulated cytoskeletal-associated protein (Arc) in the dorsal hippocampus (DH), an important area of the brain related to memory and emotional processes. We also studied whether this single injection of CBD would interfere with short-term memory. In order to assess whether the effects were exclusively associated with fear memory processing, after prolonged CBD treatment we also investigated anxiety-related behavioral responses. Finally, we studied the effects of single or continuous treatments with CBD on other types of non-fear-associated memories, such as object recognition and location memory, as well as spatial memory. A single injection of CBD was able to impair only the generalization of the fear memory, not being this effect persistent. This single injection was not able to alter the short-term contextual fear memory in these induced-T1DM animals. However, it was able to reduce the expression of Arc in the DH, indicating a reduction in the exacerbated processing of consolidation of this fear memory in these animals. Unlike acute treatment, longer treatment with CBD impaired the persistence of exacerbated conditioned fear memory and this effect may be related to the long-term memory process. Furthermore, we cannot rule out the involvement of emotional aspects in these processes, since these animals treated for another 1 week with CBD demonstrated a clear anxiolytic-like effect. In the tests of memory not associated to fear (recognition and location of objects and spatial memories), the animals with induced T1DM showed impairment in the discrimination index and neither the acute nor the continuous treatment with CBD significantly altered these indices. On these specific experimental conditions, our data indicate that CBD acts differently in these

induced T1DM animals depending on the type of memory involved and the types of treatments, such as the duration of treatment and the dose used. While the CBD treatment was able to induce beneficial effects on the fear memory processing or on the anxiety response of the animals, the same treatments did not alter the spatial and object recognition/location memories that are impaired in these animals, showing a likely deficit in the separation pattern of these induced-T1DM animals.

Keywords: streptozotocin, cannabidiol, fear memory, diabetes, elevated plus maze.

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Arc – proteína associada ao citoesqueleto regulada por atividade

BDNF – fator neurotrófico derivado do cérebro

CBD – Canabidiol

CB1R – Receptor canabinóide tipo 1

CB2R – Receptor canabinóide tipo 2

DCV – doenças cardiovasculares

DM – Diabetes *mellitus*

DM1 – Diabetes *mellitus* tipo 1

DM2 – Diabetes *mellitus* tipo 2

FAAH – enzima amida hidrolase de ácidos graxos

HbA1c – hemoglobina glicada A1c

HD – Hipocampo dorsal

ISRSs – inibidores seletivos da recaptação da serotonina

LCE – Labirinto em cruz elevado

LTD – Long-term depression

LTP – Long-term potentiation

PA – Pressão arterial

STZ – Estreptozotocina

TEPT – Transtorno de estresse pós-traumático

TTOG – Teste de tolerância oral a glicose

SUMÁRIO

1. INTRODUÇÃO

1.1O diabetes *mellitus* e suas implicações

O diabetes *mellitus* é um distúrbio metabólico crônico caracterizado pela falta de produção de insulina ou pelo uso ineficaz da insulina pelo organismo, resultando em níveis elevados de glicose sanguínea (hiperglicemia). Quando não controlada corretamente, a hiperglicemia pode causar sérios danos a muitos órgãos do corpo, levando a complicações de saúde incapacitantes e com risco de vida, como doenças cardiovasculares (DCV), danos nos nervos (neuropatia), danos nos rins (nefropatia), amputação de membros inferiores e doenças oculares (afetando principalmente a retina), resultando em perda visual e até cegueira (IDF, 2021). Existem três tipos de diabetes *mellitus* mais comuns e prevalentes na população, o tipo 1, tipo 2 e gestacional. O diagnóstico é feito quando um ou mais dos critérios listados a seguir são encontrados: glicose plasmática em jejum ≥ 7.0 mmol/L (126 mg/dL); duas horas de glicose plasmática após 75 g de carga oral de glicose (Teste de tolerância oral a glicose – TTOG) ≥ 11.1 mmol/L (200 mg/dL); hemoglobina glicada A1c (HbA1c) ≥ 48 mmol/mol (equivalente a 6.5%); glicose plasmática aleatória na presença de sintomas de hiperglicemia ≥ 11.1 mmol/L (200 mg/dL) (IDF, 2021).

O diabetes *mellitus* tipo 1 (DM1) é causado por um processo autoimune no qual o sistema imunológico do corpo ataca as células beta pancreáticas produtoras de insulina. Como resultado, o corpo produz muito pouca ou nenhuma insulina. As causas desse processo destrutivo não são totalmente compreendidas, mas uma explicação provável é que a combinação de suscetibilidade genética (conferido por muitos genes) e um gatilho ambiental, como uma infecção viral, inicia a reação autoimune (IDF, 2021; *American Diabetes Association*, 2018). A condição pode se desenvolver em qualquer idade, embora o DM1 ocorra mais frequentemente em crianças e adultos jovens. O DM1 é uma das doenças crônicas mais comuns na infância. Os sintomas típicos incluem sede excessiva (polidipsia), micção frequente (poliúria), falta de energia ou fadiga, fome constante, perda de peso repentina, visão embaçada e cetoacidose diabética (acúmulo de cetonas no corpo). O quadro clínico clássico de polidipsia, poliúria e perda de peso podem, no entanto, não estar presentes,

acarretando diagnóstico tardio ou até mesmo perdido por completo (WHO, 2021; American Diabetes Association, 2018; PAHO, 2012).

No diabetes *mellitus* tipo 2 (DM2), a hiperglicemia é o resultado, inicialmente, da incapacidade das células do corpo de responder totalmente à insulina, uma condição denominada resistência à insulina. Com o início da resistência à insulina, o hormônio é menos eficaz e, ao longo do tempo, provoca um aumento na produção de insulina. Com o tempo, a produção inadequada de insulina pode se desenvolver como resultado da falha das células beta pancreáticas em acompanhar a demanda (WHO, 2021; *American Diabetes Association*, 2018; PAHO, 2012). O DM2 pode ter sintomas semelhantes aos do DM1, mas, em geral, os sintomas são muito menos dramáticos e a condição pode ser completamente assintomática. Se o diagnóstico for adiado por um tempo prolongado, complicações como deficiência visual, úlceras de membros inferiores mal cicatrizadas, doença cardíaca ou acidente vascular cerebral podem levar ao diagnóstico. O DM2 também é observado em crianças mais velhas e sua incidência está aumentando em alguns países, à medida que o sobrepeso e a obesidade infantil se tornam mais comuns. As causas do DM2 não são completamente compreendidas, mas há uma forte ligação com sobrepeso e obesidade, aumento da idade, etnia e histórico familiar. Assim como no DM1, acredita-se que os contribuintes para o risco de DM2 incluam gatilhos poligênicos e ambientais (IDF, 2021).

A tendência global de DM2, responsável pela maioria dos casos de diabetes *mellitus*, foi semelhante à do diabetes total. De 1990 a 2017, as taxas padronizadas por idade de DM2 aumentaram de 228,5 milhões (213,7-244,3) para 279,1 (256,6-304,3) para incidência, de 4.576,7 (4.238,6-4.941,9) para 5.722,1 (5.238,2-6.291,0) para prevalência. Em relação ao DM1 de 1990 a 2017, a taxa padronizada por idade aumentou ligeiramente de 5,1 (4,6-5,6) para 5,4 (4,9-6,0) para incidência e de 161,7 (146,1-180,7) para 164,8 (148,4–184,9) para prevalência, respectivamente (Lin et al., 2020). Importante salientar que embora o DM2 seja o mais prevalente na população (OMS, 2021; IDF, 2020), cerca de 90%, nas últimas duas décadas houve um grande aumento de casos de DM1 e consequentemente dos estudos pré-clínicos e clínicos (Ribeiro et al., 2020; de Souza et al., 2018; Mobasseri et al., 2020; Ikeda et al., 2015; Gomes et al., 2012; Maahs et al., 2010). Diferentemente do DM2, o DM1 não é evitável e também

não existem recomendações de políticas públicas e mudanças nos hábitos de vida visando a redução de sua prevalência, como ocorre com o DM2. Assim, mais do que avanços políticos e mudanças de comportamento, no cenário atual, o DM1 precisa de avanços na área médica para que seu impacto na vida do paciente e no mundo seja reduzido (OMS, 2021; *American Diabetes Association*, 2018; OPAS, 2012)

Importante ressaltar que em ambos as condições de diabetes *mellitus* é de extrema importância o controle rigoroso dos níveis de glicose no sangue, além do gerenciamento dos níveis de pressão arterial (PA) e colesterol no sangue. Importante avaliar o controle desses fatores de risco regularmente, pelo menos 1 vez ao ano. A triagem regular para o desenvolvimento de complicações precoces relacionadas ao diabetes *mellitus*, como doença renal, retinopatia, neuropatia, doença arterial periférica e ulceração do pé, permitirá tratamentos preventivos, quando disponíveis, para prevenir o desenvolvimento e progressão dessas complicações (IDF, 2021). Não menos importante, muitos pacientes com diabetes *mellitus* desenvolvem doenças mentais ou problemas psicológicos e sociais, que são subdiagnosticados nesses pacientes e que podem agravar ainda mais as comorbidades e o controle rigoroso e atento da doença (Doherty, 2015). Dessa forma, a falta deste controle mais rigoroso do diabetes *mellitus* tem consequências significativas para o indivíduo otimizando a progressão das comorbidades e aumentando as taxas de morbidade e mortalidade.

1.2 Diabetes *mellitus*, alterações emocionais e mnemônicas

O estudo de comorbidades psiquiátricas associadas ao diabetes *mellitus* é um grande desafio. Sabe-se que muitas dessas psicopatologias são subdiagnosticadas ou não tratadas de forma eficaz, o que causa um maior agravamento do diabetes *mellitus per se* podendo ainda facilitar o surgimento de outras comorbidades associadas ao diabetes *mellitus* e/ou o agravamentos dessas próprias psicopatologias associadas. Das psicopatologias estudadas tanto em estudos clínicos quanto pré-clínicos com seus respectivos modelos associados estão o transtorno depressivo maior, transtorno de ansiedade generalizada e transtornos relacionados com eventos estressantes que envolvam memórias traumáticas, como o transtorno de estresse pós-traumático (TEPT).

Interessante notar que as pesquisas apontam que o risco de pacientes com diabetes *mellitus* desenvolverem depressão é de 15 a 20% maior e 40 a 60% mostram um aumento nos sintomas relacionados à ansiedade quando comparados à população não diabética (Lustman et al., 1992; Gavard et al., 1993; Talbot et al., 2000; Anderson et al., 2001; Grigsby et al., 2002; Lin et al., 2008; Maia et al., 2014). Em contrapartida, estudos relataram que alguns pacientes depressivos apresentaram níveis de glicose em jejum elevada (Kahn et al., 2011) tolerância a glicose prejudicada (Hennings et al., 2010) e resistência a ação da insulina (Okamura et al., 2000) quando comparados a outros pacientes, indicando que a depressão também é um fator de risco para desencadear algum tipo de diabetes (Eaton et al., 1996; Golden et al., 2008). Portanto, tem sido proposta e discutida uma relação bidirecional entre diabetes e estas psicopatolgias (Golden et al., 2008; Downs and Faulkner, 2015; Prabhakar et al., 2015; Moulton et al., 2015; Petrak et al., 2015; Zanoveli et al., 2015), sendo ainda desconhecido os mecanismos fisiopatológicos que causam essa relação bidirecional.

Além disso, muitos estudos mostraram que a proporção de disfunção cognitiva concomitante com diabetes *mellitus* é significativamente maior em comparação com indivíduos normais da mesma idade (Shalimova et al., 2019; Zanoveli et al., 2015; McCrimmon et al., 2012; Sima, 2010; Kodl e Seaquist, 2008) e que pacientes diabéticos apresentavam um risco aumentado de demência e doença de Alzheimer (Xue et al., 2019; Bednarik et al., 2017; Mauras et al., 2015; Matsuzawa et al., 2012; Mijnhout et al., 2006). Em estudos pré-clínicos, o prejuízo de aprendizagem envolvendo memórias espacial, de localização e reconhecimento de objetos em animais com DM1 induzido pela injeção de estreptozotocina (STZ) já está bem descrito na literatura (Li et al., 2019; Bassani et al., 2018; Delkhosh-Kasmaie et al., 2018; de Senna et al., 2017; Ghasemi et al. al., 2016), bem como uma interrupção nos processos de neurogênese (Mishra et al., 2018; Sadeghi et al., 2018; Reichelt et al., 2022; Damphousse et al., 2021). Assim, esse prejuízo no aprendizado/memória pode ser consequência da hiperglicemia resultante e da falta de um controle rigoroso, conforme observado em estudos pré-clínicos envolvendo animais com DM1 induzida (Lin et al., 2018; Zhang et al., 2018; Sukhov et al., 2016), bem como as evidências clínicas que demonstram essa associação de comprometimento da memória (demência) com DM1 (Awad et al., 2017; Reaven et al., 1990).

Interessante que tem sido descrito também uma maior predisposição do TEPT nesses pacientes (Renna et al. 2016; Bystritsky et al. 2014). Na mesma direção, estudos pré-clínicos mostraram que a memória do medo é mais pronunciada em animais com DM1 induzida, e esta pode ou não estar associada ao aumento de respostas de ansiedade (Ribeiro et al., 2020; de Souza et al. 2018; Zhang et al. 2018; da Silva Dias et al. 2016; Renna et al. 2016; Gambeta et al. 2015; Ikeda et al. 2015; Bystritsky et al. 2014).

Uma revisão de Hayes e colaboradores (2012) mostrou que, estudos clínicos envolvendo modelos cognitivos contemporâneos de TEPT, um aumento no processamento de informações é alocado para detecção de ameaças e interpretação de estímulos neutros como ameaçadores, estreitando o foco de atenção em detrimento de outras operações cognitivas. Nesta mesma revisão, em diferentes paradigmas de estudo, parece haver um *trade-off* (quando uma característica não pode aumentar sem que a outra diminua (ou vice-versa)) no desempenho cognitivo em modelos associados a memória aversiva; embora o aprendizado e reativação do medo sejam aumentados nesta condição, essa característica ocorre às custas do processamento de outros tipos de informação. Além disso, o aprendizado de extinção e o aprendizado de pistas seguras geralmente são prejudicados, a memória para informações específicas e detalhadas é geralmente ruim e os pacientes com TEPT podem ser mais propensos a lembrar falsamente de novas informações.

De particular interesse, neste estudo investigaremos a associação do DM1 com a memória de medo condicionada exacerbada desses animais, bem como com a ansiedade (também exacerbada nesses animais) e outros tipos de memórias não associadas ao medo, como de localização e reconhecimentos de objetos e memória espacial.

1.3Generalização da memória e padrão de separação

Em estudos clínicos e pré-clínicos, a generalização da memória de medo está muito presente em testes de condicionamento do medo ao contexto e pode ser caracterizada pelo aumento da expressão do medo em resposta a um estímulo que tem semelhanças, mas não é idêntico a um estímulo ameaçador, manifestando-se como expressão inadequada e incontrolável de medo em ambientes neutros e seguros decorrente da discriminação prejudicada de

ambientes seguros de ambientes aversivos ou discernimento de ameaças improváveis daquelas que são altamente prováveis. Além disso, a perda dependente do tempo de detalhes episódicos de memórias traumáticas pode contribuir para sua generalização.

O padrão de separação, um processo dependente do hipocampo, é fundamental para a discriminação de estímulos; transforma experiências ou eventos semelhantes em representações não sobrepostas de memórias distintas. Como resultado, a interferência entre uma nova informação recebida e as informações armazenadas anteriormente é minimizada (Besnard e Sahay, 2015). Devido ao processo perceptivo do padrão de separação, uma pessoa pode fazer uma distinção entre estímulos ou experiências aparentemente semelhantes e foi proposto para desempenhar um papel crítico na discriminação de estímulos seguros que apresentam algumas semelhanças com estímulos ameaçadores (Kheirbek et al., 2012). Mais especificamente, o processo de padrão de separação armazena a informação de um contexto novo e seguro em uma representação distinta da memória ameaçadora original. Desta forma, um estímulo semelhante (mas seguro) pode ser discriminado de um estímulo de ameaça. Acredita-se que os processos de padrão de separação envolvam regiões de inibição de medo, incluindo o vmPFC, mas se ocorrer falhas nesse processo, o estímulo seguro ativa a representação da memória do estímulo de ameaça, resultando no recrutamento de regiões de processamento de ameaça (Lissek et al., 2013; Dymond et al., 2014). Portanto, déficits no processo de padrão de separação podem resultar em supergeneralização (Kheirbek et al., 2012; Dymond et al., 2014; Besnard e Sahay, 2015) podendo contribuir também para quadros de ansiedade, razão pela qual faz-se necessário o melhor entendimento da ligação entre o padrão de separação e a generalização do medo (McHugh et al., 2007; Sahay et al., 2011).

Um estudo de Lange e colaboradores (2017) com 46 participantes que foram submetidos a uma tarefa comportamental de padrão de separação e um paradigma de condicionamento de medo e generalização de neuroimagem mostrou uma associação entre menor desempenho de separação de padrões comportamentais e maior generalização nos escores de expectativa de choque, ou seja, o prejuízo no padrão de separação pode ser crítico na generalização

excessiva e, portanto, pode contribuir para a fisiopatologia dos transtornos de ansiedade e TEPT.

1.4O papel do hipocampo e da plasticidade sináptica na memória

O hipocampo é uma região chave no processo de discriminação de representações contextuais semelhantes para restringir uma generalização exacerbada do medo. Por exemplo, ratos com lesões hipocampais dorsais exibem níveis normais de congelamento no contexto de condicionamento, mas estes animais apresentam prejuízo na discriminação entre contextos semelhantes mas que não estão associados a um estimulo incondicionado de medo, como o choque nas patas (Maren et al, 1997; Frankland et al, 1998; Antoniadis e McDonald, 2000). A interferência genética da plasticidade sináptica nas sinapses da via perfurante do giro denteado do hipocampo prejudica a discriminação entre um contexto no qual os camundongos foram condicionados a um choque nas patas e um contexto neutro semelhante, mas não distinto (McHugh et al, 2007). Esses estudos comportamentais demonstram mecanismos dependentes do hipocampo para minimizar a interferência entre ameaças ambíguas (contexto neutro semelhante) e memórias armazenadas (contexto de treinamento).

Um estudo clínico mediu parâmetros fisiológicos (avaliação de risco e eletromiografia) em indivíduos saudáveis e indivíduos com TEPT à medida que foram mostradas uma série de imagens que se transformaram de um estímulo de perigo condicionado (círculo grande) associado a um choque de dedo em um círculo menor que não estava associado com o choque (estímulo de segurança condicionado). Nesta tarefa, indivíduos saudáveis exibiram um declínio acentuado nas respostas fisiológicas à medida que o estímulo de perigo condicionado foi transformado no estímulo seguro indicativo de discriminação eficiente de ameaças ambíguas. Embora o grupo de indivíduos com TEPT tenha apresentado maior sensibilização ao estímulo condicionado, eles também exibiram maior percepção de ameaça para as pistas intermediárias mais próximas em semelhança com a pista de perigo condicionada refletindo o aumento da interferência entre estímulos aversivos e neutros (Lissek e Grillon, 2012).

Tendo em vista a importância do hipocampo na memória associativa e no processamento do padrão de separação bem como na generalização da memória de medo, prejuízos nessa região do encéfalo podem ser cruciais para um desempenho prejudicado em tarefas que exigem suas conexões. Alguns estudos já demonstraram em modelos animais de DM1 induzidos experimentalmente com STZ que o hipocampo possui danos relacionados com aumento de citocinas pró-inflamatórias, de estresse oxidativo, além da simplificação da ramificação neuronal e a perda de espinhas dendríticas e de apoptose. Essas alterações tem sido associadas com prejuízos emocionais observados por meio de testes comportamentais como natação forçada (comportamento do tipo depressivo) e labirinto em cruz elevado (comportamento do tipo ansioso), além de prejuízos relacionados ou não ao medo, como aumento da expressão da memória aversiva, dificuldade em extinguir essa memória de medo condicionado, bem como com prejuízo cognitivo em testes de memória espacial (de Morais et al., 2014; 2016; Ribiro et al., 2020; da Silva Dias et al. 2016; Redivo et al. 2016; Gambeta et al., 2015; Wrighten et al. 2009; Revsin et al. 2005).

Em associação ao prejuízo em tarefas relacionadas a memória e aprendizagem, estudos com marcadores moleculares de fortalecimento de sinapses e que parecem ser essenciais para a formação de memória relacionada a plasticidade sináptica, também mostraram alteração na expressão de tais proteínas como proteína quinase II dependente de cálcio/calmodulina fosforilada (p-CaMKII), fator neurotrófico derivado do cérebro (BDNF) e proteína associada ao citoesqueleto regulado por atividade (Arc) (Zoladz et al., 2012).

A proteína Arc possui grande papel na regulação da dinâmica do citoesqueleto de actina subjacente à consolidação da potenciação de longo prazo (LTP) e na regulação da endocitose do receptor de glutamato do tipo AMPA subjacente à depressão de longo prazo (LTD) e plasticidade homeostática. Esta proteína tem um papel fundamental na consolidação de formas explícitas e implícitas de memória, com trabalhos recentes também associando a proteína Arc na adaptação ao estresse, considerando assim esta proteína como um “regulador mestre” de formas de plasticidade sináptica dependentes da síntese de proteínas. Assim, a análise da expressão dessa

proteína Arc tem sido bastante utilizada como um marcador de aprendizagem/memória associativa e dependente de novidade.

1.4 Tratamento farmacológico de psicopatologias associadas ao diabetes *mellitus* tipo 1

Os antidepressivos aliados a psicoterapia são os tratamentos de primeira escolha para psicopatologias associadas ao diabetes *mellitus*. Os antidepressivos inibidores seletivos da recaptação da serotonina (ISRSs) sertralina e paroxetina são os únicos medicamentos aprovados pelo FDA para o tratamento de TEPT. Embora os ISRSs sejam tipicamente a primeira classe de medicamentos usados no tratamento (Brady et al, 2000; Marshall, Beebe, Oldham & Zaninelli, 2001), exceções podem ocorrer para pacientes com base em suas histórias individuais de efeitos colaterais, resposta, comorbidades e problemas pessoais (APA, 2017). Além disso, já que para o tratamento de ansiedade e depressão associados ao DM1 são também prescritos drogas da classe dos antidepressivos, é importante ressaltar seus desafios quanto a efetividade deste tratamento. Por exemplo, sabe-se que esses fármacos requerem um tratamento prolongado para induzir uma melhora significativa no comportamento emocional dos pacientes (Fava e Davidson, 1996; Little, 2009) e mesmo assim muitos pacientes são resistentes aos tratamentos ou respondem de modo insatisfatório ao tratamento (Trivedi et al., 2006; Warden et al., 2007; Magni et al., 2013; para uma revisão ver Zanoveli et al., 2015). Mais ainda, os antidepressivos, independentemente da classe, deveriam ser cuidadosamente escolhidos uma vez que podem atuar influenciando diretamente o controle glicêmico ou mesmo interagindo com drogas hipoglicêmicas de pacientes diabéticos (Lustman et al., 1997; Gomez et al., 2001; Bhattacharjee et al., 2013; Bystritsky et a., 2014), além de induzir alteração no peso corporal (Gomez et al., 2001; Lustman e Clouse, 2005). Assim, torna-se evidente a necessidade de estudos que busquem descobrir novos tratamentos cujo efeito desejado apareça rapidamente após o início do tratamento, com menos efeitos colaterais

1.5 Canabidiol como alternativa terapêutica

O canabidiol (CBD) tem sido identificado como um possível agente terapêutico no tratamento de psicopatologias como Ansiedade, Depressão, esquizofrenia, TEPT, bem como da própria condição diabética por apresentar

perfil antioxidante, antiinflamatório e restaurador de processos endógenos desregulados. Conhecido como o composto não psicotomimético mais abundante presente na planta *Cannabis sativa,* o CBD é um composto que vem se destacando pelo amplo potencial terapêuticos, se mostrando também eficaz em outras condições patológicas, como epilepsia e dor crônica, dentre outras (Izzo et al., 2009; Réus et al., 2011; de Mello Schier et al., 2014). Mais interessante ainda são estudos mostrando que o CBD tem sido bem tolerado e sem apresentar efeitos adversos significativos quando administrado por tempo prolongado em pacientes (Horváth et al., 2012). Em relação especificamente ao diabetes *mellitus*, apesar de todo esse perfil terapêutico em potencial, os mecanismos moleculares envolvidos nos efeitos comportamentais induzidos pelo CBD permanecem pouco esclarecidos. Sabe-se que o mecanismo de ação também é amplo e complexo podendo atuar como agonista de receptor 5-HT_{1A}, inibidor da enzima amida hidrolase de ácidos graxos (FAAH), ocasionando o aumento de endocanabinóides, e também podem atuar direta ou indiretamente em receptores canabinóides CB1 e/ou CB2 (Zanelati et al., 2010; Parolaro et al., 2010; Toth et al., 2010; Horváth et al., 2012; Sartim et al., 2016).

Estudos mostram que o sistema endocanabinóide em animais com DM1 induzida por injeção de STZ parece estar desregulado. Por exemplo, em um estudo realizado neste modelo animal, foi observada uma densidade elevada do receptor canabinóide tipo 1 (CB1R) e também de sítios de ligação de CB1R no hipocampo (Duarte et al., 2007). Um estudo do nosso grupo utilizando animais STZ também demonstrou um aumento na expressão de CB1R no hipocampo (de Morais et al., 2016). Esses dados podem representar um mecanismo compensatório endógeno relacionado a um processo fisiopatológico.

Mais especificamente em relação aos efeitos do CBD associados ao DM1, objeto de interesse do presente estudo, existem poucos estudos. Weiss et al. (2006) demonstraram que o CBD é capaz de reduzir citocinas pró-inflamatórias e retardar a insulite destrutiva no plasma de camundongos diabéticos não obesos. Além disso, Mechoulam et al. (2007) evidenciaram ações neuroprotetoras e antioxidantes do CBD na neurodegeneração induzida por uma injeção unilateral de 6-hidroxidopamina no feixe prosencéfalo medial e em um modelo de isquemia cerebral, respectivamente. Curiosamente, foi relatado que o CBD reduziu a inflamação pancreática precoce no DM1 (Lehmann et al., 2016).

Mais recentemente, nosso grupo mostrou que o tratamento prolongado em animais STZ induziu uma redução na hiperglicemia juntamente com um aumento nos níveis plasmáticos de insulina, além da melhora em parâmetros comportamentais relacionados a depressão e ansiedade e modulação de monoaminas no hipocampo e córtex pré-frontal (Chaves et al., 2020).

No entanto, em relação à memória de medo, que foi demonstrada estar exacerbada nesses animais da STZ, além do prejuízo em extinguir essa memória (Ribeiro et al., 2020; de Souza et al., 2018; Ikeda et al., 2015), nenhum estudo investigou se o CBD exerceria efeito benéfico neste processamento de memória de medo prejudicado. Curiosamente, foi relatado que em animais normoglicêmicos o tratamento com CBD é capaz de prejudicar a generalização da memória contextual do medo. Os autores propõem que a supergeneralização do medo é um processo associado à consolidação da memória (Raymundi et al., 2019; Stern et al., 2017; Gazarini et al., 2015).

Para além da melhora sob a condição diabética e suas complicações associadas, o CBD possui grande potencial em melhorar o comportamento do tipo ansioso e depressivo, associados ou não ao DM1 em estudos pré-clínicos, além de prejudicar a consolidação da memória de medo em animais normoglicêmicos. Estudos que avaliaram outros tipos de memória também observaram um efeito benéfico do CBD em testes de reconhecimento de objetos e reconhecimento social (Coles et al., 2020; Osborn et al., 2017).

2. OBJETIVO

2.1. Objetivo Geral

O presente estudo tem como objetivo avaliar se o canabidiol interfere no processamento da consolidação de uma memória de medo contextual, bem como na generalização e persistência dessa memória em animais com o DM1 induzido experimentalmente. Mais ainda, se este processamento é dependente ou não da ansiedade. Por fim, estudaremos também um possível envolvimento do CBD na mediação de outros tipos de memórias, que estão prejudicadas nestes animais com DM1 induzido, tais como a de localização e reconhecimento de objetos, bem como memória espacial.

2.2. Objetivos específicos

Avaliar em animais com DM1 induzido pela injeção de STZ:

- Resposta de medo condicionado e, no dia seguinte, a generalização dessa resposta de medo; sendo esses dois testes repetidos após 1 semana, para avaliar a persistência dessa memória de medo;
- O efeito do tratamento agudo com CBD (0, 10, 30 ou 60 mg/kg, i.p.), imediatamente após o condicionamento, sobre a expressão do medo, generalização e persistência dessa memória;
- O efeito do tratamento agudo com CBD (0, 10, 30 ou 60 mg/kg, i.p.), imediatamente após o condicionamento, sobre a memória de curta duração;
- O efeito do tratamento agudo com CBD, imediatamente após o condicionamento, sobre a consolidação da memória de medo contextual através da análise da expressão da proteína que é um produto de um gene imediato necessário para a consolidação da memória, a proteína associada ao citoesqueleto regulada pela atividade (Arc). Esta proteína será avaliada em uma área encefálica importante no processamento da memória de medo, o HD;
- O efeito do tratamento sub-crônico com CBD, iniciando após o condicionamento até o término dos testes (apenas com CBD – dose efetiva) sobre a expressão do medo, generalização e persistência dessa memória;
- Os efeitos agudos e sub-crônico do CBD com a dose efetiva sobre o índice de discriminação nos testes de localização e reconhecimento de objetos, bem como na versão espacial do labirinto em Y.

TITLE PAGE

Cannabidiol distinctly regulates fear-related memory processing in animals with experimentally induced type-1 diabetes *mellitus*

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Introduction

The study of psychiatric comorbidities associated with diabetes *mellitus* (DM), the most prevalent chronic metabolic disease in the population, is a major challenge. It is known that many of these psychopathologies are underdiagnosed or not effectively treated, which causes a further aggravation of DM *per se* and may also facilitate the emergence of other comorbidities related to DM and/or the worsening of these associated psychopathologies (American Diabetes Association, 2018; Zanoveli et al., 2015; Maia et al., 2014; Roy et al., 2012; Golden et al., 2008; Grigsby et al., 2002)

Although type-2 DM is the most prevalent in the population (WHO, 2021; IDF, 2020), in the last two decades there has been a great increase in studies involving the type-1 diabetes *mellitus* (T1DM) that presents a prevalence of around 10% (Mobasseri et al., 2020; Gomes et al., 2012; Maahs et al., 2010). It is important to highlight that T1DM is not preventable, unlike type-2 DM for which there are recommendations for public policies and changes in lifestyle habits aimed at reducing its prevalence. Thus, more than political advances and changes in behavior, in the current scenario, T1DM needs advances in the medical field so that its impact on the patient's life and on the world is reduced (WHO, 2021; American Diabetes Association, 2018; PAHO, 2012). In a longitudinal study including 915 patients with T1D and 3590 children in the reference cohort, incidences of six chronic diseases (all malignant neoplasms, anemia, mental disorders, epilepsy, migraine, obstructive pulmonary disease, non-infectious enteritis and colitis, cardiovascular disease, and thyroid disease) were significantly higher in T1DM children (Farsani et al., 2015).

A large number of studies showed that the proportion of DM concurrent cognitive dysfunction was significantly higher compared to normal individuals of same age (Shalimova et al., 2019; Zanoveli et al., 2015; McCrimmon et al., 2012; Sima, 2010; Kodl and Seaquist, 2008). Since Ott et al. (1999) firstly revealed that diabetic patients exhibited an increased risk of dementia and Alzheimer's disease, a series of clinical studies have confirmed the association of these diseases (Xue et al., 2019; Bednarik et al., 2017; Mauras et al., 2015; Matsuzawa et al., 2012; Mijnhout et al., 2006). In the same direction, preclinical studies have shown an augment in the fear memory processing, which has been related in human beings to the posttraumatic stress disorder (PTSD) associated or not to anxiety (Ribeiro et al., 2020; de Souza et al. 2018; Zhang et al. 2018; da Silva Dias et al. 2016; Renna et al. 2016; Gambeta et al. 2015; Ikeda et al. 2015; Bystritsky et al. 2014).

Since we know that hippocampus is a key brain area involved in learning/memory and emotional processes, several preclinical studies reported an increase in inflammatory and oxidative stress processes in this area (Wang et al., 2021; Shallie et al., 2020; Wang et al., 2018; Amiri et al., 2017; Kaplan et al., 2017; Elahi et al., 2016; Muriach et al., 2014). Also, simplification of neuronal branching and loss of dendritic spines and apoptosis has been demonstrated in this brain area (Albazal et al., 2021; Sanna et al., 2018; Minaz et al., 2018; Gault and Holscher, 2018; Rom et al., 2018;

Zhang et al., 2018). In the same direction, clinical studies showed significant changes in the hippocampus of diabetic individuals presenting cognitive decline (Awad et al., 2004; Reaven et al., 1990), being these changes also linked to an increased predisposition to anxiety and stress related disorders, such as PTSD (Renna et al. 2016; Bystritsky et al. 2014). In this respect, preclinical studies also observed in animals with experimentally induced T1DM, an impairment in the fear memory processing demonstrated by an overconsolidation and generalization of this fear response (Ribeiro et al., 2020). Also, a more pronounced anxiety-like behavior along with damages in the hippocampus has been noted in these induced-T1DM animals (de Morais et al., 2014; 2016; da Silva Dias et al. 2016; Redivo et al. 2016; Gambeta et al., 2015; Wrighten et al. 2009; Revsin et al. 2005).

In recent years, the endocannabinoid system has been the subject of many studies due to the various therapeutic effects demonstrated by cannabidiol (CBD), the major compound from *Cannabis sp.,* that is devoid of psychotomimetic effects (Silote et al., 2019; Moreira and Guimarães, 2005; Mechoulam, 1970). It is already known that the endocannabinoid system in animals with T1DM induced through streptozotocin injection (STZ animals) seems to be dysregulated. For example, in a study conducted in this animal model, it was observed an elevated density of the cannabinoid type-1 receptor (CB1R) and also of CB1R binding sites in the hippocampus (Duarte et al., 2007). A study from our group using STZ animals also demonstrated an increase in the expression of CB1R in the hippocampus (de Morais et al., 2016). These data may represent an endogenous compensatory mechanism related to a pathophysiological process.

More specifically in relation to the CBD effects associated to the T1DM, object of interest of the present study, there are few studies. Weiss et al. (2006) demonstrated that CBD is able to reduce pro-inflammatory cytokines and to delay destructive insulitis on non-obese diabetic mice plasma. Also, Mechoulam et al. (2007) evidenced neuroprotective and anti-oxidative actions of CBD on neurodegeneration induced by a unilateral injection of 6-hydroxydopamine into the medial forebrain bundle and in a model of cerebral ischemia, respectively. Interestingly, it was reported that CBD reduced early pancreatic inflammation in T1DM (Lehmann et al., 2016). More recently, our group showed that prolonged treatment in STZ animals induced a reduction in the hyperglycemia along with an increase in the plasma insulin levels (Chaves et al., 2020).

However, regarding the fear memory, which has been demonstrated is exacerbated in these STZ animals (Ribeiro et al., 2020; de Souza et al., 2018; Ikeda et al., 2015), no study investigated whether CBD would exert beneficial effect on this impaired fear memory processing. Interestingly, it has been reported that in normoglycemic animals CBD treatment is able to impair the contextual fear memory generalization. The authors propose that the fear overgeneralization is a process associated with the consolidation of memory (Raymundi et al., 2019; Stern et al., 2017; Gazarini et al., 2015).

Considering the exposed above, and that no study investigated the effects of CBD on fear memory in animals with induced-T1DM, the present study aimed to investigate in STZ animals whether a single injection or a more prolonged treatment (1 week) with CBD would interfere in the consolidation process of contextual fear memory, as well as in its generalization and persistence. In addition, the effects of CBD on short-term memory, and on consolidation through the expression of the activity-regulated cytoskeleton-associated (Arc) protein in the dorsal hippocampus (DH) were evaluated. It is known that DH is a brain area involved in the consolidation of this type of memory (Jeffery, 2018; Buckley, 2005; Izquierdo & Medina, 1997). Finally, we will also study a possible involvement of the CBD in mediating other types of memories, which are impaired in these animals with induced-T1DM, such as object location and recognition tests, as well as spatial memory.

Methodology

Animals

A total of 150 male *Wistar* rats (180 - 200 gr, age 45 days) were supplied by the Central *vivarium* of the Biological Sciences Sector of the Federal University of Paraná (UFPR). The animals were randomly allocated in groups of four in plexiglass cages and had free access to water and food and were kept under a 12-hour light/dark cycle (7:00 am to 7:00 pm) and controlled temperature at 22±1°C. All experiments were conducted in accordance with the rules and legislation contained by the UFPR Animal Research Ethics Committee (CEUA number #1390) with the consistency of ethical principles of the National Council for Control of Animal Experimentation (CONCEA).

Drugs

Streptozotocin (STZ, 60 mg/kg, i.p., Santa Cruz Biotechnology Inc., USA), sodium citrate (Merck SA, Brazil) and cannabidiol (CBD; 10, 30, 60 mg/kg, i.p., 99.6% pure provided by BSPG-Pharm, Sandwich, United Kingdom). STZ was freshly dissolved in citrate buffer (10 mM, pH 4.5) whereas CBD was diluted in 2% Tween 80 and 98% saline. Control group received vehicle (VEH; 2% tween 80 and 98% saline). The doses were chosen based on previous works (Chaves et al. 2021; de Gregorio et al. 2019; de Morais et al. 2014, 2016, 2018; Stern et al., 2017; Jesus et al. 2019).

Induction of type-1 diabetes *mellitus* (T1DM)

The experimental T1DM was induced by a single administration of STZ in rats previously fasted 12 hours. Hyperglycemia was confirmed three days after STZ injection, by applying a small volume of peripheral blood collected from the animals' tail (5µl) on test tapes impregnated with glucose oxidase (Accu-Check ActiveTM, Roche) and performed again at the end of behavioral tests. Animals with blood glucose equal to or greater than 250 mg/dL were considered with experimentally induced-T1DM (STZ animals) and kept in the experimental groups. In parallel, we used as control group of the diabetic condition, the normoglycemic (NGL) animals which received only citrate buffer (10 mM, pH 4.5, equivalent volume) (Chaves et al. 2021; de Morais et al. 2014, 2016;).

Contextual fear conditioning (CFC) test

The apparatus used in the experiment consisted of a rectangular chamber (context A) with three steel sidewalls and a front and celling door made of plexiglass acrylic (26 × 31.5 × 21 cm; Insight, Ribeirão Preto, SP, Brazil). The bottom of the box consisted of small metal bars attached to circuit board and a shock generator to enable the delivery of controlled electrical footshocks as detailed subsequently. For the generalization test, to offer contextual cues as different as possible from context A, we used a second chamber (context B) that consisted of four sidewalls made of plexiglass transparent acrylic (30 × 30 × 30 cm) (Ribeiro et al. 2020). Between each animal, the chamber was

cleaned with a 20% alcohol solution. The CFC obeyed the following steps (the 1st day being the day 26 after the confirmation of the diabetic condition):

1st day (day 26)– familiarization: the animals were placed in context A to explore it for 3 minutes and returned to its home cage afterward.

2nd day (day 27) – CFC session: The context A becomes a conditioned stimulus (CS) with the presentation of the unconditioned aversive stimulus (US): The animals were placed in the context A, and after 30 s, they received 3 electrical footshocks (US; 0,8 mA, lasting 3 s), with 30 s of intertrial intervals. The animals remained in this chamber 30 s more before being returned to its home cage.

3rd day (day 28) – Test A1: The animals were placed into context A (CS) and remained there for 3 min without the presence of the US.

4th day (day 29) – Test B1(generalization test): The animals were placed into context B (a neutral context different from the context A) for 3 min.

10th day (day 35) – Test A2: After 7 days of the CFC, the animals were placed into context A and remained there for 3 min without the presence of the US to investigate the persistence of the conditioned fear memory.

11th day (day 36) – Test B2: The animals were placed into context B (a neutral context different from the context A) for 3 min to investigate the persistence of the fear memory generalization.

In all tests, the time the animal remained in freezing was quantified in seconds (s). The freezing behavior of each animal was used as an aversive conditioning index. An animal was considered in freezing when it presented a stereotyped position with complete immobility, except for breathing movements. The freezing time was expressed as percentage (%) of total session time. Freezing behavior was measured by a trained observer blind to the treatments.

Elevated plus-maze test (EPMT)

The potential anxiolytic-like effect of all treatments was assessed using the EPMT as described by Pellow et al. (1985). The apparatus was made of wood and was 50 cm from the floor, consisting of 4 arms, being 2 open and 2 closed and at the cross between the arms there was a central area of 10 cm2 under incandescent light (40 W-60 lux). Each animal was placed in the center of the apparatus facing a closed arm and the session had duration of 5 minutes. As anxiety index, we evaluated the open arm time and entries in these same arms in percentage (%): % of open arm entries (%OAE = 100 × open arm entries/total entries) and % of time spent on the open arms (%OAT = 100 × time spent on open arms/[time spent on open arms + time spent on closed arms]). As index of locomotor activity, the number of entries in the enclosed arms was quantified. Between each session, the elevated plus-maze was cleaned with a 20% alcohol solution.

Object location test (OLT) and object recognition test (ORT)

Both tests took place in a squared arena, 100 cm × 100 cm × 40 cm, made of wood and painted black. It was placed in a moderately lit room (30-40 lx). A video camera was positioned over the arena, and the animals' behavior was recorded for later evaluation. The first procedure consisted of habituation of the animals in the box. Each animal was placed in the empty apparatus for 5 min for free exploration. Twenty-four hours later, a novel habituation session of 5 min was performed. After a 1 h delay, on the training session, two identical objects were placed in the apparatus in a symmetrical position about 10 cm away from the wall. The animals were allowed to explore them freely for 5 min and were then returned to their home cages.

- In the OLT, after 1 h delay, during the test session, each rat was put back into the box with one of the objects displaced 15 cm away from the original position (novel position); animals were allowed to freely explore the objects for 3 min.
- In the ORT, a new training session was conducted with two other identical objects placed in a symmetrical position about 10 cm away from the wall, and the animals were allowed to explore them freely for 5 min and were then returned to their home cages. After 1 h interval, the rats were put back into the arena for the test session; but now with a familiar one (the sample) and a new one, and the animals were allowed to freely explore the objects for 3 min.

The objects were made of plastic, glass, or ceramic. To avoid an olfactory bias, before each trial, the objects were cleaned with a 20% ethanol solution. All objects and locations were balanced to reduce potential biases due to preferences for locations or objects. A rat could not displace the objects and the subjects were always placed into the box facing the same wall. Exploration was defined as sniffing at a distance of no more than 2 cm or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects was not considered as exploratory behavior.

The measures for both - the OLT and ORT - were the time spent by the rats while exploring each object during the test session. The time spent exploring the familiar and the new object (ORT) or displaced object (OLT) was represented by 'a' and 'b', respectively. The following variables were calculated: $e = a + b$, and $d = (b - a)/e$. The 'e' variable is a measure of the total exploration time of both objects during the test session. 'd' is considered a discrimination index between the new and the familiar objects/locations, and a relative measure of discrimination that corrects for the exploratory activity (e) (Mello-Carpes et al., 2013; Bassani et al., 2017).

Spatial version of Y maze

A symmetrical Y maze was constructed of wood and painted black. It consisted of three arms (50 cm length, 27 cm height, 12 cm width) and arranged at a 120° angle relative to each other. It consisted of a training session and a test session that were performed at a 1 h interval. In the training session, one arm was made inaccessible by a removable door that was placed in front of it. Each animal was placed in one of the

other arms (*i.e.*, “start arm”), which was randomized between groups. The animal was allowed to explore these two arms for 5 min and was then returned to its home cage. After a 1 h interval, the rat was returned to its corresponding start arm, but the blockade that prevented access to the third arm (*i.e.*, “novel arm”) was removed, thus providing access to all three arms. The rat was allowed to explore the three arms for 3 min. A video camera was positioned over the Y maze to record the animals’ behavior for later evaluation. An arm entry was considered only when both hind paws were placed completely inside an arm.

The spatial memory was assessed as the time spent on the novel arm, which had to be significantly greater than 33.3% of the total time in the maze (corrected for the latency to move from the start arm to another arm and the time spent in the center of the maze). The Y maze apparatus was cleaned between sessions with a 20% ethanol solution to prevent an olfactory bias (Mello-Carpes et al., 2013; Bassani et al., 2017).

Western blotting for analysis of Arc expression

The dorsal hippocampus (DH) was quickly removed and stored at −80ºC. The DH from a group of non-STZ animals was used to record the basal expression of the Arc protein. For protein extraction, the tissues were homogenized in 0.6 ml of solubilization buffer (10-mM EDTA, 100-mM Tris pH 7.5, 0.2% protease inhibitor cocktail [PROMEGA], and 1% Triton X-100). Insoluble material was removed by centrifugation (20 min, 10000rpm, 4 ºC). The supernatant protein concentration was determined colorimetrically (Bradford Protein Assay, Bio-Rad). Tissue extracts (500 µl) were denatured in boiling water for 5 min in Laemmli buffer containing 200 mM of DTT. Protein extracts were separated by SDSPAGE, transferred onto a nitrocellulose membrane (0.45 µm; BIORAD), blocked with basal solution (20-mM Tris pH 7.6, 137-mM NaCl, and 0.025% Tween® 20) containing 3% BSA (Sigma, USA) for 2h, and then incubated with monoclonal primary antibody anti-Arc 1:500 (Santa Cruz Biotechnology Cat# sc-17839, RRID:AB_626696) overnight and secondary antibody anti-mouse 1:5,000 (Santa Cruz Biotechnology Cat# sc-516102, RRID:AB_2687626) for 1 hr. For evaluation of protein loading, all membranes were stripped and reblotted with monoclonal primary anti-GAPDH antibody 1:500 (Santa Cruz Biotechnology Cat# sc-134237, RRID:AB_2212295). After incubation with the appropriate secondary antibody conjugated with Western ECL Substrate (Bio-Rad), membranes were developed by chemiluminescence. Quantitative analysis was performed by densitometry using Scion Image software (Scion Corporation, USA). The intensities were normalized to corresponding values for GAPDH expression (Arc value of the sample of interest *100/GAPDH value of the sample of interest) and expressed with relative value to the basal expression (normoglycemic group expression) (normalization value between Arc and GAPDH of the sample of interest *100/mean of normalization value of the NGL/VEH group) (Raymundi et al., 2019).

Experimental design

All experimental sessions were recorded using a Sony® action cam 4 K for posterior analysis. In all experiments, the body weight was evaluated. The BW was expressed as weight gain (WG) calculated by subtracting the body weight taken on the last day of experiment from that one taken on day 0. All behavioral tests were performing in the afternoon period (12 p.m. – 18 p.m.) and the animals were randomly allocated to the groups based on the treatments.

- Experiment 1: We aimed to evaluate in STZ animals the effects of a single injection of CBD on the consolidation of fear memory, its generalization and persistence.

For this, the following groups were performed: NGL animals treated with vehicle (VEH), STZ animals treated with VEH or CBD (10, 30 or 60 mg/kg, i.p.). The animals were treated in the fourth week after hyperglycemia confirmation (day 27) immediately after the CFC session and were submitted to the tests in the subsequent days, as already described above in detail.

- Experiment 2: We investigated the possible effects of the CBD given immediately after CFC on the short-term fear memory, according to Stern et al. (2017).

For that, the following groups were performed: NGL animals treated with VEH, STZ animals treated VEH or CBD (60 mg/kg, i.p.). The animals received a single injection of CBD immediately after the CFC session (day 27 after hyperglycemia confirmation and 3 hs later were submitted to the test A1 followed by test B1, 30 min later.

- Experiment 3: This experiment was designed to investigate whether a single injection of CBD administered immediately after CFC (day 27 after hyperglycemia confirmation) would interfere with the consolidation process of the fear memory by analyzing the expression of Arc protein in the DH.

Thus, the following groups were performed: NGL animals (conditioned) treated with VEH, STZ animals non-conditioned (naive), STZ animals (conditioned) treated with VEH or CBD (60 mg/kg, i.p.). The animals were euthanized by decapitation 120 min after treatment.

- Experiment 4: In this experiment we studied whether a sub-chronic treatment with CBD would induce a better effect on the increased fear response related to CFC, its generalization and persistence.

So, the first injection of CBD was administered immediately after CFC (day 27 after diabetic condition confirmation) and the subsequent injection with CBD were made on days 30 to 36 (with an interval of at least 7 h after tests A1 and B2 on days 35 and 36). All groups of animals - NGL animals treated with VEH, STZ animals treated with VEH and CBD (30 or 60 mg/kg, i.p.) - were submitted to the contextual fear conditioning protocol: familiarization (day 26), contextual fear conditioning (day 27), test A1 (day 28), test B1 (day 29), test A2 (day 35) and test B2 (day 36). Because an anxiolytic-like

effect could alter the freezing behavior in the chambers (contexts A and B), we also evaluated the effects of the drug on the EPM test (day 37).

- Experiment 5: - We intended to investigate the effects of acute and sub-chronic (7 days) injection of CBD on the localization, recognition and spatial memory.

Thus, all groups of animals - NGL animals treated with VEH, STZ animals treated with VEH or CBD (60 mg/kg, i.p.) - were submitted to the tests after 4 weeks of the hyperglycemia confirmation, *i.e.* we performed the OLT at day 27, the ORT at day 28 and spatial version of Y maze at day 29.

Statistical Analysis

Shapiro-Wilk normality test was applied to ensure that the data met the criteria for performing parametric tests. Once the criteria were accepted, the results were expressed as mean ± 95% confidence interval or standard error mean (SEM). Except for experiment 3, among the group of NGL and STZ animals treated with vehicle, Student's t-test was applied to assess whether there was a significant difference between these two groups. Among the different STZ groups treated with vehicle and drugs, one-way analysis of variance (ANOVA) was applied considering the treatments (different treatment groups) as a single independent factor. For experiment 3, a one-way ANOVA was performed including all groups, being considered 1 factor the different groups. When appropriate, Newman-Keuls test was used for *post-hoc* analysis. The differences were considered statistically significant when $p<0.05$. The data were analyzed using Graph Pad Prism Software 7.0.

Results

Experiment 1 – Effects of a single injection of CBD in STZ animals on the consolidation of fear memory, its generalization and persistence.

As shown in Fig.1A, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the freezing time during tests A1 [$t = 4.151$; df = 12; $p<0.05$], B1 [$t = 3.42$; df = 12; $p<0.05$], A2 [$t = 2.795$; df = 12; $p<0.05$] and B2 [$t = 3.209$; df=12; $p<0.05$], *i.e.* STZ animals presented an increased freezing time. When all groups of STZ animals were analyzed, one-way ANOVA showed that the treatment was able to change the freezing time during test B1 [$F(4, 30) = 15.95$; $p<0.05$] and test B2 [$F(4, 30) = 8.422$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed CBD (60 mg/kg) decreased (test B1), the freezing time in the neutral context ($p<0.05$). In Fig. 1B, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the discrimination index A1/B1 [$t = 7.678$; df = 12; $p<0.05$] and A2/B2 [$t = 5.054$; df = 12; $p<0.05$]. When all groups of STZ animals were analyzed, one-way ANOVA showed that the treatment was able to change the discrimination index during A1/B1 [$F(4,30) = 8.584$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed that CBD (60 mg/kg) increased the discrimination index ($p<0.05$).

1 Student's *t*-test showed a difference between NGL/VEH and STZ/VEH animals
2 when blood glucose [t = 12.8; df = 15; $p<0.05$] and weight gain [t = 2.142; df = 15;
3 $p<0.05$] were evaluated. When STZ groups were analyzed, one-way ANOVA revealed
4 that the treatment did not alter these parameters (see Table S1 - supplementary
5 material).

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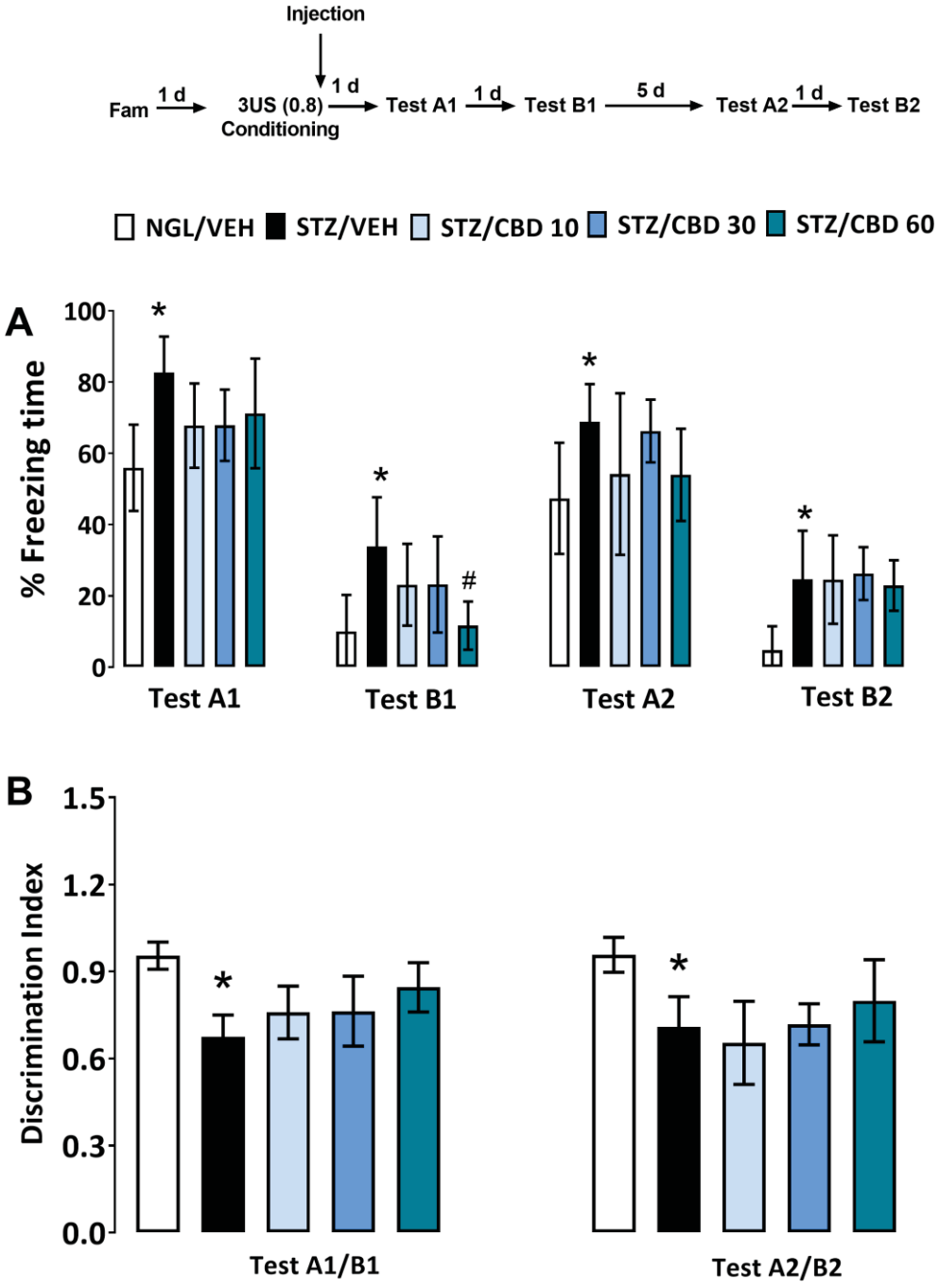

Fig. 1. Effect of a single injection with cannabidiol (CBD; 10, 30, 60 mg/kg, ip) or vehicle (VEH) immediately after the conditioning session – evaluation of the treatment on consolidation of the fear memory (Test A1), generalization (Test B1) and persistence (Test A2 and B2; panel A). Panel B represents the calculation of discrimination index. Values were expressed as mean ± 95% CI (n = 6-7). *$p<0.05$ when compared to NGL animals treated with VEH (NGL/VEH); $^{\#}p<0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

Experiment 2 - Effects of a single injection of CBD in STZ animals on short-term fear memory.

As shown in Fig. 2, one-way ANOVA showed a significant difference between the groups for tests A1 [F (2, 15) = 3.779; $p<0.05$] and B1 [F (2, 15) = 4.309; $p<0.05$]. Multiple comparisons showed that STZ/VEH presented an increased freezing time compared to NGL/VEH ($p<0.05$) and the treatment with CBD did not alter this parameter in STZ animals ($p>0.05$) neither in A1 nor B1.

One-way ANOVA showed a difference between the NGL/VEH and STZ/VEH animals when blood glucose [F (2.17) = 148.5; $p<0.05$] and weight gain [F (2.17) = 11.85; $p<0.05$] were evaluated. *Post-hoc* analysis showed a difference between all STZ groups with NGL/VEH ($p<0.05$) (see Table S1 - supplementary material).

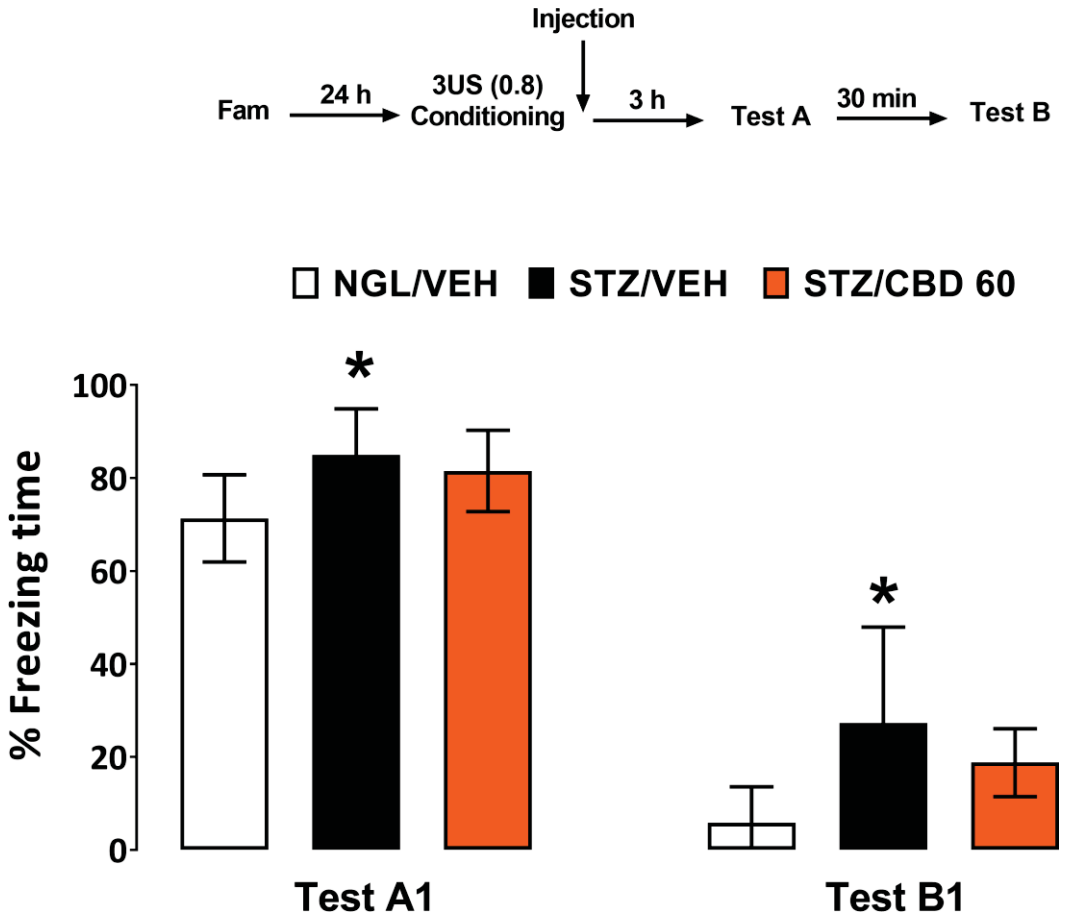

Fig. 2. Effect of a single injection with cannabidiol (CBD; 60 mg/kg, ip) or vehicle (VEH) on short-term fear memory – evaluation of the treatment on consolidation of the fear memory (Test A1) and its generalization (Test B1). Values were expressed as mean ± 95% CI (n = 5-6). *$p<0.05$ when compared to NGL animals treated with VEH (NGL/VEH); $^{\#}p<0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

Experiment 3 - Effects of a single injection of CBD on the expression of Arc protein in the dorsal hippocampus (DH) of STZ animals.

As can be seen in Fig. 3, one-way ANOVA showed difference between the groups [F (3, 23) = 22.58; p<0.05]. Newman-Keuls *post-hoc* test showed a decrease in the Arc expression into DH from all STZ animals (p<0.05).

Student's t-test showed a difference between NGL/VEH and STZ/VEH animals when blood glucose [t = 15.85; df = 13; p<0.05] and weight gain [t = 3.162; df = 12; p<0.05] were evaluated. When STZ groups were analyzed, one-way ANOVA revealed that the treatment significantly altered weight gain [F (2.21) = 5.403; p<0.05]. The Newman-Keuls *post-hoc* test showed that CBD (60 mg/kg) in STZ animals decreased the weight gain (p<0.05) (see Table S1 - supplementary material).

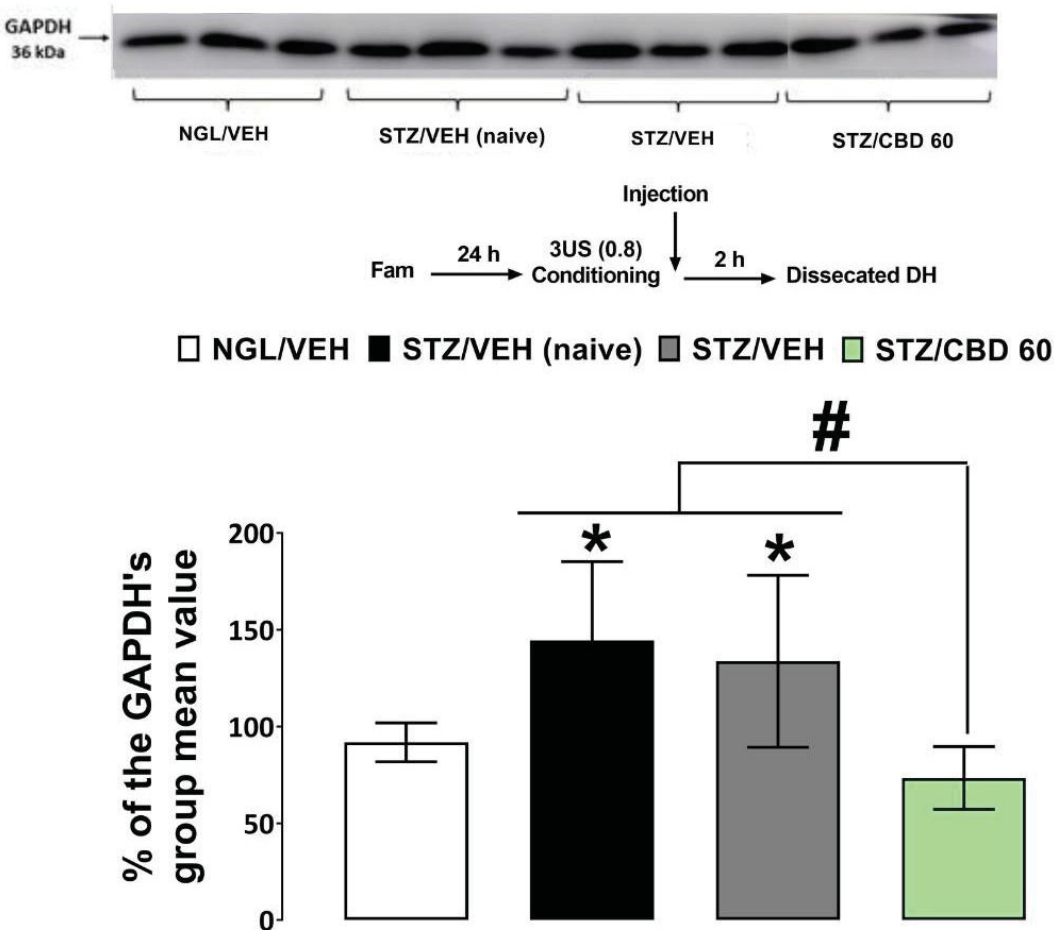

Fig. 3. Effect of a single injection with cannabidiol (CBD; 60 mg/kg, ip) or vehicle (VEH) immediately after the conditioning session on expression of Arc protein in dorsal hippocampus (DH). Values were expressed as mean ± 95% CI (n = 6-7). *p<0.05 when compared to NGL animals treated with VEH (NGL/VEH).

Experiment 4: Effects of sub-chronic treatment with CBD (30 or 60 mg/kg) in STZ animals on processes related to fear memory and anxiety-like behavior.

As shown in Fig. 4A, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the freezing time during tests A1 [$t = 3.644$; df = 11; $p<0.05$], B1 [$t = 10.75$; df = 11; $p<0.05$], A2 [$t = 6.26$; df = 11; $p<0.05$] and B2 [$t = 3.627$; df = 11; $p<0.05$], *i.e.* STZ/VEH animals spent more time in freezing behavior. When all groups of STZ animals were analyzed, one-way ANOVA showed that the treatment was able to change the freezing time during test B1 [$F(2,18) = 5.636$; $p<0.05$] and A2 [$F(2,18) = 5.753$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed that CBD (60 mg/kg) decreased the freezing time ($p<0.05$) in the neutral context (test B1) and in the conditioned context (test A2). In Fig. 4B, Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the discrimination index A1/B1 [$t = 10.23$; df = 11; $p<0.05$] and A2/B2 [$t = 4.416$; df = 11; $p<0.05$]. In that situation, STZ animals presented a reduction in this index, compared to NGL animals. When all groups of STZ animals were analyzed, one-way ANOVA showed that the treatment was able to change the discrimination index A1/B1 [$F(2,18) = 7.745$; $p<0.05$] and A2/B2 [$F(2,18) = 5.98$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed that STZ animals treated with CBD (60 mg/kg) increased the discrimination index in both cases, compared to STZ/VEH group ($p<0.05$).

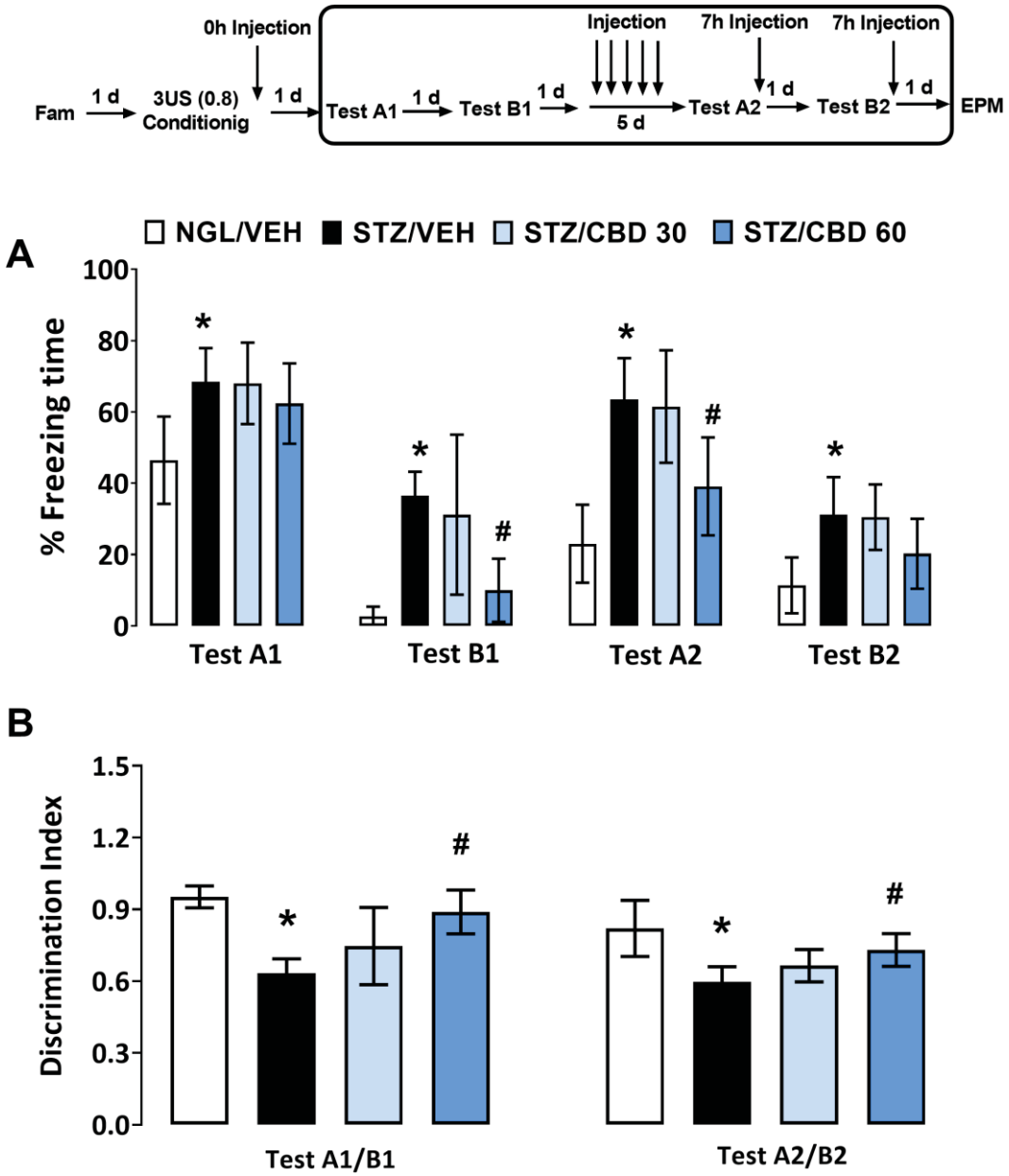

Fig.4. Effect of sub-chronic (7 days between A1/B1 and A2/B2) treatment with cannabidiol (CBD; 30, 60 mg/kg, ip), or vehicle (VEH) – evaluation of the treatment on consolidation of the fear memory (Test A1), generalization (Test B1) and persistence (Test A2 and B2; panel A). Panel B represents the calculation of discrimination index. Values were expressed as mean ± 95% CI (n = 6-7). *$p<0.05$ when compared to NGL animals treated with VEH (NGL/VEH); #$p<0.05$ when compared to STZ animals treated with VEH (STZ/VEH).

As observed in Fig. 5 (panels A, B and C), the Student's *t*-test showed a difference between the NGL/VEH and STZ/VEH animals in the time spent in the open arms [panel A: $t = 3.552$; df = 11; $p<0.05$], the number of entries in the open arms [Panel B: $t = 2.493$; df = 11; $p<0.05$] and the number of total entries into the arms [panel C: $t = 4.437$; df = 11; $p<0.05$]. When only the group of STZ animals were analyzed, the one-way ANOVA showed that the treatment was able to change the time spent in the open arms [panel A: $F(2,18) = 6.358$; $p<0.05$], the number of entries in the open arms [panel B: $F(2,18) = 4.86$; $p<0.05$] and the number of total entries into open and closed arms [panel C: $F(2,18) = 7.07$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed that CBD increased the time spent in the open arms ($p<0.05$) the number of entries in the open arms ($p<0.05$), indicative of an anxiolytic-like effect. Also, the treatment increased the number of total entries into open and closed arms, indicating an improvement on exploratory activity ($p<0.05$).

Student's *t*-test showed a difference between NGL/VEH and STZ/VEH animals when blood glucose [$t = 34.13$; df = 29; $p<0.05$] and weight gain [$t = 12.44$; df = 29; $p<0.05$] were evaluated. When STZ groups were analyzed, one-way ANOVA revealed that the treatment significantly altered weight gain [$F(2.31) = 3.453$; $p<0.05$]. The Newman-Keuls *post-hoc* test showed that CBD (60 mg/kg) when acutely, but not sub-chronically, injected in STZ animals decreased the weight gain ($p<0.05$) (see Table S1 - supplementary material).

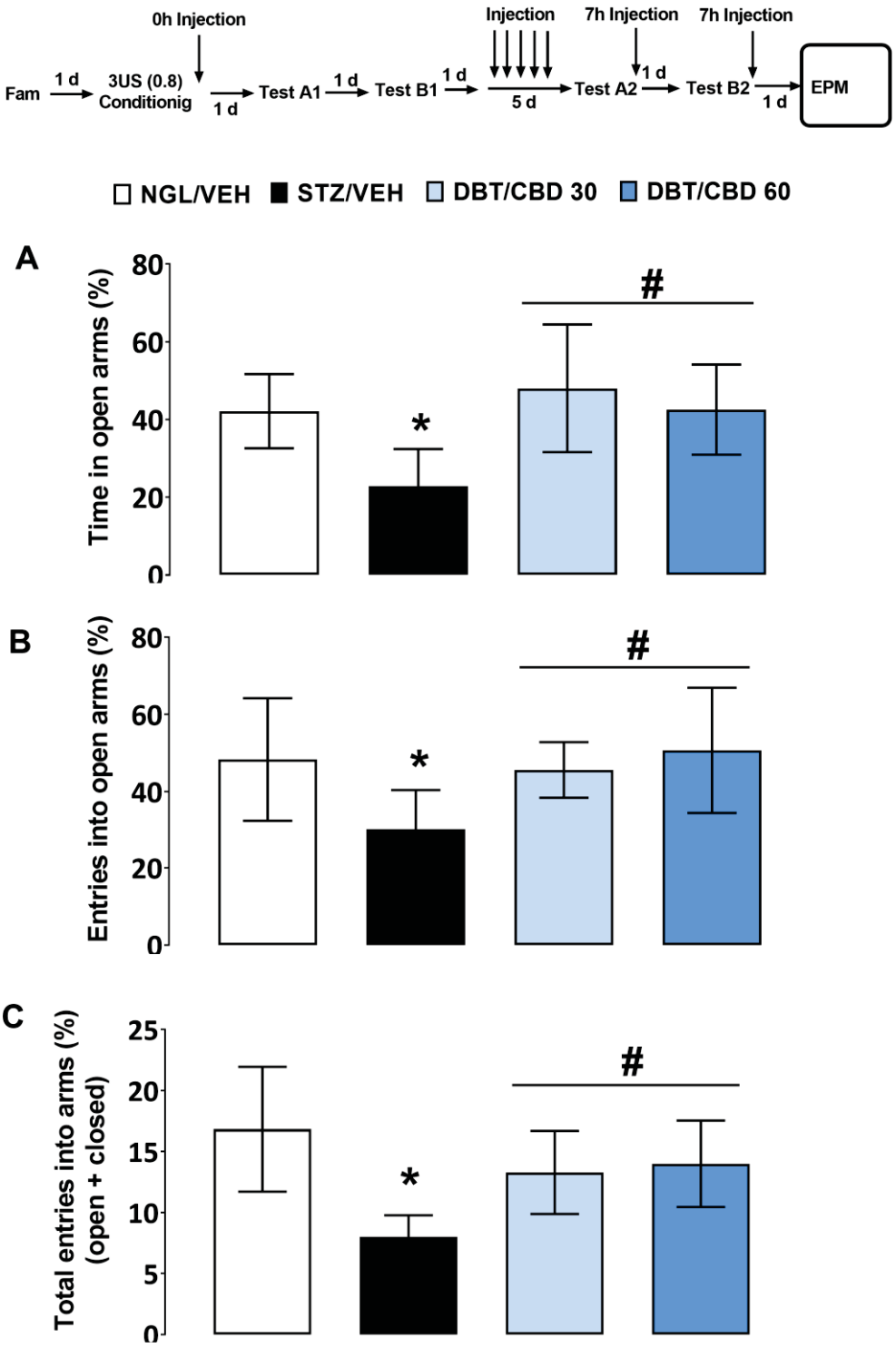

Fig. 5. Effect of sub-chronic (7 days between A1/B1 and A2/B2) treatment with cannabidiol (CBD; 30, 60 mg/kg, ip), or vehicle (VEH) on anxiety-like behavior – evaluation of time in the open arms (%, panel A), entries into open arms (%, panel B) and total entries into arms (%, open + closed, panel C) of STZ or NGL animals submitted to EPMT. Values were expressed as mean ± 95% CI (n = 6-7). * = $p<0.05$ when compared to NGL animals treated with VEH; # = $p<0.05$ when compared to STZ animals treated with VEH.

Experiment 5: Effects of single or sub-chronic treatment with CBD (30 or 60 mg/kg) in STZ animals on other types of memory.

Student's *t*-test showed a decrease in the discrimination index of STZ/VEH animals, compared to NGL/VEH [OLT – panel A: $t = 2.234$ df = 13; $p<0.05$; ORT – panel B: $t = 2.774$ df = 13; $p<0.05$; Y maze – panel C: $t = 5.116$ df = 13; $p<0.05$]. When STZ groups were analyzed, the one-way ANOVA revealed that the treatments did not alter these parameters.

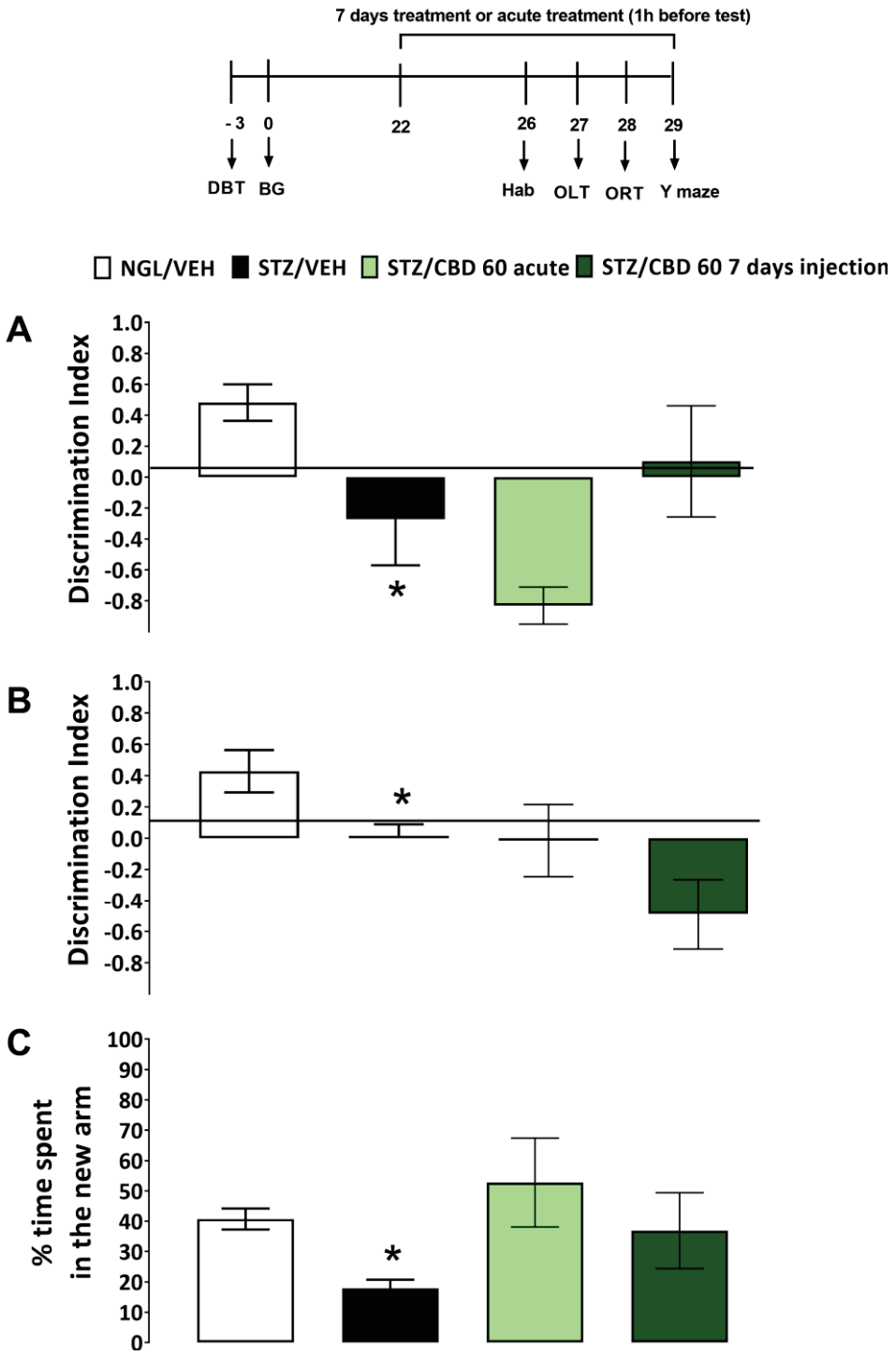

Fig. 6. Effect of a single injection or sub-chronic treatment with cannabidiol (CBD; 60 mg/kg, ip), or vehicle (VEH) – evaluation in the Object Localization Test (%, discrimination index; panel A), Object Recognition Test (%, discrimination index; panel B) and Y-maze (time spent in the new arm; panel C). Values were expressed as mean ± SEM (n = 5-8). * = $p<0.05$ when compared to NGL animals treated with VEH.

Discussion

This study was originally designed to examine in STZ animals the effects of CBD on the consolidation and generalization of contextual fear memory, as well as the persistence of effects.

Regarding to STZ animals, these animals when submitted to the CFC test presented a greater expression of freezing behavior compared to NGL animals in both contexts - the conditioned (test A1) and the neutral (test B1). This increase in the expression of conditioned fear and its generalization was persistent as it was maintained after the animals were re-tested 1 week later (tests A2 and B2, Fig. 1A). This result showing a more pronounced conditioned fear memory response associated with a generalization of this fear response corroborates previous evidence from our lab and others (Ribeiro et al., 2020; de Souza et al., 2018; Ikeda et al., 2015, 2021). Moreover, this fear memory seems to be quite resistant, once we previously demonstrate that these STZ animals presented this same pronounced freezing response even during and after an extinction training session (Ribeiro et al., 2020; Gambeta et al., 2015; de Souza et al., 2018). Important to highlight that our data demonstrated for the first time the persistence of these effects, which were maintained up to 7 days after the first tests (test A1 and B1). Thus, it is plausible to speculate that STZ animals present an overconsolidation of the fear memory. With that in mind, we designed a study to evaluate the expression of the Arc protein in DH.

It is known that the Arc protein is a product of an immediate early gene necessary for memory consolidation (Gallo et al., 2018; Korb and Finkbeiner, 2011; Plath et al., 2006). In addition, several studies show the relationship between increased Arc expression with activation/plasticity of brain areas associated with memory consolidation, like DH (Raymundi et al., 2019; Gouty-Colomer et al., 2016; Besnard et al., 2014; Lonergan et al., 2010), as well as its involvement in persistence of aversive memory, mainly through the second wave of its expression commonly reported after 12 h of memory reactivation (da Silva et al., 2020; Nakayama et al., 2015). Our results demonstrated an increase in the expression of the Arc in STZ animals (naïve and conditioned), compared to NGL animals (conditioned), indicating a facilitation on synaptic plasticity, according to our premises that these animals present an overconsolidation of the fear memory (Ribeiro et al., 2020). Although no studies have related fear memory, Arc expression, and the diabetic condition, Cai (2017) also observed a basal increase in Arc in an animal model of type 2 DM using STZ compared to normoglycemic animals. Interestingly, when we evaluated the discrimination index of these STZ animals, we observed an impairment in the processing of these memories (Fig. 1A and 1B).

The same impairment on discrimination index was observed when we evaluated memories not related to the fear throught the object location (OLT) and recognition (ORT) test, as well as a spatial memory (Y maze test) test. Thus, we observed that these STZ animals clearly demonstrated an impairment in these learnings, demonstrated by

the decreased discrimination index in these tests (Fig. 6). This learning impairment involving these memories in STZ animals is already well described in the literature (Li et al., 2019; Bassani et al., 2018; Delkhosh-Kasmaie et al., 2018; de Senna et al., 2017; Ghasemi et al., 2016), as well as a disruption in neurogenesis processes (Mishra et al., 2018; Sadeghi et al., 2018; Reichelt et al., 2022; Damphousse et al., 2021). Thus, this impairment in learning/memory may be a consequence of the resulting hyperglycemia, as observed in preclinical studies involving induced-T1DM animals (Lin et al., 2018; Zhang et al., 2018; Sukhov et al., 2016), as well as the clinical evidence that demonstrates this association of memory impairment (dementia) with T1DM (Awad et al., 2017; Reaven et al., 1990) and a greater predisposition to PTSD in these patients (Renna et al. 2016; Bystritsky et al. 2014). This also applies to those patients already diagnosed with T1DM and that are being treated with hypoglycemic drugs. It is known that a rigorous control in the management of the glycemic level by patients is quite difficult to achieve in practice.

In this way, it is evident the importance of deepening the studies in the search for a better understanding the complexity of the pathophysiology of the T1DM, as well as for a more effective treatment that can reverse or delaying in some way the emergence of these impairments in learning/memory processes. Studies conducted with non-diabetic animals have shown beneficial evidence of CBD in dealing with the impairment in fear memory formation and in facilitating or restoring learning (Stern et al., 2012, 2017; Uhernik et al., 2018). Thus, these studies demonstrated that CBD disrupts the consolidation and generalization of this type of memory, in addition to facilitating its extinction memory formation. Also, even when other types of memory were studied, CBD demonstrated beneficial effects, such as improvement on cognition when animals were submitted to the social preference or recognition tests and novel object recognition task (Assareh et al., 2020; Shallcross et al., 2019; Song et al., 2016; Cheng et al., 2014a, 2014b; Das et al., 2013; Bitencourt et al., 2008).

As to date, no study has investigated the effects of CBD on contextual fear memory in STZ animals, as well as on other types of memories; thus, in this study we investigated a likely therapeutic potential of CBD on memory processes in this animal model of T1DM. We initiated the studies by performing a single administration of CBD. Our data show that a single injection of CBD immediately after CFC session, which is within the fear memory consolidation window (Lamprecht & LeDoux, 2004; McGaugh, 2000) did not influence the consolidation of the contextual fear memory because any change was observed in the freezing time during tests A1 and A2. However, it was able to reverse, at the highest dose (60 mg/kg), the generalization of the conditioned fear response of STZ animals by reducing freezing time (test B1), not being this effect persistent (see Fig. 1A).

Regarding to CBD effects, it is interesting to note that that although the treatment did not change freezing time during tests A1 and neither its persistence (test A2, Fig. 1A), it was able to decrease the already increased Arc expression in the DH of these

STZ animals. In the same direction, Stern and coworkers (2017) showed that a single injection of CBD even in a lower dose of CBD (30 mg/kg) was effective in impairing the consolidation of aversive memory and its persistence by decreasing Arc expression at the DH. However, this study was conducted in non-diabetic animals. Curiously, we did not observe any change on discrimination index (Fig. 1B), which allow us to assume that a single injection of CBD did not exert a biologically significant effect on improving learning in that diabetic condition. What seems controversial is the association of Arc and improvement in the stabilization processes of other types of memory, such as spatial memory, but in our STZ animal model, these behavioral parameters were impaired (Sable et al., 2021; Gao et al., 2018; Morin et al., 2016; Li et al., 2016). The emotional aspect of the tasks cannot be ruled out, in addition to a possible trade-off in cognitive performance in models associated with aversive memory, more specifically between greater recruitment in information processing for threat detection and interpretation of neutral stimuli as threatening, narrowing the focus of attention to the detriment of other cognitive operations (Hayes et al., 2012).

Due to the lack of effect of a single injection of CBD in the highest dose on the persistence of fear memory expression in STZ animals, we decided to evaluate whether CBD would act preferentially through short-term memory. As our data did not show any change of single injection of CBD on short-term memory, *i.e.* did not reduce the increased freezing time of STZ animals, we conclude that CBD may be acting preferentially in long-lasting memory processes. However, further studies are needed to better understand the absence of a persistent effect of CBD when animals were re-exposed to conditioned and neutral contexts 7 days after CFC session. One hypothesis to explain the lack of effect is that the complexity of the disease would require a longer treatment.

Therefore, considering the absence of persistence of effect of CBD (after 1 week of the injection), we conducted experiments in which we performed a continued treatment with CBD in the week preceding the A2 and B2 tests. We confirmed the effect of a single injection showing again a decrease in the freezing time during test B1. In addition, the continued or sub-chronic treatment with CBD, differently of acute treatment, at the highest dose decreased the freezing time of STZ animals when re-exposed to the same context 7 days after conditioning (test A2, Fig. 4). Although there is a tendency of effect, we did not observe a significative statistically effect during B2 test, as observed previously when animals received only a single injection of CBD 1 week later. However, when we calculate the discrimination index, we can see more clearly that the sub-chronic treatment increased this parameter when the animals were re-exposed to the contexts, indicating that somehow CBD is acting in a beneficial way. Considering that an anxiolytic-like effect has already been demonstrated in STZ animals after sub-chronic treatment with CBD (Chaves et al., 2020; 2021), we cannot state that these effects are due to an exclusive improvement on contextual fear memory processing.

In order to confirm whether the effects induced by sub-chronic treatment with CBD are dependent or not of emotional processes, these same sub-chronically treated STZ animals were subjected to the EPM test, 24 hours after the B2 test (and at least 18 hours after the last CBD treatment). Our data showed that both dose - 30 and 60 mg/kg - were able to reverse the anxious-like behavior of these animals (Fig. 5). Thus, our data demonstrate that CBD acts by relieving emotional behavior such as anxiety. But it is noteworthy that the lowest dose of CBD was able to induce an anxiolytic-like effect, without inducing an improvement in fear memory processing in these animals. Thus, the anxiolytic-like effect induced by CBD is important, but it does not seem to be the only factor associated with the improvement induced by CBD on the discrimination between neutral and aversive contexts in these animals, because there was no improvement in these parameters with the lowest dose of CBD (30 mg/ kg).

In the last set of experiments, we aimed to investigate whether CBD would improve the discriminative behavior of STZ animals involving other types of memory not associated with fear. It is known that STZ animals present impairment in locating or recognizing objects, as well as impairment when submitted to a test involving spatial memory (Li et al., 2019; Delkhosh-Kasmaie et al., 2018; Senna et al., 2017; Ghasemi et al., 2016). So, our results confirm previous evidence from literature by demonstrating a decreased discrimination index when these STZ animals are exposed to the TLO, TRO and Y maze test (Fig. 6). Curiously, neither a single injection of CBD nor sub-chronic treatment was able to improve significantly this index, which indicates that in this specific experimental protocol involving STZ animals, CBD seems to preferentially act on memory related to emotional aspects, such as fear.

Based on the above and considering data already published, it is evident the difference between non-diabetic animals and STZ animals in the processing of emotions as a whole, as well as in the effect of drugs. However, it is important to emphasize that, unlike what was observed in the present study, a few reports involving preclinical studies have already demonstrated a significant improvement in the memory performance when it comes to non-diabetic individuals treated with CBD (Coles et al., 2020; Osborn et al., 2017). As this is the first study conducted in induced-diabetes' animals involving CBD treatment and its effects on memory performance, it is important that these data be confirmed.

It is important to note that CBD in the highest dose further reduced the weight gain in STZ groups under the sub-chronic or acute treatment in the experiments 4 and 5, respectively. It could be a side effect of this dose or random data and need to be better checked in future experiments. The modulation of weight gain through the endocannabinoid system, mainly through the antagonism of cannabinoid receptors, has already been widely reported even in the clinic, with the drug rimonabant, which has already been approved, but is currently no longer in circulation due to its induction of depression and anxiety (Le Foll et al., 2013). Despite the non-property of CBD to antagonize such receptors, studies focusing on inverted U-shaped dose-response curve

of this compound show that higher doses of CBD, when administered, can activate the transient receptor potential vanilloid subfamily 1 (TRPV1), the latter also participating in enhancing metabolism and energy expenditure, a possible explanation for this effect (Linares et al., 2019; Baskaran et al., 2017; Varghese et al., 2017; Zheng et al., 2017; Campos et al., 2012; Campos and Guimarães, 2009).

In conclusion, our findings demonstrate that CBD improves contextual fear memory performance, *i.e.*, impairs early and late aversive memory over expressive in STZ animals. For that, the choice of the type of treatment, if acute or sub-chronic, seems to be important. Once the treatment induces anxiolytic-like effect and these effects seems not be associated specifically with consolidation process involving DH, the emotional process cannot be discarded on these effects. Finally, further studies need to be conducted in animals with diabetes induced experimentally to better understand and discriminate the action of CBD on emotional behavior and memory associated or not with fear.

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Declaration of conflicting interests

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 has received travel support from and was medical advisor of SCBD Centre. JAC has received a grant from University Global Partnership Network (UGPN) – ''Global priorities in cannabinoid research excellence.'' JAC is member of the international advisory board of The Australian Centre for Cannabinoid Clinical and Research Excellence (ACRE), funded by

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Contributors

Janaina Menezes Zanoveli designed the study conception in collaboration with Cristina Aparecida Jark Stern. Material preparation and data collection was performed by Yane Costa Chaves, Ana Maria Raymundi and Ana Paula Farias Waltrick. Statistical analysis was performed by Janaina Menezes Zanoveli. The first draft of the manuscript was written by Yane Costa Chaves, while the following versions with English revision were made by Janaina Menezes Zanoveli. The CBD was donated by José Alexandre Crippa. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

Compliance with ethical standards

All experiments were conducted in accordance with the rules and legislation contained by the UFPR Animal Research Ethics Committee (CEUA number #1390), with the consistency of ethical principles of the National Council for Control of Animal Experimentation (CONCEA).

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Supplementary material

1 Effect of condition (NGL/VEH or STZ/VEH) and/or different treatments on
2 glycemia and weight gain.

Groups	Blood glucose (mg/dL)	Weight gain (g)
Experiment 1		
NGL/VEH	90.56 ± 2.351	100.3 ± 80.59
STZ/VEH	504.8 ± 97.44*	33.38 ± 38.07*
STZ/CBD 10	454.4 ± 118	45.25 ± 32.41
STZ/CBD 30	501.9 ± 82.99	8.625 ± 39.58
STZ/CBD 60	565.6 ± 45.6	31.71 ± 37.19
Experiment 2		
NGL/VEH	90.86 ± 2.193	114.7 ± 48.59
STZ/VEH	484.8 ± 75.58*	19.33 ± 28.83*
STZ/CBD 60	497.1 ± 46.94*	18.14 ± 44.32*
Experiment 4		
NGL/VEH	91.38 ± 2.504	112 ± 16.58
STZ/VEH	469.9 ± 67.85*	73.83 ± 28.53*
STZ/CBD 30	474.8 ± 148.5	67.56 ± 45.82
STZ/CBD 60	416.7 ± 172.2	10.33 ± 47.98#
Experiment 5		
NGL/VEH	91.25 ± 2.527	139.5 ± 24.68
STZ/VEH	568.4 ± 48.09*	23.79 ± 25.56*
STZ /CBD 60 (acute)	550.1 ± 62.56	-4.25 ± 31.3#
STZ/CBD 60 (sub-chronic)	554.6 ± 49.74	14.43 ± 14.6

Values are expressed as mean ± standard deviation; *$p < 0.05$ when compared to NGL/VEH group; #$p<0.05$ when compared to STZ animals treated with VEH (STZ/VEH) (n = 5-8/group).

3

4.**CONCLUSÃO**

Nossos achados demonstram que o CBD melhora o desempenho da memória associada ao medo, ou seja, diminui a expressão dessa memória aversiva precoce e tardia que está super expressiva em animais STZ. Todavia, estes efeitos benéficos parecem ser dependentes da escolha do tipo de tratamento, se agudo ou subcrônico, bem como a dose. Tendo em vista que o tratamento induz efeito ansiolítico, estes efeitos parecem não estar associados exclusivamente com uma melhora no processamento dessa memória de medo, como observado no processamento de consolidação envolvendo o HD. Por fim, mais estudos precisam ser realizados em animais com DM1 induzido experimentalmente para melhor compreender e discriminar a ação do CBD no comportamento emocional e na memória associada ou não ao medo.

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