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MAISA DE ARAUJO COSTA

N-FORMIL-METION IL-LEUCIL-FENILALANINA DESEMPENHA UM PAPEL NEUROPROTETOR E ANTICONVULSIVANTE NO MODELO DE STATUS EPILEPTICUS

Orientador: Prof. Dr. Olagide Wagner de Castro

Coorientadora: Profª. Dra. Adriana Ximenes da Silva

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N-formyl-methionyl-leucyl-phenylalanine plays a neuroprotective and anticonvulsant role in Status Epilepticus model

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RESUMO

O Status epilepticus (SE) é caracterizado como convulsões contínuas e autossustentáveis que levam à neurodegeneração hipocampal, inflamação e gliose. O receptor do peptídeo N-formil (FPR) tem sido associado a mecanismos inflamatórios. O peptídeo N-formil-metionil-leucil-fenilalanina (fMLP) desempenha um papel anti-inflamatório, mediado pela ativação de FPR acoplado à proteína G. Aqui avaliamos a influência do peptídeo fMLP no comportamento de crises límbicas, consolidação da memória e processo de neurodegeneração hipocampal. Ratos Wistar machos (Rattus norvegicus) receberam microinjeções de pilocarpina no hipocampo (H-PILO, 1,2mg / μL, 1μL), seguido por fMLP (1mg / mL, 1μL) ou veículo (VEH, solução salina 0,9%, 1μL). A análise comportamental das crises foi realizada por 90 minutos durante o SE. Os processos de memória e aprendizagem foram analisados pelo teste de esquiva inibitória. Após 24 horas de SE, o processo de neurodegeneração foi avaliado em áreas do hipocampo. Não houve mudança na latência e no número de WDS após a administração de fMLP. Os resultados mostraram que a infusão de fMLP foi capaz de reduzir a gravidade das crises, bem como o número de crises límbicas. Além disso, a infusão de fMLP protegeu a disfunção de memória seguida por SE. Por fim, a administração intra-hipocampal de fMLP atenuou o processo de neurodegeneração em ambos os hipocampos. Juntos, nossos dados sugerem um novo insight sobre o papel funcional do peptídeo fMLP, com implicações importantes para seu uso potencial como agente terapêutico para o tratamento de distúrbios cerebrais, como a epilepsia.

PALAVRAS-CHAVE: Receptor de N-formil Peptídeo, N-Formil-Metionil-Leucil-Fenilalanina, *Status Epilepticus*, Hipocampo, Cérebro, Epilepsia, Neurodegeneração, Morte neuronal.

Abstract

Status epilepticus (SE) is described as continuous and self-sustaining seizures, which leads to hippocampal neurodegeneration, inflammation, and gliosis. N-formyl peptide receptor (FPR) has been associated with inflammatory mechanisms. N-formyl-methionyl-leucyl-phenylalanine (fMLP) peptide plays an anti-inflammatory role, mediated by activation of G-protein-coupled FPR. Here, we evaluated the influence of fMLP peptide in behavior of limbic seizures, memory consolidation, and hippocampal neurodegeneration process. Male Wistar rats (*Rattus norvegicus*) received microinjections of pilocarpine in hippocampus (H-PILO, 1.2mg/μL, 1μL) followed by FMLP (1mg/mL, 1μL) or vehicle (VEH, saline 0.9%, 1μL). During the 90 minutes of SE, epileptic seizures were analyzed according to the Racine's Scale. After 24 hours of SE, memory impairment was assessed by the inhibitory avoidance test and the neurodegeneration process was evaluated in hippocampal areas. There was no change in latency and number of wet dog shake (WDS) after administration of fMLP. Our results showed that the infusion of fMLP was able to reduce the severity of seizures, as well as the number of limbic seizures. In addition, fMLP infusion protected memory dysfunction followed by SE. Finally, the intrahippocampal administration of fMLP attenuated the process of neurodegeneration in both hippocampi. Taken together our data suggest a new insight into the functional role of fMLP peptides, with important implications for their potential use as a therapeutic agent for the treatment of brain disorders, such as epilepsy.

KEYWORDS: N-formyl Peptide Receptor, N-Formyl-Methionyl-Leucyl-Phenylalanine, Status Epilepticus, Hippocampus, Brain, Epilepsy, Neurodegeneration, Neuronal death.

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INTRODUCTION

Status epilepticus (SE) is a common neurological emergency defined as continuous and self-sustaining seizures, with high morbidity and mortality, requiring rapid diagnosis and treatment [1-6]. SE can present different clinical manifestations and promote cognitive and memory deficits, neurodegeneration, inflammation, and gliosis in the hippocampus, which culminates in the development of epilepsy [7-10]. Typically, intrahippocampal pilocarpine (H-PILO)-induced SE triggers behavioral changes, including wet dog shake (WDS), forelimb myoclonus, rearing, and falling [11-13], as well as selective neuronal death in hilus of dentate gyrus (DG), CA3 and CA1 hippocampal subfields [9,14-16]. In addition, the inflammatory process induced by SE stands out for the proliferation of activated resident microglia [17], monocyte infiltration [18] and reactive astrocytes [19,20] in the hippocampus, as well as the increase in the level of glial fibrillary acidic protein (GFAP), cyclooxygenase-2 (COX-2), cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukins (IL-1β, IL-6, IL-10) and interferons (IFN-γ and IFN-β) (21–27). These inflammatory responses intensify neuronal and cognitive damage followed by SE [10,28,29].

About 30 to 40% of SE patients are resistant to typical antiepileptic drugs (AEDs) [6,30,31]. Therefore, it is important to conduct further research to search for new substances that have a better pharmacological performance and more efficacy in these patients who are refractory to standard treatment. Many studies have shown that several drugs with an anti-inflammatory role may be able to exert a neuroprotective and anticonvulsant effect in SE models [8,19,23,24]. Therefore, further studies on new substances with a potential anti-inflammatory effect during SE are necessary.

N-formylated peptides are derived from bacterial and mitochondrial proteins [32] and important endogenous inhibitory regulators of inflammation that is activated in response to tissue injury [33,34]. Evidence indicates that N-formylated peptides mediates most of biological functions via interaction with N-formyl peptide receptor (FPR) on mammalian phagocytes [32]. These FPR are G-protein-coupled receptors (GPCR) that activate the transduction of chemotactic signals in phagocytes and mediate host defense, as well as the onset and resolution of inflammatory processes, including degranulation, cell adhesion and migration, reactive oxygen species production and cytokine release [35-38]. In addition, it has been reported that N-formyl-methionyl-leucyl-phenylalanine (fMLP),

an N-formylated peptide, can exerts an anti-inflammatory role, mediated by activation of FPR [32,39]. Reduction of inflammation process may contribute to attenuate neurodegeneration caused by epileptic seizures. However, physiological role of fMLP during SE is unknown. The purpose of this study was to evaluate the role of the fMLP peptide in the behavior of limbic seizures, memory consolidation, and hippocampal neurodegeneration following intrahippocampal pilocarpine (H-PILO)-induced SE.

METHODS

Animals

All experimental procedures were approved and conducted by the Ethical Committee of the Federal University of Alagoas (Protocol # 04/2016), according to Ethical Principles adopted by the Brazilian College of Animal Experimentation (COBEA) and the Guide for the Care and Use of Laboratory Animals of the Brazilian Society of Laboratory Animals Science (SBCAL). Animal studies are reported in compliance with the approved guidelines. Male Wistar rats (*Rattus norvegicus* [n= 31, 240-340g, 2-3 months]) were used in all experiments and were obtained from the main breeding stock of the Federal University of Alagoas. They were maintained on a 12h/12h light/dark cycle at 21 ± 2°C, with lights on at 07:00 AM and lights off at 07:00 PM and were individually housed in plastic cages with food and water *ad libitum*. All experiments were designed to minimize animal suffering and to limit the number of animals used.

To verify signs of illness or impairment by observing the general body condition, respiration rate, dehydration, posture, immobility, social interaction and response to manipulation, the research staff monitored rats at least 2 times per day.

After submission to SE, rats received nutrient and electrolyte replacement, as described by Melo et al., 2021[40]. Clinical/behavioral pain signs were not observed in the animals.

Surgical procedure and intrahippocampal microinjections

Before surgery, animals were anesthetized with ketamine (100 mg/kg, ip), and xylazine (10 mg/kg, ip), as well as they received 0.1 mL/100g veterinary pentabiotic (Fort Dodge®, subcutaneous). Rats received local anesthetic (lidocaine with epinephrine, subcutaneous [Astra®]) after fixing on stereotaxic. Then, a cannula was implanted stereotaxically in the hilus of the dentate gyrus (DG) of the left hippocampus, according to following coordinates: - 6.30 mm anterior-posterior (AP, reference: bregma); 4.50 mm medial-lateral (ML, reference: sagittal sinus); - 4,50 mm dorsal-ventral (DV, reference: dura mater) [16,41,42]. Animals were seven days in recovery followed by surgery.

Animals received either fMLP, pilocarpine and/or vehicle (1µL) in the left hilus of the DG of hippocampus, as described below. The rats were divided into 3 experimental groups: VEH+fMLP (n=11), PILO+VEH (n=11), PILO+fMLP (n=9). Animals were gently immobilized, and the drugs microinjection were performed. VEH animals received only one administration of intrahippocampal saline 0,9% (VEH). PILO+VEH animals received microinjections of pilocarpine (1.2mg) to evoke limbic seizures followed 5 minutes later by VEH. PILO+fMLP animals received fMLP (1 mg/mL) intrahippocampal infusion after 5 minutes of pilocarpine. Manually, we used a 5µL syringe (Hamilton Company, Reno, NV, USA) at a speed of 0.5 µl/min.

After 90 minutes of SE onset, all animals that develop SE were rescued with diazepam (5 mg/kg; i.p.). In addition, rats that did not develop SE received the injection of diazepam under the same conditions.

SE seizures

Behavioral activity was recorded by video camera (Full HD Digital Camcorder Sony DCR-PJ6) for a period of 90 minutes after microinjection of intrahippocampal pilocarpine, which is enough time to observe neurodegeneration [16,42]. Epileptic seizures were analyzed according to the Racine's scale (1972) [43], based on the following classes: (0) immobility; (1) facial movements; (2) head nodding; (3) forelimb clonus; (4) rearing; (5) rearing and falling.

In addition, the latency to the beginning of the SE and the number of wet dog shakes before and during the SE were quantified. For a better understanding of the evolution of the SE, the total number and time of the seizure classes, as well as the severity of seizures, were analyzed according to Melo et al. (2020) [40].

Inhibitory Avoidance Test (IAT)

All animals were placed in an automatically operated box (40x25x25 cm) with a wall glass front in the inhibitory avoidance test (IAT). An energy generating box was coupled to a steel grid, the floor. The test consisted of the (1) learning/training and (2) test sessions before and after the SE, respectively, which were performed as described by Melo et al. (2020) [40].

Histological processing

At 24 hours after SE induction, the rodents were injected with an overdose of xylazine and ketamine to execute the histological procedures. Posteriorly, animals were transcardially perfused with phosphate-buffered saline (PBS, 0.1 M, pH 7.4), followed paraformaldehyde solution (4%, diluted in PBS). Then, the brains were collected, cryoprotected (sucrose 20%), frozen (-20 ºC; 3h) and stored (−80°C). In addition, brains were cut into sections (30 μm thickness) from the cryostat (Leica CM 1850) at a temperature ranging from -18 to -22°C. Finally, brain sections were processed for Fluoro-Jade C (FJ-C) staining.

FJ-C staining procedure

Brain sections were placed onto slides and then FJ-C staining procedure was performed as described [16,40,44]. The sections were analyzed, and images captured using a fluorescence microscope (Nikon DS RI1).

Cell counting and densitometry

Fluoro-Jade positive (FJ+) neurons were quantified by using the ImageJ software (Wayne Rasband; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). To quantify the FJ+ neurons in the hippocampal areas, different coordinates were used [45]. All FJ + cells were counted in both hippocampi. Although the rodents received intrahippocampal microinjection of pilocarpine and developed a scar around the microinjection site (42), the regions of the ipsilateral hippocampus were also analyzed.

In both hippocampi, the following different coordinates were used: CA1, CA3 and DG hilus (AP −2.56 mm; AP −3.30 mm and AP −6.30 mm), according to Melo et al. (2020) [40]. Because of the high sensitivity to the neurodegenerative process, these regions were selected.

Statistical analysis

All results were expressed as mean ± SEM. Data related to epileptic seizures and neuronal death were evaluated by unpaired t test. However, only the findings regarding memory consolidation were presented as median with interquartile range and evaluated by the Kruskal-Wallis test. The GraphPad Prism 8.0 program (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analyses, with a significance level of 5% (described as $P < 0.05$). In the figure legends, the number of animals is described.

RESULTS

The latency for SE and the number of WDS do not change after intrahippocampal administration of peptide

The model for H-PILO-induced SE is characterized by a set of behavioral changes, which include mild (immobility, facial movements, head nodding) and severe (myoclonic movements of the limbs and continuous tonic clonic convulsive seizures) seizures.

Initially, after administration of H-PILO, the animals had a latency period for SE, presenting isolated epileptic seizures. To observe whether the fMLP peptide after H-PILO interferes with the SE generation time, the animal behavior was evaluated before the SE (Fig. 1A). FMLP peptide did not change the latency for the SE when compared to the control (*t-test*, t_{14} = 0.20, P = 0.84; Fig. 1B). Commonly, during the latency period, a significant number of WDS are observed in SE models, decreasing during SE. The total number of WDS remained unchanged after infusion of the FMLP peptide after H-PILO (*t-test*, t_{15} = 0.84, P = 0.40; Fig. 1C).

Intrahippocampal peptide infusion protects against memory deficit caused by SE

To verify the effect of the fMLP peptide on memory consolidation, the inhibitory avoidance test was performed (Fig. 1A). During the training period, all animals spent little time on the platform (Fig. 1D and E). After 24h, the animals in the VEH group consolidated their long-term memory, observed during the test period. On the other hand, the PILO + VEH group had impaired memory consolidation, as expected (Kruskal-Wallis test, P = 0.0004 vs VEH; Fig. 1E). However, the intrahippocampal infusion of the fMLP peptide was able to protect against memory deficit following pilocarpine-induced SE (Kruskal-Wallis test, P = 0.0004 vs VEH; Fig. 1E).

The severity of seizures during SE is reduced by the intrahippocampal infusion of peptide

To observe the role of the peptide in seizure severity, epileptic seizures were assessed during SE according to the Racine's scale (Fig. 2A). The number and total time of classes 2 and 3 remained unchanged after administration of the fMLP peptide followed by H-PILO ($P > 0.05$; Fig. 2B-E). However, fMLP peptide was able to decrease the number (*t-test*, $t_{16} = 3.13$, $P = 0.006$; Fig. 2F) and total time (*t-test*, $t_{16} = 2.48$, $P = 0.025$; Fig. 2G) of class 4, as well as the number of class 5 compared to control (*t-test*, $t_{15} = 2.19$, $P = 0.04$; Fig. 2H). The total time of class 5 seizures remained unchanged (P > 0.05, Fig. 2I).

The severity of seizures was assessed based on the average of the most severe seizures among the 18 windows of the SE. As the previous data showed, the severity of seizures was attenuated after the infusion of fMLP peptide followed by H-PILO compared to PILO+VEH group (*t-test*, $t_{17} = 2.21$, $P = 0.04$; Fig. 2J). In other words, peptide may trigger an anticonvulsive role on the SE.

Intrahippocampal peptide infusion decreases neuronal death in the hippocampus

During H-PILO-induced SE, hippocampal and extrahippocampal areas are affected by neuronal death. In order to analyze whether brain peptide administration is able to protect against neuronal damage caused by SE, the neurodegeneration process was evaluated by FJ histochemistry. The neuroprotective role of intrahippocampal infusion of fMLP is observed in DG hilus (Fig. 3A - A', B - B', G - G', H - H'), CA3 (Fig. 4A - A', B - B', G - G', H - H'), and CA1 (Fig. 5A - A', B 214 - B', G - G', H - H') areas of both hippocampi. Qualitatively, the level of hippocampi that showed a significant effect of fMLP was represented in the photomicrograph.

In the left hippocampus, intrahippocampal administration of the fMLP peptide decreased the total number of FJ + neurons in the DG hilus (*t-test*, *t8* = 2.76, $P = 0.02$;

Fig. 3F), CA3 (*t-test*, *t9* = 2.51, *P* = 0.03; Fig. 4F) and CA1 (*t-test*, *t9* = 2.35, *P* = 0.04; Fig. 5F) areas. On the left side of the DG hilus (*t-test*, *t9* = 3.41, *P* = 0.008; Fig. 3E) and the CA3 (*t-test*, *t7* = 3.80, *P* = 0.007; Fig. 4E) region, fMLP had a more significant effect on the ventral hippocampus. On the other hand, in the left CA1 subfield, the most significant role of fMLP was in the dorsal *t test*, *t7* = 2.59, *P* = 0.04; Fig. 5C) and medial (*t-test*, *t8* = 2.37, *P* = 0.04; Fig. 5D) hippocampus. In addition, the fMLP peptide did not change the number of FJ+ neurons in DG hilus (Fig. 3C and D) and CA3 (Fig. 4C and D) of the dorsal and medial left hippocampus, as well as in CA1 (Fig. 5E) of the ventral left hippocampus (P > 0.05).

Similarly, in the right hippocampus, fMLP intrahippocampal infusion followed by H-PILO was able to reduce the total number of FJ+ neurons in the DG hilus (*t-test*, *t9* = 4.48, *P* = 0.002; Fig. 3L), CA3 (*t-test*, *t10* = 6.15, *P* = 0.0001; Fig. 4L) and CA1 (*t-test*, *t11* = 5.92, *P* = 0.0001; Fig. 5L) subfields compared to the control. This pattern was observed in the dorsal (Fig. 3I, 4I and 5I), medial (Fig. 3J, 4J and 5J) and ventral (Fig. 3K, 4K and 5K) regions of the hippocampus (*P* < 0.05).

DISCUSSION

Clinical and experimental data support the idea that the inflammatory process is a determinant of epileptic seizures and neuronal loss in the hippocampus [7–10]. Upregulation of inflammatory markers, including cytokines, chemokines, oxidative stress, can be evidenced after SE [21–27]. Given this perspective, the use of antiinflammatory substances has been the subject of many studies to decrease the impacts of inflammation resulting from this epileptogenic insult. Some molecules, such as fMLP, can exert distinct pharmacological effects depending on the concentration levels and expression of their target receptors.

Typically, a latency time precedes SE in animal models of H-PILO and its alteration can lead to the onset of epileptic seizures [16, 40, 42]. However, the administration of fMLP did not interfere with the latency period. In addition, during the latency time, WDS is observed, a behavioral pattern that can modulate the evolution of epileptic seizures [11, 16, 40, 46–48]. We observed that the number of WDS remained unchanged after infusion of the fMLP peptide.

Our results showed that intrahippocampal infusion of fMLP significantly reduced the number of class 4 and 5 seizures during the 90 min of SE. No significant differences were recorded in any of the parameters between the fMLP and control groups in classes 2 and 3. These data seem to indicate that fMLP exerts a beneficial effect on the most severe seizures of Racine's scale. Therefore, fMLP promoted a significant anticonvulsant effect, reducing the severity of seizures in relation to control. The action of other formylated peptides, such as AC2-26, on the behavior of epileptic seizures has already been described in the literature. The anti-inflammatory action of AC2-26, a mimetic peptide of annexin-A1 (ANXA1), has been well described in previous studies [49–52], however it did not change the classes of PILO-induced epileptic seizures, despite having a neuroprotective effect [53]. Therefore, the fMLP peptide showed a higher performance in reducing the severity of seizures observed in our study compared to AC2-26.

Our data demonstrated that H-PILO-induced SE caused an impairment in memory consolidation, but the administration of fMLP prevented the impairment in long-term memory consolidation after SE induction. Brain damage induced by SE is already extensively explored in animal models of temporal lobe epilepsy [16, 54–56] and in humans [57–60], including memory impairment, neurodegeneration, behavioral changes and electroencephalographic recording. Due to the well-established association between hippocampal damage and memory impairment [61], we investigated the profile of hippocampal neurodegeneration in the DG hilus, CA1 and CA3 regions. FJ analysis was performed bilaterally to analyze the effect of the peptide on both hippocampi. As expected, SE induced neuronal death in different areas of the hippocampus after 24h of the epileptogenic insult caused by H-PILO, corroborating the other studies [16, 42, 62].

Interestingly, the intrahippocampal infusion of fMLP significantly reduced the total number of FJ+ neurons in DG hilus, CA1 and CA3, indicating an important neuroprotective role. The hipocampal neuroprotection caused by the peptide was region-dependent. In the right hippocampus, there was a reduction in FJ+ cells at all three levels – dorsal, medial, and ventral. On the other hand, in the left hippocampus, the neuroprotection pattern was different. In DG hilus and CA3 region on the left side, there was a reduction of FJ+ neurons only in the ventral region. While in the CA1 area of the left hippocampus, the decrease was observed in the dorsal and medial regions. Similarly, AC2-26 also showed a neuroprotective action on the hippocampus, specifically on dorsal and ventral CA1, as well as on ventral CA3 [53]. However, our findings demonstrated

broader fMLP-mediated neuroprotection in more hippocampal areas and levels, including the DG hilus.

The neuroprotective effect of fMLP appears to be mediated by an anti-inflammatory action. The induction of SE and the maintenance of the epileptic process in rodents have been associated with inflammatory events in the hippocampus, including increased levels of pro-inflammatory and neurotoxic cytokines (IL-1β, IL-6, TNF-α), chemokines, glial migration and oxidative stress [60–64]. In addition, temporal lobe epilepsy inflammatory processes can cause changes in FPR2 expression and activation of its molecular cascades, with alterations in albumin levels and in the integrity of the blood-brain barrier (BBB) during epileptogenesis [53]. From these inflammatory cascades associated with SE, we hypothesized the anti-inflammatory role of fMLP as a fundamental property for the control of hippocampal sclerosis, since the epileptogenesis process is also induced and maintained by inflammatory action.

However, several studies have shown that fMLP has a pro-inflammatory role [65], playing importante roles in the innate immune response, as a chemotactic molecule and activator of polymorphonuclear neutrophils and macrophage, which stimulate the production of free radicals [32, 37, 66-72]. This pro-inflammatory action is highlighted in several tissues, including the brain, where it has been associated with the development of encephalitis and relevant changes in BBB permeability [73]. In addition, fMLP can promote immune changes that are related to the use of antiepileptics, since the chemotaxis of fMLP with neutrophils can be impaired by the use of traditional anticonvulsants [74–76].

There is evidence that points to the existence of a more complex functioning on the part of the formylated peptides [77]. According to these authors, ANXA1, derived from peptides such as AC2-26, presents a pattern of FPR stimulation identical to that of fMLP, although it has a discrepant effect in relation to this one. Therefore, the effects of these peptides on the FPR can be better explained as concentration-dependent. ANXA1 may have a pronounced pro-inflammatory effect at high concentrations or anti-inflammatory effects at low concentrations.

Another factor that can be considered is the participation of glial cells. FPR is strongly expressed in glial cells such as astrocytes and microglia. Microglia may trigger a neuroprotective action due to the stimulation of FPR at low levels, which could explain the anticonvulsant effect and reduction of neuronal death described above [53, 78]. It is

known that microglia play a pivotal role in CNS damage situations. There are studies that show a neuroprotective role of microglia, characterized by the release of molecules (growth factors and anti-inflammatory interleukins), capable of reducing neuroinflammation and inhibiting neuronal death [79]. However, other authors have already described that microglia can intensify neuronal death, due to the release of cytotoxic molecules (NMDA, free radicals, nitric oxide, proteases, and cytokines) [80]. In other words, the neuroprotective or neurotoxic action of microglia depends on the type of molecule secreted, the properties and vulnerabilities of the surrounding neurons, as well as the ability of these glial cells to be activated and how this activation can occur [80].

CONCLUSION

In the present study, we described significant neuroprotective action of fMLP, suggesting a critical role of this peptide in neuroinflammatory events after SE. Further studies should be conducted to specifically understand the mechanisms of action that mediate the neuroprotective/anti-inflammatory response of fMLP. Together, our data can substantially contribute in the future to the development of novel therapies for the treatment and quality of life of patients with temporal lobe epilepsy.

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FIGURES LEGENDS

Fig. 1 The intrahippocampal infusion of fMLP in the brain does not change latency to the SE or the number of WDS, but prevents against memory damage. The experimental scheme (A). Rats received fMLP microinjections 5 min after H-PILO (A). intrahippocampal infusion of fMLP after H-PILO does not change the latency to SE (*t*593 *test, t14* = 0.20, *P* = 0.84; B), as well as the number of WDS (*t-test, t15* = 0.84, *P* = 0.40; C). After 24hrs of SE, the consolidation of long-term memory was analyzed by the inhibitory avoidance test (A). Initially, all animals were submitted to an aversive stimulus (training session) before induction of the SE (D). Memory consolidation was protected by the intrahippocampal infusion of fMLP after H-PILO (Kruskal-Wallis test, *P* = 0.0004 vs VEH; E). Latency and WDS data represented the mean ± SEM of 9-11 rats. *P > 0.05*, unpaired t-test. Memory consolidation data represent the median with interquartile range. ****P < 0.001* in comparison with the VEH; one-way ANOVA with Kruskal-Wallis test with Dunn's post-hoc test. VEH, vehicle; PILO + fMLP, pilocarpine followed by intrahippocampal infusion of N-formyl-methionyl-leucyl-phenylalanine; PILO + VEH, pilocarpine followed by vehicle infusion; DZP, Diazepam; SE, status epilepticus; SEM, standard error of the mean.

Fig. 2 The intrahippocampal infusion of fMLP reduces the severity of seizures after SE induced by pilocarpine. The experimental scheme (A). Rats received fMLP microinjections 5 min after H-PILO (A). According to the Racine's scale, epileptic seizures were evaluated over the 90 minutes of SE. During SE, the total number (*t-test, t16* = 3.13, *P* = 0.006; F) and time (*t-test, t16* = 2.48, *P* = 0.025; G) of class 4 seizures were decreased when FMLP was administered to the hippocampus. Likewise, the total number of class 5 was reduced after the intrahippocampal infusion of the FMLP (*t-test, t15* = 2.19, *P* = 0.04; H). The administration of FMLP was able to decrease the severity of seizures (*t-test, t17* = 2.21, *P* = 0.04; J). Error bars indicate SEM. Data represent the mean ± SEM of 9-11 rats. **P < 0.05* and ***P < 0.01*; unpaired t-test. PILO + FMLP, pilocarpine followed by intrahippocampal infusion of N-formyl-methionyl-leucyl-phenylalanine; PILO + VEH, pilocarpine followed by vehicle infusion; DZP, Diazepam; SE, Status epilepticus; SEM, standard error of the mean.

Fig. 3 The intrahippocampal infusion of fMLP reduces neuronal death in hilus of hippocampi after H-PILO induced SE. After 24h of SE, the neurodegeneration process was evaluated by Fluoro-Jade C (FJ-C) histochemistry. The neurons of the DG hilus were marked with FJ (FJ+, green). fMLP administration after H PILO attenuated the total number of FJ+ neurons in DG hilus of both hippocampi ($P < 0.05$; F and L). In the right hippocampus, the intrahippocampal infusion of fMLP reduced the neurodegenerative process in all its regions ($P< 0.05$; I, J and K), but on the left side, the peptide was significant only in the ventral region (*t-test*, $t9 = 3.41$, $P = 0.008$; E). Representative digital zoom was made on the photomicrographs of the groups (A', B', G' and H'; see squares). Arrows represent the DG hilus. Magnification, 100×; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5-9 rats. *$P < 0.05$, **$P < 0.01$, compared with PILO+VEH; unpaired t test. PILO+fMLP, pilocarpine followed by intrahippocampal infusion of N-formyl-methionyl-leucyl-phenylalanine; PILO+VEH, pilocarpine followed by vehicle infusion; DZP, Diazepam; SE, Status epilepticus; SEM standard error of the mean.

Fig. 4 The intrahippocampal infusion of fMLP reduces neuronal death in CA3 of hippocampi after H-PILOinduced SE. The neurons of the CA3 subarea were marked with FJ (FJ+, green). The total number of FJ+ neurons in CA3 subarea was decreased after intrahippocampal infusion of fMLP peptide in both hippocampi ($P < 0.05$; F and L). In the right hippocampus, all regions showed a reduction in the pattern of neuronal death ($P < 0.05$; I, J and K); however, in the left hippocampus, only the ventral region was impacted by the intrahippocampal infusion of fMLP (*t-test*, $t7 = 3.80$, $P = 0.007$; E). Representative digital zoom was made on the photomicrographs of the groups (A', B', G' and H'; see squares). Arrows represent the CA3 region. Magnification, 100×; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5–9 rats. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with PILO+VEH; unpaired t test. PILO+fMLP, pilocarpine followed by infusion of N-formyl-methionyl-leucyl-phenylalanine; PILO+VEH, pilocarpine followed by vehicle infusion; DZP, Diazepam; SE, Status epilepticus; SEM standard error of the mean.

Fig. 5 The intrahippocampal infusion of fMLP reduces neuronal death in CA1 of hippocampi after H-PILO induced SE. The neurons of the CA1 subarea were marked with FJ (FJ+, green). In CA1 subarea of both hippocampi, the administration of fMLP after H-

PILO reduced the total number of FJ+ neurons ($P < 0.05$; F and L). In the right hippocampus, all regions showed a decrease in the neurodegenerative pattern ($P < 0.05$; I, J and K); however, on the left side, a reduction was observed in the dorsal (*t-test*, $t7 = 2.59$, $P = 0.04$; C) and medial (*t test*, $t8 = 2.37$, $P = 0.04$; D) regions. Representative digital zoom was made on the photomicrographs of the groups (A', B', G' and H'; see squares). Arrows represent the CA3 region. Magnification, 100×; scale bar, 100 μm. Error bars indicate the SEM. Data represent the mean ± S.E.M. of 5–9 rats. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with PILO+VEH; unpaired t test. PILO+fMLP, pilocarpine followed by intrahippocampal infusion of N-formyl-methionyl-leucyl-phenylalanine; PILO+VEH, pilocarpine followed by 648 vehicle infusion; DZP, Diazepam; SE, Status epilepticus; SEM, standard error of the mean.

ANEXO

Figure 1

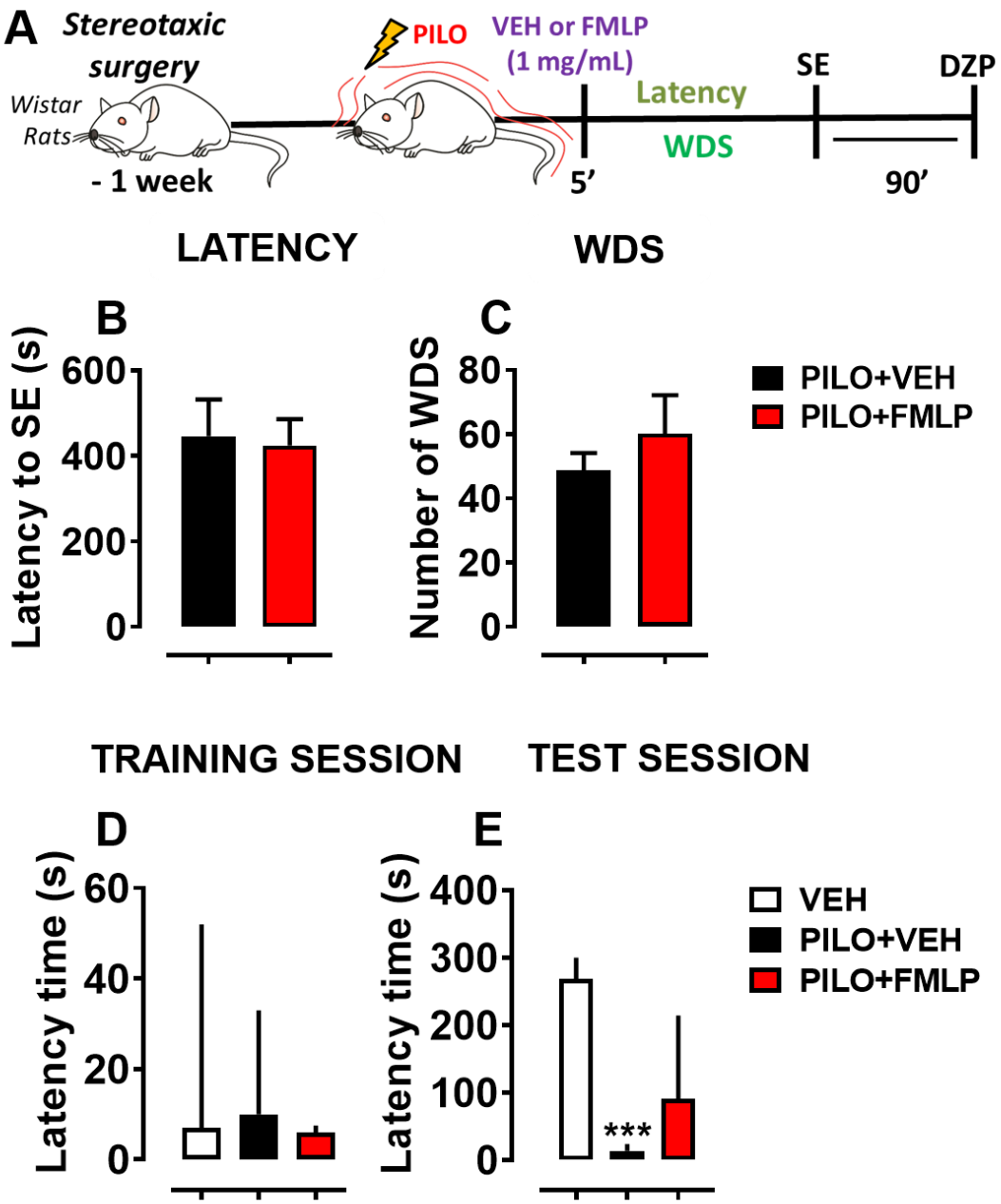


Figure 2


Stereotaxic surgery
Wistar Rats
- 1 week
PILO
VEH or FMLP (1 mg/mL)
SE
DZP
Behavioral analysis
5'
90'
CLASS 2
CLASS 3
CLASS 4
CLASS 5
B
C
D
E
F
G
H
I
Seizures
Seizures time (s)
SEVERITY OF SEIZURES
J
Seizures severity
PILO+VEH
PILO+FMLP

Fig.3

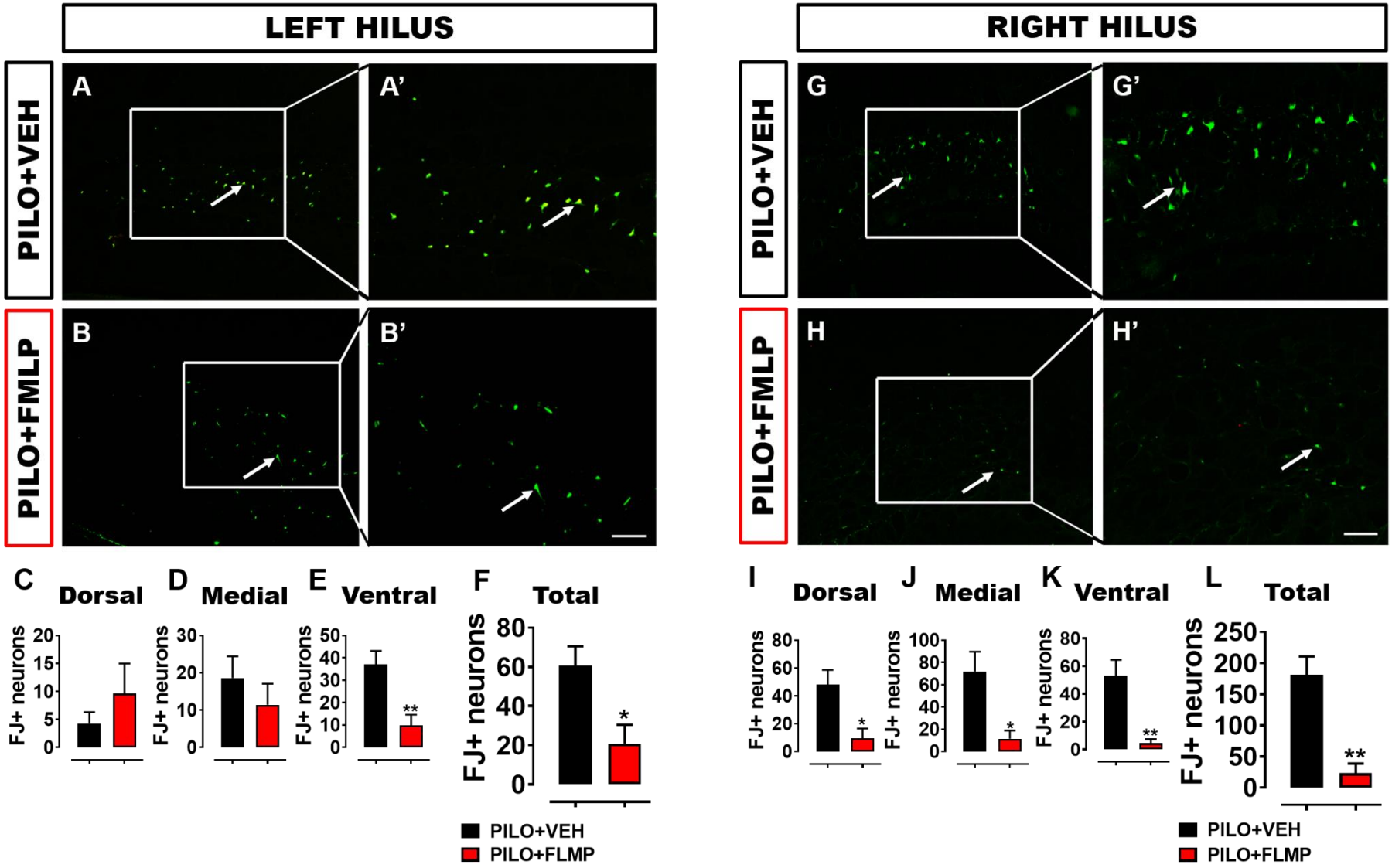

LEFT HILUS
RIGHT HILUS
PILO+VEH
PILO+FMLP
A
A'
B
B'
G
G'
H
H'
C Dorsal
D Medial
E Ventral
F Total
I Dorsal
J Medial
K Ventral
L Total
FJ+ neurons
PILO+VEH
PILO+FLMP

Fig. 4

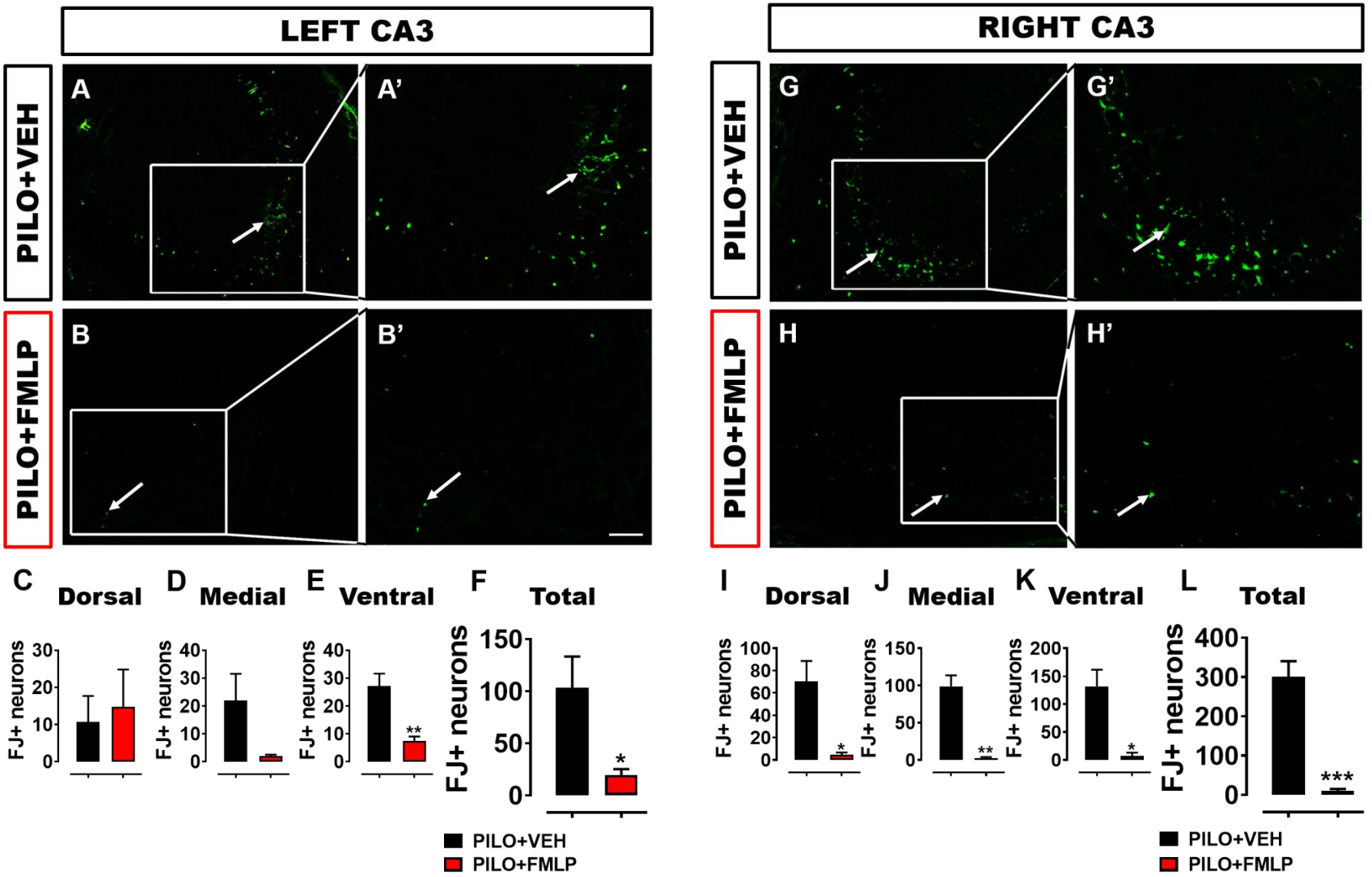
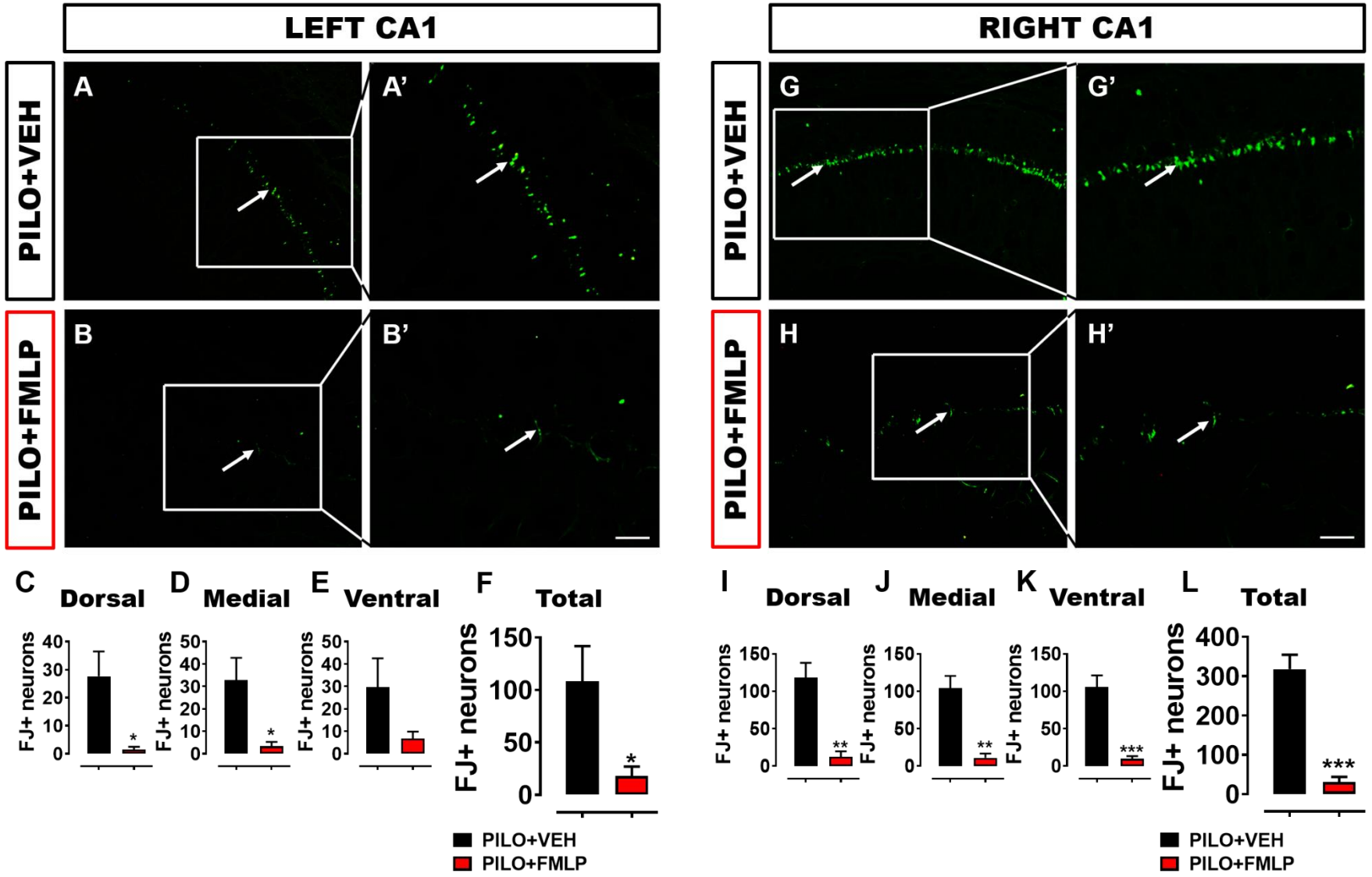

LEFT CA3
RIGHT CA3
PILO+VEH
PILO+FMLP
A
A'
B
B'
G
G'
H
H'
C Dorsal
D Medial
E Ventral
F Total
I Dorsal
J Medial
K Ventral
L Total
FJ+ neurons
PILO+VEH
PILO+FMLP

Fig. 5


LEFT CA1
RIGHT CA1
PILO+VEH
PILO+FMLP
A
A'
B
B'
G
G'
H
H'
C Dorsal
D Medial
E Ventral
F Total
I Dorsal
J Medial
K Ventral
L Total
FJ+ neurons
PILO+VEH
PILO+FMLP