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The ERK phosphorylation levels in the amygdala predict anxiety symptoms in humans and MEK/ERK inhibition dissociates innate and learned defensive behaviors in rats

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Highlights

- The P-ERK1,2/Total-ERK1,2 ratio in the amygdala correlates negatively anxiety symptoms in patients with mesial temporal lobe epilepsy.

- The P-ERK1,2/Total-ERK1,2 in the amygdala correlates negatively with the anxiety-like behavior and positively with freezing-learned behavior in naïve rats.
- ERK1,2 in the basolateral amygdala is required for learned-defensive but not for the anxiety-like behavior expression in rats.

Abstract

We demonstrate that the rate of extracellular signal-related kinase phosphorylation (P-ERK1,2/Total-ERK1,2) in the amygdala is negatively and independently associated with anxiety symptoms in 23 consecutive patients with drug-resistant mesial temporal lobe epilepsy that was surgically treated. In naive Wistar rats, the P-ERK1,2/Total-ERK1,2 ratio in the amygdala correlates negatively with innate anxiety-related behavior on the elevated plus maze (n = 20) but positively with expression of defensive-learned behavior (i.e., freezing) on Pavlovian aversive (fear) conditioning (n = 29). The microinfusion of ERK1/2 inhibitor (FR180204, n = 8–13/group) or MEK inhibitor (U0126, n = 8–9/group) into the basolateral amygdala did not affect anxiety-related behavior but impaired the evocation (anticipation) of conditioned-defensive behavior (n = 9–11/group). In conclusion, the P-ERK1,2/Total-ERK1,2 ratio in the amygdala predicts anxiety in humans and the innate anxiety- and conditioned freezing behaviors in rats. However, the ERK1/2 in the basolateral AMY is only required for the expression of defensive-learned behavior. These results support a dissociate ERK-dependent mechanism in the amygdala between innate anxiety-like responses and the anticipation of learned-defensive behavior. These findings have implications for understanding highly prevalent psychiatric disorders related to the defensive circuit manifested by anxiety and fear.

Keywords: Amygdala, anxiety, Pavlovian aversive (fear) conditioning, ERK1/2 pathway.

Introduction

Anxiety disorders are characterized by higher levels of symptoms affecting the quality of life and have an estimated lifetime prevalence rate of 31.1 % in the United States [1]. Anxiety is usually described as a psychophysiological and behavioral state induced in subjects by an actual or potential threat to wellbeing or survival [2]. In anxiety, the stressor is not always clearly identified, in contrast to what happens in fear. Anxiety is characterized by increased arousal, expectancy, autonomic and neuroendocrine activation, and specific behaviors that facilitate defense mechanisms in response to potential danger. Therefore, anxiety is defined as a more general response to a not known potentially dangerous situation or internal conflict. In contrast,

fear may be defined as the response to an explicit, specific hazard [2, 3]. Furthermore, in humans, anxiety and fear may become pathological conditions, involving maladaptive abnormalities in neural processing of threat-related stimuli and can lead to anxiety disorders. LeDoux proposed a provocative model of emotion implicating anxiety and fear as "higher-order states" engaged by cortical circuits in recent years. Unlike other hypotheses, LeDoux argues that emotions are not innately programmed into the brain but reflect cognitive states resulting from the assembly of information [4]. The brain circuitries engaged in processing anxiety and fear display great overlap, and the ultimate behavioral output circuits of defensive behavior might be mainly shared between them [2]. Although anxiety and fear are evolutionarily adaptive and closely related, these emotions are two distinct phenomena [3].

In rodents, anxiety may be assessed by behavioral measures such as avoidance, escape, or freezing behavior, which are accepted as enhanced anxiety's state parameters. Thus, in rodents, the "anxiety-related" or "anxiety-like" behavior is an acute emotional response, often elicited by tests reflecting a "state" of anxiety [3, 5, 6] rather than as anxiety disorder *per se*. Tests of anxiety-related behavior can be classified as unconditioned and conditioned paradigms. The unconditioned paradigms are based on an animal's spontaneous reactions to an aversive new environment, which concurrently evokes curiosity and fear, creating a typical approach-avoidance conflict. Thus, the inherent avoidance behavior (e.g., explore open or brightly lit areas) competes with the natural explorative drive, for example, to seek a reward such as novelty. Unconditioned behavioral paradigms such as elevated plus maze (EPM), open field (OF) are classic approaches to test inherent conflict anxiety. In contrast, the conditioned paradigms require training and involve mnemonic and motivational processes such as conditioned operant conflict tests and Pavlovian conditioning task [3, 6]. The capacity to remember past events or situations (particularly frightful or traumatic ones) and anticipate them is an adaptive advantage. However, anxiety disorder, including generalized anxiety disorder, seems to be linked to inappropriate characteristics to anticipate adverse circumstances (often without any apparent threat). In contrast, PTSD involves a deficit in repressing traumatic memories [2].

Some protein kinases including, calcium/calmodulin-dependent kinase II (CaMKII), extracellular signal-regulated kinase 1 and 2 (ERK1/2), cAMP-dependent protein kinase A (PKA), and protein kinase C (PKC), are regulated by several different intracellular and extracellular signaling pathways and are engaged in a wide-ranging of brain functions, including cell survival, synaptic plasticity, and the stress response. These protein kinases have been implicated as regulatory elements of many experimental models of psychiatric disorders, such as anxiety, stress-coping responses, the learning and memory process. CaMKII, ERK1/2, PKA, and PKC are extensively related to learning and memory and long-term potentiation

(LTP), considered the molecular substrate of learning and memory storage [7, 8]. These protein kinases and their cascade signaling are implicated with defensive behaviors, such as Pavlovian aversive (fear) conditioning and stress responses [9-12] anxiety like-behaviors [11, 13-15].

In this study, we will focus on ERK1/2, which consists of two isoforms (ERK1 and ERK2) that belongs to the family the mitogen-activated protein kinases (MAPK) cascade, that had three kinases: (i) MAP kinase kinase kinase (MAPKKK, Raf-1, and B-Raf in the ERK cascade), which activates the (ii) MAP kinase kinase (MAPKK, MEK1/2), which in turn activates (iii) MAP kinase (ERK1 and ERK2) by phosphorylation [16, 17]. Despite this complexity, one feature of the ERK1/2 cascade is that its activity is exclusively regulated by MEK1/2, the upstream dual-specificity kinase that phosphorylates the ERK [16]. Once activated, ERK1/2 phosphorylates other downstream kinases, leading to activation of transcription factors of immediate early genes or epigenetic modifications, which affect gene expression involved with neruroplasticity [8, 16,17].

A large body of evidence demonstrates that the ERK1/2 pathway is implicated with synaptic plasticity and structural plasticity in the adult brain. ERK's cascade seems essential for neuronal transcriptional events that control long-term memory including, formation, expression, and maintenance of Pavlovian aversive conditioning [8,17]. Furthermore, ERK1/2 is implicated with stress responses and depression-related behavior. Overall, chronic exposure to stress or inescapable foot-shook induces depressive-related behavior and decreases ERK1/2 phosphorylation in the hippocampus. Similarly, pharmacological inhibition of ERK1/2 induces a phenotype of depression-related in naïve rats [18]. It has been reported that antidepressants' effectiveness may be due to normalizing the ERK1/2 activity downregulated [19]. In parallel to these findings, transcriptome analyses of cortical brain tissue identified ERK1/2, a strong association between ERK1/2 and depression [20].

Some studies suggest that an increase of ERK1/2 activation in the AMY is related to anxiety-related behavior induced by stressful stimuli [34,35] because stress exposure can trigger anxiety-related and defensive behaviors in some experimental paradigms. On the other hand, both acute and chronic stress increase ERK signaling in the ventral tegmental area (VTA) [21], striatum, and cortical areas, but not in the amygdala or hippocampus [22]. Further, the upregulation of ERK2 via viral vector delivery in the dorsal hippocampus induces an anxiolytic-like phenotype [24], while overexpressing ERK2 within the VTA increases susceptibility to stress responses and anxiety-like behavior [22].

Mammalian brain regions implicated in processing anxiety and fear include the anterior HIP (aHIP) in humans, analogous to the ventral HIP in rodents, and the AMY [24], which is conserved in mammals [25, 26]. The AMY participates in a set of defensive survival circuits [4, 27],

whereas the aHIP is implicated in emotional encoding and is connected to the AMY [26]. Due to methodological limitations, the relationship between signal transduction molecules involving protein kinases in AMY with anxiety symptoms in humans has not been previously investigated. Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) is frequently a surgically treatable epileptic syndrome [28]. The surgery involves a standard resection of the mesial temporal lobe structures implicated with seizure onset and early propagation, including the HIP (head and body), AMY, entorhinal cortex, and parahippocampal gyrus [28]. The procedure offers a unique opportunity to obtain human samples of AMY and aHIP under well-controlled conditions to investigate the phosphorylation state and activity of protein kinases and their substrates.

In the current study, we firstly evaluated in human brain samples whether phosphorylation state or activities of protein kinases that are known to be involved in neuroplasticity were associated with anxiety symptoms reported by surgically treated patients with MTLE-HS. We used the clinical results to guide further experiments in rodents. Specifically, we found that only ERK1/2 phosphorylation [P-ERK(1/2) / Total-ERK(1/2) percent of ratio] in the AMY, but not the phosphorylation of CaMKII, neither PKC nor PKA activity, was associated with anxiety symptoms reported by surgically treated patients with MTLE-HS. No association was observed between anxiety symptoms and kinases phosphorylation or activity levels in the anterior HIP and the middle temporal neocortex of patients. Based on those findings, we developed a “bed to bench” approach to evaluating whether the P-ERK (1,2) levels in the AMY of naïve Wistar rats are correlated with both defensive behaviors: anxiety-like responses on EPM test and freezing expression on Pavlovian aversive (fear) conditioning. Further, independent groups of rats received a single microinfusion of an ERK1/2 inhibitor (FR180204) or an ERK1/2 phosphorylation suppressor (U0126; MEK1/2 inhibitor) into the basolateral amygdala (BLA) to investigate the role of ERK1/2 pathway signaling on both innate and learned-defensive behaviors.

Results

P-ERK/Total-ERK ratio in the amygdala negatively correlates the anxiety symptoms reported by surgically treated patients with MTLE-HS

Figure 1A shows a significant and negative correlation ($r = -0.46$, $p = 0.03$) between the P-ERK (1,2) /Total-ERK(1,2) ratio in the AMY and the anxiety symptoms determined by HADS anxiety scores reported by surgically treated MTLE-HS patients. In these patients, the Total-ERK (1,2) in the AMY correlates positively with HADS anxiety scores ($r = 0.47$, $p = 0.03$; **Figure 1B**). Interestingly, the P-ERK (1,2) does not correlate with the variation in the Total-ERK(1,2)

in the AMY (r = 0.23, p = 0.22) **(Figure C)**. No significant correlations ($p \geq 0.73$) were observed in P-CaMKII, neither the PKA nor PKC activity in the AMY, and patients' anxiety symptoms (**Figure 1D-F**). Representative Western blotting of these data was shown in **Suppl. Figure 1 A-D**).

The variable amount of gliosis and neuronal loss observed in the AMY of MTLE-HS patients [29] could represent a confounding factor in interpreting our results. The histopathological analysis is not feasible in the same samples used for western blot. Hence, we determined the immunocontent of glial fibrilar acid protein (GFAP) and the astrocytic glutamate transporters (EAAT1 and EAAT2) for controlling the gliosis distribution in our samples. Moreover, the determination of the GluA1 subunit of AMPA receptors was used to control possible variations in neuronal loss. As showed in the **Table at the bottom of Figure 1**, there were no significant correlations ($p \geq 0.21$) among the variation in the GluA1, GFAP, EAAT1, and EAAT2 in AMY, and the anxiety symptoms reported by the MTLE-HS patients (representative WB see **Suppl. Figure 1 E-K**).

Because ERK pathway activation depends on phosphorylation state, which may be influenced by BDNF binding to tropomyosin-related kinase B (Trk-B)[30], a tyrosine kinase receptor, we analyzed the correlation among the AMY levels of BDNF, the phosphorylated- or total-TrkB receptor, and HADS-anxiety score. No significant correlations ($p \geq 0.20$) were observed among the AMY levels of these proteins and our patients' anxiety symptoms. Importantly, there were no correlations between the HADS-anxiety scores and the P-ERK(1,2)/Total-ERK(1,2) neither in the anterior HIP ($p \geq 0.40$) or the middle temporal neocortex ($p \geq 0.96$; **the bottom of Figure 1)**, supporting the specificity of the observed correlation between the P-ERK/Total-ERK in AMY and the anxiety symptoms in our patients.

Considering that the negative correlation between anxiety symptoms and the P-ERK(1,2)/Total-ERK(1,2) in the AMY could be due to variations in the distribution of other demographic and clinical variables affecting both variables simultaneously, we analyzed whether the demographic, clinical, and neuroradiologic characteristics of patients and the trans-operative parameters would be correlated with the anxiety reported by the patients (**Table 1**). Overall, females had significantly higher levels of anxiety than males (p = 0.05). As expected, there was a significant and positive association between anxiety and depressive symptoms (r = 0.51, p = 0.01). We also found the HADS anxiety scores showed a trend for correlation ($p \leq 0.15$) with anesthesia duration until surgical resection of the AMY (r = 0.32, p = 0.15) and the time of brain samples storage in the -80 ^{o}C freezer (r = -0.33, p = 0.14). We performed multiple linear regressions to investigate the independent relationship between P-ERK(1,2)/Total-ERK(1,2) in the AMY and the HADS-anxiety scores (**Bottom of Table 1**). Only

the P-ERK(1,2)/Total-ERK(1,2) in AMY (B = -0.09, p = 0.05) and HADS-depression scores (B = 0.40, p = 0.02) remained independent and significantly associated with HADS-anxiety scores, and both together explained 40% (r^2 = 0.40) of the HADS-anxiety scores variation, while the P-ERK(1,2)/Total-ERK(1,2) in AMY alone predicted 23% of its variation (r^2 = 0.23). These results indicate that the association between P-ERK (1,2) in AMY was not due to the distribution of demographic, clinical, and surgical confounding bias.

P-ERK/Total-ERK ratio in the amygdala negatively correlates the anxiety-like behavior in rats but, MEK/ERK inhibition is not required for its expression.

Based on our clinical results, the question arose: could the association between P-ERK(1,2)/Total-ERK(1/2) in the AMY and anxiety symptoms be restricted to the MTLE-HS condition? To answer this critical question, we exposed 20 naïve Wistar rats to the elevated plus maze (EPM) test used to evaluate rodent's anxiety-related states. We investigated whether the P-ERK(1,2)/Total-ERK(1,2) determined in the AMY of male rats immediately after 5-minutes of exposure to the EPM test also predicts their anxiety-like behavior. Similar to observed in humans data, the P-ERK(1,2)/Total-ERK(1,2) ratio in the AMY of rats showed a significant and inverse correlation with their anxiety-related behavior levels, expressed by more time spent in closed arms (r = -0.49, p = 0.03, **Figure 2A**) from EPM test. This parameter is an "inverse" measure of the percent of time spent on open arms with is the typical parameter adopted. Thus, rats. Additionally, no correlation was observed between the Total-ERK(1,2) in the AMY and anxiety-related behavior (r = 0.18, p = 0.42, **Figure 2B**) in the animals (representative WB see **Suppl. Figure 1L**).

To investigate a possible cause-effect relationship between AMY levels of P-ERK(1,2)/Total-ERK(1,2) and anxiety-like behavior of rats, a set of animals received bilateral microinjection of FR180204 (1.0, 10, or 50 μg / 0.2 μL/side, n = 8-13 / group), a selective ERK1/2 inhibitor, into the BLA, 5 minutes before the exposure to the EPM test. As shown in **Figure 2C**, the intra-BLA inhibition of ERK1/2 did not affect the anxiety-like behavior (neither ethological behavior nor locomotor activity, see **Suppl. Figure 2A-F**). As this inhibitor does not prevent the ERK phosphorylation mediated by the upstream kinases (MEK1/2), we also investigated the effect of MEK1/2 inhibitor U0126 (1.0 μg / 0.5 μL/side, n = 8-9 / group). This drug that prevents ERK activation, into the BLA on the anxiety-related behavior of rats [31]. We show in **Figure 2D** that the intra-BLA U0126 microinfusion did not affect the anxiety-related behavior of animals (neither ethological behavior nor locomotor activity, see **Suppl. Figure 2G-L**). Furthermore, the selective ERK1/2 or the MEK signaling pathway inhibition in the BLA changed neither anxiety-related parameters nor locomotor activity in the open field test during 5 min of exposure (see **Suppl. Figure 2M-R**). In both experiments, we included an animal

group systemically treated with diazepam as a positive control for observing anxiolytic-like effects at this test. Overall, these results suggest that the pharmacological blockade of MEK/ERK1/2 signaling into the BLA did not affect the expression of anxiety-related behavior in rats, suggesting the correlation between P-ERK(1,2)/Total-ERK(1,2) in the AMY and anxiety-related behavior in rats may be an epiphenomenon.

P-ERK/Total-ERK ratio in the amygdala positively correlates to learned-defensive behavior in rats and is required for the expression of Pavlovian aversive conditioning learning and memory

Since Pavlovian threat (fear) conditioning and AMY have been implicated in anxiety and fear [24, 27]. Pavlovian aversive (fear) conditioning is an adaptive phenomenon through which a subject learns to anticipate an impending threat. Thus, we employed the Pavlovian aversive (fear) conditioning procedure to investigate whether the ERK pathway in the AMY, which is part of the brain circuit that detects threats, would involve the associative learning process that controls defense responses in anticipation of danger (i.e., freezing behavior). We evaluated whether P-ERK(1,2)/Total-ERK(1,2) in the AMY levels is correlated with the expression of a learned-defensive behavior of naïve Wistar rats (n = 29) exposed to the Pavlovian treat (fear) conditioning protocol, a 2-hour interval was given between the training and test sessions to avoid long-term plasticity. Similar to the EPM, the animals were euthanized immediately after the test. As showed in **Figure 3A**, there was a positive correlation between freezing behavior and the P-ERK(1,2)/Total-ERK(1,2) [r = 0.39, p = 0.04], but not the Total-ERK(1,2) [r = -0.13, p = 0.49, **Figure 3B**, for representative WB, see **Suppl. Figure 1M**]. Rats that spent more time in freezing showed higher levels of P-ERK1/Total-ERK1 into de AMY. Similar to the EPM, the animals were euthanized immediately after the test.

Since ERK1/2 is transiently activated in brain structures, including some AMY nuclei, upon Pavlovian treat (fear) retrieval [32, 33]. We asked if ERK1/2 activity in BLA is also implicated with the expression of defensive-learning behavior. First, we give the treatment immediately after the training to confirm the effectiveness of MEK inhibitor (U0126 1.0 µg/0.5 µL/side, n = 8-9/ group) on the consolidation of Pavlovian aversive long-term memory, and also to test the effects of different doses (0, 1.0, 10 or 50 µg/0.2 µL/side, n = 8-11/ group) of FR180204, an ERK1/2 inhibitor on memory consolidation. Exceptionally in this experiment, an interval of 24h was given between training and testing. Then, we evaluated if ERK1/2 is required for evocation (recall) of short-term Pavlovian treat (fear) conditioning. Thus, rats received bilateral intra-BLA microinfusion of both ERK1/2 inhibitor (FR180204) and MEK inhibitor (U0126) before the test, which occurred two hours after training.

One-way ANOVA with repeated measures revealed a significant effect of treatment for both inhibitors FR120204 and U0126, respectively ($F_{1, 29}$ = 3095.80, $p < 0.000001$; $F_{1, 17}$ =629.82, p = 0.000001), trial ($F_{3, 29}$ = 39.00, $p < 0.00001$; $F_{1, 17}$ =147.59, $p < 0.000001$) and an interaction between treatment and trial ($F_{3, 29}$ = 54.25, $p < 0.00001$; $F_{1, 17}$ = 99.20, $p < 0.00001$) injected immediately post-training. The higher dose of FR180204 impaired significantly the consolidation of contextual fear conditioning ($p < 0.001$; **Figure 3C**). Further, as expected, the intra-BLA microinfusion of U0126 impairs the consolidation of fear conditioning ($p < 0.001$; **Figure 3D)**, confirming the previous findings in the literature [8, 31].

The FR180204 (50 μg / 0.2 μL / side, n = 9-11 / group) or U0126 (1.0 μg / 0.5 μL / side, n = 11 / group) were bilaterally microinjected into the BLA of rats (5 or 30 minutes, respectively) before test session of the Pavlovian aversive conditioning in order to investigate the role of the ERK pathway on the retrieval component of a conditioned fear-learning task. One-way ANOVA with repeated measures revealed a significant effect of treatment for both inhibitors FR120204 and U0126, respectively ($F_{1, 18}$ =123.10, $p < 0.00001$; $F_{1, 20}$ = 95.50, $p < 0.00001$), trial ($F_{1, 18}$ = 815.77, $p < 0.00001$; $F_{1, 20}$ = 151.0, $p < 0.00001$) and an interaction between treatment and trial ($F_{1, 18 =}$ 216.12, $p < 0.000001$; $F_{1, 20 =}$ 148.25, $p < 0.000001$) injected immediately before retrieval. The results show for the first time in the literature that either selective BLA inhibition of ERK1/2 or the inhibition of the MEK/ERK pathway impairs significantly ($p < 0.01$; **Figures 3E-F**) the expression of defensive-learning behavior in rats.

To evaluate a putative relationship between the animals' emotional behaviors on the EPM test and contextual fear conditioning, we investigated if the expression of a state of anxiety-like behavior on the EPM test correlates with the subsequent fear-learned expression in the same animals. Therefore, naïve Wistar rats (n = 13) were tested on EPM apparatus; subsequently, they were subjected to a Pavlovian aversive conditioning training session, and 24 h later, we tested them to assess freezing behavior. As shown in **Figure 3G**, there was a strong and positive correlation (r = 0.62, p = 0.02) between the percentage of time spent in the closed-arms (more anxiety-like behavior expression) and the percentage of time spent in freezing (more defensive-learned behavior expression), indicating that anxiety-like behavior (an innate defensive-behavior) is a strong and positive predictor of defensive-learned behavior. These results imply that although in naïve Wistar rats, the innate and learned defensive behaviors are correlated positively, the P-ERK (1,2) / Total-ERK(1,2) percent of ratio in the AMY shows an inverse relationship with them. Furthermore, the ERK/MAPK pathway activation is only required for learning but not for the innate defensive behavior expression.

Discussion

To our knowledge, this is the first study investigating the correlations between the

phosphorylation state or activity of protein kinases in brain structures related to the defensive circuit and anxiety in humans and anxiety-like and defensive-learned behaviors in rodents. The results showed that the P-ERK(1,2)/Total-ERK(1,2) ratio was negative in the AMY. At the same time, Total-ERK was positively correlated with the HADS anxiety scores reported by MTLE-HS patients. P-ERK(1,2) does not correlate with Total-ERK(1,2), indicating that the ERK(1,2) phosphorylation did not accompany the variation of the total content of ERK(1,2). Applying a "bed to bench" approach, we demonstrated that in naïve rats, anxiety-like behavior also negatively correlates with P-ERK(1,2)/Total-ERK(1,2) ratio, similar to what was observed in our patients. The Total-ERK(1,2) in AMY was not correlated with anxiety-like behavior. We speculate that the observed correlation between the Total-ERK(1,2) in the AMY and the HADS anxiety scores could be, at least in part, attributed to chronic neurochemical adaptive mechanisms in the limbic system in our patients. There are two isoforms of ERK (ERK1 and ERK2), expressed in the mammalian brain. Both ERK 1 and ERK2 display 84% homology, have no apparent differences in subcellular localization, have the same substrate specificities, display identical activation kinetics, and are activated by the same upstream kinase [16]. Thus, we believe the P-ERK(1,2)/Total-ERK(1,2) percentage of ratio may be used to infer the global activation of the ERK1/2 pathway in the AMY, despite the limitations inherent to the WB methodology.

The ERK1/2 pathways play an important role in synaptic plasticity and emotional processing (including fear conditioning and stress), as demonstrated in various preclinical paradigms. However, studies regarding the involvement of the ERK1/2 pathway in anxiety are scarce [34-36]. Some results of previous reports are, at least in part, conflicting with our findings. For instance, the expression of phosphorylated ERK1/2 in the AMY did not differ between Wistar rat high and low responders on EPM test [36]. Nonetheless, it should be pointed out that there are notable procedural differences between this and our study protocol. It is possible that both the experimental design and the time points at which animals were sacrificed did not capture a critical period of increased phosphorylation of ERK1/2 and the slight variability between both factors ERK1/2 phosphorylation versus the percentage of time in the open arms. The exposure of rodents to acute stressors increases P-ERK levels in brain areas [22], including BLA [35]. The exposure to acute restraint stress following a weak training of Pavlovian aversive conditioning increases P-ERK2 in the BLA and enhances aversive memory retention of aversive memory in rodents. In contrast, intra-BLA or systemic injection of midazolam (a short-term benzodiazepine) before restraint attenuated both stress-induced ERK activation and acquisition defensive behavior [35]. In addition, some studies demonstrated an increase in ERK1/2 phosphorylation in the AMY linked with anxiety-related behavior induced by stressful stimuli [34, 35]. Overall, these studies indicate that activation of ERK1/2 signaling elicited by stress

is closely related to (innate and learned) defensive behaviors. However, it must be conceded that in previous studies is difficult to dissociate whether the increase of ERK1/2 phosphorylation may be due to prior stressful experience or is related to the innate anxiety-like behavior of animals.

Here, we observed a strong and positive association between the basal state of anxiety-related behavior and the subsequent expression of conditioned freezing behavior in the rats submitted to the EPM apparatus and subsequently trained on Pavlovian aversive conditioning, then tested 24 hours later. These results suggest that an innate behavior creates by a conflict between explorative behavior and avoidance response on the EPM test predicts subsequent defensive-learned responses elicited by threat anticipation during the test of Pavlovian aversive conditioning. The relationship between the time spent on open arms on EPM and subsequent freezing behavior seems to be, at least in part, resembles what occurs in anxious people, considering that anxious people often avoid situations that can seem threatening [4]. Rats displayed more conflict to avoid-explore open arms from EPM and presented more conditioned freezing in response to (footshock) threat. Thus, our finding reinforces the close relationship between innate and learned defensive behaviors.

Despite the correlation between P-ERK(1,2)/Total-ERK(1,2) ratio in the AMY with anxiety-related behavior on the EPM test, both selective inhibition of ERK1/2 and MEK/ERK inhibition did not affect the “basal” state of anxiety-related behavior (i.e., innate) of naïve rats. Previous studies implicate ERK1/2 activation in BLA and medial prefrontal cortex stress-triggered anxiety-related behavior [34, 35]. Still, it is not involved in regulating the “basal” state (i.e., innate anxiety-related behavior). Although the EPM test is an unconditioned paradigm that allows the detection of an innate state of anxiety-related behavior accompanied by changes in ERK1/2 phosphorylation in the AMY, the lack of effect of both FR180204 and U0126 intra-BLA injection exclude the cause-effect relationship between ERK1 phosphorylation in AMY and the state of anxiety-related behavior of naïve rats.

We found that ERK1/2 phosphorylation in the AMY exhibit a positive association between the expression of freezing behavior in rats after exposure to the Pavlovian aversive conditioning. Then we showed that inhibition of both kinases MEK1/2 and ERK1/2 before test impairs the expression (evocation/recall) of freezing at 2-hours short-term memory. This result is similar to the previously reported [37, 38], demonstrating that hippocampal ERK1/2 activity is critical for retrieval of Pavlovian aversive (fear) conditioning. Here, we give pre-test infusions of FR180204 and U0126 to address short-term memory. This strategy allows an investigation of ERK's role on the evocation of learned-defensive behavior, similar to what was performed for innate defensive behavior on the EPM test. Thus, our findings indicated that ERK and MEK inhibitors effectively decrease freezing expression at short-term memory tests. However, no

studies about ERK's pharmacological inhibition effect immediately before evocation of short-term aversive memory have been reported to the best of our knowledge. In most cases, the injections were given before or post-training to affect the acquisition and formation of learning and memory. These studies had reported that intra-amygdala (lateral nucleus) injection of MEK/ERK inhibitors did not interfere with short-term memory when drugs were administered before training [31, 39], or before a "reactivation" of short-term [40] Pavlovian aversive conditioning.

The pharmacological inhibition of ERK1/2 in some areas of the cerebral cortex and the HIP disrupts the retrieval of inhibitory avoidance tasks in rodents [8]. Considering that phosphorylation events and pharmacological effects of MEK inhibitors are transitory, the discrepancy between these studies and our findings regarding the effects of drugs on short-term memory may occur due to a possible specific role of ERK1/2 on different memory (formation vs. evocation) process, as well as, methodological differences. In addition, the BLA-injections of FR180204 or U0126 immediately post-training exhibited significantly less freezing 24h later. This confirms similar previous studies demonstrating that ERK1/2 activity in the lateral nucleus of AMY and hippocampus is necessary to consolidate contextual and cued fear learning conditioning [31, 39-41]. Indeed, Bernard and coauthors [42] analyzed the pattern of P-ERK1/2 in brain regions, including BLA, at different time-points (0-60 min) following training Pavlovian threat conditioning and retrieval conducted 24 h later. An increase of P-ERK1/2-positive cells in the BLA immediately after training and progressively returned to basal level within 60 min was found. In contrast, in retrieval, the number of P-ERK1/2-positive cells was increased at 30 min and returned to basal level within 60 min [42]. Furthermore, pharmacological blockade of ERK1/2 pathway in cerebral cortex and HIP impair the evocation of short-term inhibitory avoidance task in rodents [43]. Thus, despite methodological differences, our results seem to corroborate previous studies that point to a crucial role for the ERK pathway in retrieving and consolidating Pavlovian aversive memories [8,17].

Although our findings implicate ERK1/2 in the expression of learned-defensive, they do not exclude the possibility that ERK1/2 activation during evocation of short-term (or consolidation of long-term) memory initiates downstream effectors engaged for these processes. ERK1/2 regulates many neuronal processes through direct or indirect (by RSK1-4, MSK1/2, and MNK1/2 activity) through activation of transcription factors epigenetic or modifications that affect gene expression phosphorylation of the chromatin histone H3 and other epigenetic changes witch affect gene activation [16.] Since ERK's activity regulates transcription factors such as CREB (Cre-binding protein) and ELK-1, which enhance the transcription of immediate early genes (i.e., c-fos and zif268/Erg1) implicated to neuronal plasticity; mood and emotional regulation; and memory process. Therefore, it remains to be determined (i) whether downstream effectors from ERK1/2 cascade are also associated with

anxiety epileptics patients and rats; and (ii) the involvement of ERK's downstream effectors on the expression of learned defensive behavior in rats to clarify the molecular mechanisms that underlying our findings. This is similar to studies implicating ERK1/2 signaling in mood regulation and suggesting ERK's downstream, such as ELK-1, as a modulator in the physiopathology and pharmacological target of depression [18, 19].

The small sample size is a limitation in Western blot analysis, resulting in type II errors due to false-negative results about the lack of association between the analyzed neurochemical targets and our patients' anxiety symptoms. However, the observed significant association in a relatively small sample size of patients, together with the blind confirmation in animal models, strengthens the results' credibility. We would like to emphasize the strengths of the human data of our study: i) all the variables were collected prospectively according to our well-established research protocol; ii) during the interview an experienced psychiatrist in the field of psychiatric comorbidities in epilepsy the applied HADS questionnaire avoid reliance on aspects of the somatic symptoms of illness; iii) an extensive control used concerning surgical and anesthetic parameters control during brain sample collection, storage, and analysis; v) the neurochemical analysis was done blinded for the clinical variables of patients; vi) the neurochemical analysis of animals was blinded for their behavioral performance; vii) the behavioral analysis was blinded for the pharmacological treatments received by the animals; viii) the use of multivariate analysis, an approach that is seldom used in association studies using Western blot results of protein phosphorylation in the clinical scenario. Finally, the experimental protocols for all the neurochemical determinations and animals' behavior were done blindly for drug treatments.

In summary, the AMY levels of P-ERK(1,2) / Total-ERK(1,2) negatively correlate to anxiety symptoms in humans and the anxiety-like behavior but are positively associated with conditioned freezing behavior when Wistar rats respond to a threat. The ERK1/2 is required for both expression and consolidation of defensive-learned, but not for Wistar rats' innate defensive behaviors. Altogether, these findings support that a state of anxiety-related behavior on the EPM test and the expression of conditioned freezing behavior in rats show a differential ERK1/2 - dependent mechanism into the AMY. It is important to note that, despite the inhibition of MEK/ERK1/2 signaling into the BLA with a drug that makes rodents display less defensive (i.e., decrease freezing), challenging situations (threat of foot shock) will not necessarily make people feel less anxious or fearful.

Considering the point of view from LeDoux and Pine, "the two systems (conscious versus unconscious)," the emotional experience of anxiety (and fear) emerges from a distinct neuronal substrate that underlies nonconscious processes that control defense (behavioral and physiological) responses to threat elicited by conscious states of feeling fear and anxiety.

Consequently, the authors argue that behavioral and physiological defense responses (i.e., freezing) should not be used to study the subjective aspects of emotion. Thus, when rats engaging in instinctive behaviors (defensive) respond to a threat, it doesn't necessarily show that rats are experiencing the human emotions attributes to them. Therefore, despite AMY levels of P-ERK(1,2) / Total-ERK(1,2) ratio negatively correlates the anxiety symptoms in humans and the anxiety-like behavior in rats, this finding does not imply a causal relationship, not only because anxiety-like behavior was not affected by MEK/ERK inhibition, but also considering to two-system view behavioral and physiological parameters of anxiety and fear would have no ability to predict subjective experience in humans. While the clinic evaluation is based on subjective reports of anxiety symptoms that can be objectively quantified using scales (i.e., HADS score), the preclinical studies have focused on behavioral measures that are no necessarily encompassing the subjective state, which cannot be directly assessed in nonverbal species. Therefore, this point of view may explain, at least in part, the limited translation from the preclinical studies related to "anxiety disorders" and the realistic clinic scenario.

In conclusion, our results support distinct ERK1/2 pathway-dependent mechanisms between anxiety-like and expression of fear-learned behavior in the AMY, with implications for our understanding of highly prevalent psychiatric disorders related to the defensive circuit manifested by anxiety and fear.

Methods

Patients

We included 23 consecutive adult patients (≥ 18 years old) male and female with drug-resistant mesial temporal lobe epilepsy related hippocampus sclerosis (MTLE-HS) investigated at the Centro de Epilepsia de Santa Catarina (CEPESC) between August 2008 to December 2013 who participated in a previous prospective randomized trial [44]. All patients presented seizures that impaired awareness at least once a month (mean 7.5/month), despite using at least two anti-seizure medications (ASMs). Their medical history, seizure semiology, neurological examination, neuropsychological evaluation, psychiatric diagnoses, interictal and ictal video-EEG, and magnetic resonance imaging (MRI) findings were consistent with MTLE-HS [28, 44-46]. A postoperative cranial MRI was carried out up to 1 year after the surgery to confirm the successful resection of AMY, aHIP, and the middle temporal neocortex. Patients with MTLE-HS also showing non-epileptic attacks were excluded from the analysis.

Analyzed clinical variables included age, gender, marital status, occupational status, years of education, history of initial precipitating injury, age of epilepsy onset, disease duration, and the ASMs schedule, which include carbamazepine, phenobarbital, diphenylhydantoin,

valproic acid, lamotrigine, or topiramate, associated or not with benzodiazepines [28, 44-46]. The Ethics Committee approved the research protocol for Human Research of Universidade Federal de Santa Catarina (365-FR304969). Written informed consent was collected from all patients.

Psychiatric evaluation

The psychiatric assessment was performed by a psychiatrist with experience in comorbid psychiatric conditions associated with epilepsy, as previously described [28, 44-46]. All patients were interviewed alone and afterward in the presence of the caregiver. Anxiety and depressive symptoms were quantified through the Hospital Anxiety and Depression Scale (HADS) validated for Brazilian patients [44, 4 6]. HADS were applied to patients between one to three months before the surgery and indicate how the respondent has felt in "the past week" the pre-surgical psychiatric evaluation was done. The HADS was designed to measure psychological distress in non-psychiatric patients and consists of 2 sub-scales with 7 multiple-choice items for anxiety and 7 for depression. The items are rated on a 4-point Likert scale from 0 to 3, resulting in a final score ranging from 0 to 21 for anxiety and depression, being higher scores indicate worse symptoms [45]. HADS questionnaire has the advantage of avoiding reliance on aspects of the somatic symptoms of illness.

Human and rat brain tissue sampling

The samples from human brain tissue were obtained during the standard anterior and temporal lobectomy without thermo-coagulation following the recommended prospective collection model by the same neurosurgeon and the study's principal investigator (PI), as we described previously [44, 47]. A 1-cm^2 sample of the middle temporal cortex (CX) localized 3 cm posterior to the temporal lobe pole was gently dissected from the white matter. After the mesial temporal region assessment, two-thirds of the AMY (part of the central area and the entire basolateral area) was resected. Finally, the HIP head and body were removed "*en bloc*", and the anterior hippocampus (aHIP) was quickly dissected on the ice-refrigerated glass. Immediately after collection, the samples were transferred to a microfuge tube, frozen in liquid nitrogen, and stored in a −80 °C freezer until analysis. Several parameters were controlled while collecting the human brain samples, including anesthesia duration, arterial blood gases, electrolytes, hematocrit/hemoglobin, pH, mean arterial pressure, heart, and respiratory rate. Hemodynamic and respiratory parameters remained stable during all procedures, and there were no surgical complications. The PI of the study collected all variables.

The animals were euthanized by guillotine 2-3h following the behavioral evaluation for brain rat collect samples. The brains were excised from the skull, and the AMY was dissected in a frozen Petri dish and subsequently frozen in the same way.

Animals

Adult male Wistar rats (*Rattus norvegicus*) 3 months old, 300-360 g were housed in groups of 4-5 per cage and kept 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, except during the experiments. Rats were allowed to adapt to the laboratory conditions for at least one month before the experiments. Behavioral experiments were carried out during the light phase of the cycle (between 1 and 6 p.m.). Experiments were conducted following international standards of animal welfare recommended by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with experimental protocols approved by the Committee for Ethics in animal research of the Federal University of Santa Catarina (CEUA-UFSC #PP00893/2014). A total of 295 animals were used in this study. The minimum number of animals was required to obtain consistent data were used based on pilot experiments. A total of 295 animals were used in this study. Twenty-eight animals were excluded from the study, 23 with cannulas placed outside target and five for presenting ≥18 s of freezing on training (before the release of footshock), which was considered an atypical unconditioned defensive behavior. All behavioral data were collected by independent observers blinded to the treatment.

Stereotaxic surgery and microinfusion of drugs into the BLA

Rats were anesthetized with a mixture of ketamine and xylazine (75 and 7.5 mg/kg i.p., respectively) and given 2 mL of saline (s.c.) to facilitate the clearance of these drugs and prevent dehydration. The rats were placed in a stereotaxic frame (Stoelting Co, Instruments), and stainless steel guide cannulas (22-gauge, 11 mm in length) were bilaterally implanted 1 mm above the BLA (from bregma: anterior/posterior −2.8 mm; medial/lateral ± 5.0 mm; dorsal/ventral −6.5) according to the atlas of Paxinos and Watson [48]. A stainless-steel obturator (0.25 mm diameter) was inserted into the guide cannula to prevent foreign materials' entry. After surgery, the rats were allowed to recover for 5–7 days, and they were handled approximately 1 min per day for 3 days before the beginning of the behavioral test.

FR180204 (Tocris), an inhibitor of ERK1/2 (1, 10, and 50 μg / μL) was dissolved in 10% DMSO, whereas the inhibitor of MEK (U0126, 1 μg / μL) was dissolved in 5% DMSO, based on previous study [31]. A 27-gauge needle was fitted into the 22-gauge guide cannula at the time of infusion, with its tip protruding 1 mm beyond the guide cannula. Bilateral infusions of drug or an equivalent volume of the vehicle into the BLA were made by using 30-gauge injection

needles connected to 10-µL Hamilton micro syringes by polyethylene (PE-20) tubing. The injection needles protruded 1 mm beyond the cannula tips, and a manual microinjector system infused a 0.2-µl (FR180204) or 0.5-µL (U0126) injection volume per hemisphere over 60 s period. The injection needles were retained within the cannula for 30 s following drug infusion to maximize diffusion and prevent the drug's backflow along the cannula track.

The cannula placement was verified for each rat immediately after the behavior experiments by histological examination of the brains following 1% methylene blue solution injection (0.5 µL / site)[49]. After an overdose of ketamine and xylazine terminally anesthetized the rats, brains were removed quickly for subsequent histological verification of injector positioning in the BLA. The distribution of the injection sites is represented in **Supplemental Figure 3.** Only data obtained from a rat with correctly inserted cannulae were included in the statistical analysis. We used a simple randomization scheme to select the subjects according to the experimental group (only for MEK/ERK inhibitors treatment) through a single sequence of random values to guide the assignment of subjects to groups. Histological verification of cannula placements was carried out in all rats by experimenters blinded to the treatment.

Protein extraction and Western blot analysis

All human samples were homogenized by the same researcher on the same day and stored at −80 ºC until the analysis was done 23 (S.E. 1.7) months after sampling. The phosphorylation levels and total amount of target proteins were determined in a blinded manner for all clinical data by western blot (WB), as previously described [47]. Briefly, the brain samples were mechanically homogenized in buffer solution containing 50mM Tris, pH 7.0, 1 mM EDTA, 100mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3PO_4, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail and centrifuged 10 000 × g at 4 °C for 10 min. The supernatants were diluted in electrophoresis buffer. The protein content was estimated by the method described by Peterson [50]. The same amount of protein (60 µg per lane) for each sample was electrophoresed in 10% SDS–PAGE mini gels and transferred to nitrocellulose membranes using a semidry blotting apparatus (1.2 mA/cm^2; 1.5 h). The membranes were blocked with 5% skim milk in TBS (Tris 10 mM, NaCl 150 mM, pH 7.5). The total content and phosphorylation of proteins were detected after overnight incubation with specific antibodies diluted in TBS-T containing BSA 2% according to specific dilution anti-ERK1/2 (p-ERK1/2 – Sigma® M8159; 1:5000 and T-ERK1/2 - Sigma® M5670; 1:20000); anti-PKA substrates (Cell Signaling, #9624) antibody against a glial high-affinity glutamate transporter such as the excitatory amino acid transporter 1 and 2, anti-EAAT1 and anti-EAAT2 (Cell Signaling, #5684, Cell Signaling #3838, respectively) and the glial fibrillary acidic protein (anti-GFAP, Cell

Signaling, #3670) in a 1:1000 dilution. The Western blots were developed by chemiluminescent assay. For load control, all membranes were incubated with na anti-β-actin antibody (Santa Cruz Biotechnology, sc-47778, 1:2000). **Supplementary Figure 4** shows the effect of time between death and brain sampling to analyze the phosphorylation state of ERK1/2 in samples obtained from naïve rats. The result indicates that the phosphorylated-ERK1/2 content decays significantly 2 h post-euthanasia. These findings reinforce our concern about our decision, no use samples from autopsied patients due to the possibility of false negatives resulting from western blotting dosage of protein phosphorylated content by western blotting analyses. Due to the lack of brain tissue samples from healthy controls, an internal control sample was applied as a reference in all electrophoresis. This reference sample was obtained from three pooled HIP prepared as all other samples.

Immediately after behavior tests, the animals were killed by decapitation. The AMY was rapidly dissected and placed in liquid nitrogen and then stored at -80°C until the day of sample preparation. During dissection, the AMY was maintained in an ice-cold saline 0.9%. Samples were prepared as previously described in humans. Internal control was also prepared from two pooled amygdala prepared as all the other samples and applied as a reference in all electrophoresis. The same specifications for ERK1/2 antibodies were used.

For the humans and animal samples quantification, the OD ratio (phosphorylated/total or total/β-actin) for each target protein in the reference sample (internal control) was considered 100%, and the neurochemical data of each patient or animal were expressed as percentage variation from the reference sample [47]. The phosphorylation level was determined as a ratio of the phosphorylated band's optic density (OD) relative to the OD of the total band. The protein immunocontent was determined as a ratio of the OD of the protein band to the OD of the β-actin band [47]. The PKA and PKC were expressed as a percentage of their activity on the specific substrate compared to the reference sample. The experimenter performing the western-blot analyses was blinded.

Elevated plus-maze (EPM)

The elevated plus-maze consisted of two arms enclosed by 30-cm-high walls, whereas the remaining two arms were open (30 × 10 cm) mounted at an angle of 90º, all facing a central platform (10 cm x 10 cm), elevated 40 cm from the floor. A small raised lip (1.0 cm) around the open arms perimeter's to prevent the falling. The maze was situated in a room illuminated by white fluorescent lighting (200 lux). Each rat was placed on the central platform facing an enclosed arm and freely explored the apparatus for 5 min. A highly trained observer scored open and closed arm entries and time spent in the open arms. Ethological parameters such as unprotected head-dipping, open-arms end activity, and protected stretch-attend postures were

also recorded to increase the test's sensitivity of the test. The bilateral micro infusions of FR180204 or U0126 were given 5-minutes (FR180204) or 30-minutes (U0126) before the EPM test exposure.

Contextual fear conditioning

Pavlovian aversive (fear) conditioning involves an associative learning process in which a neutral conditional stimulus (CS) is paired with an unconditional stimulus (US), usually a foot-shock. Once learned, re-exposure to the CS will elicit a conditioned behavioral and physiological responses, which resemble defensive reactions that occur in the presence of danger, such as freezing in anticipation of the US [27]. Therefore, conditioned responses (i.e., freezing behavior) expression during Pavlovian conditioning reflect the imminent threat's expectation and provide an objective measure of associative learning. We used a standard conditioning chamber (26 x 26 x 30 cm; EP 107C, Insight, Brazil). On training session, rats were placed in the conditioning chamber for 3 min before receiving two unsignalled foot-shock 2-s, 0.7-mA separated by a 30-s interval. They were kept in the conditioning context for an additional 30 seconds before returning to their home cage. The test consisted of reexposing rats to the same conditioning chamber (without foot-shock) for 4 min. The interval between training and testing was 2 or 24 h depending on the experiment. Pharmacological treatment was given at different times to address different phases of Pavlovian associative learning and memory processes: an intra-BLA treatment was given immediately after conditioning training (long-term memory consolidation) or 5- or 30-minutes before the test (for FR180204 and U0126, respectively) to address the expression (evocation) of short-term memory. The behavior of the rats was recorded with a video camera mounted on top of the conditioning chambers.

Open field test

We used a standard open field (OF) made of wood-covered witch-impermeable Formica and 200 lux illumination measured in the center of the arena. Each animal was placed in the open field central area and freely explored the apparatus for 5 minutes. The total time spent in the central squares of the arena, the number of total squares crossed, and time spent in grooming was recorded. The animal's behavior was recorded by a video camera positioned above the arena, allowed recording in another room via a closed T.V. circuit.

Statistical analysis

The clinical and neurochemical variables of the patients were analyzed by uni and multivariate analysis using the SPSS 17.0. The univariate analysis of associations between the HADS anxiety scores and the neurochemical, demographic, clinical neurosurgical, trans-operative parameters was done by the Student's t-test, ANOVA, or Pearson's correlation. Multiple linear regression was done to determine the independent association between the neurochemical variables of interest and HADS anxiety scores, including all the variables showing an association with HADS anxiety scores for a $p \leq 0.15$ level univariate analysis. A $p \leq 0.05$ was accepted as statistically significant for the final model of multiple linear regression analysis. We did not determine the pre-test effect of size because this was an exploratory study including all the consecutive patients who underwent the surgical procedure during the study period.

Data from experiments with pharmacological inhibitors of MEK/ERK were analyzed by one-way ANOVA (with or without repeated measures), followed by the Newman-Keuls *post-hoc* test for multiple comparisons when pertinent. The one-sample Kolmogorov-Smirnov test is used for testing if a variable (behavioral parameters in rats) is normally distributed (see **Supplemental Figure 5**). Differences were considered significant at $p \leq 0.05$. The EPM and CAC data were performed using the software Statistica® (StaSoft Inc., Tulsa, OK, USA) version 8.0, and graphs were drawn with the software GraphPad Prism®, version 5.0.

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Conflicts of interest

All authors state that they have no financial interests or potential conflicts of interest.

Contributions

Principal Investigator (PI): Roger Walz. Study design: CRC, RBL and RW. Method development and data analysis: CRC, MWL, LCC, HMM, AAH, KL, RBL and RW. Clinical data

collection: RG, KL and RW. Human brain tissue sampling: MNL and RW. Stereotaxic surgery and rat tissue sampling: CRC, HMM, LCC and AAH. Biochemical assays of human and rat samples: MWL, LCC, AL and RBL. Experimental and analytical supervision: CRC, AAH, RDP, RBL and RW. Manuscript Writing: CRC and RW. Data interpretation: CRC, LCC, AAH, RDP, AL, ZAB, JL, RBL, and RW. Manuscript edition and approval: All authors.

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Figure and table legends

Table 1. Symbols indicate: [a] ASMs = anti-seizure medications: carbamazepine (n = 21), phenobarbital (n = 9), valproic acid (n=3), lamotrigine (n= 3), phenytoin (n = 2) and topiramate (n = 2). [b] Adjuvant benzodiazepines were clobazam (n = 3) or clonazepam (n = 8). [c] For correlation analysis the serum levels was considered zero for the patients who were not using the ASMs (carbamazepine or phenobarbital). [d] Comparisons between means were done by Student 's t-test or ANOVA. [e] Correlations were determined by Pearson's correlation test. [f] Together the HADS depression scores and P-ERK (1,2) / Total-ERK (1,2) ratio in AMY explains 39% (r^2= 0.39) of the HADS anxiety variation. [g] P-ERK (1,2) / Total-ERK (1,2) ratio in the AMY explains 23% (r^2 = 0.23) of the HADS anxiety score variation.

Figure 1: Pearson's correlation between the neurochemical parameters levels according to the brain areas and the HADS anxiety scores (n=23). A) Correlation between the HADS anxiety and the P-ERK(1,2) / Total-ERK(1,2) ratio in the AMY (r = -0.46, p = 0.03); B) Correlation between the HADS anxiety and the Total-ERK(1,2) in the AMY (r = 0.47, p = 0.03); C) Correlation between the P-ERK(1,2) / Beta Actin and the Total-ERK(1,2) / Beta Actin in the AMY (r = 0.23, p = 0.22); D) Correlation between HADS anxiety scores and the P-CaMKII/Total-CaMKII ratio in the AMY (r = 0.08, p = 0.73); E) Correlation between HADS anxiety and PKA activity in the AMY (r = -0.03, p = 0.88); F) Correlation between HADS anxiety and the PKC activity in the AMY (r = 0.07, p = 0.75). The bottom table shows the respective "r" coefficient and the "p" level of significance of the correlations among the neurochemical variables in the AMY, aHIP, and Middle Temporal Neocortex and the HADS anxiety scores. All the

neurochemical parameters are expressed in the percentage of the reference sample corrected by Beta-actin expressed in the reference sample's percentage from the respective brain structure analyzed.

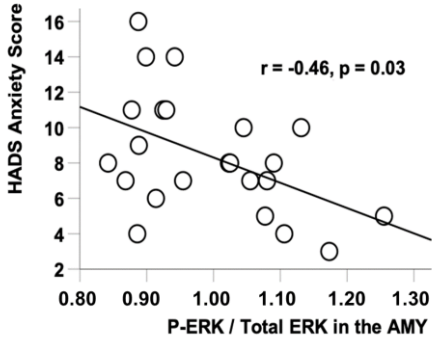
Figure 2: Pearson's correlation between the P-ERK (1,2) / Total-ERK (1,2) ratio and the Total-ERK (1,2) determined in the AMY of naïve rats (n = 20) immediately after a 5-min exposure to the EPM test (A and B). The P-ERK (1,2) and Total-ERK (1,2) were expressed in percentage of the reference sample corrected by beta-actin, also expressed in percentage of the reference sample from the respective brain structure analyzed. The higher time spent in open arms on the EPM apparatus indicates less anxiety-like behavior. A) Animals showing more anxiety-related behavior had a lower P-ERK (1,2) / Total-ERK (1,2) rate ($r= -0.49$, $p = 0.03$); B) No correlation was observed between the anxiety-like behavior and the Total-ERK (1,2) in the AMY of the animals ($r = 0.18$, $p = 0.42$). C) The bilateral intra-BLA microinfusion of FR180204 (1, 10, or 50 μg /0.2 μL /side), D) neither the U0126 (1.0 μg /0.5 μL /side) did not affect the anxiety-related behavior of rats on EPM test. Only naïve rats from control groups witch received an intraperitoneal injection of diazepam (DZP, 1.5 mg/kg i.p.) showed a significant anxiolytic-like behavior ($p < 0.05$) compared to vehicle and other groups, see other behavioral parameters in Suplemental Figure 2. The "r" is the Pearson correlation coefficient. One-way ANOVA / Newman-Keuls *post-hoc* test ($F_{4, 49}=31.67$, $p < 0.00001$; $F_{2, 22}=49.87$, $p < 0.0001$), N=8-13 / group).

Figure 3: Pearson's correlation between the percentage of time spent in freezing and the P-ERK (1,2) / Total-ERK (1,2) or Total-ERK (1,2) in the AMY determined in naïve rats (n = 29) immediately after retrieval test of Pavlovian aversive conditioning task (A and B). The P-ERK (1,2) and Total-ERK (1,2) were expressed in percentage of the reference sample corrected by beta-actin, also described in the percentage of the reference sample from the respective brain structure analyzed. A) Animals that spent more time in freezing during the test (display more learned-defensive behavior) had a higher P-ERK (1,2) / Total-ERK (1,2) ratio the AMY ($p < 0.05$) but, B) no correlation was observed between the anxiety-like behavior and the Total-ERK (1,2) in AMY. Bilateral intra-BLA microinfusion of FR180204 (1.0, 10, or 50 μg/0.2 μL/side), a selective ERK1/2 inhibitor, C) or D) U0126 (1 μg/0.5 μL/side), an inhibitor of MEK/ERK pathway activation, immediately after training impair the consolidation of contextual fear conditioning, whereas, independent groups which received the same inhibitors (FR180204 or U0126 before testing decrease significantly the evocation of conditioned-defensive behavior in rats (E-F). G) Using an

independent group of animals (n = 13), we found a strong and positive correlation (r = 0.62, p = 0.02) between the level of anxiety-related behavior (% time-spent on closed-arms from EPM test and the expression of freezing an learned-defensive behavior. The “r” is the Pearson correlation coefficient. One-way ANOVA with repeated measures/Newman-Keuls *post-hoc* test *indicates significant ($p < 0.05$) difference compared to the respective group during training, # compared to the control group in the same test (n=8-13 / group).

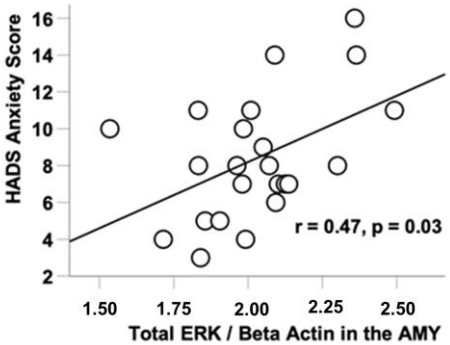
Figure 1

A Correlation between Anxiety Symptoms and the P-ERK(1,2) / Total-ERK(1,2) ratio in the AMY



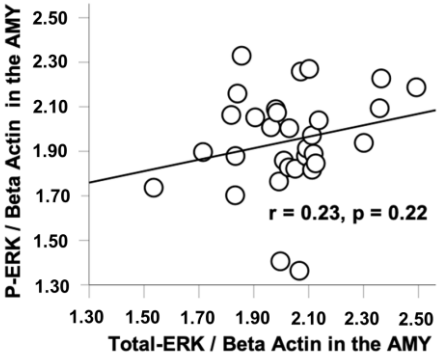
↑ Anxiety = ↓ P-ERK(1,2) / Total-ERK(1,2)

B Correlation between Anxiety Symptoms and the Total-ERK(1,2) in the AMY

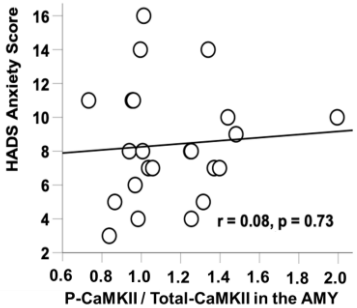


↑ Anxiety = ↑ Total-ERK(1,2) / Beta-actin

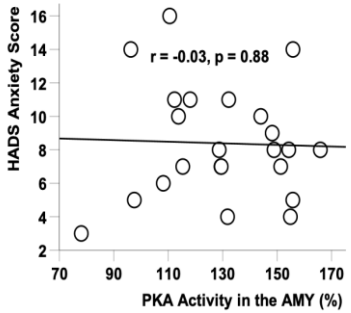
C Lack of correlation between the P-ERK and the Total-ERK(1,2) in AMY



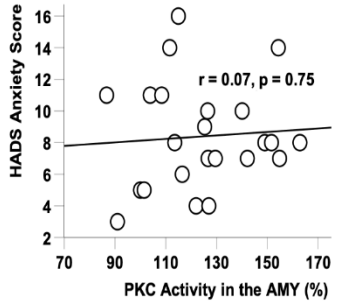
D Lack of correlation between the Anxiety Symptoms and the P-CaMKII/Total-CaMKII ratio in the AMY



E Lack of correlation between Anxiety Symptoms and the PKA activity in the AMY



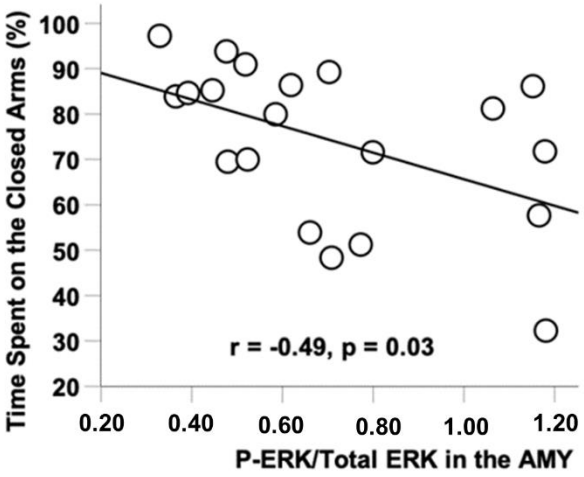
F Lack of correlation between the Anxiety Symptoms and the PKC activity in the AMY



Neurochemical parameters and brain areas analyzed	"r" coefficient	"p" level
Amygdala		
GluR1	0.273	0.21
GFAP	0.170	0.44
EAAT1	0.001	1.00
EAAT2	0.239	0.27
BDNF	0.064	0.78
Total-TrkB	0.280	0.20
P-TrkB/Total TrkB	0.319	0.15
Anterior Hippocampus		
P-ERK(1,2) / Total-ERK(1,2)	0.021	0.96
Middle temporal neocortex		
P-ERK(1,2) / Total-ERK(1,2)	0.160	0.40

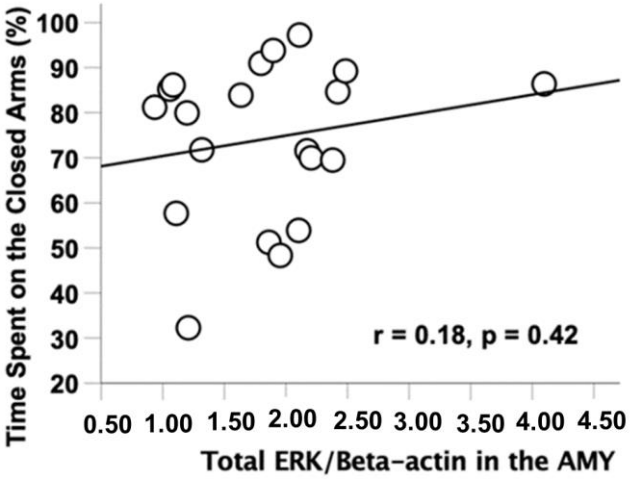
Figure 2

A) Correlation between anxiety-like behavior and the P-ERK (1,2) / Total-ERK (1,2) ratio in the AMY

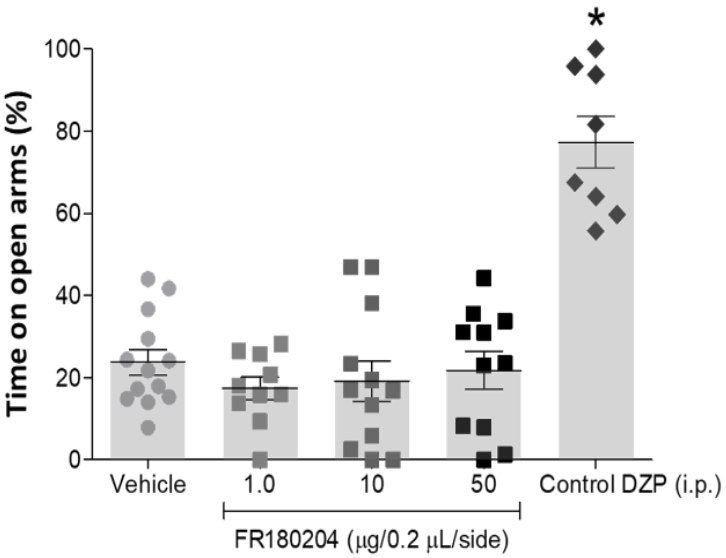


↑ Anxiety-like behavior = ↓ P-ERK (1,2) / Total-ERK (1,2)

B) Lack of correlation between anxiety-like behavior and Total-ERK (1,2) / Beta-actin ratio in the AMY



C) Intra-BLA infusion of FR180204, an ERK1/2 inhibitor, 10-min before exposure to EPM test.



D) Intra-BLA infusion of U0126, an MEK1/2 inhibitor, 30-min before exposure to EPM test.

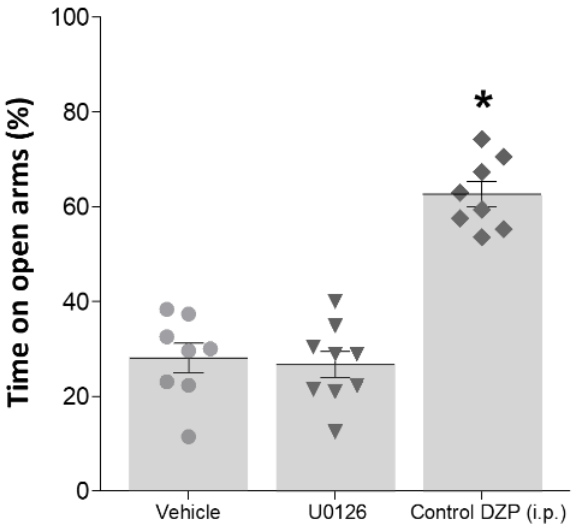
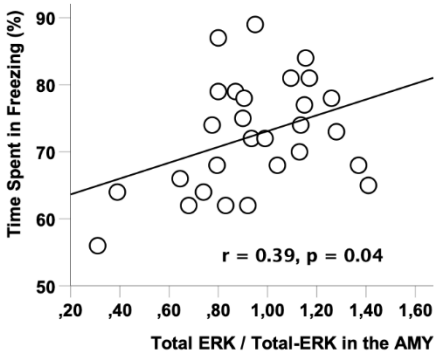
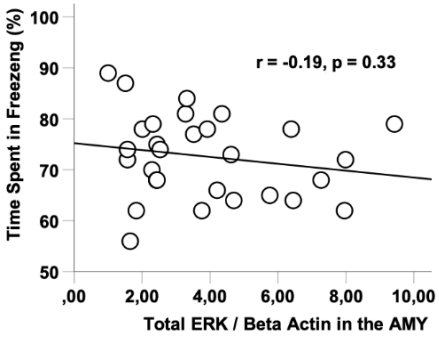


Figure 3

A. Correlation between the P-ERK / Total-ERK ratio in the AMY and learned-defensive behavior in rats.

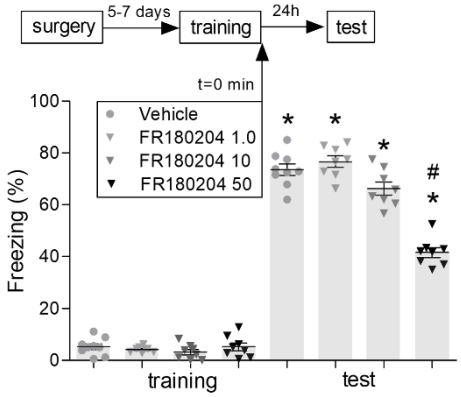


B. Lack of correlation between the AMY levels of Total-ERK / Beta-actin ratio and learned-defensive behavior in rats.

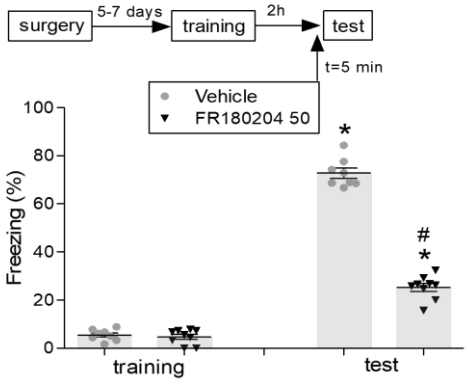


↑ freezing-learned behavior = ↑ P-ERK(1,2)/Total-ERK (1,2) ratio

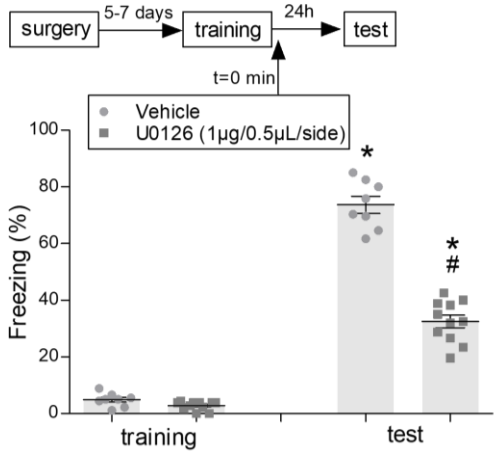
C. Intra-BLA infusion of FR180204 impairs the consolidation Pavlovian aversive long-term memory.



D. Intra-BLA infusion of FR180204 impairs the expression of learned-defensive behavior.



E. Intra-BLA infusion of U0126 impairs the consolidation of Pavlovian aversive long-term memory.



F. Intra-BLA infusion of U0126 impairs the expression of learned-defensive behavior.

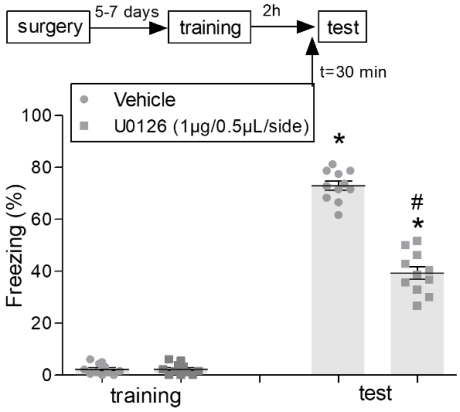
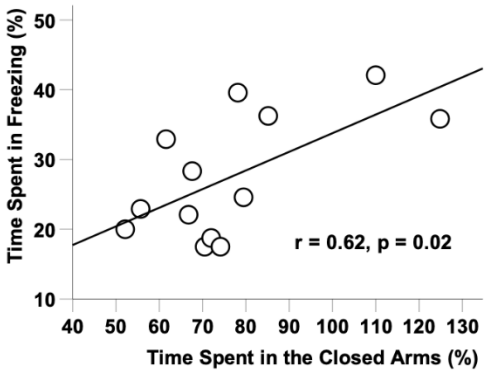


Figure 3

G. Correlation between innate (x axis) and learned-defensive (y axis) behavior in rats



↑ Anxiety-like behavior (x axis) = ↑ expression of learned-defensive behavior (y axis)

Table 1: Demographic, clinical, neuroradiologic and trans-operative characteristics of patients associated with their HADS anxiety scores.

Dependent variables	**All cases**	**HADS anxiety**	**"p"**
	n = 23 (%)	**Mean (S.E.) scores [d]**	**level**
Demographic, clinical and neuroradiologic			
Sex			
Male	07 (30.4)	6.3 (0.9)	
Female	16 (69.6)	9.3 (0.8)	**0.04**
Marital Status			
Single	12 (52.2)	9.2 (1.0)	
Married	08 (34.8)	7.0 (1.0)	
Divorced	03 (13)	8.7 (1.8)	0.36
Work activity			
Working	8 (34.8)	7.0 (0.8)	
Housewife	05 (21.7)	8.4 (2.0)	
Non-working	06 (26.1)	10.5 (1.2)	
Health insurance	4 (17.4)	8.8 (2.3)	0.41
MRI Side			
Right	11 (47.8)	8.4 (1.0)	
Left	12 (52,2)	8.1 (1.0)	0.94
ASMs schedule [a]			
Monotherapy	06 (26.1)	9.7 (1.2)	
Polytherapy	17 (73.9)	7.9 (0.8)	0.29
Adjuvant Benzodiazepines [b]			
No	12 (52.2)	8.2 (1.0)	
Yes	11 (47.8)	8.5 (1.0)	0.84
	Mean (S.E.)	**"r" coefficient [e]**	
Age, years	38.4 (2.5)	-0.09	0.67
Education Level, years	7.0 (0.6)	0.01	0.98
Disease duration, years	25.6 (2.4)	-0.01	0.98
Age of epilepsy onset, years	12.8 (1.8)	-0.12	0.59

Dependent variables	All cases	HADS anxiety	"p"
Seizures / month	10 (2.0)	0.08	0.71
The time before the last seizure, hours	258.0 (104.9)	0.08	0.75
Carbamazepine levels (n = 21) [c]	6.3 (0.7)	0.05	0.81
Phenobarbital levels (n = 09) [c]	26.5 (5.4)	-0.31	0.17
HADS depression sub-scale	7.0 (0.8)	0.52	**0.01**
Trans-operative parameters			
Dexamethasone dose (mg/Kg)	0.09 (0.01)	0.20	0.33
Anesthesia duration until AMY/HIP sampling (min)	256.6 (11.6)	0.32	0.15
Hemodynamic parameters			
Mean arterial blood pressure (mmHg),	68.5 (2.3)	0.12	0.61
Respiratory rate/minute	11.5 (0.4)	-0.06	0.77
Cardiac rate / minute	75 .1 (2.7)	-0.23	0.24
Blood gases parameters hydroeletrolitic parameters			
pH	7.41 (0.01)	0.03	0.88
PCO_2 (mmHg)	28.7 (1.0)	-0.21	0.32
PO_2 (mmHg)	219.2 (13.7)	-0.01	0.96
HCO_2 (mmHg)	20.0 (0.4)	-0.08	0.73
Total CO_2 (mmHg)	21.3 (0.6)	-0.15	0.49
Oxygen saturation (%)	219.2 (13.7)	-0.01	0.96
Hematologic parameters			
Hemoglobin (g/dL)	12.8 (1.3)	-0.08	0.72
Hematocrit (%)	35.2 (0.8)	-0.11	0.62
Biochemical parameters			
Lactic acid (mmol/L)	2.2 (0.2)	-0.32	0.17
Glucose (mg/dL)	113.7 (4.8)	-0.21	0.34
Sodium (mEq/L)	137.9 (0.7)	-0.34	0.16
Potassium (mEq/L)	4.1 (0.1)	-0.15	0.46
Magnesium (mmol/L)	0.46 (0.01)	-0.16	0.53
Calcium (mg/dl)	4.4 (0.5)	-0.27	0.26
Storage time of brain samples in the -80C freezer	23 (1.7)	-0.33	**0.14**

Multiple Linear Regressions	**Liner Regression Coefficients**			**"p" level**
Predictive Models	r	r^2	B (CI 95%)	
Model 1	0.66	0.45		0.07
Constant			15.74 (-0.04 to 31.53)	0.05
Male			-1.30 (-5.30 to 2.70)	0.50
HADS Depression			0.28 (-0.16 to 0.71)	0.19
Anesthesia duration until AMY/HIP sampling (min)			0.006 (-0.02 to 0.04)	0.65
Storage time of brain samples in the -80C freezer			-0.02 (-0.23 to 0.19)	0.84
P-ERK (1,2) / Total-ERK (1,2) ratio in the AMY			-8.95 (-22.14 to 4.23)	0.16
Model 2 [f]	0.62	0.39		0.007
Constant			16.48 (4.22 to 28.74)	0.01
HADS Depression			0.36 (0.04 to 0.70)	0.03
P-ERK / Total-ERK ratio in the AMY			-1.07 (-2.21 to 0.00)	0.05
Model 3: P-ERK (1,2) / Total-ERK (1,2) in the AMY [g]				
Constant	0.48	0.23		< 0.001
P-ERK / Total-ERK ratio in the AMY			-1.43 (-2.63 to -0.23)	0.02

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The ERK phosphorylation levels in the amygdala predict anxiety symptoms in humans and MEK/ERK inhibition dissociates innate and learned defensive behaviors in rats

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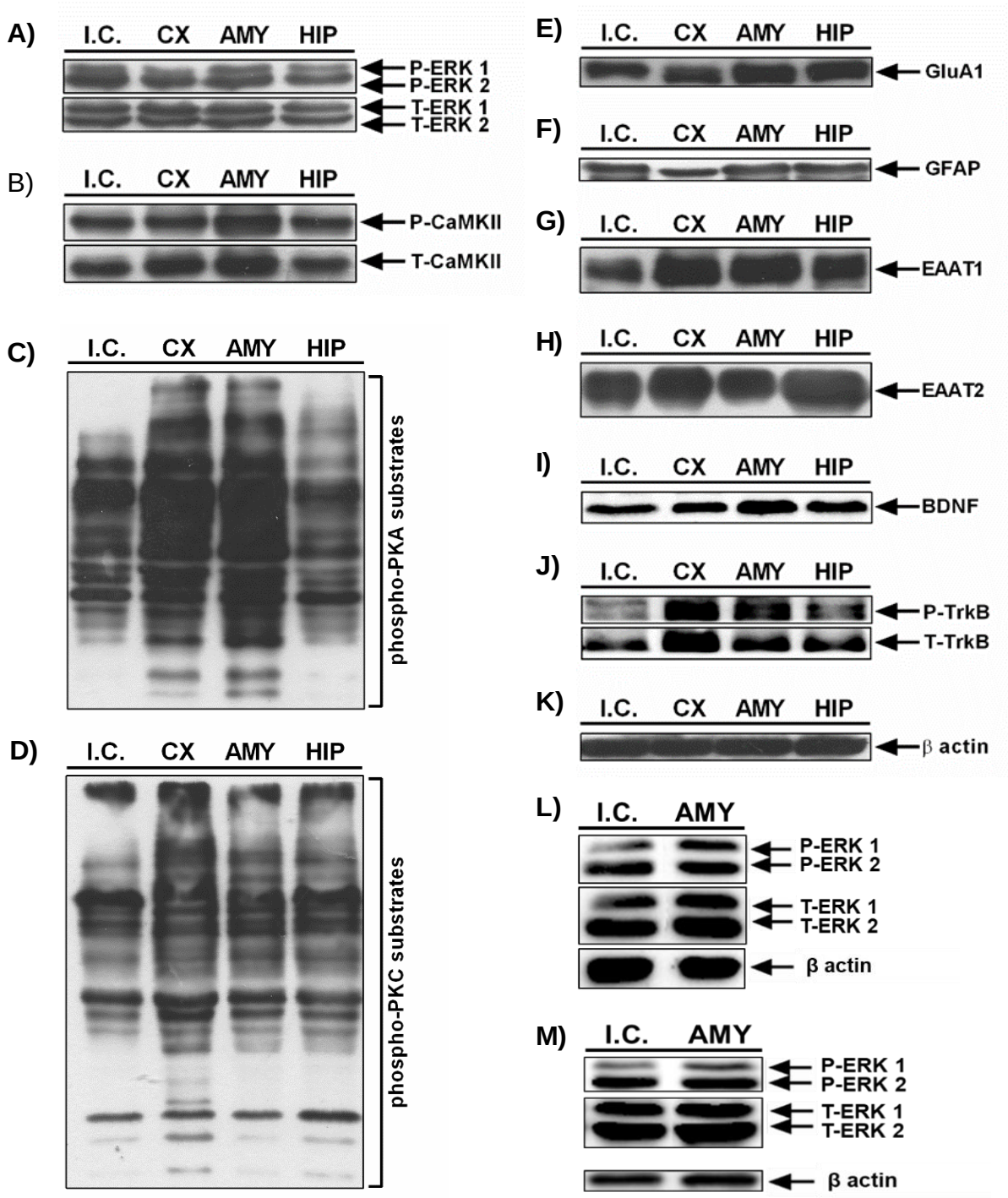
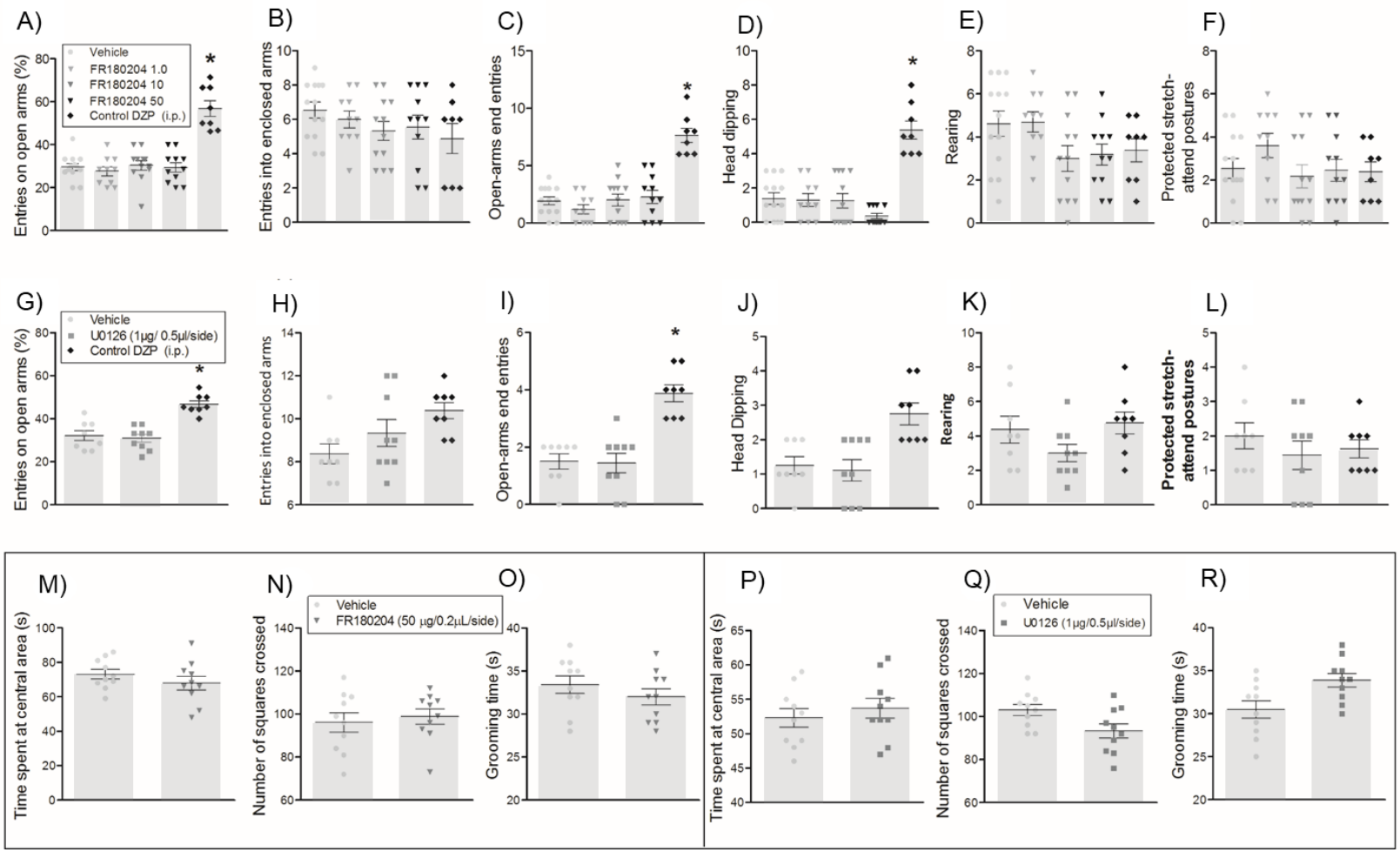
This Supplement contains: Supplemental Figures 1-5.

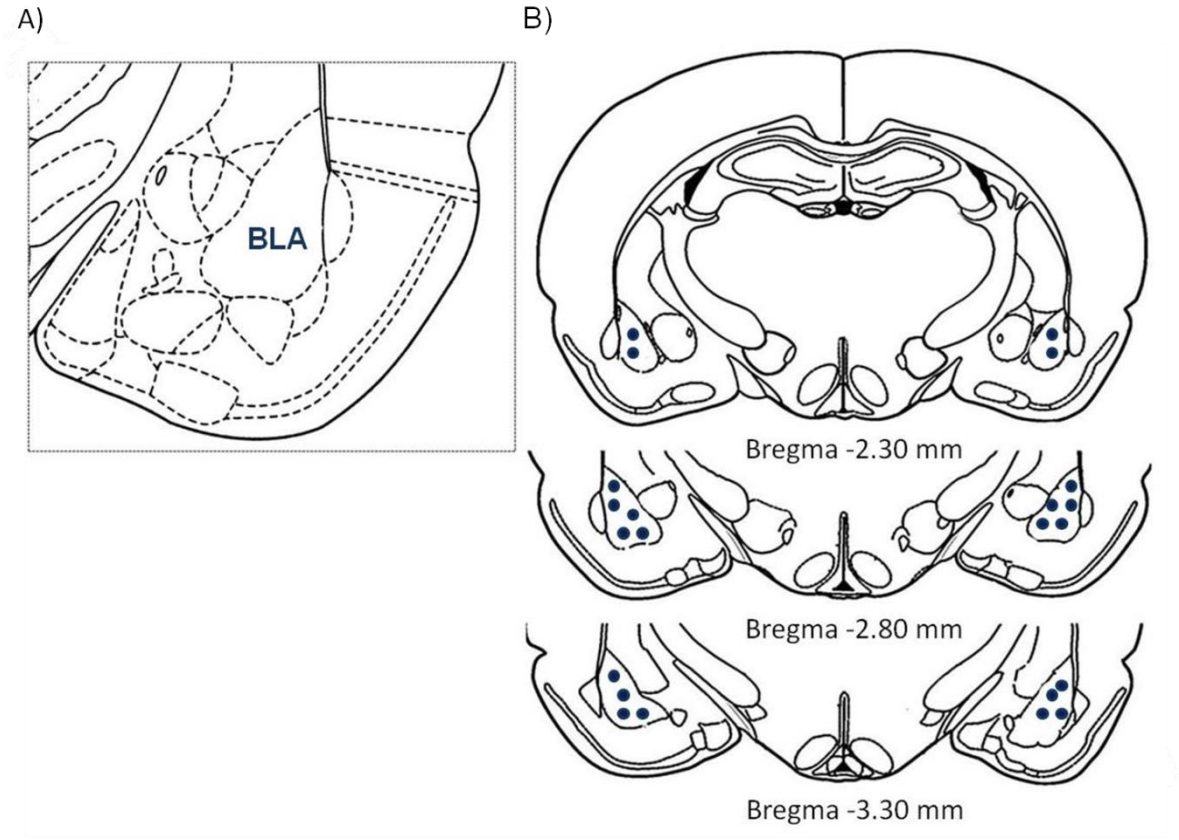
Named and ordered according to their first mention in the main text.



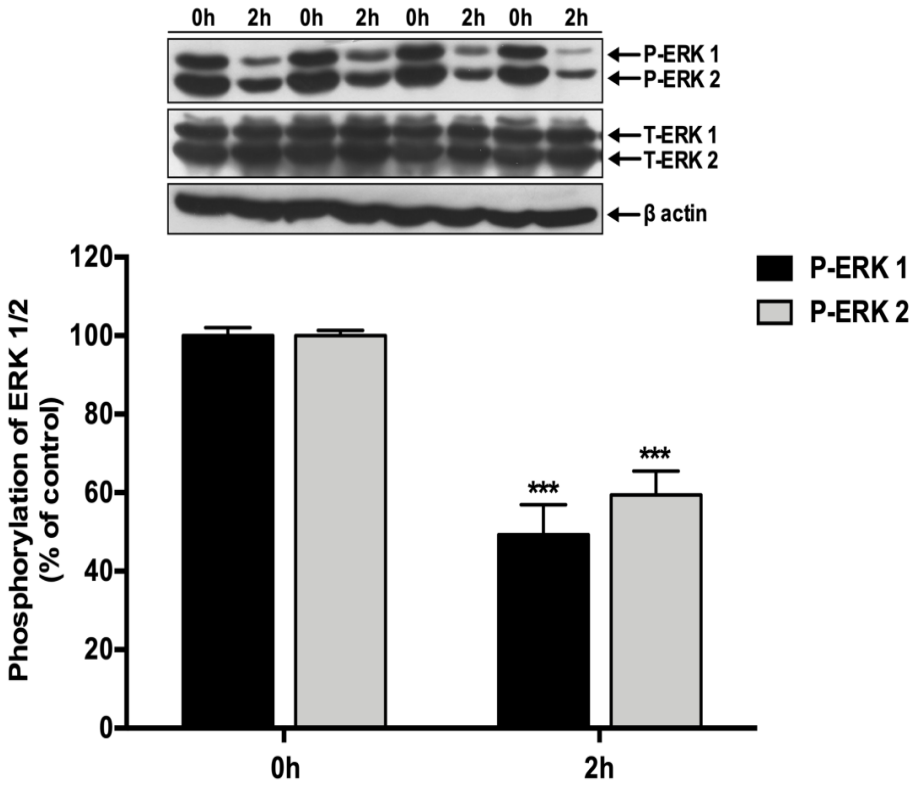
Supplemental Figure 1: Original images of representative Western blots of total- and P-ERK1/2 **(A)**, total- and P-ERK1/2 CaMKII **(B)**, P-PKA substrates **(C)**, P-PKC substrates **(D)**, GluA1 subunit of AMPA receptor **(E)**, GFAP **(F)**, EAAT1 **(G)**, EAAT2 **(H)**, BDNF **(I)**, TrkB **(J)** and β actin **(K)** in samples of the middle temporal neocortex (CX), amygdala (AMY) and anterior hippocampus (HIP) of MTLE-HS patients their internal control sample (I.C.) from both Figure 1 and Supplemental Figure 1. Representative western blots of total- and P-ERK1/2 and β actin of amygdala (AMY) and I.C. sample of rats after the exposure to EPM test **(L)** or the evocation of short-term contextual aversive conditioning task **(M)** from Figures 1-3, respectively. The images are illustrative and represent the pattern detection of targets of interest.



Supplemental Figure 2. Effects of FR180204 (ERK1/2 inhibitor) or U0126 (MEK/ERK inhibitor) infusion intra-BLA on EPM and open field tests. FR180204 and U0126 did not affect the behavioral performance of Wistar rats in the open field test. A group of naïve rats that received an intraperitoneal injection of diazepam (DZP, 1.5 mg/kg i.p.) was used as a control group test. * indicates a significant ($p < 0.05$) difference compared to the vehicle-treated group by one-way ANOVA /Newman-Keuls post-hoc test ($F_{4, 49}=23.63$, $p < 0.00001$; $F_{4, 49}=22.84$, $p < 0.00001$; $F_{4,49}=23.22$, $p < 0.00001$; $F_{2, 22}=20.53$, $p < 0.0001$; $F_{4, 49}=22.84$, $p < 0.00001$; Figures A, C, D, G and I, respectively), N=8-13/group.

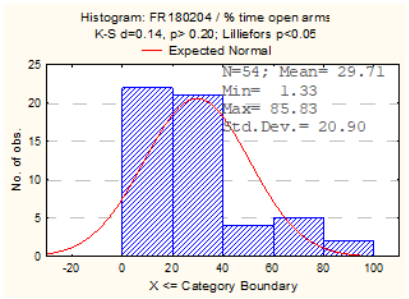


Supplemental Figure 3. (A) Diagram of rat basolateral amygdala (BLA) and adjacent structures (Paxinos and Watson 1997; 2.8 mm posterior to Bregma). **(B)** Representative data from animals that had needle tracks terminating depicting the diffusion of methylene blue in the BLA for rats included in the final statistical analysis illustrated by diagrams of rat brain sections (2.30 mm, 2.80 mm, and 3.30 mm posterior to Bregma) on each side.



Supplemental Figure 4. This figure shows the levels of ERK 1/2 phosphorylation and immunocontent in the total cerebral cortex of rats. The samples referred to as 0h were prepared as standard, being placed immediately in liquid nitrogen after isolation. The samples referred to as 2h were isolated in the same manner but were kept for 2h at refrigerated temperature (5 C°) and, after that placed in liquid nitrogen. After that, all samples were stored at -80C for further analysis 7 days later. All samples were homogenized and prepared for analysis by Western blotting according to the protocol. The representative blot shows a significant decrease of 40 to 50% in the level of P-ERK1/2 in the cortex maintained for 2 hours at 5C° temperature, in comparison to the tissue frozen immediately after the animal death. No significant changes were observed in the immunocontent of T-ERK1/2 and β-actin. The phosphorylation levels of ERK1/2 were determined by computer-assisted densitometry as a ratio of the O.D. of the phosphorylated band over the O.D. of the total band, and the data are expressed as a percentage of the control. The values are presented as mean + S.E.M. derived from 4 independent experiments. Statistical analysis was performed using Student's t-test. *** $p < 0.001$.

A



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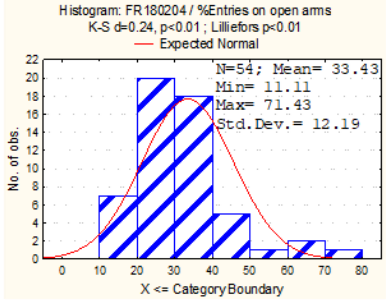

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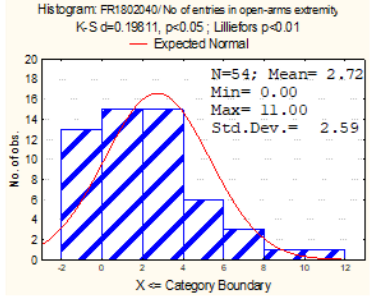

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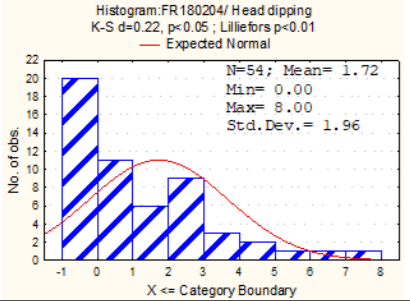

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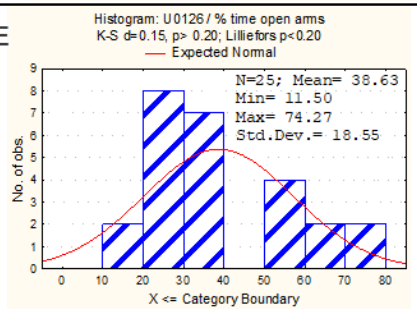

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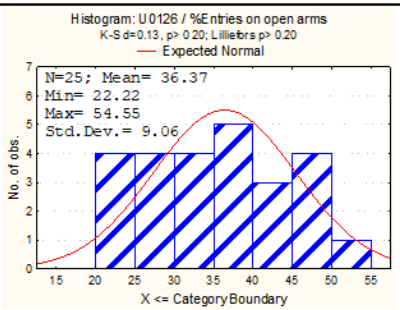

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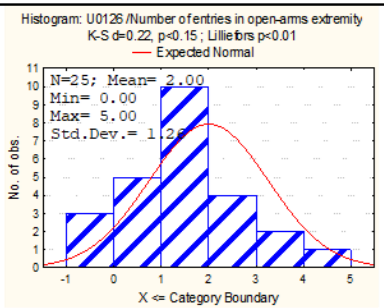

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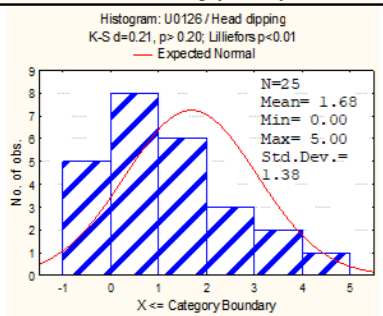

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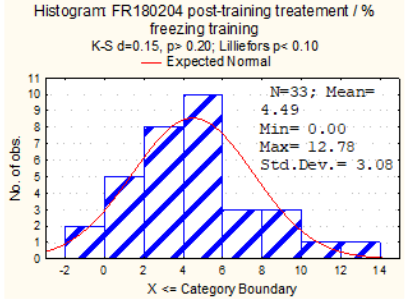

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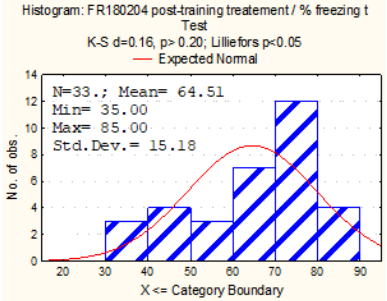

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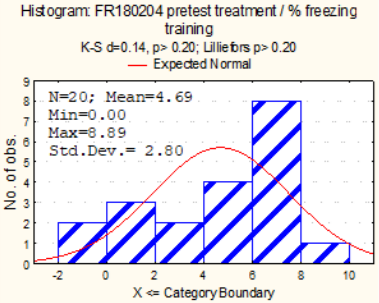

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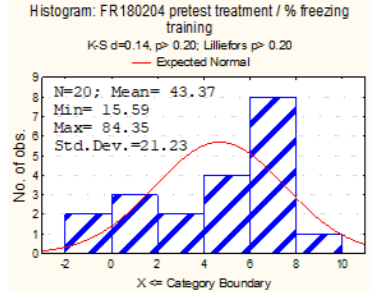

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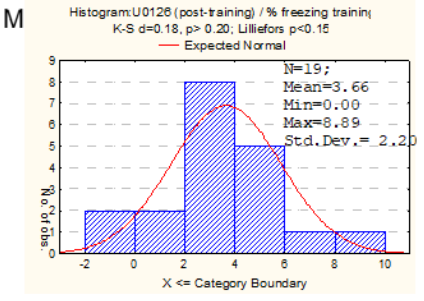

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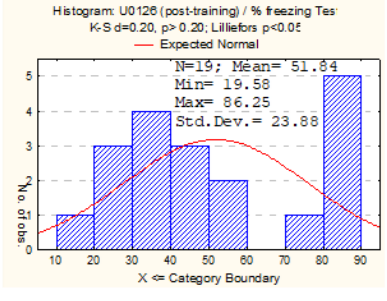

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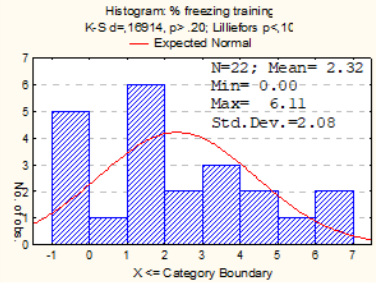

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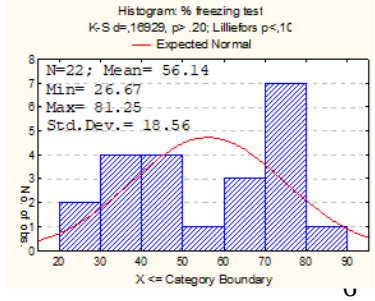

Supplemental Figure 5. Histograms from experiments with inhibitors (FR180204 and U0126) on EPM, Pavlovian aversive conditioning analyzed by Statistica software (version 8). All data have normal distribution according to Kolmogorov-Smirnov & Lilliefors test for normality. Curves are representing the expected normal distribution of the sample for each experiment. There were no outliers (according to the Grubbs' test). Animals were only excluded due to the positioning of the cannulas outside the target region (our exclusion rate is approximately 10%). Only parameters of EPM test that one-way ANOVA was displaying $p < 0.05$ (see Figures 2 C-D and Suppl. Figure 2) for FR180204 **(A-D)** and U0126 **(E-H)**; Intra-BLA treatment immediately post-training of Pavlovian aversive conditioning with FR180204 (**I, J**: training and test, respectively) or U0126 (**M, N**: training and test, respectively); Intra-BLA treatment immediately before testing of Pavlovian aversive conditioning with FR180204 (**K, L**: training and test, respectively) or U0126 (**0, P**: training and test, respectively).