ESTER SOFIA SANTOS ALVES

ROLE OF EPINEPHRINE IN FEAR MEMORY

Tese de candidatura ao grau de Doutor em Patologia e Genética Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP).

Orientador
Nome - Professora Doutora Mónica Moreira Rodrigues
Categoria - Professora Auxiliar Convidada
Afiliação - Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP)

Co-orientador
Nome - Professor Doutor Daniel Moura
Categoria - Professor Catedrático Aposentado
Afiliação - Faculdade de Medicina da Universidade do Porto (FMUP)

FUNDING

The candidate performed the experimental work supported by Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP).

Preceitos Legais

De acordo com artigo 16.º e 17.º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto, aprovado por despacho reitoral de 10 de Abril de 2015 e publicado em Diário da República, 2.ªsérie, n.º 90 de 11 de Maio de 2015, por Despacho n.º4889/2015, a autora desta tese declara que participou na concepção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redacção dos trabalhos publicados sob o nome de Ester Alves.

Publicações

Ester Alves, Nikolay Lukoyanov, Paula Serrão, Daniel Moura, Mónica Moreira-Rodrigues. Epinephrine increases contextual learning through activation of peripheral β_2-adrenoceptors. Psychopharmacology. 233: 2099, 2016.

.

“AMOR VINCIT OMNIA”

Pai

Table of contents

AGRADECIMENTOS

Antes do agradecimento individual, gostaria de dirigir um agradecimento global a todos que contribuíram para a realização desta tese e que me ajudaram, ensinaram, incentivaram e me permitiram crescer muito em termos pessoais.

Em primeiro lugar, quero agradecer à Professora Doutora Mónica Moreira Rodrigues, minha orientadora, a fundamental supervisão e todo o apoio nestes últimos anos essencial para a concretização deste trabalho. Obrigada pela paciência e por acreditar que era possível, sem a sua ajuda nada disto seria possível. E mesmo na recta final, apesar de toda a rotina e vida paralela no desporto, teve sempre coragem de corrigir a minha direção e apoiar-me. Ao Professor Doutor Daniel Moura e Professor Doutor Nikolay Lukoyanov por todo o apoio, pela disponibilidade e pelo aconselhamento.

Agradeço à Professora Doutora Fátima Gartner por ser directora do Programa Doutoral e estar disponível para todo o apoio logístico necessário.

Agradeço ao Professor Patrício Soares da Silva do Departamento de Farmacologia e Terapêutica da Faculdade de Medicina da Universidade do Porto pela disponibilidade de espaço e equipamento. Agradeço em especial à Paula Serrão e Joana Afonso a colaboração no trabalho, apoio e ensinamentos. A todos os meus professores e ao grupo fabuloso do IPATIMUP, Regina e Sara pela vossa disponibilidade e enviarem a informação no momento necessário.

Obrigada ao grupo de trabalho do Biotério, Dra. Luísa Guardão, Liliana e Zita pela paciência, por tentarem sempre dado o melhor para que nada faltasse. E mesmo quando havia falhas, tentávamos em equipa animar mesmo que fosse necessário começar tudo de novo. Foi ao longo do tempo um esforçado trabalho de equipa.

Agradeço à Dra. Elisa Ferraz bem como ao grupo de vereadores de Vila do Conde toda a motivação e apoio por terem acreditado sempre no meu trabalho académico e desportivo.

Agradeço à minha mãe ter-me aturado em momentos de cansaço e incerteza e ter sido um ombro forte a todo o momento.

A quem me motivou a treinar sempre cedo e com ânimo, para que toda a rotina, trabalho e estudo fossem possíveis.

A todos os que me acompanharam e se cruzaram com este trabalho MUITO OBRIGADA.

LIST OF ABBREVIATIONS

AADC - aromatic L-amino acid decarboxylase

ACTH – adrenocorticotropin hormone

AD(s) – adrenoceptor(s)

ADX- adrenalectomy

CS - conditioned stimulus

CR - conditioned response

CRH - corticotropin-releasing hormone

DBH - β-hydroxylase

EPI- epinephrine

HPA - hypothalamic–pituitary–adrenal axis

L-DOPA - L-3, 4-dihydroxyphenylalanine

NE - norepinephrine

NTS - nucleus tractus solitarius

PNMT/Pnmt – phenylethanolamine-*N*-methyltransferase (human/mice)

PFC - prefrontal cortex

PTSD - post-traumatic stress disorder

PVN - paraventricular nucleus

SSRIs - selective serotonin re-uptake inhibitors

TH - tyrosine hydroxylase

US- unconditioned stimulus

UR - unconditioned response

WT - wild type

ABSTRACT

Epinephrine (EPI) is a hormone that triggers body alarm systems and a stress context in all body. Phenylethanolamine-*N*-methyltransferase-knock-out (Pnmt-KO) mice are unable to synthetize EPI and present reduced contextual-fear, suggesting that EPI deficiency selectively affects contextual-fear memory. Our aim was to understand the pathway by which EPI increases contextual-fear memory. In addition, we also evaluated if time erases EPI-mediated fear memory or if this memory persists as old memories. Another aim was to understand if β_2-adrenoceptor (AD) antagonists could erase traumatic contextual memories. Finally, we propose a possible pharmacological therapy for traumatic memories in anxiety disorders, in particular in post-traumatic stress disorder (PTSD).

Wild type (WT) and Pnmt-KO (129x1/SvJ) male mice were submitted to fear conditioning procedure after specific treatments with EPI, β-AD agonists (isoprenaline and fenoterol), and antagonists (sotalol and ICI 118,551). The experiments were evaluated on day one (fear acquisition) and day two (context fear test, long-term memory), and some experiments one month after fear acquisition (context fear test, old memories). The catecholamines were separated by reverse-phase HPLC, and quantified by electrochemical detection. Blood glucose was measured by coulometry.

We showed that EPI selectively affects contextual-fear memory and not sound fear memory. In addition, EPI increases contextual-fear memory, both one day (long-term memory) and one month after fear acquisition (old memories). The mechanism by which EPI influences contextual-fear memory appears to be through peripheral β_2-AD activation. In addition, glucose may be a major EPI downstream mediator in contextual-fear memory. In WT mice, plasma EPI concentration was significantly higher after fear acquisition test compared with mice without the test. ICI 118,551 (β_2-AD antagonist) decreased contextual-fear memory in WT mice, in both one day and one month after fear acquisition.

In conclusion, EPI increases in plasma after an aversive experience, possibly improving long-term contextual-fear memory, and even old memories, by acting on peripheral β_2-ADs. We suggest that blocking β_2-ADs with antagonists may inhibit undesirable memories and may be used as a treatment for patients suffering from pathogenic memories, such as PTSD.

RESUMO

A epinefrina (EPI) é uma hormona que aciona sistemas de alarme e um contexto de stresse por todo o corpo. Os ratinhos deficientes em feniletanolamina-*N*-metiltransferase (Pnmt-KO) não sintetizam EPI e apresentam a memória do medo contextual reduzida, sugerindo que a deficiência de EPI afeta seletivamente a memória contextual do medo. O nosso objetivo foi perceber a via pela qual a EPI aumenta a memória contextual do medo. Além disso, também avaliamos se o tempo apaga a memória de medo mediada pela EPI ou se essa memória persiste. Outro objectivo foi perceber se os antagonistas dos recetores adrenérgicos β_2 permitem apagar memórias traumáticas contextuais antigas. Finalmente propomos uma possível terapia farmacológica para memórias traumáticas nas patologias de ansiedade, em particular na síndrome de stresse pós-traumático (PTSD).

Os ratinhos do tipo selvagem (WT) e Pnmt-KO (129x1 / SvJ) foram submetidos ao procedimento de condicionamento do medo após tratamentos específicos com EPI e agonistas (isoprenalina e fenoterol) e antagonistas (sotalol e ICI 118,551) dos recetores adrenérgicos β. As experiências foram avaliadas no primeiro dia (aquisição de medo) e no segundo dia (teste contextual de medo, memória a longo prazo), e algumas experiências um mês após a aquisição do medo (teste contextual de medo, memórias antigas). As catecolaminas foram separadas por HPLC de fase reversa e quantificadas por detecção electroquímica. A glicemia foi medida por colometria.

Nós mostramos que a EPI afeta seletivamente a memória contextual do medo e não a memória auditiva do medo. Além disso, a EPI aumenta a memória do medo contextual, um dia (memória de longo prazo) e um mês após a aquisição do medo (memórias antigas). O mecanismo pelo qual a EPI influencia a memória do medo contextual parece ser através da ativação de recetores adrenérgicos β_2 periféricos. Além disso, a glicose pode ser um importante mediador da EPI a jusante, na memória contextual do medo. Nos ratinhos WT, a concentração plasmática de EPI foi significativamente maior após o teste de aquisição de medo comparativamente com ratos sem o teste. O ICI 118,551 (antagonista dos

recetores adrenérgicos β_2) diminui a memória de medo contextual em ratinhos WT, tanto um dia como um mês após a aquisição do medo.
Em conclusão, a EPI aumenta no plasma após uma experiência aversiva, possivelmente melhorando a memória do medo contextual a longo prazo e até memórias antigas, atuando sobre recetores adrenérgicos β_2 periféricos. Nós sugerimos que o bloqueio de recetores adrenérgicos β_2 com antagonistas pode inibir memórias traumáticas e ser usado como um tratamento para doentes que sofrem de memórias patogénicas, como é o caso da PTSD.

CHAPTER 1

INTRODUCTION

1) Learning and memory

A very large amount of evidence suggests that norepinephrine (NE) and epinephrine (EPI) signaling facilitate cognitive processes such as learning and memory (King and Williams 2009). Learning commonly refers to acquisition of information, such as learning a new word list. In humans, it is usually evaluated using an immediate recall test. On the other hand, memory is the process by which information is encoded, stored and retrieved. There are two types of memory, the declarative (explicit) and the non-declarative (implicit) memory (Elzinga and Bremner 2002).

1.1) Declarative memory

Declarative memory encompasses the storage of consciously learned facts, and comprises both semantic and episodic knowledge (Gabrieli et al. 1988). Episodic memory generally lures upon semantic knowledge, and over time with repetition becomes semantic knowledge. Most standard verbal and visual neuropsychological memory tests evaluate episodic declarative memory. Intense research in this area suggests that the hippocampus, rhinal cortex, and areas of the neo-cortex all play an important role in declarative memory (Elzinga and Bremner 2002).

1.2) Non-declarative memory

Non-declarative memory (or implicit memory) comprises the incidental acquisition of new knowledge and is frequently tested with priming tasks. Non-declarative memory also includes the acquisition of new behaviors or skills through recurrent exposures or trials (procedural memory and motor skills) and associative learning (classical and operant conditioning, and related contextual memory). Evidence suggests that the hippocampus and the amygdala are essential for associative learning (Kim and Jung 2006).

Non-declarative memory, in particular associative learning, can be tested by pairing one stimulus with another, and later evaluate whether a subject has learned to make the association between two stimuli. An example is classical or Pavlovian conditioning, which is a paradigm developed by the Russian physiologist Pavlov. In classical conditioning, a novel stimulus (conditioned stimulus, CS; for example sound) is paired with an unconditioned stimulus (US;

for example food) that usually elicits a reflexive response (unconditioned response, UR; for example salivation). After appropriate training with contingent CS-US presentations, the CS is capable of provoking a response (conditioned response, CR), which often resembles the UR.

1.2.1) Fear Conditioning

Fear conditioning is a behavioral paradigm in which animals learn to forecast aversive events. Fear is considered a self-protective mechanism that developed because of its success in protecting animals from threats. While fear due to certain types of stimuli is innately hardwired, fear can also be learned quickly and enduringly to different stimuli, allowing animals to respond adaptively to changing environmental conditions (Kim and Jung 2006). Fear conditioning is frequently used in associative learning, in which memory for a context-shock association is stabilized via hippocampal-dependent consolidation processes, and memory for a sound-shock association is stabilized through amygdala-dependent consolidation processes (Bergstrom et al. 2013).

In fear conditioning procedure, the CS, such as a context, is paired with an aversive US, such as an electric shock. After numerous shocks in the same context, it is produced an involuntary response, such as freezing in presence of the context (in absence of the shock) (Fanselow 1980). Some studies show that the association between the context and shock is vigorous and long lasting (LeDoux 2000).

2) Emotional arousal

Emotional arousal produced by aversive stressors results in the release of glucocorticoids and catecholamines (EPI and NE) from the adrenal glands and influence memory (Roozendaal 2002). In humans, emotional stimulus are better remembered than neutral stimulus owed to actions of adrenal hormones (glucocorticoids, EPI, NE) (Akirav 2013).

The hypothalamic-pituitary-adrenal (HPA) axis coordinates the stress response. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus (PVN) secrete corticotropin-releasing hormone (CRH) from nerve terminals into circulation, which stimulates the synthesis and release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH in turn stimulates the release

of glucocorticoids from the adrenal cortex. Glucocorticoids orchestrate physiologic behavior to handle stressors (Smith and Vale 2006).

2.1) Corticosteroids and catecholamines

Glucocorticoid and EPI are important stress hormones secreted from the adrenal gland. The adrenal glucocorticoids may stimulate phenylethanolamine-*N*-methyltransferase (PNMT) to convert NE to EPI in the adrenal medulla. On the other hand, by suppressing adrenal glucocorticoid production it might also reduce the secretion of EPI (Munck et al. 1984).

Drugs that increase plasma concentrations of glucocorticoids or catecholamines during, or following learning improve memory in mice and rats (Krugers et al. 2012). A very large amount of evidence suggests that NE and EPI signaling facilitate cognitive processes (Cahill and Alkire 2003). However, the specific role of EPI in memory is less known, because of the difficulty in distinguishing the effects of EPI from those of NE.

3) Adrenal gland

3.1) Adrenal gland overview

The adrenal glands are located just medial to the upper pole of each kidney. Each adrenal gland consists of an inner medulla (produces EPI and NE) and an outer cortex (produces steroid hormones). The medulla and cortex differ in embryological origin, structure, and function. The medulla develops from neural crest tissue and the cortex from mesoderm (Rosol et al. 2001).

The adrenal medulla is constituted by groups of chromaffin cells filled with catecholamine granules, which stock large amounts of EPI and NE. The adrenal cortex is made up of sheets of cells surrounded by capillaries. It is arranged in three zones: the outer zone glomerulosa (synthesis of aldosterone), middle zone fasciculate (synthesis of cortisol), and inner zone reticularis (synthesis of androgens) (Rosol et al. 2001).

The adrenal gland is abundantly vascularised and obtains its chief arterial supply from branches of the inferior phrenic artery, renal arteries and the aorta. These small arteries form an arterial plexus beneath the capsule surrounding the adrenal and then enter a sinusoidal system that enters the cortex and the medulla draining into a single central adrenal vein. The veins drain to the

inferior vena cava and the renal vein. The blood supply is not reduced during stress (Rosol et al. 2001).

3.2) Catecholamines and Enzymes

Tyrosine hydroxylase (TH) catalyzes the formation of 3, 4-dihydroxyphenylalanine (L-dopa) from L-tyrosine. TH is the rate-limiting enzyme in catecholamine biosynthesis, it is regulated by a wide array of physiological mechanisms. These mechanisms are short or long-term and include phosphorylation, feedback inhibition by catecholamines and regulation of mRNA and protein synthesis (Zhang et al. 2014). Then aromatic L-amino acid decarboxylase (AADC) synthesizes dopamine with L-dopa as substrate. These two enzymes are present in all catecholamine-producing cells. Dopamine β-hydroxylase (DBH) catalyzes the hydroxylation of dopamine to NE. This enzyme is present in both NE and EPI producing cells. Finally, PNMT is the methyltransferase that catalyzes the formation of EPI from NE and occurs mainly in EPI-producing cells. The catecholamine phenotype is determined by the specific cell type and harmonized expression of the genes encoding the catecholamine-synthesizing enzymes (Nagatsu 2006).

3.3) Receptors

EPI and NE act at adrenoceptors (ADs). ADs are G protein coupled receptors. β-ADs are divided in β_1 (heart, blood vessels…), β_2 (bronchi, blood vessels…) and β3 (fat…). The relative potency of EPI and NE at βADs is for β_1, EPI=NE and for β_2, EPI>>NE. α-ADs are divided in α_1, (blood vessels, gut sphincters…) and α_2 (presynaptic terminals…). (MacGregor et al. 1996).

3.4) Adrenalectomy

Manuscripts showing the adverse effects of adrenalectomy (ADX) on memory provide evidence for the importance of corticosteroids and catecholamines in animal cognition. The elimination of the adrenal glands causes a dramatic reduction in endogenous circulating corticosterone and catecholamines. Adrenalectomized rats submitted to the Morris water maze show impaired spatial memory for the platform location, which was reversed by dexamethasone supplementation (Roozendaal et al. 1996). These rats also

show deficiencies in associative learning tasks, such as passive avoidance (Borrell et al. 1983) and acquired immobility response (Peeters et al. 1992). It is important to understand that no impairment was observed when only the adrenal medulla was removed (thus sparing the adrenal cortex and endogenous corticosterone levels)(Oitzl et al. 1995), however residual adrenal medulla could be sufficient to produce catecholamines. In addition, memory deficits detected resulting of ADX, which relentlessly depletes peripheral concentrations of EPI and glucocorticoids, were reversed by the infusion of NE into the amygdala (Liang et al. 1986). These studies provide evidence of the modulatory effects of corticosteroids and catecholamines on animal cognition (Mizoguchi et al. 2004).
It has been problematic to decode the role of catecholamines with the frequently used adrenal medullectomy because this process can harm the adrenal cortex, changing the release of corticosteroids, and it eliminates the release of other adrenal amines and peptides, such as NE, chromogranin A, catestatin and neuropeptide (Harrison and MacKinnon 1966).

3.5) Knock-out mice

Studies with knockout mice have been important in the identification of novel molecular mechanisms in learning and memory (Nguyen et al. 2000). Dopamine β-hydroxylase knockout mice (unable to synthesize both NE and EPI) exhibit reduced contextual-fear memory. Poor conditioned fear in these mice was restored by β-AD agonist isoprenaline (Murchison et al. 2004). However, the inquiry continued whether reduced contextual-fear memory is due to the absence of both catecholamines (NE and EPI) or if lack of EPI only could create this phenotype. Therefore, the precise part of EPI in these processes is less known, because of the difficulty in differentiating the effects of NE from those of EPI.
Another method is the use of Pnmt inhibitors to block the EPI synthesis in vivo, but most of them also hinder monoamine oxidase and β_2-ADs (Bondinell et al. 1983). These disadvantages for the clarification of the specific role of EPI on fear learning are avoided by using an EPI deficient mice model produced by knocking out the Pnmt gene (Ebert et al. 2004).
The Pnmt knockout (Pnmt-KO) mouse model ($Pnmt^{-/-}$) used in these studies was originally designed to evaluate the distribution of adrenergic cells by Ebert

and coworkers (Ebert et al. 2004). The Cre-recombinase gene was inserted into the mouse Pnmt locus, disrupting the functional expression of Pnmt (Ebert et al. 2004) .The targeted disruption of the Pnmt gene led to the creation of mice that were deficient in their capability to produce EPI. It is likely that the absence of EPI in Pnmt-KO mice will provoke some adaptive changes in the metabolism of catecholamines. Nevertheless, these mice were still able to produce NE. In fact, increased levels of NE have been found in adrenal glands of the Pnmt-KO mice (Ebert et al. 2004).

There were no gross apparent developmental defects in homozygous mice lacking EPI. In addition, they persisted to adulthood, were physically indistinguishable from wild type (WT) and heterozygous littermates and were able to breed efficiently (Ebert et al. 2004). Thus, these mice are a model for investigating the physiologic action of EPI. Afterwards, Toth et al (Toth et al. 2013) showed that Pnmt-KO mice presented reduced contextual-fear learning, suggesting that EPI deficiency selectively affects contextual-fear memory and these mice exhibit less selective memory effects on highly emotional memories.

3.6) Influence of peripheral epinephrine in the brain

Peripheral EPI increases under highly arousing states (Chen and Williams 2012). Since EPI does not pass from the peripheral circulation into the brain, there must be mechanisms that allow the influence of EPI in the brain. There are two major hypotheses about EPI specific pathway in influencing memory.

Electrophysiological and pharmacological studies suggest that the effect of EPI on memory and in facilitating NE output in the amygdala may be initiated by the stimulation of peripheral vagal fibers that project to the brain (McIntyre et al. 2012). Chen et al showed simultaneously that the intraperitoneal injection of EPI (0.3 mg/Kg) lead to the increasing firing discharge along afferent fibers of the vagus nerve mediated through β-ADs and this was accompanied by increases in extracellular concentration of NE in the basolateral amygdala (Chen and Williams 2012). The information is conducted by ascending vagal fibers to the nucleus of the solitary tract (NTS) situated in the brainstem. The memory-modulating EPI effects causes glutamatergic release in the NTS, stimulates locus coeruleus and noradrenergic release in the amygdala and hippocampus. Pharmacological studies show that the blockade of NTS

glutamatergic receptors attenuates the enhancement in memory produced by EPI injections (King and Williams 2009).

The other hypothesis is centered in glucose, which is released into the blood after activation of hepatic ADs by EPI (Gold 2014). Maintenance of glucose supply to the brain is a primacy for basic brain processes. Therefore, the brain is a chief consumer of glucose during stress, and the fact that the brain has only insignificant energy reserves makes it critically reliant on the supply from the periphery. Memory formation is limited by the hippocampus glucose supply that regulates the ability to form memories. Administration of glucose, either peripherally or directly to the hippocampus, can increase memory in a dose-dependent way (Messier 2004).

4) Potential clinical applications

The importance of this study is further enhanced due to the knowledge that in anxiety disorders contextual factors contribute to fear generalization, traumatic memory retrieval and relapse after exposure therapy (Mineka et al. 1999). Therefore, the mechanisms that lie beneath the retrieval of emotional associations due to context may have implications for the study and treatment of anxiety disorders.

The findings on memory consolidation have generated unlimited attention in the medical community, particularly among those who treat disorders that are based on pathogenic memories. Since post-traumatic stress disorder (PTSD) is characterized by the existence of strong and recurrently recalled memories (Alberini 2011), it could benefit from targeting memory consolidation, as a possible therapeutic approach. On the other hand, traumatic exposure is known to increase the risk for depression, phobias, panic disorder, obsessive-compulsive disorder and addiction (Dohrenwend 2000).

PTSD is an incapacitating and chronic disorder resulting from exposure to life frightening trauma and stress. It is frequently associated with shocking wartime experiences, but it affects both military persons and civilians (Ursano et al. 2016). The PTSD prevalence in the world is 2%-20% depending on the country and exposure to life-threatening events (Atwoli et al. 2015). In PTSD, the traumatic event (US) causes a robust hormonal stress response, which facilitates the development of a strong and enduring memory of the trauma

(UR). Subsequent recall of the event (CR) in reply to cues and reminders (CS) releases additional stress hormones. This may even promote consolidation of memory, leading to PTSD symptoms such as flashbacks, nightmares, and anxiety (Pitman and Delahanty 2005). The persistence of PTSD can be clarified in terms of trauma-induced consolidation of the memory trace. In fact, it is hypothesized that noradrenergic hyperactivity and stress hormones facilitate encoding and consolidation (O'Donnell et al. 2004).

CHAPTER 2

OBJECTIVES

Toth et al showed reduced contextual-fear learning in Pnmt-KO mice, suggesting that EPI deficiency selectively affects contextual-fear learning (Toth et al. 2013). However, they did not explain the mechanism responsible for the impaired contextual-fear memory in these mice. In view of this, one aim was to evaluate contextual-fear memory with EPI treatment in Pnmt-KO and WT mice. Another aim was to understand the pathway by which EPI increases contextual-fear memory using β-AD agonists and antagonists. In addition, we also evaluated if time erases EPI-mediated fear memory or if this memory persists in the long-term, and even as old memories. We also tested NE in the same doses as EPI to understand if its peripheral action in contextual-fear memory is similar or not. Another aim was to understand if β_2-AD antagonists could erase traumatic contextual memories. Finally, we propose a possible pharmacological therapy for traumatic memories in anxiety disorders, in particular PTSD.

CHAPTER 3

Ester Alves, Nikolay Lukoyanov, Paula Serrão, Daniel Moura, Mónica Moreira-Rodrigues. Epinephrine increases contextual learning through activation of peripheral β_2-adrenoceptors. Psychopharmacology. 233: 2099, 2016.

CHAPTER 3 - Epinephrine increases contextual learning through activation of β_2-adrenoceptors

Psychopharmacology
DOI 10.1007/s00213-016-4254-5

ORIGINAL INVESTIGATION

Epinephrine increases contextual learning through activation of peripheral β_2-adrenoceptors

Ester Alves[1,2] · **Nikolay Lukoyanov**[3] · **Paula Serrão**[2,4] · **Daniel Moura**[2,4] · **Mónica Moreira-Rodrigues**[1,2,5]

Received: 7 August 2015 / Accepted: 22 February 2016

Abstract
Rationale Phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) mice are unable to synthesize epinephrine and display reduced contextual fear. However, the precise mechanism responsible for impaired contextual fear learning in these mice is unknown.
Objectives Our aim was to study the mechanism of epinephrine-dependent contextual learning.
Methods Wild-type (WT) or Pnmt-KO (129x1/SvJ) mice were submitted to a fear conditioning test either in the absence or in the presence of epinephrine, isoprenaline (non-selective β-adrenoceptor agonist), fenoterol (selective β_2-adrenoceptor agonist), epinephrine plus sotalol (non-selective β-adrenoceptor antagonist), and dobutamine (selective β_1-adrenoceptor agonist). Catecholamines were separated by reverse-phase HPLC and quantified by electrochemical detection. Blood glucose was measured by coulometry.
Results Re-exposure to shock context induced higher freezing in WT and Pnmt-KO mice treated with epinephrine and fenoterol than in mice treated with vehicle. In addition, freezing response in Pnmt-KO mice was much lower than in WT mice. Freezing induced by epinephrine was blocked by sotalol in Pnmt-KO mice. Epinephrine and fenoterol treatment restored glycemic response in Pnmt-KO mice. Re-exposure to shock context did not induce a significant difference in freezing in Pnmt-KO mice treated with dobutamine and vehicle.
Conclusions Aversive memories are best retained if moderately high plasma epinephrine concentrations occur at the same moment as the aversive stimulus. In addition, epinephrine increases context fear learning by acting on peripheral β_2-adrenoceptors, which may induce high levels of blood glucose. Since glucose crosses the blood–brain barrier, it may enhance hippocampal-dependent contextual learning.

Keywords Epinephrine · Contextual learning · β_2-adrenoceptors · Phenylethanolamine-*N*-methyltransferase · Phenylethanolamine-*N*-methyltransferase knockout mice

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

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 Anatomy, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal
4 Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto (FMUP), Porto, Portugal
5 Laboratory of General Physiology, ICBAS, University of Porto, R. Jorge Viterbo Ferreira, 228, Building 2, Floor 4, Cabinet 22, 4050-313 Porto, Portugal

Published online: 02 March 2016

Introduction

Humans remember emotional events better than neutral ones. This is due to actions of the adrenal stress hormones (epinephrine, norepinephrine, glucocorticoids, etc.) that act on the brain structures responsible for memory (Akirav and Maroun 2013). On the other hand, in the early 80s, McGaugh et al. suggested that both peripheral and central β-adrenergic activation might influence memory consolidation (Roozendaal and McGaugh 2011).

Fear is considered a defense mechanism that evolved because of its evolutionary success in protecting animals from

danger. Fear to certain kinds of stimuli is innately hardwired, but it can also be rapidly and lastingly acquired to different stimuli, allowing animals to respond adaptively to new or changing environmental situations (Kim and Jung 2006). Fear conditioning is a behavioral paradigm by which organisms learn to predict aversive events and relate them to an innocuous stimulus, such as a specific context or tone. Fear and anxiety may develop as a response to the environmental context and to the discrete cue during fear conditioning (Grillon 2008).

Epinephrine (0.1–1.0 mg/kg, i.p.) administered immediately after training does not modulate fear conditioning to context or tone in mice (Lee et al. 2001). Since plasma epinephrine rapidly increases under stress (Goldstein and Kopin 2008), pre-training and pre-testing epinephrine treatment could likely be a better option to reveal the physiological effects of epinephrine in fear conditioning.

Epinephrine is a hydrophilic molecule and does not readily cross the blood–brain barrier. There are two major hypotheses about the specific mechanism by which epinephrine influences behavior. One hypothesis is the activation of β-adrenergic receptors in vagus nerve by epinephrine which might transmit the information to the brain through afferent neuronal axons. The other hypothesis is that glucose, released into the blood after activation of hepatic adrenoceptors, mediates the effects of epinephrine in memory (Gold 2014).

Strain and knockout mice studies have been critical to identify novel genetic and molecular mechanisms in learning and memory (Tipps et al. 2014). Dopamine β-hydroxylase knockout mice (unable to synthesize both norepinephrine and epinephrine) exhibit reduced contextual fear learning (Murchison et al. 2004). Deficient conditioned fear in these mice was restored by β-adrenoceptor agonist isoprenaline (Murchison et al. 2004). However, the question remained whether reduced contextual fear learning is due to the absence of both norepinephrine and epinephrine or if absence of epinephrine alone could originate this phenotype.

On the other hand, the role of epinephrine with the commonly used adrenal medullectomy has been difficult to decipher because this procedure can damage the adrenal cortex, altering the release of corticosteroids, and of other adrenal amines and peptides, such as norepinephrine, chromogranin A, catestatin, and neuropeptide Y (Harrison and Seaton 1966). An alternative approach is to use phenylethanolamine-*N*-methyltransferase (Pnmt) inhibitors to block epinephrine synthesis in vivo, but most of them also inhibit monoamine oxidase and β_2-adrenoceptors (Bondinell et al. 1983). These drawbacks for elucidation of the specific role of epinephrine on fear learning are avoided by using an epinephrine-deficient animal model generated by knocking out Pnmt gene (Ebert et al. 2004).

One of our aims was to evaluate fear conditioning in mice treated with epinephrine immediately before fear acquisition and tests. On the other hand, epinephrine released from adrenal glands in wild-type (WT) mice treated with vehicle during fear conditioning studies could be a confusing variable because endogenous epinephrine also acts upon β-adrenoceptors. As an alternative, Pnmt-KO mice do not have endogenous epinephrine and avoid this problem. Recently, Toth et al. (2013) showed that Pnmt-KO mice, which are unable to synthesize epinephrine, display reduced contextual fear learning (Toth et al. 2013). However, the precise mechanism responsible for impaired contextual fear learning in these mice is unknown. Another aim of this study was to define the mechanism by which epinephrine influences contextual fear learning in Pnmt-KO and WT mice.

Materials and methods

Animals All animal care and experimental protocols were carried out in accordance with the European Directive 63/2010/EU, transposed to the Portuguese legislation by the Directive Law 113/2013. Pnmt-KO mice (*Pnmt*−/−) were produced by disruption of *Pnmt* locus by insertion of Cre-recombinase in exon 1 (Ebert et al. 2004). A couple of Pnmt-KO mice were kindly provided by Steven N. Ebert, and animals were bred in our conventional vivarium. The presence of the *Pnmt*−/− alleles was verified by polymerase chain reaction of ear DNA (data not shown). Pnmt-KO ($n=116$) and WT ($n=36$) male mice (129x1/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.

Drugs Isoflurane 100 % was obtained from Abbott laboratories (Queenborough, UK). (−)-Epinephrine (+)-bitartrate salt, L-(−)-norepinephrine (+)-bitartrate salt monohydrate, isoprenaline hydrochloride, fenoterol hydrobromide, (±)-sotalol hydrochloride, and dobutamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Catecholamine assay For model verification, mice were anesthetized (isoflurane 100 %, 200 μL by inhalation) and the left adrenal gland was removed and placed in 0.2 M perchloric acid overnight, at 4 °C. Then, the supernatant of the left adrenal gland samples was diluted and centrifuged for 2 min, 4 °C, at 2700×*g*. In another group, mice were injected with epinephrine (0.1 mg/kg, i.p.) or vehicle (0.9 % NaCl), anesthetized (ketamine, 100 mg/kg and xylazine, 10 mg/kg, i.p.), and blood was collected by a left ventricle puncture. The time span since injections and collection of blood was about 5 min to predict plasma concentration of epinephrine during fear conditioning tests. Blood was centrifuged (1500×*g*, 12 min), and supernatant was collected and kept under

−20 °C until use. Catecholamines in plasma samples were concentrated by alumina. Catecholamines (epinephrine and norepinephrine) in samples were separated by reverse-phase HPLC and quantified by electrochemical detection, with a detection limit between 350 and 1000 fmol (Moreira-Rodrigues et al. 2007, 2014).

Fear conditioning procedure The fear conditioning procedure was adapted as previously described (Lukoyanov and Lukoyanova 2006; Manceau et al. 2012). The conditioning chambers consisted of a clear Plexiglas box equipped with a metal grid floor, wired to a stimulus generator. The animal's behavior was recorded with a digital video camera Sony DCR-SR58E (Sony Corporation, Japan). Freezing was defined as the absence of movement except for respiration. Conditioning was assessed by the freezing response because this response is a widely used indicator of conditioned fear (Fanselow and Kim 1994). Freezing was only scored if mice remained inactive for at least 3 s. The percentage of accumulated freezing time was then calculated. On the first day (fear acquisition; 6 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. The time between the offset of the aversive unconditioned stimulus and the onset of the innocuous conditioned stimulus of the next trial was termed intertrial interval (ITI, 40 s). On the second day (context fear test; 8 min), 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). On the first and second days, the chambers were cleaned and wiped with 1 % acetic acid. On the third day (cue fear test; 6 min), tactile, odor, and visual context was changed to minimize generalization from the conditioning context. The new chamber was composed of black Plexiglas except for the bottom that was composed of a piece of black carpet. The chamber was scented with lemon juice instead of acetic acid. Mice were undisturbed for 3 min, and then three tones (tone trials) were presented for 20 s at intervals of 40 s. The time between the offset of the innocuous conditioned stimulus and the onset of the innocuous conditioned stimulus of the next trial was termed ITI (40 s). Freezing was scored during the 3-min acclimation period and during the 3-min tone presentation period.

Behavioral experiment 1 As shown in Fig. 2, WT and Pnmt-KO mice were submitted to fear conditioning procedure after epinephrine (0.1 mg/kg, i.p., 3 min; WT, $n=10$; Pnmt-KO, $n=5$) (Introini-Collison and Baratti 1992; Lee et al. 2001) or vehicle (0.9 % NaCl; WT, $n=10$; Pnmt-KO, $n=5$) treatment, in both pre-training and pre-testing.

Behavioral experiment 2 As shown in Fig. 3a, Pnmt-KO mice were submitted to fear conditioning procedure after epinephrine (0.1 mg/kg, i.p., 3 min, $n=6$) (Introini-Collison and Baratti 1992; Lee et al. 2001), epinephrine (0.1 mg/kg, i.p., 3 min) plus sotalol (non-selective β-adrenoceptor antagonist; 2 mg/kg, i.p., 30 min, $n=7$) (Lee et al. 2001), or vehicle (0.9 % NaCl, $n=5$) treatment, in both pre-training and pre-testing.

Behavioral experiment 3 As shown in Fig. 3b, Pnmt-KO mice were submitted to fear conditioning procedure after isoprenaline (non-selective β-adrenoceptor agonist; 2 mg/kg, s.c., 30 min, $n=8$) (Sullivan et al. 1989; Yuan et al. 2000) or vehicle (0.9 % NaCl, $n=5$) treatment, in both pre-training and pre-testing.

Behavioral experiment 4 As shown in Fig. 4a, WT and Pnmt-KO mice were submitted to fear conditioning procedure after fenoterol (selective β_2-adrenoceptor agonist; 2.8 mg/kg, i.p., 10 min; WT, $n=5$; Pnmt-KO, $n=5$) (Ryall et al. 2002, 2004) or vehicle (0.9 % NaCl; WT, $n=10$; Pnmt-KO, $n=5$) treatment, in both pre-training and pre-testing.

Behavioral experiment 5 As shown in Fig. 4b, Pnmt-KO mice were submitted to fear conditioning procedure after dobutamine (selective β_1-adrenoceptor agonist, 0.02 mg/kg, i.p., 5 min, $n=6$) (Guarini et al. 1997) or vehicle (0.9 % NaCl, $n=5$) treatment, in both pre-training and pre-testing.

Behavioral experiment 6 As shown in Fig. 6, Pnmt-KO mice were submitted to fear conditioning procedure after fenoterol (selective β_2-adrenoceptor agonist; 2.8 mg/kg, i.p., 10 min) (Ryall et al. 2002, 2004) or vehicle (0.9 % NaCl, $n=9$) treatment, just pre-training (day 1, $n=5$), just pre-testing (day 2, $n=6$), and both pre-training and pre-testing (day 1 + day 2, $n=5$).

Glucose quantification Some fear conditioning experiments were repeated, and blood glucose concentration was determined before and after the fear conditioning tests in conscious animals. Afterwards, glycemic variation (Δ Glycemia) was calculated as the glucose concentration difference between after and before the fear conditioning test. Blood glucose concentration in capillary tail blood was assessed by coulometry (Alva 2008).

Statistical analysis Results are presented as means ± standard error of the means (SEM) for the indicated number of determinations. Data from the fear conditioning tests were analyzed by two-way ANOVA (one dependent variable and two independent variables) or three-way ANOVA (one dependent variable and three independent variables). We used the Newman-Keuls test for multiple comparisons. Catecholamine

concentrations and glycemic increase were analyzed by Student's *t* test. $P<0.05$ was assumed to denote a significant difference. GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) or SPSS (IBM, New York, NY, USA) statistics software packages were used for statistical analysis.

Results

Pnmt-KO mice, an epinephrine-deficient mice model

Pnmt-KO mice presented vestigial epinephrine content when compared to WT mice in both adrenal glands (53.9 ± 6.2 vs 3799.4 ± 126.5 pmol/mg) and plasma (Table 1). Representative chromatograms of catecholamines in adrenal glands of WT and Pnmt-KO mice are presented in Fig. 1. The standard solution and adrenal gland of WT mice presented two peaks corresponding to norepinephrine and epinephrine, whereas in Pnmt-KO mice, the peak corresponding to epinephrine was almost undetected (Fig. 1).

Decreased contextual fear learning in Pnmt-KO mice

On the first day of the fear conditioning test, there were no differences in the freezing response between WT and Pnmt-KO mice (F (1, 78) = 3.73, p = 0.10, Fig. 2a) during fear acquisition.

On the context fear test, the freezing response in Pnmt-KO mice was lower than in WT mice (Figs. 2a and 4a). A genotype effect was observed in Fig. 2a (F (1, 112) = 7.53, p = 0.007) and in Fig. 4a (F (1, 84) = 75.79, $p<0.0001$). The test-induced increase in glycemia was lower in Pnmt-KO than in WT mice (Fig. 5a). The basal (i.e., pre-conditioning) plasma glucose concentration in WT and Pnmt-KO mice was not different (98.7 ± 2.4 vs 118.5 ± 12.8 mg/dL).

Table 1 Blood plasma concentration of epinephrine (EPI) (pmol/mL) in mice treated with EPI (0.1 mg/kg) or vehicle (NaCl 0.9 %)

	Vehicle	EPI
WT	16.8 ± 2.1	50.1 ± 7.2[a]
Pnmt-KO	Undetectable[a]	24.6 ± 7.4[b]

Values are means ± SEM of five to eight mice per group

Pnmt-KO phenylethanolamine-*N*-methyltransferase knockout mice, *WT* wild-type mice

[a] Significantly different from correspondent values in WT mice treated with vehicle ($p<0.05$)

[b] Significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p<0.05$)

There were no significant differences in freezing between WT and Pnmt-KO mice during cue response (F (1, 75) = 3.75, p = 0.10; Fig. 2c).

Contextual freezing response in WT and Pnmt-KO mice treated with epinephrine

In most of the experiments, drugs were given before fear conditioning (pre-training) and before the animals were tested (pre-testing) because Pnmt-KO mice do not have epinephrine during fear conditioning and testing.

On the first day of the fear conditioning test, there were no differences in the freezing response between WT and Pnmt-KO mice treated with epinephrine compared to mice treated with vehicle (F (1, 78) = 1.61, p = 0.21; Fig. 2a).

On the second day, mice were re-exposed to the shock context (context fear test) and a drug effect (F (1, 112) = 114.4, $p<0.001$) was observed (Fig. 2b). There was a significant interaction between genotype and drug (F (1, 112) = 46.34, $p<0.001$) and no interaction between genotype, drug, and time (F (3, 112) = 1.05, p = 0.373) (Fig. 2b). Re-exposure to shock context induced higher freezing in both WT and Pnmt-KO mice treated with epinephrine compared to mice treated with vehicle (Fig. 2b). Plasma concentration of epinephrine in mice treated with epinephrine 0.1 mg/kg was higher than in mice treated with vehicle (Table 1).

In modified context test, there were no differences in freezing responses between WT and Pnmt-KO mice treated with epinephrine compared to mice treated with vehicle (F (1, 75) = 1.42, p = 0.24; Fig. 2c).

Contextual freezing response in WT and Pnmt-KO mice is mediated through activation of peripheral β_2-adrenoceptors

In context fear retention test, re-exposure to shock context induced lower freezing in Pnmt-KO mice treated with epinephrine plus sotalol than in Pnmt-KO mice treated with epinephrine. A significant drug effect (F (2, 15) = 58.03, $p<0.0001$; Fig. 3a), a time effect (F (3, 45) = 5.39, p = 0.003; Fig. 3a), and an interaction between drug and time (F (6, 45) = 4.63, p = 0.001; Fig. 3a) were observed. Also, re-exposure to shock context induced higher freezing in Pnmt-KO mice treated with isoprenaline than in mice treated with vehicle (F (1, 15) = 62.94, $p<0.0001$; Fig. 3b).

Re-exposure to shock context induced an increase in freezing in WT and Pnmt-KO mice treated with fenoterol in comparison to mice treated with vehicle (Fig. 4a). A drug effect (F (1, 84) = 63.58, $p<0.0001$) and a time effect (F (3, 84) = 4.73, p = 0.004) (Fig. 4a) were observed. There was not a significant interaction among genotype, drug, and time (F (3, 84) = 1.65, p = 0.184; Fig. 4a). In Pnmt-KO mice, epinephrine and fenoterol treatment caused an increase in glycemia (Fig. 5b,

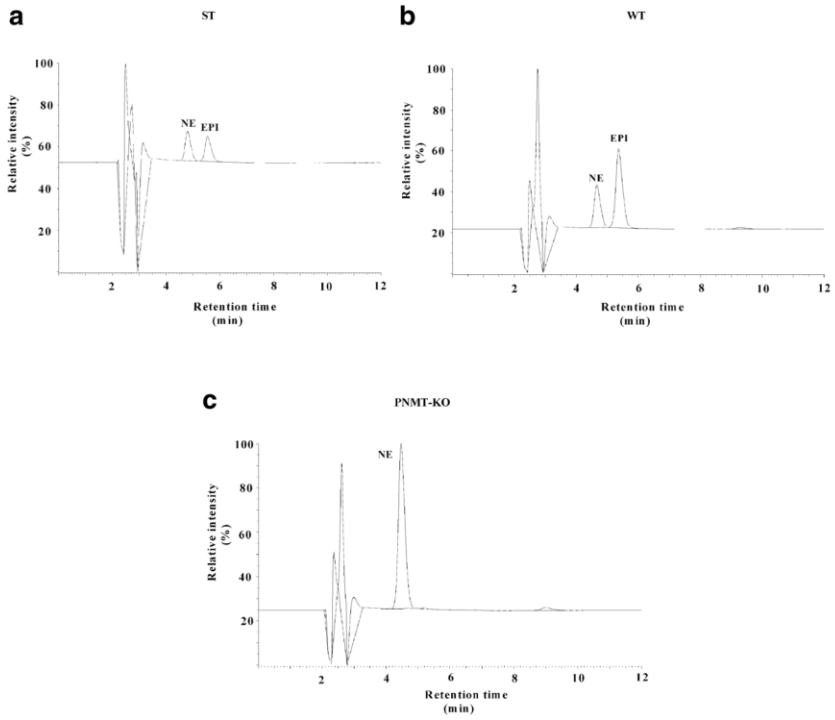


Fig. 1 Representative chromatograms of catecholamines in **a** standard (*St*) and adrenal glands of **b** wild-type (*WT*) and **c** phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice. *NE* norepinephrine, *EPI* epinephrine

c). Re-exposure to shock context did not induce a significant difference in freezing in Pnmt-KO mice treated with dobutamine and vehicle (F (1, 9) = 0.075, p = 0.79; Fig. 4b).

To understand the drug effects on encoding and retrieval, fenoterol was injected just in pre-training (day 1), just in pre-testing (day 2), and both in pre-training and pre-testing (day 1 + day 2) in Pnmt-KO mice. Re-exposure to shock context induced an increase in freezing in Pnmt-KO mice treated with fenoterol injected in pre-training, in pre-testing, and in both, in comparison to mice treated with vehicle (Fig. 6). Pnmt-KO mice injected in both days exhibited a higher increase in freezing than those injected just in pre-training or in pre-testing. A drug effect (F (3, 84) = 134.3, p < 0.0001; Fig. 6) and a time effect (F (3, 84) = 34.6, p < 0.0001; Fig. 6) as well as a significant interaction between time and drug (F (9, 84) = 10.5, p < 0.0001; Fig. 6) were observed.

Discussion

Our results show that epinephrine selectively affected contextual fear learning by acting on peripheral β_2-adrenoceptors in WT mice. In addition, the lack of physiological effects of peripheral epinephrine in Pnmt-KO mice during shock context presentations directly contributes to impaired fear conditioning. In these mice, peripheral β_2-adrenoceptors also seem to be the specific target in contextual fear learning.

Lee et al. (2001) did not observe any effects in context or tone fear with pre-training and pre-testing epinephrine treatments (0.1 mg/kg, i.p., 3 min prior to training and testing) in rats. Yet, higher epinephrine dosages were not used in these animals. In mice, post-training epinephrine (0.1–1.0 mg/kg, i.p.) treatment in fear conditioning procedure does not modulate fear conditioning to context or tone (Lee et al. 2001). However, our results showed that pre-training and pre-testing treatment with epinephrine increased contextual fear in WT mice. It seems that WT mice are capable of retaining the aversive memory better when high plasma epinephrine concentrations and the aversive stimulus occur together during acquisition and context fear test. Epinephrine selectively affected contextual fear learning since cue fear learning was not different between groups.

To evaluate fear learning, either the classical fear conditioning or the inhibitory avoidance can be used, although there are some mechanism differences between them (Wilensky et al. 2000). In mice, epinephrine (0.1 mg/kg, i.p.) facilitates retention in the inhibitory avoidance test (Introini-Collison and Baratti 1992), which is in agreement with our results in the fear conditioning test. This memory modulating effects of epinephrine can be blocked by the low lipophilic β-

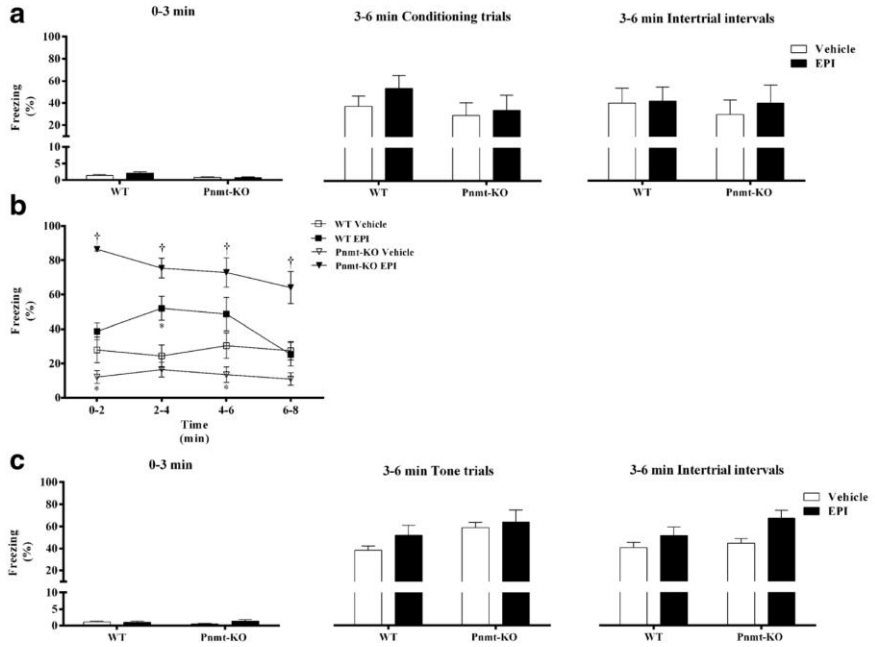


Fig. 2 Behavioral experiment 1. Freezing on the **a** first day (fear acquisition), **b** second day (context fear test), and **c** third day (cue fear) of fear conditioning procedure in wild-type (*WT*) and phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl 0.9 %) or epinephrine (EPI, 0.1 mg/kg). The time between the offset of the aversive unconditioned stimulus (or the innocuous conditioned stimulus in **c**) and the onset of the innocuous conditioned stimulus of the next trial was termed intertrial interval (*ITI*, 40 s). Each *group point* represents the mean of five to ten mice per group, and *error bars* represent SEM. *Asterisk*: significantly different from correspondent values in WT mice treated with vehicle ($p < 0.05$). *Dagger*: significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$)

antagonist sotalol, suggesting that epinephrine effects on memory are initiated by activation of peripheral β-adrenoceptors (Introini-Collison and Baratti 1992). This was one of the first hypotheses about the mechanism of epinephrine in fear learning (for review, Roozendaal and McGaugh 2011). However, to our knowledge, the influence of specific peripheral β_1 or β_2-antagonists or agonists in inhibitory avoidance test was not evaluated. In these experiments, epinephrine was given immediately after training and we did not test this possibility.

Our results in WT mice showed that pre-training and pre-testing treatments with a selective β_2-adrenoceptor agonist (fenoterol) resulted in an increased contextual fear. Although epinephrine acts as a non-selective agonist of the adrenergic receptors, it is the only biogenic catecholamine that has affinity for β_2-adrenoceptors at physiologically relevant concentrations (Lands et al. 1967). Since neither epinephrine (Weil-Malherbe et al. 1959) nor fenoterol (Rominger and Pollmann 1972) cross the blood–brain barrier, we propose that epinephrine increases context fear learning by acting on peripheral β_2-adrenoceptors.

On the other hand, dopamine β-hydroxylase knockout mice are unable to synthesize both norepinephrine and epinephrine. Dopamine β-hydroxylase knockout mice exhibited reduced contextual fear learning (Murchison et al. 2004). Otherwise, Pnmt-KO mouse, which is generated by knocking out the Pnmt gene, is deficient in epinephrine only (Ebert et al. 2004). In agreement with Sun et al. (2008), only vestigial amounts of epinephrine were found in the adrenal medulla and plasma of Pnmt-KO mice. Toth et al. (2013) suggested that epinephrine deficiency selectively decreases contextual fear learning in Pnmt-KO mice, which is in agreement with our results.

In order to explore the basis of this memory impairment in Pnmt-KO mice, epinephrine and β-agonists were injected prior to training and testing. Epinephrine, non-selective β-adrenoceptor (isoprenaline), and selective β_2-adrenoceptor agonist (fenoterol) treatments in Pnmt-KO mice restored the expression of contextual fear. Moreover, in Pnmt-KO mice, increased freezing induced by epinephrine was blocked by a β-adrenoceptor antagonist (sotalol). These results confirm that the lack of physiological effects of peripheral epinephrine during shock context presentations directly contributes to impaired fear conditioning in these mice. In addition, since selective β_1-adrenoceptor agonist (dobutamine) does not increase freezing in Pnmt-KO mice, peripheral β_2-adrenoceptors are also the specific target in contextual fear learning in these mice. On the other hand, Pnmt-KO mice

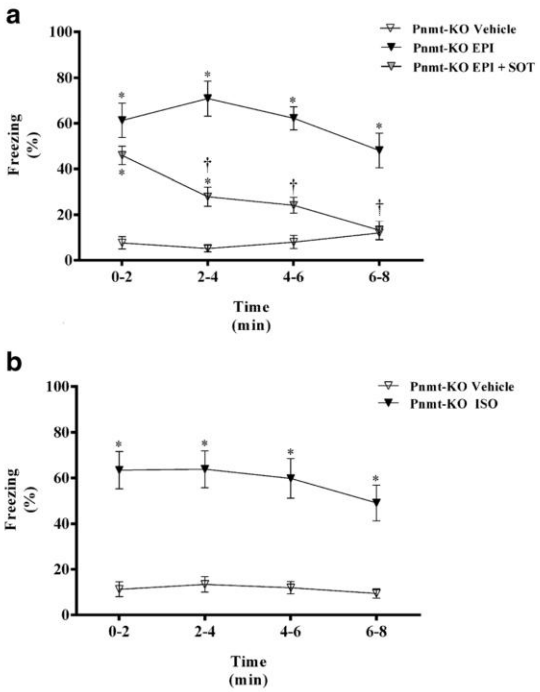


Fig. 3 Behavioral experiments 2 and 3. Freezing on the second day (context fear test) of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl 0.9 %) and **a** epinephrine (*EPI*, 0.1 mg/kg) and EPI (0.1 mg/kg) plus sotalol (*SOT*, non-selective β-adrenoceptor antagonist; 2.0 mg/kg) (behavioral experiment 2) or **b** isoprenaline (*ISO*, non-selective β-adrenoceptor agonist, 2.0 mg/kg) (behavioral experiment 3). Behavioral experiments 2 and 3 were performed and analyzed separately. Each *group point* represents the mean of five to eight mice per group, and *error bars* represent SEM. *Asterisk*: significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$). *Dagger*: significantly different from correspondent values in Pnmt-KO mice treated with EPI (0.1 mg/kg) ($p < 0.05$)

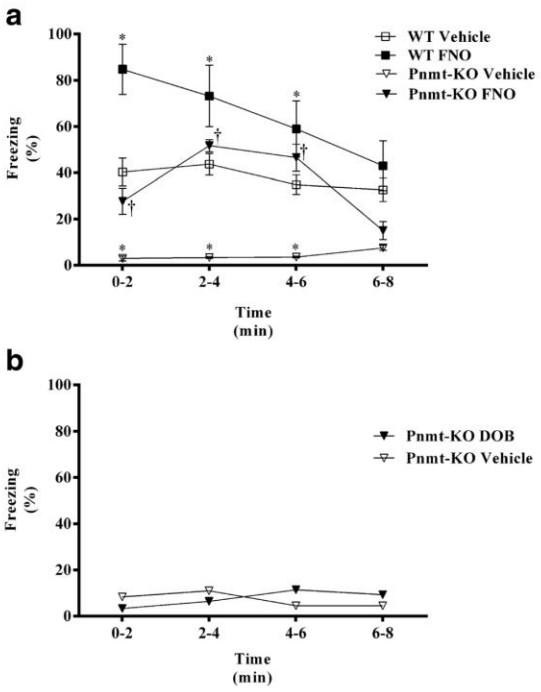


Fig. 4 Behavioral experiments 4 and 5. Freezing on the second day (context fear test) of fear conditioning procedure in wild-type (*WT*) or phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl, 0.9 %) and **a** fenoterol (*FNO*, selective β_2-adrenoceptor agonist, 2.8 mg/kg) (behavioral experiment 4) or **b** dobutamine (*DOB*, selective β_1-adrenoceptor agonist, 0.02 mg/kg) (behavioral experiment 5). Behavioral experiments 4 and 5 were performed and analyzed separately. Each *group point* represents the mean of five to ten mice per group, and *error bars* represent SEM. *Asterisk*: significantly different from correspondent values in WT mice treated with vehicle ($p < 0.05$). *Dagger*: significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$)

injected with fenoterol in both days exhibited a higher increase in freezing than those injected just in pre-training or in pre-testing, favoring the possibility that β_2-adrenoceptors are important in both encoding and retrieval.

On the other hand, epinephrine restores the glycemic response at the same time as context fear learning in Pnmt-KO mice. It is known that the brain uses glucose almost exclusively as a primary energy source and that its storage is limited. In addition, it is well established that hepatic glucose production increases in response to a surge in plasma epinephrine, which results from both stimulation of glycogenolysis and gluconeogenesis (Dufour et al. 2009; Gray et al. 1980; Rizza et al. 1980). Furthermore, John et al. (1990) showed that isoprenaline (β-adrenoceptor agonist) also elicits a hyperglycemic response which is attenuated by a selective β_2-adrenoceptor antagonist (ICI 118551) in rats (John et al. 1990). Since glucose crosses the blood–brain barrier, it may modulate memory. Raised blood glucose levels may increase acetylcholine synthesis in the hippocampus (Durkin et al. 1992; Pych et al. 2005) or provide additional energy to specific neural components and modulate neuronal excitability and neurotransmitter release (McNay and Gold 2002). On the other hand, it has been shown that glucose consumption leads to superior retention of hippocampal-dependent contextual learning (Glenn et al. 2014). Therefore, glucose may be an important down-stream mediator of epinephrine actions in contextual learning.

Also, in humans, β-adrenoceptors are involved in contextual fear conditioning, contrary to cued fear conditioning (Grillon et al. 2004). In addition, in anxiety disorders, contextual factors contribute to fear generalization, traumatic memory retrieval, and relapse after exposure therapy. The mechanisms that underlie the recovery of emotional associations due

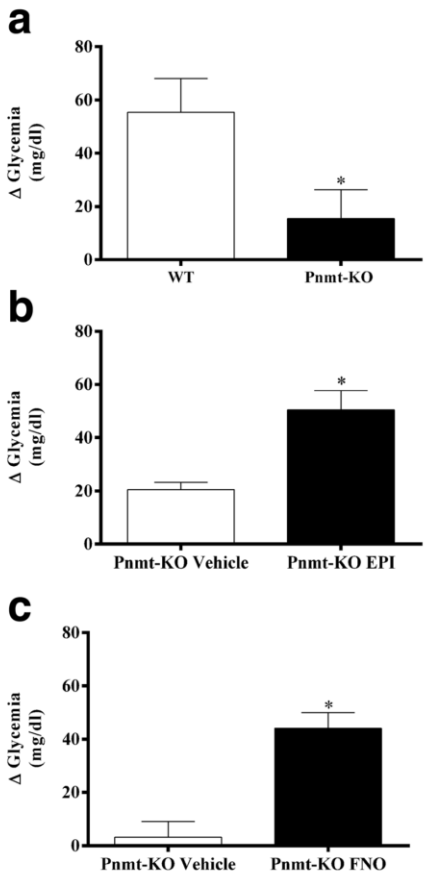


Fig. 5 Glycemic increase in replicated fear conditioning experiments. **a** Wild-type (*WT*) and phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice. Pnmt-KO mice treated with vehicle (NaCl 0.9 %) and **b** epinephrine (*EPI*, 0.1 mg/kg) or **c** FNO (2.8 mg/kg). The glycemic variation (Δ Glycemia) is the difference between the glucose concentration after and before fear conditioning test. Each *group point* represents the mean of five to seven mice per group and *error bars* represent SEM. *Asterisk*: significantly different from correspondent values ($p < 0.05$)

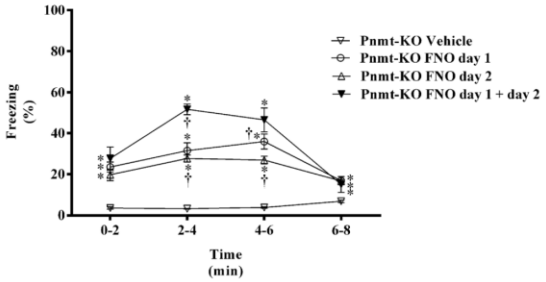


Fig. 6 Behavioral experiment 6. Freezing on the second day (context fear test) of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl, 0.9 %) or fenoterol (*FNO*, 2.8 mg/kg) injected just pre-training (day 1), just pre-testing (day 2), and both in pre-training and pre-testing (day 1 + day 2). Each *group point* represents the mean of five to nine mice per group, and *error bars* represent SEM. *Asterisk*: significantly different from correspondent values in Pnmt-KO mice treated with vehicle ($p < 0.05$). *Dagger*: significantly different from correspondent values in Pnmt-KO mice treated with FNO injected both in pre-training and pre-testing (day 1 + day 2)

to context may have implications for the study and treatment of anxiety disorders (Mineka et al. 1999). Thus, we propose a mechanism of epinephrine-dependent contextual learning that may be a potential pharmacologic target in anxiety disorders.

Context-shock and auditory cue-shock association of classical fear conditioning are mediated by different neuronal circuits. Since hippocampus is only involved in contextual and not in auditory cue fear conditioning (Rudy et al. 2004), it is possible that enhancement of contextual fear by epinephrine (and glucose as a mediator) is specific to the hippocampus. Indeed, Toth et al. also did not observe a significant effect of genotype on cued responses (Toth et al. 2013). In addition, Glenn et al. observed that glucose consumption leads to superior retention of hippocampal-dependent context learning and no effect on recall of cued conditioning (Glenn et al. 2014). Possibly, there are neuron cells that are under direct control of glucose availability (glucose-sensing neurons), which appears to be specific of the hippocampus (de Araujo 2014). To our knowledge, this mechanism is not known to occur in the amygdala.

Furthermore, it appears to occur a segregation of sensory input since different intra-amygdala circuitry may be used in conditioning to different conditional stimuli (contextual vs auditory). Contextual stimuli are processed in the hippocampus and the hippocampal afferents to the amygdala synapse primarily on basal nuclei. In fact, selective neurotoxic (ibotenate) bilateral damage to the basal nuclei disrupted contextual, but not auditory, fear conditioning (Onishi and Xavier 2010). In contrast, afferents relaying auditory information from the medial geniculate nucleus of the thalamus are thought to be the primary relay of auditory information to the amygdala, in particular neurons of the lateral amygdaloid nuclei (Nader et al. 2001).

In conclusion, aversive memories are best retained if moderately high plasma epinephrine concentrations occur at the same moment as the aversive stimulus. In addition, we propose that the mechanism by which epinephrine influences context fear learning involves peripheral β_2-adrenoceptors activation, since neither epinephrine nor fenoterol cross the blood–brain barrier. In turn, activation of peripheral β_2-adrenoceptors may induce high levels of blood glucose. Since glucose crosses the blood–brain barrier, it may enhance hippocampal-dependent contextual learning.

Acknowledgments The authors thank Oleh Mytakhir for technical support and Milaydis Sosa Napolskij for proofreading.

This work was supported by grants from the Fundação Professor Ernesto Morais (2012, Porto, Portugal) and the University of Porto (Grant IJUP2011-219).

References

Akirav I, Maroun M (2013) Stress modulation of reconsolidation. Psychopharmacology 226:747–761

Alva S (2008) FreeStyle Lite—a blood glucose meter that requires no coding. J Diabetes Sci Technol 2:546–551

Bondinell WE, Chapin FW, Frazee JS, Girard GR, Holden KG, Kaiser C, Maryanoff C, Perchonock CD, Gessner GW, Hieble JP et al (1983) Inhibitors of phenylethanolamine N-methyltransferase and epinephrine biosynthesis: a potential source of new drugs. Drug Metab Rev 14:709–721

de Araujo IE (2014) Contextual fear conditioning: connecting brain glucose sensing and hippocampal-dependent memories. Biol Psychiatry 75:834–835

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:E231–E235

Durkin TP, Messier C, de Boer P, Westerink BH (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

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 Off Publ Am Assoc Anatomists 231:849–858

Fanselow MS, Kim JJ (1994) Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. Behav Neurosci 108:210–212

Glenn DE, Minor TR, Vervliet B, Craske MG (2014) The effect of glucose on hippocampal-dependent contextual fear conditioning. Biol Psychiatry 75:847–854

Gold PE (2014) Regulation of memory—from the adrenal medulla to liver to astrocytes to neurons. Brain Res Bull 105:25–35

Goldstein DS, Kopin IJ (2008) Adrenomedullary, adrenocortical, and sympathoneural responses to stressors: a meta-analysis. Endocr Regul 42:111–119

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 29:600–608

Grillon C (2008) Models and mechanisms of anxiety: evidence from startle studies. Psychopharmacology 199:421–437

Grillon C, Cordova J, Morgan CA, Charney DS, Davis M (2004) Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. Psychopharmacology 175:342–352

Guarini S, Bazzani C, Bertolini A (1997) Resuscitating effect of melanocortin peptides after prolonged respiratory arrest. Br J Pharmacol 121:1454–1460

Harrison TS, Seaton JF (1966) Tissue content of epinephrine and norepinephrine following adrenal medullectomy. Am J Physiol 210:599–600

Introini-Collison IB, Baratti CM (1992) Memory-modulatory effects of centrally acting noradrenergic drugs: possible involvement of brain cholinergic mechanisms. Behav Neural Biol 57:248–255

John GW, Doxey JC, Walter DS, Reid JL (1990) The role of alpha- and beta-adrenoceptor subtypes in mediating the effects of catecholamines on fasting glucose and insulin concentrations in the rat. Br J Pharmacol 100:699–704

Kim JJ, Jung MW (2006) Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. Neurosci Biobehav Rev 30:188–202

Lands AM, Luduena FP, Buzzo HJ (1967) Differentiation of receptors responsive to isoproterenol. Life Sci 6:2241–2249

Lee HJ, Berger SY, Stiedl O, Spiess J, Kim JJ (2001) Post-training injections of catecholaminergic drugs do not modulate fear conditioning in rats and mice. Neurosci Lett 303:123–126

Lukoyanov NV, Lukoyanova EA (2006) Retrosplenial cortex lesions impair acquisition of active avoidance while sparing fear-based emotional memory. Behav Brain Res 173:229–236

Manceau V, Kremmer E, Nabel EG, Maucuer A (2012) The protein kinase KIS impacts gene expression during development and fear conditioning in adult mice. PLoS One 7:e43946

McNay EC, Gold PE (2002) Food for thought: fluctuations in brain extracellular glucose provide insight into the mechanisms of memory modulation. Behav Cogn Neurosci Rev 1:264–280

Mineka S, Mystkowski JL, Hladek D, Rodriguez BI (1999) The effects of changing contexts on return of fear following exposure therapy for spider fear. J Consult Clin Psychol 67:599–604

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

Moreira-Rodrigues M, Graca AL, Ferreira M, Afonso J, Serrao P, Morato M, Ferreirinha F, Correia-de-Sa P, Ebert SN, 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

Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117:131–143

Nader K, Majidishad P, Amorapanth P, LeDoux JE (2001) Damage to the lateral and central, but not other, amygdaloid nuclei prevents the acquisition of auditory fear conditioning. Learn Mem 8:156–163

Onishi BK, Xavier GF (2010) Contextual, but not auditory, fear conditioning is disrupted by neurotoxic selective lesion of the basal nucleus of amygdala in rats. Neurobiol Learn Mem 93:165–174

Pych JC, Chang Q, Colon-Rivera C, Gold PE (2005) Acetylcholine release in hippocampus and striatum during testing on a rewarded spontaneous alternation task. Neurobiol Learn Mem 84:93–101

Rizza RA, Cryer PE, Haymond MW, Gerich JE (1980) Adrenergic mechanisms of catecholamine action on glucose homeostasis in man. Metab Clin Exp 29:1155–1163

Rominger KL, Pollmann W (1972) Comparative pharmacokinetic studies on fenoterol-hydrobromide in rat, dog and man. Arzneimittelforschung 22:1190–1196

Roozendaal B, McGaugh JL (2011) Memory modulation. Behav Neurosci 125:797–824

Rudy JW, Huff NC, Matus-Amat P (2004) Understanding contextual fear conditioning: insights from a two-process model. Neurosci Biobehav Rev 28:675–685

Ryall JG, Gregorevic P, Plant DR, Sillence MN, Lynch GS (2002) Beta 2-agonist fenoterol has greater effects on contractile function of rat skeletal muscles than clenbuterol. Am J Physiol Regul Integr Comp Physiol 283:R1386–R1394

Ryall JG, Plant DR, Gregorevic P, Sillence MN, Lynch GS (2004) Beta 2-agonist administration reverses muscle wasting and improves muscle function in aged rats. J Physiol 555:175–188

Sullivan RM, Wilson DA, Leon M (1989) Norepinephrine and learning-induced plasticity in infant rat olfactory system. J Neurosci Off J Soc Neurosci 9:3998–4006

Sun P, Bao X, Elayan H, Milic M, Liu F, Ziegler MG (2008) Epinephrine regulation of hemodynamics in catecholamine knockouts and the pithed mouse. Ann N Y Acad Sci 1148:325–330

Tipps ME, Raybuck JD, Buck KJ, Lattal KM (2014) Delay and trace fear conditioning in C57BL/6 and DBA/2 mice: issues of measurement and performance. Learn Mem 21:380–393

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

Weil-Malherbe H, Axelrod J, Tomchick R (1959) Blood–brain barrier for adrenaline. Science 129:1226–1227

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 Off J Soc Neurosci 20:7059–7066

Yuan Q, Harley CW, Bruce JC, Darby-King A, McLean JH (2000) Isoproterenol increases CREB phosphorylation and olfactory nerve-evoked potentials in normal and 5-HT-depleted olfactory bulbs in rat pups only at doses that produce odor preference learning. Learn Mem 7:413–421

CHAPTER 4

Ester Alves, Ana Oliveira, Paula Serrão, and Mónica Moreira-Rodrigues. β_2-adrenoceptor antagonists decrease long term contextual-fear memory. (in preparation for submission)

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Ester Alves[1,2], Ana Oliveira[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); [2]Center for Drug Discovery and Innovative Medicines, University of Porto (MedInUP); [3] Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto (FMUP).

Running title: β_2-adrenoceptors and contextual-fear memory

*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

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Abstract

Rationale. Patients with posttraumatic stress disorder (PTSD) may have a deficit in extinction of fear learning and propranolol (peripheral and central acting β-adrenoceptor antagonist) reduces symptoms and reactivity to trauma cues. In addition, we showed that epinephrine (EPI) increases contextual-fear memory by acting specifically on peripheral β_2-adrenoreceptors. However, the influence of β_2-adrenoceptor antagonists in contextual-fear memories is still not understood.

Objectives. Therefore, our main goal was to understand if β_2-adrenoceptor antagonists can erase traumatic contextual memories.

Methods. Wild type and phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO, 129x1/SvJ) mice were submitted to fear conditioning procedure. Afterwards, the levels of catecholamines (EPI and norepinephrine, NE) were quantified in blood plasma and adrenal glands by HPLC-ED. On another group of mice freezing was evaluated after treatment with EPI, NE, EPI plus ICI 118,551 (selective β_2-adrenoreceptor antagonist), ICI 118,551 or vehicle (NaCl 0.9%), one day and one month after fear acquisition.

Results. In WT mice plasma EPI increase after fear acquisition test and intraperitoneal injection of EPI increases contextual-fear memory, contrary to NE. In Pnmt-KO mice, freezing induced by EPI was blocked by ICI 118,551, one day and one month after fear acquisition. ICI 118,551 decreases contextual-fear memory in WT mice, in both one day and one month after fear acquisition.

Conclusions. EPI increases in plasma after an aversive experience, possibly improving long-term context fear memory, and even old memories, by acting on peripheral β_2-adrenoceptors. β_2-adrenoceptor antagonists decrease long term contextual-fear memory and possibly traumatic old memories. Therefore, may be a possible treatment in anxiety diseases, particularly PTSD.

Keywords: contextual-fear learning, β_2-adrenoceptors, β_2-adrenoceptor antagonist, ICI 118,551, long term memory, old memories, posttraumatic stress disorder, anxiety disorders.

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Introduction

Stress is a state of disturbance caused by many life factors (stressors) *(Joëls et al. 2006)*. Stress leads to triggering of the stress response and activation of the central and peripheral nervous system. The stress response enables the organism to deal with challenges by mobilizing energy, increasing cardiovascular tone, inhibiting reproduction, feeding, and digestion, and by modifying immune responses (Sapolsky et al. 2000). Thus, the stress response is important for survival and is adaptive in nature. However, in some circumstances stress may cause pathology, as is the case of posttraumatic stress disorder (PTSD) and other anxiety disorders. The development of PTSD, can be conceptualized as learning under severe stress (Lissek et al. 2005). The inability to inhibit fear responses contributes to the maintenance of fear responses. Indeed, patients with PTSD show deficits in extinction of fear memory (Inslicht et al. 2013; Lissek et al. 2005).

The most distinctive attribute of long-term memory is persistence over time. Strong memories are often based on experiences that are emotionally arousing (Ochsner 2000). There is broad evidence supporting the hypothesis that the strength of long-term memories is influenced by hormonal systems (Gold et al. 1975). In addition, modulation of gene expression seems to be a requirement of consolidation and persistence of long term memories and may result in synaptic remodeling (Dudai 2002).

A valuable paradigm for studying fear memory in animals is Pavlovian fear conditioning. In fear conditioning a conditioned stimulus (CS), such as a context, is paired with an aversive unconditioned stimulus (US), such as an electric shock (Pearce and Hall 1980). After numerous such pairings, the context elicits behavioral fear responses such as freezing (Sacchetti et al. 1999). The relation between the context and the shock is strong and long lasting. In fact, it has been suggested that conditioned fear associations are unforgettable (LeDoux et al. 1989). Therefore, these associations could aid perceive and evade previously encountered dangers, throughout live.

It was shown that phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) mice are deficient in epinephrine (EPI) and have reduced contextual-fear learning (Alves et al. 2016; Toth et al. 2013). In addition, we found that aversive contextual memories are best remembered if moderately high plasma

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

EPI concentrations occur at the same moment as the aversive stimulus. In addition, EPI increases context fear memory by acting on peripheral β_2-adrenoceptors. Afterwards, high levels of blood glucose may be induced by peripheral β_2-adrenoceptor activation and enhance hippocampal-dependent contextual learning (Alves et al. 2016).

Patients with PTSD may have a deficit in extinction of fear learning and propranolol (peripheral and central acting β-adrenoceptor antagonist) reduces symptoms and reactivity to trauma cues (Brunet et al. 2008; Brunet et al. 2011). However, the influence of a β_2-adrenoceptor antagonist in contextual-fear memories is still not understood. Therefore, our main goal was to understand if β_2-adrenoceptor antagonists can erase traumatic contextual memories and provide a novel possible therapeutic approach on anxiety disorders.

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. The Pnmt-KO mice ($Pnmt^{-/-}$) were produced by the insertion of Cre-recombinase gene into the locus encoding for Pnmt enzyme, creating a functional knockout of Pnmt expression, with loss of EPI in homozygous $Pnmt^{-/-}$ (Ebert et al. 2004). 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, in ear DNA samples) by using the following primers: Primer 1, 5'-CAGGCGCCTCATCCCTCAGCAGCC-3'; Primer 2, 5'-CTGGCCAGCGTCGGAGTCAGGGTC-3'; and Primer 3, 5'-GGTGTACGGTCAGTAAATTGGACACCGTCCTC-3'. Pnmt-KO (n = 56) and wild-type (WT, n = 22) male mice (129x1/SvJ) were kept 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) and housed with the respective litter.

Fear conditioning procedure. The Fear conditioning procedure was adapted as previously described (Lukoyanov and Lukoyanova 2006 ; Manceau et al. 2012). The conditioning chambers consisted of a clear Plexiglas box equipped with a metal grid floor, wired to a stimulus generator. The animal's behavior was recorded with a digital video camera Sony HDR-CX405 (Sony Corporation, Japan). Freezing was defined as the absence of movement except for respiration. Conditioning was assessed by the freezing response because this response is a widely used indicator of conditioned fear (Fanselow and Kim 1994). Freezing was only scored if mice remained inactive for at least 3 s. The percentage of accumulated freezing time was then calculated. On the first day (fear acquisition; 6 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, short term fear memory), 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). On the first and second day the chambers were cleaned and wiped with 1% acetic acid.

Catecholamine assay. One group of Pnmt-KO and WT mice were submitted to fear acquisition test (FA; and other group without FA, control, C) and at the end of this test they were anesthetized (ketamine, 100 mg/kg and xylazine,10 mg/kg; i.p.). The blood was collected by left ventricle puncture to a heparinized tube and then the samples were centrifuged (1250xg, 2 min) and kept under -80 °C until further use. The catecholamines present in plasma were concentrated by alumina, as previously described (Moreira-Rodrigues et al. 2014). Left adrenal was collected and emerged in percloric acid (0.2 M) and frozen -80°C. Catecholamines were separated by reverse-phase HPLC and quantified by electrochemical detection. The detection limit is between 350 and 1000 fmol.

Behavioral experiments. Other groups of WT and Pnmt-KO mice were submitted to fear conditioning procedure after the following treatments: 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). Long term fear memory (context fear test, one day after fear acquisition) and old memories (context fear test, one month after fear acquisition) were evaluated.

Drugs. (−)-EPI (+)-bitartrate salt, L-(-)-NE (+)-bitartrate salt monohydrate, and ICI 118,551 were purchased from Sigma-Aldrich (St Louis, MO, USA).

Statistical analysis. Results are presented as means ± standard error of the means (SEM) for the indicated number of determinations. Catecholamine concentrations were analyzed by Student`s t-test. Data from fear conditioning tests was analyzed by two-way ANOVA. For multiple comparisons we used Turkey´s test (3 groups) and Sidak´s test (2 groups). $p<0.05$ was assumed to denote a significant difference. GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Genotyping of Pnmt-KO mice

The presence of the Pnmt$^{-/-}$ allele was verified by genotyping. Amplification reactions yielded products of 200 and 160 bp for the wild type (WT) and mutant alleles, respectively. Representative PCR products are shown, corresponding to WT, heterozygous (HZ) and Pnmt-KO mice (Figure 1).

Increased plasma EPI concentration after fear acquisition test

In the WT mice, plasma EPI concentration was significantly higher after fear acquisition test compared with mice without the test (Fig. 2A). Plasma NE concentrations were not different in both WT and Pnmt-KO mice, with or without fear acquisition test (Fig. 2B and C). EPI in the left adrenal glands and plasma of Pnmt-KO mice were undetectable. The WT mice did not present any significant differences in adrenal gland content of EPI or NE between mice with or without fear acquisition test (Table 1).

On the first day (fear acquisition) of the fear conditioning procedure there were no differences in the freezing response between groups (data not shown).

Intraperitoneal injection of NE does not increase contextual-fear memory

On the second day, mice were re-exposed to the shock context (context fear test), and it induced an increase in freezing in Pnmt-KO mice treated with EPI compared to vehicle and NE treated mice. No differences were observed in freezing between Pnmt-KO mice treated with NE and vehicle. It was observed a drug ($F(2, 14) = 16.50$, $p = 0.0002$) and a time effect ($F(3, 42) = 3.97$, $p = 0.01$; Fig. 3). There was a significant interaction between drug and time ($F(6, 42) = 4.64$, $p = 0.001$; Fig. 3).

Epinephrine induced long term and old memories are mediated through activation of β_2-adrenoceptors

One day after fear acquisition, re-exposure to shock context induced an increase in freezing in Pnmt-KO mice treated with EPI compared to vehicle and EPI plus ICI 118,551 (selective β_2-adrenoceptor antagonist) treated mice. It was observed a drug ($F(2, 24) = 45.01$, $p<0.0001$), a time ($F(3, 72) = 12.33$, $p<0.0001$) effect and a significant interaction between drug and time ($F(6, 72) = 4.13$, $p = 0.001$; Fig. 4A).

One month after fear acquisition, Pnmt-KO mice treated with EPI still showed a significant freezing compared to the other groups. A significant drug ($F(2, 15) = 11.42$, $p = 0.001$), time effect ($F(3, 45) = 8.03$, $p = 0.0002$) and interaction ($F(6, 45) = 3.36$, $p = 0.008$) was observed (Fig. 4B). Pnmt-KO mice treated with EPI presented a higher freezing in one day (long term) compared with one month after fear acquisition (old memories).

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 one day and one month after fear acquisition. It was observed a drug effect in both one day ($F(1, 10) = 14.08$, $p = 0.004$) and one month after fear acquisition ($F(1, 9) = 10.06$, $p = 0.01$) (Fig. 5A and B). It was also observed a time ($F(3, 27) = 4.94$, $p = 0.007$) effect and an interaction ($F(3, 27) = 3.45$, $p = 0.03$) one month after fear acquisition (Fig. 5B).

Discussion

The absence of PNMT expression altered catecholamine biosynthesis in the PNMT KO mouse. E was eliminated in adrenal gland, plasma, and urine, while adrenal NE increased significantly in the PNMT KO mouse. In contrast, adrenal dopamine (DA) and NE and DA in the plasma and urine were not significantly altered. Mice with disrupted Pnmt genes, shown by genotyping, do not express the enzyme responsible for EPI synthesis (Pnmt). Our results also show that EPI is absent from the adrenal gland and plasma of Pnmt-KO mice, and this is in agreement with other authors (Ebert et al. 2004; Sun et al. 2008). Therefore, the Pnmt-KO mice model is a refined model to study EPI influence in behavior because of the lack of EPI production in these mice.

It is known that memories are best retained in stressful contexts, which could be related to the fact that emotions and arousal moments increase plasma adrenal hormone concentrations (McGaugh and Roozendaal 2002). In fact, it was shown that in mice, EPI (0.1 mg/Kg, i.p.) facilitates retention in inhibitory avoidance test (Introini-Collison et al. 1992). In agreement, we found that aversive contextual memories are best retained if moderately high plasma EPI concentrations occur at the same moment as the aversive stimulus, after EPI treatment (Alves et al. 2016).

Furthermore, it was shown that dopamine β-hydroxylase knockout mice (deficient in both NE and EPI) (Murchison et al. 2004) and later Pnmt-KO mice (deficient in EPI) (Alves et al. 2016; Toth et al. 2013) have reduced contextual-fear learning. Several authors also have shown that EPI increases in plasma after emotional arousal moments, particularly, plasma EPI increases after tone plus shock exposure (McDowell et al. 2013). This is in agreement with our results, which show that EPI plasma levels increase significantly during and after fear acquisition (fear conditioning procedure) in WT mice. However, plasma NE does not. On the other hand, we now show that intraperitoneal injections of NE do not increase context fear learning, contrary to intraperitoneal injections of EPI, in similar doses. It appears that peripheral NE is not important for contextual-fear memory, opposing the known effects of central NE in memory (Tully and Bolshakov 2010).

McDowell et al also have shown that plasma EPI does not increase after just tone exposure (McDowell et al. 2013). Accordingly, we have shown previously

that EPI selectively affects contextual-fear memory and not cue fear memory (Alves et al. 2016). In addition, conditioned fear responses to a tone paired with a foot shock rapidly extinguish when the tone is presented in the absence of the shock (Quirk et al. 2000). For these reasons, we have not explored cue fear memory in these experiments.

We also have shown that EPI increases contextual-fear learning on long term memory (one day after fear acquisition), by acting on peripheral β_2-adrenoceptors (Alves et al. 2016). And now, we show that ICI 118,551, a β_2-adrenoceptor antagonist, decreases long term contextual-fear memory and possibly traumatic old memories (one month after fear acquisition). Thus, EPI seems to be an important mediator of long term consolidation of fear memory and even old memories. Mechanisms underlying long term memory integrate physiological functions of multiple organ systems to support brain processes. Intensive research suggests that this type of memory consolidation, occurring within minutes to weeks after initial learning, 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).

Epinephrine does not cross the blood-brain barrier (Weil-Malherbe et al. 1959), so there must be a mediator that takes the signal to the brain. We found that EPI activates peripheral β_2-adrenoceptors and the signal reaches the brain in contextual-fear memory. This was supported by context fear learning improvement in Pnmt-KO mice treated with EPI (Alves et al. 2016). We also showed that glucose may be a down-stream mediator of EPI actions in long term contextual learning (Alves et al. 2016), and a critical component of fear memory modulation. Hence, it also might be essential for old memories.

In humans, β-adrenoceptors also selectively affect contextual-fear memory. In healthy humans, propranolol (peripheral and central acting β-adrenoceptor antagonist) has been found to impair the consolidation of fear memory in the contextual-fear conditioning test. (Grillon et al. 2004). The PTSD is an example of a condition that may arise due to exposure to a traumatic event. Patients with this pathology may have a deficit in extinction of fear learning (Inslicht et al. 2013; Lissek et al. 2005). In addition, the administration of propranolol shortly after exposure to psychological trauma has been reported to reduce

PTSD symptom severity and reactivity to trauma cues, and may even prevent PTSD development (Brunet et al. 2008; Brunet et al. 2011).

Although propranolol may be a possible therapeutic treatment in PTSD it has significant secondary effects (fatigue, bradycardia, Raynaud phenomenon, sleep and gastrointestinal disturbances…). In addition, propranolol affected memory consolidation in non-aversive tasks (object recognition and object location) and impaired memory reconsolidation not only in most, but also in least aversive tasks Since some stress disorders as PTSD are built from activation of traumatic memories and exacerbation of EPI release (Van der Kolk 2003), we suggest that β_2-adrenoceptors antagonists might be a more specific and efficient treatment in PTSD.

In conclusion, EPI increases in plasma after an aversive experience, possibly improving long-term context fear memory, and even old memories, by acting on peripheral β_2-adrenoceptors. β_2-adrenoceptor antagonists decrease long term contextual-fear memory and possibly traumatic old memories. Therefore, may be a possible treatment in anxiety diseases, particularly PTSD.

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Acknowledgements

This work was supported by Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS/UP).

Bibliography

Alves E, Lukoyanov N, Serrao P, Moura D, Moreira-Rodrigues M (2016) Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors. Psychopharmacology 233: 2099-108.

Brunet A, Orr SP, Tremblay J, Robertson K, Nader K, Pitman RK (2008) Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. Journal of psychiatric research 42: 503-6.

Brunet A, Poundja J, Tremblay J, Bui E, Thomas E, Orr SP, Azzoug A, Birmes P, Pitman RK (2011) Trauma reactivation under the influence of propranolol decreases posttraumatic stress symptoms and disorder: 3 open-label trials. Journal of clinical psychopharmacology 31: 547-50.

Dudai Y (2002) Molecular bases of long-term memories: a question of persistence. Curr Opin Neurobiol 12: 211-6.

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: 849-58.

Fanselow MS, Kim JJ (1994) Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. Behav Neurosci 108: 210-2.

Gold PE, van Buskirk RB, McGaugh JL (1975) Effects of hormones on time-dependent memory storage processes. Prog Brain Res 42: 210-1.

Grillon C, Cordova J, Morgan CA, Charney DS, Davis M (2004) Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. Psychopharmacology 175: 342-52.

Inslicht SS, Metzler TJ, Garcia NM, Pineles SL, Milad MR, Orr SP, Marmar CR, Neylan TC (2013) Sex differences in fear conditioning in posttraumatic stress disorder. Journal of psychiatric research 47: 64-71.

Introini-Collison I, Saghafi D, Novack GD, McGaugh JL (1992) Memory-enhancing effects of post-training dipivefrin and epinephrine: involvement of peripheral and central adrenergic receptors. Brain Res 572: 81-6.

Joëls M, Pu Z, Wiegert O, Oitzl MS, Krugers HJ (2006) Learning under stress: how does it work? Trends in Cognitive Sciences 10: 152-158.

LeDoux JE, Romanski L, Xagoraris A (1989) Indelibility of subcortical emotional memories. Journal of Cognitive Neuroscience 1: 238-243.

Lee CL, Hannay J, Hrachovy R, Rashid S, Antalffy B, Swann JW (2001) Spatial learning deficits without hippocampal neuronal loss in a model of early-onset epilepsy. Neuroscience 107: 71-84.

Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, Grillon C, Pine DS (2005) Classical fear conditioning in the anxiety disorders: a meta-analysis. Behav Res Ther 43: 1391-424.

Lukoyanov NV, Lukoyanova EA (2006) Retrosplenial cortex lesions impair acquisition of active avoidance while sparing fear-based emotional memory. Behav Brain Res 173: 229-36.

Manceau V, Kremmer E, Nabel EG, Maucuer A (2012) The protein kinase KIS impacts gene expression during development and fear conditioning in adult mice. PLoS One 7: e43946.

McDowell AL, Filippone AB, Balbir A, Germain A, O'Donnell CP (2013) Mild Transient Hypercapnia as a Novel Fear Conditioning Stimulus Allowing Re-Exposure during Sleep. PLoS One 8: e67435.

McGaugh JL, Roozendaal B (2002) Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol 12: 205-10.

Moreira-Rodrigues M, Graça AL, Ferreira M, Afonso J, Serrão P, Morato M, Ferreirinha F, Correia-de-Sá P, Ebert SN, Moura D (2014) Attenuated Aortic Vasodilation and Sympathetic Prejunctional Facilitation in Epinephrine-Deficient Mice: Selective Impairment of β2-Adrenoceptor Responses. Journal of Pharmacology and Experimental Therapeutics 351: 243-249.

Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117: 131-43.

Ochsner KN (2000) Are affective events richly recollected or simply familiar? The experience and process of recognizing feelings past. Journal of Experimental Psychology: General 129: 242.

Pearce JM, Hall G (1980) A model for Pavlovian learning: variations in the effectiveness of conditioned but not of unconditioned stimuli. Psychol Rev 87: 532-52.

Quirk GJ, Russo GK, Barron JL, Lebron K (2000) The role of ventromedial prefrontal cortex in the recovery of extinguished fear. J Neurosci 20: 6225-31.

Sacchetti B, Ambroqi Lorenzini C, Baldi E, Tassoni G, Bucherelli C (1999) Memorization of contextual and CS conditioned fear response (freezing) in a one-trial acquisition paradigm. Arch Ital Biol 137: 235-48.

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21: 55-89.

Stone EA, Rhee J, Quartermain D (1996) Blockade of effect of stress on risk assessment behavior in mice by a beta-1 adrenoceptor antagonist. Pharmacology Biochemistry and Behavior 55: 215-217.

Sun P, Bao X, Elayan H, Milic M, Liu F, Ziegler MG (2008) Epinephrine regulation of hemodynamics in catecholamine knockouts and the pithed mouse. Ann N Y Acad Sci 1148: 325-30.

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: 1-9.

Tully K, Bolshakov VY (2010) Emotional enhancement of memory: how norepinephrine enables synaptic plasticity. Mol Brain 3: 15.

Van der Kolk BA (2003) Posttraumatic stress disorder and the nature of trauma. Healing trauma: Attachment, mind, body, and brain: 168-195.

Weil-Malherbe H, Axelrod J, Tomchick R (1959) Blood-brain barrier for adrenaline. Science 129: 1226-7.

Zhu Q, Gu L, Wang Y, Jia L, Zhao Z, Peng S, Lei L (2014) The role of alpha-1 and alpha-2 adrenoceptors in restraint stress-induced liver injury in mice. PloS one 9: e92125.

Tables

Table 1: Concentration of adrenal catecholamines in phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) or wild-type (WT) mice after fear acquisition test (FA).

	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 ± SEM of 5-6 mice per group. C, control mice without fear acquisition test; EPI, epinephrine; NE, norepinephrine.

Figure Legends

Figure 1. Germ-line transmission of the Pnmt$^{-/-}$ allele was verified by polymerase chain reaction (PCR). Representative agarose gel electrophoresis of PCR products are shown, corresponding to wild-type (WT) (+/+), heterozygous (HZ) (+/-), and homozygous mutant (Pnmt-KO) (-/-) mice bands. L, ladder.

Figure 2. Catecholamines in blood plasma after fear acquisition test (FA). Plasma (A) epinephrine (EPI) and (B) (C) norepinephrine (NE) in phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) or wild-type (WT) mice. Each group column represents the mean of 5-6 mice per group, and error bars represent SEM. C, control mice without fear acquisition test *, significantly different from correspondent control (C, without FA) values ($p < 0.05$).

Figure 3: Freezing on the second day (context fear test) of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) mice treated with epinephrine (EPI, 0.1 mg/kg), norepinephrine (NE, 0.1 mg/kg) or vehicle (NaCl 0.9%). Each group point represents the mean of 6-10 mice per group, and error bars represent SEM. *, 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 4: Freezing in context fear test (A) one day and (B) one month after fear acquisition of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (Pnmt-KO) mice treated with epinephrine (EPI, 0.1 mg/kg), EPI (0.1 mg/kg) plus ICI 118,551 (ICI, selective β_2-adrenoceptor antagonist, 2.0 mg/kg) or vehicle (NaCl 0.9%). Each group point represents the mean of 5-10 mice per group, and error bars represent SEM. *, 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 5: Freezing in context fear test (A) one day and (B) one month after fear acquisition of fear conditioning procedure in wild type (WT) mice treated with ICI 118,551 (ICI, selective β_2-adrenoceptor antagonist, 2.0 mg/kg) or vehicle (NaCl 0.9%). Each group point represents the mean of 6-8 mice per group, and error bars represent SEM. *Significantly different from correspondent values in WT mice treated with vehicle ($p<0.05$).

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Figure 1

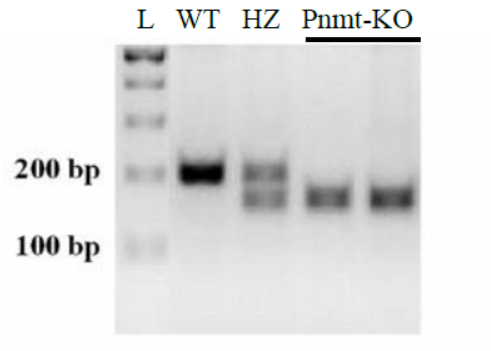
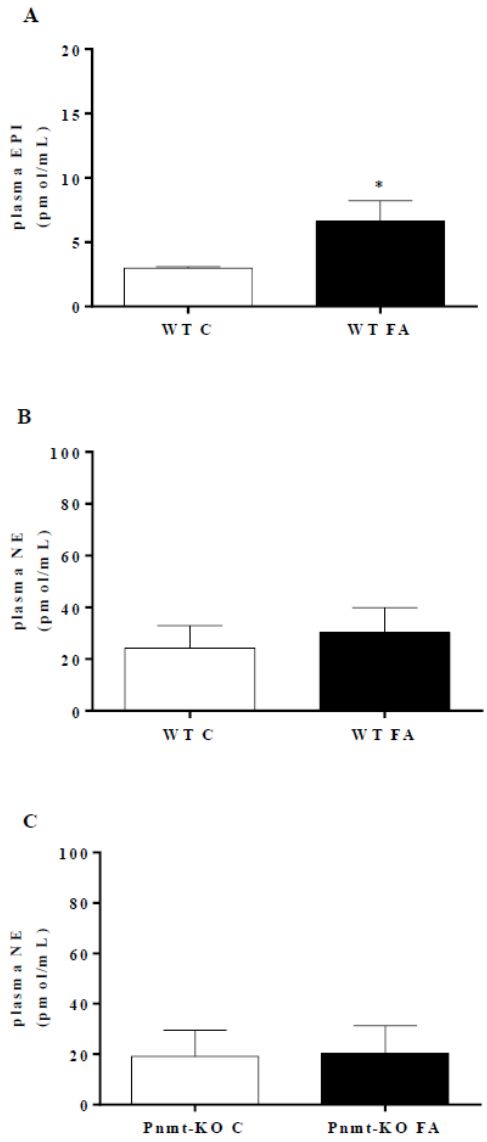


Figure 2


A
plasma EPI (pmol/mL)
20
15
10
5
0
*
WT C
WT FA
B
plasma NE (pmol/mL)
100
80
60
40
20
0
WT C
WT FA
C
plasma NE (pmol/mL)
100
80
60
40
20
0
Pnmt-KO C
Pnmt-KO FA

CHAPTER 4 - β_2-adrenoceptor antagonists decrease long term contextual-fear memory

Figure 3

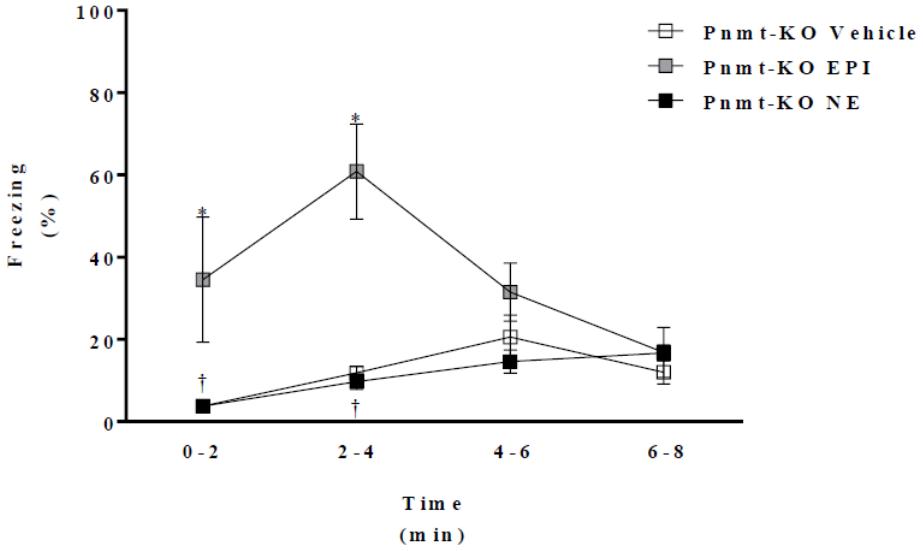


Figure 4

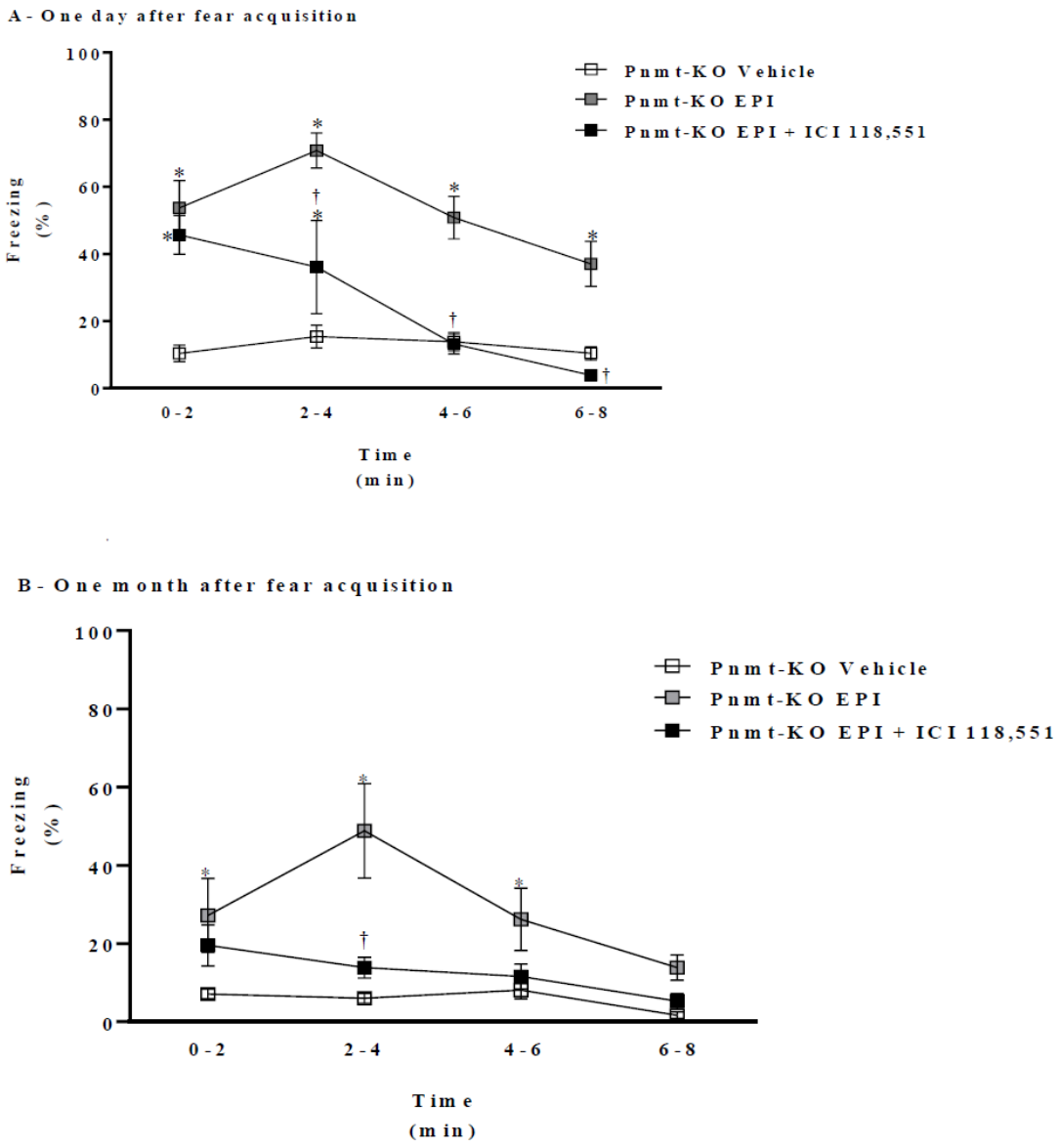
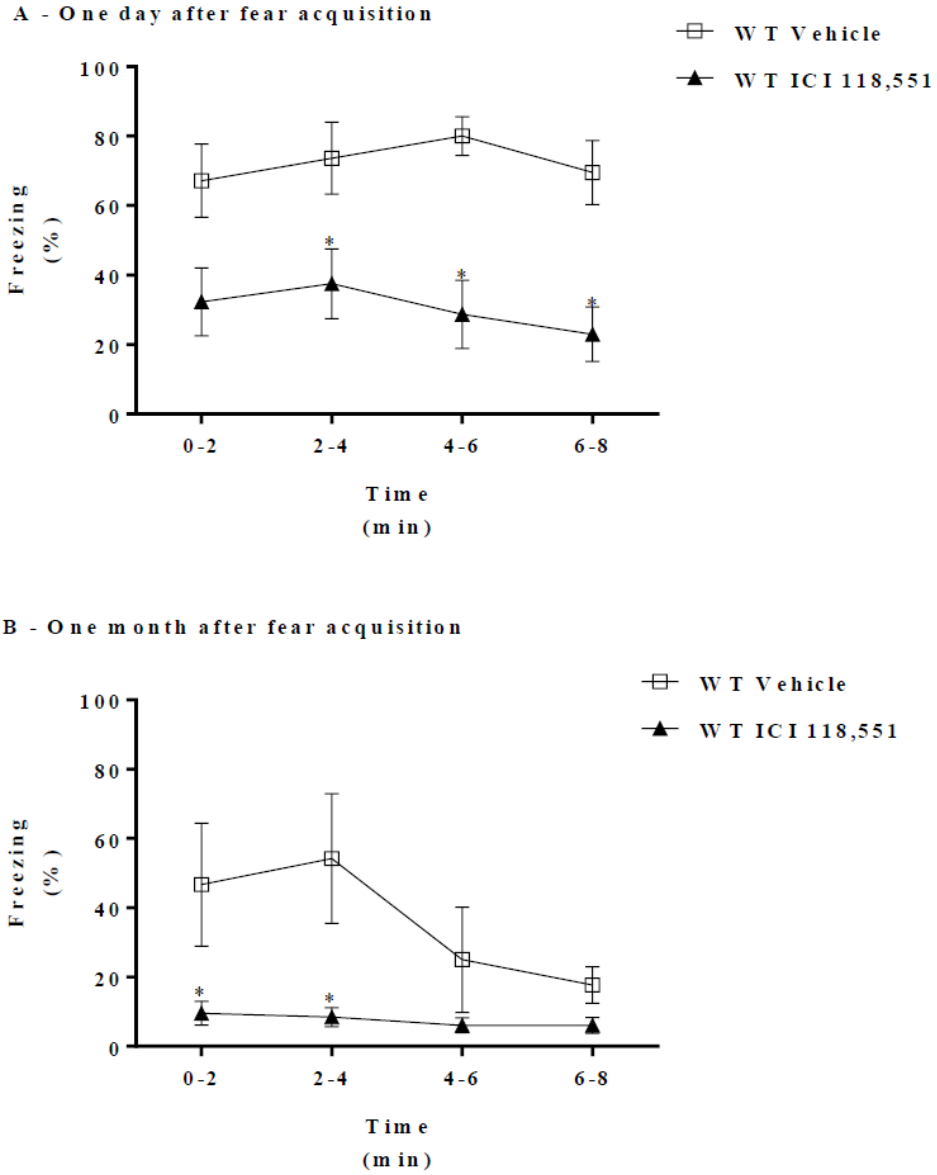

A - One day after fear acquisition
Freezing (%)
100
80
60
40
20
0
0 - 2
2 - 4
4 - 6
6 - 8
Time (min)
Pnmt-KO Vehicle
Pnmt-KO EPI
Pnmt-KO EPI + ICI 118,551
B - One month after fear acquisition
Freezing (%)
100
80
60
40
20
0
0 - 2
2 - 4
4 - 6
6 - 8
Time (min)
Pnmt-KO Vehicle
Pnmt-KO EPI
Pnmt-KO EPI + ICI 118,551

Figure 5


A - One day after fear acquisition
WT Vehicle
WT ICI 118,551
Freezing (%)
100
80
60
40
20
0
0-2
2-4
4-6
6-8
Time (min)
B - One month after fear acquisition
WT Vehicle
WT ICI 118,551
Freezing (%)
100
80
60
40
20
0
0-2
2-4
4-6
6-8
Time (min)

CHAPTER 5

DISCUSSION AND FUTURE PERSPECTIVES

CHAPTER 5 - DISCUSSION AND FUTURE PERSPECTIVES

Stress is an undisputed environmental risk factor for disease, in particular psychiatric diseases. The moment and the context of stressful stimuli are essential and determine the adaptive or maladaptive consequences (Schmidt 2010). Adrenal stress hormones have an important role in encoding alarm signals to the brain. These hormones influence memory, in fact, it has been provided widespread evidence that EPI, NE and glucocorticoids modulate long-term memory consolidation in humans (McGaugh and Roozendaal 2002).

Pnmt-KO mice are EPI deficient mice and show impairment in contextual-fear memory (Toth et al. 2013), suggesting that these mice reveal selective memory effects on highly emotional memories. To understand this better we started to explore EPI role in fear memory. We showed that contextual aversive memories are better retained in the presence of moderately high plasma EPI concentrations. In Pnmt-KO mice, we also showed restored expression of contextual-fear memory after EPI, or non-selective β-AD agonist (isoprenaline) or selective β_2-AD agonist (fenoterol) treatments. In addition, a selective β_1-AD agonist (dobutamine) does not seem to increase freezing in Pnmt-KO mice. Moreover, treatment of Pnmt-KO mice with a peripheral β-AD antagonist (sotalol) (Alves et al. 2016) blocked the increased freezing induced by EPI. In addition, EPI (Weil-Malherbe et al. 1959), AD agonists (fenoterol, (Rominger and Pollmann 1972)) and antagonists (sotalol (Mason and Angel 1983)) used do not cross the blood-brain barrier. Therefore, we showed that EPI affects contextual-fear memory by acting on peripheral β_2-ADs.

Glucose is a limited source of energy. It appears that EPI acts on hepatic glucose production by stimulation of glycogenolysis and gluconeogenesis (John et al. 1990). In addition, we showed that EPI restores the glycemic response at the same time as contextual-fear memory in Pnmt-KO mice (Alves et al. 2016). Since glucose crosses the blood–brain barrier, it may modulate memory (Gold et al. 1986). John et al. also showed that a β-AD agonist (isoprenaline) improves the hyperglycemic response and this was blocked by a selective β_2-AD antagonist (ICI 118,551) (John et al. 1990). Glucose may be a chief down-stream intermediary of EPI actions in contextual memory. In fact, it was shown that a surge in plasma glucose increases acetylcholine synthesis in the hippocampus (Durkin et al. 1992), providing supplementary energy to specific neural components, and modulate neuronal excitability and neurotransmitter

release (McNay and Gold 2002). Therefore the evaluation of acetylcholine synthesis and neurotransmitter release after fear conditioning procedure in Pnmt-KO and WT mice could be the next step to better understand this mechanism. In addition, the quantification of specific proteins in hippocampus would be interesting, since modulation of gene expression seems to be a requirement of consolidation and persistence of long-term memories, and may result in synaptic remodeling (Dudai 2006).

In both mice and humans, β-ADs selectively affect contextual-fear memory, and not auditory fear learning (Alves et al. 2016; Grillon et al. 2004; Toth et al. 2013). The pathways for contextual and auditory learning seem different. Cellular mechanisms whereby contextual-fear memory consolidation occurs are connected to the hippocampus and the amygdala, whereas tone conditioning only depends on amygdala molecular plastic changes (Phillips and LeDoux 1992).

The classic fight-or-flight response to aversive stimulus has clear survival benefits in evolutionary terms. However, the systems responsible to the perceived threat can become deregulated under some circumstances. Chronic deregulation of these systems can lead to PTSD (Sherin and Nemeroff 2011). PTSD is a psychopathological response to extreme stressors. It is characterized by the intrusive re-experiencing of the traumas and heightened physiological arousal with noticeable anxiety and depressive features persisting for at least one month. Only a percentage of those exposed to fear-producing events develop or sustain PTSD (Yehuda et al. 1998). PTSD can be a limitation to an organized life routine. There is a considerable amount of work being done to find new approaches for delivering evidence-based treatments for PTSD, such as exposure and cognitive therapy. However, the efficacy of these treatments has been limited (Kaczkurkin and Foa 2015).

There are also several pharmacotherapies for treating PTSD. Serotonin reuptake inhibitors are the most frequently prescribed pharmacological agents for the treatment of PTSD, as they are reasonably well tolerated and safe. However, they have innumerous limitations. The beginning of the therapeutic effect usually takes several weeks to occur, sometimes there are remaining symptoms, or non-response, as well as persistent undesirable side effects, such

as changes in appetite and weight, gastrointestinal disturbances, and loss of sexual drive (Kelmendi et al. 2016).

Hidrocortisone shortly after exposure to psychological trauma is thought to be effective in PTSD (de Quervain and Margraf 2008), by exerting negative feedback control of the hypothalamic-pituitary-adrenal axis. However, sustained glucocorticoid exposure has huge adverse effects in the brain and metabolism. In fact, continued glucocorticoid exposure induces the decline of dendritic branching, damage of dendritic spines and impairment of neurogenesis in hippocampus (Sherin and Nemeroff 2011).

Patients with PTSD manifest greater changes in heart rate, blood pressure, and skin conductance than controls, when exposed to trauma-related contexts. In fact, a persistent hyperactivity of autonomic sympathetic system was detected. Accordingly, increased urinary excretion of catecholamines and their metabolites, as well as, increase of EPI in plasma has been documented in PTSD (Shalev et al. 1992; Sherin and Nemeroff 2011).

In healthy humans, propranolol (peripheral and central acting β-AD antagonist) has been found to weaken the consolidation of contextual-fear memory in fear conditioning procedure. Therefore, similarly to mice, in humans, β-ADs are also implicated in contextual-fear conditioning (Grillon et al. 2004). In addition, altered amygdala and hippocampus activity, evaluated by functional magnetic resonance, is related with propranolol-induced emotional memory weakening in healthy individuals (Schwabe et al. 2012).

Administration of propranolol soon after exposure to psychological trauma has been described to reduce PTSD symptoms severity and reactivity to trauma cues, and may even prevent PTSD development (Brunet et al. 2008; Pitman et al. 2002). Reconsolidation theory defends that in order to endure, a recalled memory needs to be saved again to long-term memory storage, thereby recapitulating the process of memory consolidation. Propranolol appears to block consolidation and even reconsolidation, allowing the attenuation of emotional strength of traumatic memories (Lonergan et al. 2013). A PTSD remission of up 71 % was detected after six sessions of trauma recollection under the effect of propranolol (Brunet et al. 2011), with a significant decrease in physiological reacting to context cues, at 6 month follow-up (Brunet et al. 2014).

In mice, there are evidences that fear memory lasts for a long-term, even months (old memories) (McGaugh and Roozendaal 2002). On the other hand, we showed that fear memory may be conducted by EPI peripheral pathway trough β_2-ADs (Alves et al. 2016). In addition, our results show that this mechanism persists in the long-term (one day after fear acquisition) and even as old memories (one month after fear acquisition), by blocking freezing in Pnmt-KO EPI treated mice and in WT mice, with a β_2-AD antagonist (ICI 118,551). Thus, β_2-AD activation by EPI seems to be an important pathway for both short and long-term fear memory, and even old memories. In addition, propranolol administered systemically after retrieval (through contextual reactivation) disrupts contextual-fear memory reconsolidation, being a possible therapeutic treatment in PTSD (Muravieva and Alberini 2010). However, propranolol has significant secondary effects (fatigue, bradycardia, Raynaud phenomenon, sleep and gastrointestinal disturbances…). In addition, propranolol decreased memory consolidation in non-aversive tasks (object recognition and object location) and weakened memory reconsolidation not only in most, but also in least aversive tasks (Villain et al. 2016). Therefore, we suggest that β_2-AD antagonists may be a more successful treatment in anxiety diseases, particularly in PSTD. The efficacy of β_2-AD antagonists could be evaluated in contextual fear memory, of a PTSD mice model. To disrupt traumatic memories that may contribute to the development of PTSD, we could interfere with the reconsolidation process (Dudai 2006), though the administration of a β_2-AD antagonist (ICI 118,551 or one that does not pass the blood-brain barrier) after retrieval, though contextual reactivation.

In conclusion, EPI increases in plasma after an aversive experience, possibly improving long-term contextual fear memory, and even old memories, by acting on peripheral β_2-ADs. We suggest that blocking β_2-ADs with antagonists may inhibit undesirable memories, and be used as a treatment for patients suffering from pathogenic memories, such as PTSD.

CHAPTER 6

REFERENCES

Akirav I (2013) Cannabinoids and glucocorticoids modulate emotional memory after stress. Neurosci Biobehav Rev 37: 2554-63.

Alberini CM (2011) The Role of Reconsolidation and the Dynamic Process of Long-Term Memory Formation and Storage. Frontiers in behavioral neuroscience 5: 12.

Alves E, Lukoyanov N, Serrao P, Moura D, Moreira-Rodrigues M (2016) Epinephrine increases contextual learning through activation of peripheral beta2-adrenoceptors. Psychopharmacology (Berl) 233: 2099-108.

Atwoli L, Stein DJ, Koenen KC, McLaughlin KA (2015) Epidemiology of posttraumatic stress disorder: prevalence, correlates and consequences. Curr Opin Psychiatry 28: 307-11.

Bergstrom HC, McDonald CG, Dey S, Tang H, Selwyn RG, Johnson LR (2013) The structure of Pavlovian fear conditioning in the amygdala. Brain Struct Funct 218: 1569-89.

Bondinell WE, Chapin FW, Frazee JS, Girard GR, Holden KG, Kaiser C, Maryanoff C, Perchonock CD, Gessner GW, Hieble JP, et al. (1983) Inhibitors of phenylethanolamine N-methyltransferase and epinephrine biosynthesis: a potential source of new drugs. Drug Metab Rev 14: 709-21.

Borrell J, De Kloet ER, Versteeg DH, Bohus B (1983) Inhibitory avoidance deficit following short-term adrenalectomy in the rat: the role of adrenal catecholamines. Behav Neural Biol 39: 241-58.

Brunet A, Orr SP, Tremblay J, Robertson K, Nader K, Pitman RK (2008) Effect of post-retrieval propranolol on psychophysiologic responding during subsequent script-driven traumatic imagery in post-traumatic stress disorder. J Psychiatr Res 42: 503-6.

Brunet A, Poundja J, Tremblay J, Bui E, Thomas E, Orr SP, Azzoug A, Birmes P, Pitman RK (2011) Trauma reactivation under the influence of propranolol decreases posttraumatic stress symptoms and disorder: 3 open-label trials. Journal of clinical psychopharmacology 31: 547-50.

Brunet A, Thomas E, Saumier D, Ashbaugh AR, Azzoug A, Pitman RK, Orr SP, Tremblay J (2014) Trauma reactivation plus propranolol is associated with durably low physiological responding during subsequent script-

driven traumatic imagery. Canadian journal of psychiatry Revue canadienne de psychiatrie 59: 228-32.

Cahill L, Alkire MT (2003) Epinephrine enhancement of human memory consolidation: interaction with arousal at encoding. Neurobiol Learn Mem 79: 194-8.

Chen CC, Williams CL (2012) Interactions between epinephrine, ascending vagal fibers, and central noradrenergic systems in modulating memory for emotionally arousing events. Frontiers in behavioral neuroscience 6: 35.

de Quervain DJ, Margraf J (2008) Glucocorticoids for the treatment of post-traumatic stress disorder and phobias: a novel therapeutic approach. European journal of pharmacology 583: 365-71.

Dohrenwend BP (2000) The role of adversity and stress in psychopathology: some evidence and its implications for theory and research. J Health Soc Behav 41: 1-19.

Dudai Y (2006) Reconsolidation: the advantage of being refocused. Curr Opin Neurobiol 16: 174-8.

Durkin TP, Messier C, de Boer P, Westerink BH (1992) Raised glucose levels enhance scopolamine-induced acetylcholine overflow from the hippocampus: an in vivo microdialysis study in the rat. Behav Brain Res 49: 181-8.

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: 849-58.

Elzinga BM, Bremner JD (2002) Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? Journal of Affective Disorders 70: 1-17.

Fanselow MS (1980) Conditioned and unconditional components of post-shock freezing. Pavlov J Biol Sci 15: 177-82.

Gabrieli JDE, Cohen NJ, Corkin S (1988) The impaired learning of semantic knowledge following bilateral medial temporal-lobe resection. Brain and Cognition 7: 157-177.

Gold PE (2014) Regulation of memory - from the adrenal medulla to liver to astrocytes to neurons. Brain Res Bull 105: 25-35.

Gold PE, Vogt J, Hall JL (1986) Glucose effects on memory: behavioral and pharmacological characteristics. Behav Neural Biol 46: 145-55.

Grillon C, Cordova J, Morgan CA, Charney DS, Davis M (2004) Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. Psychopharmacology (Berl) 175: 342-52.

Harrison J, MacKinnon PC (1966) Physiological role of the adrenal medulla in the palmar anhidrotic response to stress. J Appl Physiol 21: 88-92.

John GW, Doxey JC, Walter DS, Reid JL (1990) The role of alpha- and beta-adrenoceptor subtypes in mediating the effects of catecholamines on fasting glucose and insulin concentrations in the rat. Br J Pharmacol 100: 699-704.

Kaczkurkin AN, Foa EB (2015) Cognitive-behavioral therapy for anxiety disorders: an update on the empirical evidence. Dialogues in clinical neuroscience 17: 337-346.

Kelmendi B, Adams TG, Yarnell S, Southwick S, Abdallah CG, Krystal JH (2016) PTSD: from neurobiology to pharmacological treatments. Eur J Psychotraumatol 7: 31858.

Kim JJ, Jung MW (2006) Neural circuits and mechanisms involved in Pavlovian fear conditioning: A critical review. Neuroscience & Biobehavioral Reviews 30: 188-202.

King SO, 2nd, Williams CL (2009) Novelty-induced arousal enhances memory for cued classical fear conditioning: interactions between peripheral adrenergic and brainstem glutamatergic systems. Learning & memory 16: 625-34.

Krugers HJ, Karst H, Joels M (2012) Interactions between noradrenaline and corticosteroids in the brain: from electrical activity to cognitive performance. Frontiers in Cellular Neuroscience 6: 15.

LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23: 155-84.

Liang KC, Juler RG, McGaugh JL (1986) Modulating effects of posttraining epinephrine on memory: involvement of the amygdala noradrenergic system. Brain Res 368: 125-33.

Lonergan MH, Olivera-Figueroa LA, Pitman RK, Brunet A (2013) Propranolol's effects on the consolidation and reconsolidation of long-term emotional memory in healthy participants: a meta-analysis. J Psychiatry Neurosci 38: 222-31.

MacGregor DA, Prielipp RC, Butterworth JFt, James RL, Royster RL (1996) Relative efficacy and potency of beta-adrenoceptor agonists for generating cAMP in human lymphocytes. Chest 109: 194-200.

Mason ST, Angel A (1983) Anaesthesia: the role of adrenergic mechanisms. European journal of pharmacology 91: 29-39.

McGaugh JL, Roozendaal B (2002) Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol 12: 205-10.

McIntyre CK, McGaugh JL, Williams CL (2012) Interacting Brain Systems Modulate Memory Consolidation. Neuroscience and biobehavioral reviews 36: 1750-1762.

McNay EC, Gold PE (2002) Food for thought: fluctuations in brain extracellular glucose provide insight into the mechanisms of memory modulation. Behav Cogn Neurosci Rev 1: 264-80.

Messier C (2004) Glucose improvement of memory: a review. European journal of pharmacology 490: 33-57.

Mineka S, Mystkowski JL, Hladek D, Rodriguez BI (1999) The effects of changing contexts on return of fear following exposure therapy for spider fear. J Consult Clin Psychol 67: 599-604.

Mizoguchi K, Ishige A, Takeda S, Aburada M, Tabira T (2004) Endogenous glucocorticoids are essential for maintaining prefrontal cortical cognitive function. J Neurosci 24: 5492-9.

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr Rev 5: 25-44.

Muravieva EV, Alberini CM (2010) Limited efficacy of propranolol on the reconsolidation of fear memories. Learning & memory 17: 306-13.

Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117: 131-43.

Nagatsu T (2006) The catecholamine system in health and disease —Relation to tyrosine 3-monooxygenase and other catecholamine-synthesizing

enzymes—. Proceedings of the Japan Academy Series B, Physical and Biological Sciences 82: 388-415.

Nguyen PV, Abel T, Kandel ER, Bourtchouladze R (2000) Strain-dependent Differences in LTP and Hippocampus-dependent Memory in Inbred Mice. Learning & memory 7: 170-179.

O'Donnell A, Judson I, Dowsett M, Raynaud F, Dearnaley D, Mason M, Harland S, Robbins A, Halbert G, Nutley B, Jarman M (2004) Hormonal impact of the 17alpha-hydroxylase/C(17,20)-lyase inhibitor abiraterone acetate (CB7630) in patients with prostate cancer. Br J Cancer 90: 2317-25.

Oitzl MS, van Haarst AD, Sutanto W, de Kloet ER (1995) Corticosterone, brain mineralocorticoid receptors (MRs) and the activity of the hypothalamic-pituitary-adrenal (HPA) axis: the Lewis rat as an example of increased central MR capacity and a hyporesponsive HPA axis. Psychoneuroendocrinology 20: 655-75.

Peeters BW, Smets RJ, Broekkamp CL (1992) The involvement of glucocorticoids in the acquired immobility response is dependent on the water temperature. Physiol Behav 51: 127-9.

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106: 274-85.

Pitman RK, Delahanty DL (2005) Conceptually driven pharmacologic approaches to acute trauma. CNS Spectr 10: 99-106.

Pitman RK, Sanders KM, Zusman RM, Healy AR, Cheema F, Lasko NB, Cahill L, Orr SP (2002) Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. Biol Psychiatry 51: 189-92.

Rominger KL, Pollmann W (1972) [Comparative pharmacokinetic studies on fenoterol-hydrobromide in rat, dog and man]. Arzneimittel-Forschung 22: 1190-6.

Roozendaal B (2002) Stress and Memory: Opposing Effects of Glucocorticoids on Memory Consolidation and Memory Retrieval. Neurobiology of Learning and Memory 78: 578-595.

Roozendaal B, Portillo-Marquez G, McGaugh JL (1996) Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. Behav Neurosci 110: 1074-83.

Rosol TJ, Yarrington JT, Latendresse J, Capen CC (2001) Adrenal gland: structure, function, and mechanisms of toxicity. Toxicol Pathol 29: 41-8.

Schmidt MV (2010) Molecular mechanisms of early life stress--lessons from mouse models. Neurosci Biobehav Rev 34: 845-52.

Schwabe L, Nader K, Wolf OT, Beaudry T, Pruessner JC (2012) Neural signature of reconsolidation impairments by propranolol in humans. Biol Psychiatry 71: 380-6.

Shalev AY, Orr SP, Peri T, Schreiber S, Pitman RK (1992) Physiologic responses to loud tones in Israeli patients with posttraumatic stress disorder. Arch Gen Psychiatry 49: 870-5.

Sherin JE, Nemeroff CB (2011) Post-traumatic stress disorder: the neurobiological impact of psychological trauma. Dialogues in clinical neuroscience 13: 263-278.

Smith SM, Vale WW (2006) The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in clinical neuroscience 8: 383-395.

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: 1-9.

Ursano RJ, Kessler RC, Stein MB (2016) Suicide Attempts in the US Army--Reply. JAMA Psychiatry 73: 176-7.

Villain H, Benkahoul A, Drougard A, Lafragette M, Muzotte E, Pech S, Bui E, Brunet A, Birmes P, Roullet P (2016) Effects of Propranolol, a beta-noradrenergic Antagonist, on Memory Consolidation and Reconsolidation in Mice. Frontiers in behavioral neuroscience 10: 49.

Weil-Malherbe H, Axelrod J, Tomchick R (1959) Blood-brain barrier for adrenaline. Science 129: 1226-7.

Yehuda R, McFarlane AC, Shalev AY (1998) Predicting the development of posttraumatic stress disorder from the acute response to a traumatic event. Biol Psychiatry 44: 1305-13.

Zhang S, Huang T, Ilangovan U, Hinck AP, Fitzpatrick PF (2014) The Solution Structure of the Regulatory Domain of Tyrosine Hydroxylase. Journal of molecular biology 426: 1483-1497.