U.PORTO

DOUTORAMENTO
CIÊNCIAS BIOMÉDICAS

FROM PERIPHERAL CHANGES TO CENTRAL MOLECULAR MODULATION: EXPLORING MULTIPLE PHYSIOLOGICAL PATHWAYS FOR ENHANCEMENT OF CONTEXTUAL FEAR MEMORY CONSOLIDATION

Ana Catarina Nogueira Cancela Oliveira

D

2024

From Peripheral Changes To Central Molecular Modulation: Exploring Multiple Physiological Pathways For Enhancement Of Contextual Fear Memory Consolidation

Ana Catarina Nogueira Cancela Oliveira

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

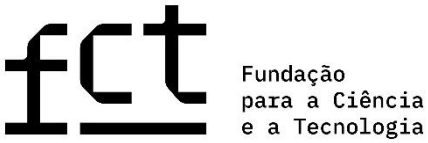
ANA CATARINA NOGUEIRA CANCELA OLIVEIRA

FROM PERIPHERAL CHANGES TO CENTRAL MOLECULAR MODULATION: EXPLORING MULTIPLE PHYSIOLOGICAL PATHWAYS FOR ENHANCEMENT OF CONTEXTUAL FEAR MEMORY CONSOLIDATION

Dissertation in fulfilment of the requirements for the PhD degree in Biomedical Sciences, submitted to ICBAS - School of Medicine and Biomedical Sciences, University of Porto

Supervisor - Prof. Doctor Mónica Moreira Rodrigues
Category - Assistant Professor
Affiliation - ICBAS - School of Medicine and Biomedical Sciences, University of Porto.

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, project UIDB/04308/2020 and UIDP/04308/2020).
The author was in receipt of PhD Studentships from FCT (POPH - QREN/FSE funding, SFRH/BD/138984/2018 and COVID/BD/152954/2022).



Ao João,

Aos meus pais,

À minha Irmã,

À minha família, principalmente à minha avó.

DECLARAÇÃO DE HONRA

Declaro que a presente tese é da minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

X Ana Oliveira

Ana Catarina Nogueira Cancela Oliveira

ACKNOWLEDGEMENTS

Ao longo deste percurso foram muitas as pessoas que, de alguma forma, me ajudaram e incentivaram a continuar, a melhorar, a aprender e a não desistir.

Gostaria de agradecer, em primeiro lugar, à Prof. Doutora Mónica Moreira Rodrigues, minha orientadora. O nosso percurso juntas começou em 2016, ano em que iniciei a realização da minha tese de mestrado no grupo de investigação da Prof. Doutora Mónica, ao qual dei continuidade neste doutoramento. Durante estes 7 anos a professora Mónica demonstrou sempre uma enorme disponibilidade e paciência para todas as alturas deste percurso. Transmitiu sempre o seu conhecimento científico ao mesmo tempo que me incentivava a querer mais e melhor. Foi uma inspiração que me fez crescer não só a nível académico como pessoal. Foi uma verdadeira mentora ao longo deste percurso e por isso não podia estar mais agradecida.

Um agradecimento muito especial às minhas colegas do Laboratório de Fisiologia Geral. Foram anos de partilhas, dúvidas, conquistas, mas também perdas que fizeram com que todas nós crescêssemos academicamente. Deste grupo de colegas um obrigada especial à Raquel Martinho, Rafaela Seixas e Márcia Azevedo, que se tornaram amigas e que me incentivaram sempre a continuar independentemente das dificuldades. Obrigada por acreditarem mais em mim do que eu própria.

Um obrigada à Engenheira Paula Serrão da MedInUP por toda a disponibilidade e ajuda para a realização deste projeto. Um obrigada à Sónia Soares e a Prof. Doutora. Elisabete Moura do Laboratório de Fisiologia Geral e ao Dr. António Carlos por toda a ajuda e disponibilidade.

Ao Professor Doutor Paulo Correia-de-Sá do Laboratório de Farmacologia do Departamento de Imuno-Fisiologia e Farmacologia do ICBAS. Por ser um profissional exímio e por acolher os membros do Laboratório de Fisiologia Geral do mesmo Departamento de forma a podermos partilhar as nossas dúvidas e discutir melhorias do nosso trabalho. Teve sempre disponibilidade para nos receber e teve sempre boas sugestões e as quais tentei sempre ter em consideração.

Obrigada a todos os colegas, colaboradores e professores do Laboratório de Farmacologia do Departamento de Imuno-Fisiologia e Farmacologia do ICBAS, que são tantos que se torna impossível nomear um a um. Tornaram este percurso mais leve.

Aos meus pais, à minha irmã, ao meu sobrinho e a toda a restante família. Em muitas situações, mesmo sem se aperceberem, foram o porto de abrigo, foram o escape que precisava para me abstrair dos problemas, dos desafios e das frustrações.

Ao João, se houve pessoa que nunca me deixou cair durante este percurso turbulento foi o João. Esteve sempre lá nos bons momentos, mas especialmente nos menos bons. Foram anos de muita partilha, muita entreajuda, muitas dúvidas, muitas confidências e muitas conquistas. Ajudou-me sempre a olhar para o lado mais positivo das situações menos boas e a aprender com elas. Incentivou-me a melhorar e a não desistir. Por tudo isto, o meu maior agradecimento é para ele.

Aos meus patudos, que estão sempre ao meu lado, que esperam que eu chegue a casa para me receberem todos os dias com a felicidade mais autêntica que existe e que nada pode substituir.

À minha avó.

TABLE OF CONTENTS

RESUMO

Memória pode ser definida como a capacidade de aprender e relembrar experiências passadas. Dentro dos diversos tipos de memórias, é geralmente aceite que um evento relacionado com emoções (como medo e stress) é melhor lembrado quando comparado com eventos neutros. Durante situações stressantes, mecanismos neuroendócrinos podem ser ativados, levando a uma resposta fisiológica ao stress. A ativação do eixo simpático-adrenal-medular (SAM) e hipotálamo-hipófise-adrenal (HPA) resulta na libertação de glucocorticóides e catecolaminas nomeadamente noradrenalina (NA) e adrenalina (Ad), para a circulação periférica.

A Ad parece ser essencial para memórias relacionadas com o stress, como é o caso da memória contextual do medo. No entanto, a influência do antagonista dos recetores β_2-adrenérgicos não foi explorada na memória contextual do medo, bem como o papel do Ad em memórias antigas. Portanto, o nosso objetivo foi avaliar se um antagonista dos recetores β_2-adrenérgicos é capaz de reduzir a memória contextual do medo fortalecida pela Ad e se a Ad contribui para o fortalecimento de memórias antigas.

No entanto, a Ad é uma hormona hidrofílica e pode não ser capaz de atravessar a barreira hematoencefálica. A Ad pode ativar os recetores β-adrenérgicos das células hepáticas, levando à liberação de glicose para a circulação periférica e a glicose pode ser um mediador para o fortalecimento da memória do medo contextual. Existe uma lacuna da literatura em separar completamente o efeito da Ad e da glicose no fortalecimento da memória contextual do medo. Portanto, utilizando um murganho que não produz Ad, pretendemos avaliar se a glicose por si só fortalece a memória contextual do medo e se existe uma sinergia entre Ad e glicose. A glicose atravessa a barreira hematoencefálica através de transportadores de glicose e pode servir como fonte de energia para a síntese e libertação de acetilcolina e desta forma participar na formação da memória contextual do medo. Portanto, outro objetivo é avaliar se a expressão dos recetores muscarínicos no hipocampo está alterada em murganhos deficientes em Ad e entender se o sistema colinérgico está envolvido no fortalecimento da memória contextual do medo induzido pela Ad periférica. Além disso, a glicemia aumentada pela ação da Ad pode induzir a libertação de insulina para a corrente sanguínea. Outro objetivo deste trabalho é elucidar o papel da insulina na memória contextual do medo usando murganhos com défice em Ad no procedimento do medo condicionamento.

Os nossos resultados indicam que a Ad fortalece as memorias contextuais do medo tanto a longo prazo como as antigas, que é revertido na presença de um antagonista do recetor β_2-adrenérgico (Manuscrito I – Capítulo 2). Além disso, o efeito de fortalecimento na

memória do medo contextual pela Ad é seguido por um aumento dos genes da família *Nr4a* no hipocampo (Manuscrito I – Capítulo 2). O que sugere alterações moleculares centrais induzidas por uma hormona periférica para justificar um aumento da memória contextual do medo. Além disso, a administração de glicose foi capaz de fortalecer a memória contextual do medo na ausência de Ad (murganhos deficientes em Ad) e também levou ao aumento da expressão de mRNA de *Nr4a3* e *Bdnf* no hipocampo (Manuscrito II – Capítulo 3). O aumento destes alvos moleculares pode ser importante para aumentar a força da memória contextual do medo observada após a administração de glicose. Além disso, doses sub-eficazes de glicose e Ad quando administradas simultaneamente também fortaleceram a memória contextual do medo (Manuscrito II – Capítulo 3). Portanto, a glicose pode ser uma parte importante da via periférica para a via central envolvida na formação da memória contextual do medo.

Murganhos deficientes em Ad apresentaram diminuição da memória contextual do medo em comparação com murganhos do tipo selvagem (WT), o que foi seguido por uma diminuição na expressão do recetor M_4 no hipocampo, e foi restaurada pela administração de Ad em murganhos deficientes em Ad (Manuscrito III – Capítulo 4). Estes resultados demonstram que a ausência de Ad leva à modificação dos padrões de expressão de recetor centrais, o que pode justificar a diminuição da memória contextual do medo. Além disso, a atropina, um antagonista do recetor muscarínico com efeitos centrais, reverte o aumento da memória do medo contextual induzida pela Ad, ao contrário da metilatropina, um antagonista do recetor muscarínico com ações periféricas (Manuscrito III – Capítulo 4). Isto pode ser concordante com o envolvimento da acetilcolina no fortalecimento da memória contextual do medo induzido pela Ad periférica. Finalmente, a insulina fortalece a memória mesmo na ausência de Ad (Manuscrito IV - Capítulo 5). Além disso, a administração de insulina em murganhos deficientes em Ad também leva à expressão de mRNA de *Bdnf* no hipocampo (Manuscrito IV – Capítulo 5), o que pode estar relacionado ao aumento da captação de glicose nos neurónios induzido pela insulina.

Em geral, este trabalho traz mais avanços nas possíveis vias fisiológicas pelas quais a Ad fortalece a memória contextual do medo. Foi possível concluir que a Ad através dos recetores β_2-adrenérgicos fortalece as memórias contextuais do medo a longo prazo e as memórias antigas, e a glicose pode ser um mediador da Ad no sistema nervoso central. O fortalecimento da memória contextual do medo pela Ad ou pela glucose é através do aumento da expressão da família de genes *Nr4a* no hipocampo. Além disso, tanto a glicose como a insulina parecem fortalecer a memória contextual do medo através do

aumento da expressão de *Bdnf* no hipocampo, o que pode estar relacionado ao aumento da captação de glicose no sistema nervoso central. Também foi possível concluir que a ausência de Ad, além de reduzir as memórias contextuais de medo, também leva a modificações moleculares centrais que podem justificar o comprometimento da memória, como o observado na expressão dos recetores muscarínicos.

ABSTRACT

Memory can be defined as the capacity to learn and recall past experiences. Among the diverse types of memories, it is generally agreed that an event related to emotions (like fear and stress) is better remembered when compared to neutral ones. During stressful situations, neuroendocrinal mechanisms may be activated, leading to a physiological response to stress. The activation of the sympathetic-adreno-medullar (SAM) and hypothalamus-pituitary-adrenal (HPA) axis results in glucocorticoids and catecholamine release, namely noradrenaline (NA), and adrenaline (Ad), to the peripheral circulation.

Ad appears to be essential for stress-related memories, such as contextual fear memory. Nevertheless, the influence of β_2-adrenergic receptor antagonist was not explored in contextual fear memory, as well as the role of Ad in old memories. Therefore, our aim was to evaluate if a β_2-adrenergic receptor antagonist is capable of reducing contextual fear memory strengthened by Ad and if Ad is capable of strengthening old memories.

However, Ad is a hydrophilic hormone and may not be capable of crossing the blood-brain barrier. Ad may activate β-adrenergic receptors in hepatic cells resulting in glucose release to the peripheral circulation and glucose may be a mediator for contextual fear memory strengthening. A literature caveat exists in completely detach the effect of Ad and glucose in the strengthening of contextual fear memory. Therefore, using an Ad-deficient mouse we aimed to evaluate if glucose alone strengthens contextual fear memory and if there is a synergic interaction between Ad and glucose. Glucose crosses the brain's blood barrier through glucose transporters and may serve as an energy source for acetylcholine synthesis and release, and then participate in contextual fear memory formation. Therefore, another purpose is to evaluate if hippocampal muscarinic receptor expression is altered in Ad-deficient mice and understand if the cholinergic system is involved in the strengthening of contextual fear memory by peripheral Ad. In addition, the blood glucose increased by Ad action may induce insulin release to the bloodstream. Another aim of this work is to elucidate the role of insulin on contextual fear memory using Ad-deficient mice in the fear conditioning procedure.

Our results indicate that Ad strengthen contextual fear long-term and old memories and those are reverted in the presence of a β_2-adrenergic receptor antagonist (Manuscript I - Chapter 2). In addition, Ad contextual fear memory strengthening effect is followed by an increase of *Nr4a* family genes in the hippocampus (Manuscript I - Chapter 2). This suggests central molecular changes induced by a peripheral hormone to justify contextual fear memory strength. Also, glucose administration was capable of strengthening contextual fear memory in Ad absence (Ad-deficient mice) and also led to increased

hippocampus *Nr4a3* and *Bdnf* mRNA expression (Manuscript II - Chapter 3). The increase of these molecular targets may be critical for contextual fear memory strengthening observed after glucose administration. In addition, the joint administration of subeffective doses of glucose and Ad also strengthen contextual fear memory (Manuscript II - Chapter 3). Therefore, glucose may be an important part of the peripheral to central pathway involved in contextual fear memory formation.

Ad-deficient mice presented decreased contextual fear memory compared to wild-type (WT) mice, which was followed by a decrease in hippocampus M_4 receptor expression, which was restored followed by Ad administration in Ad-deficient mice (Manuscript III - Chapter 4). These results demonstrate that Ad absence leads to modification of central receptor expression patterns which may justify contextual fear memory decrease. In addition, atropine, a muscarinic receptor antagonist with central effects, reverts contextual fear memory enhancement by Ad contrary to methylatropine, a peripheral muscarinic receptor antagonist (Manuscript III - Chapter 4). This may be consistent with the acetylcholine involvement in contextual fear memory strengthened by peripheral Ad. Finally, insulin strengthens memory even in Ad absence (Manuscript IV - Chapter 5). Furthermore, insulin administration in Ad-deficient mice also leads to hippocampus *Bdnf* mRNA expression (Manuscript IV - Chapter 5), which could be related to increased blood glucose uptake in neurons induced by insulin.

In general, this work brings further advances in Ad pathways to contextual fear memory strengthening. It was possible to conclude that Ad strengthens both long-term and old contextual fear memories through β_2-adrenergic receptors, and glucose may be a mediator of the hormone in the central nervous system. The contextual fear memory strengthening by Ad or glucose is throughout increased hippocampal *Nr4a* family gene expression. In addition, both glucose and insulin appear to strengthen contextual fear memory through increased hippocampal *Bdnf* expression, which could be related to increased glucose uptake in the central nervous system. It was also possible to conclude that Ad absence, beyond reducing contextual fear memories, also leads to central molecular modifications that may justify memory decrease, such as observed in muscarinic receptor expression.

ABREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
ACTH	Adrenocorticotropic hormone
Ad	Adrenaline
ANS	Autonomic nervous system
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CREB	cAMP-response element binding protein
CRF	Corticotrophin-releasing factor
CRH	Corticotropin-releasing hormone
DA	Dopamine
DBH	Dopamine β-hydroxylase
DBH-KO	Dopamine β-hydroxylase-knockout
ED	Electrochemical detection
GLUT	Glucose transporter
HPA	Hypothalamus-pituitary-adrenal
HPLC	High-performance liquid chromatography
IEG	Immediate-early genes
KO	Knockout
LC	Locus coeruleus
L-DOPA	3,4-dihydroxyphenylalanine
LTM	Long-term memory
min	Minutes
NA	Noradrenaline
NTS	Nucleus tractus solitarius
PFC	Prefrontal cortex
PKA	Protein kinase A
Pnmt	Phenylethanolamine-*N*-methyltransferase
PTSD	Post-traumatic stress disorder
SAM	Sympathetic-adreno-medullar
SAMe	S-adenosylmethionine
SNS	Sympathetic nervous system
STM	Short-term memory

TH	Tyrosine hydroxylase
WT	Wild type

CHAPTER 1 - INTRODUCTION

1.1 Memory

The concept of memory is difficult and slippery to define, is not defined by a single system and includes many brain regions and processes. In a simple way, it is the process of reproducing or recalling what has been previously learned and retained (Squire, 2009). It relates to emotions, physical sensations, and/or the environment where it was created. This ability to retrieve the learned and retained information is crucial for animal and human survival, without memory it would not be possible to learn, develop and evolve (Ortega-de San Luis and Ryan, 2022). However, such an important feature of our evolution is not static and is always being modified and reconstructed every time it is recalled. All its complexity, makes memory difficult to study, having become one of the biggest challenges in the neuroscience field. Although learning and memory are two different concepts, they are closely related. Thus, to overcome this challenge, the scientific community makes use of different learning paradigms to study the processes of memory formation (Lieberman, 2011).

1.2 Memories: formation and types

1.2.1 Processes of memory formation and modifications

The learning and memory formation processes have been discussed for several years, leading to several theories. The most consensual theory nowadays is the consolidation process, which states that a temporary and labile memory is changed into a stable and long-lasting memory upon undergoing the consolidation process (Lechner, et al., 1999, Müller and Pilzecker, 1900). Currently, it is accepted that memory formation involves four main steps: encoding, consolidation, storage, and recall/retrieving (Bisaz, et al., 2014, Ortega-de San Luis and Ryan, 2022) (summarized in Figure 1). Encoding refers to the learning or acquisition of perceived information, consolidation turns the information stable for a long period, storage is used to describe the preservation of information over time, and retrieving is the ability to access the information whenever it is needed, allowing its reactivation (McGaugh, 2000, Squire, et al., 2015).

A recent memory is volatile and sensitive to disruption; it is termed short-term memory (STM) since it can last up to seconds to a few minutes (Cowan and AuBuchon, 2008, Gibbs and Ng, 1976) and is thought to be dependent solely on the sodium pump (Gibbs and Ng, 1977). STM maintenance over time is dependent on diverse features, such as stimulus strength, the individual's attention and motivation, and the presence of some drugs or molecules (Bisaz, et al., 2014). After that consolidation takes place, and STM

may be altered to form a long-term memory (LTM). During the consolidation process new gene expression, RNA transcription, and protein synthesis take place and ultimately lead to structural and functional modifications that may occur over days, weeks, months, or a lifetime (Alberini, 2009, Izquierdo, et al., 2002). This cellular or molecular consolidation denotes the beginning of memory storage. Studies applying protein-synthesis inhibitors such as anisomycin showed that, unlike STMs, the formation of LTMs is dependent on de novo protein expression (Frankland, et al., 2004, Schafe and LeDoux, 2000). This is one of the features that allows the distinction between STM and LTM, whereas STM depends on pre-existing networks and post-translational modifications, LTM involves structural and functional modifications to neural networks that demand *de novo* gene expression (Alberini, 2009, Davis and Squire, 1984).

LTM is less susceptible to interference, however, even this stored memory can be modified. Some years after the consolidation theory, memory reconsolidation was proposed by Misanin and colleagues in 1968 and later by Sara and Doux in 1997. According to the authors' electroconvulsive shocks or drug administration (protein synthesis inhibitors and NMDA and β-adrenergic blockers) have a memory-reducing effect in the presence of memory recall (Misanin, et al., 1968, Nader, et al., 2000, Przybyslawski and Sara, 1997). During retrieval, memory may be, once again, susceptible to modifications. In other words, the neural pathways and the molecular mechanisms involved in memory consolidation are reactivated, therefore the previously consolidated memory becomes labile and may be disrupted (Auchter, et al., 2017, Nader, et al., 2000). Whenever a memory goes through retrieval to be modified and stored once again (not erased), the process is called reconsolidation (Silva and Gräff, 2023). After retrieval, the reconsolidated memory may be maintained, strengthened or impaired (Silva and Gräff, 2023) (Figure 1).

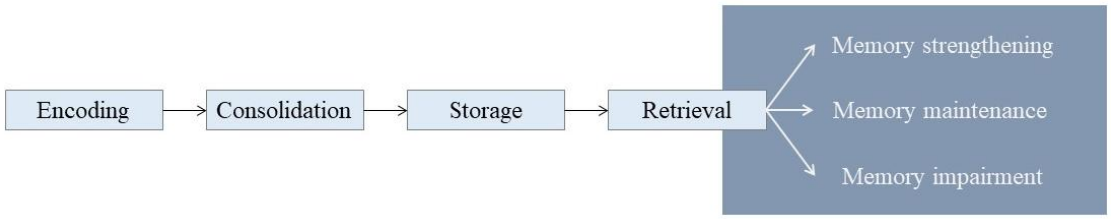


Figure 1. Schematic representation of the process of memory formation. Adapted from (Silva and Gräff, 2023).

1.2.2 Taxonomy of memory types

Memory can be divided according to different characteristics, based on duration, memory can be divided into STM and LTM, as described previously. Based on the qualitative classification, LMT can be further divided into declarative (also known as explicit) or nondeclarative (sometimes referred to as implicit or procedural) (Squire and Zola, 1996), as demonstrated in Figure 2. This classification proposed by Squire and Zola (1996) is based on different underlying networks or memory systems used in each type of memory that ultimately reflect memory behaviour manifestation. Declarative memory involves the storing and conscious retrieval of information that can be communicated through language, such as remembering a song, facts, and cell phone numbers (Squire and Zola, 1996). Nondeclarative memory refers to memories that usually are not available to consciousness, it refers to procedures, skills, or abilities, like knowing how to sing a song, how to use a cell phone, and how to ride a bike. Such differences imply different brain regions to be recruited. This will be explored hereafter (Squire and Zola, 1996).

Declarative memory division into episodic and semantic was first proposed by Tulving (1972). Episodic memory refers to the recall, in space and time, of personally experienced moments (Squire, 2004). Semantic memory is associated with general world knowledge, such as language-related information like word comprehension (Tulving, 1972). Those types of memories are dependent on the hippocampus, medial temporal lobe, and the midline diencephalon (Derner, et al., 2020, Sridhar, et al., 2023). The amygdala may also have an important role in encoding and retrieving this type of memory, particularly if it is emotionally related (Qasim, et al., 2023, Sridhar, et al., 2023).

Concerning non-declarative memories, those are subdivided into four subtypes specifically, procedural (skill and habits), priming, associative learning, which subdivides into emotional responses and skeletal responses, and non-associative learning (Squire, 2004, Squire and Zola, 1996). Non-declarative memories are dependent on the motor cortex, striatum, limbic system, cerebellum activity, and reflex pathways (Karni, et al., 1998, Sridhar, et al., 2023).

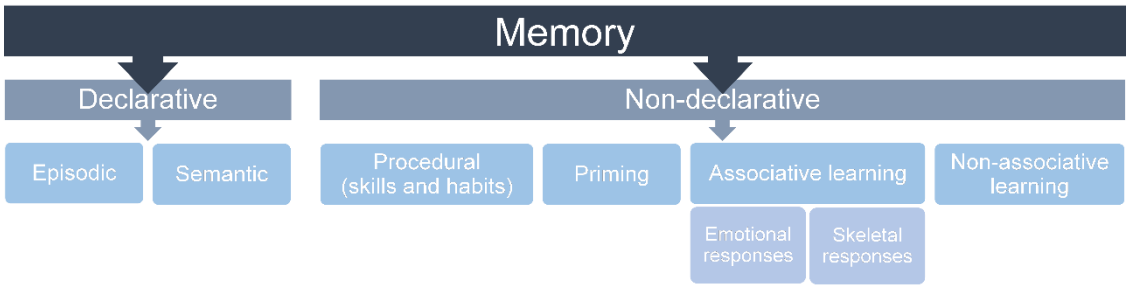


Figure 2: Memory types based on qualitative classification and the main brain regions involved. Adapted from (Squire, 2004).

1.2.3 The Pavlov model: from associative memory to fear conditioning

Pavlov´s findings in associative memory were a milestone in the neuroscience field to further memory comprehension. Around 1900, Pavlov, while studying vagus nerve innervations of the digestive tract, discovered that when two or more stimuli were presented at the same time or in the same order, it resulted in a behavioural change (Pavlov and Thompson, 1910). In this experiment, Pavlov observed that dogs salivate when food is present: as so, food was called an unconditioned stimulus. Therefore, he started to pair the presence of food with a sound, a bell tone, known as a conditioned stimulus. After the pairing of both unconditioned and conditioned stimuli the dogs start to salivate: this physiological response was then called a conditioned response. After some trials, the bell tone was no longer paired with the presence of food, however, dogs anticipated food and salivated in the same way (producing a conditioned response even in the absence of an unconditioned stimulus). This experiment proved that animals learn to associate two different stimuli and present a response (VanElzakker, et al., 2014).

Classical fear conditioning emerged from Pavlov's discovery with some modifications from his model with dogs. Similar to the Pavlov model (Maren, 2001), classical fear conditioning, a paradigm often used in rodents, also consists of a conditioned stimulus, which can be a light, a tone, or a specific chamber, paired with an unconditioned stimulus. However, this unconditioned stimulus has aversive characteristics, such as a shock. From this association also arises a conditioned response. Rodents exposed to the pairing usually display a fear response in the form of freezing behaviour. Freezing behaviour may be explained by a skeletal muscle tonic reaction which causes animal immobilization, without affecting the respiratory muscles (Izquierdo, et al., 2016, Paylor, et al., 1994). Animals may also present defecation and piloerection (Phillips and LeDoux, 1992). This indicates the formation of a contextual fear memory in response to the stressful situation

(Valentinuzzi, et al., 1998) and is a paradigm that allows the study of a subtype of non-declarative memories, namely the emotional response in the associative learning (Squire, 2004) (Figure 2).

1.3 The brain circuit for contextual fear memory

The contextual fear memory requires the division of two concepts, the context and the fear, which imply different brain regions. The hippocampus and the amygdala are important brain regions and operate together for the acquisition of this type of memories. During the memory acquisition of contextual fear conditioning, the encoding of the context, when the animal discovers the context for the first time, the dorsal hippocampus is the mainly activated area and is responsible for the "context creation", since it is where context and spatial memory is processed (Anagnostaras, et al., 2001, Maren, et al., 2013). Subsequently, the context information is communicated to the amygdala by projections from the hippocampus, more specifically to the basolateral amygdala (Anagnostaras, et al., 1999, Maren and Fanselow, 1995). The amygdala integrates the information from sensory inputs such as visual, auditory, and somatosensory stimuli (LeDoux, et al., 1991). Thus, it is in the amygdala that context information from the hippocampus is associated with fear information from the sensory inputs, leading to the acquisition of conditioned and unconditioned stimulus association (Goosens and Maren, 2001).

Both amygdala and hippocampus participate in the consolidation of contextual fear memory (Chaaya, et al., 2018). After contextual fear association, synaptic consolidation occurs in the amygdala (Chaaya, et al., 2018) and then proceeds to the ventral hippocampus (Pitkänen, et al., 2002) and finally to the dorsal CA1 and CA3 subregions, where system consolidation of contextual fear memory occurs (Riedel, et al., 2000, Sananbenesi, et al., 2002, Yavas, et al., 2019).

Although consolidation occurs in these regions, the memories are not exclusively maintained in the hippocampus or amygdala regions. The prefrontal cortex (PFC) also plays a role in contextual fear memory consolidation. Over time, this type of memory is less hippocampal-dependent and more frontal cortex-dependent (Rajbhandari, et al., 2017), which results from hippocampus and basolateral amygdala (BLA) inputs to the PFC (McDonald, et al., 1996). During retrieval these connections are constantly activated, meaning that all brain areas are recruited for memory to be recalled. The ventral PFC appears to be involved in fear memory extinction whereas the dorsal prefrontal appears to promote the expression of learned fear (Gilmartin, et al., 2014).

1.4 Stress leading to fear and to an emotional memory

Contextual fear conditioning makes use of an inherent part of an animal's evolution, the capacity to feel fear. Fear is an emotion caused by the exposure to a threatening (or a perceived threat) event, object, or situation. Emotional events, like fear, anger, love, and happiness create the most robust and long-lasting memories. This is well established in the scientific community, therefore, fear learning and memory are extensively studied.

Fear and stress are two different concepts; however, in some situations, fear can cause stress (Izquierdo, et al., 2016). Contextual fear conditioning paradigm also as the ability to initiate a stress reaction by triggering the pituitary-adrenal axis (LeDoux, 1994). The inverse may also occur, since stress may also lead to fear of learning enhancement (Izquierdo, et al., 2016).

The concept of stress is generally used to describe body reactions, through alterations in hormonal and metabolic status, in response to a change in psychological homeostasis (LeDoux, 1994). With the focus on physiological alterations, stress (a threat to the body's homeostasis) leads to catecholamine release to the blood stream by the adrenal medulla, triggering an increase in heart rate and blood pressure as well as shortness of breath (Cannon, 1915, Cannon and Lissák, 1939, Selye, 1950). Stress may have different meanings for different people and situations, normally it leads people to feel arousal and anxious. The stress-inducing factors are subdivided into acute (short-term stress) or chronic (long-term stress) (Stults-Kolehmainen and Sinha, 2014). Furthermore, intense or prolonged stress might have negative effects such as memory impairment (LaBar and Cabeza, 2006).

1.5 The sympathetic-adrenal medullary system and the hypothalamic-pituitary-adrenal axis

When a physical or psychological stressor is detected many brain regions are activated to allow the proper organism reaction (Godoy, et al., 2018), with the perception of the stressor being the first step of the stress response. After stress recognition two very important stress systems are activated, the sympathetic-adreno-medullar (SAM) axis and the hypothalamus-pituitary-adrenal (HPA) axis (Bracha, 2004), summarized in Figure 3. The major differences in SAM and HPA axis are the duration and the endocrinal mediators, whereas SAM, the first phase, is recognized as the rapid response with the release of noradrenaline (NA) and adrenaline (Ad), the HPA, the second phase, is

considered a long-lasting response with the release of corticosterone (Godoy, et al., 2018, Joëls and Baram, 2009).

After the stressors are processed in the brainstem and hypothalamic regions (Dayas, et al., 2001, Fenoglio, et al., 2006), the SAM axis is rapidly activated. The paraventricular nucleus of the hypothalamus, locus coeruleus (LC), and rostral ventrolateral medulla projects to pre-ganglionic sympathetic neurons (Godoy, et al., 2018), leading to the release of NA and Ad from the chromaffin cells of the adrenal medulla and NA from sympathetic nerves (Euler, 1946, Godoy, et al., 2018, Goldstein, et al., 2003, Takahashi, et al., 1992, Tank and Lee Wong, 2015). Sympathetic branch activation is predominant, which chains a physiological adaptation to prepare the organism for the "fight-or-flight" response, resulting in alertness, vigilance, metabolism increase through glucose recruiting from liver and fat tissue, increased oxygen consumption, and increased cardiovascular actions due to changes in blood vessels (Cannon, 1915, Godoy, et al., 2018, Nijsen, et al., 2000).

In the second phase of the stress reaction, the hypothalamus is recruited by amygdala stimulation, and the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH) (Beaulieu, et al., 1988, Matheson, et al., 1971) that in turn stimulates the anterior pituitary portion of the adenohypophysis to secrete adrenocorticotropic hormone (ACTH) to the hypophyseal portal system, allowing this hormone to reach the peripheral circulation and act on the cortical area of the adrenal gland (Antoni, et al., 1983, Gibbs, et al., 1983). ACTH then prompts the adrenal glands to produce and release glucocorticoids, like cortisol in humans, corticosterone in rodents, and mineralocorticoids (Herman, et al., 2003, Vale, et al., 1978). Those steroid hormones may regulate blood pressure and glucose availability to the brain tissue to allow the organism to react to a "fight-or-flight" response (Bucci, et al., 2016). Furthermore, they also have more prolonged actions leading to gene transcription, protein synthesis, and epigenetics modifications in the peripheral and central nervous system to promote physiological and behavioural adaptations (Godoy, et al., 2018). Glucocorticoids may also induce the activity of phenylethanolamine-*N*-methyltransferase (Pnmt), increasing Ad synthesis (Sharara-Chami, et al., 2010).

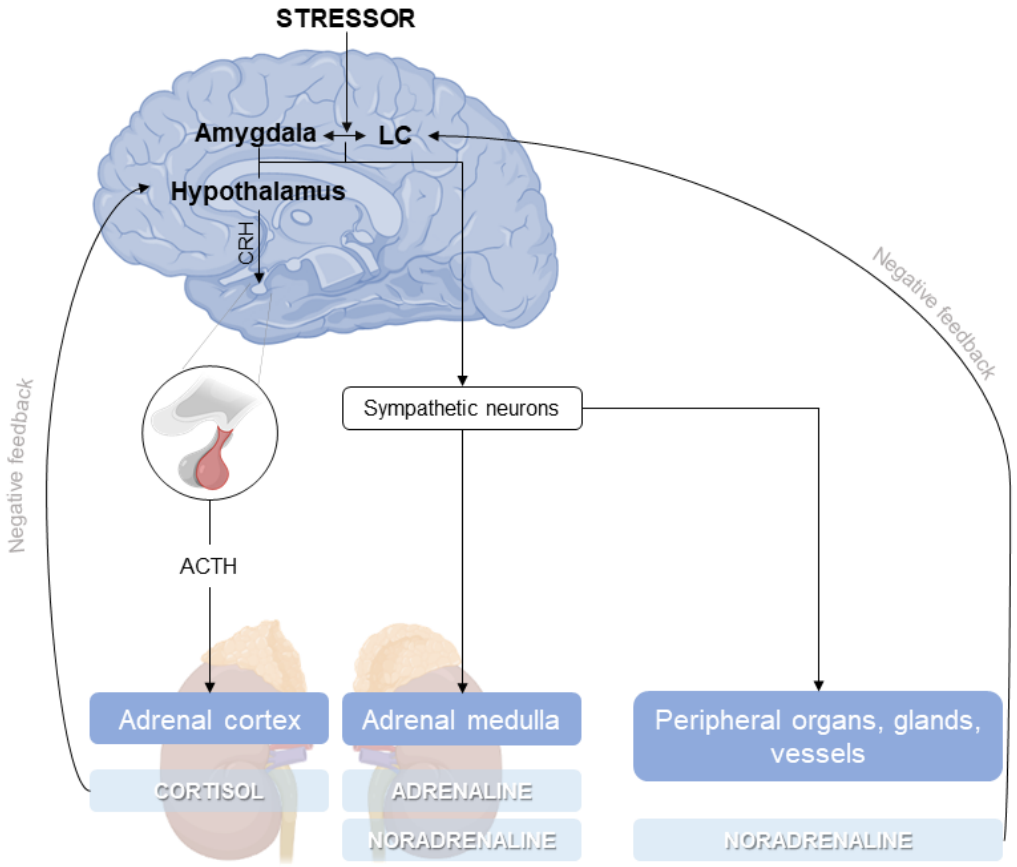


Figure 3: Stress response pathway leading to sympathetic-adreno-medullar and hypothalamic-pituitary-adrenal axis activation resulting in cortisol and catecholamines release. LC, locus coeruleus; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin hormone. Adapted from (Bucci, et al., 2016).

1.6 Catecholamines

Catecholamines are a group of hormones or neurotransmitters and their metabolites from the adrenomedullary, sympatho-neuronal and central catecholaminergic systems. The main three hormones are dopamine (DA), NA, and Ad. As stated in the previous section, these hormones are often recruited in a stressful situation, therefore they are frequently called “stress hormones”.

1.6.1 Historical Aspects

In 1856, during the study of adrenal gland circulation, Vulpian observed a differential colouration of the medulla and the cortex of the adrenal gland, whereas the medulla and the venous blood stained green, the cortex and the arterial blood did not (Vulpian, 1856). Therefore, the author proposed that the medulla of the adrenal gland releases a compound into the peripheral circulation. This was found to have physiological effects on the arterial system, such as blood pressure increase (Oliver and Schäfer, 1895). The vasoactive compound was first classified by Abel in 1897 as “epinephrine” (Abel, 1897) and was

latter isolated by Takamine in 1901 who named it “adrenaline” (Takamine, 1901). Therefore, nowadays the two terms are accepted to nominate the hormone in the science field (Aronson, 2000).

In the following years, the scientific community intensively studied Ad and other substrates of the adrenal gland, with significant advances being made in the twentieth century. Ad chemical structure was described and the term catecholamine was raised (Aldrich, 1901); it was the first hormone artificially produced (Stolz, 1904); NA was found to be an Ad precursor and also a sympathetic neurotransmitter (Euler, 1946); DA was also identified as an adrenal gland compound and as part of catecholamine’s biosynthesis pathway with specific signalling capabilities (Barger and Dale, 1910, Carlsson, et al., 1958, Hornykiewicz, 2002).

1.6.2 Adrenal gland

Localised near each kidney, the adrenal glands are one of the major endocrine glands whose products, after being released to the peripheral circulation, have important functions in several physiological processes. Morphologically, the adrenal gland is formed by three layers: an external capsule, the cortex, and the medulla (Rhodin, 1971, Rosol, et al., 2001). The cortex and the medulla produce distinct compounds with different structures and functions.

The adrenal cortex is located more externally and is the largest portion of the adrenal gland, it is subdivided into three histological zones, the glomerulosa, the fasciculata, and the reticularis (Rhodin, 1971, Rosol, et al., 2001). In the glomerulosa, the outer region of the adrenal cortex occurs the production of mineralocorticoids as is the case of aldosterone, in the fasciculata, which represents more than 70% of the cortex, the production of glucocorticoids occurs, and finally, in the reticularis, the inner layer of the cortex, is where the androgen precursors biosynthesis take place (Berger, et al., 2019, Nussdorfer, 1980, Nussdorfer, et al., 1978).

The medulla of the adrenal gland corresponds to 10% of the gland, it is histologically different from the cortex, being a modified sympathetic ganglion. The chromaffin cells are modified postganglionic neurons, a type of neuroendocrine cells, and are the main cells of the medulla of the adrenal gland (Rosol, et al., 2001). Those cells are responsible for the biosynthesis, of catecholamines, namely DA, NA, and Ad (Costa, et al., 2012, Rosol, et al., 2001) which are stored in the secretory vesicles of the chromaffin granules of chromaffin cells (Berger, et al., 2019). The release of catecholamines to the peripheral circulation occurs due to cholinergic innervation by splanchnic nerve activation and by

glucocorticoid action (Ehrhart-Bornstein and Bornstein, 2008, Wurtman and Axelrod, 1965), HPA axis activation occurs in response to stress, as stated in a previous section.

1.6.3 Catecholamines biosynthesis

The biosynthesis of catecholamines begins with the amino acid tyrosine. Tyrosine is obtained from two primary sources: the diet and the hepatic hydroxylation of the amino acid phenylalanine (Goldstein, 2010, Kvetnansky, et al., 2009). In the cytoplasm of the chromaffin cells or brain catecholaminergic nerves, tyrosine is hydrolysed by tyrosine hydroxylase (TH) to form L-3,4-dihydroxyphenylalanine (L-DOPA) (Rios, et al., 1999). This first step is called the rate-limiting step in the biosynthesis of catecholamines (Nagatsu, et al., 1964, Zigmond, et al., 1989). Therefore, TH is a biomarker of catecholamines-producing cells (Carlsson, 1959). The activity of TH enzyme begins after stimulation of the adrenal medulla or the sympathetic nerves (Costa, et al., 2012), moreover, TH activity is inhibited by negative feedback from excess catecholamines concentration (L-DOPA, DA, and NA) (Randall and Ferry, 1992).

In the cytoplasm of neuronal and adrenomedullary cells, L-DOPA is then decarboxylated by Aromatic L-amino acid decarboxylase (AADC), producing DA (Shih, et al., 2013, Verhofstad, et al., 1979). DA is then taken from the cytoplasm to storage vesicles, where dopamine β-hydroxylase (DBH) transforms DA into NA (Goldstein, 2010, Rios, et al., 1999) and this step can occur in chromaffin cells and noradrenergic neurons of the central nervous system (Shih, et al., 2013). The production of NA occurs in sympathetic nerve endings and some adrenomedullary cells that only produce NA is the final step, and the hormone stays in the granule until it is secreted.

The last step in the catecholamine biosynthesis pathway is the conversion of NA to Ad by methylation, through the action of Pnmt which requires methyl donor S-adenosylmethionine (SAMe) as a cofactor (Stanford, 2001). The adrenal glucocorticoids (synthesized in the cortical zone of the adrenal gland (Cole, et al., 1995) stimulate Pnmt enzyme activity to convert NA to Ad (Hodel, 2001). To protect from cytosolic enzyme oxidation, Ad is stored in chromaffin granules after the final step.

Pnmt can be found in the chromaffin cells of the adrenal medulla, the tissue with major expression and activity of Pnmt (Cahill, et al., 1996, Rios, et al., 1999), but it is also present and activated in the retina, heart, lung, kidney, pancreas, and some lower brainstem neurons (Kennedy, et al., 1995, Rios, et al., 1999, Stanford, 2001). The enzyme that synthesizes Ad was already detected and activated in some brain regions like medulla

oblongata and hypothalamic nuclei. Nevertheless, in the hippocampus, olfactory bulb, and cerebral cortex the Pnmt activity was not detected (Lew, et al., 1977, Pendleton, et al., 1978). The biosynthetic pathway of catecholamines is schematized in Figure 4.

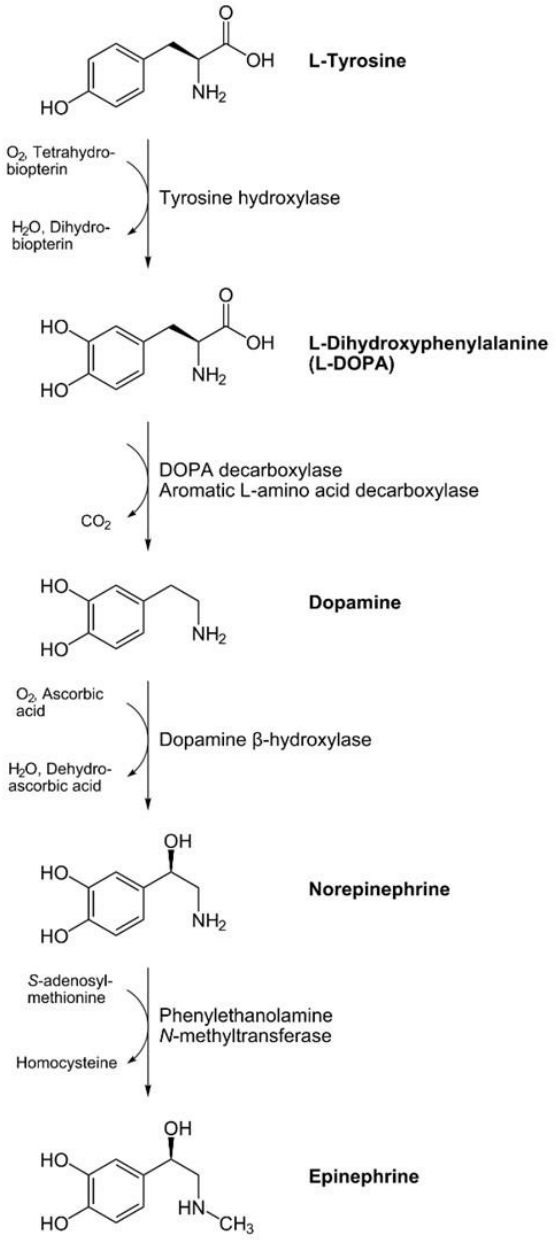


Figure 4: Catecholamines biosynthetic pathway. Adapted from (Purves, et al., 2001).

1.6.4 Catecholamines structure and functions

Catecholamines are a group of hormones that includes DA, NA, Ad, and other metabolites. This name group is due to the biochemical structure since the three have a catechol group, consisting of a benzene ring with two hydroxyl groups and a link to an

ethylamine side chain (Carlsson, 1959). They form a group of neurotransmitters and endogenous hormones that are soluble in water (Costa, et al., 2012).

DA is the most abundant catecholamine in the brain and although it has peripheral effects, it mainly acts in the central nervous system (Rios, et al., 1999) where it is found in greater amounts. The main site of DA biosynthesis in the brain is the substantia nigra. However, DA occurs in higher concentrations in the corpus striatum once it receives a big input from the substantia nigra (Sonne, et al., 2022). Moreover, DA receptors allow DA to exert its actions, and those are present in the brain, in the kidney, and in the renal, coronary and intracerebral arteries (Moreira-Rodrigues, et al., 2010, Moreira-Rodrigues, et al., 2012, Moreira-Rodrigues, et al., 2007, Moreira-Rodrigues, et al., 2009, Yanowitch and Coccaro, 2011). In the central nervous system this neurotransmitter is involved in body movement coordination, motivation, reward, cognitive function, and maternal and reproductive behaviours (Klein, et al., 2019). In the periphery, DA regulates blood pressure by inducing vasodilatation and increasing natriuresis (Carey, 2013, Chugh, et al., 2013, Horita, et al., 2013, Moreira-Rodrigues, et al., 2010, Moreira-Rodrigues, et al., 2012, Moreira-Rodrigues, et al., 2007, Moreira-Rodrigues, et al., 2009). In stressful situations, circulating DA may increase, but in lower magnitude when compared to NA and Ad (Bühler, et al., 1978).

NA is a neurotransmitter found in the central and peripheral nervous system (Bylund and Bylund, 2014, Costa, et al., 2012). This catecholamine is synthesized mainly in the brainstem nuclei, LC and subceruleus, sympathetic neurons and adrenal gland (Costa, et al., 2012, Schwarz and Luo, 2015, Wang, et al., 1999). Centrally, NA is involved in a diversity of functions such as locomotion control, attention, perception, and memory formation (Axelrod and Kopin, 1969, Xu, et al., 2000). In the periphery, NA leads to heart rate and blood pressure increase and vasoconstriction (Costa, et al., 2012, Wang, et al., 1999). In stressful situations the SNS and NA chromaffin cells release NA into circulation, however, in most situations, the increase of Ad in the peripheral circulation is more significant (Tank and Lee Wong, 2015).

The Ad found in circulation is derived mostly from chromaffin cells of the medulla of the adrenal gland where nearly 80% of Ad production occurs (Kvetnansky, et al., 2013). Ad is also synthesized in blood vessels and the heart (Axelrod, 1962, Spatz, et al., 1982). Catecholamines are polar molecules and therefore do not cross the blood-brain barrier, however, there is some evidence of Ad production in the brain as referred before (Goodchild, et al., 1984). Quantification of DA, NA, and Ad by high-performance liquid

chromatography with electrochemical detection (HPLC-ED) in different brain regions concluded that Ad is not present in physiological amounts in most of the brain areas, such as amygdaloid nuclei, limbic cortex (including ventral and dorsal hippocampus) and olfactory tubercle. Even so, in the nucleus of stria terminalis and hypothalamic nuclei, Ad was detected but in very small amounts (Kilts and Anderson, 1986).

Regarding Ad physiological functions, this catecholamine has dose-dependent effects on the cardiovascular system, where it has positive chronotropic and inotropic effects, causes vasoconstriction, and increases the respiratory rate (Furnival, et al., 1971, Tank and Lee Wong, 2015). In metabolism, Ad is responsible for inducing glycogenolysis resulting in blood glucose increase (Gold, 2014, Talley, et al., 2000). During a stress response, Ad is the primary recruited catecholamine, and although it does not cross the blood-brain barrier it has effects on the central nervous system such as learning and memory enhancement (Tank and Lee Wong, 2015), a topic that will be further explored.

1.7 Adrenergic receptors

To exert their action, NA, and Ad act in a group of cell surface G-protein coupled receptors identified as adrenergic receptors. In 1948, Ahlquist classified the adrenergic receptors into two major subtypes: α and β, based on their response to Ad, NA, and isoproterenol, an adrenergic agonist (Ahlquist, 1948). The α and β adrenergic receptors are further divided into several subtypes and both NA and Ad act on both classes of receptors however with different affinities (Costa, et al., 2012).

1.7.1 α-adrenoceptors

The α-adrenergic receptors where first subdivided into α_1- and α_2- adrenergic receptors, nevertheless the existence of more subtypes, namely α1A, α1B, α1D, α2A, α2B, and α2C is currently accepted (Langer, 1974, McGrath, 1982).

α_1-adrenergic receptors mediate their response via a $G_{q/11}$ protein, leading to phospholipase C activation (Cotecchia, et al., 1990). The subsequent cascade ultimately ends with an intracellular Ca^{2+} increase which results in smooth muscle contraction (Hoffman and Lefkowitz, 1980, Starke, 1981). They mediate an excitatory response and are found at post-synaptic junctions of the sympathetic nerves in cardiac muscle, smooth muscle blood vessels, bronchi, gastrointestinal tract, uterus, and bladder(Costa, et al., 2012, Tank and Lee Wong, 2015). In general, they cause vasoconstriction, gastrointestinal relaxation, and salivary secretion. Although both NA and Ad have an

affinity to α_1-adrenergic receptors, they differ in the potency: Ad ≥ NA > Isoprenaline (Tank and Lee Wong, 2015).

Contrary to α_1-adrenergic receptors, α_2-adrenergic receptors are coupled to an inhibitory G-protein in the presynaptic nerve terminals causing negative feedback to NA release in sympathetic nerves (Langer, 1997, Starke, 2001, Wise, et al., 1997). The activated Gi protein results in decreased production of cyclic adenosine monophosphate (cAMP) by adenyl cyclase, which induces the K^+ channels to open, increasing the time of hyperpolarization (Cotecchia, et al., 1990). Nevertheless, the postsynaptic expression of α_2-adrenergic was also demonstrated, in vascular smooth muscle cells and the brain (Gyires, et al., 2009). The regulation of blood pressure, hypothermia, cognitive function, lipolysis suppression, and contraction of vascular smooth muscle are some of the effects of α_2-adrenergic receptors (Costa, et al., 2012, Tank and Lee Wong, 2015). The affinity and potency of NA and Ad to α_2-adrenergic receptors are similar to α_1.

1.7.2 β-adrenoceptors

The β-ARs class is subdivided into β_1, β_2, and β_3, all of which are part of the superfamily of G-protein-coupled receptors (Dixon, et al., 1986, Emorine, et al., 1989, Frielle, et al., 1989). Ad has excellent affinity for both α- and β-adrenergic receptors but is more potent at β-adrenergic receptors (Tank and Lee Wong, 2015). β_1-adrenergic receptors have the same affinity for Ad and NA (Isoprenaline > Ad ≥ NA) while the affinity order for β_2-adrenergic receptors is Isoprenaline > Ad >> NA (Tank and Lee Wong, 2015). Compared to β_1- and β_2-adrenergic receptors, β_3 has a lower affinity for both NA and Ad (Lafontan, et al., 1997).

β-adrenergic receptors are coupled to G_s proteins (Chruscinski, et al., 1999) and when activated induce adenylate cyclase to convert ATP into cAMP, which acts as a second messenger to activate protein kinase A (PKA) (Frielle, et al., 1989, Tresguerres, et al., 2011).

β_1-adrenergic receptors are essentially distributed throughout the heart muscle where they predominately control cardiac function mediating positive inotropic and chronotropic responses (Brodde, 1991, Brodde, 1993). Additionally, β_1-adrenergic receptors activation induces relaxation of peripheral vessels, coronary arteries, and pulmonary arteries (O'Donnell, et al., 1984, Toda and Okamura, 1990, Vatner, et al., 1985).

Considering β_2-adrenergic receptors, they are predominantly expressed in vascular smooth muscle and bronchial tissue. The activation of β_2-adrenergic receptors leads to

peripheral vasodilation and bronchodilation (Bao, et al., 2007, Brodde, 1991). As it was previously stated, catecholamines (NA and Ad) and corticoids are involved in the "fight or flight" response (Bracha, 2004). The increase in blood concentration of NA and Ad after a stressful situation causes physiological changes, namely by β_2-adrenergic receptors, to allow the organism to be prepared to react (Cannon, 1915). The physiological changes include alertness maintenance, pupillary dilatation, piloerection, aggressive or escape behaviour, metabolic actions (increased glucose via glycogenolysis and gluconeogenesis, lipolysis, increased oxygen consumption and thermogenesis) and cardiovascular actions (Fuchs, et al., 1985).

β_3-adrenergic receptors are principally expressed in adipose tissue and its activation induces lipolysis in white adipose tissue and induces thermogenesis in brown adipose tissue (Arch, et al., 1984, Emorine, et al., 1989).

1.8 Role of peripheral adrenaline in fear memory

The SAM and HPA axis activation during stressful situations, as previously explained, leads to subsequent catecholamines (NA and Ad) and glucocorticoid blood increase. Other than all the physiological actions these hormones have, they also seem to be involved in cognitive aspects, more specifically in the enhancement of stressful-related memories (McGaugh and Roozendaal, 2002). From an evolutionary point of view, this response is necessary for survival in order to avoid dangerous situations, since emotional-related events, such as fear-related ones, are better remembered and long-lasting than neutral ones (Buchanan, 2007).

Further investigation was conducted to better elucidate the role of Ad as an enhancer of the memory formation process. The administration of peripheral Ad after a learning-based aversive paradigm, the inhibitory avoidance, resulted in memory enhancement (Gold and van Buskirk, 1978b, Gold and Van Buskirk, 1975). These results where latter reproduced in other aversive paradigms (Alves, et al., 2016, Introini-Collison and McGaugh, 1986, Sternberg, et al., 1985, Toth, et al., 2013), beyond the inhibitory avoidance, reinforcing the significance of Ad in this subject.

Ad effects in aversive memory enhancement are not a monotonic function but a so-called U-inverted shape dose response. This means that levels below a certain threshold have no memory impact, whereas those above that threshold improve memory, however, an even higher dose impairs memory (Gold and Van Buskirk, 1975).

Several studies, in both rodents and humans, using pharmacological manipulation of β-adrenergic receptors through propranolol and sotalol administration (β-adrenergic receptors antagonists) demonstrated that Ad exerts its actions through this class of adrenergic receptors (Gold and van Buskirk, 1978a, Grillon, et al., 2004, King Ii and Williams, 2009, Parfitt, et al., 2012). Additionally, this also reinforces that the memory enhancement effect of Ad is through peripheral actions since sotalol is a β-adrenergic receptor antagonist with peripheral effects. Later, in a fear conditioning paradigm, a study specified which β-adrenergic receptor was involved in fear memory enhancement using selective agonists. The use of fenoterol, a selective β_2-adrenergic receptor agonist enhanced contextual fear memory, but the same outcome was not observed with a β_1-adrenergic receptor agonist, dobutamine (Alves, et al., 2016).

1.8.1 Animal models for adrenaline functional studies: the adrenaline deficient mice

The surgical removal of the medulla of the adrenal gland was one of the first approaches to study the influence of Ad on memory. In fact, studies with this model where pioneers in trying to exclude the effects of adrenal catecholamines from glucocorticoids. In adrenalectomized rats (adrenal gland removed by surgical procedure), it was observed an impairment of fear memory (Borrell, et al., 1984, Borrell, et al., 1983, Colucci, et al., 2021, Oitzl and de Kloet, 1992). Nevertheless, this approach can not differentiate if the observed effect is due to NA or Ad depletion, since both NA and Ad are synthetized in the adrenal medulla. Furthermore, the surgery may lead to adrenal cortex damage and alter the liberation of glucocorticoids, pancreastatin, catestatin, chromogranin A, and neuropeptide Y (Harrison and Seaton, 1966).

An additional approach was to inhibit the conversion of DA into NA, by DBH pharmacological inhibitors or by a genetic knockout (DBH-KO) of the enzyme. In behavioural experiments, the DBH-KO mice presented reduced contextual fear memory (Murchison, et al., 2004) and the mice in which DBH was pharmacologically inhibited with Nepicastat also presented reduced contextual traumatic memories (Martinho, et al., 2021a). Notwithstanding it is uncertain if the effects are due to the action of NA or Ad, or both, since, in both models the synthesis of the two catecholamines where inhibited.

To specifically study the Ad influence, a new alternative emerged created by Ebert, et al. (2004). By inserting a Cre-recombinase and a Neomycin-resistant gene in the Pnmt locus, mice became unable to express a functional Pnmt enzyme and therefore could not convert NA into Ad, becoming Ad-deficient (Ebert, et al., 2004). In Ad-deficient mice, adrenal Ad is not detected but adrenal NA is significantly higher. Nevertheless, Ad-deficient mice

were morphologically indistinguishable from wild-type (WT) and were able to reproduce successfully (Bao, et al., 2007, Ebert, et al., 2004). Ad-deficient mice have normal heart rate at rest and in acute exercise. In addition, they have normal blood pressure in rest but increased blood pressure during acute exercise compared to WT mice, possibly due to lack of vasodilation which is mediated by Ad action on β_2-adrenergic receptors (Bao, et al., 2007, Mendes, et al., 2018, Moreira-Rodrigues, et al., 2014, Sun, et al., 2008).

Ad-deficient mice are a reliable model to better understand Ad effects on contextual fear memory, bringing interesting advances in the field. Using the contextual fear conditioning paradigm, Toth was the first author to demonstrate a memory contextual fear impairment in Ad-deficient mice, reinforcing the role of Ad in this type of memory (Toth, et al., 2013). In addition, in other non-aversive behavioural paradigms (novel object preference), memory related with recognition was not affected in Ad-deficient mice (Toth, et al., 2013). Later, Ad or a β_2-adrenergic receptor agonist administration in Ad-deficient mice showed the restore of contextual fear memory (Alves, et al., 2016). Moreover, in both studies no differences were observed in memory acquisition day, meaning that pain perception and memory acquisition are not affected by Ad's absence (Alves, et al., 2016, Toth, et al., 2013).

Another significant finding of these studies was the absence of differences in a modified context to evaluate auditory cue fear conditioning, meaning that Ad does not affect auditory fear memory (Alves, et al., 2016, Toth, et al., 2013). Since context and auditory fear memory involve different brain circuits (de Araujo, 2014, Grillon, et al., 2004), being hippocampus responsible for context-fear conditioning (Rudy, et al., 2004), but not auditory fear conditioning, Ad contextual fear memory enhancement may be occurring in the hippocampus. In human studies, it was also demonstrated the involvement of β-adrenergic receptors in contextual fear conditioning, without influencing cue fear conditioning (Grillon, et al., 2004). Altogether, these results suggest that the enhancement effects of Ad in contextual fear memory appear to be mainly through peripheral β-adrenergic receptors and in particular β_2-adrenoceptors activation (Alves, et al., 2016, Toth, et al., 2013).

More recently, using a post-traumatic stress disorder (PTSD) mouse model, Ad-deficient mice also presented decreased contextual traumatic memories and anxiety-like behaviour compared to WT mice (Martinho, et al., 2020), which implies the Ad relevance in this pathology. Catecholamines where already described in PTSD, with people diagnosed with this pathology having increased NA and Ad in plasma and urine (Delahanty, et al.,

2005, Lemieux and Coe, 1995, Yehuda, et al., 1992), which was also demonstrated by (Martinho, et al., 2020) in a PTSD mouse model. Moreover, nepicastat (a DBH inhibitor) and sotalol (a peripheral β-adrenergic receptor), which respectively reduce NA and Ad concentration and inhibit Ad action, decrease mice PTSD contextual traumatic memories and related signs (Martinho, et al., 2021a, Martinho, et al., 2021b).

Despite the described evidence that peripheral Ad produced effects in the central nervous system, this hormone does not cross the blood-brain barrier (Weil-Malherbe, et al., 1959) and it is not present in the central nervous system (Kilts and Anderson, 1986). Together with outcomes showing that intra-cerebroventricular infusions with Ad do not lead to memory enhancement (de Almeida, et al., 1983), some theories have emerged to justify peripheral Ad´s actions, in order to allow the occurrence of downstream molecular processes for memory formation. The two main perspectives will be described in the following subsections, however, both hypotheses may converge and are not exclusive to justify Ad actions.

1.8.2 The glucose theory

A first hypothesis described as "glucose theory", refers to Ad metabolic effects. It states that upon a stressful situation and Ad being released to the peripheral circulation, β-adrenergic receptors will be activated. The activation of β-adrenergic receptors in the hepatocytes will result in glycogenolysis and gluconeogenesis enhancement (Gold, 2014, Talley, et al., 2000), and the hepatic glucose storage will be released to the peripheral circulation, causing an increase in blood glucose (Coker, et al., 1997) (Figure 5). Contrary to Ad, the highly hydrophilic glucose molecule can pass through the endothelial cells of the blood-brain barrier, in which the mainly expressed glucose transporter (GLUT) is the GLUT1 (Cornford, et al., 1994, Dobrogowska and Vorbrodt, 1999, Farrell and Pardridge, 1991, Vorbrodt, et al., 2001). In the central nervous system glucose in rapidly transported to astrocytes, microglia, and neuron cells (Patching, 2017). In astrocytes, the primarily expressed GLUT is also GLUT1 (Morgello, et al., 1995, Yu and Ding, 1998), in microglia is GLUT5 (Horikoshi, et al., 2003, Payne, et al., 1997), and in neurons is GLUT3 (Gerhart, et al., 1992, Maher, et al., 1996). Once in the brain cells glucose may enhance the synthesis of neuromodulators, which will activate signalling pathways and influence the process of memory formation (Ragozzino, et al., 1996).

Studies with glucose administration show some similarities between glucose and Ad in memory enhancement which reinforces glucose as an Ad mediator. Similar to Ad, glucose also enhances memory in a U-inverted shape response in both rodents and humans

(Messier and Destrade, 1988, Parsons and Gold, 1992). Memory enhancement occurs with blood glucose levels identical to those induced by Ad (Gold, 1986, Hall and Gold, 1986). Additionally, although β-adrenergic receptors prevent Ad stressful fear memory enhancement effect (Alves, et al., 2016, Gold and van Buskirk, 1978a), the memory enhancement effect by glucose remains (Gold, et al., 1986). These results support the hypothesis that increases in blood glucose following Ad injection or release mediate the effects of the hormone on memory. It is important to notice, that contrary to Ad (de Almeida, et al., 1983), both systemic and central administration of D-glucose enhances memory for appetitive, aversive, habituation, and spatial tasks in rodents (Gold, 1986, Stefani, et al., 1999).

After the fear conditioning paradigm, WT mice had higher glycaemic variation than Ad-deficient mice (Alves, et al., 2016), possibly due to Ad release. Stimulation of hepatic cells by Ad leads to blood glucose increases and may contribute to neuronal glucose availability. Two primary substrates for brain energy are available to neurons, both of which start with circulating glucose. The first is the direct entrance of glucose into neurons, and the second is glucose uptake into astrocytes to increase glycogen and enable lactate production (Newman, et al., 2011). Direct glucose into neurons and astrocytic storage of glycogen provide supplemental energy to neurons in high-demand situations (Newman, et al., 2011). Those may provide the necessary energy for the processes involved in fear memory formation (Brown, et al., 2004, Suzuki, et al., 2011). Thus, peripheral Ad and glucose availability may impact hippocampus-dependent contextual fear memory (Alves, et al., 2016).

As already stated, the hippocampus is an important brain region for learning and memory formation (Costa, et al., 2022) including contextual fear memory, a process associated with high-energy demand neurons (Kong, et al., 2017). In the hippocampus, in addition to GLUT3 neuronal expression also occurs GLUT4 expression (Apelt, et al., 1999, El Messari, et al., 2002), where both GLUT3 and GLUT4 are up-regulated when an increase energy demand occurs during hippocampus neuronal activation (El Messari, et al., 1998, Leloup, et al., 1996), as is the case of emotional stress and learning (Koepsell, 2020, Pearson-Leary, et al., 2018, Pearson-Leary and McNay, 2016). The increased energy requirement in these situations is addressed by lactate and glucose transportation to neurons through transporters overexpression (Newman, et al., 2011, Pearson-Leary, et al., 2018, Pearson-Leary and McNay, 2016).

1.8.3 The vagus nerve theory

Another hypothesis states that Ad may mediate its actions by activation of peripheral β-adrenergic receptors expressed in the ascending vagal fibres. Also, the vagus nerve expresses β_2-adrenoreceptors (Lawrence, et al., 1995, Schreurs, et al., 1986) and an increase in peripheral Ad might lead to vagal activation (Miyashita and Williams, 2006) (Figure 5). As a consequence of vagus nerve activation, the neurons of the nucleus tractus solitarius (NTS), which receive the vagal inputs (Sumal, et al., 1983), release NA into the amygdala (Petrov, et al., 1993). Additionally, noradrenergic cells from NTS also stimulate the brainstem and the LC (Schwarz and Luo, 2015, Van Bockstaele, et al., 1999), the main noradrenergic brain regions which also provides the main NA innervation to the amygdala (Fallon and Moore, 1978), to release NA into BLA (Chen and Williams, 2012). The triggering of those brain regions results in hippocampus and pre-frontal cortex activation (Fallon, et al., 1978, Richter-Levin and Akirav, 2003). As already described, NTS, locus coeruleus, BLA, and hippocampus consist of the memory learning and consolidation circuit (Wong, et al., 2012).

For instance, Ad effects on memory enhancement are blunted when the amygdala or NTS are lesioned (Cahill and McGaugh, 1990, Clayton and Williams, 2000, Liang, et al., 1990, Roozendaal and McGaugh, 1996) or when β-adrenergic receptors are directly delivered into amygdala (Liang, et al., 1986, Quirarte, et al., 1997). Also, an increase in extracellular concentrations of NA in the BLA may improve memory formation after emotionally arousing experiences (Chen and Williams, 2012). In addition, bilateral subdiaphragmatic vagotomy resulted in decreased NA concentration in the brainstem (Kobrzycka, et al., 2019), and vagotomized animals demonstrate impaired memory in aversive tasks such as inhibitory avoidance (Flood and Morley, 1988, Nogueira, et al., 1994, Talley, et al., 2002, Williams and Jensen, 1991), reinforcing the idea of the vagus nerve in transmitting peripheral signalling to the central nervous system to strengthen memory.

Another approach to studying the vagus nerve in memory is by electrical stimulation. Moderate-intensity vagus nerve electrical stimulation resulted in an amygdala NA increase (Hassert, et al., 2004) and also improves rodent and human performance on memory-related tasks (Clark, et al., 1995, Clark, et al., 1999, Clark, et al., 1998). In both cases, inverted-U-shaped functions were similar as those observed with peripheral Ad (Gold and Van Buskirk, 1975).

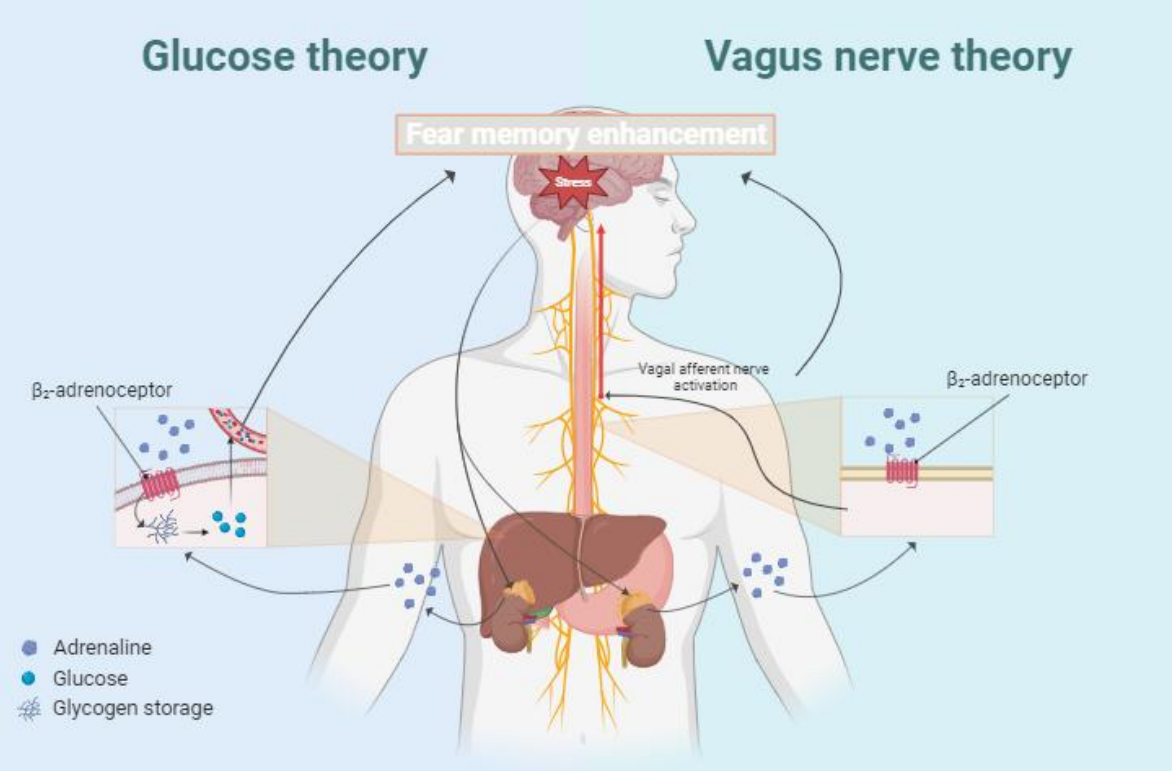


Figure 5: The glucose and the vagus nerve theories for adrenaline effects in contextual fear memory enhancement. Upon adrenaline release from adrenal gland due to stress stimulation, β_2-adrenergic receptors on hepatocytes and vagus nerve may be activated. Leading to glucose release and vagal nerve afferent activation, respectively. Both pathways may ultimately lead to contextual fear memory enhancement.

1.9 The cholinergic system and its relationship with peripheral adrenaline in contextual fear memory

One of the mechanisms, already described previously, for contextual fear memory enhancement by Ad is through glucose. In the brain, glucose can by directly utilized by neurons or be stored in glia cells for further uses in high-energy demand conditions. The glucose utilization under these circumstances may be used to the synthesis of neurotransmitters in the brain, as is the case of acetylcholine and glutamate (Kaufman, et al., 1991, Schousboe, et al., 1993, Tucek and Cheng, 1974). Acetylcholine synthesis is dependent on choline and acetyl coenzyme A. The latter comes from glucose metabolism from the Krebs cycle, after pyruvate oxidation (Gibson and Blass, 1976, Gibson, et al., 1978, Gibson and Shimada, 1980). In addition, the release of acetylcholine during learning and memory tasks appears to be regulated by glucose, since for example during a spatial working memory task glucose administration increases acetylcholine in the hippocampus and enhances memory (Pych, et al., 2005, Ragozzino, et al., 1996).

1.9.1 The cholinergic receptors

Acetylcholine is considered a neurotransmitter and a neuromodulator. It exerts its function by acting in two distinct classes of receptors, the nicotinic and the muscarinic receptors (Albuquerque, et al., 2009). The nicotinic receptors are excitatory ligand-gated ion channels and are expressed in the neuromuscular junction, in the ganglions of the autonomic nervous system (ANS), and in the central nervous system (CNS) (Albuquerque, et al., 2009). In the central nervous system, several nicotinic receptor subtypes are expressed, each receptor has a total of five α and/or β subunits, and the combination of the α (α_2-α_{10}) and β (β_2-β_4) subunits determines the receptor subtype pharmacological and physiological properties (Elgoyhen, et al., 1994, Liu, et al., 2012, Millar and Gotti, 2009).

The muscarinic receptors family are divided into five subtypes, M_1, M_2, M_3, M_4, and M_5 (Bonner, et al., 1987, Bonner, et al., 1988, Kubo, et al., 1986a, Kubo, et al., 1986b, Peralta, et al., 1987). The M_1, M_3, and M_5 are coupled to a G_q protein and are excitatory receptors. The M_2 and M_4 receptors are coupled to a $G_{i/o}$ protein and are considered inhibitory receptors (Felder, 1995, Scarr, 2012). The five muscarinic receptors are expressed in the central nervous system; however, muscarinic receptors may have different expression patterns according to different brain regions.

The muscarinic M_1 subtype is expressed throughout the brain, however in cortical regions and the hippocampus this receptor may be found in the highest amounts (Flynn, et al., 1995). The M_2 subtype can be found in the nucleus basalis, occipital cortex, hippocampus, and other cortical regions, but the highest amounts occur in the nucleus basalis and occipital cortex (Flynn, et al., 1995, Mrzljak, et al., 1993). Regarding M_3, those may be found in the same regions as M_1 but in lower amounts (Levey, et al., 1994). M_4 expression occurs in the hippocampus, where together with M_1, the receptor subtype in higher amounts, may also be found in the cortex (Falk, et al., 2020, Flynn, et al., 1995). Finally, M_5 expression occurs in the hippocampus and in the ventral tegmental area (Vilaró, et al., 1990).

1.9.2 Cholinergic system and contextual fear memory induced by adrenaline

Many pharmacological studies have shown the significance of these receptors in memory modulation. Treatment with oxotremorine (non-selective muscarinic receptor agonist) enhances the consolidation of contextual fear memory, whereas the administration of scopolamine (non-selective muscarinic receptor antagonist) disrupts it (Cangioli, et al., 2002, Passani, et al., 2001).

Few studies relate the peripheral effect of Ad, glucose, and central acetylcholine and its receptor modulation in memory. With the scope to study the interaction of adrenal hormones and muscarinic receptor expression in the central nervous system, Mulugueta and colleagues demonstrated that adrenalectomized rats presented a decrease in hippocampus M_4 receptor expression one month after surgery compared to sham mice (Mulugeta, et al., 2006). Nevertheless, the surgery approach does not allow the distinction of which adrenal gland hormone interferes with cholinergic receptor expression. Furthermore, in two memory tasks, namely inhibitory-avoidance and y-maze, the facilitatory effect of Ad is blocked by atropine (non-selective muscarinic receptor antagonist) (Introini-Collison and McGaugh, 1988). In addition, oxotremorine (a muscarinic receptors agonist) significantly facilitated retention in the inhibitory avoidance test when administered simultaneously with a sub-effective dose of Ad (Introini-Collison and McGaugh, 1988). Interestingly, in another study, post-training administration of atropine but not methylatropine (peripheral muscarinic antagonist) prevented the facilitatory effects of β-adrenoceptor agonist clenbuterol (non-selective β-adrenoceptor agonist) (Introini-Collison and Baratti, 1992), which reinforces the peripheral role of Ad and the central acetylcholine in fear memory.

In addition, glucose administration during cognitive tasks appears to increase hippocampal acetylcholine release (Ragozzino, et al., 1996). Furthermore, both systemic doses of glucose and choline enhance memory in inhibitory avoidance tasks, contrary to sub-effective doses of each compound (Kopf, et al., 2001). In addition, when sub-effective doses of choline and glucose were given at the same time, memory enhancement was reestablished and acetylcholine in the hippocampus increased (Kopf, et al., 2001).

Still, none of these studies fully investigated Ad, glucose and acetylcholine possible pathways. Therefore, the use of an Ad-deficient mouse may be a step forward in this subject. Additionally, none of these studies relates to fear memory using the fear conditioning paradigm (Introini-Collison and Baratti, 1992, Introini-Collison and McGaugh, 1988).

1.10 Insulin role in contextual fear memory

Another outcome of Ad-induced blood glucose increase is the insulin release by pancreatic cells. Hyperglycaemia is sensed by GLUT2 in the pancreatic cells which in turn stimulates insulin secretion. On the other hand, insulin-induced hypoglycaemia can be detected by glucosensors in the periphery and in the central nervous system (Levin, et

al., 1999, Oomura, et al., 1969), activating the sympathoadrenal system, inducing glucocorticoids and Ad release (Fisher, et al., 2005). As a result, there is a feedback loop between hypoglycaemia, Ad, and insulin production. Catecholamines (NA and Ad) increase after hyperinsulinemia (Tesfaye and Seaquist, 2010, Voss, et al., 2017).

Since it was discovered that insulin crosses the blood-brain barrier (Poduslo, et al., 1994, Schwartz, et al., 1991) along with the discovery of insulin receptors in various brain regions (Posner, et al., 1974, Szabo and Szabo, 1972), the research on insulin's role in cognitive processes has increased. Immunoblotting and immunohistochemistry experiments indicated a widespread insulin receptor distribution in the hypothalamus, cerebellar cortex (Spinelli, et al., 2019), and limbic system, for instance, the amygdala, and hippocampus (Zhao, et al., 1999). In addition, these brain structures are both involved in memory (Yavas, et al., 2019) and more specifically, fear memory (Izquierdo, et al., 2016). Zhao and colleagues used water-maze training to investigate the changes in insulin receptor expression and associated signalling molecules in the rat hippocampus (Zhao, et al., 1999). They concluded that insulin receptors may play a role in the regulation of synaptic activities during spatial memory formation.

However, to our knowledge, no studies reporting the effects of acute insulin administration in the fear conditioning paradigm have been published. In paradigms also associated with emotional fear-motivated memory insulin administration appears to enhance rat's performance in the passive avoidance task (Kopf and Baratti, 1995, Park, et al., 2000, Schwarzberg, et al., 1989). Inhibitory avoidance is a complex form of contextual fear conditioning that requires learning through both classical and operant conditioning. Similarly to the fear conditioning procedure, a subject learns to associate a specific context with an aversive stimulus, however, this experience is contingent on the subject’s choice to move into that context, studying both associative and non-associative memory (Liang, 2009, Ögren and Stiedl, 2015). The main difference between both behavioural tests is that the fear conditioning procedure analysis only requires learning through associative memory. Therefore, the use of inhibitory avoidance conditioning can not establish if the findings were due to both associative and non-associative memory or only to one of these types of memory. Moreover, in humans, intranasal administration of insulin strengthens declarative memory (Benedict, et al., 2004).

The precise insulin action in contextual fear memory, especially in the physiological loop between Ad and insulin secretion, makes it difficult to separate the effect of both

hormones in memory, namely in contextual fear memory. Ad-deficient mice might be a valuable tool to uncover this literature gap.

1.11 Molecular pathway for fear memory consolidation

Looking into the process of memory formation, it is well established that a long-lasting memory is dependent on changes at a molecular level (Alberini, 2009). For instance, fear memory consolidation depends on new gene transcription, protein synthesis, changes in cell surface proteins, and synaptic remodelling (Alberini, 2009, Davis and Squire, 1984). The first studies showing this dependence made use of RNA or protein synthesis inhibitors which prevent LTM formation (Agranoff, et al., 1965, Frey, et al., 1996, Sangha, et al., 2003, Schafe and LeDoux, 2000).

cAMP-response element binding protein (CREB) is a transcription factor and it is considered a molecular marker for memory. Once activated by phosphorylation (Yamamoto, et al., 1988), pCREB binds to regulatory sequences of DNA and activates the expression of many genes with CRE sequences (Viola, et al., 2000). The CREB pathway's role in fear memory has been well-established at this point. However, the downstream effectors of this response should then be better understood.

1.11.1 Nuclear receptor 4a family

Considering CREB targets, the NR4A family, a group of transcription factors and immediate-early genes (IEG), has already been implicated in memory formation, particularly in contextual fear memory (Hawk, et al., 2012, Martinho, et al., 2020). This family of nuclear receptors consists of three members, *Nr4a1* (also denominated as *NGF-B/Nur77*), *Nr4a2* (also known as *NURR1*), and *Nr4a3* (*NOR1*) (Hazel, et al., 1988, Law, et al., 1992, Ohkura, et al., 1996). NR4A family are rapidly expressed after a stimulus and are presented in many types of tissues that regulate the expression of several genes (Safe, et al., 2016).

NR4A genes have already been implicated in inflammatory and immune responses, cardiovascular and metabolic diseases, and neurological functions (Safe, et al., 2016). In the central nervous system, the three members of the *Nr4a* family belong to the same cluster (Bookout, et al., 2006). *Nr4a1*, *Nr4a2*, and *Nr4a3* expression occurs in the CA1 and CA3 regions of the hippocampus (Xiao, et al., 1996).

Considering memory formation, *Nr4a* gene expression occurs in the hippocampus after learning paradigms (Bridi and Abel, 2013, Xiao, et al., 1996), including after contextual fear conditioning (Malkani and Rosen, 2000). For instance, *Nr4a1* expression increases

in the hippocampus CA1 region during contextual fear memory consolidation (Malkani and Rosen, 2000, von Hertzen and Giese, 2005), *Nr4a2* expression increases in CA1 and CA3 hippocampal subregions during the acquisition of a spatial discrimination task (Peña de Ortiz, et al., 2000). Furthermore, a *Nr4a2*-deficient mouse showed impaired contextual fear memory formation (Hawk, et al., 2012). In a PTSD mouse model *Nr4a2* and *Nr4a3* hippocampus expression are increased after PTSD induction compared to the control group (Martinho, et al., 2020). Besides, Ad-deficient mice were also used in this study and had a lower hippocampal expression of the *Nr4a2* and *Nr4a3* genes compared to WT mice induced with PTSD. Furthermore, sotalol (a peripheral β-adrenergic receptor antagonist) decreases PTSD traumatic memory and signs, and also the expression of the *Nr4a1* gene in the hippocampus (Martinho, et al., 2021b).

The NR4A transcription factor induces the expression of several target genes. The brain-derived neurotrophic factor (*Bdnf*) gene is one of the targets of NR4A transcription factors.

1.11.2 Brain-derived neurotrophic factor

The *Bdnf* gene encodes for the BDNF protein which is implicated in neurogenesis, cell proliferation, and neuronal survival and maturation (Katoh-Semba, et al., 2002, Lee, et al., 2007, Lindsay, et al., 1994). Furthermore, BDNF also supports spine morphology modifications by increasing the number, size and complexity of dendritic spines (Alonso, et al., 2004, Horch and Katz, 2002), possibly being involved in synaptic consolidation in memory formation (Bramham and Messaoudi, 2005). Beyond neurons, BDNF can also be found in glial cells, muscles, heart, and lungs (Lindsay, et al., 1994, Maisonpierre, et al., 1990).

In the central nervous system, *Bdnf* expression occurs in many regions, like the cerebellum, cerebral cortex, and amygdala (Dugich-Djordjevic, et al., 1995, Hofer, et al., 1990, Kawamoto, et al., 1996). Notwithstanding, the hippocampus is where it is expressed the most. However, the expression of this neurotrophin, and the consequent BDNF release, is stimuli-induced and does not occur constitutively (Lewin and Barde, 1996). Stimuli like epileptic seizures, ischemia, stress, and exercise, among others, have been shown to increase *Bdnf* gene expression (Isackson, et al., 1991, Lauterborn, et al., 1998, Metsis, et al., 1993, Oliff, et al., 1998, Smith, et al., 1995).

Regarding memory formation, the *Bdnf* gene is suggested to play an important role in hippocampus-dependent memory including contextual fear conditioning. Inside contextual fear conditioning studies, contextual fear memory strength was already related

to increased *Bdnf* gene expression in the CA1 hippocampal subregion after context day of the contextual fear conditioning paradigm (Hall, et al., 2000). Additionally, interfering with *Bdnf* gene expression in the hippocampus was shown to decrease contextual fear memory without interfering with acquisition day of contextual fear paradigm (Heldt, et al., 2007). In the mouse model for PTSD, PTSD-induced mice showed increased contextual traumatic memory followed by increased hippocampus *Bdnf* gene expression (Takei, et al., 2011).

AIMS

Exposure to stressful situations can lead to the formation of contextual traumatic memories. Aversive memories are best retained if Ad is present at the same time as the aversive stimulus. Ad-deficient mice have reduced contextual fear memory 24 hours after training (Alves, et al., 2016, Toth, et al., 2013). However, it is not understood if Ad deficiency affects old memories. Our first aim was to elucidate the role of Ad in the formation of contextual old memories (one month after fear acquisition) (Manuscript I – Chapter II).

Furthermore, past reports from our group also showed that Ad may increase blood glucose levels and enhance contextual fear memory by acting selectively on peripheral β_2-adrenoceptors (Alves, et al., 2016). However, the influence of β_2-adrenoceptor antagonists in contextual fear memory was still unclear. Accordingly, it was also an objective of this work to evaluate if a β_2-adrenoceptor antagonist (ICI 118,551) could prevent glucose release into the bloodstream after the fear acquisition, and consequently, impair contextual fear memory (Manuscript I – Chapter II).

On the other hand, molecular pathways may influence contextual fear memory. The process for memory formation and the conversion of STM into a LTM implicate new mRNA and protein synthesis (Dudai, 2002). The learning and memory consolidation processes usually involves the expression of several genes, such as the NR4A family of transcription factors, which have been implicated in the formation of contextual fear memory (Hawk, et al., 2012). Thus, another aim of this work was to understand if Ad modulates the transcription of *Nr4a1*, *Nr4a2,* and *Nr4a3* genes after the fear conditioning procedure in hippocampus samples (Manuscript I – Chapter II).

Although all the evidence indicates the possible involvement of Ad in strengthening contextual fear memory, Ad does not appears to cross from the peripheral circulation into the central nervous system (Weil-Malherbe, et al., 1959), and therefore, is not expected to have a direct effect on strengthening contextual fear memory. One proposed mechanism suggests glucose as the mediator of Ad central nervous effects (Morris and Gold, 2013). It was demonstrated that Ad may increase blood glucose concentration by the activation of β_2-adrenoceptors (Alves, et al., 2016). However, no previous study truly separated the effect of Ad and glucose in the strengthening of contextual fear memory. Therefore, this study aimed to use Ad-deficient mice to evaluate if glucose alone strengthens contextual fear memory and if there is a synergic interaction between Ad and glucose in strengthening contextual fear memory (Manuscript II – Chapter III). Simultaneously, it was also a purpose of this work to evaluate the effects of glucose in

the hippocampus expression of contextual fear memory-related genes, such as *Nr4a1*, *Nr4a2*, *Nr4a3*, and *Bdnf* (Manuscript II – Chapter III).

One mechanism by which glucose may be a mediator of peripheral Ad in contextual fear memory strengthening is by acting as a substrate for the synthesis of neuromodulators such as acetylcholine (Ragozzino, et al., 1998, Ragozzino, et al., 1996). Muscarinic receptors activation by acetylcholine enhances learning and memory formation (Cangioli, et al., 2002, Passani, et al., 2001, Power, et al., 2003). Few studies relate the effect of peripheral Ad and central acetylcholine in memory strengthening (Introini-Collison and Baratti, 1992, Introini-Collison and McGaugh, 1988). However, none of them was capable of accurately evaluating the acetylcholine effect independently of Ad. Therefore, in this rationale, we also aimed to evaluate if the relative mRNA expression of hippocampus *M1*, *M2*, and *M4* muscarinic receptors is altered in Ad-deficient mice (Manuscript III – Chapter IV). Additionally, another aim was to understand if the cholinergic system is involved in the strengthening of contextual fear memory by peripheral Ad, using a non-selective muscarinic receptor antagonist (atropine) in the contextual fear conditioning procedure (Manuscript III – Chapter IV).

The increase in blood glucose induces the release of insulin into the bloodstream, and insulin itself appears to enhance memory and improve cognitive performance (Zhao, et al., 1999). Moreover, there seems to be a close interaction between blood glucose concentration attained during acute insulin-induced hypoglycaemia and the rise in plasma Ad (Galbo, et al., 1979). Taking it all together, it has been difficult to detach the Ad and insulin action in contextual fear memory. Therefore, another aim of this work was to elucidate the role of insulin on contextual fear memory using Ad-deficient mice in the fear conditioning procedure (Manuscript IV– Chapter V). Furthermore, the molecular mechanisms by which insulin affects contextual fear memory was also an objective of this work, therefore the hippocampus expression of contextual fear memory-related genes, such as *Nr4a1*, *Nr4a2*, *Nr4a3*, and *Bdnf* were also evaluated (Manuscript IV– Chapter V).

MANUSCRIPTS

The results obtained in this thesis were published as original research papers in peer-reviewed JCR international journals, as follows:

Manuscript 1: **Oliveira, A**.*, Martinho, R., Serrão, P., & Moreira-Rodrigues, M. (2018). Epinephrine released during traumatic events may strengthen contextual fear memory through increased hippocampus mRNA expression of *Nr4a* transcription factors. Frontiers in molecular neuroscience, 11, 334. Q1 in Cellular and Molecular Neuroscience and Molecular Biology.

Manuscript 2: **Oliveira, A**.*, Azevedo, M., Seixas, R., Martinho, R., Serrão, P., & Moreira-Rodrigues, M. (2023). Glucose may Contribute to Retrieval and Reconsolidation of Contextual Fear Memory Through Hippocampal *Nr4a3* and *Bdnf* mRNA Expression and May Act Synergically with Adrenaline. Molecular Neurobiology, 12035. Q1 in Neurosciences.

Manuscript 4: **Oliveira, A**.*, Seixas, R., Pereira, F., Azevedo, M., Martinho, R., Serrão, P., & Moreira-Rodrigues, M. (2023). Insulin enhances contextual fear memory independently of its effect in increasing plasma adrenaline. Life Sciences, 121881. Q1 in Medicine, Research and Experimental and Pharmacology and Pharmacy.

*Oliveira A contributed significantly to the experimental design, data acquisition, and interpretation of the results obtained, as well as in writing and revising the manuscript.

CHAPTER 2 - MANUSCRIPT I

EPINEPHRINE RELEASED DURING TRAUMATIC EVENTS MAY STRENGTHEN CONTEXTUAL FEAR MEMORY THROUGH INCREASED HIPPOCAMPUS mRNA EXPRESSION OF *NR4A* TRANSCRIPTION FACTORS

Front Mol Neurosci. 2018 Sep. 25; 11:334.
doi: 10.3389/fnmol.2018.00334.
Q1 in Cellular and Molecular Neuroscience and Molecular Biology.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 25 September 2018
doi: 10.3389/fnmol.2018.00334

Epinephrine Released During Traumatic Events May Strengthen Contextual Fear Memory Through Increased Hippocampus mRNA Expression of *Nr4a* Transcription Factors

Ana Oliveira[1,2], *Raquel Martinho*[1,2], *Paula Serrão*[2,3] *and Mónica Moreira-Rodrigues*[1,2]*

[1]*Laboratory of General Physiology, Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS/UP), Porto, Portugal,* [2]*Center for Drug Discovery and Innovative Medicines, University of Porto (MedInUP), Porto, Portugal,* [3]*Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal*

Epinephrine (EPI) strengthens contextual fear memories by acting on peripheral β_2-adrenoceptors. Phenylethanolamine-*N*-methyltransferase-knockout (Pnmt-KO) mice are EPI-deficient mice and have reduced contextual fear learning. Our aim was to evaluate the molecular mechanisms by which peripheral EPI strengthens contextual fear memory and if a β_2-adrenoceptor antagonist can erase contextual fear memories. Pnmt-KO and wild-type (WT) mice were submitted to fear conditioning (FC) procedure after treatment with EPI, norepinephrine (NE), EPI plus ICI 118,551 (selective β_2-adrenoceptor antagonist), ICI 118,551 or vehicle (NaCl 0.9%). Catecholamines were separated and quantified by high performance liquid chromatography-electrochemical detection (HPLC-ED). Blood glucose was measured by coulometry. Real-time polymerase chain reaction (qPCR) was used to evaluate mRNA expression of nuclear receptor 4a1 (*Nr4a1*), *Nr4a2* and *Nr4a3* in hippocampus samples. In WT mice, plasma EPI concentration was significantly higher after fear acquisition (FA) compared with mice without the test. NE did not increase in plasma after FA and did not strengthen contextual fear memory, contrary to EPI. Freezing induced by EPI was blocked by ICI 118,551 in Pnmt-KO mice. In WT mice, ICI 118,551 blocked blood glucose release into the bloodstream after FA and decreased contextual fear memory. *Nr4a1*, *Nr4a2* and *Nr4a3* mRNA expression decreased in Pnmt-KO mice compared with WT mice after FC procedure. In Pnmt-KO mice, EPI induced an increase in mRNA expression of *Nr4a2* compared to vehicle. In conclusion, EPI increases in plasma after an aversive experience, possibly improving long-term and old memories, by acting on peripheral β_2-adrenoceptors. Glucose could be the mediator of peripheral EPI in the central nervous system, inducing the expression of *Nr4a* transcription factor genes involved in consolidation of contextual fear memories.

Keywords: epinephrine, contextual fear memory, traumatic experience, β_2-adrenoceptors, *Nr4a* transcription factors, phenylethanolamine-*N*-methyltransferase-knockout mice

OPEN ACCESS

Edited by:
Ramiro Angel Maria Freudenthal, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Clarissa Carlin Parker, Middlebury College, United States
Jeffrey B. Rosen, University of Delaware, United States

***Correspondence:**
Mónica Moreira-Rodrigues
mirodrigues@icbas.up.pt

Received: *18 May 2018*
Accepted: *27 August 2018*
Published: *25 September 2018*

Citation:
Oliveira A, Martinho R, Serrão P and Moreira-Rodrigues M (2018) Epinephrine Released During Traumatic Events May Strengthen Contextual Fear Memory Through Increased Hippocampus mRNA Expression of Nr4a Transcription Factors. Front. Mol. Neurosci. 11:334. doi: 10.3389/fnmol.2018.00334

INTRODUCTION

During and after a traumatic experience, fear is a natural reaction. The fear and stress responses enable the organism to react, being responsible for "fight-or-flight" response (Fuller et al., 2010). This response is important for survival and adaptation in nature. Fear conditioning (FC) is a good paradigm to study emotional memories (Quillfeldt, 2016). In this behavioral experiment, animals learn to associate an aversive stimulus (electric shock) to a specific context (Pearce and Hall, 1980). This procedure can be used to study the re-experiencing phenomenon upon exposure to reminders of the trauma (but not the traumatic event itself; VanElzakker et al., 2014).

Stress leads to activation of the sympathetic nervous system which results in an increase of heart rate, contractility, vasoconstriction and release of hormones to bloodstream (Ayada et al., 2015). It has been suggested in some studies that epinephrine (EPI) is an important hormone for memory consolidation in animals and human subjects (Cahill and Alkire, 2003; Dornelles et al., 2007). Furthermore, we also have shown that mice deficient in EPI (phenylethanolamine-*N*-methyltransferase-knockout, Pnmt-KO mice) have reduced contextual fear learning (Toth et al., 2013; Alves et al., 2016). In addition, we found that peripheral EPI strengthens contextual fear memory 1 day after fear acquisition (FA), by acting specifically on peripheral β_2-adrenoceptors (Alves et al., 2016).

However, EPI is a hydrophilic hormone and does not cross the blood-brain barrier (Weil-Malherbe et al., 1959). One of the hypotheses is that glucose may mediate EPI action in the central nervous system (Morris and Gold, 2013). Indeed, we showed that after FA, wild-type (WT) mice had higher glycemic variation than Pnmt-KO mice, possibly due to EPI release in WT mice after the traumatic event (Alves et al., 2016). EPI may induce liver cells to release glucose into the bloodstream (Hall and Gold, 1986, 1992) and glucose may lead to a subsequent increase of the energy source in the central nervous system and may enhance contextual fear learning (McNay and Gold, 2002; Kong et al., 2017). Downstream molecular processes seem to be a requirement for consolidation and persistence of long-term memories and may result in synaptic remodeling (Dudai, 2002; de la Fuente et al., 2015). Nuclear receptor 4a (*Nr4a*) is a family of transcription factors that has been widely implicated in fear learning and memory (Hawk and Abel, 2011; McNulty et al., 2012). Hawk and Abel showed an increase of the expression of the *Nr4a* family genes in the hippocampus after contextual fear learning (Hawk et al., 2012).

The present study focuses in understanding the mechanism of EPI in strengthening contextual long-term memory (1 day after FA) and old memories (1 month after FA).

MATERIALS AND METHODS

Animals

All animal care and experimental protocols were carried out in accordance with European Directive number 63/2010/EU, transposed to Portuguese legislation by Directive Law 113/2013, and approved by the Organism Responsible for Animal Welfare of Faculty of Medicine, University of Porto. The Pnmt-KO mice ($Pnmt^{-/-}$) were produced by the insertion of Cre-recombinase gene into the locus encoding for Pnmt enzyme, creating a functional KO of Pnmt expression, with loss of EPI in homozygous $Pnmt^{-/-}$. Steven N. Ebert kindly provided a couple of Pnmt-KO mice and animals were bred in our conventional vivarium. Genotypes at the Pnmt locus were identified by polymerase chain reaction (PCR) of ear DNA, as previously described (Ebert et al., 2004). Pnmt-KO (n = 92) and WT (n = 70) male mice (129×1/SvJ) were kept under controlled environmental conditions (12 h light/dark cycle, room temperature 23 ± 1°C, humidity 50%, autoclaved drinking water, mice diet 4RF25/I and 4RF21/A; Mucedola, Porto, Portugal) and housed with the respective litter.

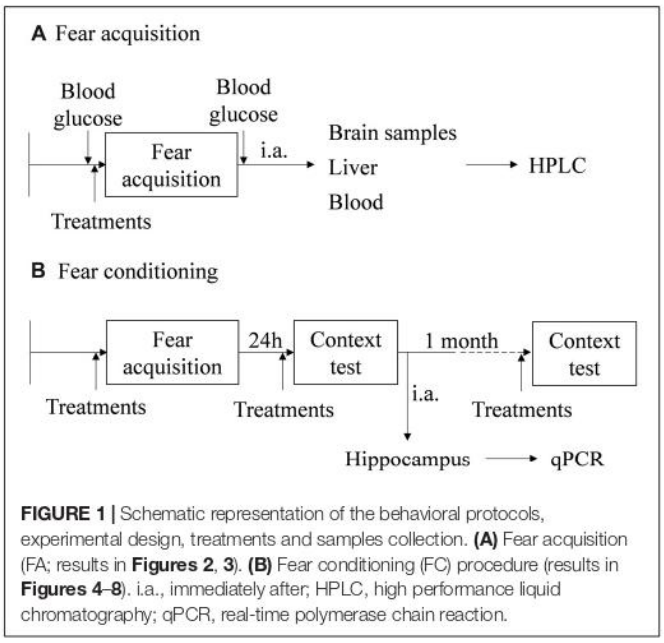


FIGURE 1 | Schematic representation of the behavioral protocols, experimental design, treatments and samples collection. **(A)** Fear acquisition (FA; results in **Figures 2**, **3**). **(B)** Fear conditioning (FC) procedure (results in **Figures 4**–**8**). i.a., immediately after; HPLC, high performance liquid chromatography; qPCR, real-time polymerase chain reaction.

Fear Conditioning Procedure

The FC procedure was performed as previously described (Lukoyanov and Lukoyanova, 2006; Manceau et al., 2012). The conditioning chamber consisted of a clear Plexiglas box equipped with a metal grid floor, wired to a stimulus generator. The mice behavior was recorded with a digital video camera Sony HDR-CX405 (Sony Corporation, Japan). On the first day (FA;

TABLE 1 | Primers used in gene expression analysis.

Gene	Primer (5'→3')
Nr4a1	F: AAAATCCCTGGCTTCATTGAG
	R: TTTAGATCGGTATGCCAGGCG
Nr4a2	F: CGGTTTCAGAAGTGCCTAGC
	R: TTGCCTGGAACCTGGAATAG
Nr4a3	F: GTGGCTCGACTCCATTAAAGAC
	R: GTGCATAGCTCCTCCACTCTCT
Gapdh	F: CCATCACCATCTTCGAGGAG
	R: GCATGGACTGTGGTCATGAG

Nr4, Nuclear receptor 4; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; F, forward primer and R, reverse primer.

6 min (min)), mice had a period of 3 min undisturbed followed by a tone (conditioned stimulus: 80 dB; 2.8 kHz) for 20 s that co-terminated with a foot shock (unconditioned stimulus: 2 s; 0.5 mA). Three tone-shock pairings (conditioning trials) were presented at intervals of 40 s. On the second day (context fear test; 8 min, long-term memory) or 1 month later (context fear test; 8 min, old memories), mice were re-exposed to the conditioning chamber with identical contextual features and no shocks or tones were presented (freezing was scored for the duration of the session). Freezing was defined as the absence of movement except for respiration for at least 3 s (Valentinuzzi et al., 1998; Curzon et al., 2009). The percentage of accumulated freezing time was then calculated. Vocalization response was defined as the audible vocalization in response to the shock, and jump response was defined as the removal of at least three paws from the grid floor (Rocinholi et al., 1997). On the first and second day the chambers were cleaned and wiped with 1% acetic acid.

Behavioral Treatments

Freezing behavior was quantified in WT and Pnmt-KO mice submitted or not to FA. Pnmt-KO mice was submitted to FA after pre-training treatment with EPI (0.1 mg/kg, i.p., 3 min; Lee et al., 2001), ICI 118,551 (2.0 mg/kg, i.p., 30 min; Stone et al., 1996; Zhu et al., 2014) or with vehicle (0.9% NaCl). Another group of WT and Pnmt-KO mice were submitted to FC procedure after EPI (0.1 mg/kg, i.p., 3 min; Lee et al., 2001), norepinephrine (NE, 0.1 mg/Kg, i.p., 3 min; Murchison et al., 2004), EPI (0.1 mg/kg, i.p., 3 min) plus ICI 118,551 (2.0 mg/kg, i.p., 30 min), ICI 118,551 (2.0 mg/kg, i.p., 30 min; Stone et al., 1996; Zhu et al., 2014) or vehicle (0.9% NaCl) treatment, in both pre-training and pre-testing. Long-term memory (context fear test, 1 day after FA) and old memories (context fear test, 1 month after FA) were evaluated. The control groups received the same number of vehicle (0.9% NaCl) injections with the same time interval of the treated groups. A diagram of the experimental design, treatments and samples collection is in **Figure 1**.

Catecholamine Assay

Immediately after FA, mice were anesthetized (ketamine, 100 mg/kg and xylazine, 10 mg/kg; i.p.) and the left adrenal was collected and emerged in percloric acid (PCA; 0.2 M) overnight, at 4°C. Afterwards, the supernatant was centrifuged for 2 min (2,700× *g* and 4°C) and diluted. In some experiments blood was collected by left ventricle puncture to a heparinized tube and the samples were centrifuged (15 min, 4°C and 1,000× *g*) and kept under −80 °C until further use. In another experiment, and immediately after FA, liver, hippocampus, amygdala and frontal lobe were collected and emerged in PCA (0.2 M) overnight, at 4°C. The catecholamines present in plasma and tissue samples were concentrated by

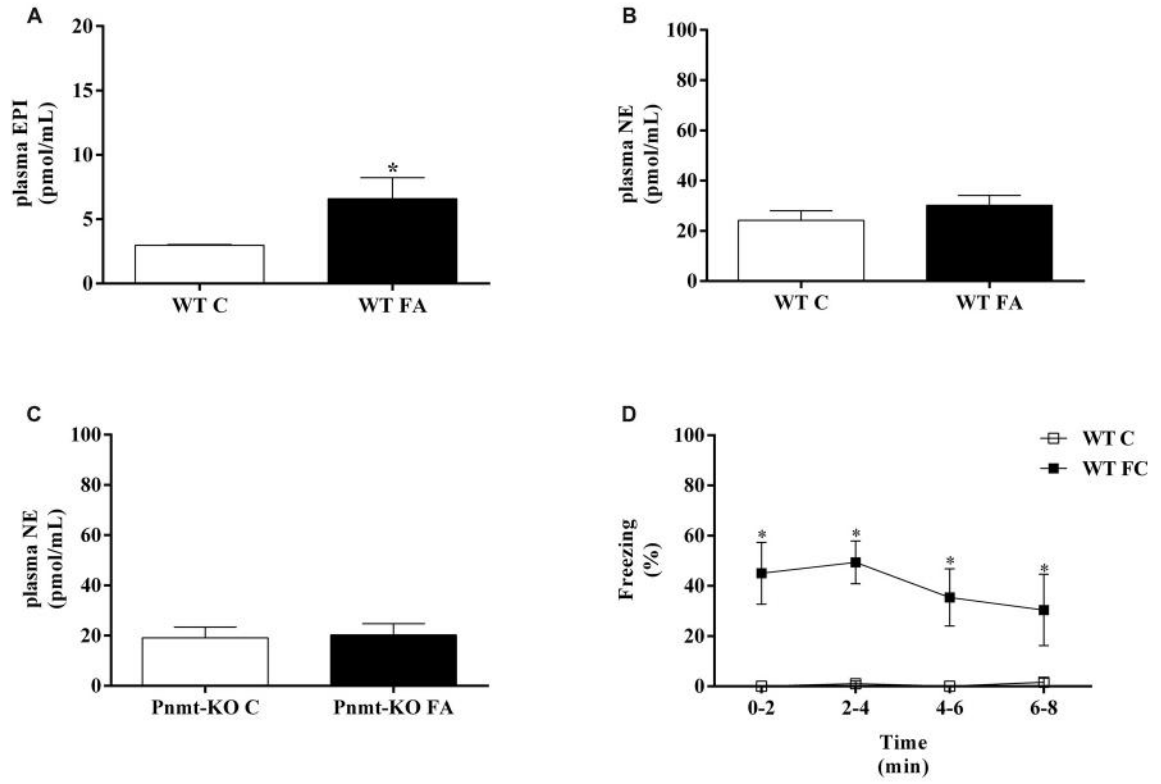


FIGURE 2 | Plasma **(A)** epinephrine (EPI) and **(B,C)** norepinephrine (NE) in wild-type (WT) or phenylethanolamine-*N*-methyltransferase-knockout (Pnmt-KO) mice submitted or not (control, C) to FA of FC procedure. **(D)** Freezing on context fear test in WT mice submitted or not (control, C) to FC procedure. Values are means ± standard error of the means (SEM) of 4–6 mice per group. *Significantly different from correspondent control (C, without FA and FC) values ($p < 0.05$).

TABLE 2 | Concentration of adrenal catecholamines in phenylethanolamine-*N*-methyltransferase-knockout (Pnmt-KO) or wild-type (WT) mice after FA of fear conditioning (FC) procedure.

	EPI (pmol/mL)		NE (pmol/mL)	
	C	FA	C	FA
WT	19.42 ± 3.50	16.91 ± 3.24	8.86 ± 1.73	8.34 ± 2.07
Pnmt-KO	Undetectable	Undetectable	17.42 ± 1.93*	16.85 ± 3.05*

*Values are means ± standard error of the means (SEM) of 5–6 mice per group. C, control mice without fear acquisition test; FA, fear acquisition; EPI, epinephrine; NE, norepinephrine. *Significantly different from correspondent values in WT mice ($p < 0.05$).*

alumina, as previously described (Moreira-Rodrigues et al., 2010, 2014). Catecholamines were separated by reverse-phase high performance liquid chromatography (HPLC) and quantified by electrochemical detection (ED). The detection limit was between 350 fmol and 1,000 fmol.

Blood Glucose

Blood glucose concentration was determined immediately before and after the FA in conscious animals in rear paw, by coulometry (FreeStyle Freedom Lite; Alva, 2008). Afterwards, glycemic variation (ΔGlycemia) was calculated as the glucose concentration difference after and before the FA.

RNA Isolation and Relative Quantification of mRNA Expression

Real-time PCR (qPCR) was performed in hippocampus samples collected immediately after context fear test of FC procedure, as previously described (Moreira-Rodrigues et al., 2007; Mendes et al., 2018). Total RNA isolation was carried out with the SV Total RNA Isolation System kit (Promega, Fitchburg, WI, USA). Concentration and purity of the isolated RNA were measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription was performed in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) using a Reverse Transcription kit (Thermo Scientific, Waltham, MA, USA). qPCR reactions were carried out in StepOne™ RT-PCR System (Applied BioSystems, Waltham, MA, USA). Gene-specific primers (5 μM), Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA), Nuclease-free H_2O (Thermo Scientific, Waltham, MA, USA) were mixed and cDNA was added (1:20). Instead of cDNA, Nuclease-free water (Thermo Scientific, Waltham, MA, USA) was added as a negative control. Gene specific primers are in **Table 1**. Results of mRNA quantification are expressed in an arbitrary unit (AU) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Drugs

(−)-EPI (+)-bitartrate salt, L-(-)-NE (+)-bitartrate salt monohydrate, and ICI 118,551 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ketamine (Imalgene 1000, Merial, Lisboa, Portugal) and xilazine (Rompum 2%, Bayer, Lisboa, Portugal).

Statistical Analysis

Results are presented as means ± standard error of the means (SEM) for the indicated number of determinations. Catecholamine concentrations, blood glycemic variation and qPCR results were analyzed by unpaired Student's *t*-test. Behavioral data was analyzed by two-way ANOVA. For multiple comparisons we used Sidak's test (two groups) or Tukey's test (three groups). $p < 0.05$ was assumed to denote a significant difference. GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analysis.

RESULTS

Plasma EPI Increased After Fear Acquisition of Fear Conditioning Procedure

In the WT mice, plasma EPI concentration was significantly higher after FA compared with mice without the test ($t_{(8)} = 4.97$, $p = 0.001$; **Figure 2A**). However, plasma NE concentrations were

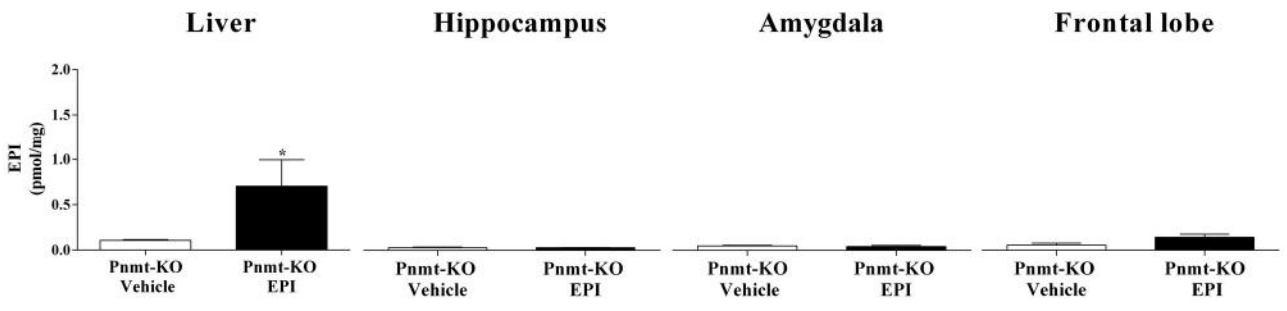


FIGURE 3 | Concentration of EPI in liver, hippocampus, amygdala and frontal lobe after FA of FC procedure in Pnmt-KO mice treated with EPI (0.1 mg/kg) or vehicle (0.9% NaCl). Values are means ± SEM of five mice per group. *Significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$). EPI content in both treatments of cerebral tissues and in vehicle treatment in liver samples correspond to baseline levels of HPLC technique.

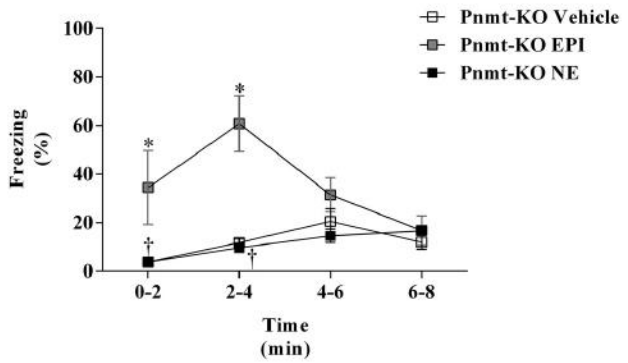


FIGURE 4 | Freezing on the second day (context fear test) of FC procedure in Pnmt-KO mice treated with EPI (0.1 mg/kg), NE (0.1 mg/kg) or vehicle (NaCl 0.9%). Values are means ± SEM of 6–10 mice per group. *Significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice treated with EPI ($p < 0.05$).

not different in both WT and Pnmt-KO mice, with or without FA (**Figures 2B,C**). WT mice submitted to FC procedure showed a significant increase in freezing behavior in FA day of FC procedure compared to WT mice not submitted to FC. It was observed a group ($F_{(1,7)} = 16.8$, $p < 0.04$), time ($F_{(3,21)} = 21.7$, $p < 0.0001$), and a significant interaction between group and time ($F_{(3,21)} = 20.5$, $p < 0.0001$). When re-exposed to the shock context (context fear test) WT mice submitted to FC procedure showed a higher freezing response compared to WT mice not submitted (WT control, **Figure 2D**). It was observed a group effect ($F_{(1,7)} = 20.7$, $p = 0.003$; **Figure 2D**). EPI in plasma and in left adrenal glands (**Table 2**) of Pnmt-KO mice were undetectable. Adrenal NE was significantly increased in Pnmt-KO mice compared to WT mice in both control and FA groups (**Table 2**). WT mice with or without FA did not present any significant differences in adrenal gland content of EPI or NE (**Table 2**).

EPI Does Not Seem to Cross the Blood Brain Barrier Into the Brain Tissue After Fear Acquisition of Fear Conditioning Procedure

Pnmt-KO mice treated with EPI (0.1 mg/Kg; i.p.) submitted to FA showed a significant increase of EPI in plasma (61.4 ± 9.3 vs. 1.0 ± 0.1 pmol/mL; $p = 0.0002$) and liver (**Figure 3**) compared to Pnmt-KO treated with vehicle. Furthermore, no differences were observed in EPI levels in hippocampus, amygdala and frontal lobe (**Figure 3**).

Intraperitoneal Injection of NE Does Not Increase Contextual Fear Memory

On the first day of FC test, no differences were observed between treatments in Pnmt-KO mice. It was only observed a time effect ($F_{(3,45)} = 90.9$, $p = 0.0001$). On the

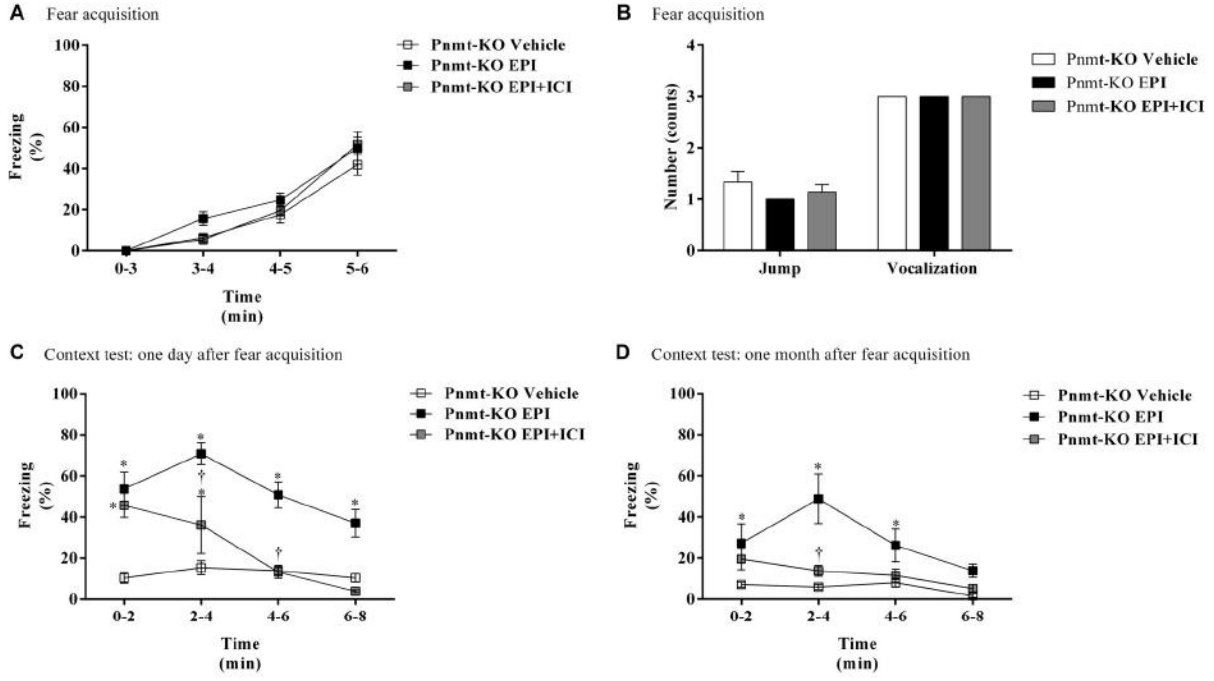


FIGURE 5 | (A) Freezing, **(B)** shock responsivity in FA day, and freezing in context fear test **(C)** 1 day and **(D)** 1 month after FA of FC procedure in Pnmt-KO mice treated with EPI (0.1 mg/kg), EPI plus ICI 118,551 (ICI, selective β_2-adrenoceptor antagonist, 2.0 mg/kg) or vehicle (NaCl 0.9%). Values are means ± SEM of 5–10 mice per group. *Significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$). †Significantly different from correspondent values in Pnmt-KO mice treated with EPI ($p < 0.05$).

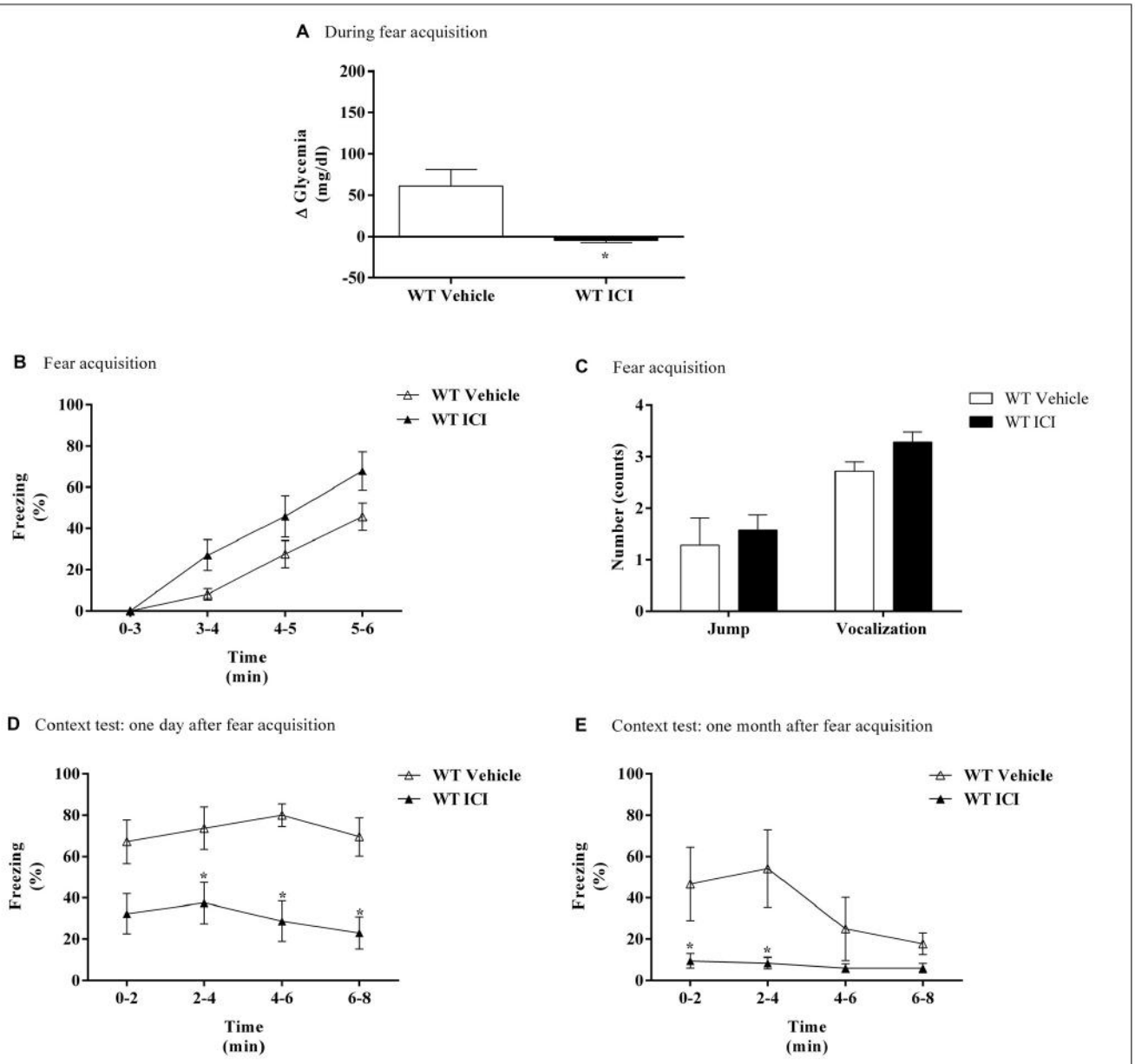


FIGURE 6 | (A) Glycemic variation (Δ Glycemia) after FA, **(B)** freezing, **(C)** shock responsivity in FA day, and freezing in context fear test **(D)** 1 day and **(E)** 1 month after FA of FC procedure in WT mice treated with ICI 118,551 (ICI, selective β_2-adrenoceptor antagonist, 2.0 mg/kg) or vehicle (NaCl 0.9%). Values are means ± SEM of 6–8 mice per group. *Significantly different from correspondent values in WT mice treated with vehicle ($p < 0.05$).

second day of FC procedure, mice were re-exposed to the shock context which induced an increase in freezing in Pnmt-KO mice treated with EPI (0.1 mg/Kg; i.p.) compared to vehicle (**Figure 4**). However, no differences were observed in freezing behavior between Pnmt-KO mice treated with NE (0.1 mg/Kg; i.p.) and vehicle (**Figure 4**). It was observed an effect of drug ($F_{(2,14)} = 16.5$, $p = 0.0002$), time ($F_{(3,42)} = 4.0$, $p = 0.01$) and a significant interaction between drug and time ($F_{(6,42)} = 4.6$, $p = 0.001$; **Figure 4**).

EPI Seems to Strengthen Long-Term and Old Memories Through Activation of β_2-Adrenoceptors

On the first day of FC test, there were no differences in freezing response between treatments (**Figure 5A**). It was only observed a time effect ($F_{(3,45)} = 122.7$, $p < 0.0001$). No differences were observed in shock responsivity (vocalization and jump) between treatments (**Figure 5B**). One day after FA, re-exposure to the shock context induced an increase in freezing in Pnmt-KO mice treated with EPI compared to vehicle and EPI plus ICI

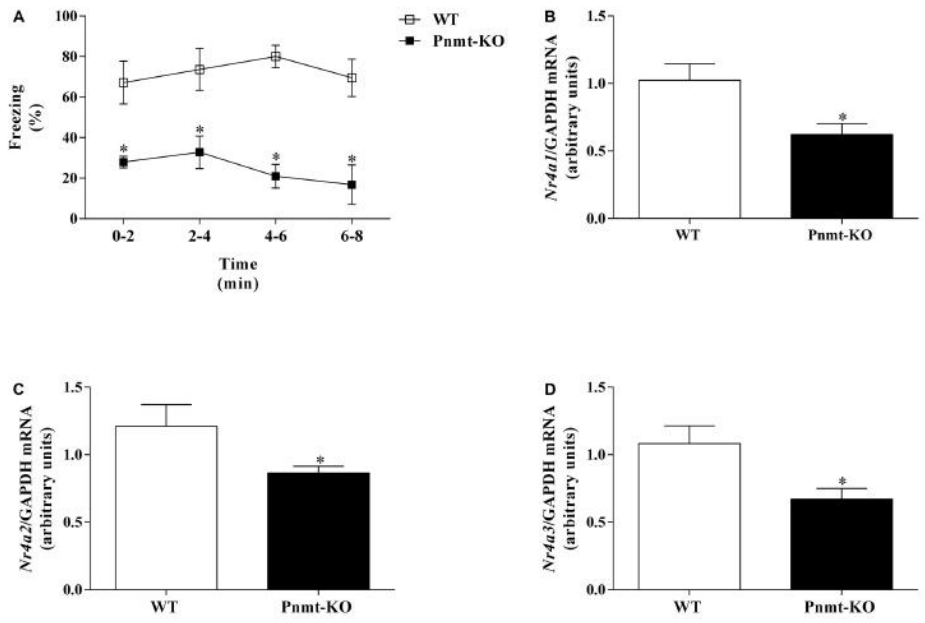


FIGURE 7 | (A) Freezing on context fear test and hippocampus mRNA expression of nuclear receptor 4 (Nr4), namely, **(B)** *Nr4a1*, **(C)** *Nr4a2* and **(D)** *Nr4a3* after context fear test of FC procedure in Pnmt-KO and WT mice. Values are means ± SEM of six mice per group. Results of mRNA are expressed as arbitrary units (AUs) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *Significantly different from correspondent values in WT mice ($p < 0.05$).

118,551 (selective β_2-adrenoceptor antagonist) treated mice. It was observed an effect of drug ($F_{(2,24)} = 45.0$, $p < 0.0001$), time ($F_{(3,72)} = 12.3$, $p < 0.0001$), and a significant interaction between drug and time ($F_{(6,72)} = 4.1$, $p = 0.001$; **Figure 5C**). One month after FA, Pnmt-KO mice treated with EPI still showed a significant freezing compared to the other groups. A significant effect of drug ($F_{(2,15)} = 11.4$, $p = 0.001$), time ($F_{(3,45)} = 8.0$, $p = 0.0002$), and an interaction ($F_{(6,45)} = 3.4$, $p = 0.008$) was observed (**Figure 5D**).

In WT mice, treatment with ICI 118,551 caused a significantly decrease in blood glycemic variation compared with WT mice treated with vehicle after FA (**Figure 6A**). On the first day of FC test no differences were observed in freezing response between treatments (**Figure 6B**). It was only observed a time effect ($F_{(3,39)} = 49.17$, $p < 0.0001$). No differences were observed in shock responsivity (vocalization and jump) between treatments (**Figure 6C**). In context fear test, after re-exposure to shock context the freezing was lower in WT mice treated with ICI 118,551 when compared with WT mice treated with vehicle, in both 1 day and 1 month after FA (**Figures 6D,E**). It was observed a drug effect in both 1 day ($F_{(1,10)} = 14.1$, $p = 0.004$) and 1 month after FA ($F_{(1,9)} = 10.1$, $p = 0.01$; **Figures 6D,E**). It was also observed a time ($F_{(3,27)} = 4.9$, $p = 0.007$) effect and an interaction ($F_{(3,27)} = 3.4$, $p = 0.03$) 1 month after FA (**Figure 6E**).

Peripheral EPI Appears to Enhance Gene Expression in Hippocampus Involved in Consolidation of Contextual Fear Memories

In FA day no differences were observed in freezing response between groups. It was only observed a time effect ($F_{(3,36)} = 59.65$, $p < 0.0001$). One day after FA, Pnmt-KO mice showed a lower freezing behavior compared to WT mice after re-exposure to the shock context (**Figure 7A**). It was observed a genotype ($F_{(1,9)} = 27.5$, $p = 0.0005$) effect (**Figure 7A**). In the hippocampus, mRNA expression of *Nr4a1* (**Figure 7B**), *Nr4a2* (**Figure 7C**) and *Nr4a3* (**Figure 7D**) was significantly decreased in Pnmt-KO mice compared to WT mice.

On the first day of FC test no differences were observed in freezing response between treatments. It was only observed a time effect ($F_{(3,33)} = 128.2$, $p < 0.0001$). One day after FA, re-exposure to shock context induced an increase in freezing in Pnmt-KO mice treated with EPI compared to vehicle treated mice (**Figure 8A**). It was observed a drug effect ($F_{(1,11)} = 48.2$, $p < 0.0001$; **Figure 8A**). In the hippocampus, mRNA expression of *Nr4a2* was significantly increased in Pnmt-KO mice treated with EPI compared to Pnmt-KO mice treated with vehicle (**Figure 8C**). No differences were observed in mRNA expression of *Nr4a1* (**Figure 8B**) and *Nr4a3* (**Figure 8D**) between groups.

DISCUSSION

In WT mice the aversive stimulus was sufficient to induce an increase of EPI plasmatic concentration compared to those not submitted to FA. However, plasma NE did not increase in the same conditions. In addition, the increase of plasmatic EPI after FA was accompanied by a strengthening in contextual fear memory compared to those not submitted to FC procedure. Taking together, these results suggest that after stress EPI is released to the bloodstream, possibly from adrenal medulla, and

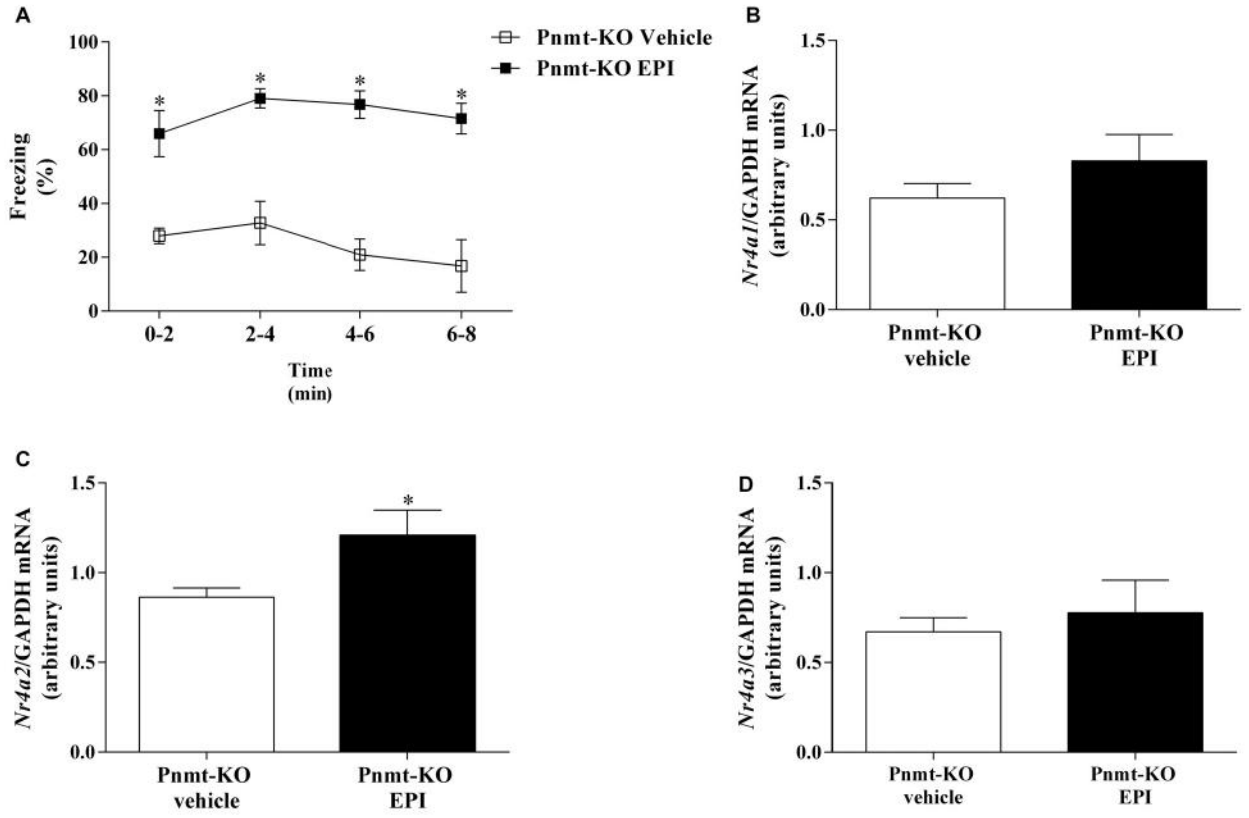


FIGURE 8 | (A) Freezing on contextual fear test and hippocampus mRNA expression of Nr4, namely, **(B)** *Nr4a1*. **(C)** *Nr4a2* and **(D)** *Nr4a3* after context fear test of FC procedure in Pnmt-KO mice treated with EPI (0.1 mg/kg) or vehicle (0.9% NaCl). Values are means ± SEM of 6–7 mice per group. Results of mRNA are expressed as AUs after normalization for Gapdh. *Significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$).

facilitates the formation of emotional contextual fear memories. Accordingly, in mice, i.p. injections of EPI (0.1 mg/kg, i.p.) facilitate retention in inhibitory avoidance test (Introini-Collison et al., 1992). We now show that i.p. injections of EPI strengthen contextual fear memory, contrary to i.p. injections of NE, in similar doses. It appears that peripheral NE is not important for contextual fear memory, opposing the known effects of central NE in memory (McGaugh, 2002; Tully and Bolshakov, 2010).

Furthermore, it was shown that dopamine β-hydroxylase KO mice (deficient in both NE and EPI; Murchison et al., 2004) and later Pnmt-KO mice (deficient in EPI; Toth et al., 2013; Alves et al., 2016) have reduced contextual fear learning. In addition, we found that EPI strengthens contextual fear learning on long-term memory (1 day after FA), by acting specifically on peripheral β_2-adrenoceptors (Alves et al., 2016). Our results show that pre-training and pre-testing treatments with ICI 118,551, a β_2-adrenoceptor antagonist, decreases long-term memory (1 day after FA) and possibly 1-month old fear memories. Thus, EPI seems to be an important mediator of long-term consolidation of fear memory and even old memories.

EPI is a polar molecule that does not cross the blood-brain barrier (Weil-Malherbe et al., 1959) and Pnmt has almost no expression and enzymatic activity in the brain (Ho et al., 1974; Lew et al., 1977). However, there are some evidences that intense stressful situations can lead to physiological changes in blood-brain barrier permeability (Skultétyová et al., 1998; Sántha et al., 2016) and consequently in molecules that normally do not cross the blood-brain barrier (as is the case of EPI) might cross (Hayes et al., 1985). For that reason, we performed an experiment to understand if EPI could cross the blood-brain barrier to the brain tissue after our stress conditions using an EPI deficient (Pnmt-KO) mice. Pnmt-KO mice were submitted to FA and EPI was found in plasma and liver samples in mice injected i.p. with this molecule, but not in Pnmt-KO treated with vehicle. These results indicate that EPI (i.p.) goes into the bloodstream and reaches the hepatic tissue of EPI deficient (Pnmt-KO) mice. However, we found that after FA (stress event), Pnmt-KO mice treated with EPI did not present this catecholamine in many brain regions, namely hippocampus, amygdala, and frontal lobe. Therefore, our results show that EPI does not appear to cross the blood-brain barrier after the acute stress event in our experimental conditions.

In FA (first day of FC procedure) we did not observe any differences in freezing, vocalization and jump responses between treatments, in both Pnmt-KO and WT mice. Our results suggest that pain perception of the foot shocks is not affected

by treatments. These results are in agreement with previous manuscripts (Toth et al., 2013; Alves et al., 2016).

In response to an increase of plasmatic EPI concentration, glycogenolysis and gluconeogenesis processes are stimulated and hepatic glucose production increases (Gray et al., 1980; Dufour et al., 2009). We already showed that i.p. injections of EPI induces an increase in glycemic variation in Pnmt-KO mice, suggesting that glucose may be a downstream mediator of EPI in long-term memory formation and a critical component of fear memory modulation (Alves et al., 2016). Furthermore, we now show in WT mice that i.p. injections of ICI 118,551 (peripheral β_2-adrenoceptor antagonist) is effective in blocking the action of endogenous EPI, not occurring an increase of blood glucose levels nor contextual fear memory formation. Thus, because glucose easily crosses the blood-brain barrier, we emphasize that it can be a mediator of EPI in the central nervous system, since the increase of blood glucose levels may provide additional energy for specific memory mechanisms, leading to an increase of contextual fear learning (Durkin et al., 1992; Pych et al., 2005).

Intensive research suggests that memory consolidation occurs within minutes to weeks after initial learning and may reflect the ongoing changes in the intracellular signaling pathways, gene expression, and new protein synthesis by leading to synaptic modifications and gene plasticity (Dudai, 2002). As we already showed, Pnmt-KO mice have a decrease of contextual fear memory in comparison to WT mice (Alves et al., 2016), and we now show a significant decrease in hippocampus mRNA expression of the three members of *Nr4a* family (*Nr4a1*, *Nr4a2* and *Nr4a3*). This suggests that essential molecular pathways for contextual fear memory formation are not activated due to the absence of EPI release after a traumatic event, and possibly the lack of glucose availability in the brain as the energy source for neuronal mechanisms. When we restored EPI levels with i.p. injections in Pnmt-KO mice, we found an increase in contextual fear memory and a significant increase only in *Nr4a2* mRNA expression in Pnmt-KO mice. This could suggest that the *Nr4a2* gene expression may be sufficient to strengthen contextual fear memory by peripheral EPI. *Nr4a* transcription factors family is increased 2 h after acquisition (Vecsey et al., 2007) and are necessary to contextual fear memory formation (Hawk et al., 2012). We show that *Nr4a* gene family continues upregulated 24 h after fear learning, which suggests their requirement for long-lasting memory storage. Actually, *Nr4a* gene family may function in memory to activate secondary waves of transcription (Vecsey et al., 2007).

Previous reports indicate that the lack of EPI is enough to mimic the impairment of contextual fear learning in the context-dependent conditioned fear, without affecting cue-dependent conditioned fear (Toth et al., 2013; Alves et al., 2016). As EPI, glucose also improves retention of contextual fear learning (but not cued learning; Glenn et al., 2014; Alves et al., 2016). Neuron cells that are under direct control of glucose availability, that appear to be specific to hippocampus (de Araujo, 2014) could be the reason for the elevated mRNA expression of *Nr4a* family genes in hippocampus after FC procedure in mice with peripheral EPI (WT and Pnmt-KO treated with EPI). Our and other results are a strong evidence that EPI presence in a stressful situation leads to an increase of blood glycemia (Sutherland et al., 1968). In addition, the increase of mRNA expression of *Nr4a2* in hippocampus only occurs in mice with EPI (WT mice and in Pnmt-KO mice treated with EPI).

In conclusion, EPI increases in plasma after an aversive situation which may cause an increase in blood glucose by acting in peripheral β_2-adrenoceptors. In turn, glucose may be a mediator of EPI in the central nervous system and could be essential to induce the expression of the *Nr4a* family genes involved in contextual fear memories. β_2-adrenoceptor antagonists decrease long-term contextual fear memory and possibly old memories.

AUTHOR CONTRIBUTIONS

MM-R conceived the study. AO and MM-R performed most of the experiments. RM and PS performed some experiments. AO, MM-R and PS analyzed and interpreted the results and performed the statistical analysis. AO and MM-R wrote the manuscript. MM-R and RM revised the manuscript. All authors have reviewed and approved the final manuscript.

FUNDING

This work was supported by Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS/UP) and Portuguese Foundation for Science and Technology (Grant SFRH/BD/138984/2018 to AO and Grant SFRH/BD/133860/2017 to RM).

ACKNOWLEDGMENTS

We would like to thank Dr. Ester Alves, Dr. António Carlos Ferreira, and Professor Miguel Soares for technical support.

REFERENCES

Alva, S. (2008). FreeStyle lite—a blood glucose meter that requires no coding. *J. Diabetes Sci. Technol.* 2, 546–551. doi: 10.1177/193229680800200402

Alves, E., Lukoyanov, N., Serrão, P., Moura, D., and Moreira-Rodrigues, M. (2016). Epinephrine increases contextual learning through activation of peripheral β2-adrenoceptors. *Psychopharmacology* 233, 2099–2108. doi: 10.1007/s00213-016-4254-5

Ayada, C., Toru, Ü., and Korkut, Y. (2015). The relationship of stress and blood pressure effectors. *Hippokratia* 19, 99–108.

Cahill, L., and Alkire, M. T. (2003). Epinephrine enhancement of human memory consolidation: interaction with arousal at encoding. *Neurobiol. Learn. Mem.* 79, 194–198. doi: 10.1016/s1074-7427(02)00036-9

Curzon, P., Rustay, N. R., and Browman, K. E. (2009). "Cued and contextual fear conditioning for rodents," in *Methods of Behavior Analysis in Neuroscience*, 2nd Edn. ed. J. J. Buccafusco (Boca Raton, FL: CRC Press), 20–37.

de Araujo, I. E. (2014). Contextual fear conditioning: connecting brain glucose sensing and hippocampal-dependent memories. *Biol. Psychiatry* 75, 834–835. doi: 10.1016/j.biopsych.2014.03.024

de la Fuente, V., Federman, N., Zalcman, G., Salles, A., Freudenthal, R., and Romano, A. (2015). NF-κB transcription factor role in consolidation and reconsolidation of persistent memories. *Front. Mol. Neurosci.* 8:50. doi: 10.3389/fnmol.2015.00050

Dornelles, A., de Lima, M. N., Grazziotin, M., Presti-Torres, J., Garcia, V. A., Scalco, F. S., et al. (2007). Adrenergic enhancement of consolidation of object recognition memory. *Neurobiol. Learn. Mem.* 88, 137–142. doi: 10.1016/j.nlm.2007.01.005

Dudai, Y. (2002). Molecular bases of long-term memories: a question of persistence. *Curr. Opin. Neurobiol.* 12, 211–216. doi: 10.1016/s0959-4388(02)00305-7

Dufour, S., Lebon, V., Shulman, G. I., and Petersen, K. F. (2009). Regulation of net hepatic glycogenolysis and gluconeogenesis by epinephrine in humans. *Am. J. Physiol. Endocrinol. Metab.* 297, E231–E235. doi: 10.1152/ajpendo.00222.2009

Durkin, T. P., Messier, C., de Boer, P., and Westerink, B. H. (1992). Raised glucose levels enhance scopolamine-induced acetylcholine overflow from the hippocampus: an *in vivo* microdialysis study in the rat. *Behav. Brain Res.* 49, 181–188. doi: 10.1016/s0166-4328(05)80163-9

Ebert, S. N., Rong, Q., Boe, S., Thompson, R. P., Grinberg, A., and Pfeifer, K. (2004). Targeted insertion of the Cre-recombinase gene at the phenylethanolamine n-methyltransferase locus: a new model for studying the developmental distribution of adrenergic cells. *Dev. Dyn.* 231, 849–858. doi: 10.1002/dvdy.20188

Fuller, M. D., Emrick, M. A., Sadilek, M., Scheuer, T., and Catterall, W. A. (2010). Molecular mechanism of calcium channel regulation in the fight-or-flight response. *Sci. Signal.* 3:ra70. doi: 10.1126/scisignal.2001152

Glenn, D. E., Minor, T. R., Vervliet, B., and Craske, M. G. (2014). The effect of glucose on hippocampal-dependent contextual fear conditioning. *Biol. Psychiatry* 75, 847–854. doi: 10.1016/j.biopsych.2013.09.022

Gray, D., Lickley, H., and Vranic, M. (1980). Physiologic effects of epinephrine on glucose turnover and plasma free fatty acid concentrations mediated independently of glucagon. *Diabetes* 29, 600–609. doi: 10.2337/diab.29.8.600

Hall, J. L., and Gold, P. E. (1986). The effects of training, epinephrine, and glucose injections on plasma glucose levels in rats. *Behav. Neural Biol.* 46, 156–167. doi: 10.1016/s0163-1047(86)90640-0

Hall, J. L., and Gold, P. E. (1992). Plasma glucose levels predict the disrupting effects of adrenoceptor antagonists on enhancement of memory storage. *Eur. J. Pharmacol.* 221, 365–370. doi: 10.1016/0014-2999(92)90724-i

Hawk, J. D., and Abel, T. (2011). The role of NR4A transcription factors in memory formation. *Brain Res. Bull.* 85, 21–29. doi: 10.1016/j.brainresbull.2011.02.001

Hawk, J. D., Bookout, A. L., Poplawski, S. G., Bridi, M., Rao, A. J., Sulewski, M. E., et al. (2012). NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors. *J. Clin. Invest.* 122, 3593–3602. doi: 10.1172/jci64145

Hayes, R., Pechura, C., Povlishock, J., and Becker, D. (1985). "Changes in blood-brain barrier function associated with conditioned fear in rats," in *Brain Edema*, eds Y. Inaba, I. Klatzo and M. Spatz (Berlin, Heidelberg: Springer), 224–227.

Ho, T., Fuxe, K., Goldstein, M., and Johansson, O. (1974). Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res.* 66, 235–251. doi: 10.1016/0006-8993(74)90143-7

Introini-Collison, I., Saghafi, D., Novack, G. D., and McGaugh, J. L. (1992). Memory-enhancing effects of post-training dipivefrin and epinephrine: involvement of peripheral and central adrenergic receptors. *Brain Res.* 572, 81–86. doi: 10.1016/0006-8993(92)90454-h

Kong, L., Zhao, Y., Zhou, W.-J., Yu, H., Teng, S.-W., Guo, Q., et al. (2017). Direct neuronal glucose uptake is required for contextual fear acquisition in the dorsal hippocampus. *Front. Mol. Neurosci.* 10:388. doi: 10.3389/fnmol.2017.00388

Lee, H. J., Berger, S. Y., Stiedl, O., Spiess, J., and Kim, J. J. (2001). Post-training injections of catecholaminergic drugs do not modulate fear conditioning in rats and mice. *Neurosci. Lett.* 303, 123–126. doi: 10.1016/s0304-3940(01)01733-5

Lew, J., Matsumoto, Y., Pearson, J., Goldstein, M., Hökfelt, T., and Fuxe, K. (1977). Localization and characterization of phenylethanolamine N-methyl transferase in the brain of various mammalian species. *Brain Res.* 119, 199–210. doi: 10.1016/0006-8993(77)90100-7

Lukoyanov, N. V., and Lukoyanova, E. A. (2006). Retrosplenial cortex lesions impair acquisition of active avoidance while sparing fear-based emotional memory. *Behav. Brain Res.* 173, 229–236. doi: 10.1016/j.bbr.2006.06.026

Manceau, V., Kremmer, E., Nabel, E. G., and Maucuer, A. (2012). The protein kinase KIS impacts gene expression during development and fear conditioning in adult mice. *PLoS One* 7:e43946. doi: 10.1371/journal.pone.0043946

McGaugh, J. L. (2002). Memory consolidation and the amygdala: a systems perspective. *Trends Neurosci.* 25, 456–461. doi: 10.1016/s0166-2236(02)02211-7

McNay, E. C., and Gold, P. E. (2002). Food for thought: fluctuations in brain extracellular glucose provide insight into the mechanisms of memory modulation. *Behav. Cogn. Neurosci. Rev.* 1, 264–280. doi: 10.1177/1534582302238337

McNulty, S. E., Barrett, R. M., Vogel-Ciernia, A., Malvaez, M., Hernandez, N., Davatolhagh, M. F., et al. (2012). Differential roles for *Nr4a1* and *Nr4a2* in object location vs. object recognition long-term memory. *Learn. Mem.* 19, 588–592. doi: 10.1101/lm.026385.112

Mendes, P., Martinho, R., Leite, S., Maia-Moço, L., Leite-Moreira, A. F., Lourenco, A. P., et al. (2018). Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice. *Int. J. Cardiol.* 253, 113–119. doi: 10.1016/j.ijcard.2017.10.014

Moreira-Rodrigues, M., Graça, A. L., Ferreira, M., Afonso, J., Serrão, P., Morato, M., et al. (2014). Attenuated aortic vasodilation and sympathetic prejunctional facilitation in epinephrine-deficient mice: selective impairment of β2-adrenoceptor responses. *J. Pharmacol. Exp. Ther.* 351, 243–249. doi: 10.1124/jpet.114.217281

Moreira-Rodrigues, M., Quelhas-Santos, J., Serrão, P., Fernandes-Cerqueira, C., Sampaio-Maia, B., and Pestana, M. (2010). Glycaemic control with insulin prevents the reduced renal dopamine D1 receptor expression and function in streptozotocin-induced diabetes. *Nephrol. Dial. Transplant.* 25, 2945–2953. doi: 10.1093/ndt/gfq150

Moreira-Rodrigues, M., Roncon-Albuquerque, R. Jr., Henriques-Coelho, T., Lourenco, A. P., Sampaio-Maia, B., Santos, J., et al. (2007). Cardiac remodeling and dysfunction in nephrotic syndrome. *Kidney Int.* 71, 1240–1248. doi: 10.1038/sj.ki.5002204

Morris, K. A., and Gold, P. E. (2013). Epinephrine and glucose modulate training-related CREB phosphorylation in old rats: relationships to age-related memory impairments. *Exp. Gerontol.* 48, 115–127. doi: 10.1016/j.exger.2012.11.010

Murchison, C. F., Zhang, X. Y., Zhang, W. P., Ouyang, M., Lee, A., and Thomas, S. A. (2004). A distinct role for norepinephrine in memory retrieval. *Cell* 117, 131–143. doi: 10.1016/s0092-8674(04)00259-4

Pearce, J. M., and Hall, G. (1980). A model for Pavlovian learning: variations in the effectiveness of conditioned but not of unconditioned stimuli. *Psychol. Rev.* 87, 532–552. doi: 10.1037/0033-295x.87.6.532

Pych, J. C., Chang, Q., Colon-Rivera, C., Haag, R., and Gold, P. E. (2005). Acetylcholine release in the hippocampus and striatum during place and response training. *Learn. Mem.* 12, 564–572. doi: 10.1101/lm.33105

Quillfeldt, J. A. (2016). "Behavioral methods to study learning and memory in rats," in *Rodent Model as Tools in Ethical Biomedical Research*, eds M. L. Andersen and S. Tufik (Switzerland: Springer International Publishing), 271–311.

Rocinholi, L. F., Almeida, S. S., and De-Oliveira, L. M. (1997). Response threshold to aversive stimuli in stimulated early protein-malnourished rats. *Braz. J. Med. Biol. Res.* 30, 407–413. doi: 10.1590/s0100-879x1997000300016

Sántha, P., Veszelka, S., Hoyk, Z., Mészáros, M., Walter, F. R., Tóth, A. E., et al. (2016). Restraint stress-induced morphological changes at the blood-brain barrier in adult rats. *Front. Mol. Neurosci.* 8:88. doi: 10.3389/fnmol.2015.00088

Skultétyová, I., Tokarev, D., and Jezová, D. (1998). Stress-induced increase in blood-brain barrier permeability in control and monosodium glutamate-treated rats. *Brain Res. Bull.* 45, 175–178. doi: 10.1016/s0361-9230(97)00335-3

Stone, E. A., Rhee, J., and Quartermain, D. (1996). Blockade of effect of stress on risk assessment behavior in mice by a β-1 adrenoceptor antagonist. *Pharmacol. Biochem. Behav.* 55, 215–217. doi: 10.1016/s0091-3057(96)00070-6

Sutherland, E. W., Robison, G. A., and Butcher, R. W. (1968). Some aspects of the biological role of adenosine 3′, 5′-monophosphate (cyclic AMP). *Circulation* 37, 279–306. doi: 10.1161/01.cir.37.2.279

Toth, M., Ziegler, M., Sun, P., Gresack, J., and Risbrough, V. (2013). Impaired conditioned fear response and startle reactivity in epinephrine-deficient mice. *Behav. Pharmacol.* 24, 1–9. doi: 10.1097/fbp.0b013e32835cf408

Tully, K., and Bolshakov, V. Y. (2010). Emotional enhancement of memory: how norepinephrine enables synaptic plasticity. *Mol. Brain* 3:15. doi: 10.1186/1756-6606-3-15

Valentinuzzi, V. S., Kolker, D. E., Vitaterna, M. H., Shimomura, K., Whiteley, A., Low-Zeddies, S., et al. (1998). Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ × C57BL/6J)F_2 mice. *Learn. Mem.* 5, 391–403.

VanElzakker, M. B., Dahlgren, M. K., Davis, F. C., Dubois, S., and Shin, L. M. (2014). From Pavlov to PTSD: the extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiol. Learn. Mem.* 113, 3–18. doi: 10.1016/j.nlm.2013.11.014

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., et al. (2007). Histone deacetylase inhibitors enhance memory and synaptic plasticity via CREB: CBP-dependent transcriptional activation. *J. Neurosci.* 27, 6128–6140. doi: 10.1523/JNEUROSCI.0296-07.2007

Weil-Malherbe, H., Axelrod, J., and Tomchick, R. (1959). Blood-brain barrier for adrenaline. *Science* 129, 1226–1227. doi: 10.1126/science.129.3357.1226

Zhu, Q., Gu, L., Wang, Y., Jia, L., Zhao, Z., Peng, S., et al. (2014). The role of α-1 and α-2 adrenoceptors in restraint stress-induced liver injury in mice. *PLoS One* 9:e92125. doi: 10.1371/journal.pone.0092125

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CHAPTER 3 – MANUSCRIPT II

GLUCOSE MAY CONTRIBUTE TO RETRIEVAL AND RECONSOLIDATION OF CONTEXTUAL FEAR MEMORY THROUGH HIPPOCAMPAL *NR4A3* AND *BDNF* MRNA EXPRESSION AND MAY ACT SYNERGICALLY WITH ADRENALINE

Molecular Neurobiology. 2023 Oct. 27; 12035.
doi: 10.1007/s12035-023-03745-6.
Q1 in Neurosciences.

Molecular Neurobiology
https://doi.org/10.1007/s12035-023-03745-6

Glucose may Contribute to Retrieval and Reconsolidation of Contextual Fear Memory Through Hippocampal *Nr4a3* and *Bdnf* mRNA Expression and May Act Synergically with Adrenaline

Ana Oliveira[1,2] · Márcia Azevedo[1,2] · Rafaela Seixas[1,2] · Raquel Martinho[1,2] · Paula Serrão[2,3] · Mónica Moreira-Rodrigues[1,2]

Received: 16 August 2023 / Accepted: 27 October 2023

Abstract

Adrenaline (Ad) and glucose released into the bloodstream during stress may strengthen contextual fear memory. However, no previous studies have detached the effects of glucose from Ad in this paradigm. Using Ad-deficient mice, we aimed to evaluate the effect of glucose on contextual fear memory when endogenous Ad is absent. Fear conditioning was performed in wild-type (WT) and Ad-deficient mice (129 × 1/SvJ) administered with glucose (30 or 10 mg/kg; i.p.) or/and Ad (0.01 mg/kg; i.p.) or vehicle (0.9% NaCl; i.p.). Catecholamines were quantified using HPLC-ED. Real-time qPCR was used to assess mRNA expression of hippocampal genes. WT and Ad-deficient mice display increased contextual fear memory when administered with glucose both in acquisition and context days when compared to vehicle. Also, *Nr4a3* and *Bdnf* mRNA expression increased in glucose-administered Ad-deficient mice. Sub-effective doses of glucose plus Ad administered simultaneously to Ad-deficient mice increased contextual fear memory, contrary to independent sub-effective doses. Concluding, glucose may be an important part of the peripheral to central pathway involved in the retrieval and reconsolidation of fear contextual memories independently of Ad, possibly due to increased hippocampal *Nr4a3* and *Bdnf* gene expression. Furthermore, Ad and glucose may act synergically to strengthen contextual fear memory.

Keywords Fear conditioning · Contextual fear memory · Glucose · Adrenaline · Adrenaline-deficient mice

Introduction

Physiological reactions to stress are an essential tool for preserving the homeostasis of the organism: when one is faced with a threat, fear and stress responses enable the organism to react, being responsible for the "fight, flight, or freeze" responses [1]. Humans, as well as other animals, benefit from the ability to retain and use information gained remotely, in order to remember what situations pose a threat to them so they can successfully avoid those situations in the future [2].

However, the processes involved in the acquisition and consolidation of emotional information associated with fear are not always correctly activated. Comprehending how emotional memories, such as fear memories, are formed and stored in the brain allows a window of opportunity for the treatment of pathologies associated with unpleasant and traumatic memories. In fact, while automatic reactions to stress are an important survival tool, when improperly enabled or uncontrolled they can lead to prolonged stress, ultimately leading to a chronic overactivation of the adrenergic system. This overactivation may be implicated in the development of psychiatric disorders such as anxiety, depression, post-traumatic stress disorder (PTSD), and other disorders [3].

✉ Mónica Moreira-Rodrigues
mirodrigues@icbas.up.pt

1 Department of Immuno-physiology and Pharmacology, Laboratory of General Physiology, School of Medicine and Biomedical Sciences (ICBAS), University of Porto (UP), R. Jorge Viterbo Ferreira, 228, Building 2, Floor 4, Cabinet 22, Porto 4050-313, Portugal

2 Center for Drug Discovery and Innovative Medicines, University of Porto (MedInUP), Porto, Portugal

3 Department of Biomedicine, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal

Published online: 08 November 2023

Contextual fear conditioning is a behavioural paradigm based on classical conditioning, allowing the association of an aversive stimulus (such as mild-intensity electric shocks) with a specific context. Fear conditioning animal models are frequently used in research to study the neurobiological basis of adverse memories, being able to indicate the presence of fear and anxiety. These behaviour variations possibly reflect alterations in certain brain regions such as the hippocampus and the amygdala, which interplay together for contextual fear memory formation [4]. For instance, memory acquisition of the context occurs in the hippocampus and the memory acquisition of the emotional information (fear in the case of fear conditioning) takes place in the amygdala [5–9].

Contextual fear conditioning provokes stress and activates the hypothalamus-pituitary-adrenal axis, which triggers the release of catecholamines, in particular noradrenaline (NA) and adrenaline (Ad) from the adrenal gland into the bloodstream [10]. Moreover, several authors have shown that catecholamines are important for long-term memory consolidation in human and animal studies, in which Ad administration immediately after training sessions increased memory retention compared to vehicle administration [11, 12]. Furthermore, in previous studies, we and others have demonstrated that mice not capable of expressing the enzyme phenylethanolamine-*N*-methyltransferase (Pnmt), which catalyses the conversion of NA to Ad, and, therefore, are adrenaline-deficient (Ad-deficient, Pnmt-KO) [13], have reduced contextual fear memory [14, 15]. In addition, Ad strengthens contextual fear memory and induces, in the hippocampus, the expression of genes related to contextual fear memory consolidation, such as transcription factor nuclear receptor 4a2 (*Nr4a2*) [10].

Despite all the evidence for Ad enhancement of contextual fear memory, as a hydrophilic catecholamine, peripheral Ad does not cross the blood-brain barrier (BBB) which prevents it from acting in the central nervous system (CNS) [16]. Therefore, the role of Ad in contextual fear memory appears to be due to its peripheral actions rather than direct central ones. This suggestion led several authors to propose that this catecholamine may exert its central actions through glucose. Consistently with this theory, Morris and Gold proposed the hypothesis that glucose may mediate Ad action in the CNS [17].

Since Ad induces blood glucose increase, it is difficult to evaluate the effects of glucose detached from those of Ad in contextual fear memory. Thus, using Ad-deficient mice (do not synthesize Ad), which is a reliable model to better understand Ad and glucose effects alone on contextual fear memory, the main aim of this study was to detach the effects of Ad from those of glucose and evaluate their action on contextual fear memory strengthening through possible effects in the CNS. Therefore, we attempted to evaluate if glucose administration enhances contextual fear memory in an Ad-deficient mouse model, as well as to evaluate a possible synergy between Ad and glucose. Lastly, another aim of this study was to evaluate the hippocampal gene expression implications of glucose administration in Ad-deficient mice after fear conditioning.

Methods

Animals

The animal care and experimental protocols were performed in accordance with Portuguese legislation by Directive Law 113/2013 and 1/2019 and approved by the Organism Responsible for Animal Welfare (ORBEA) in the Faculty of Medicine of the University of Porto and National Authority for Animal Health (DGAV) which is a transposition of the European Directive number 2010/63/EU. In the vivarium, Ad-deficient (Pnmt-KO) ($n=49$) and WT ($n=27$) male mice (129×1/SvJ) were bred under controlled environmental conditions (12 h light/dark cycle, room temperature 23 ± 1 °C, 50% of humidity, *ad libitum* autoclaved drinking water and mice diet 4RF25/I and 4RF21/A; Mucedola, Milan, Italy) in the same room and housed with the respective litter, in cages with two to five animals.

A Cre-recombinase gene and a Neomycin resistance gene were inserted into exon 1 of the locus encoding for *Pnmt* gene, resulting in the Ad-deficient ($Pnmt^{-/-}$, Pnmt-KO) mice. These $Pnmt^{-/-}$ mice are unable to express the Pnmt enzyme and, therefore, unable to produce Ad [13]. This was confirmed by polymerase chain reaction (PCR) of DNA ear samples, as previously shown [13].

Blood Glucose Quantification

Initially, a group of WT mice was administered with glucose (30 mg/kg; intraperitoneally, i.p.) or vehicle (NaCl 0.9%, i.p.). Ten minutes after administration and following local anaesthesia (Lidonostrum®) applied on the site of the needle prick, a blood glucose drop was collected in a blood glucose test strip (FreeStyle®) connected to a glucose meter (FreeStyle®) for blood glucose concentration (mg/dl) quantification (Fig. 1A) [18].

Conditioning Fear Test

The procedure followed for the conditioned fear test was adapted from previous studies [10, 14]. The conditioning chamber consisted of a clear Plexiglas box (26.5 cm x 26.5 cm x 26.5 cm) equipped with a metal grid floor with

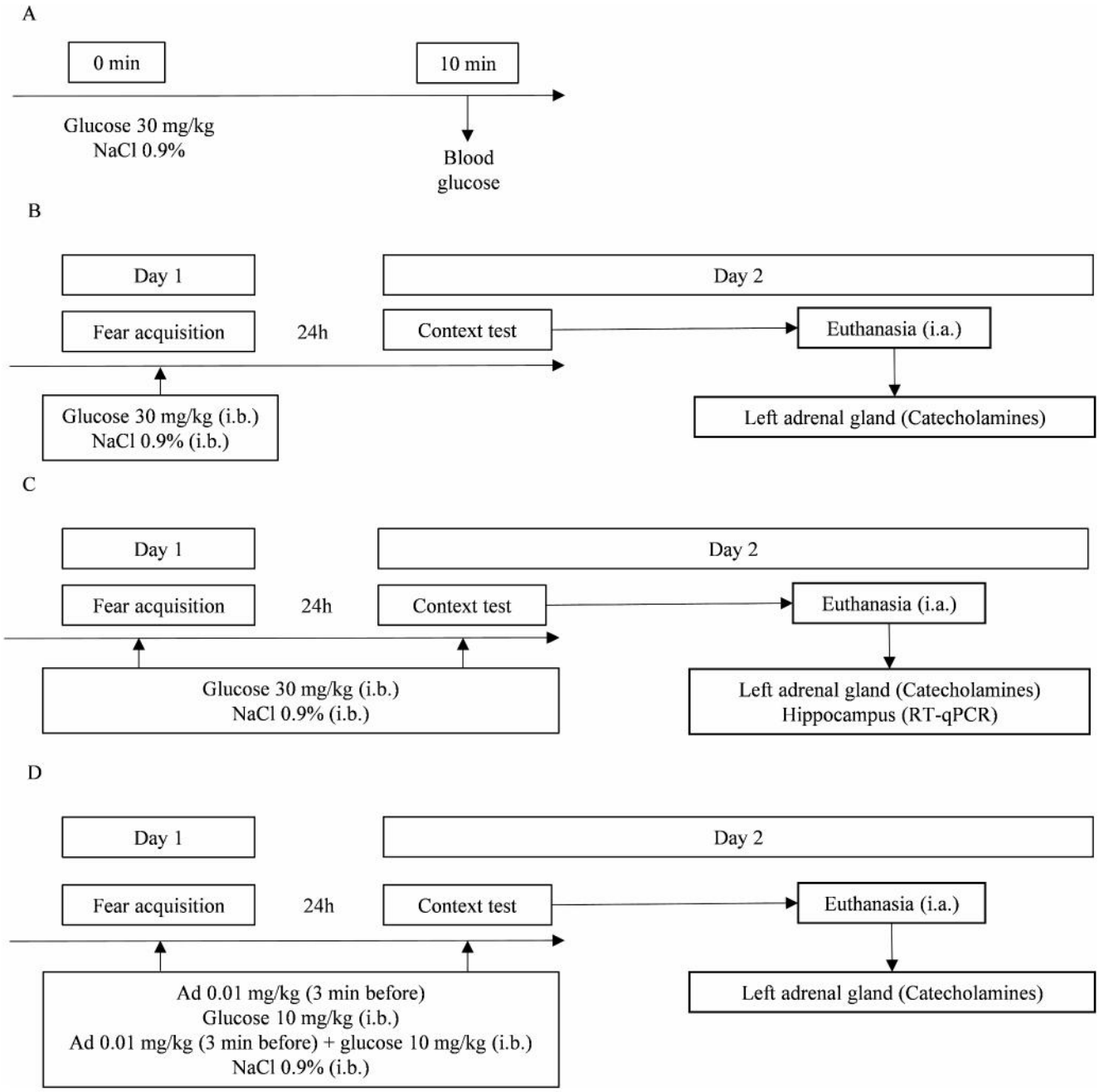


Fig. 1 Schematic representation of the experimental design, behavioural protocols, treatments, and sample collection. **(A)** Initially, a group of wild-type (WT) mice followed protocol A being administered with glucose (30 mg/kg; intraperitoneal, i.p.) or vehicle (0.9% NaCl; i.p.) and 10 min after blood glucose was measured. Afterwards, mice were submitted to fear conditioning protocol with different experimental protocols: **(B)** A group of WT mice followed protocol B being administered with glucose (30 mg/kg; i.p., immediately before, i.b.) or vehicle (0.9% NaCl; i.p., i.b.) only on the fear acquisition day. **(C)** Subsequently, a group of WT and Ad-deficient (Pnmt-KO) mice followed protocol C with glucose (30 mg/kg; i.p.; i.b.) or vehicle (0.9% NaCl; i.p., i.b.) administration in both the fear acquisition and context days. **(D)** Finally, a group of Ad-deficient (Pnmt-KO) mice followed protocol D administered in both the fear acquisition and context days with sub-effective doses of Ad (0.01 mg/kg; i.p., 3 min), glucose (10 mg/kg; i.p., i.b.), Ad (0.01 mg/kg; i.p., 3 min) plus glucose (10 mg/kg; i.p., i.b.), or vehicle (0.9% NaCl; i.p., i.b.). RT-qPCR, real-time polymerase chain reaction. i.a, immediately after

32 stainless steel bars (placed 5 mm apart) wired to a stimulus generator. The first day of the conditioned fear test (fear acquisition) had a duration of 6 min: the first 3 min consisted of a habituation period (mice left undisturbed), after which 3-foot shocks (duration: 2 s, intensity: 0.6 mA) were delivered at 60 s intervals. After 24 h, mice were re-exposed to the same apparatus with no foot shocks delivered during the total duration of the context test (10 min; context). The

10-minute duration of the context was chosen to allow hippocampal gene expression after re-exposure [19]. A timer was positioned on top of the contextual chamber and both days were video recorded with a digital video camera Sony HDR-CX405 (Sony Corporation, Japan) to allow the posterior evaluation of the mice's behaviour. All analyses were conducted under manual and blind protocols. On acquisition and context days, freezing behaviour, defined as the lack of movements excluding those related to respiration for at least 3 s, was evaluated [20]. Additionally, on the acquisition day, vocalization (high-pitched squeak) and jump (removal of 3 paws from the grid floor) responses to the foot shock were also quantified [21]. On both days, the conditioned chamber was cleaned with alcohol 70% and smeared with 1% acetic acid between trials.

Behavioural Treatments

WT and Ad-deficient (Pnmt-KO) mice were submitted to the fear conditioning protocol with different experimental designs as displayed in Fig. 1. A group of WT mice were administered pre-acquisition with glucose (30 mg/kg, i.p.; immediately before, i.b.) or vehicle (0.9% NaCl, i.p., i.b.) (Fig. 1B). Next, a group of WT or Ad-deficient (Pnmt-KO) mice were administered pre-training and pre-context with glucose (30 mg/kg, i.p., i.b.) or vehicle (0.9% NaCl, i.p., i.b.) (Fig. 1C). Finally, a group of Ad-deficient (Pnmt-KO) mice were administered with Ad (0.01 mg/kg, i.p., 3 min), glucose (10 mg/kg, i.p., i.b.), Ad (0.01 mg/kg, i.p., 3 min) plus glucose (10 mg/kg, i.p., i.b.), or vehicle (0.9% NaCl, i.p., i.b.) (Fig. 1D).

Quantification of Catecholamines

Mice were anaesthetized (ketamine, 100 mg/kg and xylazine, 10 mg/kg; i.p.) immediately after the end of the fear conditioning procedure for samples and tissues collection. The left adrenal gland was collected and emerged in perchloric acid (PCA) 0.2 M overnight, at 4 ºC, and frozen at -80 ºC until further use. The PCA of each sample was transferred to tubes with filters and centrifuged at 1250 g for 2 min at 4 ºC to quantify catecholamines in the adrenal gland. An external standard solution (125 mg/µl for purified Ad, NA, and DA (Sigma-Aldrich, St. Louis, USA) was used. Afterwards, 50 µL of the PCA of each sample or standard solution were mixed with the mobile phase (citric acid monohydrate 100 mM, sodium acetate 100 mM, 1-octanesulfonic acid sodium salt 1.6 mM, ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate 0.15 mM, methanol 8%, di-n-butylamine 1 mM, pH was set for 3.5 with $HClO_4$ 70%) and injected in a reverse-phase high-performance liquid chromatography (HPLC) (Gilson Medical Electronics, Villiers le Bel, France) machine. The components of interest in each sample or in the external standard solution were separated in the column (SPHERI-5 C18 4.6×250 mm, 5 µm; BROWNLEE™, Waltham, MA, USA), and quantified by electrochemical detection (ED). The limits of detection and quantification for the HPLC (Gilson Medical Electronics, Villiers le Bel, France) employed for adrenal gland samples were 350–1000 fmol and 700–2000 fmol, respectively. NA = 6.60 min, Ad = 7.81 min, and DA = 15.95 min were the retention times. Catecholamine content was calculated per whole adrenal gland (nmol/AG).

RNA Isolation and Relative Quantification of mRNA Expression

Real-time PCR (qPCR) was performed in hippocampus samples collected immediately after the context day of the fear conditioning protocol, as described in previous works [10, 22, 23]. Total RNA isolation was carried out with Mini Kit illustra™ Isolate II RNA (Bioline, London, United Kingdom), according to the manufacturer's instructions. In, brief, ethanol (70%) was added to the tubes containing the RNA. Afterwards, desalting was performed by adding a Membrane Desalting Buffer (MEM) (Bioline, London, United Kingdom) to the tubes. For DNA digestion, DNase I (Bioline, London, United Kingdom) was added to the Reaction Buffer for DNase I (RDN) (Bioline, London, United Kingdom). Next, the DNase I reaction mixture was applied directly to the center of the silica membrane. Three washes were then performed. At first, the RNA was washed with Wash Buffer RW1 (Bioline, London, United Kingdom). For the second and third washes it was used Wash Buffer RW2 (Bioline, London, United Kingdom). Finally, RNA was eluted with RNase-free water (Bioline, London, United Kingdom). The concentration and purity of the isolated RNA were measured with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, United States). A T100™ Thermal Cycler (Bio-Rad, Hercules, United States) was used to carry out reverse transcription, applying a Reverse Transcription kit (NZY First-Strand cDNA Synthesis Kit NZYTech — Genes and Enzymes, Lisbon, Portugal). StepOne™ real-time PCR System (Applied BioSystems, Waltham, United States) allowed the conduction of qPCR reactions. Gene-specific primers (10 µM), Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, United States), RNase-free H_2O (Bioline, London, United Kingdom) were mixed and cDNA was added (1:20). Instead of cDNA, RNase-free H_2O (Bioline, London, United Kingdom) was added as a negative control. Gene-specific primers are presented in Table 1. Results of mRNA quantification were expressed in an arbitrary unit (AU) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1 Primers used in gene expression analysis

Gene	Primer (5'→ 3')
Nr4a1	F: AAAATCCCTGGCTTCATTGAG
	R: TTTAGATCGGTATGCCAGGCG
Nr4a2	F: CGGTTTCAGAAGTGCCTAGC
	R: TTGCCTGGAACCTGGAATAG
Nr4a3	F: GTGGCTCGACTCCATTAAAGAC
	R: GTGCATAGCTCCTCCACTCTCT
Bdnf	F: GGACATATCCATGACCAGAAAGAAA
	R: GCAACAAACCACAACATTATCGAG
Gapdh	F: CCATCACCATCTTCCAGGAG
	R: GCATGGACTGTGGTCATGAG

Nr4, Nuclear receptor 4; Bdnf, Brain-derived neurotrophic factor; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; F, forward primer and R, reverse primer

Drugs

D (+)-Glucose was purchased from EMD Millipore Corporation (Germany); (-) - adrenaline, L-(-)-noradrenaline, dopamine hydrochloride, and PCA were purchased from Sigma-Aldrich (St. Louis, United States). Ketamine (Imalgene 1,000, Merial, Lisbon, Portugal) and xylazine (Rompum 2%, Bayer, Lisbon, Portugal) were purchased from Agrofauna (Vila Nova de Gaia, Portugal). Lidocaine hydrochloride (Lidonostrum® 2%, Sidefarma, Lisbon, Portugal).

Statistical Analysis

An online Sample Size Calculator was used to establish the minimum sample size for each experimental protocol (https://clincalc.com/Stats/SampleSize.aspx). All statistical analysis was performed using the program GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), and all data was displayed as means with standard error of the means (SEM). Freezing behaviour was analysed by Two-Way Analysis of Variance (ANOVA) repeated measures, followed by multiple comparison corrections with Sidak's (two groups) or Tukey´s (three or more groups) post-hoc tests using treatment as a "between-subjects factor" and time as "within-subjects factor" (repeated measure) (α was set at 0.05). Other behavioural outcomes (vocalization and jump responses), catecholamines concentration, and qPCR were examined using Student's t-test (2 groups) or One-Way ANOVA (three or more groups). For One-Way ANOVA the post-hoc Tukey test was used for multiple comparisons correction (α was set at 0.05). Effect sizes were estimated by calculating Cohen's *d* test for t-test comparisons and partial eta squared (η_2^P) for ANOVA. The statistically significant differences observed between groups in the different time points or between groups in Two-way and One-way ANOVA, respectively, were based on the adjusted

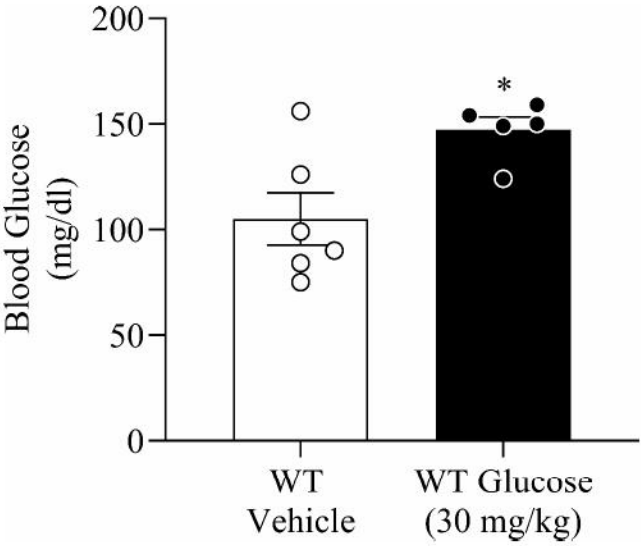


Fig. 2 Blood glucose concentration in wild-type mice ten minutes after glucose administration. Wild-type (WT) mice were administered with glucose (30 mg/kg; intraperitoneal, i.p.) or vehicle (NaCl 0.9%; i.p.). Values are means ± SEM of 5–6 mice per group. WT Glucose, wild-type mice administered with glucose; WT Vehicle, wild-type mice administered with vehicle. *, significantly different from correspondent values in WT Vehicle mice ($p < 0.05$)

p-value < 0.05. The statistically significant difference for unpaired t-test analysis was set at $p < 0.05$.

Results

Hyperglycaemic State is Attained 10 Min After Glucose Administration

In an initial approach (Fig. 1A), it was verified a significant increase in blood glucose when compared with vehicle-treated mice ($t_{(9)} = 2.85$, $p = 0.02$, Cohens' $d = 1.97$, Fig. 2). Therefore, it was confirmed that glucose-administered WT mice attained a moderate hyperglycaemic state 10 min after glucose (30 mg/kg) administration. This preliminary evaluation gave us information for all the subsequent protocols which were carried out considering the glucose concentration and time point identified.

Glucose Administration Only Before the Fear Acquisition Day Does not Strengthen Contextual Fear Memory in Wild-Type Mice

Figure 3A shows the effects in freezing behaviour and shock responsiveness of glucose (30 mg/kg) administration only on the fear acquisition day, in WT mice (Fig. 1B). In acquisition day, it was observed a time effect ($F_{(3, 27)} = 58.39$, $p = 6.26 \times 10^{-12}$, $\eta_p^2 = 0.87$, Fig. 3A1), but no significant effect of treatment ($F_{(1, 9)} = 0.13$, $p = 0.72$, $\eta_p^2 = 0.01$,

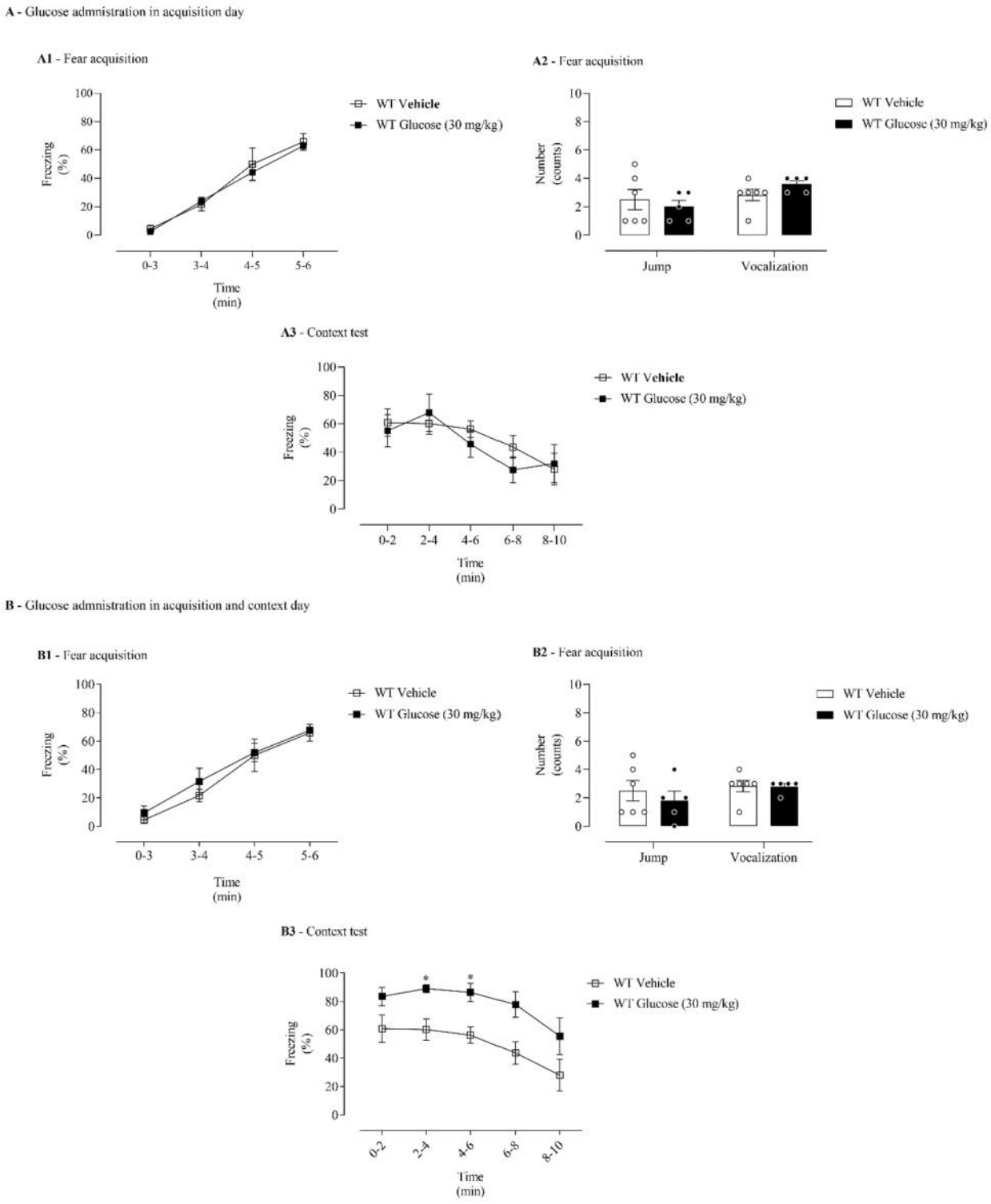


Fig. 3 Fear behaviour in wild-type mice after glucose administration. Glucose (30 mg/kg; intraperitoneal, i.p.) or vehicle (NaCl, 0.9%; i.p.) administration **(A)** only on the fear acquisition day and **(B)** in both the fear acquisition and context days. (A1 and B1) Freezing and (A2 and B2) shock responsivity in fear acquisition day. (A3 and B3) Freezing response in re-exposure to the context. Values are means ± SEM of 5–6 mice per group. WT Glucose, wild-type mice administered with glucose; WT Vehicle, wild-type mice administered with vehicle. *, significantly different from correspondent values in WT Vehicle mice (adjusted p-value < 0.05)

Fig. 3A1) or time x treatment interaction ($F_{(3, 27)}=0.22$, $p=0.88$, $\eta_p^2=0.02$, Fig. 3A1) were present. No differences between glucose or vehicle-administered WT mice were observed in the freezing response (Fig. 3A1). Also, no differences were observed in vocalization ($t_{(9)}=1.55$, $p=0.16$, Cohens' $d=1.06$, Fig. 3A2) and jump ($t_{(9)}=0.56$, $p=0.59$, Cohens' $d=0.38$, Fig. 3A2) responses on the first day of the fear conditioning protocol (training day).

On context day, after re-exposure to shock context, a significant effect of time ($F_{(4, 36)}=16.69$, $p=8.02\times10^{-8}$, $\eta_p^2=0.65$, Fig. 3A3) was observed, but no effect of treatment ($F_{(1, 9)}=0.11$, $p=0.74$, $\eta_p^2=0.01$, Fig. 3A3) or time x treatment interaction ($F_{(4, 36)}=1.95$, $p=0.12$, $\eta_p^2=0.18$, Fig. 3A3) were present. Administration with glucose on acquisition day does not alter freezing behaviour in WT mice compared with vehicle-administered WT mice (Fig. 3A3).

No differences were observed in dopamine (DA) ($t_{(9)}=1.80$, $p=0.10$, Cohens' $d=1.22$, Table 2) and NA ($t_{(9)}=1.86$, $p=0.09$, Cohens' $d=1.28$, Table 2) content in the adrenal gland between groups. Ad increased in glucose-administered WT mice compared to vehicle ($t_{(9)}=2.53$, $p=0.03$, Cohens' $d=1.73$, Table 2).

Glucose Administration Both Before the Fear Acquisition and Context Days Appears to Strengthen Contextual Fear Memory in Wild-Type Mice

Figure 3B shows the effects in freezing behaviour and shock responsiveness of glucose (30 mg/kg) administrated both in the fear acquisition and context days (Fig. 1C), in WT mice. In acquisition day, it was observed a time effect ($F_{(3, 27)}=40.82$, $p=3.60\times10^{-10}$, $\eta_p^2=0.82$, Fig. 3B1), but no significant effect of treatment ($F_{(1, 9)}=0.57$, $p=0.47$,, $\eta_p^2=0.06$, Fig. 3B1) or time x treatment interaction ($F_{(3, 27)}=0.21$, $p=0.89$, $\eta_p^2=0.02$, Fig. 3B1) were present. No differences between the groups were observed in the freezing response (Fig. 3B1). Also, no differences were observed in vocalization ($t_{(9)}=0.07$, $p=0.95$, Cohens' $d=0.05$, Fig. 3B2) and jump ($t_{(9)}=0.70$, $p=0.50$, Cohens' $d=0.48$, Fig. 3B2) responses on the first day of the fear conditioning protocol.

On context day, during re-exposure to shock context, a significant effect of time ($F_{(4, 36)}=14.20$, $p=4.76\times10^{-7}$, $\eta_p^2=0.61$, Fig. 3B3) and treatment ($F_{(1, 9)}=7.84$, $p=0.02$, $\eta_p^2=0.47$, Fig. 3B3) was observed. Glucose (30 mg/kg) administration increased freezing behaviour in WT mice compared with vehicle-treated WT mice (Fig. 3B3).

No differences were observed in DA ($t_{(9)}=0.30$, $p=0.77$, Cohens' $d=0.21$, Table 2), NA ($t_{(9)}=0.12$, $p=0.91$, Cohens' $d=0.08$, Table 2), and Ad ($t_{(9)}=1.94$, $p=0.08$, Cohens' $d=1.28$, Table 2) content in the adrenal gland when comparing the experimental groups.

Glucose Administration Appears to Strengthen Contextual Fear Memory in Adrenaline-Deficient Mice

To understand the individual effect of glucose independently from Ad, Ad-deficient (Pnmt-KO) mice were administered pre-acquisition and pre-context with glucose (30 mg/kg) and submitted to contextual fear conditioning protocol (Fig. 1C). On acquisition day, a time effect ($F_{(3, 27)}=59.56$, $p=4.97\times10^{-12}$, $\eta_p^2=0.87$, Fig. 4A) was observed. No significant effect of treatment ($F_{(1, 9)}=0.16$, $p=0.70$, $\eta_p^2=0.02$, Fig. 4A) and time x treatment interaction ($F_{(3, 27)}=0.23$, $p=0.87$, $\eta_p^2=0.02$, Fig. 4A) was observed. No differences were observed in freezing response between treatments on the fear acquisition day (Fig. 4A). Also on the fear acquisition day, vocalization ($t_{(9)}=0.16$, $p=0.88$, Cohens' $d=0.10$, Fig. 4B) and jump ($t_{(9)}=0.00$, $p=1.00$, Cohens' $d=0.00$, Fig. 4B) responses were not different between treatment groups.

Once re-exposed to shock context, a treatment ($F_{(1, 9)}=13.40$, $p=0.005$, $\eta_p^2=0.60$, Fig. 4C) and time ($F_{(4, 36)}=7.08$, $p=0.0003$, $\eta_p^2=0.44$, Fig. 4C) effect, and a significant time x treatment interaction between treatment and time ($F_{(4, 36)}=3.04$, $p=0.003$, $\eta_p^2=0.25$, Fig. 4C) was observed. Ad-deficient (Pnmt-KO) mice administered with glucose presented an increase in freezing behaviour

Table 2 Adrenal gland catecholamines' concentration in wild-type (WT) mice administered with glucose before (A) only the acquisition day and (B) both the acquisition and context days

Administration days	Treatment groups	DA (nmol/AG)	NA (nmol/AG)	Ad (nmol/AG)
(A) Acquisition	WT Vehicle (NaCl 0.9%)	0.29 ± 0.04	6.39 ± 0.76	14.27 ± 1.62
	WT Glucose (30 mg/kg)	0.42 ± 0.06	8.11 ± 0.43	19.45 ± 1.11*
(B) Acquisition and context	WT Vehicle (NaCl 0.9%)	0.28 ± 0.05	6.18 ± 0.90	13.77 ± 1.88
	WT Glucose (30 mg/kg)	0.30 ± 0.03	6.08 ± 0.35	17.56 ± 0.86

Values are means ± standard error of the means (SEM) of 5–6 mice per group. WT Glucose, wild-type mice administered with glucose; WT Vehicle, wild-type mice administered with vehicle; DA, dopamine; NA, noradrenaline; Ad, adrenaline. *, significantly different from correspondent values in WT Vehicle mice ($p<0.05$)

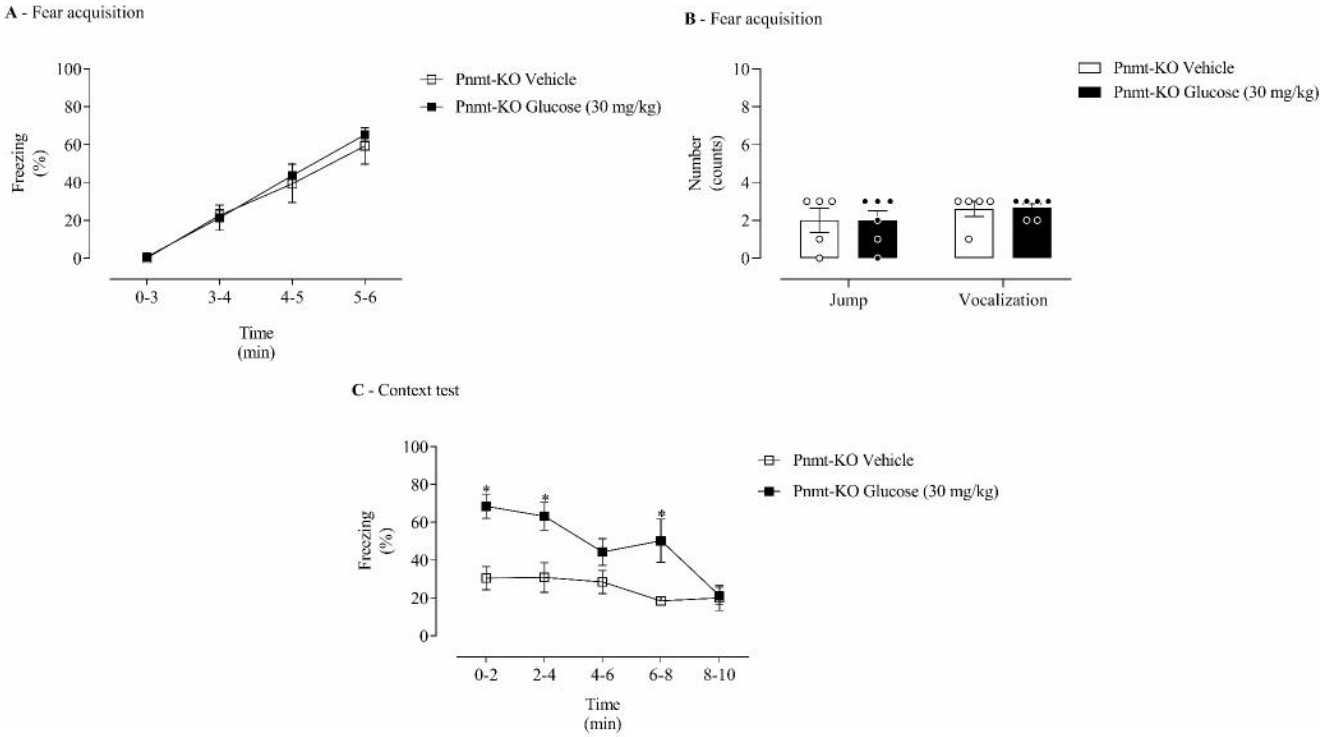


Fig. 4 Fear behaviour in adrenaline-deficient mice after glucose administration in both the fear acquisition and context days. **(A)** Freezing and **(B)** shock responsivity on fear acquisition day, and **(C)** freezing response in re-exposure to the context in Ad-deficient (Pnmt-KO) mice administered pre-training and pre-testing with glucose (30 mg/kg; intraperitoneal, i.p.) or vehicle (NaCl, 0.9%; i.p.). Values are means ± SEM of 5–6 mice per group. Pnmt-KO Glucose, adrenaline-deficient mice treated with glucose; Pnmt-KO Vehicle, adrenaline-deficient mice treated with vehicle. *, significantly different from correspondent values in Pnmt-KO Vehicle mice (adjusted p-value < 0.05)

Table 3 Adrenal gland catecholamines' concentration in adrenaline-deficient (Pnmt-KO) mice administered with glucose before both the acquisition and context days

Treatment groups	DA (nmol/AG)	NA (nmol/AG)	Ad (nmol/AG)
Pnmt-KO Vehicle (NaCl 0.9%)	0.23 ± 0.02	16.20 ± 1.14	Undetectable
Pnmt-KO Glucose (30 mg/kg)	0.20 ± 0.02	14.41 ± 1.07	Undetectable

Values are means ± standard error of the means (SEM) of 5–6 mice per group. Pnmt-KO Glucose, adrenaline-deficient mice administered with glucose; Pnmt-KO vehicle, adrenaline-deficient mice administered with vehicle; DA, dopamine; NA, noradrenaline; Ad, adrenaline

compared to vehicle-treated Ad-deficient (Pnmt-KO) mice (Fig. 4C).

Across groups, there were no differences in DA ($t_{(9)} = 0.76$, $p = 0.11$, Cohens' $d = 1.18$, Table 3) and NA content ($t_{(9)} = 0.71$, $p = 0.50$, Cohens' $d = 0.48$, Table 3) in the adrenal gland. In Ad-deficient mice (Pnmt-KO) no Ad was detected by HPLC-ED (Table 3).

Glucose Administration Increases *Nr4a3* and *Bdnf* mRNA Hippocampal Gene Expression in Adrenaline-Deficient Mice

The influence of glucose in the hippocampal gene expression independently from Ad was evaluated (Fig. 1C). No differences were observed in hippocampus mRNA expression of *Nr4a1* ($t_{(11)} = 0.29$, $p = 0.77$, Cohens' $d = 0.18$, Fig. 5A) and *Nr4a2* ($t_{(11)} = 1.96$, $p = 0.08$, Cohens' $d = 1.18$, Fig. 5B) between groups. However, the administration of Glucose (30 mg/kg) in Ad-deficient (Pnmt-KO) mice increased hippocampus mRNA expression of *Nr4a3* ($t_{(11)} = 2.18$, $p = 0.05$, Cohens' $d = 1.31$, Fig. 5C) and *Bdnf* ($t_{(11)} = 2.99$, $p = 0.01$, Cohens' $d = 1.82$, Fig. 5D) compared with vehicle-treated Ad-deficient (Pnmt-KO) mice.

Glucose and Adrenaline Appear to Act Synergically to Strengthen Contextual Fear Memory in Adrenaline-Deficient Mice

To infer an interaction between glucose and Ad in contextual fear memory, Ad-deficient (Pnmt-KO) mice were submitted to fear conditioning protocol after treatment with sub-effective doses (Fig. 1D). On the fear acquisition day, it

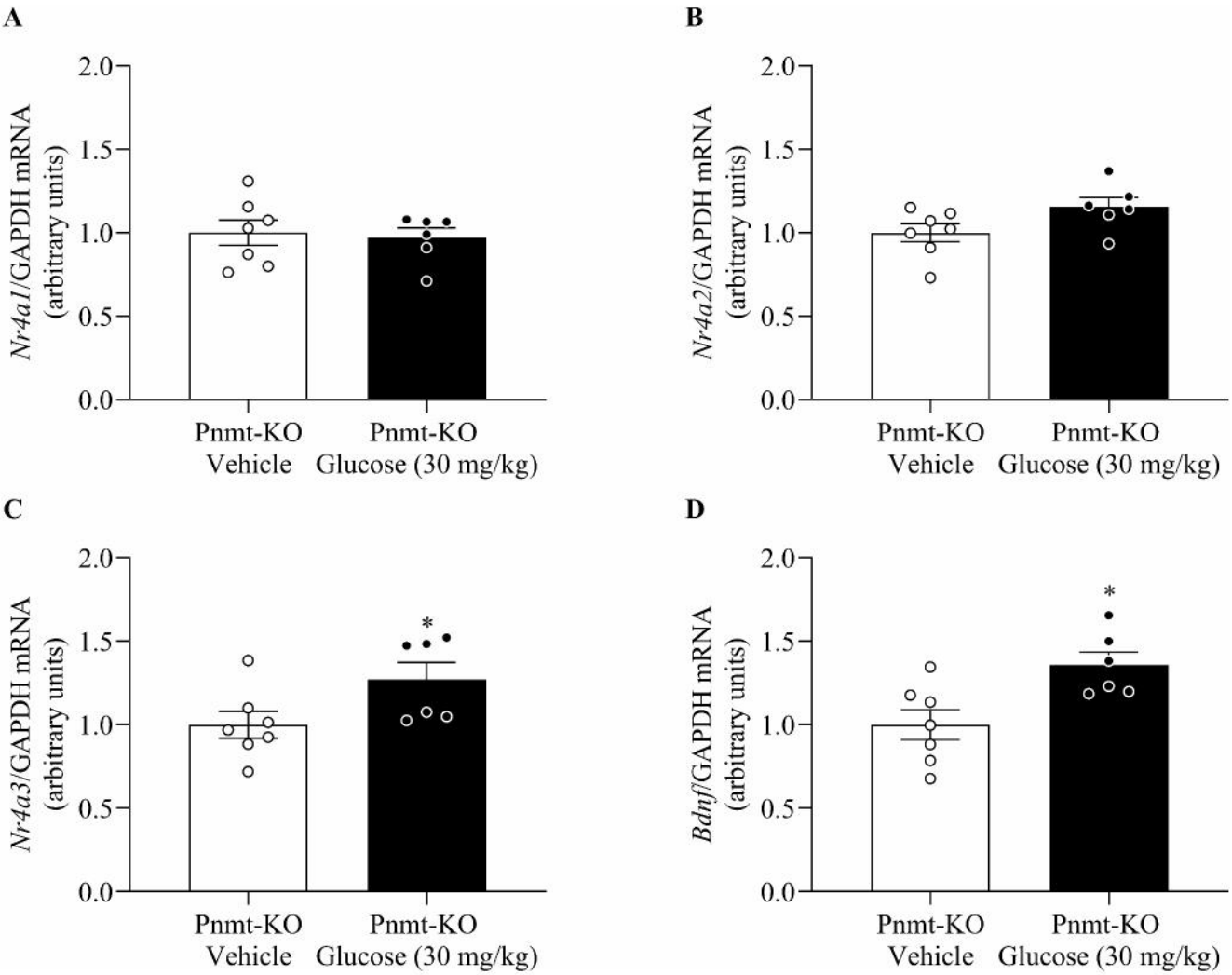


Fig. 5 Hippocampus mRNA expression of transcription factor genes after glucose administration in adrenaline-deficient mice. Hippocampus mRNA expression of **(A)** Nuclear receptor 4 (Nr4) a1, **(B)***Nr4a2*, **(C)***Nr4a3*, and **(D)***Bdnf* in adrenaline-deficient (Pnmt-KO) mice after glucose (30 mg/kg; i.p.) or vehicle (NaCl 0.9%; i.p.) administration in both the fear acquisition and context days. Values are means ± SEM of 6–7 mice per group. Results of mRNA are expressed as arbitrary units (AUs) after normalization for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Pnmt-KO Glucose, adrenaline-deficient mice treated with glucose; Pnmt-KO Vehicle, adrenaline-deficient mice treated with vehicle. *, significantly different from correspondent values in Pnmt-KO vehicle mice ($p < 0.05$)

was observed a time effect ($F_{(3, 63)} = 156.5$, $p = 6.81 \times 10^{-36}$, $\eta_p^2 = 0.88$, Fig. 6A). No significant effect of treatment ($F_{(3, 21)} = 1.11$, $p = 0.37$, $\eta_p^2 = 0.14$, Fig. 6A) and time x treatment interaction ($F_{(9, 63)} = 1.33$, $p = 0.24$, $\eta_p^2 = 0.03$, Fig. 6A) was observed. No differences were observed in the freezing response between groups (Fig. 6A). Furthermore, no differences were observed in vocalization ($F_{(3, 21)} = 0.62$, $p = 0.61$, $\eta_p^2 = 0.08$, Fig. 6B) and jump ($F_{(3, 21)} = 0.57$, $p = 0.63$, $\eta_p^2 = 0.08$, Fig. 6B) responses between treatments.

After re-exposure to the shock context, a treatment ($F_{(3, 21)} = 5.96$, $p = 0.004$, $\eta_p^2 = 0.46$, Fig. 6C) and time ($F_{(4, 84)} = 18.35$, $p = 7.23 \times 10^{-11}$, $\eta_p^2 = 0.47$, Fig. 6C) effect were observed. No differences were observed between Ad-deficient (Pnmt-KO) mice treated with a sub-effective dose of Ad (0.01 mg/kg) or Glucose (10 mg/kg) and vehicle (Fig. 6C). However, when Ad-deficient (Pnmt-KO) mice were treated simultaneously with Ad (0.01 mg/kg) and Glucose (10 mg/kg) it was observed an increase in freezing behaviour when compared to Ad-deficient (Pnmt-KO) mice only treated with Ad (0.01 mg/kg), or Glucose (10 mg/kg) or vehicle (Fig. 6C).

Concerning the adrenal glands, no differences were observed in DA ($F_{(3, 21)} = 1.27$, $p = 0.31$, $\eta_p^2 = 0.15$, Table 4) or NA content ($F_{(3, 21)} = 0.43$, $p = 0.74$, $\eta_p^2 = 0.06$, Table 4) between groups. In Ad-deficient mice (Pnmt-KO) no Ad was detected by HPLC-ED (Table 3).

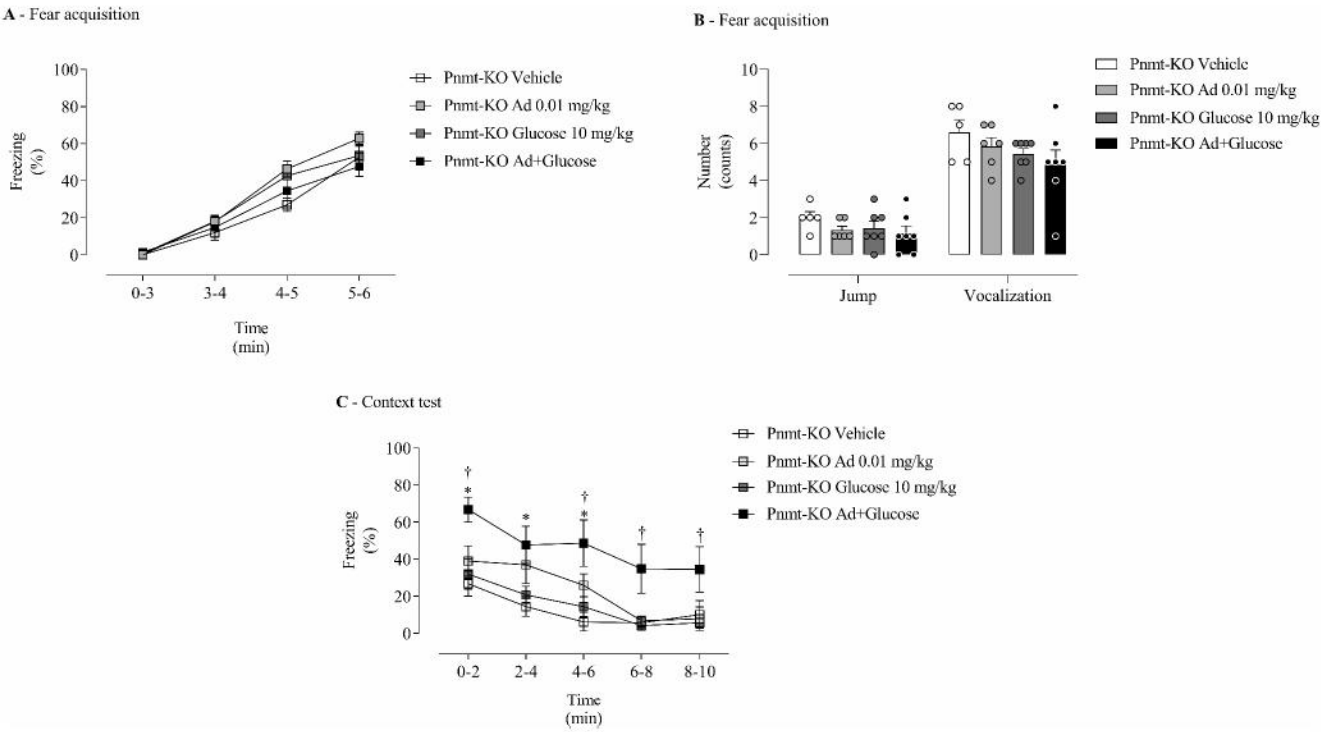


Fig. 6 Fear behaviour in adrenaline-deficient mice after glucose or/and adrenaline sub-effective dose administration. **(A)** Freezing and **(B)** shock responsivity on fear acquisition day, and **(C)** freezing response in re-exposure to the context in Ad-deficient (Pnmt-KO) mice administered pre-training and pre-testing with glucose (10 mg/kg; intraperitoneal, i.p.), Ad (0.01 mg/kg; i.p.), glucose (10 mg/kg; i.p.) plus Ad (0.01 mg/kg; i.p.), or vehicle (NaCl, 0.9%; i.p.). Values are means ± SEM of 5–7 mice per group. Pnmt-KO Glucose, adrenaline-deficient mice treated with glucose; Pnmt-KO Ad, adrenaline-deficient mice treated with adrenaline; Pnmt-KO Glucose + Ad, adrenaline-deficient mice treated with glucose plus adrenaline; Pnmt-KO Vehicle, adrenaline-deficient mice treated with vehicle. *, significantly different from correspondent values in Pnmt-KO vehicle mice (adjusted p-value < 0.05). †, significantly different from correspondent values in Pnmt-KO Ad mice (adjusted p-value < 0.05)

Table 4 Adrenal gland catecholamines' concentration in adrenaline-deficient (Pnmt-KO) mice administered with sub-effective doses of glucose and/or adrenaline before both the acquisition and context days

Treatment groups	DA (nmol/AG)	NA (nmol/AG)	Ad (nmol/AG)
Pnmt-KO Vehicle (NaCl 0.9%)	0.13 ± 0.05	8.41 ± 1.06	Undetectable
Pnmt-KO Ad (0.01 mg/kg)	0.04 ± 0.01	11.55 ± 1.80	Undetectable
Pnmt-KO Glucose (10 mg/kg)	0.13 ± 0.04	0.30 ± 0.03	Undetectable
Pnmt-KO Ad (0.01 mg/kg) + Glucose (10 mg/kg)	0.08 ± 0.04	0.31 ± 0.03	Undetectable

Values are means ± standard error of the means (SEM) of 5–7 mice per group. Pnmt-KO Glucose, adrenaline-deficient mice treated with glucose; Pnmt-KO Ad, adrenaline-deficient mice administered with adrenaline; Pnmt-KO glucose + Ad, adrenaline-deficient mice administered with glucose plus adrenaline; Pnmt-KO vehicle, adrenaline-deficient mice administered with vehicle; DA, dopamine; NA, noradrenaline; Ad, adrenaline

Discussion

Briefly, the presented results reinforce the view that glucose may have an important role in contextual fear memory even when Ad is absent. In addition, glucose and Ad appear to act in synergy in contextual fear memory strengthening.

After a stressful event, Ad is released into the bloodstream and appears to be essential for fear and traumatic memories strengthening. This has been demonstrated in behavioural models such as inhibitory avoidance [24], contextual fear conditioning [14, 15], and PTSD mouse models [25, 26]. However, due to the biochemical proprieties of this catecholamine, preventing it from crossing the BBB, its actions and effects are thought to be restricted to the periphery [16]. As reviewed in Gold [17], glucose is one of the proposed mediators for Ad effects on memory strengthening. According to this theory, we have shown that Ad released from the medulla zone of the adrenal gland acts in peripheral β_2-adrenoceptors, activating them [10, 14], and may induce glucose synthesis and release from hepatic tissue [27, 28].

Glucose, the primary energy substrate of the brain, is carried by glucose transport proteins (GLUT-1) across the BBB, where they are found in high density [29]. In the brain, glucose may serve as an energy source for the increased production and release of neurotransmitters into the brain areas highly associated with fear memory, namely the hippocampus or the amygdala, possibly enhancing it. Another set of evidence supporting glucose as a possible Ad mediator in the CNS is that Ad fails to facilitate rat's performance in a spontaneous alternation task, when the animals were submitted to food-restriction protocols and thus were hypoglycaemic [30]. Besides, adrenergic antagonists are capable of preventing contextual fear memory-strengthening effects induced by Ad [10, 14] but not by glucose [31].

Under the circumstances of our experimental conditions (protocol in Fig. 1A), the chosen dose of glucose induced a moderate hyperglycaemic state in our animals 10 min after administration, as previously described [32]. In mice, the administration of glucose (30 mg/kg) immediately after inhibitory avoidance training strengthens the aversive memory of the aversive situation [32], however, the passive avoidance task does not allow for an accurate investigation of associative memory, contrary to fear conditioning [33].

In the experimental protocol of Fig. 1B, WT mice were administered with glucose (30 mg/kg) only before the fear acquisition session of the fear conditioning protocol, and no differences were observed in fear memory when mice were re-exposed to context. However, when glucose was administered both before the fear acquisition day and re-exposure to the context day (Fig. 1C) the outcome was different: WT mice administered with glucose presented increased contextual fear memory when compared to WT mice administered with the vehicle. This could indicate that glucose in both fear acquisition and context re-exposition may be necessary for fear contextual memory consolidation and reconsolidation. It has been proposed that each phase of the fear conditioning paradigm may be associated with a phase of memory establishment: the training day appears to be strongly related to acquisition (learning), while the context day is more intertwined with retrieval and reconsolidation processes [34, 35]. Because we only observed significant effects when glucose was administered on both days of the paradigm, and not when it was only administered before the acquisition day, we propose that glucose may be crucial for the recall of contextual fear memory.

Considering the Ad levels in the adrenal gland, which is the production site of about 80% of Ad [36], mice administered with glucose only on the acquisition day showed increased levels of this catecholamine when compared with mice administered with the vehicle. However, WT mice administered with glucose on both training and context days showed no differences in adrenal gland Ad levels. These results are in line with previous reports showing that during hyperglycaemia, the production and release of Ad from the adrenal gland is not increased [37]. In our experimental design, glucose-treated mice on fear acquisition and context days were evaluated while still hyperglycaemic, explaining our findings.

A low blood glucose sugar level triggers the release of Ad to induce glucose release to the bloodstream. Therefore, Ad is considered a hyperglycaemic agent [38, 39]. On the other hand, our previous reports showed that blood glucose increased in WT mice after the fear acquisition day of the fear conditioning procedure, which was not observed in Ad-deficient mice [14]. Additionally, exogenous administration of Ad in Ad-deficient mice reverts the observed contextual fear memory impairment [14, 15]. Although several interactions between Ad and glucose exist, none of the previously published works isolate the specific role of glucose. After blood glucose increment, astrocytes increase the glucose uptake for the synthesis of glycogen stores. During learning, the activation of neurotransmitter membrane receptors on those cells triggers glycogenolysis and the production of lactate [40]. Therefore, the role of glucose in memory enhancement may be connected with the astrocytic supply of lactate to neurons as a supplemental energy source [41, 42]. To our knowledge, this is the first study that accurately separates the effect of Ad from that of glucose. Following the contextual fear memory paradigm, Ad-deficient mice administered with glucose in both the acquisition and context days presented a strengthening of contextual fear memory when compared with the respective vehicle group. Therefore, glucose may revert the Ad-deficient mice contextual fear memory impairment, being necessary for consolidation and/or recall of contextual fear memory. Correspondingly, glucose appears to have an important role in contextual fear memory even when Ad is absent.

As previously cited, glucose plays a role as a general fuel for brain cells [40, 41]. Accordingly, glucose administration resulted in the release of acetylcholine in the hippocampus during inhibitory avoidance training and spontaneous alternation [43, 44]. This evidence suggests that some memory neurotransmitter modulators, such as acetylcholine, might operate on astrocyte receptors instead of neuronal receptors [45]. Therefore, our findings might follow this evidence: glucose may trigger the hippocampus's production of acetylcholine, which could subsequently act on astrocyte receptors to enhance contextual fear memory.

Long-term memory formation and enhancement have been related to the NR4A family of transcription factors encoding genes, that comprise the *Nr4a1*, *Nr4a2*, and *Nr4a3* genes [46]. Furthermore, studies confirmed that emotional memory formation is compromised in *Nr4a2*-deficient mutant mice and hippocampal expression of the three NR4A

family members has been shown to increase after contextual fear conditioning [46, 47]. The NR4A family of genes code for transcription factors that can trigger the expression of other genes, including the brain-derived neurotrophic factor (*Bdnf*), which has been linked to long-term memory formation [46, 48]. BDNF (peptide encoded by the *Bdnf* gene) is needed for the formation of brain circuits, the development and preservation of neuronal morphology, brain architecture, as well as synaptic and neuronal network plasticity [49–51]. Concerning the formation of fear memories, it has also been shown that fear conditioning causes an increase in *Bdnf* gene expression in the amygdala and is also widely expressed in the hippocampus, implying that this neurotrophin is involved in contextual fear memory and traumatic memories [49, 52].

Our group has previously demonstrated that Ad-deficient mice presenting less recall of a fearful event also presented lower levels of hippocampal *Nr4a* mRNA expression on the fear acquisition day of the contextual fear paradigm. Furthermore, when Ad was administered to the Ad-deficient mice, it was observed an increase in *Nr4a2* mRNA expression [10]. In this work, we assessed the hippocampal mRNA expression of *Nr4a1*, *Nr4a2*, *Nr4a3*, and *Bdnf* after a fear conditioning procedure in Ad-deficient mice administered with glucose (30 mg/kg). *Nr4a3* and *Bdnf* mRNA expression in the hippocampus of Ad-deficient mice treated with glucose (30 mg/kg) was significantly increased in comparison with those administered with the respective vehicle. These results are in line with a previously published article in which hippocampal mRNA expression of *Bdnf* as well as contextual fear memory were increased following insulin administration in Ad-deficient mice [53]. We propose that this happens due to an increase in glucose uptake in the CNS. In addition, our group has shown a possible involvement of the *Nr4a* gene family in the persistence of PTSD symptoms and signs [25], verifying a significant downregulation of the *Nr4a1* gene expression in the hippocampus of WT mice administrated with sotalol, a peripheral β-adrenoceptor antagonist, when compared to vehicle-treated mice [54].

In another set of experiments aiming to evaluate a possible synergy between Ad and glucose, Ad-deficient mice were submitted to the same fear conditioning protocol described above and administered with both sub-effective doses of Ad (0.01 mg/kg) and/or glucose (10 mg/kg) (Fig. 1D). The separate administration of these sub-effective doses was insufficient to strengthen contextual fear memory. However, the simultaneous administration of these sub-effective doses of Ad and glucose proved to strengthen contextual fear memory in Ad-deficient mice. These results lead us to suggest that Ad and glucose might act synergically to improve memory acquisition and consolidation. Altogether, this could indicate a synergy between Ad and glucose when animals are submitted to aversive tasks (such as fear conditioning procedure), contrary to non-aversive tasks which will not induce the release of Ad from the adrenal medulla.

In conclusion, our results reinforce the hypothesis that glucose may be important for the recall of contextual fear memories and a crucial part of the peripheral to the central pathway for the retrieval and reconsolidation of fear memories independently of Ad. These effects possibly occur through the upregulation of *Nr4a3* and *Bdnf* mRNA expression in the hippocampus which may be directly related to central molecular mechanism changes. In addition, Ad and glucose appear to act in synergy to enhance contextual fear memory establishment and persistence.

Acknowledgements The authors thank Sónia Soares and António Carlos Ferreira for their technical support.

Author Contributions Conceptualization: Ana Oliveira, Rafaela Seixas, and Mónica Moreira-Rodrigues; Methodology: Ana Oliveira, Márcia Azevedo, and Rafaela Seixas; Writing - original draft preparation: Ana Oliveira, Rafaela Seixas, and Mónica Moreira-Rodrigues; Writing - review and editing: Ana Oliveira, Márcia Azevedo, Rafaela Seixas, Raquel Martinho, and Mónica Moreira-Rodrigues; Funding acquisition: Ana Oliveira, Raquel Martinho, and Mónica Moreira-Rodrigues; Resources: Paula Serrão and Mónica Moreira-Rodrigues; Supervision: Paula Serrão and Mónica Moreira-Rodrigues.

Funding This work was partly supported by Foundation for Science and Technology (FCT, project UIDB/04308/2020 and UIDP/04308/2020). FCT provided PhD studentships to Ana Oliveira (SFRH/BD/138984/2018 and COVID/BD/152954/2022) and Raquel Martinho (SFRH/BD/133860/2017).
Open access funding provided by FCT|FCCN (b-on).

Data Availability All data generated or analyzed in this study are available in the published article.

Ethics Declarations

Ethics Approval All animal studies presented in this article were reviewed and approved by the Organism Responsible for Animal Welfare in Faculty of Medicine of University of Porto and National Authority for Animal Health.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

References

1. Fuller MD, Emrick MA, Sadilek M, Scheuer T, Catterall WA (2010) Molecular mechanism of calcium channel regulation in the fight-or-flight response. Sci Signal 3(141):ra70. https://doi.org/10.1126/scisignal.2001152
2. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, Myers B (2016) Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr Physiol 6(2):603–621. https://doi.org/10.1002/cphy.c150015
3. Chu B, Marwaha K, Sanvictores T, Ayers D (2022) Physiology, stress reaction. StatPearls. StatPearls Publishing Copyright © 2022. StatPearls Publishing LLC., Treasure Island (FL)
4. Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106(2):274–285. https://doi.org/10.1037//0735-7044.106.2.274
5. Anagnostaras SG, Gale GD, Fanselow MS (2001) Hippocampus and contextual fear conditioning: recent controversies and advances. Hippocampus 11(1):8–17. https://doi.org/10.1002/1098-1063(2001)11:1%3C8::Aid-hipo1015%3E3.0.Co;2-7
6. Anagnostaras SG, Maren S, Fanselow MS (1999) Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. J Neurosci 19(3):1106–1114. https://doi.org/10.1523/jneurosci.19-03-01106.1999
7. Goosens KA, Maren S (2001) Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. Learn Mem 8(3):148–155. https://doi.org/10.1101/lm.37601
8. Maren S, Fanselow MS (1995) Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. J Neurosci 15(11):7548–7564. https://doi.org/10.1523/jneurosci.15-11-07548.1995
9. Maren S, Phan KL, Liberzon I (2013) The contextual brain: implications for fear conditioning, extinction and psychopathology. Nat Rev Neurosci 14(6):417–428. https://doi.org/10.1038/nrn3492
10. Oliveira A, Martinho R, Serrao P, Moreira-Rodrigues M (2018) Epinephrine Released during traumatic events may strengthen contextual fear memory through increased Hippocampus mRNA expression of Nr4a transcription factors. Front Mol Neurosci 11:334. https://doi.org/10.3389/fnmol.2018.00334
11. Cahill L, Alkire MT (2003) Epinephrine enhancement of human memory consolidation: interaction with arousal at encoding. Neurobiol Learn Mem 79(2):194–198. https://doi.org/10.1016/s1074-7427(02)00036-9
12. Dornelles A, de Lima MN, Grazziotin M, Presti-Torres J, Garcia VA, Scalco FS, Roesler R, Schroder N (2007) Adrenergic enhancement of consolidation of object recognition memory. Neurobiol Learn Mem 88(1):137–142. https://doi.org/10.1016/j.nlm.2007.01.005
13. Ebert SN, Rong Q, Boe S, Thompson RP, Grinberg A, Pfeifer K (2004) Targeted insertion of the cre-recombinase gene at the phenylethanolamine n-methyltransferase locus: a new model for studying the developmental distribution of adrenergic cells. Dev Dyn 231(4):849–858. https://doi.org/10.1002/dvdy.20188
14. Alves E, Lukoyanov N, Serrao P, Moura D, Moreira-Rodrigues M (2016) Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors. Psychopharmacology 233(11):2099–2108. https://doi.org/10.1007/s00213-016-4254-5
15. Toth M, Ziegler M, Sun P, Gresack J, Risbrough V (2013) Impaired conditioned fear response and startle reactivity in epinephrine-deficient mice. Behav Pharmacol 24(1):1–9. https://doi.org/10.1097/FBP.0b013e32835cf408
16. Weil-Malherbe H, Axelrod J, Tomchick R (1959) Blood-brain barrier for adrenaline. Science 129(3357):1226–1227. https://doi.org/10.1126/science.129.3357.1226
17. Gold PE (2014) Regulation of memory - from the adrenal medulla to liver to astrocytes to neurons. Brain Res Bull 105:25–35. https://doi.org/10.1016/j.brainresbull.2013.12.012
18. Alva S (2008) FreeStyle Lite–a blood glucose meter that requires no coding. J Diabetes Sci Technol 2(4):546–551. https://doi.org/10.1177/193229680800200402
19. Ishikawa R, Fukushima H, Frankland PW, Kida S (2016) Hippocampal neurogenesis enhancers promote forgetting of remote fear memory after hippocampal reactivation by retrieval. Elife 5. https://doi.org/10.7554/eLife.17464
20. Valentinuzzi VS, Kolker DE, Vitaterna MH, Shimomura K, Whiteley A, Low-Zeddies S, Turek FW, Ferrari EA, Paylor R, Takahashi JS (1998) Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ x C57BL/6J)F2 mice. Learn Mem 5(4–5):391–403
21. Rocinholi LF, Almeida SS, De-Oliveira LM (1997) Response threshold to aversive stimuli in stimulated early protein-malnourished rats. Braz J Med Biol Res 30(3):407–413. https://doi.org/10.1590/s0100-879x1997000300016
22. Mendes P, Martinho R, Leite S, Maia-Moco L, Leite-Moreira AF, Lourenco AP, Moreira-Rodrigues M (2018) Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice. Int J Cardiol 253:113–119. https://doi.org/10.1016/j.ijcard.2017.10.014
23. Moreira-Rodrigues M, Roncon-Albuquerque R Jr., Henriques-Coelho T, Lourenco AP, Sampaio-Maia B, Santos J, Pestana M, Leite-Moreira AF (2007) Cardiac remodeling and dysfunction in Nephrotic Syndrome. Kidney Int 71(12):1240–1248. https://doi.org/10.1038/sj.ki.5002204
24. Introini-Collison IB, Baratti CM (1992) Memory-modulatory effects of centrally acting noradrenergic Drugs: possible involvement of brain cholinergic mechanisms. Behav Neural Biol 57(3):248–255. https://doi.org/10.1016/0163-1047(92)90234-u
25. Martinho R, Oliveira A, Correia G, Marques M, Seixas R, Serrao P, Moreira-Rodrigues M (2020) Epinephrine May contribute to the persistence of traumatic memories in a post-traumatic stress disorder animal model. Front Mol Neurosci 13:588802. https://doi.org/10.3389/fnmol.2020.588802
26. Moreira-Rodrigues M, Grubisha MJ (2022) Editorial: molecular mechanisms of neuropsychiatric Diseases. Front Mol Neurosci 15:1102296. https://doi.org/10.3389/fnmol.2022.1102296
27. Dufour S, Lebon V, Shulman GI, Petersen KF (2009) Regulation of net hepatic glycogenolysis and gluconeogenesis by epinephrine in humans. Am J Physiol Endocrinol Metab 297(1):E231–235. https://doi.org/10.1152/ajpendo.00222.2009
28. Gray DE, Lickley HL, Vranic M (1980) Physiologic effects of epinephrine on glucose turnover and plasma free fatty acid concentrations mediated independently of glucagon. Diabetes 29(8):600–608. https://doi.org/10.2337/diab.29.8.600
29. McAllister MS, Krizanac-Bengez L, Macchia F, Naftalin RJ, Pedley KC, Mayberg MR, Marroni M, Leaman S, Stanness KA, Janigro D (2001) Mechanisms of glucose transport at the blood-brain barrier: an in vitro study. Brain Res 904(1):20–30. https://doi.org/10.1016/s0006-8993(01)02418-0

30. Talley CE, Kahn S, Alexander LJ, Gold PE (2000) Epinephrine fails to enhance performance of food-deprived rats on a delayed spontaneous alternation task. Neurobiol Learn Mem 73(1):79–86. https://doi.org/10.1006/nlme.1999.3920
31. Gold PE, Vogt J, Hall JL (1986) Glucose effects on memory: behavioral and pharmacological characteristics. Behav Neural Biol 46(2):145–155. https://doi.org/10.1016/s0163-1047(86)90626-6
32. Kopf SR, Buchholzer ML, Hilgert M, Loffelholz K, Klein J (2001) Glucose plus choline improve passive avoidance behaviour and increase hippocampal acetylcholine release in mice. Neuroscience 103(2):365–371. https://doi.org/10.1016/s0306-4522(01)00007-0
33. Wilensky AE, Schafe GE, LeDoux JE (2000) The amygdala modulates memory consolidation of fear-motivated inhibitory avoidance learning but not classical fear conditioning. J Neurosci 20(18):7059–7066. https://doi.org/10.1523/JNEUROSCI.20-18-07059.2000
34. Quillfeldt JA (2016) Behavioral methods to study learning and memory in rats. Rodent model as tools in ethical biomedical research. Springer, pp 271–311
35. Izquierdo I, Quillfeldt JA, Zanatta MS, Quevedo J, Schaeffer E, Schmitz PK, Medina JH (1997) Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. Eur J Neurosci 9(4):786–793. https://doi.org/10.1111/j.1460-9568.1997.tb01427.x
36. Kvetnansky R, Lu X, Ziegler MG (2013) Stress-triggered changes in peripheral catecholaminergic systems. Adv Pharmacol 68:359–397. https://doi.org/10.1016/B978-0-12-411512-5.00017-8
37. Cohen WR, Deutsch J (1994) Hyperglycemia suppresses the adrenal medullary response to hypoxemia. Diabetes 43(5):645–648. https://doi.org/10.2337/diab.43.5.645
38. Moratinos J, Olmedilla B, de Pablos I, Vigueras MD (1986) Alpha-adrenoceptor involvement in catecholamine-induced hyperglycaemia in conscious fasted rabbits. Br J Pharmacol 89(1):55–66. https://doi.org/10.1111/j.1476-5381.1986.tb11120.x
39. Verberne AJ, Korim WS, Sabetghadam A, Llewellyn-Smith IJ (2016) Adrenaline: insights into its metabolic roles in hypoglycaemia and Diabetes. Br J Pharmacol 173(9):1425–1437. https://doi.org/10.1111/bph.13458
40. Brown AM, Baltan Tekkok S, Ransom BR (2004) Energy transfer from astrocytes to axons: the role of CNS glycogen. Neurochem Int 45(4):529–536. https://doi.org/10.1016/j.neuint.2003.11.005
41. Gold PE, Newman LA, Scavuzzo CJ, Korol DL (2013) Modulation of multiple memory systems: from neurotransmitters to metabolic substrates. Hippocampus 23(11):1053–1065. https://doi.org/10.1002/hipo.22182
42. Qiu L-L, Tan X-X, Yang J-J, Ji M-H, Zhang H, Zhao C, Xia J-Y, Sun J (2023) Lactate improves long-term cognitive impairment Induced by repeated neonatal sevoflurane exposures through SIRT1-mediated regulation of adult hippocampal neurogenesis and synaptic plasticity in male mice. Mol Neurobiol. https://doi.org/10.1007/s12035-023-03413-9
43. Morris KA, Chang Q, Mohler EG, Gold PE (2010) Age-related memory impairments due to reduced blood glucose responses to epinephrine. Neurobiol Aging 31(12):2136–2145. https://doi.org/10.1016/j.neurobiolaging.2008.12.003
44. Ragozzino ME, Pal SN, Unick K, Stefani MR, Gold PE (1998) Modulation of hippocampal acetylcholine release and spontaneous alternation scores by intrahippocampal glucose injections. J Neurosci 18(4):1595–1601. https://doi.org/10.1523/JNEUROSCI.18-04-01595.1998
45. Bekar LK, He W, Nedergaard M (2008) Locus coeruleus alpha-adrenergic-mediated activation of cortical astrocytes in vivo. Cereb Cortex 18(12):2789–2795. https://doi.org/10.1093/cercor/bhn040
46. Hawk JD, Bookout AL, Poplawski SG, Bridi M, Rao AJ, Sulewski ME, Kroener BT, Manglesdorf DJ, Abel T (2012) NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors. J Clin Invest 122(10):3593–3602. https://doi.org/10.1172/JCI64145
47. Rojas P, Joodmardi E, Hong Y, Perlmann T, Ogren SO (2007) Adult mice with reduced Nurr1 expression: an animal model for schizophrenia. Mol Psychiatry 12(8):756–766. https://doi.org/10.1038/sj.mp.4001993
48. Cunha C, Brambilla R, Thomas KL (2010) A simple role for BDNF in learning and memory? Front Mol Neurosci 3:1. https://doi.org/10.3389/neuro.02.001.2010
49. Hall J, Thomas KL, Everitt BJ (2000) Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci 3(6):533–535. https://doi.org/10.1038/75698
50. Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res 65(1):11–22. https://doi.org/10.1016/j.neures.2009.06.004
51. Lee TH, Ahadullah, Christie BR, Lin K, Siu PM-f, Zhang L, Yuan T-f, Komal P, Xu A, So K-f, Yau S-y (2021) Chronic AdipoRon Treatment mimics the effects of Physical Exercise on restoring hippocampal neuroplasticity in Diabetic mice. Mol Neurobiol 58(9):4666–4681. https://doi.org/10.1007/s12035-021-02441-7
52. Sun H, Zhang X, Kong Y, Gou L, Lian B, Wang Y, Jiang L, Li Q, Sun H, Sun L (2021) Maternal separation-Induced histone acetylation correlates with BDNF-Programmed synaptic changes in an animal model of PTSD with sex differences. Mol Neurobiol 58(4):1738–1754. https://doi.org/10.1007/s12035-020-02224-6
53. Oliveira A, Seixas R, Pereira F, Azevedo M, Martinho R, Serrao P, Moreira-Rodrigues M (2023) Insulin enhances contextual fear memory independently of its effect in increasing plasma adrenaline. Life Sci 328:121881. https://doi.org/10.1016/j.lfs.2023.121881
54. Martinho R, Seixas R, Azevedo M, Oliveira A, Serrao P, Moreira-Rodrigues M (2021) Sotalol Treatment May Interfere with Retrieval, expression, and/or reconsolidation processes thus disrupting traumatic Memories in a post-traumatic stress disorder mice Model. Front Pharmacol 12:809271. https://doi.org/10.3389/fphar.2021.809271

CHAPTER 4 – MANUSCRIPT III

MUSCARINIC SUBTYPE M4 MRNA EXPRESSION IN THE HIPPOCAMPUS MAY INFLUENCE CENTRAL CHOLINERGIC ACTIVITY THUS CONTRIBUTING TO THE STRENGTHENING EFFECT OF CONTEXTUAL FEAR MEMORY BY PERIPHERAL ADRENALINE

Muscarinic subtype M4 receptor mRNA expression in the hippocampus may influence central cholinergic activity thus contributing to the strengthening effect of contextual fear memory by peripheral adrenaline

Ana Oliveira[1,2], Márcia Azevedo[1,2], Rafaela Seixas[1,2], Soraia Silva[1,2], Raquel Martinho[1,2], Paula Serrão[2,3], Elisabete Silva[4,5], and Mónica Moreira-Rodrigues*[1,2]

[1]Department of Immuno-physiology and Pharmacology, Laboratory of General Physiology, School of Medicine and Biomedical Sciences (ICBAS), University of Porto (UP), Porto, Portugal
[2]Center for Drug Discovery and Innovative Medicines, University of Porto (MedInUP), Porto, Portugal
[3]Department of Biomedicine, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal
[4]Ageing and Stress Group; i3S– Institute for Research and Innovation in Health, University of Porto, Porto, Portugal
[5]Faculty of Veterinary Medicine, Lusófona University, Lisboa, Porto

ORCID:
Ana Oliveira – 0000-0003-3080-7625

Márcia Azevedo- 0000-0002-5755-1132

Rafaela Seixas - 0000-0003-2566-4383

Raquel Martinho - 0000-0002-2755-4965

Paula Serrão - 0000-0002-3663-7500

Elisabete Silva- 0000-0002-6736-3469

Mónica Moreira-Rodrigues - 0000-0003-1373-9202

Short Title: Adrenaline strengthening effect of contextual fear memory by M4 expression in the hippocampus

*Correspondence:
Address correspondence to M. Moreira-Rodrigues, Laboratory of General Physiology, ICBAS, University of Porto, R. Jorge Viterbo Ferreira, 228, Building 2, Floor 4, Cabinet 22, 4050-313, Porto, Portugal. Tel: 00351 22 0428 217.

E-mail: mirodrigues@icbas.up.pt

Keywords: fear conditioning, contextual fear memory, muscarinic receptor, adrenaline, adrenaline-deficient mice

Abstract

Adrenaline (Ad)-deficient mice have reduced contextual fear memory. Ad strengthens contextual fear memory due to peripheral actions which culminate in blood glucose increase. Raised blood glucose levels may increase acetylcholine synthesis in the hippocampus which may strengthen memory. Nevertheless, it is not known if the cholinergic system is influenced by Ad contributing to the strengthening effect of contextual fear memory. Our aim was to evaluate if muscarinic receptor expression is modulated by peripheral Ad and if the cholinergic system is involved in the strengthening of contextual fear memory by peripheral Ad.

Wild-type (WT) and Ad-deficient (129x1/SvJ) male mice were submitted to the fear conditioning procedure. Mice were administered pre-training and pre-context with Ad (0.1 mg/kg, i.p., 3 minutes (min)), atropine (10 mg/kg, i.p., 5 min), methylatropine (0.5 mg/kg, i.p., 5 min), Ad (0.1 mg/kg, i.p., 3 min) plus atropine (10 mg/kg, i.p., 5 min) or vehicle (NaCl, 0.9%, i.p.). Mice responsiveness to shock was evaluated on training day and freezing was evaluated during both fear acquisition and context day. Hippocampus samples were collected and relative quantification of M_1, M_2, and M_4 mRNA expression was evaluated.

In Ad-deficient mice, hippocampal muscarinic M_4 subtype receptor mRNA expression was decreased compared to WT mice. In Ad-deficient mice administered with Ad, it was observed an increase in hippocampal muscarinic M_4 subtype receptor mRNA expression compared with vehicle-administered Ad-deficient mice. No differences were observed in mRNA expression of the M_1 or M_2 receptor subtype between groups. On the context day, WT mice administered with atropine presented a decrease in freezing behaviour when compared to WT mice administered with vehicle or methylatropine. Moreover, administration of Ad plus atropine in Ad-deficient mice leads to a decrease in freezing behaviour when compared to Ad-administered Ad-deficient mice.

In conclusion, the present study suggests that contextual fear memory impairment observed in Ad-deficient mice could be related to the down expression of the hippocampal muscarinic M_4 subtype receptor, which is reverted when Ad is present. This may be related with contextual fear memory acquisition, consolidation, or retrieval induced by peripheral Ad. Furthermore, the peripheral Ad strengthening effect of contextual fear memory might be due to the increase in muscarinic subtype M4 expression in the

hippocampus, which may contribute to an increase of the cholinergic activity in the central nervous system.

Introduction

Animals benefit from the ability to retain and use information gained remotely, as is the case of emotional memories, which can help them avoid risky situations in the future. Comprehending how emotional memories are formed and stored allows a window of opportunity for the treatment of pathologies associated with unpleasant and traumatic memories.

Contextual fear conditioning is a behavioural paradigm based on Pavlov's classical conditioning. It allows the association of an aversive stimulus (such as mild-intensity electric shocks) with a specific context. The fear conditioning paradigm is frequently utilized in research of the neurobiological basis for adverse memory and allows the study of fear and anxiety (Maren, 2001).

Exposure to fear conditioning leads to adrenaline (Ad) secretion from the medulla of the adrenal gland and Ad appears to be important for contextual fear memory strengthening (Toth et al., 2013; Alves et al., 2016; Oliveira et al., 2018). A genetically modified mouse lacking expression of the phenylethanolamine-N methyltransferase (Pnmt) gene, thus unable to synthesize Ad (Ebert et al., 2004), showed decreased contextual fear memory compared to wild-type (WT) mice (Toth et al., 2013; Alves et al., 2016). However, fear memory was restored upon administration of Ad (Alves et al., 2016). Furthermore, Ad appears to strengthen contextual fear memory by selectively acting on peripheral β2-adrenoceptors (β2-Ars) (Alves et al., 2016; Oliveira et al., 2018). However, despite all the evidence of Ad's importance in strengthening contextual fear memory, this catecholamine does not cross the peripheral circulation into the central nervous system, even in extremely stressful conditions (Weil-Malherbe et al., 1959; Oliveira et al., 2018). Ad-mediated strengthening effect of contextual fear memory may be due to glucose release from hepatic glucogenesis and gluconeogenesis storage (Talley et al., 2000; Gold, 2014). Glucose is transported through the blood-brain barrier (Cornford et al., 1994; Dobrogowska and Vorbrodt, 1999; Vorbrodt et al., 2001) and may be a precursor for acetylcholine synthesis (Gibson and Blass, 1976; Gibson et al., 1978), which has memory enhancement effects (Ragozzino et al., 1996; Pych et al., 2005).

Acetylcholine is an important neurotransmitter for attention, learning, and synaptic plasticity (Gold, 2003; Sarter et al., 2003). This neuromodulator can act on two different families of cholinergic receptors, the muscarinic (G protein-coupled receptor) and nicotinic receptors (a ligand-gated ion channel) (Albuquerque et al., 2009). Many

pharmacological studies have shown the importance of these receptors in memory modulation. For instance, oxotremorine (a muscarinic receptor agonist) enhances the consolidation of contextual fear memory, whereas the administration of scopolamine (a muscarinic receptor antagonist) disrupts it (Passani et al., 2001; Cangioli et al., 2002), suggesting a role of muscarinic receptors in this type of memory.

Although the independent role of Ad (Toth et al., 2013; Alves et al., 2016) and muscarinic receptors (Passani et al., 2001; Cangioli et al., 2002) is well established in contextual fear memory, only a few studies demonstrate that stressful situations can lead to acetylcholine release in the hippocampus (Billewicz-Stankiewicz et al., 1975; Górny et al., 1975) and regulate the expression of muscarinic receptors (Mulugeta et al., 2006), however without showing a direct relation with Ad. For instance, it was shown an impairment in the expression of muscarinic receptors in the hippocampus in adrenalectomized rats (Mulugeta et al., 2006), and this may be due to the inability to synthesize corticosteroids and/or catecholamines. In two memory tasks, namely inhibitory avoidance (associative memory) and y-maze (spatial memory), the facilitatory effect of Ad in memory strengthening was reverted by atropine (a muscarinic antagonist) (Introini-Collison and McGaugh, 1988). Also, oxotremorine facilitated retention in the inhibitory avoidance test when administered simultaneously with a sub-effective dose of Ad (Introini-Collison and McGaugh, 1988). Nevertheless, the inhibitory avoidance paradigm does not allow the precise study of associative memory like contextual fear memory test (Liang, 2009; Ogren and Stiedl, 2015).

The above mentioned studies do not demonstrate the direct role of Ad in influencing the cholinergic system and contributing to Ad strengthening effect of contextual fear memory. Therefore, the present study uses Ad-deficient mice aiming to understand if the muscarinic receptors expression in the hippocampus is modulated by Ad and if the cholinergic system is involved in the strengthening of contextual fear memory by peripheral Ad, using the fear conditioning procedure.

Methods

Animals

The animal care and experimental protocols were carried out in accordance with European Directive number 2010/63/EU, transposed to Portuguese legislation by Directive Law 113/2013 and 1/2019, and approved by the Organism Responsible for Animal Welfare (ORBEA) in the Faculty of Medicine of University of Porto and National Authority for Animal Health (DGAV). Ad-deficient (129x1/SvJ, n = 31) and WT (129x1/SvJ, n = 29) male mice (8 to 12 weeks old) were bred under controlled environmental conditions (12 hour light / dark cycle, room temperature 23 ± 1°C, humidity 50%, autoclaved drinking water, mice diet 4RF25/I and 4RF21/A; Mucedola, Porto, Portugal) in the same room and housed with the respective litter.

Ad-deficient (*Pnmt*-/-) mice were produced by the insertion of the Cre-recombinase gene and a Neomycin resistance gene into exon 1 of the locus encoding for Pnmt enzyme. Consequently, *Pnmt*-/- mice are unable to express the Pnmt enzyme and to synthesize Ad (Ebert et al., 2004). Polymerase chain reaction (PCR) of DNA from ear samples, was used to confirm the genotype, as previously described (Ebert et al., 2004).

Conditioned fear test

The protocol for the contextual fear conditioning paradigm was adapted from previous studies (Alves et al., 2016; Oliveira et al., 2023). The conditioning chamber consisted of a clear Plexiglas box (26.5 cm x 26.5 cm x 26.5 cm) equipped with a metal grid floor with 32 stainless steel bars (placed 5 mm apart) wired to a stimulus generator. The first day of the contextual fear conditioning paradigm (fear acquisition) had a duration of 6 minutes (min). In the first 3 mins, mice had an undisturbed habituation period to allow the exploration of the conditioning chamber. Afterwards, 3-foot shocks (duration: 2 s, intensity: 0.6 mA) were delivered at 60-second intervals. After 24 hours mice were re-exposed to the same conditioning chamber in the same apparatus (context test) for 10 min, but no foot shocks were delivered. On both days, a timer was placed on the top of the conditioning chamber and mice were recorded with a digital video camera Sony HDR-CX405 (Sony Corporation, Japan) for posterior mice behaviour evaluation. The analysis of the video records was performed under manual and blind protocols. On acquisition day, jump (removal of 3 paws from the grid floor) and vocalization (audible high-pitched squeak) were quantified (Rocinholi et al., 1997). Freezing behaviour was defined as the absence of movement excluding respiration for at least 3 seconds (Valentinuzzi et al.,

1998), and was evaluated in both fear acquisition and context test day. The conditioned chamber was cleaned with alcohol 70% and smeared with 1% acetic acid between trials.

Behavioural treatments

Mice were submitted to the fear conditioning paradigm, with different drug administration protocols, as displayed in Figure 1. Initially, protocol A consisted in a group of Ad-deficient and WT mice, and in another set of experiments a group of Ad-deficient mice administered with Ad pre-training and pre-context (0.1 mg/mg, i.p., 3 min (Alves et al., 2016)) or vehicle (0.9 % NaCl; i.p., 3 min) (Figure 1A). Afterwards, a group of WT mice was administered pre-training and pre-context with atropine (10 mg/kg, i.p., 5 min (Baratti et al., 1984; Introini-Collison and Baratti, 1992)), methylatropine (10 mg/kg, i.p., 5 min (Baratti et al., 1984; Introini-Collison and Baratti, 1992)) or vehicle (0.9 % NaCl; i.p., 3 min) (Figure 1B). Finally, a group of Ad-deficient mice were administered pre-training and pre-context with Ad (0.1 mg/kg, i.p., 3 min), Ad (0.1 mg/kg, i.p., 3 min) plus atropine (10 mg/kg, i.p., 5 min) or vehicle (0.9% NaCl, i.p.) (Figure 1C).

Catecholamines quantification

Following fear conditioning paradigm mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg; i.p.) for left adrenal collection. The left adrenal gland collected was emerged in perchloric acid (PCA) 0.2 M overnight, at 4 ºC, and frozen at -80 ºC until further use. To quantify catecholamines in the adrenal gland, the PCA of each sample was transferred to tubes with filters and centrifuged at 1,250 g, for 2 min, at 4 ºC. A solution with 125 mg/ml for purified Ad, NA, and DA (Sigma-Aldrich, St. Louis, USA) was used as standard solution. Subsequently, 50 µL of standard solution and PCA of each sample were mixed with the mobile phase (Citric acid monohydrate 100 mM, Sodium acetate 100 mM, 1-Octanesulfonic acid sodium salt 1.6 mM, Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) 0.15 mM, Methanol 4%, Di-n-butylamine 1 mM, pH set for 3,5 with HclO4 70 %) and injected in a reverse-phase high-performance liquid chromatography (HPLC) (Gilson Medical Electronics, Villiers le Bel, France). The components of interest in each sample or in the external standard solution were separated in the column (Spheri-5 C18 4.6x250 mm, 5µm; BrownleeTM, Waltham, MA USA) and quantified by electrochemical detection (ED). The limits of detection and quantification for the HPLC (Gilson Medical Electronics, Villiers le Bel, France) employed for adrenal

gland samples were 350 to 1000 fmol and 700 to 2000 fmol, respectively. NA = 6.60 min, Ad = 7.81 min, and DA = 15.95 min were the retention times. Catecholamine content was calculated per whole adrenal gland (nmol/AG).

RNA isolation and relative quantification of mRNA expression

Real-time PCR (qPCR) was performed in hippocampus samples collected immediately after the context day of the fear conditioning protocol, as described in previous works (Moreira-Rodrigues et al., 2007; Mendes et al., 2018; Oliveira et al., 2023). Total RNA isolation was carried out with Mini Kit illustra™ Isolate II RNA (Bioline, London, United Kingdom). The concentration and purity of the isolated RNA were measured with NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, United States). A T100™ Thermal Cycler (Bio-Rad, Hercules, United States) was used to carry out reverse transcription, applying a Reverse Transcription kit (NZY First-Strand cDNA Synthesis Kit NZYTech — Genes and Enzymes, Lisbon, Portugal). StepOne™ real-time PCR System (Applied BioSystems, Waltham, United States) allowed the conduction of qPCR reactions. Gene-specific primers (10 µM), Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, United States), Rnase-free H_2O (Bioline, London, United Kingdom) were mixed and cDNA was added (1:20). Instead of cDNA, Rnase-free H_2O (Bioline, London, United Kingdom) was added as a negative control. Gene-specific primers are presented in Table 1. Results of mRNA quantification were expressed in an arbitrary unit (AU) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Drugs

-Adrenaline, L-(-)-noradrenaline, dopamine hydrochloride, atropine, methylatropine, and PCA were purchased from Sigma-Aldrich (St. Louis, United States). Ketamine (Imalgene 1,000, Merial, Lisbon, Portugal) and xylazine (Rompum 2%, Bayer, Lisbon, Portugal) were purchased from Agrofauna (Vila Nova de Gaia, Portugal).

Statistical analysis

An online Sample Size Calculator was used to establish the minimum sample size for each experimental protocol (https://clincalc.com/Stats/SampleSize.aspx). All statistical analysis was performed using the program GraphPad Prism 8 (GraphPad Software Inc.,

La Jolla, CA, USA), and all data was displayed as means with standard error of the means (SEM).

Freezing behaviour was analysed by Two-Way Analysis of Variance (ANOVA) repeated measures. When differences in ANOVA analysis were observed multiple comparison corrections by Sidak's (two groups) or Tukey´s (three or more groups) post-hoc tests were performed (α was set at 0.05). Vocalization and jump responses were examined using Student's t-test (2 groups) or One-Way ANOVA (three or more groups). For One-Way ANOVA the post-hoc Tukey test was used for multiple comparisons correction (α was set at 0.05). Catecholamines concentration and qPCR results were analysed using Student's t-test. Effect sizes were estimated by calculating Cohen's *d* test for t-test comparisons and partial eta squared (η_2^p) for ANOVA.

The statistically significant differences observed between groups in the different time points or between groups in Two-Way and One-Way ANOVA, respectively, were based on the adjusted *p*-value < 0.05. The statistically significant difference for unpaired t-test analysis was set at $p < 0.05$.

Results

Adrenaline-deficient mice presented both impaired contextual fear memory and hippocampal M_4 gene expression

Figure 2 compares WT and Ad-deficient mice in freezing, vocalization, jump behaviour, and hippocampus gene expression of muscarinic receptors (M_1, M_2, and M_4). On fear acquisition day, a time effect ($F_{(3, 24)} = 30.27$, $p < 0.0001$, $\eta_p^2 = 0.79$, Figure 2A) was observed, but no genotype effect ($F_{(1, 8)} = 0.47$, $p = 0.5130$, $\eta_p^2 = 0.06$, Figure 2A) or time versus genotype interaction ($F_{(3, 24)} = 0.80$, $p = 0.5049$, $\eta_p^2 = 0.09$, Figure 2A) were observed. No differences in freezing behaviour were observed between WT and Ad-deficient mice (Figure 2A). In addition, no differences were observed in jump ($t_{(8)} = 1.81$, $p = 0.1080$, Cohens' $d = 1.76$, Figure 2B) and vocalization ($t_{(8)} = 0.67$, $p = 0.5237$, Cohens' $d = 0.57$, Figure 2B) responses in the first day of the fear conditioning paradigm. On context day of fear conditioning paradigm, a significant effect of time ($F_{(4, 32)} = 8.28$, $p = 0.0001$, $\eta_p^2 = 0.51$, Figure 2C) and an interaction between time versus genotype ($F_{(4, 32)} = 4.67$, $p = 0.0044$, $\eta_p^2 = 0.37$, Figure 2C) was observed. Ad-deficient mice presented a decrease in freezing behaviour when compared to WT mice (Figure 2C).

Considering hippocampus gene expression of muscarinic receptors (M_1, M_2, and M_4) after fear conditioning paradigm, it was observed a diminished mRNA expression of M_4 subtype receptors ($t_{(8)} = 2.37$, $p = 0.0452$; Cohens' $d = 1.68$, Figure 2F) in Ad-deficient mice when compared to WT mice. However, no differences were observed in mRNA expression of M_1 ($t_{(8)} = 0.97$, $p = 0.3583$; Cohens' $d = 0.47$, Figure 2D) and M_2 subtype receptors ($t_{(8)} = 0.95$, $p = 0.3714$; Cohens' $d = 0.05$, Figure 2E) between groups.

Regarding catecholamines concentration in the adrenal gland, Ad was not detected, by HPLC-ED, in Ad-deficient mice, as expected. In addition, an increase in noradrenaline (NA) was observed in Ad-deficient mice compared to WT mice ($t_{(8)} = 5.12$, $p = 0.0009$, Cohens' $d = 7.23$, Table 2).

Adrenaline administration restores contextual fear memory and hippocampal expression of mRNA M_4 subtype receptors in adrenaline-deficient mice

The effect of Ad administration in freezing, vocalization, and jump behaviour, and hippocampus gene expression of muscarinic receptors (M_1, M_2, and M_4) was explored in Ad-deficient mice. During fear acquisition day a time effect ($F_{(3, 27)} = 45.12$, $p < 0.0001$, $\eta_p^2 = 0.83$, Figure 3A) was observed, however no treatment ($F_{(1, 9)} = 0.55$, $p = 0.4758$, $\eta_p^2 = 0.06$, Figure 3A) and time versus treatment interaction ($F_{(3, 27)} = 0.64$, $p = 0.5955$, η_p^2

= 0.07, Figure 3A) effect was observed. During training day no differences in freezing behaviour were observed between Ad-deficient mice administered with Ad compared to those administered with vehicle. Also, no differences were observed regarding jump ($t_{(9)}$ = 1.01, p = 0.3384, Cohens' d = 0.41, Figure 3B) and vocalization ($t_{(9)}$ = 0.53, p = 0.6072, Cohens' d = 0.37, Figure 3B) behaviours between groups.

Regarding context day of fear conditioning paradigm, a significant effect of treatment ($F_{(1, 9)}$ = 13.99, p = 0.0046, η_p^2 = 0.61, Figure 3C) was observed. No effect of time ($F_{(4, 36)}$ = 1.33, p = 0.2764, η_p^2 = 0.13, Figure 3C) was observed. Ad-deficient mice administered with Ad presented increased freezing behaviour when compared to Ad-deficient mice administered with vehicle (Figure 3C).

Regarding hippocampus gene expression of muscarinic receptors (M_1, M_2, and M_4), an increase in mRNA expression of M_4 subtype receptors ($t_{(9)}$ = 2.32, p = 0.0453; Cohens' d = 1.62, Figure 3F) was observed in Ad-deficient mice administered with Ad when compared to those administered with vehicle. However, no differences were observed in mRNA expression of M_1 ($t_{(9)}$ = 0.44, p = 0.6737; Cohens' d = 0.49, Figure 3D) and M_2 subtype receptors ($t_{(8)}$ = 0.92, p = 0.3858; Cohens' d = 0.08, Figure 3E) between groups.

Regarding catecholamines concentration in the adrenal gland, Ad was not detected, by HPLC-ED, in Ad-deficient mice, as expected. In addition, no differences in NA were observed in Ad-deficient mice administered with vehicle compared to Ad-deficient mice administered with Ad ($t_{(9)}$ = 0.49, p = 0.6374, Cohens' d = 0.66, Table 3).

Central but not peripheral muscarinic receptor antagonist appears to be involved in contextual fear memory

Figure 4 exhibits the effects of atropine (a peripheral and central muscarinic antagonist) and methylatropine (a peripheral muscarinic antagonist) in freezing behaviour and shock responsiveness in WT mice. On acquisition day it was observed a time effect ($F_{(3, 63)}$ = 193.3, $p < 0.0001$, η_p^2 = 0.90, Figure 4A), but no differences were observed in treatment ($F_{(2, 21)}$ = 0.04, p = 0.9636, η_p^2 = 0.004, Figure 4A) or interaction between time x treatment ($F_{(6, 63)}$ = 0.30, p = 0.9327, η_p^2 = 0.03, Figure 4A). No differences in freezing behaviour between groups were observed (Figure 4A). Additionally, no differences were observed in jump ($F_{(2, 21)}$ = 1.11, p = 0.3482; η_p^2 = 0.10, Figure 4B) and vocalization ($F_{(2, 21)}$ = 1.71, p = 0.2053; η_p^2 = 0.14, Figure 4B) responses between groups.

On context day of fear conditioning paradigm, a significant treatment ($F_{(2, 21)}$ = 12.23, p = 0.0003, η_p^2 = 0.54, Figure 4C) and time ($F_{(4, 84)}$ = 13.11, $p < 0.0001$, η_p^2 = 0.38, Figure

4C) effect was observed. Atropine-administered WT mice presented a decreased in freezing behaviour compared to WT mice administered with vehicle or methylatropine. Furthermore, no differences were observed between WT mice administered with methylatropine and those administered with vehicle (Figure 4C).

In the adrenal glands no differences were observed in NA ($F_{(2, 21)} = 0.08$, $p = 0.9247$, $\eta_p^2 = 0.008$, Table 4) or Ad ($F_{(2, 21)} = 1.09$, $p = 0.3524$, $\eta_p^2 = 0.09$, Table 4) between groups.

Central muscarinic receptor antagonist reverts contextual fear memory enhancement induced by adrenaline in adrenaline-deficient mice

Regarding the effect of atropine in Ad-deficient mice administered with Ad in fear conditioning, it was analysed jump, vocalization, and freezing behaviour. During acquisition day of fear conditioning paradigm, it was observed a time ($F_{(3, 36)} = 76.38$, ,$p < 0.0001$, $\eta_p^2 = 0.86$, Figure 5A) effect but no differences in treatment ($F_{(2, 12)} = 0.01$, $p = 0.9866$, $\eta_p^2 = 0.01$, Figure 5A) or interaction between time x treatment ($F_{(6, 36)} = 0.44$, $p = 0.8503$, $\eta_p^2 = 0.07$, Figure 5A) was observed. Also, no differences were observed in freezing behaviour between groups (Figure 5A). Besides, no differences were observed in jump ($F_{(2, 21)} = 3.25$, $p = 0.0745$; $\eta_p^2 = 0.24$, Figure 5B) and vocalization ($F_{(2, 12)} = 0.86$, $p = 0.4488$; $\eta_p^2 = 0.13$, Figure 5B) responses between treatments.

On context day of fear conditioning paradigm, a treatment ($F_{(2, 12)} = 11.92$, $p = 0.0014$, $\eta_p^2 = 0.67$, Figure 5C) and a time ($F_{(4, 48)} = 7.92$, $p < 0.0001$, $\eta_p^2 = 0.40$, Figure 5C) effect was observed. Ad-deficient mice administered with Ad showed an increase in freezing behaviour compared to those administered with vehicle or Ad plus atropine (Figure 5C).

Concerning catecholamines concentration in the adrenal glands, no differences were observed in NA ($F_{(2, 12)} = 0.11$, $p = 0.8988$, $\eta_p^2 = 0.02$, Table 5) between groups. In Ad-deficient mice no Ad was detected by HPLC-ED (Table 5).

Discussion

Stressful situations induce a response in any living organism, forcing the body to react. This reaction is important for the organism's survival and evokes the release of catecholamines by the medulla zone of the adrenal glands, specifically NA and Ad (Euler, 1946; Oliveira et al., 2018; Martinho et al., 2020). Catecholamines induce an adaptive physiological response to allow for an organism's reaction, namely "freeze, fight or flight" (Bracha, 2004). In fact, Ad increases memory associated with stressful and fearful events, including traumatic memories, activating peripheral β_2-aRs (Alves et al., 2016; Oliveira et al., 2018; Martinho et al., 2021). Furthermore, mice unable to produce endogenous Ad, as those used in this study, exhibit a decrease in contextual and traumatic memory of conditioned fear and post-traumatic stress disorder (PTSD) mouse model (Toth et al., 2013; Alves et al., 2016; Martinho et al., 2020).

In this study, WT and Ad-deficient mice were used. In the first protocol, these animals were subjected to the contextual fear conditioning paradigm. As expected, no differences were observed on training day in freezing behaviour or shock responsiveness between WT and Ad-deficient mice treatment groups, which may indicate that pain perception of foot shocks is not affected in Ad-deficient mice or by Ad administration. On context day, Ad-deficient mice showed contextual fear memory impairment in comparison with WT mice, further verifying the role of endogenous Ad in the strengthening of contextual fear memory, as previously reported by us and others (Toth et al., 2013; Alves et al., 2016). Afterwards, catecholamines in adrenal glands were evaluated and no Ad was detected in Ad-deficient mice by HPLC-ED, as previously shown (Alves et al., 2016; Martinho et al., 2020; Oliveira et al., 2023). Ad does not cross the blood-brain barrier (Skultetyova et al., 1998; Habgood et al., 2007; Oliveira et al., 2018) and therefore does not activate adrenergic receptors on the central nervous system, hence, another mechanism mediated by Ad may be responsible for the strengthening of contextual fear memory. At the periphery Ad may activate β-adrenoceptors expressed in hepatocytes, leading to the release of glucose into the bloodstream (Talley et al., 2000; Gold, 2014). In the brain, under high-energy demand conditions (such as fear condition memory formation) (Kong et al., 2017), glucose can be used to the synthesis of acetylcholine (Tucek and Cheng, 1974; Kaufman et al., 1991; Schousboe et al., 1993). In addition, the release of acetylcholine in the hippocampus during spatial working learning and memory tasks appears to be regulated by glucose (Ragozzino et al., 1996; Pych et al., 2005). Therefore,

glucose could contribute for memory consolidation through acetylcholine release. Although Ad-mediated responses seem to be essential for the strengthening of contextual fear memory it is still necessary to clarify which neuronal processes are activated for this strengthening to occur.

Acetylcholine is an important neurotransmitter in cognitive processes and hippocampus-dependent learning, such as in the case of conditioned fear (Nail-Boucherie et al., 2000; Kopf et al., 2001). Accordingly, previous studies have found that both systemic administrations of Ad or glucose lead to an increase in acetylcholine levels in the cortex and hippocampus (Billewicz-Stankiewicz et al., 1975; Durkin et al., 1992; Power et al., 2003). Still, none of these studies fully investigated Ad, glucose, and acetylcholine possible pathways using the contextual fear conditioning paradigm (Introini-Collison and McGaugh, 1988; Introini-Collison and Baratti, 1992). Therefore, the use of an Ad-deficient mouse may be a step forward in this subject.

In the present study, an animal model of the fear conditioning paradigm was used to evaluate whether the expression of muscarinic receptors in the hippocampus is altered in association with the contextual fear memory of animals unable to produce endogenous Ad. In the hippocampus of Ad-deficient mice mRNA expression of muscarinic M_4 receptor was decreased, when compared to WT mice. In addition, administration of 0.1 mg/kg of Ad which resulted in an Ad plasma concentration of 24 pmol/ml (Alves et al., 2016) was able to reverse the memory impairment in contextual fear memory previously observed in Ad-deficient mice and increase hippocampal mRNA expression of muscarinic M_4 subtype receptor. It was previously reported that 0.1 mg/kg of Ad (the same dose used in the present study) administered intraperitoneally increased acetylcholine content in the cerebral cortex (Billewicz-Stankiewicz et al., 1975; Górny et al., 1975) thus suggesting that peripheral Ad may influence the acetylcholine synthesis in the central nervous system. Now, we demonstrate that peripheral Ad influences hippocampal muscarinic receptors. To our knowledge, this is the first study reporting the association between peripheral Ad and hippocampal M_4 subtype receptor expression in fear conditioning paradigm.

The muscarinic receptors have been shown to play a crucial role in memory consolidation, specifically in the hippocampus-dependent learning and memory processes (Anagnostaras et al., 2003; Pych et al., 2005). Additionally, pharmacological studies have also demonstrated the involvement of M_4 subtype receptors in memory. Administration of a small molecule that enhances the activity of the muscarinic acetylcholine M_4 subtype

receptor demonstrated that allosteric potentiation can improve cognitive performance in rodents, highlighting the potential of M_4 subtype receptor modulation as a therapeutic approach for cognitive impairment (Shirey et al., 2008). Also, activation of the M_4 subtype muscarinic receptor with VU0152100 improved recognition memory performance in rats (Galloway et al., 2014). Furthermore, the M_4 subtype receptor can modulate the activity of other neurotransmitter systems involved in memory consolidation, such as the dopamine system (Gomeza et al., 1999). Accordingly, our results show that impairment of contextual fear memory acquisition, consolidation, and/or retrieval in Ad-deficient mice may be related to the downregulation of the M4 subtype muscarinic receptor in the hippocampus, which is reversed by Ad administration. Further studies targeting this receptor to explore therapeutic potential in the treatment of stress memory disorders, such as anxiety and post-traumatic stress disorder are needed.

Similarly, to M_4, M_1 and M_2 receptors are highly expressed in the hippocampus brain region (Mrzljak et al., 1993; Flynn et al., 1995; Falk et al., 2020) and could also be involved in contextual fear memory. For instance, studies have shown that M_1 subtype receptor knockout mice exhibit deficits in spatial memory and contextual fear conditioning (Gerber et al., 2001; Miyakawa et al., 2001; Anagnostaras et al., 2003), and systemic administration of a M_1 antagonist impairs contextual fear memory (Fornari et al., 2000). Regarding M_2 subtype receptors, M_2 knockout mice displayed decreased long-term potentiation (Seeger et al., 2004; Zheng et al., 2012), impaired working memory and impaired memory in a passive avoidance task (Bainbridge et al., 2008). However, memory in contextual fear memory was not affected in both cued and contextual tests (Bainbridge et al., 2008). However, in the present study, no differences were observed in M_1 and M_2 subtype receptor expression in the hippocampus between WT and Ad-deficient mice and between Ad-deficient mice administered with vehicle or Ad. Accordingly, mice undergoing adrenalectomy (unable to synthesize catecholamines and in particular Ad) presented decreased hippocampal M_4 expression but normal hippocampal M_1 expression one month after the surgery (Mulugeta et al., 2006). Therefore, it is possible that specifically M_4 subtype receptor may be more involved in contextual fear memory strengthening induced by peripheral Ad.

As decreases in the expression of the M_4 subtype receptor were observed suggesting an important role of muscarinic receptors in our model, the animals were then administered with an antagonist of this type of receptor. The hypothesis that a muscarinic receptor antagonist is able to reverse the Ad memory-strengthening effect, previously observed

(Toth et al., 2013; Alves et al., 2016; Oliveira et al., 2018) was also studied. In this new set of experiments, after the administration of non-selective muscarinic receptor antagonists (atropine and methylatropine), Ad, or vehicle, mice were subjected to the same contextual fear conditioning protocol for subsequent evaluation of contextual memory. No differences were observed in freezing behaviour or shock responsiveness on the training day in WT and Ad-deficient mice after these drugs administration, which may indicate that pain perception of foot shocks was not affected. WT mice administered with atropine (peripheral and central muscarinic antagonist) showed a decrease in contextual fear memory compared to those administered with vehicle. Nevertheless, when WT mice were administered with methylatropine (only peripheral muscarinic antagonist) contextual fear memory acquisition, consolidation, and/or retrieval was not affected. These observations may indicate a central effect of acetylcholine in contextual fear memory.

In Ad-deficient mice administered with Ad, it was observed the strengthening of contextual fear memories that was reverted by atropine administration. This observation could indicate that a strengthening effect of peripheral Ad on contextual fear memory could be facilitated by the release of acetylcholine in the central nervous system. Accordingly, previous studies suggested the block by atropine of a facilitatory effect of Ad in Y-maze discrimination response and inhibitory avoidance (Introini-Collison and Baratti, 1992). Notwithstanding, even though these studies are in agreement with the results obtained in this work, to our knowledge this is the first study relating peripheral Ad and cholinergic mechanisms in contextual fear memories paradigm. Although inhibitory avoidance and contextual fear conditioning paradigms have overlapping characteristics, the contextual fear memory test, contrary to the inhibitory avoidance paradigm, allows the precise study of associative memory (Liang, 2009; Ogren and Stiedl, 2015).

In conclusion, the present study suggests a role of the muscarinic receptor M4 subtype during contextual fear conditioning paradigm, unlike the subtypes M_1 and M_2. This may be related with contextual fear memory acquisition, consolidation, and/or retrieval induced by peripheral Ad. In addition, a central muscarinic receptor antagonist is capable to revert contextual fear memory induced by peripheral Ad, contrary to a peripheral muscarinic receptor antagonist. Altogether, the peripheral Ad strengthening effect in contextual fear memory might be due to the increase of cholinergic activity in the central nervous system.

Table 1. Primers used in gene expression analysis.

Gene	**Primer (5'→ 3')**	
M_1	F: GTCCCATGGAAACCCTGAATCC R: ATGTTGGGACTGACAGCAGG	(Mahboob et al., 2016)
M_2	F: TCTTCCTGAAAGCCAGATCCAG R: GTCACTGACTTAGTCGCCCG	
M_4	F: CACCTGTCTCCAGTTGGGTTC R: ATGAGATCTGCACACGCCAG	
Gapdh	F: CCATCACCATCTTCCAGGAG R: GCATGGACTGTGGTCATGAG	(Oliveira et al., 2018)

M, muscarinic receptor 1, 2, and 4; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; F, forward primer and R, reverse primer.

Table 2. Adrenal gland catecholamines concentration in wild-type (WT) and Ad-deficient mice after fear conditioning procedure

Treatment groups	NA (nmol/AG)	Ad (nmol/AG)
WT	4.82 ± 0.86	14.87 ± 3.13
Ad-deficient	16.71 ± 2.16*	Undetectable

Values are means ± standard error of the means (SEM) of 5 mice per group. WT, wild-type mice; Ad-deficient, Adrenaline-deficient mice; NA, noradrenaline; Ad, adrenaline. *, significantly different from correspondent values in WT mice ($p < 0.05$)

Table 3. Adrenal gland catecholamines concentration in Ad-deficient mice administered with Adrenaline after fear conditioning procedure

Treatment groups	NA (nmol/AG)	Ad (nmol/AG)
Ad-deficient Vehicle	16.71 ± 2.16	Undetectable
Ad-deficient Ad	15.52 ± 1.33	Undetectable

Values are means ± standard error of the means (SEM) of 5-6 mice per group. Ad-deficient Vehicle, Adrenaline-deficient mice administered with Vehicle; Ad-deficient Ad, Adrenaline-deficient mice administered with adrenaline; NA, noradrenaline; Ad, adrenaline.

Table 4. Adrenal gland catecholamines concentration in wild-type (WT) mice administered with Atropine or Methylatropine after fear conditioning procedure

Treatment groups	NA (nmol/AG)	Ad (nmol/AG)
WT Vehicle	6.99 ± 0.48	11.65 ± 0.99
WT Atropine	6.75 ± 0.46	13.31 ± 0.60
WT Methylatropine	6.93 ± 0.40	12.26 ± 0.76

Values are means ± standard error of the means (SEM) of 8 mice per group. WT Vehicle, Wild-type mice administered with Vehicle; WT Atropine, Wild-type mice administered with Atropine; WT Methylatropine, Wild-type mice administered with Methylatropine; NA, noradrenaline; Ad, adrenaline.

Table 5. Adrenal gland catecholamines concentration in Adrenaline-deficient mice administered with Adrenaline or Atropine after fear conditioning procedure

Treatment groups	NA (nmol/AG)	Ad (nmol/AG)
Ad-deficient Vehicle	28.83 ± 3.27	Undetectable
Ad-deficient Ad	32.51 ± 7.27	Undetectable
Ad-deficient Ad + Atropine	31.53 ± 6.12	Undetectable

Values are means ± standard error of the means (SEM) of 5 mice per group. Ad-deficient Vehicle, Adrenaline-deficient mice administered with Vehicle; Ad-deficient Ad, Adrenaline-deficient mice administered with Adrenaline; Ad-deficient Ad + Atropine, Adrenaline-deficient mice administered with Adrenaline and Atropine; NA, noradrenaline; Ad, adrenaline.

Figure captions

Figure 1. Schematic representation of the experimental design, behavioural protocols, treatments, and sample collection. (A) A group of wild-type (WT) and Ad-deficient mice, and a group of Adrenaline (Ad)-deficient mice administered with adrenaline (Ad, 0.1mg/kg, intraperitoneal, i.p., 3 min) or vehicle (0.9 % NaCl; i.p., 3 min) in both fear acquisition (day 1) and context test (day 2) of fear conditioning procedure followed protocol A. (B) A group of WT mice followed protocol B being administered with atropine (10 mg/kg; i.p., 5 min), methylatropine (0.5 mg/kg; i.p., 5 min) or vehicle (0.9 % NaCl; i.p., 5 min) in both fear acquisition and context test of fear conditioning procedure. (C) Finally, a group of Ad-deficient mice followed protocol C with atropine (10 mg/kg; i.p., 5 min) plus Ad (0.1 mg/kg, i.p., 3 min), Ad (0.1 mg/kg, i.p., 3 min) or vehicle (0.9 % NaCl; i.p., 5 min) administration in both the fear acquisition and context test. qPCR, real-time polymerase chain reaction; i.a, immediately after.

Figure 2. Behaviour and hippocampal muscarinic receptors expression in wild-type (WT) mice and Ad-deficient mice. (A) Freezing and (B) shock responsiveness in fear acquisition day, (C) freezing response in re-exposure to context, (D) muscarinic M_1, (E) M_2, and (F) M_4 subtype receptor expression in hippocampus after re-exposure to context in wild-type (WT) and Adrenaline (Ad)-deficient mice. Values are means ± SEM of 5 mice per group. Results of mRNA are expressed as arbitrary units after normalization for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *, significantly different from correspondent values in WT mice ($p < 0.05$).

Figure 3. Behaviour and hippocampal muscarinic receptors expression in Ad-deficient mice after Ad administration. (A) Freezing and (B) shock responsiveness in fear acquisition day, (C) freezing response in re-exposure to context, (D) muscarinic M_1, (E) M_2, and (F) M_4 subtype receptor expression in hippocampus after re-exposure to context in Adrenaline (Ad)-deficient mice administered with Ad or vehicle (NaCl, 0.9%). Values are means ± SEM of 5-6 mice per group. Ad-deficient Ad, Adrenaline-deficient mice administered with adrenaline; Ad-deficient Vehicle, Adrenaline-deficient mice administered with vehicle. Results of mRNA are expressed as arbitrary units after normalization for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *, significantly different from correspondent values in Ad-deficient Ad mice ($p < 0.05$).

Figure 4. Behaviour in wild-type (WT) mice after muscarinic agonists administration. (A) Freezing and (B) shock responsiveness on fear acquisition day, and (C) freezing response in re-exposure to the context in WT mice administered with Atropine (a peripheral and central muscarinic antagonist), Methylatropine (a peripheral muscarinic antagonist), or vehicle (NaCl, 0.9%). Values are means ± SEM of 8 mice per group. WT atropine, wild-type mice administered with atropine; WT methylatropine, wild-type mice administered with methylatropine; WT vehicle, wild type mice administered with vehicle. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$). †, significantly different from correspondent values in WT atropine mice ($p < 0.05$).

Figure 5. Behaviour in Ad-deficient mice after central muscarinic agonist and/or adrenaline administration. (A) Freezing and (B) shock responsiveness in fear acquisition day, and (C) freezing response in re-exposure to context in Adrenaline (Ad)-deficient mice administered with adrenaline (Ad), Ad plus Atropine or vehicle (NaCl, 0.9%). Values are means ± SEM of 5 mice per group. Ad-deficient Ad, Adrenaline-deficient mice administered with Ad; Ad-deficient Ad+Atropine, Adrenaline-deficient mice administered with Ad plus atropine; Ad-deficient vehicle, Adrenaline-deficient mice administered with vehicle. *, significantly different from correspondent values in Ad-deficient vehicle mice ($p < 0.05$). †, significantly different from correspondent values in Ad-deficient Ad mice ($p < 0.05$).

Figure 1

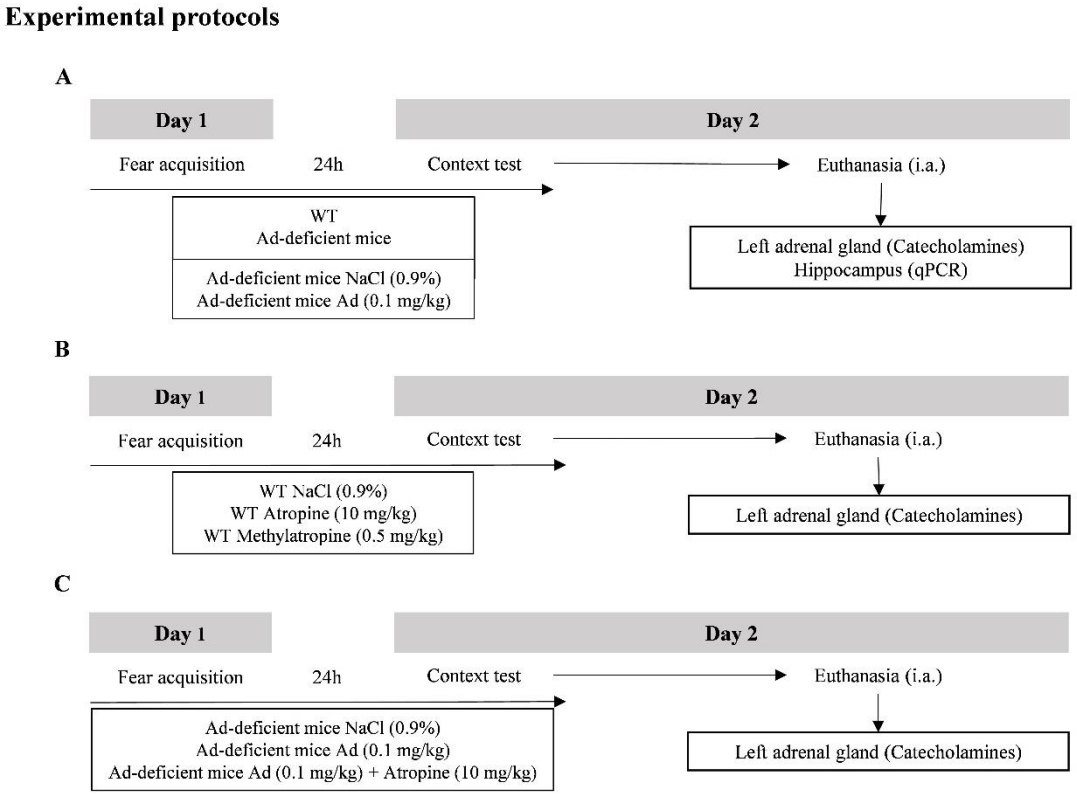

Experimental protocols
A
Day 1
Day 2
Fear acquisition
24h
Context test
Euthanasia (i.a.)
WT
Ad-deficient mice
Ad-deficient mice NaCl (0.9%)
Ad-deficient mice Ad (0.1 mg/kg)
Left adrenal gland (Catecholamines)
Hippocampus (qPCR)
B
Day 1
Day 2
Fear acquisition
24h
Context test
Euthanasia (i.a.)
WT NaCl (0.9%)
WT Atropine (10 mg/kg)
WT Methylatropine (0.5 mg/kg)
Left adrenal gland (Catecholamines)
C
Day 1
Day 2
Fear acquisition
24h
Context test
Euthanasia (i.a.)
Ad-deficient mice NaCl (0.9%)
Ad-deficient mice Ad (0.1 mg/kg)
Ad-deficient mice Ad (0.1 mg/kg) + Atropine (10 mg/kg)
Left adrenal gland (Catecholamines)

Figure 2

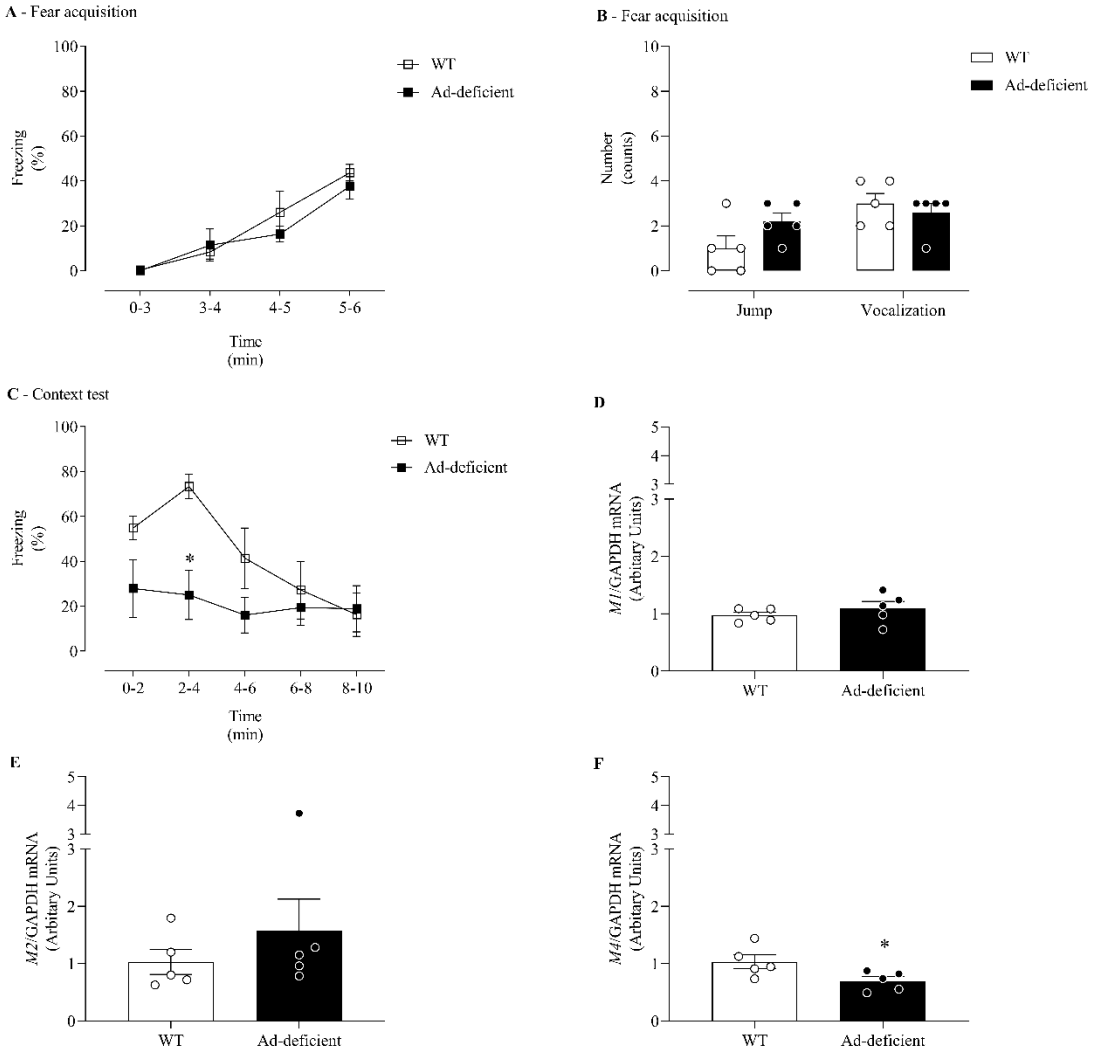

A - Fear acquisition
Freezing (%)
Time (min)
0-3
3-4
4-5
5-6
WT
Ad-deficient
B - Fear acquisition
Number (counts)
Jump
Vocalization
WT
Ad-deficient
C - Context test
Freezing (%)
Time (min)
0-2
2-4
4-6
6-8
8-10
WT
Ad-deficient
D
M1/GAPDH mRNA (Arbitary Units)
WT
Ad-deficient
E
M2/GAPDH mRNA (Arbitary Units)
WT
Ad-deficient
F
M4/GAPDH mRNA (Arbitary Units)
WT
Ad-deficient

Figure 3

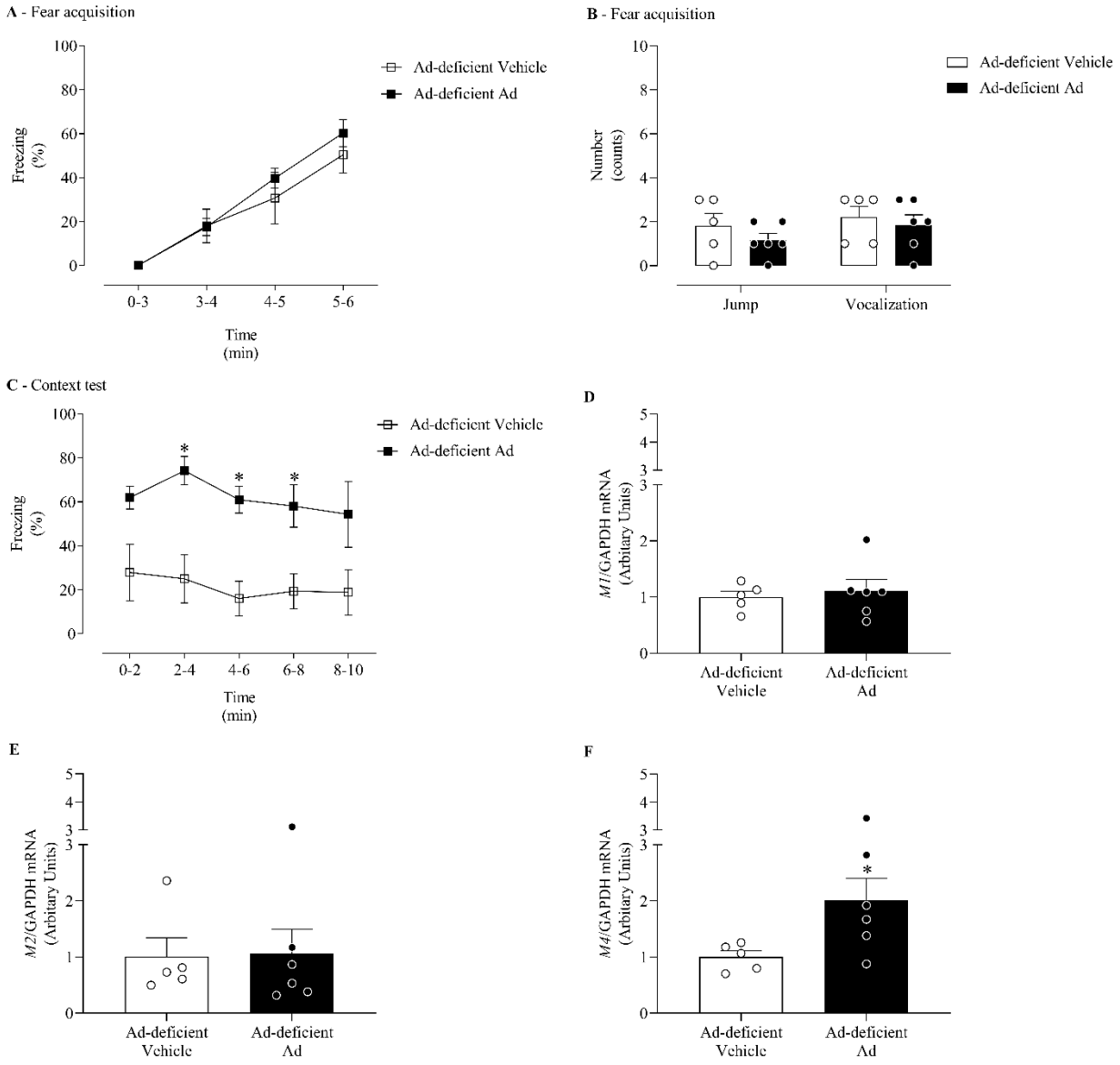

A - Fear acquisition
Freezing (%)
Time (min)
0-3
3-4
4-5
5-6
Ad-deficient Vehicle
Ad-deficient Ad
B - Fear acquisition
Number (counts)
Jump
Vocalization
C - Context test
0-2
2-4
4-6
6-8
8-10
D
M1/GAPDH mRNA (Arbitary Units)
E
M2/GAPDH mRNA (Arbitary Units)
F
M4/GAPDH mRNA (Arbitary Units)

Figure 4

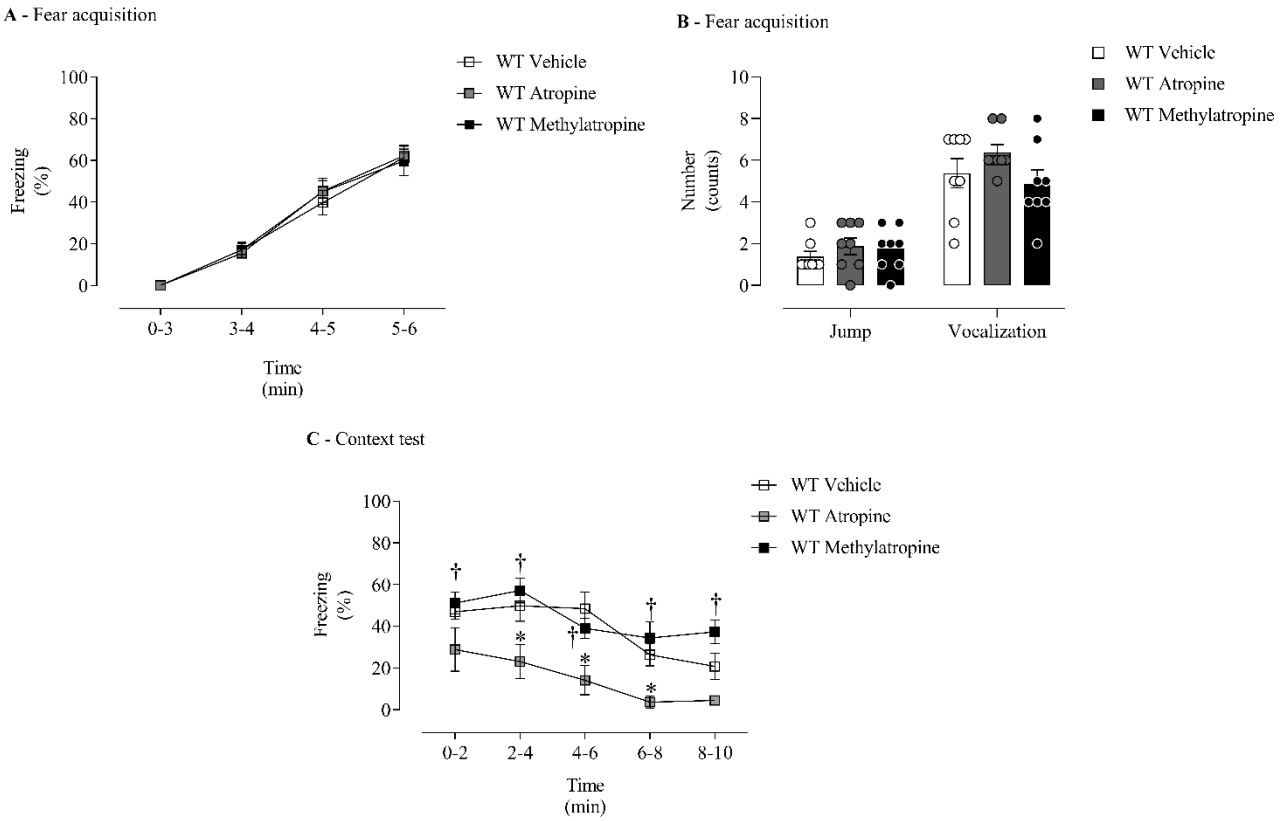
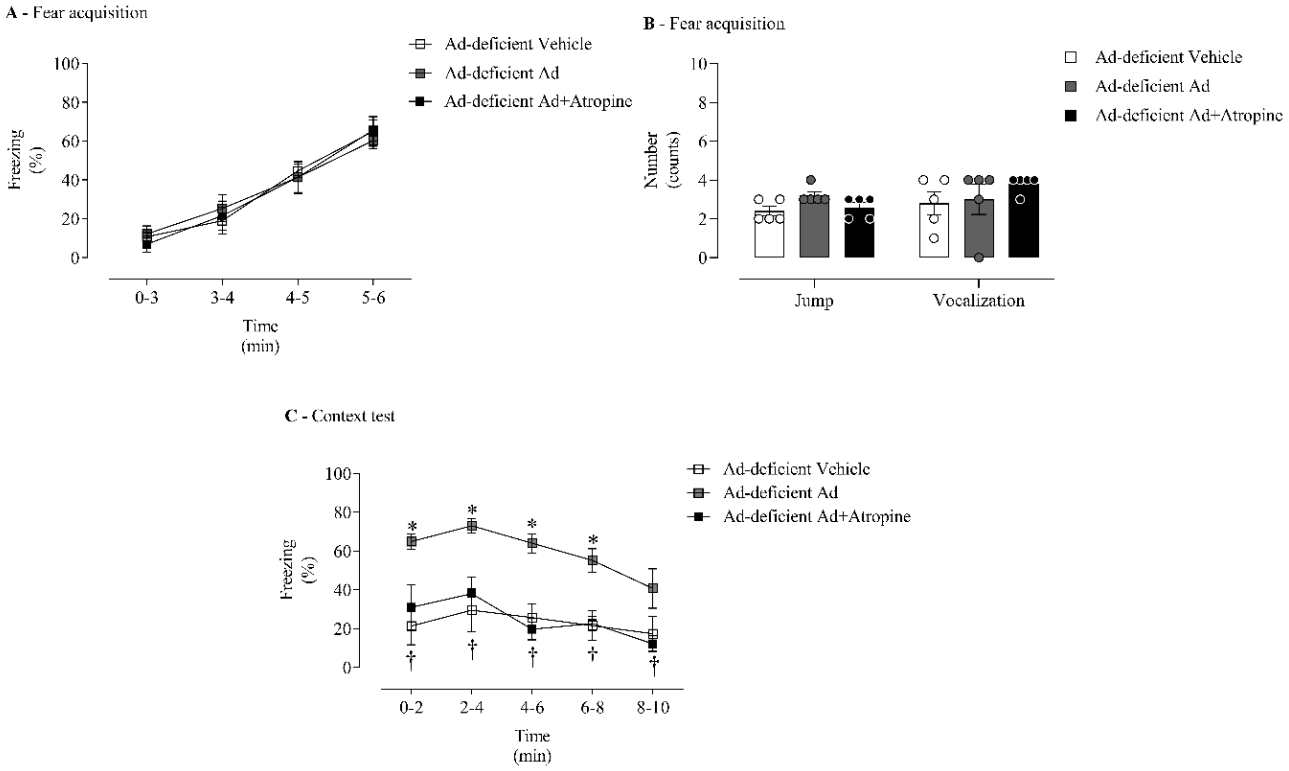

A - Fear acquisition
WT Vehicle
WT Atropine
WT Methylatropine
Freezing (%)
100
80
60
40
20
0
0-3
3-4
4-5
5-6
Time (min)
B - Fear acquisition
WT Vehicle
WT Atropine
WT Methylatropine
Number (counts)
10
8
6
4
2
0
Jump
Vocalization
C - Context test
WT Vehicle
WT Atropine
WT Methylatropine
Freezing (%)
100
80
60
40
20
0
0-2
2-4
4-6
6-8
8-10
Time (min)

Figure 5


A - Fear acquisition
Ad-deficient Vehicle
Ad-deficient Ad
Ad-deficient Ad+Atropine
Freezing (%)
100
80
60
40
20
0
0-3
3-4
4-5
5-6
Time (min)
B - Fear acquisition
Ad-deficient Vehicle
Ad-deficient Ad
Ad-deficient Ad+Atropine
Number (counts)
10
8
6
4
2
0
Jump
Vocalization
C - Context test
Ad-deficient Vehicle
Ad-deficient Ad
Ad-deficient Ad+Atropine
Freezing (%)
100
80
60
40
20
0
0-2
2-4
4-6
6-8
8-10
Time (min)

Funding

This work was partly supported by Foundation for Science and Technology (FCT, project UIDB/04308/2020 and UIDP/04308/2020). FCT provided PhD studentships to Ana Oliveira (SFRH/BD/138984/2018 and COVID/BD/152954/2022) and Raquel Martinho (SFRH/BD/133860/2017).

Author Contributions

Conceptualization: Ana Oliveira, Márcia Azevedo, and Mónica Moreira-Rodrigues; Methodology: Ana Oliveira, Márcia Azevedo, and Rafaela Seixas; Writing - original draft preparation: Ana Oliveira, Márcia Azevedo, and Mónica Moreira-Rodrigues; Writing - review and editing: Ana Oliveira, Márcia Azevedo, Rafaela Seixas, Raquel Martinho, and Mónica Moreira-Rodrigues; Funding acquisition: Ana Oliveira, Raquel Martinho, and Mónica Moreira-Rodrigues; Resources: Paula Serrão, Elisabete Silva, and Mónica Moreira-Rodrigues; Supervision: Paula Serrão, Elisabete Silva, and Mónica Moreira-Rodrigues.

Ethics approval

All animal studies presented in this article were reviewed and approved by the Organism Responsible for Animal Welfare in Faculty of Medicine of University of Porto and National Authority for Animal Health.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

Acknowledgements

The authors thank Sónia Soares for their technical support.

References

Albuquerque, E.X., Pereira, E.F., Alkondon, M., and Rogers, S.W. (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89(1), 73-120. doi: 10.1152/physrev.00015.2008.

Alves, E., Lukoyanov, N., Serrao, P., Moura, D., and Moreira-Rodrigues, M. (2016). Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors. Psychopharmacology (Berl) 233(11), 2099-2108. doi: 10.1007/s00213-016-4254-5.

Anagnostaras, S.G., Murphy, G.G., Hamilton, S.E., Mitchell, S.L., Rahnama, N.P., Nathanson, N.M., et al. (2003). Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nature Neuroscience 6(1), 51-58. doi: 10.1038/nn992.

Bainbridge, N.K., Koselke, L.R., Jeon, J., Bailey, K.R., Wess, J., Crawley, J.N., et al. (2008). Learning and memory impairments in a congenic C57BL/6 strain of mice that lacks the M2 muscarinic acetylcholine receptor subtype. Behav Brain Res 190(1), 50-58. doi: 10.1016/j.bbr.2008.02.001.

Baratti, C.M., Introini, I.B., and Huygens, P. (1984). Possible interaction between central cholinergic muscarinic and opioid peptidergic systems during memory consolidation in mice. Behav Neural Biol 40(2), 155-169. doi: 10.1016/s0163-1047(84)90255-3.

Billewicz-Stankiewicz, J., Górny, D., and Zajaczkowska, M. (1975). The effects of adrenaline, reserpine, and atropine on acetylcholine content of the brain and peripheral ganglia in stress. Acta Physiol Pol 26(3), 289-297.

Bracha, H.S. (2004). Freeze, flight, fight, fright, faint: adaptationist perspectives on the acute stress response spectrum. CNS Spectr 9(9), 679-685.

Cangioli, I., Baldi, E., Mannaioni, P.F., Bucherelli, C., Blandina, P., and Passani, M.B. (2002). Activation of histaminergic H3 receptors in the rat basolateral amygdala improves expression of fear memory and enhances acetylcholine release. Eur J Neurosci 16(3), 521-528. doi: 10.1046/j.1460-9568.2002.02092.x.

Cornford, E.M., Hyman, S., and Swartz, B.E. (1994). The human brain GLUT1 glucose transporter: ultrastructural localization to the blood-brain barrier endothelia. J Cereb Blood Flow Metab 14(1), 106-112. doi: 10.1038/jcbfm.1994.15.

Dobrogowska, D.H., and Vorbrodt, A.W. (1999). Quantitative immunocytochemical study of blood-brain barrier glucose transporter (GLUT-1) in four regions of mouse brain. J Histochem Cytochem 47(8), 1021-1030. doi: 10.1177/002215549904700806.

Durkin, T.P., Messier, C., de Boer, P., and Westerink, B.H. (1992). Raised glucose levels enhance scopolamine-induced acetylcholine overflow from the hippocampus: an in vivo microdialysis study in the rat. Behav Brain Res 49(2), 181-188. doi: 10.1016/s0166-4328(05)80163-9.

Ebert, S.N., Rong, Q., Boe, S., Thompson, R.P., Grinberg, A., and Pfeifer, K. (2004). Targeted insertion of the Cre-recombinase gene at the phenylethanolamine n-methyltransferase locus: a new model for studying the developmental distribution of adrenergic cells. Dev Dyn 231(4), 849-858. doi: 10.1002/dvdy.20188.

Euler, U.v. (1946). A specific sympathomimetic ergone in adrenergic nerve fibres (sympathin) and its relations to adrenaline and nor-adrenaline. Acta physiologica scandinavica 12(1), 73-97.

Falk, S., Lund, C., and Clemmensen, C. (2020). Muscarinic receptors in energy homeostasis: Physiology and pharmacology. Basic & Clinical Pharmacology & Toxicology 126(S6), 66-76. doi: https://doi.org/10.1111/bcpt.13311.

Flynn, D.D., Ferrari-DiLeo, G., Mash, D.C., and Levey, A.I. (1995). Differential regulation of molecular subtypes of muscarinic receptors in Alzheimer's disease. J Neurochem 64(4), 1888-1891. doi: 10.1046/j.1471-4159.1995.64041888.x.

Fornari, R.V., Moreira, K.M., and Oliveira, M.G. (2000). Effects of the selective M1 muscarinic receptor antagonist dicyclomine on emotional memory. Learn Mem 7(5), 287-292. doi: 10.1101/lm.34900.

Galloway, C.R., Lebois, E.P., Shagarabi, S.L., Hernandez, N.A., and Manns, J.R. (2014). Effects of selective activation of M1 and M4 muscarinic receptors on object recognition memory performance in rats. Pharmacology 93(1-2), 57-64.

Gerber, D.J., Sotnikova, T.D., Gainetdinov, R.R., Huang, S.Y., Caron, M.G., and Tonegawa, S. (2001). Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M1 muscarinic acetylcholine receptor-deficient mice. Proceedings of the National Academy of Sciences 98(26), 15312-15317. doi: doi:10.1073/pnas.261583798.

Gibson, G.E., and Blass, J.P. (1976). Impaired synthesis of acetylcholine in brain accompanying mild hypoxia and hypoglycemia. J Neurochem 27(1), 37-42. doi: 10.1111/j.1471-4159.1976.tb01540.x.

Gibson, G.E., Blass, J.P., and Jenden, D.J. (1978). Measurement of acetylcholine turnover with glucose used as precursor: evidence for compartmentation of glucose metabolism in brain. J Neurochem 30(1), 71-76. doi: 10.1111/j.1471-4159.1978.tb07036.x.

Gold, P.E. (2003). Acetylcholine modulation of neural systems involved in learning and memory. Neurobiol Learn Mem 80(3), 194-210. doi: 10.1016/j.nlm.2003.07.003.

Gold, P.E. (2014). Regulation of memory - from the adrenal medulla to liver to astrocytes to neurons. Brain research bulletin 105, 25-35. doi: 10.1016/j.brainresbull.2013.12.012.

Gomeza, J., Zhang, L., Kostenis, E., Felder, C., Bymaster, F., Brodkin, J., et al. (1999). Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M₄ muscarinic acetylcholine receptor knockout mice. Proceedings of the National Academy of Sciences 96(18), 10483-10488. doi: doi:10.1073/pnas.96.18.10483.

Górny, D., Billewicz-Stankiewicz, J., Zajaczkowska, M., and Kutarski, A. (1975). The effect of adrenaline on acetylcholine synthesis, choline acetylase and cholinesterase activity. Acta Physiol Pol 26(1), 45-54.

Habgood, M.D., Bye, N., Dziegielewska, K.M., Ek, C.J., Lane, M.A., Potter, A., et al. (2007). Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. Eur J Neurosci 25(1), 231-238. doi: 10.1111/j.1460-9568.2006.05275.x.

Introini-Collison, I.B., and Baratti, C.M. (1992). Memory-modulatory effects of centrally acting noradrenergic drugs: possible involvement of brain cholinergic mechanisms. Behavioral and neural biology 57(3), 248-255.

Introini-Collison, I.B., and McGaugh, J.L. (1988). Modulation of memory by post-training epinephrine: involvement of cholinergic mechanisms. Psychopharmacology 94(3), 379-385. doi: 10.1007/bf00174693.

Kaufman, D.L., Houser, C.R., and Tobin, A.J. (1991). Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct

intraneuronal distributions and cofactor interactions. J Neurochem 56(2), 720-723. doi: 10.1111/j.1471-4159.1991.tb08211.x.

Kong, L., Zhao, Y., Zhou, W.-J., Yu, H., Teng, S.-W., Guo, Q., et al. (2017). Direct Neuronal Glucose Uptake Is Required for Contextual Fear Acquisition in the Dorsal Hippocampus. Frontiers in Molecular Neuroscience 10. doi: 10.3389/fnmol.2017.00388.

Kopf, S.R., Buchholzer, M.L., Hilgert, M., Löffelholz, K., and Klein, J. (2001). Glucose plus choline improve passive avoidance behaviour and increase hippocampal acetylcholine release in mice. Neuroscience 103(2), 365-371. doi: 10.1016/s0306-4522(01)00007-0.

Liang, K.-C. (2009). Involvement of the amygdala and its connected structures in formation and expression of inhibitory avoidance memory: issues and implications. Chin J Physiol 52(4), 196-214.

Mahboob, A., Farhat, S.M., Iqbal, G., Babar, M.M., Zaidi, N.-u.-S.S., Nabavi, S.M., et al. (2016). Alpha-lipoic acid-mediated activation of muscarinic receptors improves hippocampus- and amygdala-dependent memory. Brain Research Bulletin 122, 19-28. doi: https://doi.org/10.1016/j.brainresbull.2016.02.014.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci 24, 897-931. doi: 10.1146/annurev.neuro.24.1.897.

Martinho, R., Oliveira, A., Correia, G., Marques, M., Seixas, R., Serrao, P., et al. (2020). Epinephrine May Contribute to the Persistence of Traumatic Memories in a Post-traumatic Stress Disorder Animal Model. Front Mol Neurosci 13, 588802. doi: 10.3389/fnmol.2020.588802.

Martinho, R., Seixas, R., Azevedo, M., Oliveira, A., Serrao, P., and Moreira-Rodrigues, M. (2021). Sotalol Treatment may Interfere With Retrieval, Expression, and/or Reconsolidation Processes Thus Disrupting Traumatic Memories in a Post-Traumatic Stress Disorder Mice Model. Front Pharmacol 12, 809271. doi: 10.3389/fphar.2021.809271.

Mendes, P., Martinho, R., Leite, S., Maia-Moço, L., Leite-Moreira, A.F., Lourenço, A.P., et al. (2018). Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice. Int J Cardiol 253, 113-119. doi: 10.1016/j.ijcard.2017.10.014.

Miyakawa, T., Yamada, M., Duttaroy, A., and Wess, J. (2001). Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic

acetylcholine receptor. J Neurosci 21(14), 5239-5250. doi: 10.1523/jneurosci.21-14-05239.2001.

Moreira-Rodrigues, M., Sampaio-Maia, B., Moura, M., and Pestana, M. (2007). Renal dopaminergic system activity in uninephrectomized rats up to 26 weeks after surgery. Am J Nephrol 27(3), 232-239. doi: 10.1159/000101368.

Mrzljak, L., Levey, A.I., and Goldman-Rakic, P.S. (1993). Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: morphological evidence for cholinergic modulation of excitatory neurotransmission. Proc Natl Acad Sci U S A 90(11), 5194-5198. doi: 10.1073/pnas.90.11.5194.

Mulugeta, E., Chandranath, I., Karlsson, E., Winblad, B., and Adem, A.J.E.b.r. (2006). Temporal and region-dependent changes in muscarinic M4 receptors in the hippocampus and entorhinal cortex of adrenalectomized rats. 173(2), 309-317.

Nail-Boucherie, K., Dourmap, N., Jaffard, R., and Costentin, J. (2000). Contextual fear conditioning is associated with an increase of acetylcholine release in the hippocampus of rat. Cognitive Brain Research 9(2), 193-197. doi: https://doi.org/10.1016/S0926-6410(99)00058-0.

Ogren, S., and Stiedl, O. (2015). "Passive avoidance."), 1220-1227.

Oliveira, A., Martinho, R., Serrao, P., and Moreira-Rodrigues, M. (2018). Epinephrine Released During Traumatic Events May Strengthen Contextual Fear Memory Through Increased Hippocampus mRNA Expression of Nr4a Transcription Factors. Front Mol Neurosci 11, 334. doi: 10.3389/fnmol.2018.00334.

Oliveira, A., Seixas, R., Pereira, F., Azevedo, M., Martinho, R., Serrao, P., et al. (2023). Insulin enhances contextual fear memory independently of its effect in increasing plasma adrenaline. Life Sci 328, 121881. doi: 10.1016/j.lfs.2023.121881.

Passani, M.B., Cangioli, I., Baldi, E., Bucherelli, C., Mannaioni, P.F., and Blandina, P. (2001). Histamine H3 receptor-mediated impairment of contextual fear conditioning and in-vivo inhibition of cholinergic transmission in the rat basolateral amygdala. Eur J Neurosci 14(9), 1522-1532. doi: 10.1046/j.0953-816x.2001.01780.x.

Power, A.E., Vazdarjanova, A., and McGaugh, J.L. (2003). Muscarinic cholinergic influences in memory consolidation. Neurobiol Learn Mem 80(3), 178-193. doi: 10.1016/s1074-7427(03)00086-8.

Pych, J.C., Chang, Q., Colon-Rivera, C., Haag, R., and Gold, P.E. (2005). Acetylcholine release in the hippocampus and striatum during place and response training. Learning & memory (Cold Spring Harbor, N.Y.) 12(6), 564-572. doi: 10.1101/lm.33105.

Ragozzino, M.E., Unick, K.E., and Gold, P.E. (1996). Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. Proceedings of the National Academy of Sciences of the United States of America 93(10), 4693-4698. doi: 10.1073/pnas.93.10.4693.

Rocinholi, L.F., Almeida, S.S., and De-Oliveira, L.M. (1997). Response threshold to aversive stimuli in stimulated early protein-malnourished rats. Braz J Med Biol Res 30(3), 407-413. doi: 10.1590/s0100-879x1997000300016.

Sarter, M., Bruno, J.P., and Givens, B. (2003). Attentional functions of cortical cholinergic inputs: what does it mean for learning and memory? Neurobiol Learn Mem 80(3), 245-256. doi: 10.1016/s1074-7427(03)00070-4.

Schousboe, A., Westergaard, N., Sonnewald, U., Petersen, S.B., Huang, R., Peng, L., et al. (1993). Glutamate and glutamine metabolism and compartmentation in astrocytes. Dev Neurosci 15(3-5), 359-366. doi: 10.1159/000111356.

Seeger, T., Fedorova, I., Zheng, F., Miyakawa, T., Koustova, E., Gomeza, J., et al. (2004). M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. J Neurosci 24(45), 10117-10127. doi: 10.1523/jneurosci.3581-04.2004.

Shirey, J.K., Xiang, Z., Orton, D., Brady, A.E., Johnson, K.A., Williams, R., et al. (2008). An allosteric potentiator of M4 mAChR modulates hippocampal synaptic transmission. Nature chemical biology 4(1), 42-50.

Skultetyova, I., Tokarev, D., and Jezova, D. (1998). Stress-induced increase in blood-brain barrier permeability in control and monosodium glutamate-treated rats. Brain Res Bull 45(2), 175-178. doi: 10.1016/s0361-9230(97)00335-3.

Talley, C.E., Kahn, S., Alexander, L.J., and Gold, P.E. (2000). Epinephrine fails to enhance performance of food-deprived rats on a delayed spontaneous alternation task. Neurobiol Learn Mem 73(1), 79-86. doi: 10.1006/nlme.1999.3920.

Toth, M., Ziegler, M., Sun, P., Gresack, J., and Risbrough, V. (2013). Impaired conditioned fear response and startle reactivity in epinephrine-deficient mice. Behav Pharmacol 24(1), 1-9. doi: 10.1097/FBP.0b013e32835cf408.

Tucek, S., and Cheng, S.C. (1974). Provenance of the acetyl group of acetylcholine and compartmentation of acetyl-CoA and Krebs cycle intermediates in the brain in vivo. J Neurochem 22(6), 893-914. doi: 10.1111/j.1471-4159.1974.tb04314.x.

Valentinuzzi, V.S., Kolker, D.E., Vitaterna, M.H., Shimomura, K., Whiteley, A., Low-Zeddies, S., et al. (1998). Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ x C57BL/6J)F2 mice. Learn Mem 5(4-5), 391-403.

Vorbrodt, A.W., Dobrogowska, D.H., and Tarnawski, M. (2001). Immunogold study of interendothelial junction-associated and glucose transporter proteins during postnatal maturation of the mouse blood-brain barrier. J Neurocytol 30(8), 705-716. doi: 10.1023/a:1016581801188.

Weil-Malherbe, H., Axelrod, J., and Tomchick, R. (1959). Blood-brain barrier for adrenaline. Science 129(3357), 1226-1227. doi: 10.1126/science.129.3357.1226.

Zheng, F., Wess, J., and Alzheimer, C. (2012). M2 muscarinic acetylcholine receptors regulate long-term potentiation at hippocampal CA3 pyramidal cell synapses in an input-specific fashion. J Neurophysiol 108(1), 91-100. doi: 10.1152/jn.00740.2011.

CHAPTER 5 - MANUSCRIPT IV

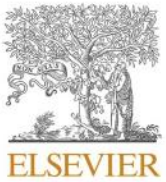
INSULIN ENHANCES CONTEXTUAL FEAR MEMORY INDEPENDENTLY OF ITS EFFECT IN INCREASING PLASMA ADRENALINE

Life Sciences. 2023 June 22; 328: 121881.

doi: 10.1016/j.lfs.2023.121881.

Q1 in Medicine, Research and Experimental and in Pharmacology And Pharmacy.

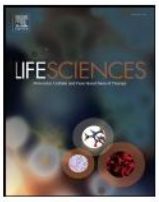
Life Sciences 328 (2023) 121881



Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



Insulin enhances contextual fear memory independently of its effect in increasing plasma adrenaline

Ana Oliveira [a,b], Rafaela Seixas [a,b], Francisca Pereira [a,b], Márcia Azevedo [a,b], Raquel Martinho [a,b], Paula Serrão [b,c], Mónica Moreira-Rodrigues [a,b,*]

[a] Department of Immuno-physiology and Pharmacology, Laboratory of General Physiology, School of Medicine and Biomedical Sciences (ICBAS), University of Porto (UP), Porto, Portugal
[b] Center for Drug Discovery and Innovative Medicines, University of Porto (MedInUP), Porto, Portugal
[c] Department of Biomedicine, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal

ARTICLE INFO

Keywords:
Fear conditioning
Contextual fear memory
Insulin
Adrenaline-deficient mice
Bdnf

ABSTRACT

Aims: Adrenaline enhances contextual fear memory consolidation possibly by activating liver β_2-adrenoceptors causing transient hyperglycaemia. Contrastingly, insulin-induced hypoglycaemia may culminate in blood adrenaline increment, hidering the separation of each hormone's action in contextual fear memory. Therefore, an adrenaline-deficient mouse model was used aiming to investigate if contextual fear memory consolidation following insulin administration requires or not subsequent increases in plasma adrenaline, which occurs in response to insulin-induced hypoglycemia.

Main methods: Fear conditioning was performed in wild-type (WT) and adrenaline-deficient (Pnmt-KO) male mice (129 × 1/SvJ) treated with insulin (2 U/kg, intraperitoneal (i.p.)) or vehicle (0.9 % NaCl (i.p.)). Blood glucose was quantified. Catecholamines were quantified using HPLC with electrochemical detection. Quantitative real-time polymerase chain reaction was used to assess mRNA expression of hippocampal *Nr4a1*, *Nr4a2*, *Nr4a3*, and *Bdnf genes*.

Key findings: Insulin-treated WT mice showed increased freezing behaviour when compared to vehicle-treated WT mice. Also, plasma dopamine, noradrenaline, and adrenaline increased in this group. Insulin-treated Pnmt-KO animals showed increased freezing behaviour when compared with respective vehicle. However, no changes in plasma or tissue catecholamines were identified in insulin-treated Pnmt-KO mice when compared with respective vehicle. Furthermore, insulin-treated Pnmt-KO mice presented increased *Bdnf* mRNA expression when compared to vehicle-treated Pnmt-KO mice.

Significance: Concluding, enhanced freezing behaviour after insulin treatment, even in adrenaline absence, may indicate a key role of insulin in contextual fear memory. Insulin may cause central molecular changes promoting contextual fear memory formation and/or retrieval. This work may indicate a further role of insulin in the process of contextual fear memory modulation.

1. Introduction

Whenever an individual faces an episode of distress, such as fear, it may culminate in intensified stress levels. When a threat is present, fear and stress reactions trigger the "freeze, fight or flight" response [1]. While these physiological reactions are essential for preserving homeostasis, when unbalanced or uncontrolled they can lead to multiple system dysfunctions such as heart disease, stomach ulcers, sleep dysregulation, and psychiatric disorders (anxiety, post-traumatic stress disorder (PTSD) or depression) [2].

Stress hormones, namely noradrenaline (NA) and adrenaline, have an important role in memory consolidation [3–5]. Previous studies showed that mice uncapable of expressing the phenylethanolamine-*N*-methyltransferase (Pnmt) enzyme (Pnmt-KO mice), which catalyses the conversion of NA to adrenaline, are adrenaline-deficient and have reduced contextual fear memory [6,7]. Furthermore, adrenaline

* Corresponding author at: Laboratory of General Physiology, ICBAS, University of Porto, R. Jorge Viterbo Ferreira, 228, Building 2, Floor 4, Cabinet 22, 4050-313 Porto, Portugal.
E-mail address: mirodrigues@icbas.up.pt (M. Moreira-Rodrigues).

https://doi.org/10.1016/j.lfs.2023.121881
Received 22 December 2022; Received in revised form 14 June 2023; Accepted 22 June 2023
Available online 23 June 2023

strengthens contextual fear memory and induces hippocampal genes expression correlated with contextual fear memory [8] and traumatic memory in a mice model for PTSD [9]. Since adrenaline does not cross the blood-brain barrier, adrenaline may induce gluconeogenesis in hepatic tissue as well as vagus nerve stimulation, resulting in blood glucose increase and vagus nerve stimulation carrying this information to the brain [10–12]. Both theories may explain the adrenaline role in fear memory strengthening. Interestingly, both blood glucose increase and vagus nerve stimulation results in insulin secretion [13].

Besides insulin relevance in glucose metabolism, insulin also has memory enhancement effect, since animal [14,15] and human studies [16] showed improved memory and cognitive performance after acute insulin administration. Insulin receptors are present in hippocampus, amygdala, and cerebellar cortex [17] which are brain structures involved in memory [18] and fear memory [19]. In healthy and in Alzheimer's disease subjects, intranasal insulin administration improved memory performance [16,20]. In rodents, intracerebroventricular injections of insulin seem to facilitate memory in passive avoidance [15] and improve spatial memory [21]. However, to our knowledge, no studies reporting insulin effects in a fear conditioning paradigm in a mice model were so far disclosed. Contrary to contextual fear conditioning, passive-avoidance does not allow to accurately study associative memory [22].

There seems to be a close interaction between blood glucose achieved during acute insulin administration and a rise in plasma adrenaline [23]. Low blood glucose attained after hyperinsulinemia is detected by the glucosensors in hepatic portal vein and brain by glucose-sensing neurons [24,25], which may trigger the sympathoadrenal system, inducing glucocorticoids and adrenaline release [26]. In healthy humans, acute episodes of insulin-induced hypoglycaemia resulted in plasma adrenaline and NA increase [27,28]. Therefore, these insulin physiologic actions in glucose homeostasis and blood stress hormones concentration make the role of insulin in memory difficult to interpret by itself. Therefore, we intend to understand if contextual fear memory consolidation following insulin administration requires or not subsequent increases in plasma adrenaline, which occurs in response to insulin-induced hypoglycemia.

The difficulty in separating the actions of adrenaline from those of insulin remains a knowledge barrier in understanding the real role of both hormones in contextual fear memory formation. The adrenaline-deficient mice (Pnmt-KO) is a valuable approach to separate the actions of insulin from those of adrenaline. We aim to comprehend how insulin administration influences contextual fear memory and the respective molecular mechanisms, in particular in adrenaline-deficient mice.

2. Methods

2.1. Animals

All animal care and experimental protocols were carried out in accordance with European Directive number 2010/63/EU, which was transposed to Portuguese legislation by Directive Laws 113/2013 and 1/2019 and approved by the Organism Responsible for Animal Welfare (ORBEA) in the Faculty of Medicine of University of Porto and National Authority for Animal Health (DGAV). The Cre-recombinase gene and a Neomycin resistance gene were inserted into the locus encoding for Pnmt enzyme in C57/BL/6 mice to produce Pnmt−/− mice (Pnmt-KO) and these mice were fully bred in 129 × 1/SvJ background. Pnmt−/− mice are homozygous and do not express Pnmt nor produce endogenous adrenaline. Steven N. Ebert kindly provided a couple of Pnmt-KO (Pnmt−/−) and heterozygous (Pnmt+/−) mice which were bred in our conventional vivarium [29]. Wild-type (WT, $n = 26$) and Pnmt-KO ($n = 27$) adult male mice were kept in the same room and housed with respective litter under controlled environmental conditions (12 h light / dark cycle, room temperature 23 ± 1 °C, humidity 50 %, autoclaved drinking water, mice diet 4RF21/A; Mucedola, Milan, Italy).

2.2. Conditioning fear test

The conditioned fear test approach was derived from earlier research [7,8]. A translucent Plexiglas box with a metal grid floor attached to a stimulus generator served as the conditioning chamber. The first day of the conditioned fear test, fear acquisition (Fig. 1), had a duration of 6 min per animal. In the first 3 min, mice had a habituation period after which 3-ft shocks (duration: 2 s, intensity: 0.6 mA) were delivered at 60-s intervals. After 24 h, mice were re-exposed to the same apparatus, for the context test (Fig. 1), with no foot shocks delivered. On both days a timer was placed on the top of the conditioning chamber. The timer was turned on at the same time as the digital video camera Sony HDR-CX405 (Sony Corporation, Japan) to allow the posterior counting of freezing time during the entire duration of the protocol. Vocalization and jump responses were also evaluated. Freezing behaviour was defined as the absence of movement for at least 3 s, excluding physiologic respiratory movements [30]. An audible squeak was characterized as vocalization, while the jump was defined as removing at least three paws off the grid floor [31]. The percentage of freezing and the number of vocalization and jump responses were determined. On both days, the conditioned chamber was cleaned with 1 % acetic acid between trials. The assessments were performed manually. All quantifications were carried out blind.

2.3. Behavioural treatments

WT and Pnmt-KO mice were treated with insulin (2 U/kg, i.p. [32]) or vehicle (NaCl 0.9 %, i.p.) 20 min before fear acquisition and context tests of fear conditioning procedure. Access to food was *ad libitum*. A diagram of the experimental protocols is in Fig. 1. Initially, a group of WT mice followed protocol A (Fig. 1A). Afterwards, a group of WT mice followed protocol B (Fig. 1B). Subsequently, Pnmt-KO mice followed protocol B (Fig. 1B). At last, a group of Pnmt-KO followed protocol C (Fig. 1C).

2.4. Quantification of glucose

To determine when the mice became hypoglycaemic after insulin administration, blood glucose levels were assessed in the rear paw vein by coulometry, (FreeStyle Freedom Lite, United States of America) [33], 0, 10, 20, 30, 40, 60, 80, and 100 min after insulin administration (Fig. 1A).

Mice were anaesthetized (ketamine 100 mg/kg and xylazine 10 mg/kg; i.p.) immediately after the context day of the fear conditioning paradigm (Fig. 1B). Blood was collected by left ventricle puncture to a heparinized tube. To evaluate hypoglycaemia, blood glucose levels were quantified immediately after the fear conditioning procedure by coulometry (FreeStyle Freedom Lite, United States of America) [33].

2.5. Quantification of catecholamines

Blood samples were centrifuged, and plasma was stored at −80 °C until further use. The left adrenal gland, prefrontal cortex, and hippocampus were extracted and immersed in 0.2 M perchloric acid (PCA) overnight, at 4 °C, and frozen at −80 °C. For catecholamine quantification in the adrenal gland, the PCA of each sample was transferred to tubes with filters and centrifuged at 1250 ×*g*, for 2 min at 4 °C. The catecholamines present in plasma, the prefrontal cortex, and the hippocampus were concentrated using the alumina technique, as reported earlier [8,34–38]. Afterwards, 50 μL of the PCA of samples of left adrenal gland, plasma, prefrontal cortex, and hippocampus were injected, separated by reverse-phase high-performance liquid chromatography (HPLC), and quantified by electrochemical detection. The HPLC-ED (Gilson Medical Electronics, Villiers le Bel, France) used for adrenal

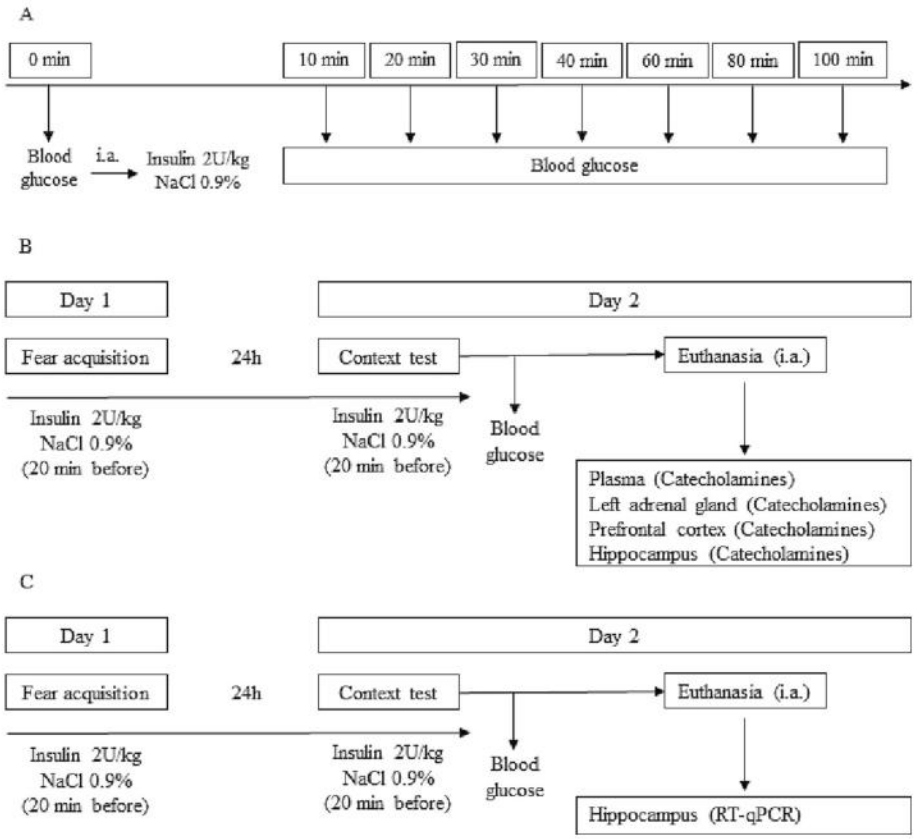


Fig. 1. Schematic representation of the behavioural protocol, experimental design, treatments, and samples collection. (A) Initially, a group of wild-type (WT) followed protocol A (B) Afterwards, a group of WT followed protocol B and later a group of adrenaline-deficient mice (Pnmt-KO) followed protocol B. (C) At last, a group of Pnmt-KO followed protocol C. i.a., immediately after; RT-qPCR, real-time polymerase chain reaction.

gland samples has a limit of detection between 350 and 1000 fmol and a limit of quantification between 700 and 2000 fmol. The HPLC-ED (ESA Coulochem II, Chelmsford, MA, USA) used for plasma and other tissues has a limit of detection between 2.7 and 3.3 fmol and a limit of quantification between 13.7 and 16.3 fmol. Retention times were as follows: NA = 6.60 min, adrenaline = 7.81 min, and DA = 15.95 min. Catecholamine content was calculated per whole adrenal gland or per mg of tissue for other tissues. This is due to the fact that mice's adrenal glands are extremely small, and it is difficult to completely remove the adipose tissue, which could erroneously and heterogeneously affect adrenal gland weight.

2.6. RNA isolation and relative quantification of mRNA expression

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed in hippocampus samples obtained immediately after the context test of fear conditioning procedure (Fig. 1), as previously described [39,40]. Total RNA isolation was carried out with the illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). The NanoDrop 2000 spectrophotometer was used to determine the concentration and purity of the extracted RNA (Thermo Scientific, Waltham, MA, USA). Reverse transcription was carried out using a Reverse Transcription kit (NZY First-Strand cDNA Synthesis Kit NZYTech - Genes & Enzymes, Lisbon, Portugal), and a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). RT-qPCR reactions were performed in StepOne™ real-time PCR System (Applied BioSystems, Waltham, MA, USA). Gene-specific primers (10 μM), Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, USA), and Nuclease-free H_2O (Thermo Scientific, Waltham, MA, USA) were mixed, and cDNA was added (1:20). As a negative control, nuclease-free water (Thermo Scientific, Waltham, MA, USA) was used as a substitute of cDNA. Table 1 lists gene-specific primers. Results of mRNA quantification are expressed in arbitrary units (AU) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.7. Drugs

Actrapid® Insulin was purchased from Novo Nordisk (Bargsvaerd, Denmark). (−)-adrenaline, L-(−)-noradrenaline, dopamine hydrochloride, 2, 3-dihydroxybenzoic acid, and perchloric acid were purchased from Sigma-Aldrich (St. Louis, USA). Ketamine (Imalgene 1000, Merial, Lisboa, Portugal) and xylazine (Rompum 2 %, Bayer, Lisboa, Portugal) were purchased from Agrofauna (Gaia, Portugal).

Table 1
Primers used in gene expression analysis.

Gene	Primer (5′ → 3′)
Nr4a1	F: AAAATCCCTGGCTTCATTGAG R: TTTAGATCGGTATGCCAGGCG
Nr4a2	F: CGGTTTCAGAAGTGCCTAGC R: TTGCCTGGAACCTGGAATAG
Nr4a3	F: GTGGCTCGACTCCATTAAAGAC R: GTGCATAGCTCCTCCACTCTCT
Bdnf	F: GGACATATCCATGACCAGAAAGAAA R: GCAACAAACCACAACATTATCGAG
Gapdh	F: CCATCACCATCTTCCAGGAG R: GCATGGACTGTGGTCATGAG

Nr4, Nuclear receptor 4; *Bdnf*, Brain-derived neurotrophic factor, *Gapdh*, Glyceraldehyde 3-phosphate dehydrogenase; F, forward primer and R, reverse primer.

2.8. Statistical analysis

An online Sample Size Calculator was used to determine the number of animals necessary in each group for the experimental methods (https://clincalc.com/Stats/SampleSize.aspx). All data was presented as means with standard error of the means (SEM). GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. Results related to vocalization, jump, blood glucose concentration, catecholamine concentration, and RT-qPCR were analysed by Student's *t*-test. Results of freezing and blood glucose concentration variation with time were analysed by Two-Way Analysis of Variance (ANOVA) repeated measures, followed by Sidak's *post-hoc* test using treatment as "between-subjects factor" and time as "within-subjects factor" (repeated measure). The presence of outliers was evaluated using GraphPad Prism 6. The threshold for statistically significance was established at $p < 0.05$.

3. Results

3.1. Insulin treatment strengthens contextual fear memory

Insulin-treated WT mice reached hypoglycaemic values at 10 min after injection, which was sustained until 60 min post injection, when compared to vehicle-treated WT mice which maintained euglycemic values for the entire duration of the experiment. Insulin-treated mice reached glycaemic baseline levels at 80–100 min post insulin injection. Return to baseline levels was physiologically expected and may be further explained since symptoms like hunger were likely to develop, and the food was available for the entire protocol. It was observed a significant time ($F_{(7, 63)} = 3.8$, $p = 0.002$; Fig. 2) and treatment ($F_{(1, 9)} = 86.0$, $p < 0.0001$; Fig. 2) effect, and a significant interaction between treatment and time ($F_{(7, 63)} = 4.8$; $p = 0.0002$; Fig. 2).

On the first day of the conditioned fear test, fear acquisition day, no differences were observed in freezing response between insulin-treated and vehicle-treated WT mice (Fig. 3A). Two-way ANOVA repeated measures showed a time effect ($F_{(3, 36)} = 89.1$, $p < 0.0001$, Fig. 3A), and no treatment effect ($F_{(1,12)} = 1.9$, $p = 0.196$, Fig. 3A). Furthermore, no differences were observed in vocalization ($t_{(12)} = 1.1$, $p = 0.297$, Fig. 3B) and jump ($t_{(12)} = 0.1$, $p = 0.944$, Fig. 3B) response between insulin-treated and vehicle-treated WT mice.

In context fear test, insulin-treated WT mice showed an increase in freezing behaviour compared to vehicle-treated WT mice. It was observed a treatment ($F_{(1, 12)} = 14.8$, $p = 0.002$, Fig. 3C) and time ($F_{(4, 48)} = 5.4$, $p = 0.001$, Fig. 3C) effect, and an interaction between treatment and time ($F_{(4, 48)} = 2.9$, $p = 0.032$, Fig. 3C). Afterwards, blood glucose was evaluated and insulin-treated WT mice showed 79.9 ± 3.4 % significant decrease in blood glucose concentration when compared to vehicle-treated WT mice ($t_{(13)} = 13.4$, $p < 0.0001$).

3.2. Insulin treatment increases catecholamines in plasma

After context fear test of the fear conditioning procedure, in insulin-treated WT mice it was observed an increase of adrenal gland DA ($t_{(13)} = 3.4$, $p = 0.005$; Fig. 4A), compared to vehicle-treated WT mice. No differences were observed in NA ($t_{(13)} = 1.1$, $p = 0.311$; Fig. 4B) or adrenaline ($t_{(13)} = 1.8$, $p = 0.101$; Fig. 4C) in the adrenal gland between groups. The DA/NA adrenal ratio increased in insulin-treated WT mice compared to vehicle-treated WT mice ($t_{(13)} = 1.4$, $p = 0.0001$; Fig. 4D). Also, in insulin-treated WT mice it was observed an increase in plasma DA ($t_{(13)} = 2.2$, $p = 0.047$; Fig. 4E), NA ($t_{(12)} = 2.5$, $p = 0.029$; Fig. 4F), and adrenaline ($t_{(13)} = 2.4$, $p = 0.033$; Fig. 4G) compared with vehicle-treated WT mice. Moreover, DA decreased in hippocampus ($t_{(12)} = 2.4$, $p = 0.036$; Fig. 5A) and NA increased in prefrontal cortex ($t_{(13)} = 2.2$, $p = 0.047$; Fig. 5D) of insulin-treated WT mice after context fear test of fear conditioning procedure compared with vehicle-treated WT mice.

3.3. Treatment with insulin strengthens contextual fear memory in adrenaline-deficient mice

On the first day of conditioned fear test, fear acquisition day, no differences were observed in freezing response between insulin-treated Pnmt-KO mice and vehicle-treated Pnmt-KO mice (Fig. 6A). Two-way ANOVA repeated measures showed a time ($F_{(3,33)} = 89.7$, $p < 0.0001$, Fig. 6A) effect. No treatment ($F_{(1,11)} = 1.5$, $p = 0.245$, Fig. 6A) effect or a treatment x time interaction ($F_{(3, 33)} = 1.8$, $p = 0.162$, Fig. 6A) were observed. Furthermore, no differences were observed in vocalization ($t_{(11)} = 1.6$, $p = 0.147$; Fig. 6B) and jump ($t_{(11)} = 0.99$, $p = 0.343$; Fig. 6B) response between insulin-treated Pnmt-KO mice and vehicle-treated Pnmt-KO mice.

In context fear test (24 h after fear acquisition day), insulin-treated Pnmt-KO mice showed an increase in freezing behaviour when compared to vehicle-treated Pnmt-KO mice. It was observed a time ($F_{(4, 44)} = 3.2$, $p = 0.022$, Fig. 6C) and a treatment ($F_{(1, 11)} = 28.3$, $p < 0.0002$, Fig. 6C) effect. No treatment x time interaction ($F_{(4, 44)} = 0.0$, $p = 0.998$, Fig. 6C) was observed. Afterwards, blood glucose was evaluated and insulin-treated Pnmt-KO mice showed 71.4 ± 10.4 %

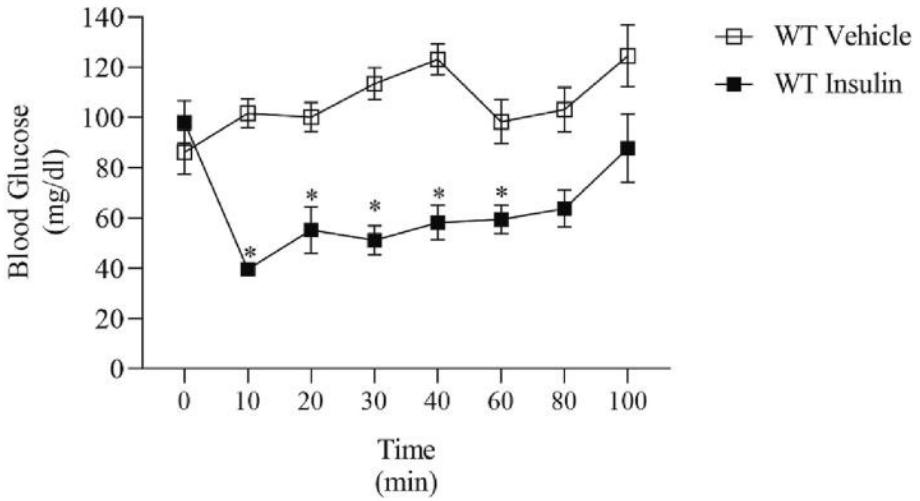


Fig. 2. Blood glucose concentration 0, 10, 20, 30, 40, 60, 80, and 100 min after insulin (2 U/kg) or vehicle (0.9 % NaCl) administration (Protocol of Fig. 1A). Values are means ± SEM of 5–6 mice per group. WT Insulin, wild-type insulin-treated mice; WT Vehicle, wild-type vehicle-treated mice. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$).

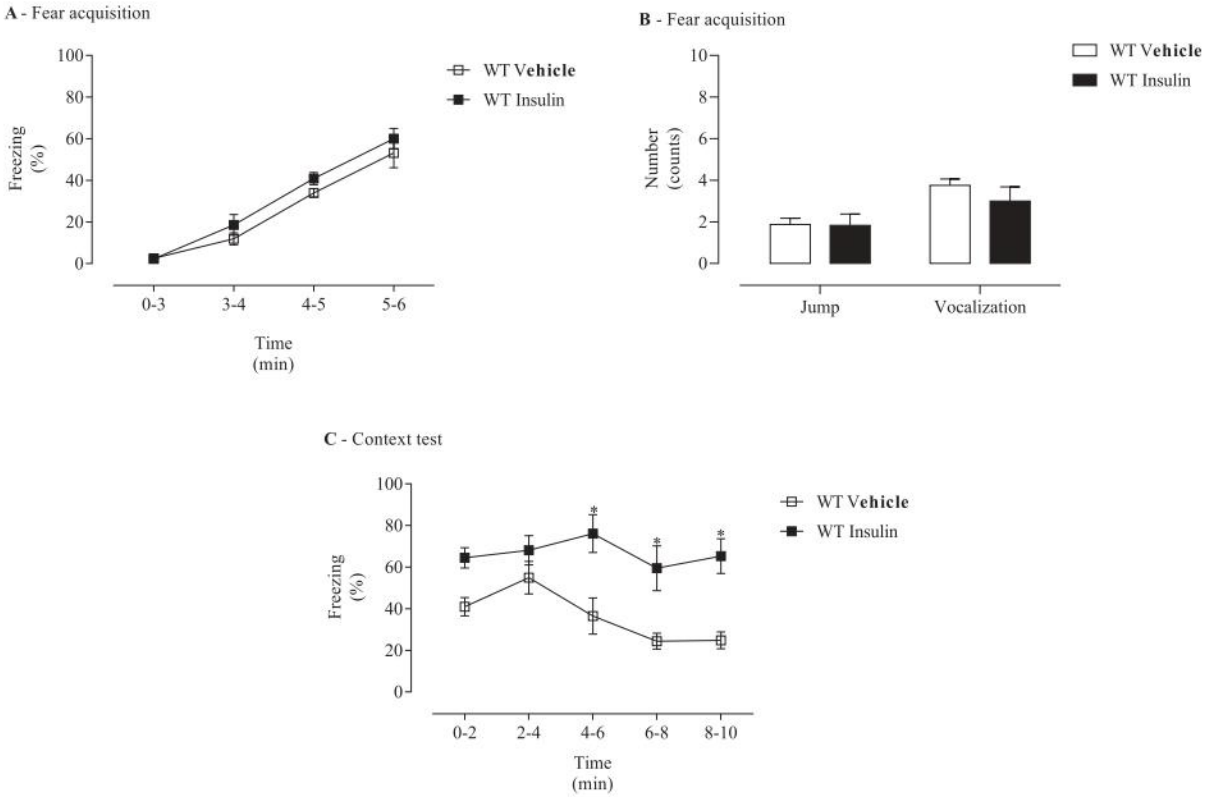


Fig. 3. Freezing behaviour in wild-type (WT) mice after insulin treatment (Protocol of Fig. 1B). (A) freezing and (B) shock responsivity in fear acquisition day, and (C) freezing response in re-exposure to context in WT insulin-treated (2 U/kg) or WT vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 7–8 mice per group. WT Insulin, wild-type insulin-treated mice; WT Vehicle, wild-type vehicle-treated mice. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$).

significant decrease in blood glucose concentration when compared to vehicle-treated Pnmt-KO mice ($t_{(11)} = 6.2$, $p < 0.0001$) mice.

3.4. Treatment with insulin does not increase catecholamine plasma levels in adrenaline-deficient mice

After context fear test of fear conditioning procedure, insulin-treated Pnmt-KO mice showed an increase in adrenal gland DA ($t_{(11)} = 3.3$, $p = 0.007$; Fig. 7A). Insulin-treated Pnmt-KO mice showed a decrease in adrenal gland NA ($t_{(11)} = 2.5$, $p = 0.027$; Fig. 7B) compared to Pnmt-KO vehicle-treated mice. The DA/NA adrenal ratio increased in insulin-treated Pnmt-KO mice compared to Pnmt-KO vehicle-treated mice ($t_{(11)} = 6.1$, $p < 0.0001$; Fig. 7C). In addition, in insulin-treated Pnmt-KO mice no statistical differences were observed in plasma DA ($t_{(11)} = 1.3$, $p = 0.222$, Fig. 7D) and NA ($t_{(11)} = 0.0$, $p = 0.999$, Fig. 7E) after context fear test, when compared with Pnmt-KO vehicle-treated mice. No adrenaline was detected by HPLC, as expected, in these adrenaline-deficient mice. The detection limit is between 350 and 1000 fmol. No significant differences were found in DA and NA levels in insulin-treated Pnmt-KO mice's hippocampus ($t_{(11)} = 0.1$, $p = 0.902$, Fig. 8A; $t_{(11)} = 0.7$, $p = 0.471$, Fig. 8B) and pre-frontal cortex ($t_{(11)} = 0.9$, $p = 0.385$, Fig. 8C; $t_{(11)} = 1.1$, $p = 0.314$, Fig. 8D), respectively, when compared to Pnmt-KO-vehicle treated mice.

3.5. Insulin increases Bdnf gene expression in hippocampus in adrenaline-deficient mice

No differences were detected in hippocampal gene expression of *Nr4a1* ($t_{(12)} = 0.1$, $p = 0.917$, Fig. 9A), *Nr4a2* ($t_{(12)} = 0.4$, $p = 0.714$, Fig. 9B), and *Nr4a3* ($t_{(12)} = 0.1$, $p = 0.889$, Fig. 9C) across treatments. However, hippocampal mRNA expression of *Bdnf* ($t_{(12)} = 2.4$, $p = 0.035$, Fig. 9D) was significantly increased in insulin-treated Pnmt-KO mice when compared with vehicle-treated Pnmt-KO mice.

4. Discussion

Stress hormones are important for the formation of strong consolidated memories [41,42]. In WT mice, the fear acquisition day of fear conditioning procedure leads to an increase of plasma adrenaline in comparison to WT mice not submitted to fear conditioning procedure [8]. This adrenaline increase was associated with contextual fear memory strengthening, showing that after stress, adrenaline released into circulation may support the establishment of emotional contextual fear memories [8].

However, adrenaline is a polar molecule and does not pass the blood-brain barrier [43]. Glucose is one of the proposed mediators for the hormone's memory-strengthening effect [44]. During a stressful event adrenaline is released from the medullar zone of the adrenal gland, acts in peripheral β_2-adrenoceptors, and may induce glucose release from hepatic tissue. Martinho el al have described the importance of β-adrenoceptors in the context of PTSD, with a β-adrenoceptor antagonist decreasing PTSD symptoms and signs [45]. After a stressful event such as the fear acquisition day of fear conditioning procedure, blood glucose increases in WT mice, but not in adrenaline-deficient mice (Pnmt-KO mice) [6]. Additionally, Pnmt-KO mice present contextual fear memory impairment compared to WT mice [6,7]. It is well established that hepatic glucose production increases in response to a surge in plasma adrenaline, which results from glycogenolysis and gluconeogenesis [10,11,46]. Nonetheless, it should be noted that hyperglycaemia can stimulate the release of insulin from β-pancreatic cells [47] making it difficult to detach the effect of adrenaline from insulin in mediating an improving action in fear memory.

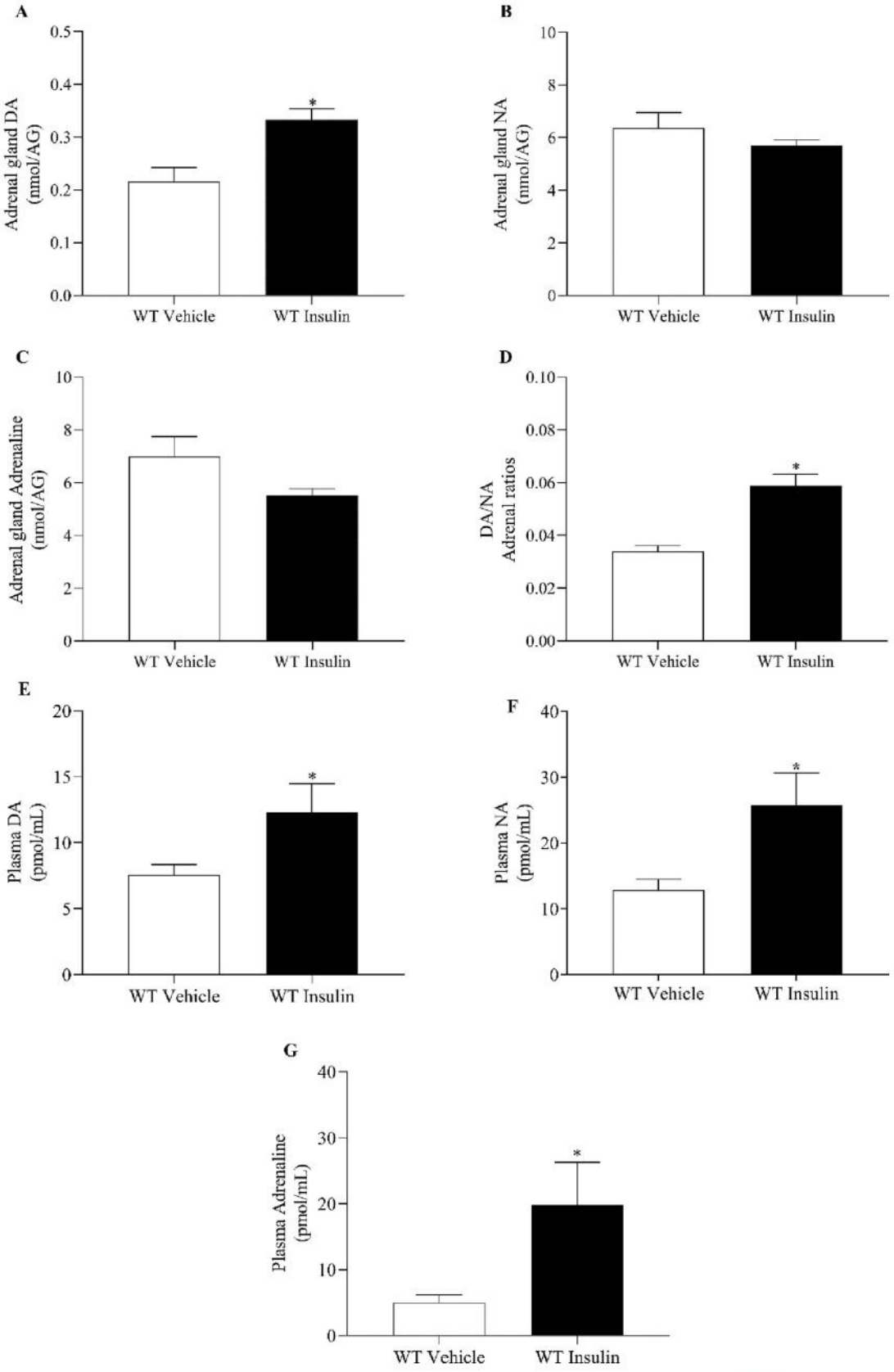


Fig. 4. Adrenal gland and plasma catecholamine concentration in wild-type (WT) mice after insulin treatment (Protocol of Fig. 1B). (A, E) dopamine (DA), (B, F) noradrenaline (NA), (C, G) adrenaline, and DA/NA ratios in WT insulin-treated (2 U/kg) or WT vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 7–8 mice per group. WT Insulin, wild-type insulin-treated mice; WT Vehicle, wild-type vehicle-treated mice; AG, adrenal gland. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$).

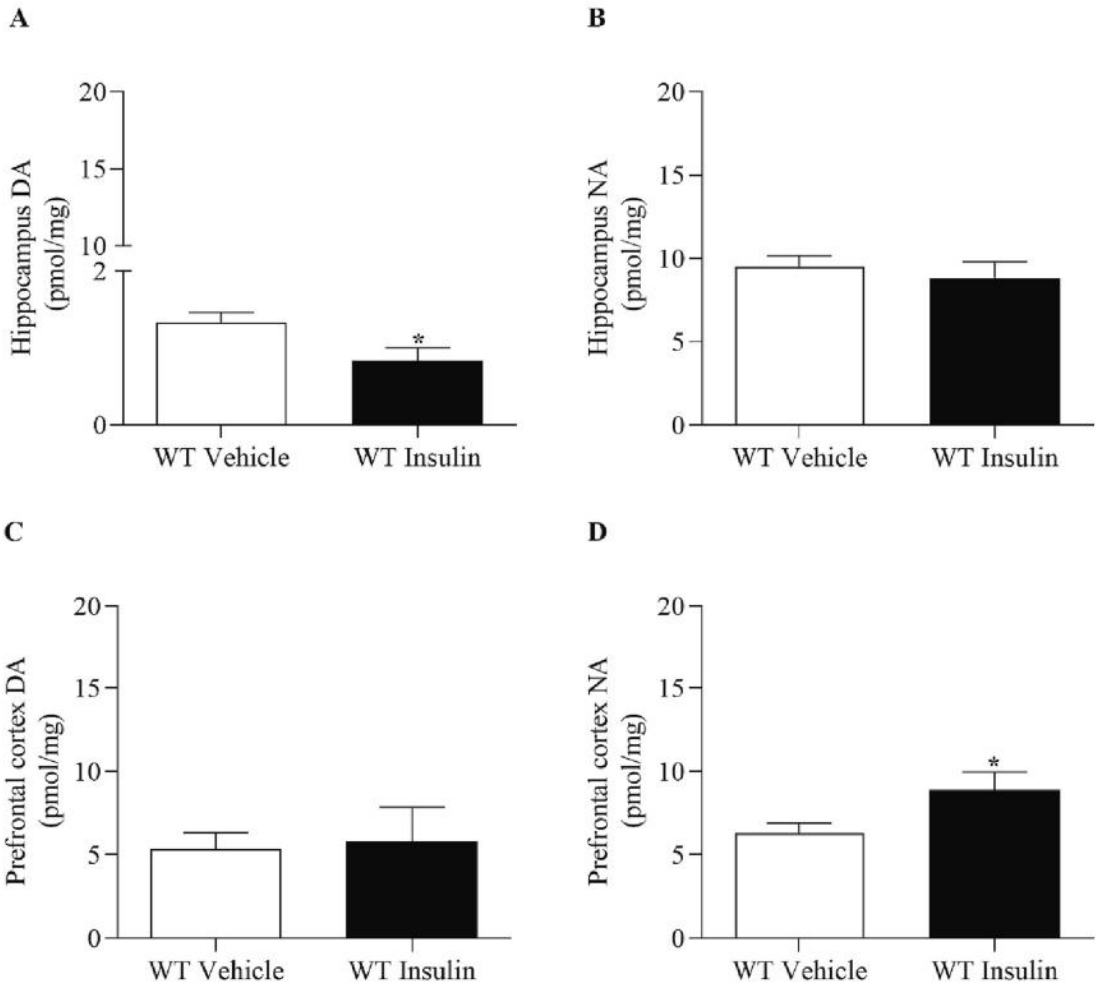


Fig. 5. Brain tissues catecholamines concentration in wild-type (WT) mice after insulin treatment (Protocol of Fig. 1B). (A) dopamine (DA) and (B) noradrenaline (NA) in hippocampus, and (C) DA and (D) NA in prefrontal cortex in WT insulin-treated (2 U/kg) or WT vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 7–8 mice per group. WT Insulin, wild-type insulin-treated mice; WT Vehicle, wild-type vehicle-treated mice. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$).

Insulin increases memory in both non-aversive and aversive tasks, such as spatial memory and passive avoidance task, respectively [15,21]. However, contrary to contextual fear conditioning, passive-avoidance does not allow to accurately study associative memory [22]. Furthermore, small doses of insulin (0.8 IU/kg, subcutaneously) reverse the amnesia in a working memory behavioural test produced by scopolamine (a muscarinic antagonist) treatment [48] and, in animal models for Alzheimer's disease, insulin or a insulin-like action substance reverts memory impairment [49,50]. In our experimental conditions, one dose of insulin (2 U/kg) was capable of producing moderate hypoglycaemia 10 min after administration which was sustained for 60 min. At the same time, insulin seems to facilitate the retention of aversive contextual fear memory when compared to vehicle-treated WT mice. With the referred dose, several hypoglycaemia processes are activated, including the release of insulin, when blood glucose concentration falls below 70 mg/dL [32,51].

Insulin therapy is widespread as an efficient therapy for diabetes, however, hypoglycaemia is the most common side effect of insulin-treated diabetes and remains the major barrier to achieving the proven benefits of intensive insulin therapy in people with type 1 diabetes [52–54]. More recently, the emerging practice of intermittent fasting has increased the incidence of acute episodes of hypoglycaemic in the general population [55]. Therefore, having in mind these extreme situations the effects of the insulin dosage used may be relevant. At this point we cannot exclude the fact that administering extra insulin to mice that already produces their own insulin could cause a confounding effect. However, since it is the same molecule that is being injected and possibly endogenously produced the observed effects will probably be additive.

There is a close correlation between blood glucose concentration attained during induced hypoglycaemia and plasma adrenaline increase [56]. Therefore, we evaluated plasma catecholamine levels in insulin-treated WT mice after the contextual fear test. Plasma DA, NA, and adrenaline increased in insulin-treated WT mice when compared with vehicle-treated WT mice. A catecholamine increase was expected in both vehicle-treated and insulin-treated WT mice, as demonstrated in previous studies where the same fear conditioning model was carried out [6,8]. This increase, however, appears to be even more significant for the insulin-administered WT mice when compared with those administered with vehicle. This reinforces the contribution of insulin and possibly hypoglycaemia to the catecholamine surge and agrees with previous reports that insulin-induced hypoglycaemia results in increased levels of catecholamines [27,28]. These outcomes, together with the evidence that i.p. injections of adrenaline strengthen the retention of aversive contextual fear memories [6], may justify the increased freezing behaviour observed in the contextual fear test in insulin-administered WT mice. Taking all into account, it appears that both adrenaline and insulin may have central roles in fear memory formation. Therefore, we intended to detach the effect of both hormones using adrenaline-deficient mice as described below.

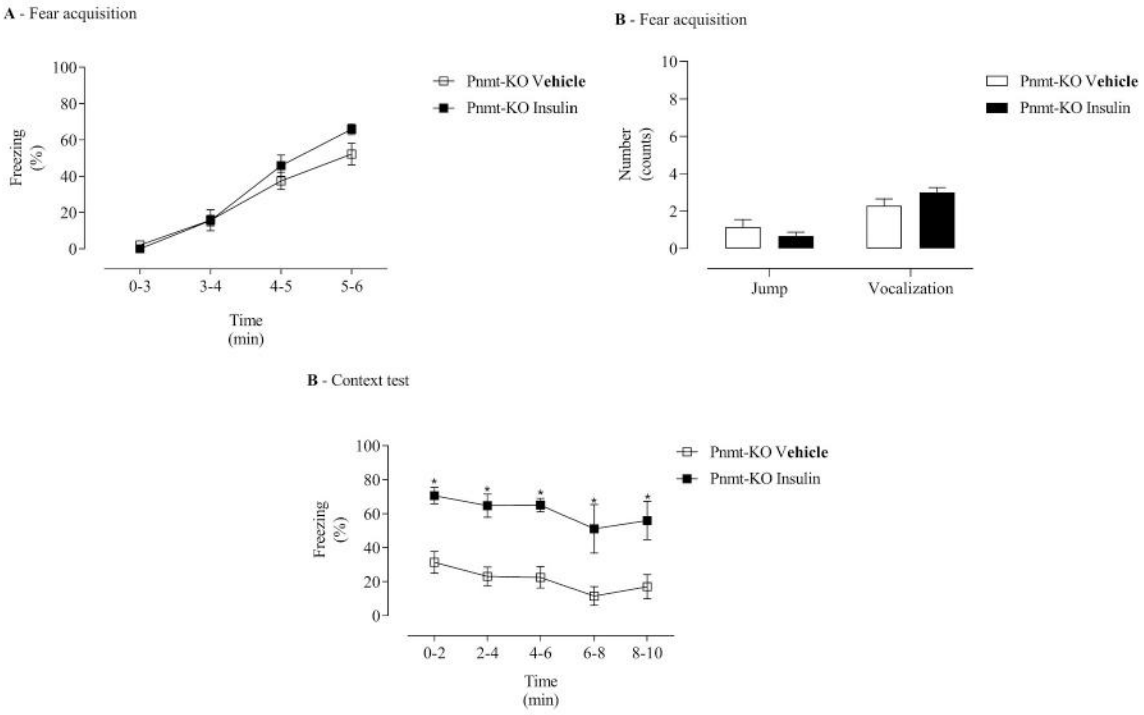


Fig. 6. Freezing behaviour in adrenaline-deficient mice (Pnmt-KO) after insulin treatment (Protocol of Fig. 1B and C). (A) Freezing and (B) shock responsivity in fear acquisition day, and (C) freezing response in re-exposure to context in Pnmt-KO insulin-treated (2 U/kg) or vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 6–7 mice per group. Pnmt-KO Insulin, adrenaline-deficient insulin-treated mice; Pnmt-KO Vehicle adrenaline-deficient vehicle-treated mice. *, significantly different from correspondent values in Pnmt-KO vehicle mice ($p < 0.05$).

The relationship between insulin and central monoamine metabolism (such as NA and DA) is not new [57,58]. In our results, we observed a decrease in hippocampal DA and an increase of NA in the prefrontal cortex. Other studies suggest that high levels of synaptic DA result in reduced contextual fear memory [59]. Accordingly, in our hands, acute administration of insulin resulted in reduced levels of synaptic DA and enhanced contextual fear memory. Moreover, this may be explained by increased uptake of DA by its transporters, caused by enhanced activation of IRs [58]. Previous articles also report that the activation of IRs by insulin administration may activate several signalling cascades, culminating in an enhanced DA transporter surface expression and therefore enhanced uptake activity [60]. In addition, as previously published [8], adrenaline was not detected in hippocampus and pre-frontal cortex brain samples possibly because this hormone does not cross the blood-brain barrier and Pnmt has almost no activity in the brain [61,62].

Moreover, acute insulin administration decreases the expression of NA transporter in the cell membrane, inhibiting NA reuptake from the synaptic cleft [63]. An animal model for type 1 diabetes (unable to produce insulin) has reduced fear and spatial memory which was accompanied by a decrease in cortical NA [57]. Additionally, since cortex NA appears to be important for memory enhancement and synaptic plasticity [59,64,65], the increase of this monoamine in the prefrontal cortex of insulin-treated WT mice can be a possible explanation for memory enhancement in the context of fear conditioning paradigm and could suggest that local cerebral energy in both the hippocampus and cortex is insulin sensitive. Simultaneously, according to the vagus nerve or the glucose theory, increased release of adrenaline after a stressful event may activate the vagus nerve receptors and/or induce glycogenolysis in the liver, respectively, which in turn may culminate in the release of NA in the amygdala, hippocampus, and prefrontal cortex [12,66]. All these events may contribute to increase contextual memory formation along with increased NA in prefrontal cortex in insulin administration group.

Insulin signalling activates two major pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt and the RAS/mitogen-activated protein kinase (MAPK) pathways [67]. Activation of the Akt pathways mobilizes the translocation of glucose transporter 4 (GLUT-4) vesicles to the cell surface, enhancing the brain's glucose uptake [67,68]. This reinforces the idea that insulin's action may be in part due to enhanced local cerebral energy utilization. Furthermore, high levels of IR in the hippocampus [69–71], a brain area extensively related to memory and learning, supports the theory that insulin could be playing an important role in synaptic plasticity mechanisms and memory formation. In turn, the activation of the MAPK-dependent branch of insulin signalling pathway has been reported to increase the expression of genes that modulate biological processes such as differentiation and proliferation, as well as increase cell proliferation and decrease levels of apoptosis [47,72]. Furthermore, studies not mentioning the role of insulin, report that hyperactivation of the MAPK pathway in the hippocampus during the retrieval stage of contextual memory could enhance fear memory [73,74]. Altogether, insulin activation of both these pathways, Akt and MAPK, could explain the hormone's contextual fear memory enhancement effects observed in our experimental procedures. Nevertheless, functional significance of the referred pathways, as well as related genes and proteins deserves future investigation, which is beyond the scope of this study.

To distinguish the effects of insulin and adrenaline in contextual fear memory, adrenaline-deficient mice (Pnmt-KO) were submitted to the fear conditioning paradigm after insulin administration. We found contextual fear memory strengthening in insulin-treated Pnmt-KO mice when compared to vehicle-treated Pnmt-KO mice. Therefore, even in adrenaline absence, insulin appears to enhance contextual fear memory formation. Additionally, adrenal DA increased and adrenal NA was decreased in insulin-treated Pnmt-KO mice when compared to vehicle-treated Pnmt-KO mice. The DA/NA tissue ratio (an indirect measure of DBH activity, substrate/product) was increased in insulin-treated WT and Pnmt-KO mice compared with vehicle-treated mice, which may

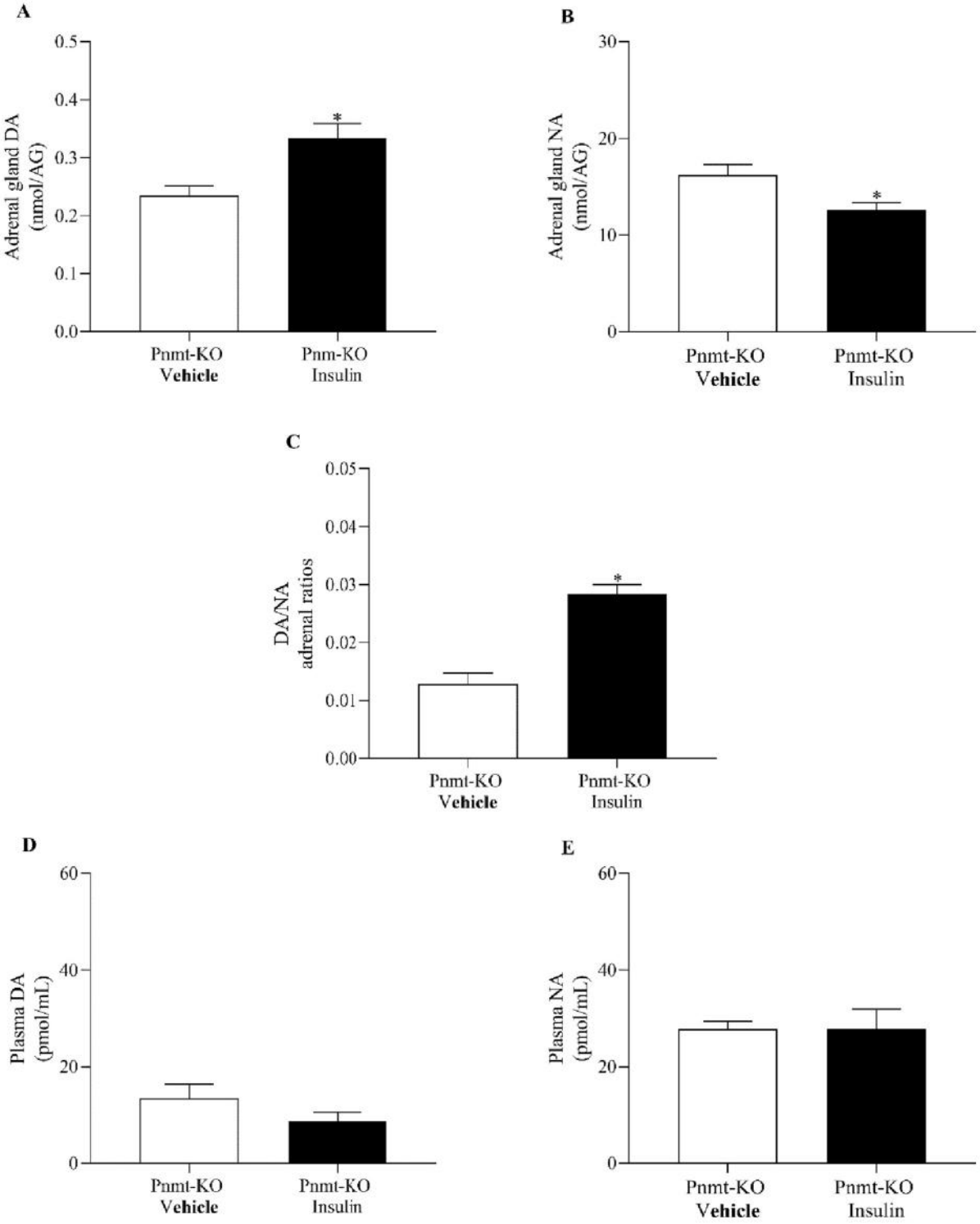


Fig. 7. Adrenal gland and plasma catecholamine concentration in adrenaline-deficient mice (Pnmt-KO) after insulin treatment (Protocol of Fig. 1B and C). (A, D) Dopamine (DA), (B, E) noradrenaline (NA), and (C) DA/NA ratios in Pnmt-KO insulin-treated (2 U/kg) or vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 6–7 mice per group. Pnmt-KO Insulin, adrenaline-deficient insulin-treated mice; Pnmt-KO Vehicle, adrenaline-deficient vehicle-treated mice; AG, adrenal gland. *, significantly different from correspondent values in WT vehicle mice ($p < 0.05$).

indicate a decrease of DBH activity promoted by insulin, as previously implicated for other conditions [75]. This agrees with a previous study which reported a decrease in plasma DBH activity of diabetic rats by insulin [76]. The decrease of adrenal DBH activity in adrenaline-deficient mice administered with insulin seems to have a higher effect in plasma catecholamines than in WT mice, since it was observed an absence of significant differences between groups in plasma DA and NA. These results differ from those obtained in WT mice, probably due to the absence of adrenaline. Contrary to adrenaline-deficient mice, in WT mice, during stress, adrenaline may be incorporated in noradrenergic transmitter stores and operate as a cotransmitter [77], resulting in elevated blood catecholamines with stress repetition.

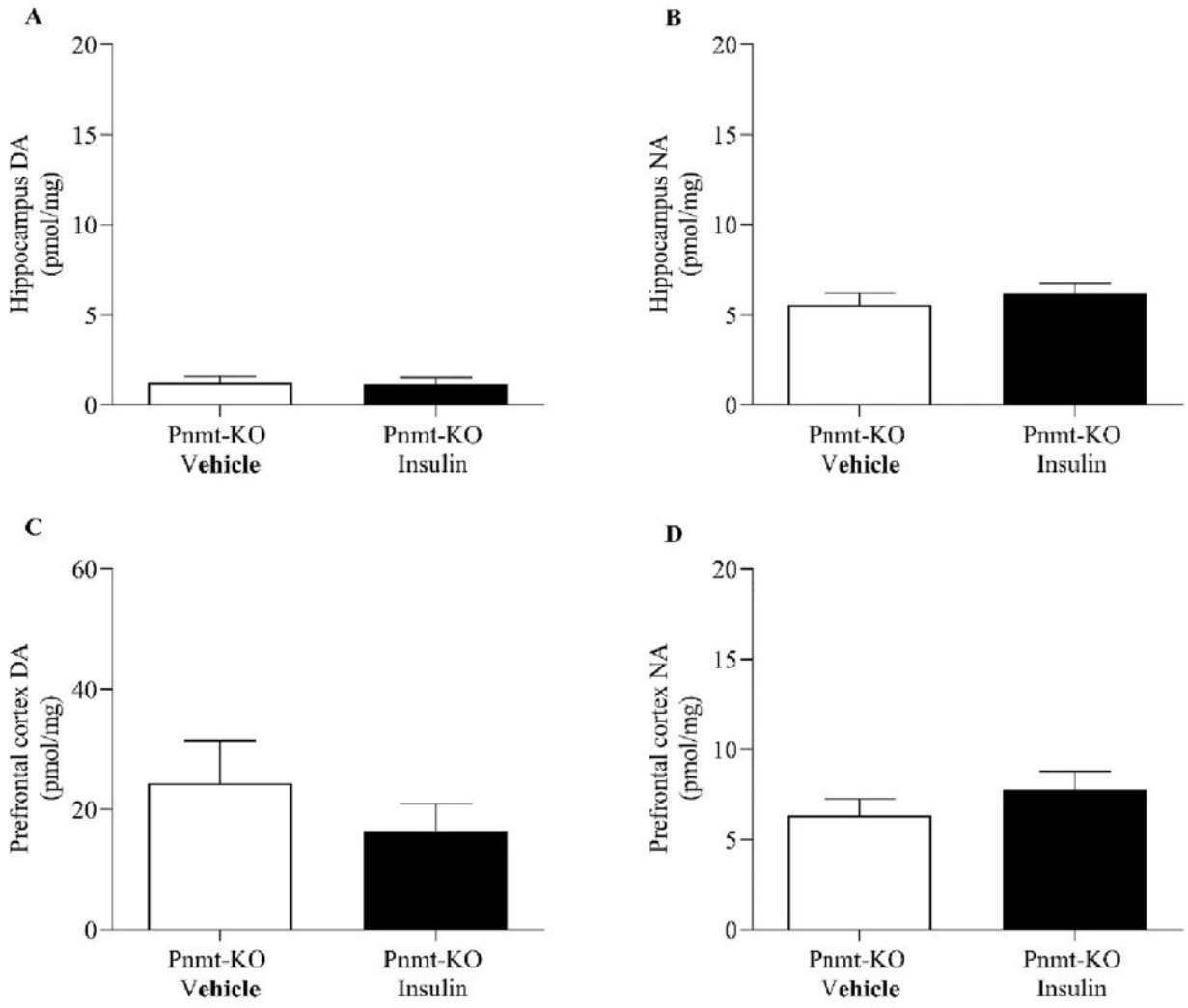


Fig. 8. Brain tissues catecholamines concentration in adrenaline-deficient mice (Pnmt-KO) mice after insulin treatment (Protocol of Fig. 1B and C). (A) dopamine (DA) and (B) noradrenaline (NA) in hippocampus, and (C) DA and (D) NA in prefrontal cortex of Pnmt-KO insulin-treated (2 U/kg) or vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 6–7 mice per group. Pnmt-KO Insulin, adrenaline-deficient insulin-treated mice; Pnmt-KO Vehicle, adrenaline-deficient vehicle-treated mice.

Insulin's action may be due to local cerebral energy utilization: when insulin levels are increased peripherally it leads to an increase in its binding to IR in several brain areas and a subsequent increase in glucose utilization [78,79]. This might have been determinant in strengthening contextual fear memory of insulin-treated groups. Moreover, hormones that interact with insulin in the periphery, such as adrenaline, may influence the cognitive effects of insulin. Taken together, our results suggest a contextual fear memory-enhancing effect of insulin, which appears to be independent of the effects of increased plasma adrenaline. However, our results can not dissociate if insulin influences contextual fear memory enhancement formation or retrieval, since insulin was administered in both days (training and context days).

Considering that in our experimental design insulin administration increased contextual fear memory in adrenaline-deficient mice, we decided to investigate hippocampus gene transcription of immediate early genes to learn more about molecular events that may be involved in these effects on fear memory. Contextual fear conditioning leads to an increase in the *Bdnf* gene expression in hippocampus and amygdala [80–82]. BDNF is implicated in neuronal survival [83,84] and long-term potentiation [85]. Hippocampal mRNA expression of *Bdnf* was increased in insulin-treated Pnmt-KO mice compared to Pnmt-KO vehicle-treated mice, suggesting an up-regulation of this gene associated with an exogenous source of insulin and increased contextual fear memory. In agreement, insulin administration reverts spatial memory impairment and increases hippocampus *Bdnf* gene expression [86]. However, to our knowledge, this had never been described for contextual fear memory. Moreover, insulin promotes Glut-4 translocation to the membrane of hippocampus cells and Glut-4 inhibition with Indinavir impaired memory in inhibitory avoidance paradigm and reduced hippocampal *Bdnf* expression [87]. This could indicate that Glut-4 translocation may be an effector of glucose cognitive enhancer actions, reinforcing the idea that insulin's action may be due to enhanced local cerebral energy utilization. Putting all together, these results suggest a key role of insulin in behaviour and central molecular changes that promote contextual fear memory formation and/or retrieval. It is important to note that the present study used male mice only, even though differences in the expression of immediate-early genes like *Bdnf* following fear conditioning have been reported to be different between female and male subjects [88]. Therefore, it would be interesting to evaluate sexual molecular differences in our paradigm following insulin administration. Unfortunately, this is beyond the scope of this study.

In the memory formation process, short-term memory is firstly formed and can last up to 30 min. Then, the second phase of memory formation, consolidation, occurs forming the so-called long-term memory (LTM) [89]. The formation of LTM is dependent on new protein expression [90,91]. It is known that the hippocampus is involved in the

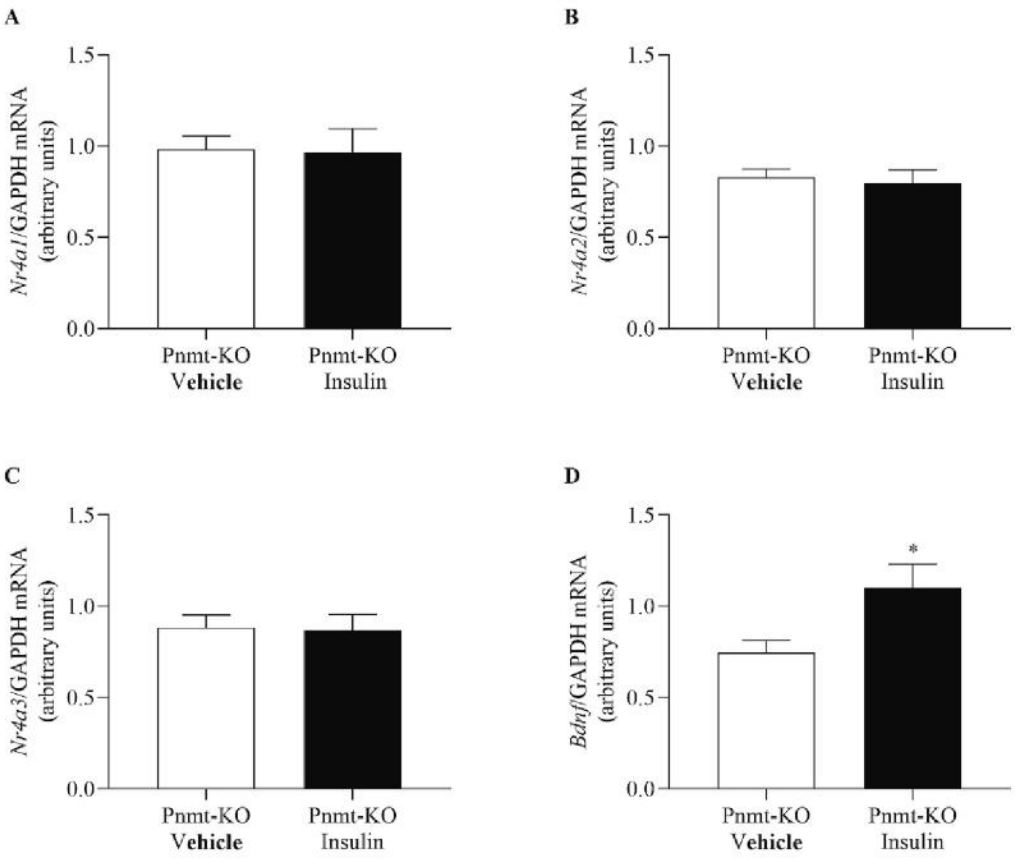


Fig. 9. Hippocampus mRNA expression of NR4A family genes and *Bdnf* in adrenaline-deficient mice (Pnmt-KO) after insulin treatment (Protocol of Fig. 1C). (A) *Nr4a1*, (B) *Nr4a2*, (C) *Nr4a3*, and (D) *Bdnf* after context fear test in Pnmt-KO insulin-treated (2 U/kg) or vehicle-treated mice (NaCl, 0.9 %). Values are means ± SEM of 7 mice per group. Pnmt-KO Insulin, adrenaline-deficient insulin-treated mice; Pnmt-KO Vehicle, adrenaline-deficient vehicle-treated mice. Results of mRNA are expressed as arbitrary units (AUs) after normalization for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *, significantly different from correspondent values in Pnmt-KO vehicle mice ($p < 0.05$).

formation of contextual fear memories and also that in order to form long-term memories there is a requirement for new gene transcription and protein synthesis [92]. *Nr4a* genes are considered to be immediate early genes because their expression can be rapidly induced by different stimuli [93] and previous studies showed *Nr4a* subfamily genes expression in the hippocampus to be upregulated after training of fear conditioning procedure contributing to contextual memory consolidation on the next day [94–96]. However, previously published studies demonstrate the increased expression of *Nr4a* gene family and *Bdnf* genes in fear conditioning and other paradigms compared to naïve groups after the acquisition day [8,88,96–98]. We did not observe any differences in the mRNA expression of *Nr4a1*, *Nr4a2*, and *Nr4a3* after fear conditioning procedure in insulin-treated Pnmt-KO mice. Keeping in mind that expression of Nr4a immediate early genes increases after a few hours of the stimulus, it may influence other target genes such as brain-derived neurotrophic factor (*Bdnf*) gene [88], proto-oncogene c-Rel [99] or others which have important roles in hippocampus-dependent contextual fear memory [88,94]. This may suggest that analyses could have been performed out of the detecting point for *Nr4a* genes since they are immediate early genes and vulnerable to expression changes a few hours after the stimulus, quickly returning to baseline levels [93].

5. Conclusion

In conclusion, enhanced freezing behaviour after insulin treatment, even in the absence of endogenous adrenaline, may indicate a key role of insulin causing central molecular changes that may promote contextual fear memory formation and/or retrieval. Fear conditioning is an excellent tool for examining cognitive components of anxious behaviour. This work may indicate a further role of insulin in these processes, as well as, in memory modulation, which may be important in diabetes.

Ethics statement

All animal studies presented in this article were reviewed and approved by the Organism Responsible for Animal Welfare in Faculty of Medicine of University of Porto and National Authority for Animal Health.

CRediT authorship contribution statement

AO: conceptualization, investigation, writing - original data, data-review and editing, visualization, funding acquisition; RS: conceptualization, investigation, writing - original data; FP: investigation; MA: investigation; RM: investigation, funding acquisition; PS: validation, resources; MMR: conceptualization, investigation, data-review and editing, visualization, supervision, project administration, funding acquisition.

Funding

This work was partially supported by Foundation for Science and Technology (FCT, project UIDB/04308/2020 and UIDP/04308/2020). Ana Oliveira and Raquel Martinho were in receipt of Ph.D. studentships from FCT (SFRH/BD/138984/2018 and SFRH/BD/133860/2017, respectively).

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be interpreted as a potential conflict of interest.

Acknowledgments

The authors thank Sónia Soares and António Carlos Ferreira for their technical support.

References

[1] M.D. Fuller, M.A. Emrick, M. Sadilek, T. Scheuer, W.A. Catterall, Molecular mechanism of calcium channel regulation in the fight-or-flight response, Sci. Signal. 3 (2010) ra70, https://doi.org/10.1126/scisignal.2001152.
[2] B. Chu, K. Marwaha, T. Sanvictores, D. Ayers, Physiology, Stress Reaction, StatPearls, 2021.
[3] L. Cahill, M.T. Alkire, Epinephrine enhancement of human memory consolidation: interaction with arousal at encoding, Neurobiol. Learn. Mem. 79 (2003) 194–198, https://doi.org/10.1016/s1074-7427(02)00036-9.
[4] A. Dornelles, M.N. de Lima, M. Grazziotin, J. Presti-Torres, V.A. Garcia, F.S. Scalco, R. Roesler, N. Schroder, Adrenergic enhancement of consolidation of object recognition memory, Neurobiol. Learn. Mem. 88 (2007) 137–142, https://doi.org/10.1016/j.nlm.2007.01.005.
[5] C.L. Williams, D. Men, E.C. Clayton, P.E. Gold, Norepinephrine release in the amygdala after systemic injection of epinephrine or escapable footshock: contribution of the nucleus of the solitary tract, Behav. Neurosci. 112 (1998) 1414–1422, https://doi.org/10.1037//0735-7044.112.6.1414.
[6] E. Alves, N. Lukoyanov, P. Serrao, D. Moura, M. Moreira-Rodrigues, Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors, Psychopharmacology 233 (2016) 2099–2108, https://doi.org/10.1007/s00213-016-4254-5.
[7] M. Toth, M. Ziegler, P. Sun, J. Gresack, V. Risbrough, Impaired conditioned fear response and startle reactivity in epinephrine-deficient mice, Behav. Pharmacol. 24 (2013) 1–9, https://doi.org/10.1097/FBP.0b013e32835cf408.
[8] A. Oliveira, R. Martinho, P. Serrao, M. Moreira-Rodrigues, Epinephrine released during traumatic events may strengthen contextual fear memory through increased hippocampus mRNA expression of Nr4a transcription factors, Front. Mol. Neurosci. 11 (2018) 334, https://doi.org/10.3389/fnmol.2018.00334.
[9] R. Martinho, A. Oliveira, G. Correia, M. Marques, R. Seixas, P. Serrao, M. Moreira-Rodrigues, Epinephrine may contribute to the persistence of traumatic memories in a post-traumatic stress disorder animal model, Front. Mol. Neurosci. 13 (2020), 588802, https://doi.org/10.3389/fnmol.2020.588802.
[10] S. Dufour, V. Lebon, G.I. Shulman, K.F. Petersen, Regulation of net hepatic glycogenolysis and gluconeogenesis by epinephrine in humans, Am. J. Physiol. Endocrinol. Metab. 297 (2009) E231–E235, https://doi.org/10.1152/ajpendo.00222.2009.
[11] D.E. Gray, H.L. Lickley, M. Vranic, Physiologic effects of epinephrine on glucose turnover and plasma free fatty acid concentrations mediated independently of glucagon, Diabetes 29 (1980) 600–608, https://doi.org/10.2337/diab.29.8.600.
[12] T. Miyashita, C.L. Williams, Epinephrine administration increases neural impulses propagated along the vagus nerve: role of peripheral beta-adrenergic receptors, Neurobiol. Learn. Mem. 85 (2006) 116–124, https://doi.org/10.1016/j.nlm.2005.08.013.
[13] A. Kaneto, E. Miki, K. Kosaka, Effects of vagal stimulation on glucagon and insulin secretion, Endocrinology 95 (1974) 1005–1010, https://doi.org/10.1210/endo-95-4-1005.
[14] M. Moosavi, N. Naghdi, N. Maghsoudi, S. Zahedi Asl, The effect of intrahippocampal insulin microinjection on spatial learning and memory, Horm. Behav. 50 (2006) 748–752, https://doi.org/10.1016/j.yhbeh.2006.06.025.
[15] C.R. Park, R.J. Seeley, S. Craft, S.C. Woods, Intracerebroventricular insulin enhances memory in a passive-avoidance task, Physiol. Behav. 68 (2000) 509–514, https://doi.org/10.1016/s0031-9384(99)00220-6.
[16] C. Benedict, M. Hallschmid, A. Hatke, B. Schultes, H.L. Fehm, J. Born, W. Kern, Intranasal insulin improves memory in humans, Psychoneuroendocrinology 29 (2004) 1326–1334, https://doi.org/10.1016/j.psyneuen.2004.04.003.
[17] M. Spinelli, S. Fusco, C. Grassi, Brain insulin resistance and hippocampal plasticity: mechanisms and biomarkers of cognitive decline, Front. Neurosci. 13 (2019) 788, https://doi.org/10.3389/fnins.2019.00788.
[18] E. Yavas, S. Gonzalez, M.S. Fanselow, Interactions Between the Hippocampus, Prefrontal Cortex, and Amygdala Support Complex Learning and Memory, F1000Res 8, 2019, https://doi.org/10.12688/f1000research.19317.1.
[19] I. Izquierdo, C.R. Furini, J.C. Myskiw, Fear memory, Physiol. Rev. 96 (2016) 695–750, https://doi.org/10.1152/physrev.00018.2015.
[20] J. Freiherr, J.N. Lundstrom, U. Habel, K. Reetz, Multisensory integration mechanisms during aging, Front. Hum. Neurosci. 7 (2013) 863, https://doi.org/10.3389/fnhum.2013.00863.
[21] V. Haj-ali, G. Mohaddes, S.H. Babri, Intracerebroventricular insulin improves spatial learning and memory in male Wistar rats, Behav. Neurosci. 123 (2009) 1309–1314, https://doi.org/10.1037/a0017722.
[22] A.E. Wilensky, G.E. Schafe, J.E. LeDoux, The amygdala modulates memory consolidation of fear-motivated inhibitory avoidance learning but not classical fear conditioning, J. Neurosci. 20 (2000) 7059–7066.
[23] H. Galbo, J.J. Holst, N.J. Christensen, The effect of different diets and of insulin on the hormonal response to prolonged exercise, Acta Physiol. Scand. 107 (1979) 19–32, https://doi.org/10.1111/j.1748-1716.1979.tb06438.x.
[24] Y. Oomura, T. Ono, H. Ooyama, M.J. Wayner, Glucose and osmosensitive neurones of the rat hypothalamus, Nature 222 (1969) 282–284, https://doi.org/10.1038/222282a0.
[25] B.E. Levin, A.A. Dunn-Meynell, V.H. Routh, Brain glucose sensing and body energy homeostasis: role in obesity and diabetes, Am. J. Phys. 276 (1999) R1223–R1231, https://doi.org/10.1152/ajpregu.1999.276.5.R1223.
[26] S.J. Fisher, J.C. Bruning, S. Lannon, C.R. Kahn, Insulin signaling in the central nervous system is critical for the normal sympathoadrenal response to hypoglycemia, Diabetes 54 (2005) 1447–1451, https://doi.org/10.2337/diabetes.54.5.1447.
[27] N. Tesfaye, E.R. Seaquist, Neuroendocrine responses to hypoglycemia, Ann. N. Y. Acad. Sci. 1212 (2010) 12–28, https://doi.org/10.1111/j.1749-6632.2010.05820.x.
[28] T.S. Voss, M.H. Vendelbo, U. Kampmann, J.R. Hingst, J.F.P. Wojtaszewski, M. V. Svart, N. Moller, N. Jessen, Acute hypoglycemia in healthy humans impairs insulin-stimulated glucose uptake and glycogen synthase in skeletal muscle: a randomized clinical study, Diabetes 66 (2017) 2483–2494, https://doi.org/10.2337/db16-1559.
[29] S.N. Ebert, Q. Rong, S. Boe, R.P. Thompson, A. Grinberg, K. Pfeifer, Targeted insertion of the Cre-recombinase gene at the phenylethanolamine n-methyltransferase locus: a new model for studying the developmental distribution of adrenergic cells, Dev. Dyn. 231 (2004) 849–858, https://doi.org/10.1002/dvdy.20188.
[30] V.S. Valentinuzzi, D.E. Kolker, M.H. Vitaterna, K. Shimomura, A. Whiteley, S. Low-Zeddies, F.W. Turek, E.A. Ferrari, R. Paylor, J.S. Takahashi, Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ x C57BL/6J)F2 mice, Learn. Mem. 5 (1998) 391–403.
[31] L.F. Rocinholi, S.S. Almeida, L.M. De-Oliveira, Response threshold to aversive stimuli in stimulated early protein-malnourished rats, Braz. J. Med. Biol. Res. 30 (1997) 407–413, https://doi.org/10.1590/s0100-879x1997000300016.
[32] S.E. Canada, S.A. Weaver, S.N. Sharpe, B.A. Pederson, Brain glycogen supercompensation in the mouse after recovery from insulin-induced hypoglycemia, J. Neurosci. Res. 89 (2011) 585–591, https://doi.org/10.1002/jnr.22579.
[33] S. Alva, FreeStyle Lite—a blood glucose meter that requires no coding, J. Diabetes Sci. Technol. 2 (2008) 546–551, https://doi.org/10.1177/193229680800200402.
[34] M. Moreira-Rodrigues, A.L. Graca, M. Ferreira, J. Afonso, P. Serrao, M. Morato, F. Ferreirinha, P. Correia-de-Sa, S.N. Ebert, D. Moura, Attenuated aortic vasodilation and sympathetic prejunctional facilitation in epinephrine-deficient mice: selective impairment of beta2-adrenoceptor responses, J. Pharmacol. Exp. Ther. 351 (2014) 243–249, https://doi.org/10.1124/jpet.114.217281.
[35] R. Martinho, G. Correia, R. Seixas, A. Oliveira, S. Silva, P. Serrao, C. Fernandes-Lopes, C. Costa, M. Moreira-Rodrigues, Treatment with Nepicastat decreases contextual traumatic memories persistence in post-traumatic stress disorder, Front. Mol. Neurosci. 14 (2021), 745219, https://doi.org/10.3389/fnmol.2021.745219.
[36] M. Moreira-Rodrigues, J. Quelhas-Santos, R. Roncon-Albuquerque, P. Serrao, A. Leite-Moreira, B. Sampaio-Maia, M. Pestana, Blunted renal dopaminergic system in a mouse model of diet-induced obesity, Exp. Biol. Med. (Maywood) 237 (2012) 949–955, https://doi.org/10.1258/ebm.2012.012077.
[37] M. Moreira-Rodrigues, J. Quelhas-Santos, P. Serrao, C. Fernandes-Cerqueira, B. Sampaio-Maia, M. Pestana, Glycaemic control with insulin prevents the reduced renal dopamine D1 receptor expression and function in streptozotocin-induced diabetes, Nephrol. Dial. Transplant. 25 (2010) 2945–2953, https://doi.org/10.1093/ndt/gfq150.
[38] M. Moreira-Rodrigues, B. Sampaio-Maia, M. Moura, M. Pestana, Renal dopaminergic system activity in uninephrectomized rats up to 26 weeks after surgery, Am. J. Nephrol. 27 (2007) 232–239, https://doi.org/10.1159/000101368.
[39] P. Mendes, R. Martinho, S. Leite, L. Maia-Moco, A.F. Leite-Moreira, A.P. Lourenco, M. Moreira-Rodrigues, Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice, Int. J. Cardiol. 253 (2018) 113–119, https://doi.org/10.1016/j.ijcard.2017.10.014.
[40] M. Moreira-Rodrigues, R. Roncon-Albuquerque Jr., T. Henriques-Coelho, A. P. Lourenco, B. Sampaio-Maia, J. Santos, M. Pestana, A.F. Leite-Moreira, Cardiac remodeling and dysfunction in nephrotic syndrome, Kidney Int. 71 (2007) 1240–1248, https://doi.org/10.1038/sj.ki.5002204.
[41] L. Cahill, B. Prins, M. Weber, J.L. McGaugh, Beta-adrenergic activation and memory for emotional events, Nature 371 (1994) 702–704, https://doi.org/10.1038/371702a0.
[42] J. L. McGaugh, Memory–a century of consolidation, Science. 287 (2000) 248–251.
[43] H. Weil-Malherbe, J. Axelrod, R. Tomchick, Blood-brain barrier for adrenaline, Science 129 (1959) 1226–1227, https://doi.org/10.1126/science.129.3357.1226.
[44] P.E. Gold, Regulation of memory - from the adrenal medulla to liver to astrocytes to neurons, Brain Res. Bull. 105 (2014) 25–35, https://doi.org/10.1016/j.brainresbull.2013.12.012.
[45] R. Martinho, R. Seixas, M. Azevedo, A. Oliveira, P. Serrão, M. Moreira-Rodrigues, Sotalol treatment may interfere with retrieval, expression, and/or reconsolidation processes thus disrupting traumatic memories in a post-traumatic stress disorder mice model, Front. Pharmacol. 12 (2022) 809271, https://doi.org/10.3389/fphar.2021.809271.
[46] R.A. Rizza, P.E. Cryer, M.W. Haymond, J.E. Gerich, Adrenergic mechanisms for the effects of epinephrine on glucose production and clearance in man, J. Clin. Invest. 65 (1980) 682–689, https://doi.org/10.1172/JCI109714.
[47] Z. Fu, E.R. Gilbert, D. Liu, Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes, Curr. Diabetes Rev. 9 (2013) 25–53.
[48] J.G. Blanchard, P.M. Duncan, Effect of combinations of insulin, glucose and scopolamine on radial arm maze performance, Pharmacol. Biochem. Behav. 58 (1997) 209–214, https://doi.org/10.1016/s0091-3057(97)00064-6.

[49] Z. He, S. Han, H. Zhu, X. Hu, X. Li, C. Hou, C. Wu, Q. Xie, N. Li, X. Du, J. Ni, Q. Liu, The protective effect of vanadium on cognitive impairment and the neuropathology of Alzheimer's disease in APPSwe/PS1dE9 mice, Front. Mol. Neurosci. 13 (2020) 21, https://doi.org/10.3389/fnmol.2020.00021.
[50] I. Kazkayasi, G. Telli, E. Nemutlu, S. Uma, Intranasal metformin treatment ameliorates cognitive functions via insulin signaling pathway in ICV-STZ-induced mice model of Alzheimer's disease, Life Sci. 299 (2022), 120538, https://doi.org/10.1016/j.lfs.2022.120538.
[51] G. Languren, T. Montiel, A. Julio-Amilpas, L. Massieu, Neuronal damage and cognitive impairment associated with hypoglycemia: an integrated view, Neurochem. Int. 63 (2013) 331–343, https://doi.org/10.1016/j.neuint.2013.06.018.
[52] P.E. Cryer, Hypoglycemia-associated autonomic failure in diabetes, Am. J. Physiol. Endocrinol. Metab. 281 (2001) E1115–E1121, https://doi.org/10.1152/ajpendo.2001.281.6.E1115.
[53] L.A. Donnelly, A.D. Morris, B.M. Frier, J.D. Ellis, P.T. Donnan, R. Durrant, M. M. Band, G. Reekie, G.P. Leese, D. M. Collaboration, Frequency and predictors of hypoglycaemia in Type 1 and insulin-treated Type 2 diabetes: a population-based study, Diabet. Med. 22 (2005) 749–755, https://doi.org/10.1111/j.1464-5491.2005.01501.x.
[54] S.R. Heller, S. Colagiuri, S. Vaaler, B.H. Wolffenbuttel, K. Koelendorf, H.H. Friberg, K. Windfeld, A. Lindholm, Hypoglycaemia with insulin aspart: a double-blind, randomised, crossover trial in subjects with type 1 diabetes, Diabet. Med. 21 (2004) 769–775, https://doi.org/10.1111/j.1464-5491.2004.01244.x.
[55] E.C. McNay, V.E. Cotero, Mini-review: impact of recurrent hypoglycemia on cognitive and brain function, Physiol. Behav. 100 (2010) 234–238, https://doi.org/10.1016/j.physbeh.2010.01.004.
[56] N.J. Christensen, K.G. Alberti, O. Brandsborg, Plasma catecholamines and blood substrate concentrations: studies in insulin induced hypoglycaemia and after adrenaline infusions, Eur. J. Clin. Investig. 5 (1975) 415–423, https://doi.org/10.1111/j.1365-2362.1975.tb00473.x.
[57] L.W. Lin, F.S. Tsai, W.T. Yang, S.C. Lai, C.C. Shih, S.C. Lee, C.R. Wu, Differential change in cortical and hippocampal monoamines, and behavioral patterns in streptozotocin-induced type 1 diabetic rats, Iran J. Basic Med. Sci. 21 (2018) 1026–1034, https://doi.org/10.22038/IJBMS.2018.29810.7197.
[58] F. Fiory, G. Perruolo, I. Cimmino, S. Cabaro, F.C. Pignalosa, C. Miele, F. Beguinot, P. Formisano, F. Oriente, The relevance of insulin action in the dopaminergic system, Front. Neurosci. 13 (2019) 868, https://doi.org/10.3389/fnins.2019.00868.
[59] C. Stubbendorff, C.W. Stevenson, Dopamine regulation of contextual fear and associated neural circuit function, Eur. J. Neurosci. 54 (2021) 6933–6947, https://doi.org/10.1111/ejn.14772.
[60] K.T. Jones, C. Woods, J. Zhen, T. Antonio, K.D. Carr, M.E. Reith, Effects of diet and insulin on dopamine transporter activity and expression in rat caudate-putamen, nucleus accumbens, and midbrain, J. Neurochem. 140 (2017) 728–740, https://doi.org/10.1111/jnc.13930.
[61] T. Hökfelt, K. Fuxe, M. Goldstein, O. Johansson, Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain, Brain Res. 66 (1974) 235–251, https://doi.org/10.1016/0006-8993(74)90143-7.
[62] J.Y. Lew, Y. Matsumoto, J. Pearson, M. Goldstein, T. Hökfelt, K. Fuxe, Localization and characterization of phenylethanolamine N-methyl transferase in the brain of various mammalian species, Brain Res. 119 (1977) 199–210, https://doi.org/10.1016/0006-8993(77)90100-7.
[63] S.D. Robertson, H.J. Matthies, W.A. Owens, V. Sathananthan, N.S. Christianson, J. P. Kennedy, C.W. Lindsley, L.C. Daws, A. Galli, Insulin reveals Akt signaling as a novel regulator of norepinephrine transporter trafficking and norepinephrine homeostasis, J. Neurosci. 30 (2010) 11305–11316, https://doi.org/10.1523/JNEUROSCI.0126-10.2010.
[64] M.E. Andres, G. Bustos, K. Gysling, Regulation of [3H]norepinephrine release by N-methyl-D-aspartate receptors in minislices from the dentate gyrus and the CA1-CA3 area of the rat hippocampus, Biochem. Pharmacol. 46 (1993) 1983–1987, https://doi.org/10.1016/0006-2952(93)90640-i.
[65] H. Hu, E. Real, K. Takamiya, M.G. Kang, J. Ledoux, R.L. Huganir, R. Malinow, Emotion enhances learning via norepinephrine regulation of AMPA-receptor trafficking, Cell. 131 (2007) 160–173, https://doi.org/10.1016/j.cell.2007.09.017.
[66] R.W. Roosevelt, D.C. Smith, R.W. Clough, R.A. Jensen, R.A. Browning, Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat, Brain Res. 1119 (2006) 124–132, https://doi.org/10.1016/j.brainres.2006.08.048.
[67] M.C. Petersen, G.I. Shulman, Mechanisms of insulin action and insulin resistance, Physiol. Rev. 98 (2018) 2133–2223, https://doi.org/10.1152/physrev.00063.2017.
[68] R.J. Schulingkamp, T.C. Pagano, D. Hung, R.B. Raffa, Insulin receptors and insulin action in the brain: review and clinical implications, Neurosci. Biobehav. Rev. 24 (2000) 855–872, https://doi.org/10.1016/s0149-7634(00)00040-3.
[69] F.G. De Felice, C. Benedict, A key role of insulin receptors in memory, Diabetes 64 (2015) 3653–3655, https://doi.org/10.2337/dbi15-0011.
[70] W.Q. Zhao, D.L. Alkon, Role of insulin and insulin receptor in learning and memory, Mol. Cell. Endocrinol. 177 (2001) 125–134, https://doi.org/10.1016/s0303-7207(01)00455-5.
[71] J.M. Hill, M.A. Lesniak, C.B. Pert, J. Roth, Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas, Neuroscience. 17 (1986) 1127–1138, https://doi.org/10.1016/0306-4522(86)90082-5.
[72] J. Bloemer, S. Bhattacharya, R. Amin, V. Suppiramaniam, Impaired insulin signaling and mechanisms of memory loss, Prog. Mol. Biol. Transl. Sci. 121 (2014) 413–449, https://doi.org/10.1016/B978-0-12-800101-1.00013-2.
[73] C. Mazzucchelli, R. Brambilla, Ras-related and MAPK signalling in neuronal plasticity and memory formation, Cell. Mol. Life Sci. 57 (2000) 604–611, https://doi.org/10.1007/PL00000722.
[74] S. Phasuk, T. Pairojana, P. Suresh, C.H. Yang, S. Roytrakul, S.P. Huang, C.C. Chen, N. Pakaprot, S. Chompoopong, S. Nudmamud-Thanoi, I.Y. Liu, Enhanced contextual fear memory in peroxiredoxin 6 knockout mice is associated with hyperactivation of MAPK signaling pathway, Mol. Brain 14 (2021) 42, https://doi.org/10.1186/s13041-021-00754-1.
[75] D.N. Catelas, M.P. Serrao, P. Soares-Da-Silva, Effects of nepicastat upon dopamine-beta-hydroxylase activity and dopamine and norepinephrine levels in the rat left ventricle, kidney, and adrenal gland, Clin. Exp. Hypertens. 42 (2020) 118–125, https://doi.org/10.1080/10641963.2019.1583245.
[76] R.E. Schmidt, D.M. Geller, E.M. Johnson Jr., Characterization of increased plasma dopamine-beta-hydroxylase activity in rats with experimental diabetes, Diabetes 30 (1981) 416–423, https://doi.org/10.2337/diab.30.5.416.
[77] M.J. Rand, H. Majewski, Adrenaline mediates a positive feedback loop in noradrenergic transmission: its possible role in development of hypertension, Clin. Exp. Hypertens. A 6 (1984) 347–370, https://doi.org/10.3109/10641968409062570.
[78] N. Henneberg, S. Hoyer, Short-term or long-term intracerebroventricular (i.c.v.) infusion of insulin exhibits a discrete anabolic effect on cerebral energy metabolism in the rat, Neurosci. Lett. 175 (1994) 153–156, https://doi.org/10.1016/0304-3940(94)91102-9.
[79] C.R. Park, Cognitive effects of insulin in the central nervous system, Neurosci. Biobehav. Rev. 25 (2001) 311–323, https://doi.org/10.1016/s0149-7634(01)00016-1.
[80] J. Hall, K.L. Thomas, B.J. Everitt, Rapid and selective induction of BDNF expression in the hippocampus during contextual learning, Nat. Neurosci. 3 (2000) 533–535, https://doi.org/10.1038/75698.
[81] L.M. Rattiner, M. Davis, C.T. French, K.J. Ressler, Brain-derived neurotrophic factor and tyrosine kinase receptor B involvement in amygdala-dependent fear conditioning, J. Neurosci. 24 (2004) 4796–4806, https://doi.org/10.1523/JNEUROSCI.5654-03.2004.
[82] P. Kumari, K. Roy, M. Wadhwa, G. Chauhan, S. Alam, K. Kishore, K. Ray, U. Panjwani, Fear memory is impaired in hypobaric hypoxia: role of synaptic plasticity and neuro-modulators in limbic region, Life Sci. 254 (2020), 117555, https://doi.org/10.1016/j.lfs.2020.117555.
[83] A. Ghosh, J. Carnahan, M.E. Greenberg, Requirement for BDNF in activity-dependent survival of cortical neurons, Science 263 (1994) 1618–1623, https://doi.org/10.1126/science.7907431.
[84] M.P. Sahu, Y. Pazos-Boubeta, A. Steinzeig, K. Kaurinkoski, M. Palmisano, O. Borowecki, T.P. Piepponen, E. Castren, Depletion of TrkB receptors from adult serotonergic neurons increases brain serotonin levels, enhances energy metabolism and impairs learning and memory, Front. Mol. Neurosci. 14 (2021), 616178, https://doi.org/10.3389/fnmol.2021.616178.
[85] C. Cunha, R. Brambilla, K.L. Thomas, A simple role for BDNF in learning and memory? Front. Mol. Neurosci. 3 (2010) 1, https://doi.org/10.3389/neuro.02.001.2010.
[86] C.B. Haas, E. Kalinine, E.R. Zimmer, G. Hansel, A.W. Brochier, J.P. Oses, L. V. Portela, A.P. Muller, Brain insulin administration triggers distinct cognitive and neurotrophic responses in young and aged rats, Mol. Neurobiol. 53 (2016) 5807–5817, https://doi.org/10.1007/s12035-015-9494-6.
[87] J. Pearson-Leary, E.C. McNay, Novel roles for the insulin-regulated glucose transporter-4 in hippocampally dependent memory, J. Neurosci. 36 (2016) 11851–11864, https://doi.org/10.1523/JNEUROSCI.1700-16.2016.
[88] K. Mizuno, E. Dempster, J. Mill, K.P. Giese, Long-lasting regulation of hippocampal Bdnf gene transcription after contextual fear conditioning, Genes Brain Behav. 11 (2012) 651–659, https://doi.org/10.1111/j.1601-183X.2012.00805.x.
[89] I. Izquierdo, D.M. Barros, T. Mello e Souza, M.M. de Souza, L.A. Izquierdo, J. H. Medina, Mechanisms for memory types differ, Nature 393 (1998) 635–636, https://doi.org/10.1038/31371.
[90] P.W. Frankland, S.A. Josselyn, S.G. Anagnostaras, J.H. Kogan, E. Takahashi, A. J. Silva, Consolidation of CS and US representations in associative fear conditioning, Hippocampus 14 (2004) 557–569, https://doi.org/10.1002/hipo.10208.
[91] G.E. Schafe, J.E. LeDoux, Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala, J. Neurosci. 20 (2000) RC96, https://doi.org/10.1523/JNEUROSCI.20-18-j0003.2000.
[92] J.B. Flexner, L.B. Flexner, E. Stellar, Memory in mice as affected by intracerebral puromycin, Science 141 (1963) 57–59, https://doi.org/10.1126/science.141.3575.57.
[93] J.I. Morgan, T. Curran, Stimulus-transcription coupling in neurons: role of cellular immediate-early genes, Trends Neurosci. 12 (1989) 459–462, https://doi.org/10.1016/0166-2236(89)90096-9.
[94] J.D. Hawk, A.L. Bookout, S.G. Poplawski, M. Bridi, A.J. Rao, M.E. Sulewski, B. T. Kroener, D.J. Manglesdorf, T. Abel, NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors, J. Clin. Invest. 122 (2012) 3593–3602, https://doi.org/10.1172/JCI64145.
[95] J.D. Hawk, C. Florian, T. Abel, Post-training intrahippocampal inhibition of class I histone deacetylases enhances long-term object-location memory, Learn. Mem. 18 (2011) 367–370, https://doi.org/10.1101/lm.2097411.
[96] L.S. von Hertzen, K.P. Giese, Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation, J. Neurosci. 25 (2005) 1935–1942, https://doi.org/10.1523/JNEUROSCI.4707-04.2005.

[97] A.S. Aguiar Jr., A.A. Castro, E.L. Moreira, V. Glaser, A.R. Santos, C.I. Tasca, A. Latini, R.D. Prediger, Short bouts of mild-intensity physical exercise improve spatial learning and memory in aging rats: involvement of hippocampal plasticity via AKT, CREB and BDNF signaling, Mech. Ageing Dev. 132 (2011) 560–567, https://doi.org/10.1016/j.mad.2011.09.005.

[98] P.B. Mello-Carpes, L. da Silva de Vargas, M.C. Gayer, R. Roehrs, I. Izquierdo, Hippocampal noradrenergic activation is necessary for object recognition memory consolidation and can promote BDNF increase and memory persistence, Neurobiol. Learn. Mem. 127 (2016) 84–92, https://doi.org/10.1016/j.nlm.2015.11.014.

[99] H.J. Ahn, C.M. Hernandez, J.M. Levenson, F.D. Lubin, H.C. Liou, J.D. Sweatt, c-Rel, an NF-kappaB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation, Learn. Mem. 15 (2008) 539–549, https://doi.org/10.1101/lm.866408.

CHAPTER 6 - DISCUSSION AND CONCLUSION

The fear conditioning procedure is a behavioural paradigm valuable to study the neurobiology of learning and memory. Fear conditioning can be applied to both rodents and humans and the resulting memory may be stored for a long period of time (Poulos, et al., 2009). The focus of this project is to clarify the involvement of Ad in the formation and strengthening of contextual fear memory, exploring different possible pathways by which a peripheral stress hormone influences the central nervous system ultimately leading to contextual fear memory strength.

It was previously shown that Ad strengthens contextual fear memory in Ad-deficient mice (Alves, et al., 2016, Toth, et al., 2013). We now show that moderate doses of Ad at the same moment of the aversive stimulus are important for the retention and strengthening of contextual fear memories in Ad-deficient mice, contrarily to i.p. injections of NA. Taking into account that Ad administration strengthens contextual fear memory twenty-four hours after the training session (fear acquisition day), we proposed to understand if this Ad effect could prevail for longer periods of time. Ad-deficient mice administered with Ad were submitted to fear acquisition day and exposed to shock context 1 month later. Our results showed preservation of contextual fear memory in Ad-deficient mice administered with Ad, compared with Ad-deficient mice administered with vehicle, even 1 month after fear acquisition day, showing AD's effect in old memories (Manuscript I – Chapter II) (Oliveira, et al., 2018).

Using a non-selective peripheral antagonist of β-adrenoceptors, sotalol, and a selective agonist for β_2-adrenoceptors, fenoterol, Alves, et al. (2016) showed that Ad induces the formation of long-term contextual fear memory acting specifically in peripheral β_2-adrenoceptors (Alves, et al., 2016). In this study, we showed that administration of selective β_2-adrenoceptor antagonist, ICI 118,551, is effective in decreasing the contextual fear memory formation, both 1 day and 1 month after the fear acquisition test (Manuscript I – Chapter II) (Oliveira, et al., 2018).

Memory consolidation processing, namely in contextual fear memory, requires the regulation of gene expression (Kim and Kaang, 2017). The expression of NR4A transcription factors family gene and *Bdnf* have been associated with memory formation (Gorski, et al., 2003, Guzowski, 2002, Hawk, et al., 2012, Ploski, et al., 2011). In the present study (Manuscript I – Chapter II) (Oliveira, et al., 2018), the expression of *Nr4a1*, *Nr4a3,* and *Nr4a3* in the hippocampus was decreased in Ad-deficient mice, which may have contributed to impair contextual fear memory in these mice compared to WT mice.

Taking all results together, Ad may be important for a strong and durable context fear memory and its effect can be reverted using a β_2-adrenoceptor antagonist.

Still, Ad, which is mainly produced in the adrenal gland, due to its biochemical characteristics does not appears to cross the blood-brain barrier (Gold, 2014, Habgood, et al., 2007, Skultetyova, et al., 1998). One proposed mechanism suggests glucose as the mediator of Ad in strengthening contextual fear memory. Ad released from the adrenal gland to the bloodstream as a response to stress (fear acquisition day) can activate β_2-adrenoceptors (Alves, et al., 2016) in the liver which can lead to glycogenolysis and increases glucose blood levels, leading to a moderate hyperglycaemia. Glucose increment can be used for glycogen stores in astrocytes allowing lactate production (Newman, et al., 2011). Lactate and glucose may be energy sources for neurons to support the processes involved in memory formation (Brown, et al., 2004, Suzuki, et al., 2011). In an important region for contextual fear memory formation, the hippocampus, there are neuronal cells under the control of glucose availability that seem to be important for contextual fear memory formation (de Araujo, 2014). This was the rationale for the second manuscript (Manuscript II – Chapter III) (Oliveira, et al., 2023a) in which we aimed to better elucidate glucose theory described above.

Glucose uptake has been implicated in hippocampal memory processing (McNay, et al., 2000, Pearson-Leary, et al., 2018) and the hippocampus has been demonstrated to be a central region for contextual fear memory formation and consolidation (Phillips and LeDoux, 1992). On the first day of the fear conditioning paradigm, there were no differences in freezing behaviour nor in shock responsiveness (jump and vocalization responses), which suggests that glucose availability does not affect the perception of the aversive stimulus. On context day, i.p. injections of glucose (30 mg/kg) in WT mice strengthen contextual fear memory compared to WT mice administered with vehicle. During contextual fear conditioning, the stress leads to Ad release to the bloodstream which induces a hyperglycaemia state, thus it was not possible to separate the Ad effects from glucose. Therefore, we replicated the experimental design using Ad-deficient mice. In Ad-deficient mice, both glucose (30 mg/kg) and Ad (0.1 mg/kg) administration led to a strengthening in contextual fear memory compared to Ad-deficient mice administered with vehicle, which was similar. Therefore, glucose may act on contextual fear memory independently from Ad (Manuscript II – Chapter III) (Oliveira, et al., 2023a).

Both Ad and glucose appear to strengthen several types of memory in a U-inverted manner (Gold, 1986, Gold, 2014, Yerkes). Moderate doses of both may strengthen

memory but lower or higher doses can have the opposite effect, and the latter can even result in retrograde amnesia in a spatial working memory task (Stone, et al., 1992). In the present study, it was also used sub-effective doses of both Ad (0.01 mg/kg) and glucose (10 mg/kg) which alone were insufficient to strengthen contextual fear memory in Ad-deficient mice. However, when both were administered together it produced an improvement in contextual fear memory (Manuscript II – Chapter III) (Oliveira, et al., 2023a). Since Ad released into the bloodstream inherently leads to an increase in glucose levels (Alves, et al., 2016) and glucose may then stimulate acetylcholine synthesis (Durkin, et al., 1992), our results could indicate that low doses of glucose and Ad may act in synergy to improve contextual fear memory formation.

In addition to the action of Ad in increasing the release of glucose into the bloodstream from the liver, Ad has other physiological actions such as increasing heart rate and contraction and consequently increasing blood flow to the brain (Osborne, et al., 2015). The chronotropy and inotropy positive effects of Ad lead to systolic blood pressure increase (Furnival, et al., 1971, Tank and Lee Wong, 2015) which is detected by the baroreceptors present in aortic arch and carotid body (Dampney, 1994, Gianaros, et al., 2012). The glossopharyngeal nerve, a vagus nerve branch, receives this sensory information and transmits it to the brainstem (Fahim, 2003, Saku, et al., 2014). This may also contribute to NTS and amygdala activation during stressful and fearful situations (Garfinkel and Critchley, 2016, Gray, et al., 2009). Besides, an increase of neuronal activity is observed in learning paradigms such as the fear conditioning procedure (Zelikowsky, et al., 2014) and Ad induces the production of neuronal neurotransmitters (acetylcholine or glutamate) which in turn evokes Ca^{2+} increases in astrocytes (Schipke and Kettenmann, 2004, Sun, et al., 2013). In turn, Ca^{2+} promotes the release of K^{+} to the extracellular space causing arterioles smooth muscle hyperpolarization resulting in vasodilation (Filosa, et al., 2006, Lind, et al., 2013), which increases local blood flow and glucose uptake at the blood-brain barrier. All these processes culminate in increased glucose supply to meet the energy demands of stress-related hippocampal memory.

On glucose-administered Ad-deficient mice, hippocampal mRNA expression of the *Nr4a3* was increased in comparison to vehicle-administered Ad-deficient mice (Manuscript II – Chapter III) (Oliveira, et al., 2023a), suggesting an upregulation of these genes related to increased glucose levels after a contextual fear memory procedure and may compensate for the effect produced by Ad absence. Likewise, *Bdnf* mRNA expression was also increased in glucose-administered Ad-deficient mice compared to

vehicle-administered Ad-deficient mice (Manuscript II – Chapter III) (Oliveira, et al., 2023a). Since the NR4A family of genes regulates *Bdnf* gene expression (Hawk and Abel, 2011), our results may indicate that glucose induces the mRNA expression of *Nr4a3*, which in turn induces the mRNA expression of *Bdnf* contributing to contextual fear memory strengthening independently of Ad.

Using biochemical and imaging studies during a response to elevated energy demands, *Bdnf* stimulation resulted in overexpression of the neuronal GLUT3 to promote glucose utilization (Burkhalter, et al., 2003). Additional analysis should be performed to verify if this glucose transporter may be associated with the mechanism underlying contextual fear memory formation in Ad-deficient mice under the influence of glucose. Taken together, these results suggest that after a stressful situation, glucose may be an important part of the peripheral to central pathway for the strengthening of contextual fear memory.

Regarding glucose theory as a central mechanism for Ad action in contextual fear memory, allied that glucose may be a precursor for acetylcholine synthesis in the hippocampus (Ragozzino, et al., 1998, Ragozzino, et al., 1996), we decided to study the influence of Ad in muscarinic receptors in contextual fear memory. In adrenalectomized rats, it was observed a down expression of the M_4 muscarinic receptor in hippocampus samples (Mulugeta, et al., 2006). However, since the adrenal gland is the main organ responsible to produce several hormones, for instance mineralocorticoids, corticosteroids, androgens, and catecholamines, this approach fails to determine which hormones may be responsible for this outcome. The Ad-deficient mice do not express Pnmt and do not produce Ad (Ebert, et al., 2004), allowing the study of Ad action in muscarinic receptor expression in the hippocampus. Regarding genetic background, Ad-deficient mice which have reduced contextual fear memory compared to WT mice (Toth, et al., 2013), also presented a decrease in M_4 muscarinic receptor mRNA expression when compared to WT mice (Manuscript III – Chapter IV). When Ad was administered in Ad-deficient mice, this peripheral hormone, was capable to restore contextual fear memory impairment observed in Ad-deficient mice (Oliveira, et al., 2018, Toth, et al., 2013) and also increased M_4 muscarinic receptor mRNA expression in hippocampus samples (Manuscript III – Chapter IV). Our data may suggest a key role of Ad, a peripheral hormone, in modulating neurotransmitter receptors which have an important function in contextual fear memory.

However, our work failed to determine a more direct relation between Ad, glucose, and acetylcholine receptors association in contextual fear memory. Therefore, since Ad-deficient mice do not present blood glucose increase after a stressful event due to a lack

of Ad stimulation, it would be interesting to administer Ad-deficient mice with glucose and sub-effective doses of glucose and Ad to evaluate the expression of muscarinic receptors in the hippocampus.

Few published studies have explored an association between increased Ad (Billewicz-Stankiewicz, et al., 1975, Górny, et al., 1975) and glucose (Ragozzino, et al., 1998, Ragozzino, et al., 1996) levels that could lead to an increased synthesis of acetylcholine in the central nervous system. After two systemic administrations of Ad at a dose capable of strengthening contextual fear memory (0.1 mg/kg) (Alves, et al., 2016, Oliveira, et al., 2018), the content of acetylcholine levels in the brain increased (Billewicz-Stankiewicz, et al., 1975, Górny, et al., 1975).

We used a pharmacological approach to modulate the activation of muscarinic receptors by acetylcholine. No differences were observed in freezing behaviour or shock responsiveness on the training day in WT and Ad-deficient treatment groups, which may indicate that pain perception of foot shocks is not affected by treatments. In this study, WT mice administered with atropine (peripheral and central muscarinic antagonist) showed a decrease in contextual fear memory compared to mice administered with vehicle. However, when WT mice were administered with methylatropine (peripheral muscarinic antagonist) contextual fear memory was not affected (Manuscript III – Chapter IV) which may indicate a central effect of acetylcholine in contextual fear memory. In Ad-deficient mice administered with Ad, it was observed contextual fear memory strengthening which was reverted by atropine administration (Manuscript III – Chapter IV). A possible explanation for these results is that the strengthening effect of peripheral Ad on contextual fear memory could be facilitated by the release of acetylcholine in the central nervous system. This is in agreement with literature observations suggesting the facilitatory effect of Ad in inhibitory avoidance (associative memory) and Y-maze discrimination (spatial memory). These results are reverted by atropine administration (Introini-Collison and McGaugh, 1988). Nevertheless, even though these studies are in agreement with the results obtained in this work, to our knowledge this is the first study correlating peripheral Ad and cholinergic mechanisms in the contextual fear memory paradigm. The contextual fear memory test, contrary to the inhibitory avoidance paradigm, allows the precise study of associative memory (Liang, 2009, Ögren and Stiedl, 2015).

Glucose breakdown may lead to enhanced levels of acetyl CoA, which is necessary for acetylcholine synthesis. This may comprise a possible explanation for the glucose's

strengthening effect on memory in Ad-deficient mice (Manuscript III – Chapter IV). It has been demonstrated that the efficacy of glucose in acetylcholine release modulation requires activated cholinergic neurons (Ragozzino, et al., 1998). Hence, it would be relevant to replicate this study using Ad-deficient mice administered with glucose, Ad, and vehicle submitted to the fear conditioning procedure and analyse acetylcholine content in the hippocampus using *in vivo* microdialysis. It will also be interesting to evaluate brain glucose uptake in Ad-deficient mice submitted or not to fear conditioning procedure using radiolabelled or fluorescent glucose.

When glucose is ingested, about 30% is absorbed by the liver to be stored as glycogen and 70% is disposed for other tissues under the stimulating influence of insulin (Messier, 2004). Blood glucose concentration increases in fear conditioning procedure (Manuscript I – Chapter II) (Oliveira, et al., 2018), and glucose may strength contextual fear memory (Manuscript II – Chapter III) (Oliveira, et al., 2023a). However, hyperglycaemia (as a consequence of Ad action or glucose administration) culminates, in normal situations, with insulin secretion from the pancreas. This makes it difficult to separate the effect of glucose from that of insulin in memory improvement. Intracerebroventricular injection of insulin seems to facilitate memory, namely in the passive-avoidance task (Park, et al., 2000), and also improves spatial memory (Haj-ali, et al., 2009). Furthermore, two previous studies have shown the ability of small doses of insulin to reverse the spatial memory amnesia produced by scopolamine (a muscarinic antagonist) i.p. injection (Blanchard and Duncan, 1997, Messier and Destrade, 1994). In our experimental conditions, one dose of insulin, capable of creating moderate hypoglycaemia, seemed to strengthen contextual fear memory when compared to WT mice administered with vehicle (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). This is in accordance with the outcomes previously described: (1) glucose also strengths contextual fear memory (Manuscript II – Chapter III); (2) activation of insulin receptors in the central nervous system may lead to the translocation of GLUT-4 containing vesicles to the cell surface, and this may enhance the brain's glucose uptake (El Messari, et al., 1998).

One obvious question that arises is that exogenous insulin injections reduce blood glucose and may lead to severe hypoglycaemia (when blood glucose decreases to values below 40 mg/dl (Languren, et al., 2013)), which is commonly associated with impaired memory (Kopf and Baratti, 1994, Kopf and Baratti, 1995, Santucci, et al., 1990). Nevertheless, in our experimental conditions, 2U/kg of insulin administration produced moderate hypoglycaemia in both Ad-deficient and WT mice. However, there is a close correlation

between blood glucose concentration attained during induced hypoglycaemia and a rise in plasma Ad (Christensen, et al., 1975). Plasma catecholamines quantification after the fear conditioning procedure showed an increase in plasma concentration of DA, NA, and Ad in insulin-administered WT mice compared with vehicle-administered WT mice (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). This outcome, together with the evidence that i.p. injections of Ad strengthen the retention of aversive contextual fear memory and that the insulin dosage used induced moderate hypoglycaemia, could be a possible explanation for the fear memory enhancement observed in the contextual fear test in insulin-administered WT mice, possibly due to an increase in cell glucose uptake. The observed increase in hippocampus DA and frontal cortex NA in insulin-administered WT mice compared with those administered with vehicle (Manuscript IV – Chapter V) (Oliveira, et al., 2023b) may be important for contextual fear memory enhancement and also for synaptic plasticity as shown by others (Andrés, et al., 1993, Hu, et al., 2007, Stubbendorff and Stevenson, 2020). Insulin decreases the NA uptake from synaptic cleft (Robertson, et al., 2010) and in long-term diabetes type 1 animal models (unable to synthesize insulin) showed decreased NA and DA in the cortex and hippocampus, respectively, which was followed by a reduced spatial and fear memory (de Souza, et al., 2019, Lin, et al., 2018). In addition, Ad may activate the vagus nerve leading to NA release in the amygdala and hippocampus (Chen and Williams, 2012). Those results may explain the contextual fear memory enhancement observed in insulin-administered WT mice, however, they are not sufficient to detach the effect of the two hormones.

Ad-deficient mice were used to separate Ad and insulin effects in contextual fear memory. Insulin-administered Ad-deficient mice demonstrated contextual fear memory strengthening compared to vehicle-administered Ad-deficient mice (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). Even in the absence of Ad, insulin promotes contextual fear memory formation. However, no differences were observed in plasma catecholamines between the two groups. Altogether, these results suggest a contextual fear memory-enhancing effect of insulin, which can be independent of the effects of increased plasma Ad.

In the insulin-administered Ad-deficient mice, hippocampal mRNA expression of *Bdnf* was increased compared to vehicle-administered Ad-deficient mice, suggesting an upregulation of this gene associated with insulin administration (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). This result is in agreement with the effect of *Bdnf* stimulation on GLUT3 overexpression (Burkhalter, et al., 2003), the major neuron glucose transporter

(Gerhart, et al., 1992, Maher and Simpson, 1994) since insulin can also regulate glucose uptake by promoting the translocation of Glut3 (Uemura and Greenlee, 2006). Moreover, insulin can promote Glut-4 translocation to the membrane of hippocampus cells. Inhibition of Glut-4 with Indinavir impaired memory in an inhibitory avoidance paradigm and reduced hippocampal BDNF expression (Pearson-Leary and McNay, 2016). Glut-4 is the only insulin-dependent glucose transporter in the brain and is mainly found in high-energy demanding neurons, like the hippocampus (El Messari, et al., 2002, El Messari, et al., 1998, McNay and Pearson-Leary, 2020). Therefore, the evaluation of GLUT3 and GLUT4 mRNA expression in the hippocampus could give interesting insights into this context. In conclusion, the results of this study indicate a possible role of insulin in fear behaviour and central molecular changes that promote contextual fear memory strengthening.

Considering the fundamental purpose of the present thesis, which aimed to investigate possible pathways by which a peripheral hormone such as Ad may strengthen contextual fear memory, our study focus was to evaluate contextual fear memory formation in a mouse model lacking endogenous Ad to accurately investigate Ad-related pathways and also hippocampal gene mRNA expression.

It was first demonstrated that Ad strengthens contextual fear memory, in both long-term and old memories, by acting in a β_2-adrenergic receptor, which may be related to higher glycaemic variation after fear acquisition day compared to those administered with vehicle (Manuscript I – Chapter II) (Oliveira, et al., 2018). To our knowledge, this was the first study to demonstrate Ad relevance in long-lasting memories. Furthermore, insulin appears to strengthen contextual fear memory independently of Ad (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). We also concluded that Ad and glucose may act in synergy to strengthen contextual fear memory (Manuscript II – Chapter III) (Oliveira, et al., 2023a). Furthermore, a non-selective muscarinic antagonist is capable of impairing contextual fear memory and revert contextual fear memory enhancement induced by Ad (Manuscript III – Chapter IV).

Regarding molecular changes in brain areas considered relevant for contextual fear memory, namely the hippocampus, we demonstrate that the *Nr4a* family gene and the downstream related gene, *Bdnf*, may have a relevant role in the contextual fear conditioning paradigm (Manuscript I – Chapter II) (Oliveira, et al., 2018), (Manuscript II – Chapter III) (Oliveira, et al., 2023a), and (Manuscript IV – Chapter V) (Oliveira, et al., 2023b). We show that the three members of the *Nr4a* family could be important for

contextual fear memory formation and specifically *Nr4a2* may be the most important related to the Ad effect in contextual fear memory strengthening (Manuscript I – Chapter II) (Oliveira, et al., 2018). Also, after glucose (Manuscript II – Chapter III) (Oliveira, et al., 2023a) or insulin (Manuscript IV – Chapter V) (Oliveira, et al., 2023b) administration hippocampus mRNA *Bdnf* expression increased, which may indicate that *Bdnf* may be relevant for glucose contextual fear memory enhancement. Therefore, the same pathway may be activated by insulin and glucose to enhance contextual fear memory, leading to the expression of the same molecular targets, since both situations contribute to increase the availability of glucose to the brain. However, insulin administration does not lead to differences in *Nr4a* mRNA expression (Manuscript IV – Chapter V) (Oliveira, et al., 2023b), contrary to Ad (Manuscript I – Chapter II) (Oliveira, et al., 2018) and glucose (Manuscript II – Chapter III) (Oliveira, et al., 2023a). Since *Nr4a* genes are immediate early genes vulnerable to expression a few hours after the stimulus and quickly recover to baseline levels, one argument is that analyses may have been carried outside of the detecting point for these genes (Morgan and Curran, 1989). The contextual fear memory impairment observed in Ad-deficient mice may be correlated to the down expression of hippocampal M_4 muscarinic receptor, which was reverted by systemic Ad administration (Manuscript III – Chapter IV).

In conclusion, the set of results presented in this work brings new insights into Ad's significance in contextual fear memory strengthening. A selective β_2-adrenoceptor antagonist is capable of reverting contextual fear memory strengthening in both long-term and old memories through the increase of *Nr4a* family genes in the hippocampus (Manuscript I – Chapter II). Furthermore, glucose (Manuscript II – chapter III) (Oliveira, et al., 2023a) appears to strengthen contextual fear memory through the increase in hippocampal *Bdnf* expression. Glucose may be involved in acetylcholine synthesis in the hippocampus, leading to muscarinic receptor activation and contextual fear memory strengthening (Manuscript III – chapter IV). Finally, insulin (Manuscript IV – chapter V) (Oliveira, et al., 2023b) appears to strengthen contextual fear memory through the increase in hippocampal *Bdnf* expression, which could be related to an increase in brain glucose uptake in the central nervous system.

REFERENCES

Abel, J.J., 1897. On the blood-pressure-raising constituent of the suprarenal capsule.

Agranoff, B.W., Davis, R.E. and Brink, J.J., 1965. Memory fixation in the goldfish. Proc Natl Acad Sci U S A. 54, 788-793. 10.1073/pnas.54.3.788.

Ahlquist, R.P., 1948. A study of the adrenotropic receptors. Am J Physiol. 153, 586-600. 10.1152/ajplegacy.1948.153.3.586.

Alberini, C.M., 2009. Transcription factors in long-term memory and synaptic plasticity. Physiological reviews. 89, 121-145. 10.1152/physrev.00017.2008.

Albuquerque, E.X., Pereira, E.F., Alkondon, M. and Rogers, S.W., 2009. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev. 89, 73-120. 10.1152/physrev.00015.2008.

Aldrich, T., 1901. A preliminary report on the active principle of the suprarenal gland. American Journal of Physiology-Legacy Content. 5, 457-461.

Alonso, M., Medina, J.H. and Pozzo-Miller, L., 2004. ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons. Learning & memory. 11, 172-178.

Alves, E., Lukoyanov, N., Serrao, P., Moura, D. and Moreira-Rodrigues, M., 2016. Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors. Psychopharmacology (Berl). 233, 2099-2108. 10.1007/s00213-016-4254-5.

Anagnostaras, S.G., Gale, G.D. and Fanselow, M.S., 2001. Hippocampus and contextual fear conditioning: recent controversies and advances. Hippocampus. 11, 8-17. 10.1002/1098-1063(2001)11:1<8::Aid-hipo1015>3.0.Co;2-7.

Anagnostaras, S.G., Maren, S. and Fanselow, M.S., 1999. Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. J Neurosci. 19, 1106-1114. 10.1523/jneurosci.19-03-01106.1999.

Andrés, M.E., Bustos, G. and Gysling, K., 1993. Regulation of [3H]norepinephrine release by N-methyl-D-aspartate receptors in minislices from the dentate gyrus and the CA1-CA3 area of the rat hippocampus. Biochem Pharmacol. 46, 1983-1987. 10.1016/0006-2952(93)90640-i.

Antoni, F.A., Holmes, M.C. and Jones, M.T., 1983. Oxytocin as well as vasopressin potentiate ovine CRF in vitro. Peptides. 4, 411-415. 10.1016/0196-9781(83)90041-4.

Apelt, J., Mehlhorn, G. and Schliebs, R., 1999. Insulin-sensitive GLUT4 glucose transporters are colocalized with GLUT3-expressing cells and demonstrate a chemically distinct neuron-specific localization in rat brain. J Neurosci Res. 57, 693-705.

Arch, J.R., Ainsworth, A.T., Cawthorne, M.A., Piercy, V., Sennitt, M.V., Thody, V.E., Wilson, C. and Wilson, S., 1984. Atypical beta-adrenoceptor on brown adipocytes as target for anti-obesity drugs. Nature. 309, 163-165. 10.1038/309163a0.

Aronson, J.K., 2000. Where name and image meet " — the argument for " adrenaline. Bmj. 320, 506-509.

Auchter, A.M., Shumake, J., Gonzalez-Lima, F. and Monfils, M.H., 2017. Preventing the return of fear using reconsolidation updating and methylene blue is

differentially dependent on extinction learning. Scientific Reports. 7, 46071. 10.1038/srep46071.

Axelrod, J., 1962. Purification and properties of phenylethanolamine-N-methyl transferase. J Biol Chem. 237, 1657-1660.

Axelrod, J. and Kopin, I.J., 1969. The uptake, storage, release and metabolism of noradrenaline in sympathetic nerves. Prog Brain Res. 31, 21-32. 10.1016/s0079-6123(08)63224-0.

Bao, X., Lu, C.M., Liu, F., Gu, Y., Dalton, N.D., Zhu, B.Q., Foster, E., Chen, J., Karliner, J.S., Ross, J., Jr., Simpson, P.C. and Ziegler, M.G., 2007. Epinephrine is required for normal cardiovascular responses to stress in the phenylethanolamine N-methyltransferase knockout mouse. Circulation. 116, 1024-1031. 10.1161/circulationaha.107.696005.

Barger, G. and Dale, H.H., 1910. Chemical structure and sympathomimetic action of amines. The Journal of physiology. 41, 19.

Beaulieu, S., Gagné, B. and Barden, N., 1988. Glucocorticoid regulation of proopiomelanocortin messenger ribonucleic acid content of rat hypothalamus. Mol Endocrinol. 2, 727-731. 10.1210/mend-2-8-727.

Benedict, C., Hallschmid, M., Hatke, A., Schultes, B., Fehm, H.L., Born, J. and Kern, W., 2004. Intranasal insulin improves memory in humans. Psychoneuroendocrinology. 29, 1326-1334.

Berger, I., Werdermann, M., Bornstein, S.R. and Steenblock, C., 2019. The adrenal gland in stress – Adaptation on a cellular level. The Journal of Steroid Biochemistry and Molecular Biology. 190, 198-206. 10.1016/j.jsbmb.2019.04.006.

Billewicz-Stankiewicz, J., Górny, D. and Zajaczkowska, M., 1975. The effects of adrenaline, reserpine, and atropine on acetylcholine content of the brain and peripheral ganglia in stress. Acta Physiol Pol. 26, 289-297.

Bisaz, R., Travaglia, A. and Alberini, C.M., 2014. The neurobiological bases of memory formation: from physiological conditions to psychopathology. Psychopathology. 47, 347-356. 10.1159/000363702.

Blanchard, J.G. and Duncan, P.M., 1997. Effect of combinations of insulin, glucose and scopolamine on radial arm maze performance. Pharmacol Biochem Behav. 58, 209-214. 10.1016/s0091-3057(97)00064-6.

Bonner, T., Buckley, N., Young, A. and Brann, M., 1987. Identification of a family of muscarinic acetylcholine receptor genes. Science. 237, 527-532.

Bonner, T.I., Young, A.C., Bran, M.R. and Buckley, N.J., 1988. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron. 1, 403-410.

Bookout, A.L., Jeong, Y., Downes, M., Ruth, T.Y., Evans, R.M. and Mangelsdorf, D.J., 2006. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 126, 789-799.

Borrell, J., de Kloet, E.R. and Bohus, B., 1984. Corticosterone decreases the efficacy of adrenaline to affect passive avoidance retention of adrenalectomized rats. Life Sci. 34, 99-104. 10.1016/0024-3205(84)90336-9.

Borrell, J., De Kloet, E.R., Versteeg, D.H. and Bohus, B., 1983. Inhibitory avoidance deficit following short-term adrenalectomy in the rat: the role of adrenal catecholamines. Behav Neural Biol. 39, 241-258. 10.1016/s0163-1047(83)90910-x.

Bracha, H.S., 2004. Freeze, flight, fight, fright, faint: adaptationist perspectives on the acute stress response spectrum. CNS Spectr. 9, 679-685.

Bramham, C.R. and Messaoudi, E., 2005. BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. Progress in Neurobiology. 76, 99-125. 10.1016/j.pneurobio.2005.06.003.

Bridi, M.S. and Abel, T., 2013. The NR4A orphan nuclear receptors mediate transcription-dependent hippocampal synaptic plasticity. Neurobiol Learn Mem. 105, 151-158. 10.1016/j.nlm.2013.06.020.

Brodde, O.E., 1991. Beta 1- and beta 2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. Pharmacol Rev. 43, 203-242.

Brodde, O.E., 1993. Beta-adrenoceptors in cardiac disease. Pharmacol Ther. 60, 405-430. 10.1016/0163-7258(93)90030-h.

Brown, A.M., Baltan Tekkok, S. and Ransom, B.R., 2004. Energy transfer from astrocytes to axons: the role of CNS glycogen. Neurochem Int. 45, 529-536. 10.1016/j.neuint.2003.11.005.

Bucci, M., Marques, S.S., Oh, D. and Harris, N.B., 2016. Toxic Stress in Children and Adolescents. Adv Pediatr. 63, 403-428. 10.1016/j.yapd.2016.04.002.

Buchanan, T.W., 2007. Retrieval of emotional memories. Psychol Bull. 133, 761-779. 10.1037/0033-2909.133.5.761.

Bühler, H.U., da Prada, M., Haefely, W. and Picotti, G.B., 1978. Plasma adrenaline, noradrenaline and dopamine in man and different animal species. J Physiol. 276, 311-320. 10.1113/jphysiol.1978.sp012235.

Burkhalter, J., Fiumelli, H., Allaman, I., Chatton, J.Y. and Martin, J.L., 2003. Brain-derived neurotrophic factor stimulates energy metabolism in developing cortical neurons. J Neurosci. 23, 8212-8220. 10.1523/jneurosci.23-23-08212.2003.

Bylund, D.B. and Bylund, K.C., 2014. Norepinephrine. Oxford. 614-616. 10.1016/B978-0-12-385157-4.00047-6.

Cahill, A.L., Eertmoed, A.L., Mangoura, D. and Perlman, R.L., 1996. Differential regulation of phenylethanolamine N-methyltransferase expression in two distinct subpopulations of bovine chromaffin cells. J Neurochem. 67, 1217-1224. 10.1046/j.1471-4159.1996.67031217.x.

Cahill, L. and McGaugh, J.L., 1990. Amygdaloid complex lesions differentially affect retention of tasks using appetitive and aversive reinforcement. Behav Neurosci. 104, 532-543. 10.1037//0735-7044.104.4.532.

Cangioli, I., Baldi, E., Mannaioni, P.F., Bucherelli, C., Blandina, P. and Passani, M.B., 2002. Activation of histaminergic H3 receptors in the rat basolateral amygdala improves expression of fear memory and enhances acetylcholine release. Eur J Neurosci. 16, 521-528. 10.1046/j.1460-9568.2002.02092.x.

Cannon, W.B., 1915. Bodily changes in pain, hunger, fear and rage: An account of recent researches into the function of emotional excitement. New York, NY, US. xiii, 311-xiii, 311. 10.1037/10013-000.

Cannon, W.B. and Lissák, K., 1939. EVIDENCE FOR ADRENALINE IN ADRENERGIC NEURONES. American Journal of Physiology-Legacy Content. 125, 765-777. 10.1152/ajplegacy.1939.125.4.765.

Carey, R.M., 2013. The intrarenal renin-angiotensin and dopaminergic systems: control of renal sodium excretion and blood pressure. Hypertension. 61, 673-680. 10.1161/hypertensionaha.111.00241.

Carlsson, A., 1959. The occurrence, distribution and physiological role of catecholamines in the nervous system. Pharmacol Rev. 11, 490-493.

Carlsson, A., Lindqvist, M., Magnusson, T. and Waldeck, B., 1958. On the presence of 3-hydroxytyramine in brain. Science. 127, 471-471.

Chaaya, N., Battle, A.R. and Johnson, L.R., 2018. An update on contextual fear memory mechanisms: Transition between Amygdala and Hippocampus. Neurosci Biobehav Rev. 92, 43-54. 10.1016/j.neubiorev.2018.05.013.

Chen, C.C. and Williams, C.L., 2012. Interactions between epinephrine, ascending vagal fibers, and central noradrenergic systems in modulating memory for emotionally arousing events. Front Behav Neurosci. 6, 35. 10.3389/fnbeh.2012.00035.

Christensen, N.J., Alberti, K.G. and Brandsborg, O., 1975. Plasma catecholamines and blood substrate concentrations: studies in insulin induced hypoglycaemia and after adrenaline infusions. Eur J Clin Invest. 5, 415-423. 10.1111/j.1365-2362.1975.tb00473.x.

Chruscinski, A.J., Rohrer, D.K., Schauble, E., Desai, K.H., Bernstein, D. and Kobilka, B.K., 1999. Targeted disruption of the beta2 adrenergic receptor gene. J Biol Chem. 274, 16694-16700. 10.1074/jbc.274.24.16694.

Chugh, G., Pokkunuri, I. and Asghar, M., 2013. Renal dopamine and angiotensin II receptor signaling in age-related hypertension. Am J Physiol Renal Physiol. 304, F1-7. 10.1152/ajprenal.00441.2012.

Clark, K.B., Krahl, S.E., Smith, D.C. and Jensen, R.A., 1995. Post-training unilateral vagal stimulation enhances retention performance in the rat. Neurobiol Learn Mem. 63, 213-216. 10.1006/nlme.1995.1024.

Clark, K.B., Naritoku, D.K., Smith, D.C., Browning, R.A. and Jensen, R.A., 1999. Enhanced recognition memory following vagus nerve stimulation in human subjects. Nat Neurosci. 2, 94-98. 10.1038/4600.

Clark, K.B., Smith, D.C., Hassert, D.L., Browning, R.A., Naritoku, D.K. and Jensen, R.A., 1998. Posttraining electrical stimulation of vagal afferents with concomitant vagal efferent inactivation enhances memory storage processes in the rat. Neurobiol Learn Mem. 70, 364-373. 10.1006/nlme.1998.3863.

Clayton, E.C. and Williams, C.L., 2000. Noradrenergic receptor blockade of the NTS attenuates the mnemonic effects of epinephrine in an appetitive light-dark discrimination learning task. Neurobiol Learn Mem. 74, 135-145. 10.1006/nlme.1999.3946.

Coker, R.H., Krishna, M.G., Lacy, D.B., Bracy, D.P. and Wasserman, D.H., 1997. Role of hepatic α- and β-adrenergic receptor stimulation on hepatic glucose production during heavy exercise. American Journal of Physiology-Endocrinology and Metabolism. 273, E831-E838. 10.1152/ajpendo.1997.273.5.E831.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K. and Schütz, G., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608-1621. 10.1101/gad.9.13.1608.

Colucci, P., Santori, A., Romanelli, L., Zwergel, C., Mai, A., Scaccianoce, S. and Campolongo, P., 2021. Amphetamine Modulation of Long-Term Object Recognition Memory in Rats: Influence of Stress. Frontiers in Pharmacology. 12, 10.3389/fphar.2021.644521.

Cornford, E.M., Hyman, S. and Swartz, B.E., 1994. The human brain GLUT1 glucose transporter: ultrastructural localization to the blood-brain barrier endothelia. J Cereb Blood Flow Metab. 14, 106-112. 10.1038/jcbfm.1994.15.

Costa, M., Lozano-Soldevilla, D., Gil-Nagel, A., Toledano, R., Oehrn, C.R., Kunz, L., Yebra, M., Mendez-Bertolo, C., Stieglitz, L., Sarnthein, J., Axmacher, N., Moratti, S. and Strange, B.A., 2022. Aversive memory formation in humans involves an amygdala-hippocampus phase code. Nature Communications. 13, 6403. 10.1038/s41467-022-33828-2.

Costa, V., Carvalho, F., Lourdes Bastos, M., Carvalho, R., Carvalho, M. and Remião, F., 2012. Adrenaline and Noradrenaline: Partners and Actors in the Same Play. 10.5772/36070.

Cotecchia, S., Kobilka, B.K., Daniel, K.W., Nolan, R.D., Lapetina, E.Y., Caron, M.G., Lefkowitz, R.J. and Regan, J.W., 1990. Multiple second messenger pathways of alpha-adrenergic receptor subtypes expressed in eukaryotic cells. J Biol Chem. 265, 63-69.

Cowan, N. and AuBuchon, A.M., 2008. Short-term memory loss over time without retroactive stimulus interference. Psychonomic Bulletin & Review. 15, 230-235.

Dampney, R.A., 1994. Functional organization of central pathways regulating the cardiovascular system. Physiol Rev. 74, 323-364. 10.1152/physrev.1994.74.2.323.

Davis, H.P. and Squire, L.R., 1984. Protein synthesis and memory: a review. Psychol Bull. 96, 518-559.

Dayas, C.V., Buller, K.M., Crane, J.W., Xu, Y. and Day, T.A., 2001. Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. Eur J Neurosci. 14, 1143-1152. 10.1046/j.0953-816x.2001.01733.x.

de Almeida, M.A., Kapczinski, F.P. and Izquierdo, I., 1983. Memory modulation by post-training intraperitoneal, but not intracerebroventricular, administration of ACTH or epinephrine. Behav Neural Biol. 39, 277-283. 10.1016/s0163-1047(83)90961-5.

de Araujo, I.E., 2014. Contextual fear conditioning: connecting brain glucose sensing and hippocampal-dependent memories. Biol Psychiatry. 75, 834-835. 10.1016/j.biopsych.2014.03.024.

de Souza, C.P., Gambeta, E., Stern, C.A.J. and Zanoveli, J.M., 2019. Posttraumatic stress disorder-type behaviors in streptozotocin-induced diabetic rats can be prevented by prolonged treatment with vitamin E. Behavioural Brain Research. 359, 749-754. 10.1016/j.bbr.2018.09.008.

Delahanty, D.L., Nugent, N.R., Christopher, N.C. and Walsh, M., 2005. Initial urinary epinephrine and cortisol levels predict acute PTSD symptoms in child trauma victims. Psychoneuroendocrinology. 30, 121-128. 10.1016/j.psyneuen.2004.06.004.

Derner, M., Dehnen, G., Chaieb, L., Reber, T.P., Borger, V., Surges, R., Staresina, B.P., Mormann, F. and Fell, J., 2020. Patterns of single-neuron activity during associative recognition memory in the human medial temporal lobe. NeuroImage. 221, 117214. 10.1016/j.neuroimage.2020.117214.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G., Lefkowitz, R.J. and Strader, C.D., 1986. Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature. 321, 75-79. 10.1038/321075a0.

Dobrogowska, D.H. and Vorbrodt, A.W., 1999. Quantitative immunocytochemical study of blood-brain barrier glucose transporter (GLUT-1) in four regions of mouse brain. J Histochem Cytochem. 47, 1021-1030. 10.1177/002215549904700806.

Dudai, Y., 2002. Molecular bases of long-term memories: a question of persistence. Curr Opin Neurobiol. 12, 211-216. 10.1016/s0959-4388(02)00305-7.

Dugich-Djordjevic, M.M., Peterson, C., Isono, F., Ohsawa, F., Widmer, H.R., Denton, T.L., Bennett, G.L. and Hefti, F., 1995. Immunohistochemical visualization of brain-derived neurotrophic factor in the rat brain. Eur J Neurosci. 7, 1831-1839. 10.1111/j.1460-9568.1995.tb00703.x.

Durkin, T.P., Messier, C., de Boer, P. and Westerink, B.H., 1992. Raised glucose levels enhance scopolamine-induced acetylcholine overflow from the hippocampus: an in vivo microdialysis study in the rat. Behav Brain Res. 49, 181-188. 10.1016/s0166-4328(05)80163-9.

Ebert, S.N., Rong, Q., Boe, S., Thompson, R.P., Grinberg, A. and Pfeifer, K., 2004. Targeted insertion of the Cre-recombinase gene at the phenylethanolamine n-methyltransferase locus: a new model for studying the developmental distribution of adrenergic cells. Dev Dyn. 231, 849-858. 10.1002/dvdy.20188.

Ehrhart-Bornstein, M. and Bornstein, S.R., 2008. Cross-talk between adrenal medulla and adrenal cortex in stress. Annals of the New York Academy of Sciences. 1148, 112-117.

El Messari, S., Aït-Ikhlef, A., Ambroise, D.H., Penicaud, L. and Arluison, M., 2002. Expression of insulin-responsive glucose transporter GLUT4 mRNA in the rat brain and spinal cord: an in situ hybridization study. J Chem Neuroanat. 24, 225-242. 10.1016/s0891-0618(02)00058-3.

El Messari, S., Leloup, C., Quignon, M., Brisorgueil, M.J., Penicaud, L. and Arluison, M., 1998. Immunocytochemical localization of the insulin-responsive glucose transporter 4 (Glut4) in the rat central nervous system. J Comp Neurol. 399, 492-512. 10.1002/(sici)1096-9861(19981005)399:4<492::aid-cne4>3.0.co;2-x.

Elgoyhen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E. and Heinemann, S., 1994. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell. 79, 705-715. 10.1016/0092-8674(94)90555-x.

Emorine, L.J., Marullo, S., Briend-Sutren, M.-M., Patey, G., Tate, K., Delavier-Klutchko, C. and Strosberg, A.D., 1989. Molecular characterization of the human β3-adrenergic receptor. Science. 245, 1118-1121.

Euler, U.v., 1946. A specific sympathomimetic ergone in adrenergic nerve fibres (sympathin) and its relations to adrenaline and nor-adrenaline. Acta physiologica scandinavica. 12, 73-97.

Fahim, M., 2003. Cardiovascular sensory receptors and their regulatory mechanisms. Indian J Physiol Pharmacol. 47, 124-146.

Falk, S., Lund, C. and Clemmensen, C., 2020. Muscarinic receptors in energy homeostasis: Physiology and pharmacology. Basic & Clinical Pharmacology & Toxicology. 126, 66-76. 10.1111/bcpt.13311.

Fallon, J.H., Koziell, D.A. and Moore, R.Y., 1978. Catecholamine innervation of the basal forebrain. II. Amygdala, suprarhinal cortex and entorhinal cortex. J Comp Neurol. 180, 509-532. 10.1002/cne.901800308.

Fallon, J.H. and Moore, R.Y., 1978. Catecholamine innervation of the basal forebrain. IV. Topography of the dopamine projection to the basal forebrain and neostriatum. J Comp Neurol. 180, 545-580. 10.1002/cne.901800310.

Farrell, C.L. and Pardridge, W.M., 1991. Blood-brain barrier glucose transporter is asymmetrically distributed on brain capillary endothelial lumenal and ablumenal membranes: an electron microscopic immunogold study. Proceedings of the National Academy of Sciences. 88, 5779-5783.

Felder, C.C., 1995. Muscarinic acetylcholine receptors: signal transduction through multiple effectors. The FASEB Journal. 9, 619-625.

Fenoglio, K.A., Brunson, K.L. and Baram, T.Z., 2006. Hippocampal neuroplasticity induced by early-life stress: functional and molecular aspects. Front Neuroendocrinol. 27, 180-192. 10.1016/j.yfrne.2006.02.001.

Filosa, J.A., Bonev, A.D., Straub, S.V., Meredith, A.L., Wilkerson, M.K., Aldrich, R.W. and Nelson, M.T., 2006. Local potassium signaling couples neuronal activity to vasodilation in the brain. Nat Neurosci. 9, 1397-1403. 10.1038/nn1779.

Fisher, S.J., Brüning, J.C., Lannon, S. and Kahn, C.R., 2005. Insulin Signaling in the Central Nervous System Is Critical for the Normal Sympathoadrenal Response to Hypoglycemia. Diabetes. 54, 1447-1451. 10.2337/diabetes.54.5.1447.

Flood, J.F. and Morley, J.E., 1988. Effects of bombesin and gastrin-releasing peptide on memory processing. Brain Res. 460, 314-322. 10.1016/0006-8993(88)90375-7.

Flynn, D.D., Ferrari-DiLeo, G., Mash, D.C. and Levey, A.I., 1995. Differential regulation of molecular subtypes of muscarinic receptors in Alzheimer's disease. J Neurochem. 64, 1888-1891. 10.1046/j.1471-4159.1995.64041888.x.

Frankland, P.W., Josselyn, S.A., Anagnostaras, S.G., Kogan, J.H., Takahashi, E. and Silva, A.J., 2004. Consolidation of CS and US representations in associative fear conditioning. Hippocampus. 14, 557-569. 10.1002/hipo.10208.

Frey, U., Frey, S., Schollmeier, F. and Krug, M., 1996. Influence of actinomycin D, a RNA synthesis inhibitor, on long-term potentiation in rat hippocampal neurons in

vivo and in vitro. The Journal of Physiology. 490, 703-711. 10.1113/jphysiol.1996.sp021179.

Frielle, T., Caron, M.G. and Lefkowitz, R.J., 1989. Properties of the beta 1- and beta 2-adrenergic receptor subtypes revealed by molecular cloning. Clin Chem. 35, 721-725.

Fuchs, S.A., Edinger, H.M. and Siegel, A., 1985. The organization of the hypothalamic pathways mediating affective defense behavior in the cat. Brain Res. 330, 77-92. 10.1016/0006-8993(85)90009-5.

Furnival, C.M., Linden, R.J. and Snow, H.M., 1971. The inotropic and chronotropic effects of catecholamines on the dog heart. J Physiol. 214, 15-28. 10.1113/jphysiol.1971.sp009416.

Galbo, H., Holst, J. and Christensen, N., 1979. The effect of different diets and of insulin on the hormonal response to prolonged exercise. Acta Physiologica Scandinavica. 107, 19-32.

Garfinkel, S.N. and Critchley, H.D., 2016. Threat and the Body: How the Heart Supports Fear Processing. Trends Cogn Sci. 20, 34-46. 10.1016/j.tics.2015.10.005.

Gerhart, D.Z., Broderius, M.A., Borson, N.D. and Drewes, L.R., 1992. Neurons and microvessels express the brain glucose transporter protein GLUT3. Proc Natl Acad Sci U S A. 89, 733-737. 10.1073/pnas.89.2.733.

Gianaros, P.J., Onyewuenyi, I.C., Sheu, L.K., Christie, I.C. and Critchley, H.D., 2012. Brain systems for baroreflex suppression during stress in humans. Hum Brain Mapp. 33, 1700-1716. 10.1002/hbm.21315.

Gibbs, D.M., Stewart, R.D., Vale, W., Rivier, J. and Yen, S.S., 1983. Synthetic corticotropin-releasing factor stimulates secretion of immunoreactive beta-endorphin/beta-lipotropin and ACTH by human fetal pituitaries in vitro. Life Sci. 32, 547-550. 10.1016/0024-3205(83)90150-9.

Gibbs, M.E. and Ng, K.T., 1976. Memory formation: A new three-phase model. Neuroscience Letters. 2, 165-169.

Gibbs, M.E. and Ng, K.T., 1977. Psychobiology of memory: Towards a model of memory formation. Biobehavioral Reviews. 1, 113-136.

Gibson, G.E. and Blass, J.P., 1976. Impaired synthesis of acetylcholine in brain accompanying mild hypoxia and hypoglycemia. J Neurochem. 27, 37-42. 10.1111/j.1471-4159.1976.tb01540.x.

Gibson, G.E., Blass, J.P. and Jenden, D.J., 1978. Measurement of acetylcholine turnover with glucose used as precursor: evidence for compartmentation of glucose metabolism in brain. J Neurochem. 30, 71-76. 10.1111/j.1471-4159.1978.tb07036.x.

Gibson, G.E. and Shimada, M., 1980. Studies on the metabolic pathway of the acetyl group for acetylcholine synthesis. Biochem Pharmacol. 29, 167-174. 10.1016/0006-2952(80)90325-1.

Gilmartin, M.R., Balderston, N.L. and Helmstetter, F.J., 2014. Prefrontal cortical regulation of fear learning. Trends Neurosci. 37, 455-464. 10.1016/j.tins.2014.05.004.

Godoy, L.D., Rossignoli, M.T., Delfino-Pereira, P., Garcia-Cairasco, N. and de Lima Umeoka, E.H., 2018. A Comprehensive Overview on Stress Neurobiology: Basic

Concepts and Clinical Implications. Frontiers in Behavioral Neuroscience. 12, 10.3389/fnbeh.2018.00127.

Gold, P.E., 1986. Glucose modulation of memory storage processing. Behavioral and Neural Biology. 45, 342-349. 10.1016/S0163-1047(86)80022-X.

Gold, P.E., 2014. Regulation of memory - from the adrenal medulla to liver to astrocytes to neurons. Brain research bulletin. 105, 25-35. 10.1016/j.brainresbull.2013.12.012.

Gold, P.E. and van Buskirk, R., 1978a. Effects of α- and β-adrenergic receptor antagonists on post-trial epinephrine modulation of memory: Relationship to post-training brain norepinephrine concentrations. Behavioral Biology. 24, 168-184. 10.1016/S0091-6773(78)93045-6.

Gold, P.E. and van Buskirk, R., 1978b. Posttraining brain norepinephrine concentrations: correlation with retention performance of avoidance training and with peripheral epinephrine modulation of memory processing. Behav Biol. 23, 509-520. 10.1016/s0091-6773(78)91614-0.

Gold, P.E. and Van Buskirk, R.B., 1975. Facilitation of time-dependent memory processes with posttrial epinephrine injections. Behav Biol. 13, 145-153. 10.1016/S0091-6773(75)91784-8.

Gold, P.E., Vogt, J. and Hall, J.L., 1986. Glucose effects on memory: behavioral and pharmacological characteristics. Behav Neural Biol. 46, 145-155. 10.1016/s0163-1047(86)90626-6.

Goldstein, D.S., 2010. Adrenaline and Noradrenaline. 10.1002/9780470015902.a0001401.pub2.

Goldstein, D.S., Eisenhofer, G. and Kopin, I.J., 2003. Sources and significance of plasma levels of catechols and their metabolites in humans. J Pharmacol Exp Ther. 305, 800-811. 10.1124/jpet.103.049270.

Goodchild, A.K., Moon, E.A., Dampney, R.A.L. and Howe, P.R.C., 1984. Evidence that adrenaline neurons in the rostral ventrolateral medulla have a vasopressor function. Neuroscience Letters. 45, 267-272. 10.1016/0304-3940(84)90237-4.

Goosens, K.A. and Maren, S., 2001. Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. Learn Mem. 8, 148-155. 10.1101/lm.37601.

Górny, D., Billewicz-Stankiewicz, J., Zajaczkowska, M. and Kutarski, A., 1975. The effect of adrenaline on acetylcholine synthesis, choline acetylase and cholinesterase activity. Acta Physiol Pol. 26, 45-54.

Gorski, J.A., Balogh, S.A., Wehner, J.M. and Jones, K.R., 2003. Learning deficits in forebrain-restricted brain-derived neurotrophic factor mutant mice. Neuroscience. 121, 341-354. 10.1016/s0306-4522(03)00426-3.

Gray, M.A., Rylander, K., Harrison, N.A., Wallin, B.G. and Critchley, H.D., 2009. Following One's Heart: Cardiac Rhythms Gate Central Initiation of Sympathetic Reflexes. The Journal of Neuroscience. 29, 1817-1825. 10.1523/jneurosci.3363-08.2009.

Grillon, C., Cordova, J., Morgan, C.A., Charney, D.S. and Davis, M., 2004. Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. Psychopharmacology. 175, 342-352.

Guzowski, J.F., 2002. Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. Hippocampus. 12, 86-104. 10.1002/hipo.10010.

Gyires, K., Zádori, Z.S., Török, T. and Mátyus, P., 2009. alpha(2)-Adrenoceptor subtypes-mediated physiological, pharmacological actions. Neurochem Int. 55, 447-453. 10.1016/j.neuint.2009.05.014.

Habgood, M.D., Bye, N., Dziegielewska, K.M., Ek, C.J., Lane, M.A., Potter, A., Morganti-Kossmann, C. and Saunders, N.R., 2007. Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. Eur J Neurosci. 25, 231-238. 10.1111/j.1460-9568.2006.05275.x.

Haj-ali, V., Mohaddes, G. and Babri, S.H., 2009. Intracerebroventricular insulin improves spatial learning and memory in male Wistar rats. Behav Neurosci. 123, 1309-1314. 10.1037/a0017722.

Hall, J., Thomas, K.L. and Everitt, B.J., 2000. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. Nat Neurosci. 3, 533-535. 10.1038/75698.

Hall, J.L. and Gold, P.E., 1986. The effects of training, epinephrine, and glucose injections on plasma glucose levels in rats. Behav Neural Biol. 46, 156-167. 10.1016/s0163-1047(86)90640-0.

Hassert, D.L., Miyashita, T. and Williams, C.L., 2004. The effects of peripheral vagal nerve stimulation at a memory-modulating intensity on norepinephrine output in the basolateral amygdala. Behav Neurosci. 118, 79-88. 10.1037/0735-7044.118.1.79.

Hawk, J.D. and Abel, T., 2011. The role of NR4A transcription factors in memory formation. Brain Research Bulletin. 85, 21-29. 10.1016/j.brainresbull.2011.02.001.

Hawk, J.D., Bookout, A.L., Poplawski, S.G., Bridi, M., Rao, A.J., Sulewski, M.E., Kroener, B.T., Manglesdorf, D.J. and Abel, T., 2012. NR4A nuclear receptors support memory enhancement by histone deacetylase inhibitors. J Clin Invest. 122, 3593-3602. 10.1172/jci64145.

Hazel, T.G., Nathans, D. and Lau, L.F., 1988. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. Proc Natl Acad Sci U S A. 85, 8444-8448. 10.1073/pnas.85.22.8444.

Heldt, S.A., Stanek, L., Chhatwal, J.P. and Ressler, K.J., 2007. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. Mol Psychiatry. 12, 656-670. 10.1038/sj.mp.4001957.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C. and Cullinan, W.E., 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol. 24, 151-180. 10.1016/j.yfrne.2003.07.001.

Hodel, A., 2001. Effects of Glucocorticoids on Adrenal Chromaffin Cells. Journal of Neuroendocrinology. 13, 216-220. 10.1111/j.1365-2826.2001.00628.x.

Hofer, M., Pagliusi, S.R., Hohn, A., Leibrock, J. and Barde, Y.A., 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. Embo j. 9, 2459-2464. 10.1002/j.1460-2075.1990.tb07423.x.

Hoffman, B.B. and Lefkowitz, R.J., 1980. Alpha-adrenergic receptor subtypes. N Engl J Med. 302, 1390-1396. 10.1056/nejm198006193022504.

Horch, H.W. and Katz, L.C., 2002. BDNF release from single cells elicits local dendritic growth in nearby neurons. Nature Neuroscience. 5, 1177-1184. 10.1038/nn927.

Horikoshi, Y., Sasaki, A., Taguchi, N., Maeda, M., Tsukagoshi, H., Sato, K. and Yamaguchi, H., 2003. Human GLUT5 immunolabeling is useful for evaluating microglial status in neuropathological study using paraffin sections. Acta Neuropathol. 105, 157-162. 10.1007/s00401-002-0627-4.

Horita, S., Seki, G., Yamada, H., Suzuki, M., Koike, K. and Fujita, T., 2013. Roles of renal proximal tubule transport in the pathogenesis of hypertension. Current hypertension reviews. 9, 148-155.

Hornykiewicz, O., 2002. Dopamine miracle: from brain homogenate to dopamine replacement. Movement disorders: official journal of the Movement Disorder Society. 17, 501-508.

Hu, H., Real, E., Takamiya, K., Kang, M.-G., Ledoux, J., Huganir, R.L. and Malinow, R., 2007. Emotion enhances learning via norepinephrine regulation of AMPA-receptor trafficking. Cell. 131, 160-173.

Introini-Collison, I.B. and Baratti, C.M., 1992. Memory-modulatory effects of centrally acting noradrenergic drugs: possible involvement of brain cholinergic mechanisms. Behav Neural Biol. 57, 248-255.

Introini-Collison, I.B. and McGaugh, J.L., 1986. Epinephrine modulates long-term retention of an aversively motivated discrimination. Behav Neural Biol. 45, 358-365. 10.1016/s0163-1047(86)80024-3.

Introini-Collison, I.B. and McGaugh, J.L., 1988. Modulation of memory by post-training epinephrine: involvement of cholinergic mechanisms. Psychopharmacology. 94, 379-385. 10.1007/bf00174693.

Isackson, P.J., Huntsman, M.M., Murray, K.D. and Gall, C.M., 1991. BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. Neuron. 6, 937-948. 10.1016/0896-6273(91)90234-q.

Izquierdo, I., Furini, C.R. and Myskiw, J.C., 2016. Fear Memory. Physiol Rev. 96, 695-750. 10.1152/physrev.00018.2015.

Izquierdo, L.A., Barros, D.M., Vianna, M.R.M., Coitinho, A., de Silva, T.D., Choi, H., Moletta, B., Medina, J.H. and Izquierdo, I., 2002. Molecular Pharmacological Dissection of Short- and Long-Term Memory. Cellular and Molecular Neurobiology. 22, 269-287. 10.1023/A:1020715800956.

Joëls, M. and Baram, T.Z., 2009. The neuro-symphony of stress. Nat Rev Neurosci. 10, 459-466. 10.1038/nrn2632.

Karni, A., Meyer, G., Rey-Hipolito, C., Jezzard, P., Adams, M.M., Turner, R. and Ungerleider, L.G., 1998. The acquisition of skilled motor performance: fast and slow experience-driven changes in primary motor cortex. Proceedings of the National Academy of Sciences. 95, 861-868.

Katoh-Semba, R., Asano, T., Ueda, H., Morishita, R., Takeuchi, I.K., Inaguma, Y. and Kato, K., 2002. Riluzole enhances expression of brain-derived neurotrophic factor

with consequent proliferation of granule precursor cells in the rat hippocampus. Faseb j. 16, 1328-1330. 10.1096/fj.02-0143fje.

Kaufman, D.L., Houser, C.R. and Tobin, A.J., 1991. Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem. 56, 720-723. 10.1111/j.1471-4159.1991.tb08211.x.

Kawamoto, Y., Nakamura, S., Nakano, S., Oka, N., Akiguchi, I. and Kimura, J., 1996. Immunohistochemical localization of brain-derived neurotrophic factor in adult rat brain. Neuroscience. 74, 1209-1226. 10.1016/0306-4522(96)00245-X.

Kennedy, B., Bigby, T.D. and Ziegler, M.G., 1995. Nonadrenal epinephrine-forming enzymes in humans. Characteristics, distribution, regulation, and relationship to epinephrine levels. J Clin Invest. 95, 2896-2902. 10.1172/jci117996.

Kilts, C.D. and Anderson, C.M., 1986. The simultaneous quantification of dopamine, norepinephrine and epinephrine in micropunched rat brain nuclei by on-line trace enrichment HPLC with electrochemical detection: Distribution of catecholamines in the limbic system. Neurochem Int. 9, 437-445. 10.1016/0197-0186(86)90086-0.

Kim, S. and Kaang, B.-K., 2017. Epigenetic regulation and chromatin remodeling in learning and memory. Experimental & Molecular Medicine. 49, e281-e281. 10.1038/emm.2016.140.

King Ii, S.O. and Williams, C.L., 2009. Novelty-induced arousal enhances memory for cued classical fear conditioning: Interactions between peripheral adrenergic and brainstem glutamatergic systems. Learning & Memory. 16, 625-634. 10.1101/lm.1513109.

Klein, M.O., Battagello, D.S., Cardoso, A.R., Hauser, D.N., Bittencourt, J.C. and Correa, R.G., 2019. Dopamine: Functions, Signaling, and Association with Neurological Diseases. Cell Mol Neurobiol. 39, 31-59. 10.1007/s10571-018-0632-3.

Kobrzycka, A., Napora, P., Pearson, B.L., Pierzchała-Koziec, K., Szewczyk, R. and Wieczorek, M., 2019. Peripheral and central compensatory mechanisms for impaired vagus nerve function during peripheral immune activation. Journal of Neuroinflammation. 16, 150. 10.1186/s12974-019-1544-y.

Koepsell, H., 2020. Glucose transporters in brain in health and disease. Pflugers Arch. 472, 1299-1343. 10.1007/s00424-020-02441-x.

Kong, L., Zhao, Y., Zhou, W.-J., Yu, H., Teng, S.-W., Guo, Q., Chen, Z. and Wang, Y., 2017. Direct Neuronal Glucose Uptake Is Required for Contextual Fear Acquisition in the Dorsal Hippocampus. Frontiers in Molecular Neuroscience. 10, 10.3389/fnmol.2017.00388.

Kopf, S.R. and Baratti, C.M., 1994. Memory-improving actions of glucose: involvement of a central cholinergic muscarinic mechanism. Behav Neural Biol. 62, 237-243.

Kopf, S.R. and Baratti, C.M., 1995. The impairment of retention induced by insulin in mice may be mediated by a reduction in central cholinergic activity. Neurobiol Learn Mem. 63, 220-228. 10.1006/nlme.1995.1026.

Kopf, S.R., Buchholzer, M.L., Hilgert, M., Löffelholz, K. and Klein, J., 2001. Glucose plus choline improve passive avoidance behaviour and increase hippocampal

acetylcholine release in mice. Neuroscience. 103, 365-371. 10.1016/s0306-4522(01)00007-0.

Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A. and Kangawa, K., 1986a. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. Nature. 323, 411-416.

Kubo, T., Maeda, A., Sugimoto, K., Akiba, I., Mikami, A., Takahashi, H., Haga, T., Haga, K., Ichiyama, A. and Kangawa, K., 1986b. Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. FEBS letters. 209, 367-372.

Kvetnansky, R., Lu, X. and Ziegler, M.G., 2013. Stress-triggered changes in peripheral catecholaminergic systems. Adv Pharmacol. 68, 359-397. 10.1016/b978-0-12-411512-5.00017-8.

Kvetnansky, R., Sabban, E.L. and Palkovits, M., 2009. Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiol Rev. 89, 535-606. 10.1152/physrev.00042.2006.

LaBar, K.S. and Cabeza, R., 2006. Cognitive neuroscience of emotional memory. Nature Reviews Neuroscience. 7, 54-64. 10.1038/nrn1825.

Lafontan, M., Barbe, P., Galitzky, J., Tavernier, G., Langin, D., Carpéné, C., Bousquet-Melou, A. and Berlan, M., 1997. Adrenergic regulation of adipocyte metabolism. Hum Reprod. 12 Suppl 1, 6-20. 10.1093/humrep/12.suppl_1.6.

Langer, S.Z., 1974. Presynaptic regulation of catecholamine release. Biochem Pharmacol. 23, 1793-1800. 10.1016/0006-2952(74)90187-7.

Langer, S.Z., 1997. 25 years since the discovery of presynaptic receptors: present knowledge and future perspectives. Trends Pharmacol Sci. 18, 95-99. 10.1016/s0165-6147(96)01034-6.

Languren, G., Montiel, T., Julio-Amilpas, A. and Massieu, L., 2013. Neuronal damage and cognitive impairment associated with hypoglycemia: An integrated view. Neurochemistry International. 63, 331-343. 10.1016/j.neuint.2013.06.018.

Lauterborn, J.C., Poulsen, F.R., Stinis, C.T., Isackson, P.J. and Gall, C.M., 1998. Transcript-specific effects of adrenalectomy on seizure-induced BDNF expression in rat hippocampus. Brain Res Mol Brain Res. 55, 81-91. 10.1016/s0169-328x(97)00368-9.

Law, S.W., Conneely, O.M., DeMayo, F.J. and O'Malley, B.W., 1992. Identification of a new brain-specific transcription factor, NURR1. Mol Endocrinol. 6, 2129-2135. 10.1210/mend.6.12.1491694.

Lawrence, A.J., Watkins, D. and Jarrott, B., 1995. Visualization of beta-adrenoceptor binding sites on human inferior vagal ganglia and their axonal transport along the rat vagus nerve. J Hypertens. 13, 631-635. 10.1097/00004872-199506000-00009.

Lechner, H.A., Squire, L.R. and Byrne, J.H., 1999. 100 years of consolidation—remembering Müller and Pilzecker. Learning & Memory. 6, 77-87.

LeDoux, J.E., 1994. The amygdala: contributions to fear and stress. Seminars in Neuroscience. 6, 231-237. 10.1006/smns.1994.1030.

LeDoux, J.E., Farb, C.R. and Romanski, L.M., 1991. Overlapping projections to the amygdala and striatum from auditory processing areas of the thalamus and cortex. Neurosci Lett. 134, 139-144. 10.1016/0304-3940(91)90526-y.

Lee, S.H., Kim, Y.J., Lee, K.M., Ryu, S. and Yoon, B.W., 2007. Ischemic preconditioning enhances neurogenesis in the subventricular zone. Neuroscience. 146, 1020-1031. 10.1016/j.neuroscience.2007.02.058.

Leloup, C., Arluison, M., Kassis, N., Lepetit, N., Cartier, N., Ferré, P. and Pénicaud, L., 1996. Discrete brain areas express the insulin-responsive glucose transporter GLUT4. Brain Res Mol Brain Res. 38, 45-53. 10.1016/0169-328x(95)00306-d.

Lemieux, A.M. and Coe, C.L., 1995. Abuse-related posttraumatic stress disorder: evidence for chronic neuroendocrine activation in women. Psychosom Med. 57, 105-115. 10.1097/00006842-199503000-00002.

Levey, A.I., Edmunds, S.M., Heilman, C.J., Desmond, T.J. and Frey, K.A., 1994. Localization of muscarinic m3 receptor protein and M3 receptor binding in rat brain. Neuroscience. 63, 207-221. 10.1016/0306-4522(94)90017-5.

Levin, B.E., Dunn-Meynell, A.A. and Routh, V.H., 1999. Brain glucose sensing and body energy homeostasis: role in obesity and diabetes. Am J Physiol. 276, R1223-1231. 10.1152/ajpregu.1999.276.5.R1223.

Lew, J., Matsumoto, Y., Pearson, J., Goldstein, M., Hökfelt, T. and Fuxe, K., 1977. Localization and characterization of phenylethanolamine N-methyl transferase in the brain of various mammalian species. Brain research. 119, 199-210.

Lewin, G.R. and Barde, Y.-A., 1996. Physiology of the Neurotrophins. Annual Review of Neuroscience. 19, 289-317. 10.1146/annurev.ne.19.030196.001445.

Liang, K.-C., 2009. Involvement of the amygdala and its connected structures in formation and expression of inhibitory avoidance memory: issues and implications. Chin J Physiol. 52, 196-214.

Liang, K.C., Juler, R.G. and McGaugh, J.L., 1986. Modulating effects of posttraining epinephrine on memory: Involvement of the amygdala noradrenergic system. Brain Research. 368, 125-133. 10.1016/0006-8993(86)91049-8.

Liang, K.C., McGaugh, J.L. and Yao, H.Y., 1990. Involvement of amygdala pathways in the influence of post-training intra-amygdala norepinephrine and peripheral epinephrine on memory storage. Brain Res. 508, 225-233. 10.1016/0006-8993(90)90400-6.

Lieberman, D.A., 2011. Learning and memory.

Lin, L.W., Tsai, F.S., Yang, W.T., Lai, S.C., Shih, C.C., Lee, S.C. and Wu, C.R., 2018. Differential change in cortical and hippocampal monoamines, and behavioral patterns in streptozotocin-induced type 1 diabetic rats. Iran J Basic Med Sci. 21, 1026-1034. 10.22038/ijbms.2018.29810.7197.

Lind, B.L., Brazhe, A.R., Jessen, S.B., Tan, F.C. and Lauritzen, M.J., 2013. Rapid stimulus-evoked astrocyte Ca2+ elevations and hemodynamic responses in mouse somatosensory cortex in vivo. Proc Natl Acad Sci U S A. 110, E4678-4687. 10.1073/pnas.1310065110.

Lindsay, R.M., Wiegand, S.J., Anthony Altar, C. and DiStefano, P.S., 1994. Neurotrophic factors: from molecule to man. Trends in Neurosciences. 17, 182-190. 10.1016/0166-2236(94)90099-X.

Liu, Q., Huang, Y., Shen, J., Steffensen, S. and Wu, J., 2012. Functional α7β2 nicotinic acetylcholine receptors expressed in hippocampal interneurons exhibit high sensitivity to pathological level of amyloid β peptides. BMC Neurosci. 13, 155. 10.1186/1471-2202-13-155.

Maher, F., Davies-Hill, T.M. and Simpson, I.A., 1996. Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons. Biochem J. 315 (Pt 3), 827-831. 10.1042/bj3150827.

Maher, F. and Simpson, I., 1994. The GLUT3 glucose transporter is the predominant isoform in primary cultured neurons: assessment by biosynthetic and photoaffinity labelling. Biochemical Journal. 301, 379-384.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. and Yancopoulos, G.D., 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science. 247, 1446-1451. 10.1126/science.247.4949.1446.

Malkani, S. and Rosen, J.B., 2000. Induction of NGFI-B mRNA following contextual fear conditioning and its blockade by diazepam. Brain Res Mol Brain Res. 80, 153-165. 10.1016/s0169-328x(00)00130-3.

Maren, S., 2001. Neurobiology of Pavlovian fear conditioning. Annu Rev Neurosci. 24, 897-931. 10.1146/annurev.neuro.24.1.897.

Maren, S. and Fanselow, M.S., 1995. Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. J Neurosci. 15, 7548-7564. 10.1523/jneurosci.15-11-07548.1995.

Maren, S., Phan, K.L. and Liberzon, I., 2013. The contextual brain: implications for fear conditioning, extinction and psychopathology. Nat Rev Neurosci. 14, 417-428. 10.1038/nrn3492.

Martinho, R., Correia, G., Seixas, R., Oliveira, A., Silva, S., Serrao, P., Fernandes-Lopes, C., Costa, C. and Moreira-Rodrigues, M., 2021a. Treatment With Nepicastat Decreases Contextual Traumatic Memories Persistence in Post-traumatic Stress Disorder. Front Mol Neurosci. 14, 745219. 10.3389/fnmol.2021.745219.

Martinho, R., Oliveira, A., Correia, G., Marques, M., Seixas, R., Serrao, P. and Moreira-Rodrigues, M., 2020. Epinephrine May Contribute to the Persistence of Traumatic Memories in a Post-traumatic Stress Disorder Animal Model. Front Mol Neurosci. 13, 588802. 10.3389/fnmol.2020.588802.

Martinho, R., Seixas, R., Azevedo, M., Oliveira, A., Serrao, P. and Moreira-Rodrigues, M., 2021b. Sotalol Treatment may Interfere With Retrieval, Expression, and/or Reconsolidation Processes Thus Disrupting Traumatic Memories in a Post-Traumatic Stress Disorder Mice Model. Front Pharmacol. 12, 809271. 10.3389/fphar.2021.809271.

Matheson, G.K., Branch, B.J. and Taylor, A.N., 1971. Effects of amygdaloid stimulation on pituitary-adrenal activity in conscious cats. Brain Research. 32, 151-167. 10.1016/0006-8993(71)90160-0.

McDonald, A.J., Mascagni, F. and Guo, L., 1996. Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience. 71, 55-75. 10.1016/0306-4522(95)00417-3.

McGaugh, J.L., 2000. Memory--a century of consolidation. Science. 287, 248-251.

McGaugh, J.L. and Roozendaal, B., 2002. Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol. 12, 205-210.

McGrath, J.C., 1982. Evidence for more than one type of post-junctional alpha-adrenoceptor. Biochem Pharmacol. 31, 467-484. 10.1016/0006-2952(82)90147-2.

McNay, E.C., Fries, T.M. and Gold, P.E., 2000. Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. Proceedings of the National Academy of Sciences. 97, 2881-2885. 10.1073/pnas.050583697.

McNay, E.C. and Pearson-Leary, J., 2020. GluT4: A central player in hippocampal memory and brain insulin resistance. Experimental Neurology. 323, 113076. 10.1016/j.expneurol.2019.113076.

Mendes, P., Martinho, R., Leite, S., Maia-Moco, L., Leite-Moreira, A.F., Lourenco, A.P. and Moreira-Rodrigues, M., 2018. Chronic exercise induces pathological left ventricular hypertrophy in adrenaline-deficient mice. Int J Cardiol. 253, 113-119. 10.1016/j.ijcard.2017.10.014.

Messier, C., 2004. Glucose improvement of memory: a review. Eur J Pharmacol. 490, 33-57. 10.1016/j.ejphar.2004.02.043.

Messier, C. and Destrade, C., 1988. Improvement of memory for an operant response by post-training glucose in mice. Behav Brain Res. 31, 185-191. 10.1016/0166-4328(88)90022-8.

Messier, C. and Destrade, C.J.P., 1994. Insulin attenuates scopolamine-induced memory deficits. 22, 16-21.

Metsis, M., Timmusk, T., Arenas, E. and Persson, H., 1993. Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation. Proc Natl Acad Sci U S A. 90, 8802-8806. 10.1073/pnas.90.19.8802.

Millar, N.S. and Gotti, C., 2009. Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology. 56, 237-246. 10.1016/j.neuropharm.2008.07.041.

Misanin, J.R., Miller, R.R. and Lewis, D.J., 1968. Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. Science. 160, 554-555. 10.1126/science.160.3827.554.

Miyashita, T. and Williams, C.L., 2006. Epinephrine administration increases neural impulses propagated along the vagus nerve: Role of peripheral beta-adrenergic receptors. Neurobiol Learn Mem. 85, 116-124. 10.1016/j.nlm.2005.08.013.

Moreira-Rodrigues, M., Graca, A.L., Ferreira, M., Afonso, J., Serrao, P., Morato, M., Ferreirinha, F., Correia-de-Sa, P., Ebert, S.N. and Moura, D., 2014. Attenuated aortic vasodilation and sympathetic prejunctional facilitation in epinephrine-deficient mice: selective impairment of beta2-adrenoceptor responses. J Pharmacol Exp Ther. 351, 243-249. 10.1124/jpet.114.217281.

Moreira-Rodrigues, M., Henriques-Coelho, T., Moura, C., Vasques-Novoa, F., Sampaio-Maia, B., Pestana, M. and Leite-Moreira, A.F., 2010. Cardiac dysfunction in HgCl2-induced nephrotic syndrome. Exp Biol Med (Maywood). 235, 392-400. 10.1258/ebm.2009.009147.

Moreira-Rodrigues, M., Quelhas-Santos, J., Roncon-Albuquerque, R., Serrao, P., Leite-Moreira, A., Sampaio-Maia, B. and Pestana, M., 2012. Blunted renal dopaminergic system in a mouse model of diet-induced obesity. Exp Biol Med (Maywood). 237, 949-955. 10.1258/ebm.2012.012077.

Moreira-Rodrigues, M., Sampaio-Maia, B., Moura, M. and Pestana, M., 2007. Renal dopaminergic system activity in uninephrectomized rats up to 26 weeks after surgery. Am J Nephrol. 27, 232-239. 10.1159/000101368.

Moreira-Rodrigues, M., Sampaio-Maia, B. and Pestana, M., 2009. Renal dopaminergic system activity in rat remnant kidney up to twenty-six weeks after surgery. Life Sci. 84, 409-414. 10.1016/j.lfs.2008.12.018.

Morgan, J.I. and Curran, T., 1989. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. Trends in Neurosciences. 12, 459-462. 10.1016/0166-2236(89)90096-9.

Morgello, S., Uson, R.R., Schwartz, E.J. and Haber, R.S., 1995. The human blood-brain barrier glucose transporter (GLUT1) is a glucose transporter of gray matter astrocytes. Glia. 14, 43-54. 10.1002/glia.440140107.

Morris, K.A. and Gold, P.E., 2013. Epinephrine and glucose modulate training-related CREB phosphorylation in old rats: relationships to age-related memory impairments. Exp Gerontol. 48, 115-127. 10.1016/j.exger.2012.11.010.

Mrzljak, L., Levey, A.I. and Goldman-Rakic, P.S., 1993. Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: morphological evidence for cholinergic modulation of excitatory neurotransmission. Proc Natl Acad Sci U S A. 90, 5194-5198. 10.1073/pnas.90.11.5194.

Müller, G.E. and Pilzecker, A., 1900. Experimentelle beiträge zur lehre vom gedächtniss. 1,

Mulugeta, E., Chandranath, I., Karlsson, E., Winblad, B. and Adem, A.J.E.b.r., 2006. Temporal and region-dependent changes in muscarinic M4 receptors in the hippocampus and entorhinal cortex of adrenalectomized rats. 173, 309-317.

Murchison, C.F., Zhang, X.Y., Zhang, W.P., Ouyang, M., Lee, A. and Thomas, S.A., 2004. A distinct role for norepinephrine in memory retrieval. Cell. 117, 131-143. 10.1016/s0092-8674(04)00259-4.

Nader, K., Schafe, G.E. and Le Doux, J.E., 2000. Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. Nature. 406, 722-726. 10.1038/35021052.

Nagatsu, T., Levitt, M. and Udenfriend, S., 1964. Tyrosine hydroxylase: the initial step in norepinephrine biosynthesis. Journal of Biological Chemistry. 239, 2910-2917.

Newman, L.A., Korol, D.L. and Gold, P.E., 2011. Lactate produced by glycogenolysis in astrocytes regulates memory processing. PLoS One. 6, e28427. 10.1371/journal.pone.0028427.

Nijsen, M.J.M.A., Croiset, G., Diamant, M., Stam, R., Kamphuis, P.J.G.H., Bruijnzeel, A., de Wied, D. and Wiegant, V.M., 2000. Endogenous corticotropin-releasing hormone inhibits conditioned-fear-induced vagal activation in the rat. European Journal of Pharmacology. 389, 89-98. 10.1016/S0014-2999(99)00870-5.

Nogueira, P.J., Tomaz, C. and Williams, C.L., 1994. Contribution of the vagus nerve in mediating the memory-facilitating effects of substance P. Behav Brain Res. 62, 165-169. 10.1016/0166-4328(94)90024-8.

Nussdorfer, G.G., 1980. Cytophysiology of the adrenal zona glomerulosa. International Review of Cytology. 64, 307-368.

Nussdorfer, G.G., Mazzocchi, G. and Meneghelli, V., 1978. Cytophysiology of the adrenal zona fasciculata. International review of cytology. 55, 291-365.

O'Donnell, J.M., Wolfe, B.B. and Frazer, A., 1984. Agonist interactions with beta adrenergic receptors in rat brain. J Pharmacol Exp Ther. 228, 640-647.

Ögren, S.O. and Stiedl, O., 2015. Passive Avoidance. Berlin, Heidelberg. 1220-1228. 10.1007/978-3-642-36172-2_160.

Ohkura, N., Ito, M., Tsukada, T., Sasaki, K., Yamaguchi, K. and Miki, K., 1996. Structure, mapping and expression of a human NOR-1 gene, the third member of the Nur77/NGFI-B family. Biochim Biophys Acta. 1308, 205-214. 10.1016/0167-4781(96)00101-7.

Oitzl, M.S. and de Kloet, E.R., 1992. Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behavioral Neuroscience. 106, 62-71. 10.1037/0735-7044.106.1.62.

Oliff, H.S., Berchtold, N.C., Isackson, P. and Cotman, C.W., 1998. Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. Brain Res Mol Brain Res. 61, 147-153. 10.1016/s0169-328x(98)00222-8.

Oliveira, A., Azevedo, M., Seixas, R., Martinho, R., Serrao, P. and Moreira-Rodrigues, M., 2023a. Glucose may Contribute to Retrieval and Reconsolidation of Contextual Fear Memory Through Hippocampal Nr4a3 and Bdnf mRNA Expression and May Act Synergically with Adrenaline. Mol Neurobiol. 10.1007/s12035-023-03745-6.

Oliveira, A., Martinho, R., Serrao, P. and Moreira-Rodrigues, M., 2018. Epinephrine Released During Traumatic Events May Strengthen Contextual Fear Memory Through Increased Hippocampus mRNA Expression of Nr4a Transcription Factors. Front Mol Neurosci. 11, 334. 10.3389/fnmol.2018.00334.

Oliveira, A., Seixas, R., Pereira, F., Azevedo, M., Martinho, R., Serrao, P. and Moreira-Rodrigues, M., 2023b. Insulin enhances contextual fear memory independently of its effect in increasing plasma adrenaline. Life Sci. 328, 121881. 10.1016/j.lfs.2023.121881.

Oliver, G. and Schäfer, E.A., 1895. The Physiological Effects of Extracts of the Suprarenal Capsules. J Physiol. 18, 230-276. 10.1113/jphysiol.1895.sp000564.

Oomura, Y., Ono, T., Ooyama, H. and Wayner, M.J., 1969. Glucose and Osmosensitive Neurones of the Rat Hypothalamus. Nature. 222, 282-284. 10.1038/222282a0.

Ortega-de San Luis, C. and Ryan, T.J., 2022. Understanding the physical basis of memory: Molecular mechanisms of the engram. Journal of Biological Chemistry. 298, 101866. 10.1016/j.jbc.2022.101866.

Osborne, D.M., Pearson-Leary, J. and McNay, E.C., 2015. The neuroenergetics of stress hormones in the hippocampus and implications for memory. Frontiers in neuroscience. 9, 164.

Parfitt, G.M., Barbosa Â, K., Campos, R.C., Koth, A.P. and Barros, D.M., 2012. Moderate stress enhances memory persistence: are adrenergic mechanisms involved? Behav Neurosci. 126, 729-734. 10.1037/a0029861.

Park, C.R., Seeley, R.J., Craft, S. and Woods, S.C., 2000. Intracerebroventricular insulin enhances memory in a passive-avoidance task. Physiol Behav. 68, 509-514. 10.1016/s0031-9384(99)00220-6.

Parsons, M.W. and Gold, P.E., 1992. Glucose enhancement of memory in elderly humans: an inverted-U dose-response curve. Neurobiol Aging. 13, 401-404. 10.1016/0197-4580(92)90114-d.

Passani, M.B., Cangioli, I., Baldi, E., Bucherelli, C., Mannaioni, P.F. and Blandina, P., 2001. Histamine H3 receptor-mediated impairment of contextual fear conditioning and in-vivo inhibition of cholinergic transmission in the rat basolateral amygdala. Eur J Neurosci. 14, 1522-1532. 10.1046/j.0953-816x.2001.01780.x.

Patching, S.G., 2017. Glucose Transporters at the Blood-Brain Barrier: Function, Regulation and Gateways for Drug Delivery. Molecular Neurobiology. 54, 1046-1077. 10.1007/s12035-015-9672-6.

Pavlov, I.P. and Thompson, W.H., 1910. The work of the digestive glands: lectures. (No Title).

Paylor, R., Tracy, R., Wehner, J. and Rudy, J.W., 1994. DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. Behav Neurosci. 108, 810-817. 10.1037//0735-7044.108.4.810.

Payne, J., Maher, F., Simpson, I., Mattice, L. and Davies, P., 1997. Glucose transporter glut 5 expression in microglial cells. Glia. 21, 327-331. 10.1002/(SICI)1098-1136(199711)21:3<327::AID-GLIA7>3.0.CO;2-1.

Pearson-Leary, J., Jahagirdar, V., Sage, J. and McNay, E.C., 2018. Insulin modulates hippocampally-mediated spatial working memory via glucose transporter-4. Behav Brain Res. 338, 32-39. 10.1016/j.bbr.2017.09.033.

Pearson-Leary, J. and McNay, E.C., 2016. Novel Roles for the Insulin-Regulated Glucose Transporter-4 in Hippocampally Dependent Memory. J Neurosci. 36, 11851-11864. 10.1523/jneurosci.1700-16.2016.

Peña de Ortiz, S., Maldonado-Vlaar, C.S. and Carrasquillo, Y., 2000. Hippocampal expression of the orphan nuclear receptor gene hzf-3/nurr1 during spatial discrimination learning. Neurobiol Learn Mem. 74, 161-178. 10.1006/nlme.1999.3952.

Pendleton, R., Gessner, G. and Sawyer, J., 1978. Studies on the distribution of phenylethanolamine N-methyltransferase and epinephrine in the rat. Research Communications in Chemical Pathology and Pharmacology. 21, 315-325.

Peralta, E.G., Ashkenazi, A., Winslow, J., Smith, D., Ramachandran, J. and Capon, D.J., 1987. Distinct primary structures, ligand-binding properties and tissue-

specific expression of four human muscarinic acetylcholine receptors. The EMBO journal. 6, 3923-3929.

Petrov, T., Krukoff, T.L. and Jhamandas, J.H., 1993. Branching projections of catecholaminergic brainstem neurons to the paraventricular hypothalamic nucleus and the central nucleus of the amygdala in the rat. Brain Research. 609, 81-92. 10.1016/0006-8993(93)90858-K.

Phillips, R.G. and LeDoux, J.E., 1992. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci. 106, 274-285. 10.1037//0735-7044.106.2.274.

Pitkänen, A., Kelly, J.L. and Amaral, D.G., 2002. Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the entorhinal cortex in the macaque monkey. Hippocampus. 12, 186-205. 10.1002/hipo.1099.

Ploski, J.E., Monsey, M.S., Nguyen, T., DiLeone, R.J. and Schafe, G.E., 2011. The neuronal PAS domain protein 4 (Npas4) is required for new and reactivated fear memories. PLoS One. 6, e23760. 10.1371/journal.pone.0023760.

Poduslo, J.F., Curran, G.L. and Berg, C.T., 1994. Macromolecular permeability across the blood-nerve and blood-brain barriers. Proc Natl Acad Sci U S A. 91, 5705-5709. 10.1073/pnas.91.12.5705.

Posner, B.I., Kelly, P.A., Shiu, R.P. and Friesen, H.G., 1974. Studies of insulin, growth hormone and prolactin binding: tissue distribution, species variation and characterization. Endocrinology. 95, 521-531. 10.1210/endo-95-2-521.

Poulos, A.M., Li, V., Sterlace, S.S., Tokushige, F., Ponnusamy, R. and Fanselow, M.S., 2009. Persistence of fear memory across time requires the basolateral amygdala complex. Proceedings of the National Academy of Sciences of the United States of America. 106, 11737-11741. 10.1073/pnas.0905257106.

Power, A.E., Vazdarjanova, A. and McGaugh, J.L., 2003. Muscarinic cholinergic influences in memory consolidation. Neurobiol Learn Mem. 80, 178-193.

Przybyslawski, J. and Sara, S.J., 1997. Reconsolidation of memory after its reactivation. Behav Brain Res. 84, 241-246. 10.1016/s0166-4328(96)00153-2.

Purves, D., Augustine, G., Fitzpatrick, D., Katz, L., LaMantia, A., McNamara, J. and Williams, S., 2001. Neuroscience 2nd edition. sunderland (ma) sinauer associates. Types of Eye Movements and Their Functions.

Pych, J.C., Chang, Q., Colon-Rivera, C., Haag, R. and Gold, P.E., 2005. Acetylcholine release in the hippocampus and striatum during place and response training. Learning & memory (Cold Spring Harbor, N.Y.). 12, 564-572. 10.1101/lm.33105.

Qasim, S.E., Mohan, U.R., Stein, J.M. and Jacobs, J., 2023. Neuronal activity in the human amygdala and hippocampus enhances emotional memory encoding. Nature Human Behaviour. 7, 754-764. 10.1038/s41562-022-01502-8.

Quirarte, G.L., Roozendaal, B. and McGaugh, J.L., 1997. Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. Proc Natl Acad Sci U S A. 94, 14048-14053. 10.1073/pnas.94.25.14048.

Ragozzino, M.E., Pal, S.N., Unick, K., Stefani, M.R. and Gold, P.E., 1998. Modulation of hippocampal acetylcholine release and spontaneous alternation scores by

intrahippocampal glucose injections. J Neurosci. 18, 1595-1601. 10.1523/jneurosci.18-04-01595.1998.

Ragozzino, M.E., Unick, K.E. and Gold, P.E., 1996. Hippocampal acetylcholine release during memory testing in rats: augmentation by glucose. Proceedings of the National Academy of Sciences of the United States of America. 93, 4693-4698. 10.1073/pnas.93.10.4693.

Rajbhandari, A.K., Tribble, J.E. and Fanselow, M.S., 2017. Neurobiology of fear memory.

Randall, D.J. and Ferry, S.F., 1992. 4 Catecholamines. 12, 255-300. 10.1016/S1546-5098(08)60011-4.

Rhodin, J.A., 1971. The ultrastructure of the adrenal cortex of the rat under normal and experimental conditions. Journal of ultrastructure research. 34, 23-71.

Richter-Levin, G. and Akirav, I., 2003. Emotional tagging of memory formation—in the search for neural mechanisms. Brain Research Reviews. 43, 247-256. 10.1016/j.brainresrev.2003.08.005.

Riedel, G., Casabona, G., Platt, B., Macphail, E.M. and Nicoletti, F., 2000. Fear conditioning-induced time- and subregion-specific increase in expression of mGlu5 receptor protein in rat hippocampus. Neuropharmacology. 39, 1943-1951. 10.1016/s0028-3908(00)00037-x.

Rios, M., Habecker, B., Sasaoka, T., Eisenhofer, G., Tian, H., Landis, S., Chikaraishi, D. and Roffler-Tarlov, S., 1999. Catecholamine synthesis is mediated by tyrosinase in the absence of tyrosine hydroxylase. Journal of Neuroscience. 19, 3519-3526.

Robertson, S.D., Matthies, H.J., Owens, W.A., Sathananthan, V., Christianson, N.S., Kennedy, J.P., Lindsley, C.W., Daws, L.C. and Galli, A., 2010. Insulin reveals Akt signaling as a novel regulator of norepinephrine transporter trafficking and norepinephrine homeostasis. J Neurosci. 30, 11305-11316. 10.1523/jneurosci.0126-10.2010.

Roozendaal, B. and McGaugh, J.L., 1996. Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. Neurobiol Learn Mem. 65, 1-8. 10.1006/nlme.1996.0001.

Rosol, T.J., Yarrington, J.T., Latendresse, J. and Capen, C.C., 2001. Adrenal gland: structure, function, and mechanisms of toxicity. Toxicol Pathol. 29, 41-48. 10.1080/019262301301418847.

Rudy, J.W., Huff, N.C. and Matus-Amat, P., 2004. Understanding contextual fear conditioning: insights from a two-process model. Neurosci Biobehav Rev. 28, 675-685. 10.1016/j.neubiorev.2004.09.004.

Safe, S., Jin, U.H., Morpurgo, B., Abudayyeh, A., Singh, M. and Tjalkens, R.B., 2016. Nuclear receptor 4A (NR4A) family - orphans no more. J Steroid Biochem Mol Biol. 157, 48-60. 10.1016/j.jsbmb.2015.04.016.

Saku, K., Kishi, T., Sakamoto, K., Hosokawa, K., Sakamoto, T., Murayama, Y., Kakino, T., Ikeda, M., Ide, T. and Sunagawa, K., 2014. Afferent vagal nerve stimulation resets baroreflex neural arc and inhibits sympathetic nerve activity. Physiol Rep. 2, 10.14814/phy2.12136.

Sananbenesi, F., Fischer, A., Schrick, C., Spiess, J. and Radulovic, J., 2002. Phosphorylation of Hippocampal Erk-1/2, Elk-1, and p90-Rsk-1 during Contextual Fear Conditioning: Interactions between Erk-1/2 and Elk-1. Molecular and Cellular Neuroscience. 21, 463-476. 10.1006/mcne.2002.1188.

Sangha, S., Scheibenstock, A. and Lukowiak, K., 2003. Reconsolidation of a Long-Term Memory in Lymnaea Requires New Protein and RNA Synthesis and the Soma of Right Pedal Dorsal 1. The Journal of Neuroscience. 23, 8034-8040. 10.1523/jneurosci.23-22-08034.2003.

Santucci, A.C., Schroeder, H. and Riccio, D.C., 1990. Homeostatic disruption and memory: effect of insulin administration in rats. Behav Neural Biol. 53, 321-333. 10.1016/0163-1047(90)90184-8.

Scarr, E., 2012. Muscarinic Receptors: Their Roles in Disorders of the Central Nervous System and Potential as Therapeutic Targets. CNS Neuroscience & Therapeutics. 18, 369-379. 10.1111/j.1755-5949.2011.00249.x.

Schafe, G.E. and LeDoux, J.E., 2000. Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. J Neurosci. 20, Rc96. 10.1523/JNEUROSCI.20-18-j0003.2000.

Schipke, C.G. and Kettenmann, H., 2004. Astrocyte responses to neuronal activity. Glia. 47, 226-232. 10.1002/glia.20029.

Schousboe, A., Westergaard, N., Sonnewald, U., Petersen, S.B., Huang, R., Peng, L. and Hertz, L., 1993. Glutamate and glutamine metabolism and compartmentation in astrocytes. Dev Neurosci. 15, 359-366. 10.1159/000111356.

Schreurs, J., Seelig, T. and Schulman, H., 1986. Beta 2-adrenergic receptors on peripheral nerves. J Neurochem. 46, 294-296. 10.1111/j.1471-4159.1986.tb12961.x.

Schwartz, M.W., Bergman, R.N., Kahn, S.E., Taborsky, G.J., Jr., Fisher, L.D., Sipols, A.J., Woods, S.C., Steil, G.M. and Porte, D., Jr., 1991. Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. J Clin Invest. 88, 1272-1281. 10.1172/jci115431.

Schwarz, L.A. and Luo, L., 2015. Organization of the Locus Coeruleus-Norepinephrine System. Current Biology. 25, R1051-R1056. 10.1016/j.cub.2015.09.039.

Schwarzberg, H., Bernstein, H.G., Reiser, M. and Günther, O., 1989. Intracerebroventricular administration of insulin attenuates retrieval of a passive avoidance response in rats. Neuropeptides. 13, 79-81. 10.1016/0143-4179(89)90002-4.

Selye, H., 1950. Stress and the General Adaptation Syndrome. British Medical Journal. 1, 1383-1392. 10.1136/bmj.1.4667.1383.

Sharara-Chami, R.I., Joachim, M., Pacak, K. and Majzoub, J.A., 2010. Glucocorticoid treatment--effect on adrenal medullary catecholamine production. Shock. 33, 213-217. 10.1097/SHK.0b013e3181af0633.

Shih, D.F., Hsiao, C.D., Min, M.Y., Lai, W.S., Yang, C.W., Lee, W.T. and Lee, S.J., 2013. Aromatic L-amino acid decarboxylase (AADC) is crucial for brain development and motor functions. PLoS One. 8, e71741. 10.1371/journal.pone.0071741.

Silva, B.A. and Gräff, J., 2023. Face your fears: attenuating remote fear memories by reconsolidation-updating. Trends in Cognitive Sciences.

Skultetyova, I., Tokarev, D. and Jezova, D., 1998. Stress-induced increase in blood-brain barrier permeability in control and monosodium glutamate-treated rats. Brain Res Bull. 45, 175-178. 10.1016/s0361-9230(97)00335-3.

Smith, M.A., Makino, S., Kvetnansky, R. and Post, R.M., 1995. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci. 15, 1768-1777. 10.1523/jneurosci.15-03-01768.1995.

Sonne, J., Reddy, V. and Beato, M.R., 2022. Neuroanatomy, substantia nigra.

Spatz, M., Nagatsu, I., Maruki, C., Yoshida, M., Kondo, Y. and Bembry, J., 1982. The presence of phenylethanolamine-N-methyltransferase in cerebral microvessel and endothelial cultures. Brain Research. 240, 191-194. 10.1016/0006-8993(82)90663-1.

Spinelli, M., Fusco, S. and Grassi, C., 2019. Brain Insulin Resistance and Hippocampal Plasticity: Mechanisms and Biomarkers of Cognitive Decline. Frontiers in Neuroscience. 13, 10.3389/fnins.2019.00788.

Squire, L.R., 2004. Memory systems of the brain: a brief history and current perspective. Neurobiol Learn Mem. 82, 171-177. 10.1016/j.nlm.2004.06.005.

Squire, L.R., 2009. Memory and brain systems: 1969-2009. J Neurosci. 29, 12711-12716. 10.1523/jneurosci.3575-09.2009.

Squire, L.R., Genzel, L., Wixted, J.T. and Morris, R.G., 2015. Memory consolidation. Cold Spring Harb Perspect Biol. 7, a021766. 10.1101/cshperspect.a021766.

Squire, L.R. and Zola, S.M., 1996. Structure and function of declarative and nondeclarative memory systems. Proc Natl Acad Sci U S A. 93, 13515-13522. 10.1073/pnas.93.24.13515.

Sridhar, S., Khamaj, A. and Asthana, M.K., 2023. Cognitive neuroscience perspective on memory: overview and summary. Frontiers in Human Neuroscience. 17, 10.3389/fnhum.2023.1217093.

Stanford, S.C., 2001. Adrenaline and Noradrenaline: Introduction. 10.1002/9780470015902.a0000271.pub3.

Starke, K., 1981. Alpha-adrenoceptor subclassification. Rev Physiol Biochem Pharmacol. 88, 199-236.

Starke, K., 2001. Presynaptic autoreceptors in the third decade: focus on alpha2-adrenoceptors. J Neurochem. 78, 685-693. 10.1046/j.1471-4159.2001.00484.x.

Stefani, M.R., Nicholson, G.M. and Gold, P.E., 1999. ATP-sensitive potassium channel blockade enhances spontaneous alternation performance in the rat: a potential mechanism for glucose-mediated memory enhancement. Neuroscience. 93, 557-563. 10.1016/S0306-4522(99)00128-1.

Sternberg, D.B., Isaacs, K.R., Gold, P.E. and McGaugh, J.L., 1985. Epinephrine facilitation of appetitive learning: attenuation with adrenergic receptor antagonists. Behav Neural Biol. 44, 447-453. 10.1016/s0163-1047(85)90856-8.

Stolz, F., 1904. Über adrenalin und alkylaminoacetobrenzcatechin. Ber. dtsch. chem. Ges. 37, 416.

Stone, W.S., Rudd, R.J. and Gold, P.E., 1992. Glucose attenuation of scopolamine-and age-induced deficits in spontaneous alternation behavior and regional brain [3 H] 2-deoxyglucose uptake in mice. Psychobiology. 20, 270-279.

Stubbendorff, C. and Stevenson, C.W., 2020. Dopamine regulation of contextual fear and associated neural circuit function. European Journal of Neuroscience.

Stults-Kolehmainen, M.A. and Sinha, R., 2014. The effects of stress on physical activity and exercise. Sports Med. 44, 81-121. 10.1007/s40279-013-0090-5.

Sumal, K.K., Blessing, W.W., Joh, T.H., Reis, D.J. and Pickel, V.M., 1983. Synaptic interaction of vagal afferents and catecholaminergic neurons in the rat nucleus tractus solitarius. Brain Res. 277, 31-40. 10.1016/0006-8993(83)90904-6.

Sun, P., Bao, X., Elayan, H., Milic, M., Liu, F. and Ziegler, M.G., 2008. Epinephrine regulation of hemodynamics in catecholamine knockouts and the pithed mouse. Ann N Y Acad Sci. 1148, 325-330. 10.1196/annals.1410.078.

Sun, W., McConnell, E., Pare, J.F., Xu, Q., Chen, M., Peng, W., Lovatt, D., Han, X., Smith, Y. and Nedergaard, M., 2013. Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. Science. 339, 197-200. 10.1126/science.1226740.

Suzuki, A., Stern, S.A., Bozdagi, O., Huntley, G.W., Walker, R.H., Magistretti, P.J. and Alberini, C.M., 2011. Astrocyte-neuron lactate transport is required for long-term memory formation. Cell. 144, 810-823. 10.1016/j.cell.2011.02.018.

Szabo, O. and Szabo, A.J., 1972. Evidence for an insulin-sensitive receptor in the central nervous system. Am J Physiol. 223, 1349-1353. 10.1152/ajplegacy.1972.223.6.1349.

Takahashi, L.K., Turner, J.G. and Kalin, N.H., 1992. Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats. Brain Res. 574, 131-137. 10.1016/0006-8993(92)90809-n.

Takamine, J., 1901. The isolation of the active principle of the suprarenal gland. 27,

Takei, S., Morinobu, S., Yamamoto, S., Fuchikami, M., Matsumoto, T. and Yamawaki, S., 2011. Enhanced hippocampal BDNF/TrkB signaling in response to fear conditioning in an animal model of posttraumatic stress disorder. Journal of Psychiatric Research. 45, 460-468. 10.1016/j.jpsychires.2010.08.009.

Talley, C.E., Kahn, S., Alexander, L.J. and Gold, P.E., 2000. Epinephrine fails to enhance performance of food-deprived rats on a delayed spontaneous alternation task. Neurobiol Learn Mem. 73, 79-86. 10.1006/nlme.1999.3920.

Talley, C.P., Clayborn, H., Jewel, E., McCarty, R. and Gold, P.E., 2002. Vagotomy attenuates effects of L-glucose but not of D-glucose on spontaneous alternation performance. Physiol Behav. 77, 243-249. 10.1016/s0031-9384(02)00850-8.

Tank, A.W. and Lee Wong, D., 2015. Peripheral and central effects of circulating catecholamines. Compr Physiol. 5, 1-15. 10.1002/cphy.c140007.

Tesfaye, N. and Seaquist, E.R., 2010. Neuroendocrine responses to hypoglycemia. Annals of the New York Academy of Sciences. 1212, 12-28. 10.1111/j.1749-6632.2010.05820.x.

Toda, N. and Okamura, T., 1990. Comparison of the response to 5-carboxamidotryptamine and serotonin in isolated human, monkey and dog coronary arteries. J Pharmacol Exp Ther. 253, 676-682.

Toth, M., Ziegler, M., Sun, P., Gresack, J. and Risbrough, V., 2013. Impaired conditioned fear response and startle reactivity in epinephrine-deficient mice. Behav Pharmacol. 24, 1-9. 10.1097/FBP.0b013e32835cf408.

Tresguerres, M., Levin, L.R. and Buck, J., 2011. Intracellular cAMP signaling by soluble adenylyl cyclase. Kidney Int. 79, 1277-1288. 10.1038/ki.2011.95.

Tucek, S. and Cheng, S.C., 1974. Provenance of the acetyl group of acetylcholine and compartmentation of acetyl-CoA and Krebs cycle intermediates in the brain in vivo. J Neurochem. 22, 893-914. 10.1111/j.1471-4159.1974.tb04314.x.

Tulving, E., 1972. Episodic and semantic memory. Organization of memory. 1, 1.

Uemura, E. and Greenlee, H.W., 2006. Insulin regulates neuronal glucose uptake by promoting translocation of glucose transporter GLUT3. Experimental neurology. 198, 48-53.

Vale, W., Rivier, C., Yang, L., Minick, S. and Guillemin, R., 1978. Effects of purified hypothalamic corticotropin-releasing factor and other substances on the secretion of adrenocorticotropin and beta-endorphin-like immunoactivities in vitro. Endocrinology. 103, 1910-1915. 10.1210/endo-103-5-1910.

Valentinuzzi, V.S., Kolker, D.E., Vitaterna, M.H., Shimomura, K., Whiteley, A., Low-Zeddies, S., Turek, F.W., Ferrari, E.A., Paylor, R. and Takahashi, J.S., 1998. Automated measurement of mouse freezing behavior and its use for quantitative trait locus analysis of contextual fear conditioning in (BALB/cJ x C57BL/6J)F2 mice. Learn Mem. 5, 391-403.

Van Bockstaele, E.J., Peoples, J. and Telegan, P., 1999. Efferent projections of the nucleus of the solitary tract to peri-locus coeruleus dendrites in rat brain: evidence for a monosynaptic pathway. J Comp Neurol. 412, 410-428.

VanElzakker, M.B., Kathryn Dahlgren, M., Caroline Davis, F., Dubois, S. and Shin, L.M., 2014. From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. Neurobiology of Learning and Memory. 113, 3-18. 10.1016/j.nlm.2013.11.014.

Vatner, S.F., Knight, D.R. and Hintze, T.H., 1985. Norepinephrine-induced beta 1-adrenergic peripheral vasodilation in conscious dogs. Am J Physiol. 249, H49-56. 10.1152/ajpheart.1985.249.1.H49.

Verhofstad, A.A., Hökfelt, T., Goldstein, M., Steinbusch, H.W. and Joosten, H.W., 1979. Appearance of tyrosine hydroxylase, aromatic amino-acid decarboxylase, dopamine beta-hydroxylase and phenylethanolamine N-methyltransferase during the ontogenesis of the adrenal medulla: an immunohistochemical study in the rat. Cell Tissue Res. 200, 1-13. 10.1007/bf00236882.

Vilaró, M.T., Palacios, J.M. and Mengod, G., 1990. Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. Neurosci Lett. 114, 154-159. 10.1016/0304-3940(90)90064-g.

Viola, H., Furman, M., Izquierdo, L.A., Alonso, M., Barros, D.M., de Souza, M.M., Izquierdo, I. and Medina, J.H., 2000. Phosphorylated cAMP response element-

binding protein as a molecular marker of memory processing in rat hippocampus: effect of novelty. J Neurosci. 20, Rc112. 10.1523/JNEUROSCI.20-23-j0002.2000.

von Hertzen, L.S. and Giese, K.P., 2005. Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation. J Neurosci. 25, 1935-1942. 10.1523/jneurosci.4707-04.2005.

Vorbrodt, A.W., Dobrogowska, D.H. and Tarnawski, M., 2001. Immunogold study of interendothelial junction-associated and glucose transporter proteins during postnatal maturation of the mouse blood-brain barrier. J Neurocytol. 30, 705-716. 10.1023/a:1016581801188.

Voss, T.S., Vendelbo, M.H., Kampmann, U., Hingst, J.R., Wojtaszewski, J.F.P., Svart, M.V., Møller, N. and Jessen, N., 2017. Acute Hypoglycemia in Healthy Humans Impairs Insulin-Stimulated Glucose Uptake and Glycogen Synthase in Skeletal Muscle: A Randomized Clinical Study. Diabetes. 66, 2483-2494. 10.2337/db16-1559.

Vulpian, E.F.A., 1856. Note sur quelques réactions propres à la substance des capsules surrénales. CR Acad Sci. 43, 663-665.

Wang, Y.M., Xu, F., Gainetdinov, R.R. and Caron, M.G., 1999. Genetic approaches to studying norepinephrine function: knockout of the mouse norepinephrine transporter gene. Biol Psychiatry. 46, 1124-1130. 10.1016/s0006-3223(99)00245-0.

Weil-Malherbe, H., Axelrod, J. and Tomchick, R., 1959. Blood-brain barrier for adrenaline. Science. 129, 1226-1227. 10.1126/science.129.3357.1226.

Williams, C.L. and Jensen, R.A., 1991. Vagal afferents: A possible mechanism for the modulation of memory by peripherally acting agents. Peripheral Signaling of the Brain in Neural-Immune and Cognitive Function. Hogrefe and Huber, New York. 467-472.

Wise, A., Watson-Koken, M.A., Rees, S., Lee, M. and Milligan, G., 1997. Interactions of the alpha2A-adrenoceptor with multiple Gi-family G-proteins: studies with pertussis toxin-resistant G-protein mutants. Biochem J. 321 (Pt 3), 721-728. 10.1042/bj3210721.

Wong, D.L., Tai, T.C., Wong-Faull, D.C., Claycomb, R., Meloni, E.G., Myers, K.M., Carlezon, W.A., Jr. and Kvetnansky, R., 2012. Epinephrine: a short- and long-term regulator of stress and development of illness : a potential new role for epinephrine in stress. Cell Mol Neurobiol. 32, 737-748. 10.1007/s10571-011-9768-0.

Wurtman, R.J. and Axelrod, J., 1965. Adrenaline synthesis: control by the pituitary gland and adrenal glucocorticoids. Science. 150, 1464-1465.

Xiao, Q., Castillo, S.O. and Nikodem, V.M., 1996. Distribution of messenger RNAs for the orphan nuclear receptors Nurr1 and Nur77 (NGFI-B) in adult rat brain using in situ hybridization. Neuroscience. 75, 221-230. 10.1016/0306-4522(96)00159-5.

Xu, F., Gainetdinov, R.R., Wetsel, W.C., Jones, S.R., Bohn, L.M., Miller, G.W., Wang, Y.M. and Caron, M.G., 2000. Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nat Neurosci. 3, 465-471. 10.1038/74839.

Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H. and Montminy, M.R., 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature. 334, 494-498. 10.1038/334494a0.

Yanowitch, R. and Coccaro, E.F., 2011. The neurochemistry of human aggression. Advances in genetics. 75, 151-169.

Yavas, E., Gonzalez, S. and Fanselow, M., 2019. Interactions between the hippocampus, prefrontal cortex, and amygdala support complex learning and memory [version 1; peer review: 3 approved]. F1000Research. 8, 10.12688/f1000research.19317.1.

Yehuda, R., Southwick, S., Giller, E.L., Ma, X. and Mason, J.W., 1992. Urinary catecholamine excretion and severity of PTSD symptoms in Vietnam combat veterans. J Nerv Ment Dis. 180, 321-325. 10.1097/00005053-199205000-00006.

Yerkes, R., Dodson. JD (1908). The relation of strength of stimulus to rapidity of habit formation. Journal of Comparative and Neurological Psychology. 459-482.

Yu, S. and Ding, W.G., 1998. The 45 kDa form of glucose transporter 1 (GLUT1) is localized in oligodendrocyte and astrocyte but not in microglia in the rat brain. Brain Res. 797, 65-72. 10.1016/s0006-8993(98)00372-2.

Zelikowsky, M., Hersman, S., Chawla, M.K., Barnes, C.A. and Fanselow, M.S., 2014. Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. J Neurosci. 34, 8462-8466. 10.1523/jneurosci.3624-13.2014.

Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M.J. and Alkon, D.L., 1999. Brain insulin receptors and spatial memory. Correlated changes in gene expression, tyrosine phosphorylation, and signaling molecules in the hippocampus of water maze trained rats. J Biol Chem. 274, 34893-34902. 10.1074/jbc.274.49.34893.

Zigmond, R.E., Schwarzschild, M.A. and Rittenhouse, A.R., 1989. Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annu Rev Neurosci. 12, 415-461. 10.1146/annurev.ne.12.030189.002215.