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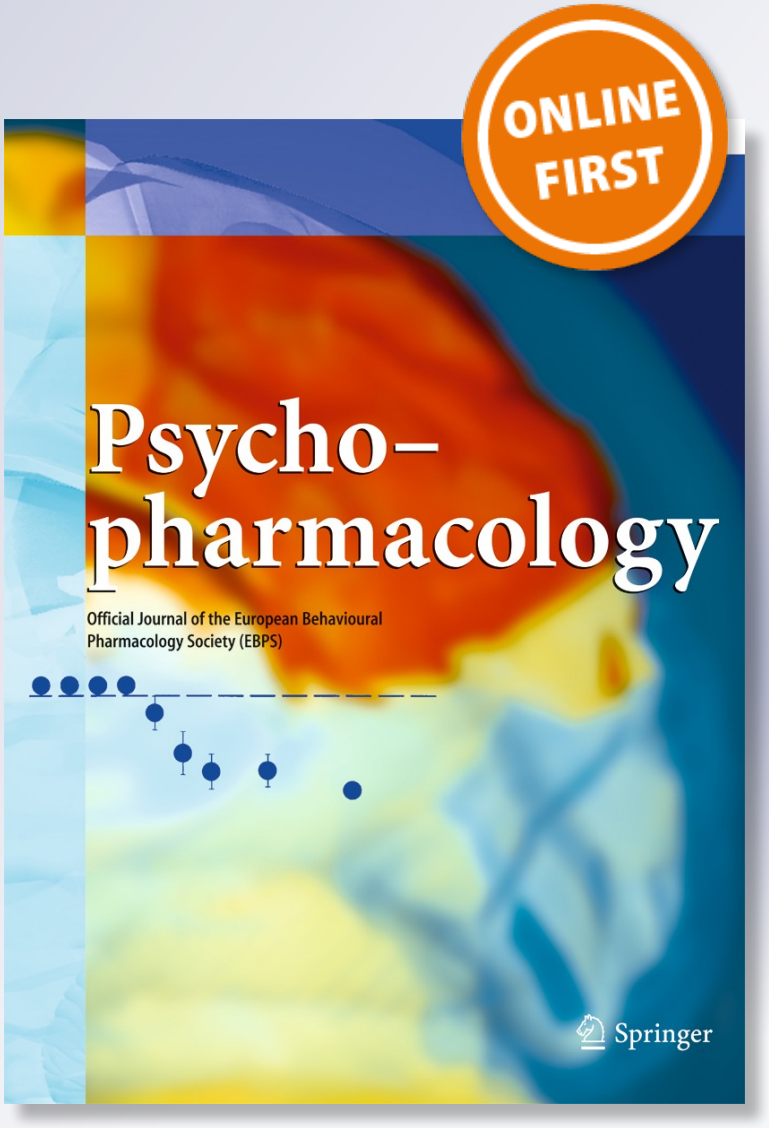

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ORIGINAL INVESTIGATION

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]

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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

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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

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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,

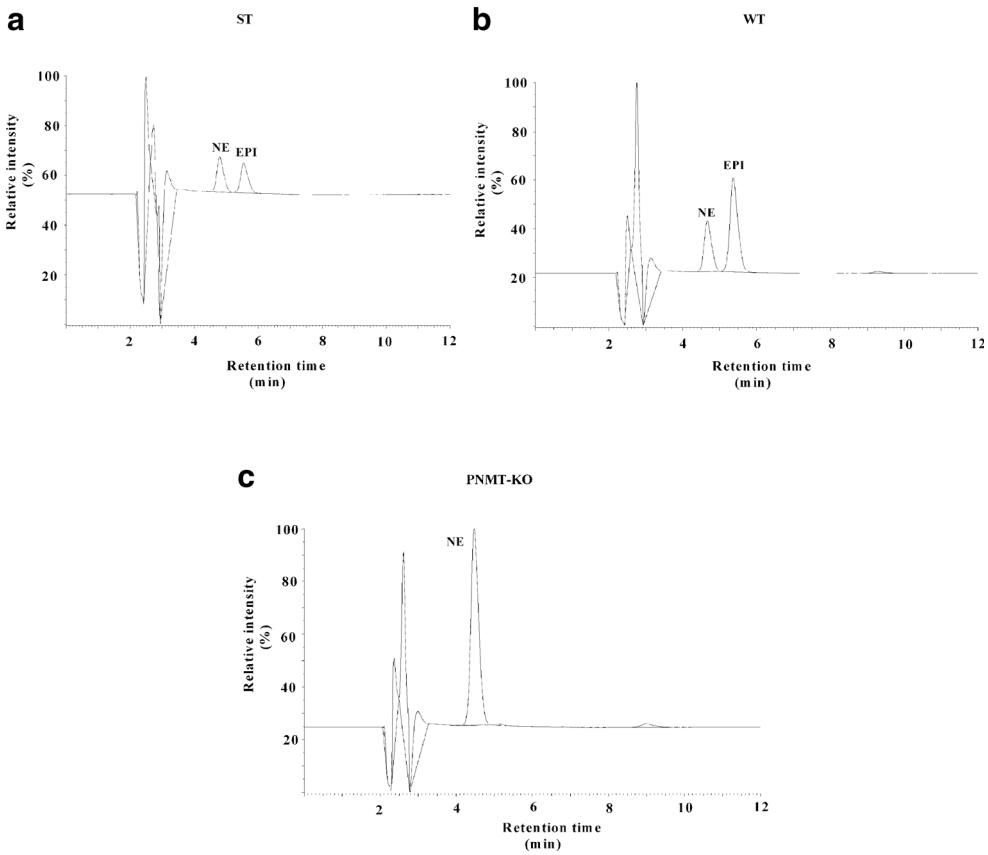

Fig. 1 Representative chromatograms of catecholamines in **a** standard (*St*) and adrenal glands of **b** wild-type (*WT*) and **c** phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice. *NE* norepinephrine, *EPI* epinephrine

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-

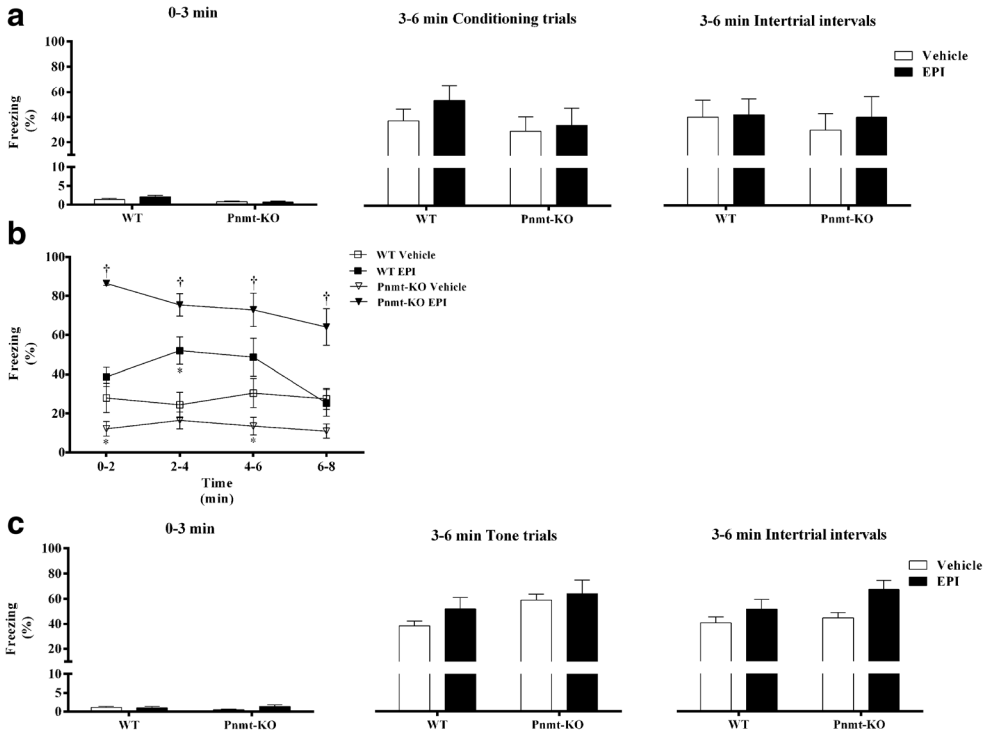

Fig. 2 Behavioral experiment 1. Freezing on the **a** first day (fear acquisition), **b** second day (context fear test), and **c** third day (cue fear) of fear conditioning procedure in wild-type (*WT*) and phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl 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

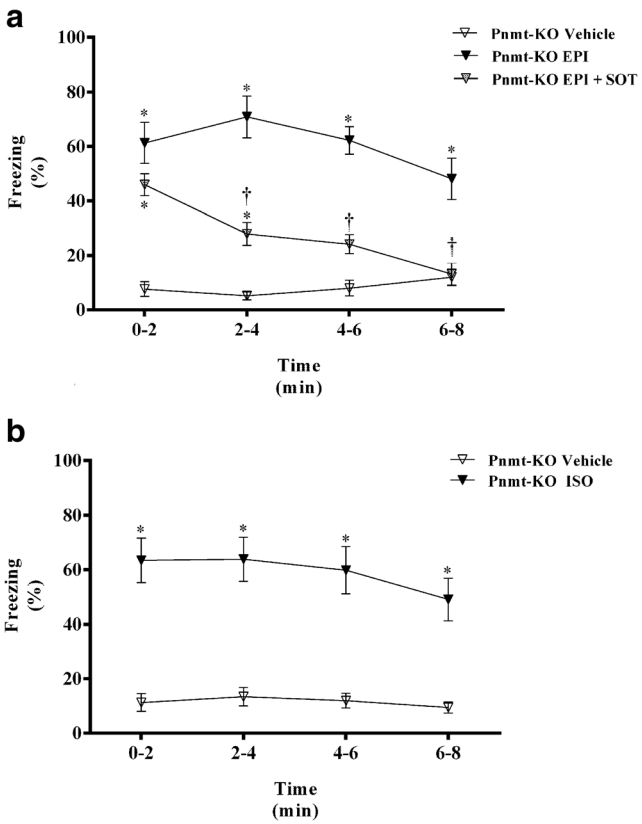

Fig. 3 Behavioral experiments 2 and 3. Freezing on the second day (context fear test) of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl 0.9 %) and **a** epinephrine (*EPI*, 0.1 mg/kg) and EPI (0.1 mg/kg) plus sotalol (*SOT*, 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) ($p < 0.05$)

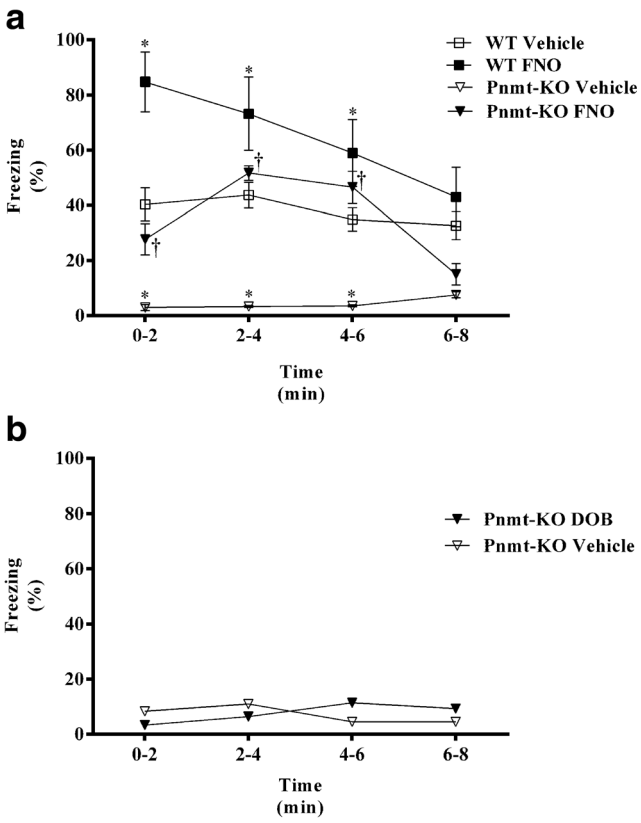

Fig. 4 Behavioral experiments 4 and 5. Freezing on the second day (context fear test) of fear conditioning procedure in wild-type (*WT*) or phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl, 0.9 %) and **a** fenoterol (*FNO*, selective β_2-adrenoceptor agonist, 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

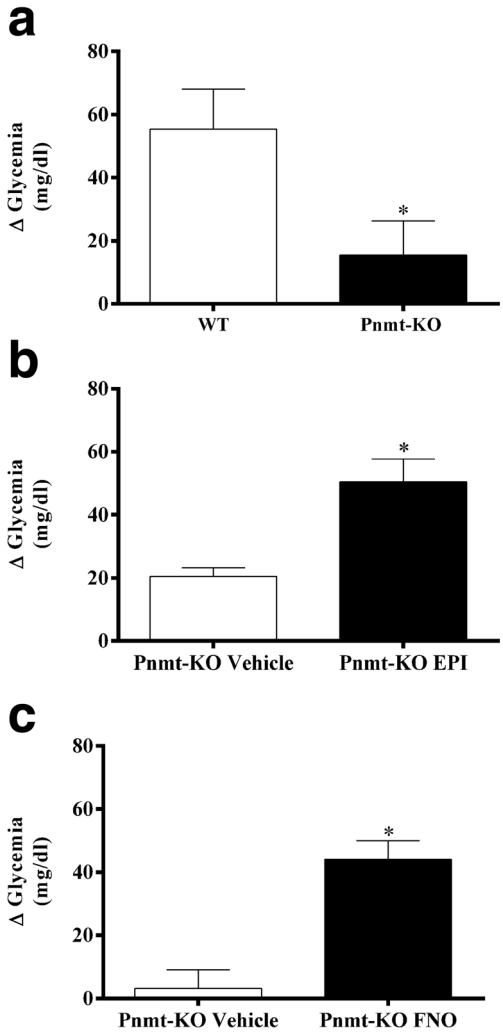

Fig. 5 Glycemic increase in replicated fear conditioning experiments. **a** Wild-type (*WT*) and phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice. Pnmt-KO mice treated with vehicle (NaCl 0.9 %) and **b** epinephrine (*EPI*, 0.1 mg/kg) or **c** FNO (2.8 mg/kg). The glycemic variation (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 ($p < 0.05$)

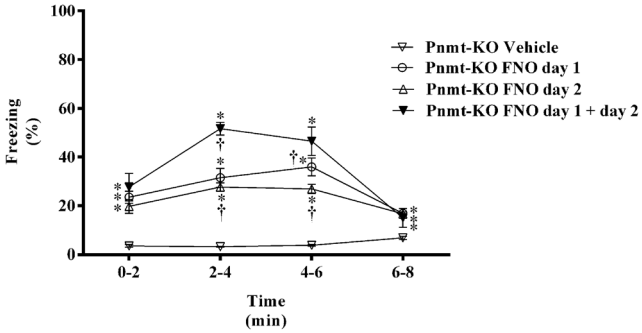

Fig. 6 Behavioral experiment 6. Freezing on the second day (context fear test) of fear conditioning procedure in phenylethanolamine-*N*-methyltransferase knockout (*Pnmt-KO*) mice treated with vehicle (NaCl, 0.9 %) or fenoterol (*FNO*, 2.8 mg/kg) injected just pre-training (day 1), just pre-testing (day 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.

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