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

Hoeller AA[1,2,3], Lach G[2,4], Costa APR[2], Walz R[1,5], Bortolotto ZA[3], De Lima TCM[2]

[1] Postgraduate Program in Medical Science, Center of Health Sciences, University Hospital, Federal University of Santa Catarina, Florianópolis, SC, 88040-970, Brazil
[2] Department of Pharmacology, Center of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88049-900, Brazil
[3] Centre for Synaptic Plasticity, School of Physiology, Pharmacology and Neuroscience, University of Bristol, Dorothy Hodgkin Building, Bristol BS1 3NY, UK
[4] APC Microbiome Institute, University College Cork, Cork, Ireland
[5] Department of Clinical Medicine, Center of Health Sciences, University Hospital, Federal University of Santa Catarina, Florianópolis, SC, 88040-970, Brazil

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**Corresponding author:**

Dr. Alexandre A. Hoeller

Federal University of Santa Catarina (UFSC)

Laboratory of Medical Sciences

Department of Clinical Medicine, University Hospital

Florianópolis, Santa Catarina, 88040-970, Brazil

Phone: +55 048 3721-2210

Email: alexandrehoeller@gmail.com

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## Abstract

The regulation of muscarinic acetylcholine receptors (mAChR) critically influences emotional outcomes. Previous researches indicate that a single systemic injection of pilocarpine – a mAChR agonist – displays long-term defensive behaviors in rats evaluated in distinct unconditioned tests up to 3 months following treatment. However, it is not clear whether these effects share underlying behavioral phenotypes involved in conditioned responses. With this in mind, we examined whether mAChR activation modulates contextual fear conditioning (CFC) and/or hippocampal synaptic plasticity. Adult male Wistar rats were injected with pilocarpine (150 mg/kg) and behaviorally evaluated in the CFC test or followed by synaptic plasticity (LTP/LTD) investigation in CA1 stratum radiatum of hippocampal slices. There was no difference between groups in the quantification of freezing behavior during the test period (24 h after treatment) besides a decrease of freezing 1 month later. Similarly, no changes were observed in rats conditioned 24 h later and tested 1 month after. Synaptic plasticity investigation following short- or long-term treatment revealed no differences between control and treated subjects. In summary, our results show that hippocampus-dependent fear behavior and memory consolidation mediated by hippocampal cholinergic inputs are not sensitive to activation of mAChR by a systemic nonconvulsant dose of pilocarpine. Therefore, we suggest that the long-term defensive behaviors and anxiogenic-like features displayed by pilocarpine observed in rats are mediated by different underlying mechanisms and or set of synapses.


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

## 1. Introduction

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

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

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

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

## 2. Material and Methods

### 2.1 Animals

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

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

## 2.2 Drugs and treatments

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

## 2.3 Contextual fear conditioning

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

## 2.4 *In vitro* extracellular recordings (LTP)

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

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

## 2.5 Experimental procedures

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

## 2.6 Statistical analysis

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

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

## 3. Results

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

**INSERT FIGURE 1 HERE**

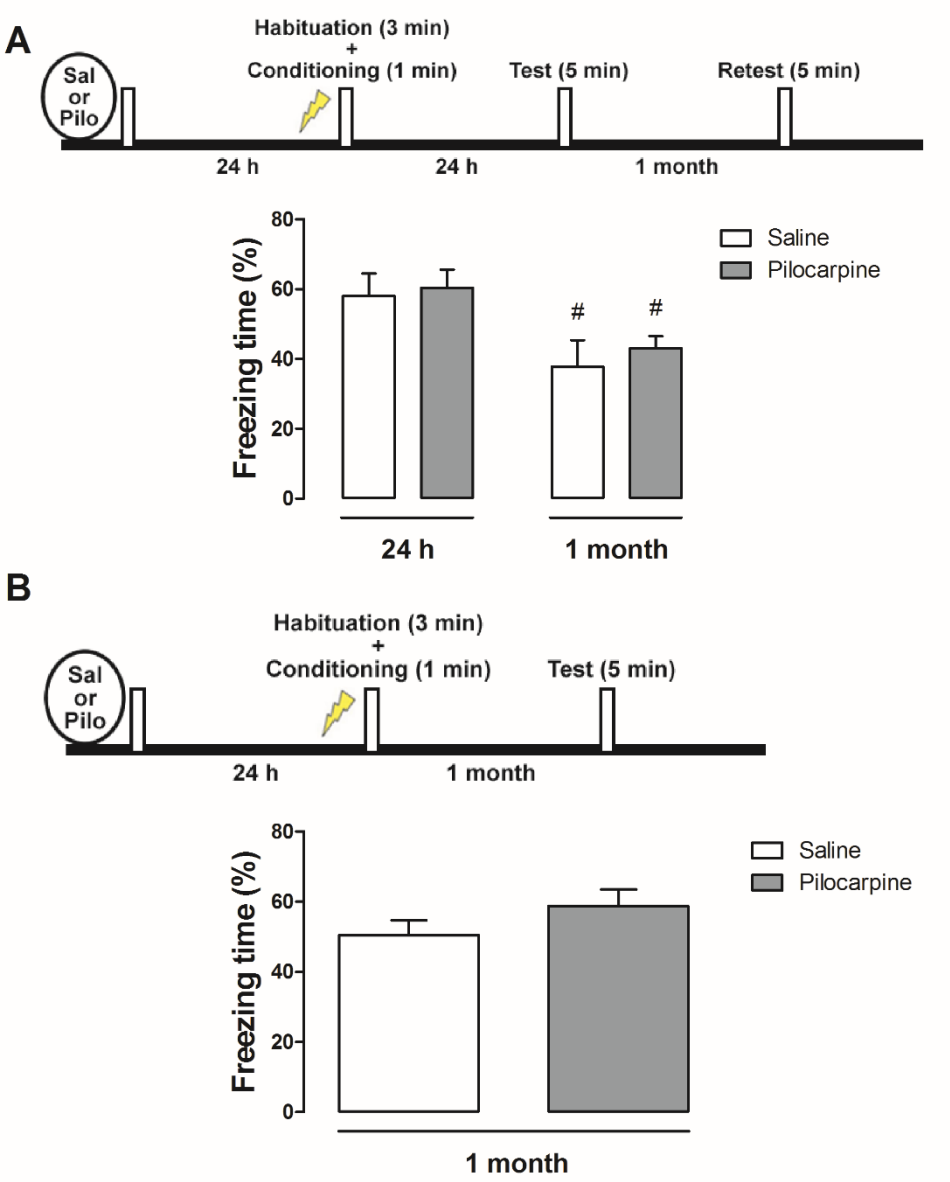


**Figure 1** – Effects of pilocarpine on freezing behavior of rats evaluated in the contextual fear conditioned task following mAChR activation. A) Animals were placed in the conditioning chamber 24 h after injections (3-min period for habituation plus 1 min-period following 1.5 mA/1 s footshock stimulation). 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.

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

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.

**INSERT FIGURE 2 HERE**

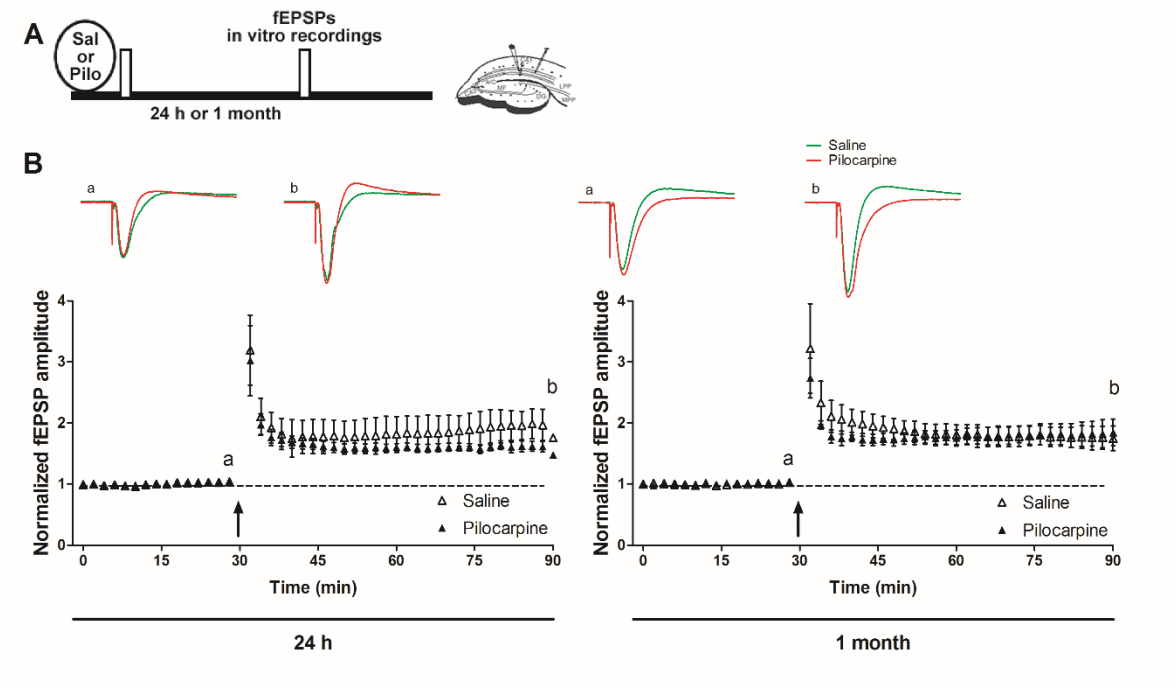


**Figure 2** – Effects of pilocarpine in CA1 synaptic plasticity. A) Rats were treated with methyl-scopolamine bromide and pilocarpine or saline and hippocampal fEPSPs recordings are carried out 24 h or 1 month following treatment. B) Upper traces represent fEPSP responses elicited by CA1 stimulation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

## 4. Discussion

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

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

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

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

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

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

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

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

## 5. Conclusion

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

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

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