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1 **Hippocampus-dependent fear conditioning is not sensitized by muscarinic**
2 **receptor activation following systemic injection of pilocarpine**

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1

2 **Abstract**

3

4 The regulation of muscarinic acetylcholine receptors (mAChR) critically influences
5 emotional outcomes. Previous researches indicate that a single systemic injection of
6 pilocarpine – a mAChR agonist – displays long-term defensive behaviors in rats
7 evaluated in distinct unconditioned tests up to 3 months following treatment. However,
8 it is not clear whether these effects share underlying behavioral phenotypes involved in
9 conditioned responses. With this in mind, we examined whether mAChR activation
10 modulates contextual fear conditioning (CFC) and/or hippocampal synaptic plasticity.

11 **Adult male Wistar rats were injected with pilocarpine (150 mg/kg) and behaviorally**
12 **evaluated in the CFC test or followed by synaptic plasticity (LTP/LTD) investigation in**
13 **CA1 stratum radiatum of hippocampal slices.** There was no difference between groups
14 in the quantification of freezing behavior during the test period (24 h after treatment)
15 besides a decrease of freezing 1 month later. Similarly, no changes were observed in
16 rats conditioned 24 h later and tested 1 month after. Synaptic plasticity investigation
17 following short- or long-term treatment revealed no differences between control and
18 treated subjects. In summary, our results show that hippocampus-dependent fear
19 behavior and memory consolidation mediated by hippocampal cholinergic inputs are
20 not sensitive to activation of mAChR by a systemic nonconvulsant dose of pilocarpine.

21 **Therefore, we suggest that the long-term defensive behaviors and anxiogenic-like**
22 **features displayed by pilocarpine observed in rats are mediated by different underlying**
23 **mechanisms and or set of synapses.**

24

25 **Keywords:** muscarinic receptors; fear conditioning; synaptic plasticity; long-term
26 anxiety

1

2 **1. Introduction**

3 Anxiety and fear are critical to survival, occurring by species-specific reactions
4 through the expression of a wide range of adaptive or defensive behaviors (Steimer,
5 2002). Epidemiological studies show that anxiety is prevalent nearly 33.7% during
6 lifetime of the American adult population (Kessler, Petukhova, Sampson, Zaslavsky, &
7 Wittchen, 2012) and 14.5% of Europeans (Alonso, Lépine, & Committee, 2007),
8 making it one of the most incident mental illness. In this sense, translational
9 investigations have identified the functions of the basal forebrain and implicated the
10 cholinergic system as crucial to better understand the mechanisms involved in these
11 disorders (Duarte et al., 2010; Fedoce, Ferreira-Junior, Reis, Corrêa, & Resstel, 2016;
12 Knox, 2016; McGaughy, Koene, Eichenbaum, & Hasselmo, 2005).

13 Acetylcholine enhances hippocampus-dependent learning by regulating the
14 induction and maintenance of signaling transmission (Hasselmo, 2006), a process that
15 requires the activation of muscarinic acetylcholine receptors (mAChR) (Mitsushima,
16 Sano, & Takahashi, 2013). Levels of acetylcholine in the hippocampus and cortex
17 increase considerably throughout stress events (Power & Sah, 2008). **However, the**
18 **way in which cholinergic function converges to regulate hippocampal projections in**
19 **response to it is still unknown. In addition, glucocorticoid and mineralocorticoid**
20 **receptors are highly expressed in the hippocampus where they play important role on**
21 **stress control (Jacobson & Sapolsky, 1991; McEwen, 2000).**

22 Recently, Ledoux and Pine (2016) have proposed the “two-systems” framework
23 to characterize different neural circuits supporting behavioral and physiological
24 responses to threats (unconscious defensive reactions) and feeling states (conscious
25 reactions). These systems involve subcortical regions such as the amygdala and
26 hippocampus and cortical areas, respectively (Maren, Phan, & Liberzon, 2013). Indeed,
27 this observation provides new substrates for translational research of anxiety disorders
28 (LeDoux & Pine, 2016).

1 Previous findings revealed the ability of mAChRs to regulate long-term
2 anxiogenic-like behavioral features in rats evaluated in different unconditioned tests
3 (Duarte et al., 2013). These effects are mainly mediated by prosencephalic
4 connections, with the involvement of the fimbria-fornix and post-commissural fornix
5 pathways (Duarte et al., 2010), associated with alterations of hippocampal theta rhythm
6 (Hoeller et al., 2013), downregulation of hippocampal NMDA and glucocorticoid
7 receptors, besides augmentation of hormonal release long-after the treatment with
8 pilocarpine – a mAChR agonist (Hoeller et al., 2016). However, it is unclear whether
9 these chronic effects elicited by the activation of mAChRs are also related with
10 behavioral phenotypes linked with implicit memory consolidation and/or specific
11 molecular memory formation. Therefore, the present study examined whether mAChRs
12 activation by pilocarpine treatment, known to induce experimental anxiogenic-like
13 responses in rats, may also affect contextual fear conditioning behavior and/or
14 hippocampal synaptic plasticity. We thought that this approach could reveal the
15 participation of the cholinergic system in the regulation of distinct processes involving
16 conscious (conditioned responses) and nonconscious feelings (unconditioned
17 responses).

18

19 **2. Material and Methods**

20 **2.1 Animals**

21 Adult male Wistar rats (2-3 months old, weighing 200–300 g) were housed in a
22 room with controlled temperature ($22 \pm 2^\circ\text{C}$) and a 12-h light/dark cycle (lights on at
23 07:00 a.m.) with free access to food and water. Rats were allowed to habituate to the
24 laboratory conditions for one week before the experiments. Experiments were carried
25 out during the light phase of the cycle. All experiments were conducted in accordance
26 with international standards of animal welfare recommended by the Brazilian Law
27 (#11.794–10/08/2008) and Animals (Scientific Procedures) Act 1986, with experimental
28 protocols approved by the Committee for Ethics in Animal Research of the Federal

1 University of Santa Catarina (CEUA-UFSC #23080.025621/2009-03) and the UK
2 Animals Scientific Procedures Act 1986.

3 4 2.2 Drugs and treatments

5 Pilocarpine hydrochloride (a non-selective mAChR agonist; Sigma-Aldrich Co.,
6 St. Louis, USA, 150mg/kg, i.p.) was injected intraperitoneally whereas methyl-
7 scopolamine bromide (a mAChR antagonist; RBI, USA, 1 mg/kg, s.c.) was given
8 subcutaneously and used to prevent the peripheral cholinomimetic effects elicited by
9 pilocarpine. All drugs were freshly dissolved in saline solution (NaCl 0.9 %), which was
10 used as control solution as well, in a volume injection of 2 ml/kg. All doses were used
11 according with previous studies with similar protocols. **Important to note, pilocarpine**
12 **effects (at 150 mg/kg) are not associated with any electrographic or behavioral**
13 **epileptiform activity aside its long-term anxiogenic effects (Duarte et al., 2013; Duarte**
14 **et al., 2010; Hoeller et al., 2013; Hoeller et al., 2016).**

15 16 2.3 Contextual fear conditioning

17 The contextual fear conditioning (CFC) protocol used here is an adaptation of
18 the Pavlovian fear conditioning protocol. Rats were placed in the conditioning chamber
19 for 3 min under a 10 lux light. After that, an unconditioned stimulus (US) is presented
20 as a 1 s electric footshock (1.5 mA) followed by contextualization for an additional
21 minute in the chamber (conditioned stimulus, CS). Rats were re-exposed in the
22 chamber twenty-four hours later for 5 min and time of freezing behavior was scored
23 according **with studies of Lach and colleagues (2016) and Lach & de Lima (2013).**

24 25 2.4 *In vitro* extracellular recordings (LTP)

26 Twenty-four hours or 1 month after pilocarpine treatment, rats were
27 anesthetized with isoflurane, killed by decapitation and the hippocampal tissue
28 prepared for extracellular recordings, according with Bortolotto and colleagues (2011).

1 Following preparation, slices were allowed to recover for, at least, 2 h in oxygenated
2 aCSF at room temperature. Field potential recordings were made using
3 microelectrodes containing 4 M NaCl and placed in the CA1 region. Synaptic
4 responses were evoked by stimulation at 0.033 Hz. The presence of synaptic
5 facilitation was established at the beginning of the experiment to confirm that the
6 responses were CA1 in origin, and stimulation intensity was adjusted so that basal field
7 excitatory post-synaptic potentials (fEPSP) amplitude was 50 to 60 % of maximum.
8 LTP was induced by delivering a single tetanus (100 Hz, 1 s) and responses were
9 collected and analyzed on-line using the WinLTP software (Anderson & Collingridge,
10 2001). All data were normalized to the baseline condition.

11

12 2.5 Experimental procedures

13 Experiments were performed in two main designs: In experiment 1, we
14 hypothesized that long-term anxiogenic-like features elicited by mAChR activation
15 might act as a conditioning factor facilitating contextual fear memories, a hippocampal-
16 dependent task. Therefore, rats (n=12 per group) were treated with pilocarpine and
17 submitted to the CFC 24 h and/or 1 month following injection (see experimental design
18 depicted in Fig 1 for detailed information). In experiment 2, we hypothesized that long-
19 term effects elicited by mAChR activation might reflect changes on hippocampal
20 synaptic plasticity. Rats (n=5 per group) were euthanized 24 h or 1 month following
21 pilocarpine treatment and fEPSPs recorded from *in vitro* preparation of hippocampal
22 slices (see protocol depicted in Fig 2 for detailed information).

23

24 2.6 Statistical analysis

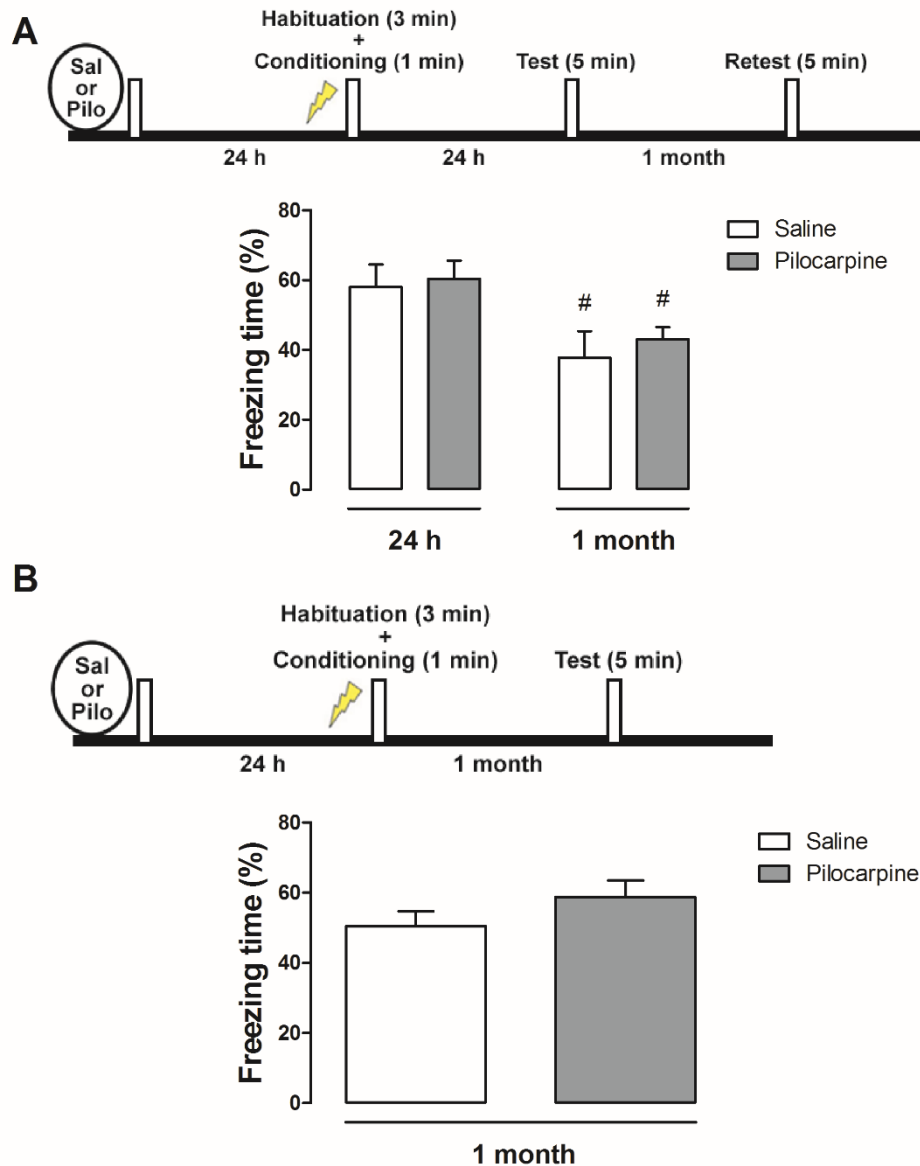
25 All values are expressed as means \pm S.E.M. Data of experiments were
26 analyzed by unpaired two-tailed Student's *t* test or ANOVA when the treatment and/or
27 period of the condition were used as factors, followed by the Student Newman–Keuls'
28 post hoc test for multiple comparisons when appropriate. Differences were considered

1 significant at $p < 0.05$. All tests were performed using the software Statistica® (StaSoft
2 Inc., Tulsa, USA), version 8.0 and graphs were drawn with the software GraphPad
3 Prism®, version 5.0.

4 5 **3. Results**

6 The acute and long-term effects elicited by the activation of mAChRs with
7 pilocarpine on contextual fear memory were investigated. As observed in Fig 1,
8 following ANOVA with repeated measures, there is no difference between treated
9 groups in the time of freezing behavior during the test period (24 h after treatment)
10 besides a significant decrease of this behavior during the retest period 1 month after
11 injections [$R1:F(1,22)=24,40$; $p < 0,0001$] when compared with the prior evaluation.
12 Similarly, Student's t test did not reveal any differences in the time of freezing behavior
13 of rats treated with pilocarpine and submitted to fear conditioning process 24 h later
14 and tested 1 month after (Fig. 1B, $t(22)=1.3$, $p > 0.05$). Important to say, a similar
15 protocol was applied with a lower stimulus during conditioning period (0.7 mA applied
16 during 1 s) but no significant alterations in the time of freezing behavior were observed
17 (data not shown).

18
19 **INSERT FIGURE 1 HERE**



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Figure 1 – Effects of pilocarpine on freezing behavior of rats evaluated in the contextual fear conditioned task following mAChR activation. A) Animals were placed in the conditioning chamber 24 h after injections (3-min period for habituation plus 1 min-period following 1.5 mA/1 s footshock stimulation). Rats were treated with methyl-scopolamine bromide and pilocarpine or saline and exposed in the conditioning chamber 24 h after injections (5-min period) and retested 1 month later (5-min period). B) Animals were treated with methyl-scopolamine bromide and pilocarpine or saline and exposed in the conditioning chamber 24 h after injections (3-min period for habituation and 1-min period following stimulus, 1.5 mA applied during 1 s) and tested 1 month later (5-min period). Values are expressed as mean±S.E.M. of time of freezing behavior expressed in percentage (n=12 animals/group). # $p < 0.05$ in relation to animals tested 24 h after treatments by ANOVA with repeated measures. Sal: Saline; Pilo: pilocarpine.

13 The effects of mAChR activation following pilocarpine administration on
14 hippocampal plasticity were investigated short- and long-term after treatment. As
15 shown in Fig. 2, Student's t test did not reveal differences between hippocampal
16 fEPSPs amplitude of rats treated with pilocarpine or saline 24 h ($t(6)=0.4$, $p > 0.05$) or 1

1 month after the treatment ($t(5)=0.8$, $p>0.05$), and no alterations on evoked potentials following LTP induction were observed (24 h: $t(5)=1.5$, $p>0.05$; 1 month: $t(5)=0.05$, $p>0.05$). However, an alteration of the evoked fEPSP's shape/profile following LTP induction can be observed in Pilocarpine treated group, 24 h, figure 2 B, red traces. Notice that the changes in the fEPSP traces are not present in the same group of animals 1 month later to parallel with the behavioral fear conditioning responses. We believe that those changes are mediated by inhibitory activity generated by interneurons.

9 Noteworthy, the treatment with pilocarpine did not interfere with LTD induction (900 shocks at 1 Hz) *in vitro* (data not shown) or LTP induction (100 Hz, 1 s) *in vivo* (data not shown). Therefore, these results show that under our conditions pilocarpine treatment (150 mg/Kg) did not alter the ability of treated animals to develop synaptic plasticity although there were alterations of the membrane properties of the recorded neurons.

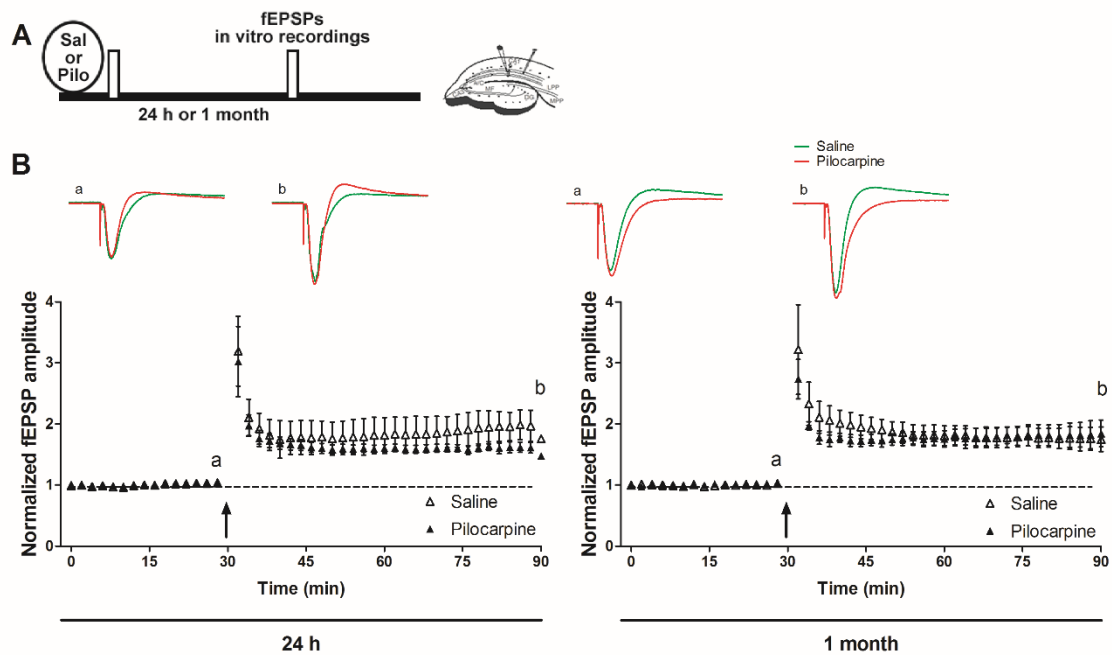
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INSERT FIGURE 2 HERE

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2

3 **Figure 2** – Effects of pilocarpine in CA1 synaptic plasticity. A) Rats were treated with **methyl-scopolamine**
 4 **bromide** and pilocarpine or saline and hippocampal fEPSPs recordings are carried out 24 h or 1 month
 5 following treatment. B) Upper traces represent fEPSP responses elicited by CA1 stimulation at baseline
 6 period (a) and 1-h following LTP induction (b). Values are expressed as the average of 4 consecutive
 7 fEPSP responses (n=5 animals/group) obtained during baseline (0-30 min) and after the tetanic stimulus
 8 (arrow, 30-90 min). Sal: Saline; Pilo: Pilocarpine.

9

10 **4. Discussion**

11 The concept of innate fear has long been known as a dichotomy of a conscious
 12 feeling triggered by a threat, behavioral and physiological events elicited by it (LeDoux
 13 & Pine, 2016). Here, the present study shows that mAChR activation does not trigger
 14 long-term hippocampus-dependent fear-conditioned behavior, nor interfere with
 15 synaptic plasticity changes, differing with previous findings that denote the clear
 16 induction of unconditioned enduring anxiogenic-like behaviors (i.e., elevated plus-maze
 17 and open field tests) and sharp hormonal changes in rats following the treatment with
 18 pilocarpine (Duarte et al., 2013; Duarte et al., 2010; Hoeller et al., 2016; Hoeller et al.,
 19 2013).

20 The hippocampus is protagonist on contextual fear conditioning (Phillips &
 21 LeDoux, 1992) and was implicated on longstanding experimental anxiogenesis
 22 following the injection of pilocarpine (Duarte et al., 2010), reflecting an increased

1 hippocampal theta activity (Duarte et al., 2013) and reduction of glucocorticoid
2 receptors expression (Hoeller et al., 2016). Nevertheless, the implication of aversive
3 memory formation on these processes was not correlated once the behavior of rats
4 evaluated in the step-down avoidance or in the elevated T-maze tests were not
5 affected (Duarte et al., 2013). Investigations regarding the modulation of the cholinergic
6 system on Pavlovian conditioning have shown discrepancies. The injections of NMDA
7 or cholinergic receptor antagonists in the hippocampus during training in fear
8 conditioning tests may impair contextual fear, but not auditory-cue conditioned memory
9 (Gale, Anagnostaras, & Fanselow, 2001; Young, Bohenek, & Fanselow, 1994),
10 suggesting that the involvement of the hippocampus on fear conditioning may be
11 related to specific paired-stimulus classes than the association between conditioned
12 and unconditioned stimulus (for review, see Fendt & Fanselow, 1999). Systemic
13 administration of scopolamine – a mAChR antagonist – prior to training decreases the
14 acquisition of CFC in rats at doses that do not change auditory conditioning, in a
15 protocol with multiple conditioning parameters. Furthermore, the immediate or 24-h
16 after intervention in training did not alter freezing duration, showing a modulatory role of
17 the cholinergic system in acquisition, but not in consolidation of aversive memories
18 (Anagnostaras, Maren, & Fanselow, 1995). In contrast, injections of scopolamine
19 before or up to 3 h after Pavlovian conditioning alter auditory-cue and contextual
20 conditioning when a single footshock is delivered. However, when multiple footshock
21 sessions were applied, none effect following training was observed in auditory-cue fear
22 conditioning, pointing that the number of footshock sessions may explain the way that
23 cholinergic drugs modulate Pavlovian conditioning (Rudy, 1996).

24 Important to note, the absence of behavioral changes (i.e. time of freezing) in
25 fear conditioning tests may not reflect the interoceptive sense of rats – the integrative
26 information regarding pain sensation, temperature, itch, muscular tension and gastric
27 distress (Paulus & Stein, 2010). Along with the prefrontal cortex and the insula
28 (considered the encephalic interoceptive center) detects pronounced emotional stimuli

1 besides acting on the generation and regulation of feedback responses (Phillips,
2 Drevets, Rauch, & Lane, 2003). In this sense, we believe that a systematic
3 investigation with different experimental protocols (e.g. different footshock parameters
4 and conditioning time) or the insertion of different conditioning factors (e.g. olfactory or
5 auditory aversive conditioning, fear potentiated startle) may clearly express the effects
6 of the cholinergic system in anxiety and conditioned fear responses.

7 Associative learning is closely related to synaptic plasticity, and LTP properties,
8 such as fast induction and associativity, are pointed as ideal candidates in the
9 codification of aversive memories (Maren, 2005). There is broad evidence showing the
10 regulatory action of the cholinergic system on hippocampal-dependent memory
11 (Auerbach & Segal, 1994; Blitzer, Gil, & Landau, 1990; Shinoe, Matsui, Taketo, &
12 Manabe, 2005), an effect possibly modulated by M1 subtype mAChRs (Boddeke, Enz,
13 & Shapiro, 1992; Burgard & Sarvey, 1990). However, M1 null mutant mice display only
14 mild impairment of learning and hippocampal LTP (Anagnostaras et al., 2003).
15 Recently, Dennis and colleagues (2016) showed a vigorous potentiation of
16 glutamatergic synaptic transmission onto CA1 pyramidal neurons following M1 subtype
17 mAChRs activation, revealing a synergistic NMDAR-dependent mechanism that bi-
18 directionally occludes LTP. Importantly, LTP induction is triggered by NMDAR at most
19 CNS synapses and it is critically involved in many sorts of learning and memory (Bliss,
20 Collingridge, & Morris, 2014). Further, the administration of different NMDAR
21 antagonists into the hippocampus promotes anxiolytic-like responses in animals tested
22 in different unconditioned tests of anxiety (Barkus et al., 2010). These effects appear to
23 be hippocampus-dependent and NMDAR subtypes-dependent since mice that do not
24 express the NR1 subunit of the NMDAR in the granule cells of the dentate gyrus exhibit
25 normal LTP in the CA1 region, although presenting an anxiolytic profile in anxiety trials
26 (Niewoehner et al., 2007). Moreover, mice that do not express the NR2B subunit in the
27 pyramidal and granular hippocampal cells also exhibit anxiolytic-like responses (von
28 Engelhardt et al., 2008). **Interestingly, an anxiogenic dose of pilocarpine (150 mg/kg)**

1 can downregulate the expression of hippocampal NMDARs long-after treatment
2 (Hoeller et al., 2016), revealing that it can play only a marginal effect on key-substrates
3 of neuroplasticity which are unable to alter the LTP induction or magnitude in the CA1
4 area.

5 In fact, the hippocampus is well established as a contextual encoder of
6 conditioned fear of rodents besides broadly projecting to the amygdala and prefrontal
7 cortex (Shin & Liberzon, 2010). The phosphorylation pattern of Ser-831 and -845 of
8 GluA1 subunit of glutamate receptors are related with fear memory and LTP induction
9 in the CA1 area (Shukla, Kim, Blundell, & Powell, 2007). Interestingly, hippocampal
10 levels of p-GluA1-Ser831 are prone to be associated with conditioned learning when
11 compared with non-associated aversive stimuli, such as foot shock stimulus or novelty
12 exposure (Bevilaqua, Medina, Izquierdo, & Cammarota, 2005; Cammarota, Bernabeu,
13 Levi De Stein, Izquierdo, & Medina, 1998; Shukla et al., 2007). Further, P-GluA1-Ser-
14 845 levels are decreased during LTD and associated with downregulation of AMPA
15 receptors (Ehlers, 2000), whereas these receptors are synaptically upregulated during
16 LTP (Huganir & Nicoll, 2013; Lee, Barbarosie, Kameyama, Bear, & Huganir, 2000).
17 Similarly, a decrease in phosphorylation of Ser-831 in the hippocampus and GluA1
18 subunit in the amygdala was observed in patients with unilateral mesial temporal lobe
19 epilepsy with ictal fear aura when compared with those with other kinds of aura,
20 highlighting a distinct pattern of interface between ictal fear and fear conditioning (Leal
21 et al., 2018), corresponding with the classical view where the hippocampus plays
22 distinct function on modulation of fear and anxiety responses (Strange, Witter, Lein, &
23 Moser, 2014).

24 25 **5. Conclusion**

26 Altogether, our results demonstrated that hippocampus-dependent fear
27 behavior and memory processes mediated by cholinergic inputs in the CA1 region are
28 inadequately activated by pilocarpine under our conditions, suggesting a distinct

1 modulation between long-term fear-related responses and experimental anxiety. We
 2 expect that additional investigation with alternative approaches will provide results that
 3 will allow better understand the adopted chronic coping strategies in both conditioned
 4 and unconditioned challenges. Also, it cannot be discard a possible involvement of
 5 post-transcriptional regulation in the mediation of the long-term phenotypic responses
 6 triggered by mAChRs activation. Additionally, the role of GABAergic transmission
 7 should be considered since there is a clear evidence in our recordings that the
 8 inhibitory component of the fEPSP is modified after treatment and it may underlay fear-
 9 related responses and experimental anxiety.

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