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ORIGINAL ARTICLE

Silencing of P2X7R by RNA interference in the hippocampus can attenuate morphological and behavioral impact of pilocarpine-induced epilepsy

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Abstract Cell signaling mediated by P2X7 receptors (P2X7R) has been suggested to be involved in epileptogenesis, via modulation of intracellular calcium levels, excitotoxicity, activation of inflammatory cascades, and cell death, among other mechanisms. These processes have been described to be involved in pilocarpine-induced status epilepticus (SE) and contribute to hyperexcitability, resulting in spontaneous and recurrent seizures. Here, we aimed to investigate the role of P2X7R in epileptogenesis in vivo using RNA interference (RNAi) to inhibit the expression of this receptor. Small interfering RNA (siRNA) targeting P2X7R mRNA was injected into the lateral ventricles (icv) 6 h after SE. Four groups were studied: Saline-Vehicle, Saline-siRNA, Pilo-Vehicle, and Pilo-siRNA. P2X7R was quantified by western blotting and neuronal death assessed by Fluoro-Jade B histochemistry. The hippocampal volume (edema) was determined 48 h following RNAi. Behavioral parameters as latency to the appearance of spontaneous seizures and the number of seizures were determined until 60 days after the SE onset. The Saline-siRNA and Pilo-siRNA groups showed a 43 and 37% reduction, respectively, in P2X7R protein levels compared to respective vehicle groups. Neuroprotection was observed in CA1 and CA3 of the Pilo-siRNA group compared to Pilo-Vehicle. P2X7R silencing in pilocarpine group reversed the increase in the edema detected in the hilus, suprapyramidal dentate gyrus, CA1, and CA3; reduced mortality rate following SE; increased the time to onset of spontaneous seizure; and reduced the number of seizures, when compared to the Pilo-Vehicle group. Therefore, our data highlights the potential of P2X7R as a therapeutic target for the adjunct treatment of epilepsy.

Keywords Temporal lobe epilepsy · P2X7 purinergic receptors · RNA interference · Pilocarpine · Hippocampus

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Introduction

Epilepsy is a group of neurological diseases with a common phenotypic manifestation, epileptic seizures. Temporal lobe epilepsy (TLE) is the most common form of epileptic conditions in adult humans [1]. Hippocampal sclerosis, gliosis, synaptic reorganization, and granular cell dispersion are the main pathophysiological hallmarks in mesial temporal lobe epilepsy (MTLE) [2, 3]. The epilepsy model induced by intraperitoneal injection with pilocarpine reproduces the main pathophysiological findings related to human MTLE, including the appearance of drug-resistant seizures [4, 5]. The pilocarpine model of epilepsy has been widely used in research and is of major value to identify the molecular and cellular basis and players involved in epileptogenesis [4, 5].

The neuroinflammatory condition triggered by seizures has been considered an important player in hyperexcitability, a modulator of seizure threshold, and has been involved in key processes leading to cell death cascades [6]. New therapy approaches of TLE have been focused on brain inflammation, highlighting the interest of purinergic P2X7 receptors (P2X7R) as potent mediator of neuroinflammation in the epileptic brain [7–18]. P2X7R are trimeric non-selective ligand-gated ion channels activated by ATP, permeable to mono- and divalent cations (permeability: $Ca^{2+} > Na^{+} > K^{+}$), resulting in the rapid depolarization of the membrane [19, 20]. P2X7R activation under pathological condition, i.e., under high level of ATP, occurring during seizures, can induce high level of intracellular calcium concentration, intensifying glutamate and GABA release, promoting pro-inflammatory cytokines release and cell death by apoptosis or necrosis [7, 9, 18, 21–23].

P2X7R have been considered an important therapeutic target in many injuries and neurological disorders, including neuropathic pain [24, 25], spinal cord injury [26], ischemia [27, 28], intracerebral hemorrhage, traumatic brain injury [29], and neurodegenerative diseases such as Alzheimer's disease [30], Huntington's disease [31], Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and depression [18], and also in epilepsy [19, 32, 33].

P2X7R levels are increased in the hippocampus of animal models of experimental model of epilepsy and in human patients with TLE [12, 34–37]. There are many studies aimed at elucidating the role of P2X7R in epilepsy using agonists and antagonists. The activation of P2X7R with Bz-ATP (P2X7R agonist) causes microglial activation, enhances TNF-α immunoreactivity, reduces astrocytes, and intensifies seizures expression [19, 38–40]. Conversely, P2X7R blockage promotes anticonvulsant effects and reduces electroencephalographic and behavioral seizures, IL-1β production, microglial activation, recruitment and infiltration of neutrophils into the frontoparietal cortex, and damage resulting from seizure [14, 15, 33, 38, 41–44]. Surprisingly, P2X7R antagonists have been described to exacerbate seizures and enhance cell death in the hippocampal CA3 subfield in the pilocarpine and intraamygdala kainic acid models, but do not change behavioral pattern in the intraperitoneal kainic acid and picrotoxin models of epilepsy [39, 41, 45]. Data obtained from P2X7R knockout mice (Pfizer) [46] are controversial as the severity of their seizures is reduced when compared to wild-type mice [33], but they show increased susceptibility to seizures induced by pilocarpine [45].

The disparities observed in epilepsy studies targeting the pharmacology of P2X7R can be attributed to several factors, including pharmacokinetics and pharmacodynamics of drugs, and the different animals models and experimental conditions used. RNA interference (RNAi) is a method that reduces the expression of the receptor of interest, allowing studying the direct impact in behavioral or morphological parameters besides excluding pharmacological effects [47].

Despite the evidence of a positive association between P2X7R activation, excitability, and excitotoxicity related to epilepsy, the role of purinergic signaling needs to be further clarified. In this study, we used in vivo RNAi intracerebral infusion to reduce the expression of P2X7R in pilocarpine-induced epileptic rat brain, in order to investigate the involvement of this receptor in brain alterations resulting from seizures, i.e., hippocampal damage, edema, and spontaneous and recurrent seizure expression.

Methods

Animals

Adult 2-month-old male Wistar rats weighting 200–250 g were used in this study. The animals were maintained under standard housing conditions, with free access to water and food, with light/dark cycle of 12 h (light from 7 a.m. to 19 p.m.), and with environment temperature kept constant between 21 ± 1 °C.

All experimental procedures were performed under the supervision and with the approval of our internal Ethics Committee (Federal University of São Paulo, CEP N. 0961/10). Animal protocols were conducted in accordance with national and international legislation (Guidelines of the Brazilian College of Animal Experimentation, COBEA; NIH Guide for Care and Use of Laboratory Animals), and the experiments followed the principles outlined in the Basel Declaration [48].

Bilateral cannulas implantation

For intracerebroventricular (icv) administration of small interfering RNA (siRNA) or vehicle, rats were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and fixed to a stereotaxic apparatus. Cannulas (12 mm in length and 0.55 mm in diameter) were stereotaxically implanted into the lateral ventricles (from bregma: anteroposterior, −0.08 mm; mediolateral, ±0.14 mm; dorsoventral, −0.3 mm) and fixed to the skull with dental cement [49]. After 15 days, the rats were injected i.p. with pilocarpine or with saline.

Pilocarpine model protocol

Animals were pretreated with methylscopolamine nitrate (1 mg/kg, subcutaneous (sc), Sigma) to minimize peripheral cholinergic effects of pilocarpine (diarrhea, piloerection, orofacial automatisms associated with salivation, wink, yawning, and vibrissae contractions) [50]. Thirty minutes after pretreatment, they received a systemic injection of pilocarpine hydrochloride (370 mg/kg, i.p., Merck, USA) for SE

induction. Five hours after the SE onset, rats were treated with diazepam (1 mg/kg, sc, Santisa) and sodium pentobarbital (30 mg/kg, i.p., Cristália) to minimize behavioral seizures and reduce mortality rate. Six hours after the SE onset, rats received siRNA.

P2X7R siRNA:RVG-9DR preparation and administration in vivo

The siRNA targeting P2X7R (antisense, 5′ [Phos] CUUUAACGUCGGCUUGGGCUC [dT] [dT]-3′, and sense, 5′ [Phos] GCCCAAGCCGACGUUAAAGUA [dT] [dT]-3′) was planned on the basis of Thomas Tuschl protocol [51], and it was synthesized by Sigma Company.

Lyophilized single-stranded RNA oligonucleotides were re-suspended at 100 μM in sterile RNase free water (0.1%, *v*/*v*, DEPC in pure water), denatured, aligned (heated at 95 °C for 5 min), and annealed through slow decrease of *T* °C, obtaining double-stranded siRNA at 50 μM.

Before use, P2X7R siRNA was complexed with RVG-9DR (a peptide sequence derived from rabies virus glycoprotein with nine arginine residues in the carboxy terminal) in a 1:10 M ratio (siRNA:RVG-9DR) to transfect the siRNA, protocol developed by Kumar and colleagues in 2007 [52].

Six hours after the onset of SE, 2 μl containing 0.5 μg of siRNA:RVG-9DR were administered bilaterally icv at a flow rate of 1 μl/min, totaling 1 μg of siRNA per animal. Control rats received the same volume of vehicle instead of siRNA. The dose of siRNA was chosen based on a previous study in rats showing maximal effect in reducing the expression of P2X7R (60%) in the hippocampus, without any sign of neurotoxicity (hind-limb paralysis, vocalization, food intake, or neuroanatomical damage).

Western blotting

Western blot analysis was used for quantifying P2X7R in rat hippocampi. Following the 48-h period of siRNA or vehicle delivery, rats were decapitated and their hippocampi quickly dissected on an iced plate, washed with cold saline to remove blood, weighted, and stored at −80 °C.

Tissues were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.02% sodium azide, and 1% protease inhibitor cocktail (Sigma-Aldrich). Protein content was determined by the Bradford method [53].

Samples (40 μg) were mixed with Laemmli buffer containing 0.125 M Tris (pH 6.8), 20% glycerol, 10% beta-mercaptoethanol, 4% SDS, and 0.002% bromophenol blue, and heated at 95 °C for 5 min. Protein was loaded on a 10% SDS-PAGE gel and separated by electrophoresis using a Bio-Rad system with molecular weight standards (Rainbow-GE) at 50 V for 20 min and 90 V for 1 h. Proteins were transferred to a polyvinylidene fluoride membrane (PVDF, Amersham Pharmacia Biotech—Hybond P) at 110 V for 90 min (Mini-Protean, Bio-Rad). Membranes were washed with 0.1 M Tris–Tween 20, blocked with 0.1 M Tris containing 5% skimmed milk, and then incubated with the primary antibody rabbit anti-P2X7 receptor (1:1000, RPA-004—Alomone Labs) at 4 °C overnight. After rinsing, the membranes were incubated with the corresponding secondary antibody (goat anti-rabbit IgG, Calbiochem) at a dilution of 1:2000 in 0.1 M Tris containing 2% fetal bovine serum for 2 h at room temperature. After washing them twice with 0.1 M Tris, membranes were ready for the blocking stage to re-probing with the monoclonal anti-β-actin immunoglobulin (1:2000, A3854—Sigma-Aldrich) used as internal control of the reaction. After rinsing, bands were detected by chemiluminescence using West Pico Super Signal® kit (Thermo Scientific), revealed in photo documentation system (Uvitec, Cambridge) and band intensity was quantified using the UvitecBand software analysis. The P2X7R protein level was reported as normalized β-actin loading control.

Perfusion

Following the 48-h period after the siRNA or vehicle infusion, rats were anesthetized with 90 mg/kg of ketamine and 10 mg/kg of xylazine (i.p.), and subjected to transcardiac perfusion with buffered paraformaldehyde (PFA) to fix the brain. Using a peristaltic pump adjusted to a flow rate of 10 mL/min, saline was perfused during 1 min followed by 250 ml of 4% PFA. The brain tissue was post-fixed in the same solution overnight at 4 °C, and then cryoprotected in 30% sucrose in phosphate buffer during 3 days at 4 °C. The brains were frozen quickly in dry ice and cut into coronal slices with a cryostat (Leica). Slices were used for Fluoro-Jade B staining (40 μm) and for hippocampal volume analysis (50 μm).

Fluoro-Jade B protocol

To study neuronal degeneration, we used the anionic dye Fluoro-Jade B (FJ-B). Brain sections containing areas of interest were fixed on gelatin-coated slides and dried at room temperature. Then, the slides were immersed in absolute ethanol (5 min), 70% ethanol (2 min), and distilled water (2 min) and protected from light, in 0.06% potassium permanganate (15 min), under gentle shaking: distilled water (2 min), 0.01% FJ-B solution plus 0.1% acetic acid (30 min), and distilled water (three times for 2 min). Slides were dried at 50 °C, during 10 min, in a hot plate, dehydrated in absolute ethanol (2 min), cleared in xylene (2 min) and mounted with “Vecta Mount” (Vector), and coverslipped, based on Schmued and Hopkins, 2000 protocol [54].

Volumetric study of hippocampal formation

Volumetric study was performed in slices obtained from a segment of hippocampal formation (−1.72 to −3.84, from bregma) from rats of both groups, 48 h after infusion of siRNA or vehicle [55]. The studied subregions were: hilus, suprapyramidal dentate gyrus, infrapyramidal dentate gyrus, CA1, and CA3.

Brain slices (50 μm) obtained with 300-μm interval were selected and incubated free floating with Hoechst 33,342 (1:10,000, Life Technologies) in PBS containing 0.2% Triton for 3 h under shaking and protected from light. The sections were washed, fixed on gelatin-coated slides, mounted with Fluormont (Abcam), and coverslipped. Images were captured in an epifluorescence microscope (Axioskop 2 plus, Zeiss), with 5× lens using Axiovision software (Zeiss). The image processing and measurements of the regions of interest were made in ImageJ and Fiji-ImageJ (NIH), respectively. The volume of selected brain regions was estimated using Table Curve 2D v5.01 software.

Behavioral analysis

Two days after SE induction, rats were kept in individual acrylic boxes, and video monitored 24 h, for 60 days. The behavioral parameters analyzed were latency to the appearance of the first spontaneous seizure, the number of seizures, and the severity of seizures. The severity of seizures was determined based on behavioral changes according to the scale of Racine: (1) mouth and facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing, and (5) rearing and falling [56].

Statistical analysis

Statistical analysis of the first spontaneous seizure latency and the number of seizures were performed by "*t*" test (unpaired). In the absence of normality, data were standardized by *Z* score and in the absence of homogeneity (Levene's test) were corrected by the Welch test. The protein quantification, hippocampal volume, and severity of seizures were analyzed by two-way ANOVA, followed by Bonferroni post-test. The results with values $p < 0.05$ were considered significant.

Results

P2X7 protein level

According to our western blotting analysis, the Saline-siRNA and Pilo-siRNA experimental groups showed a 43 and 37% reduction, respectively, in P2X7R protein levels in the hippocampus compared to their respective control vehicle groups (siRNA versus Vehicle, F (1, 16) = 57.71, $p < 0.0001$; Bonferroni: Saline-Vehicle, $p = 0.0002$; Pilo-Vehicle, $p = 0.0008$). No significant differences were observed in the levels of P2X7R when comparing the Pilo and Saline groups (F (1, 16) = 0.03151, $p = 0.8613$) (Fig. 1).

Neurodegeneration

Pilo-siRNA animals showed fewer FJ-B positive cells in CA1 and CA3 pyramidal cell layers as compared with the same regions in Pilo-Vehicle treated rats, suggesting that P2X7R knockdown caused neuroprotection in these areas, especially in CA3 (Fig. 2). Interestingly, no significant differences were observed in the number of FJ-B stained cells in the amygdala, entorhinal cortex, and piriform cortex of the Pilo-siRNA group compared to Pilo-Vehicle treated rats, indicating no neuroprotection by siRNA in these regions (data not shown).

Hippocampal volumetry

Hippocampal formation of the Pilo-Vehicle group showed an increased volume compared to the Saline-Vehicle group (Bonferroni: $p = 0.0249$, 32%). The P2X7R siRNA did not change the total hippocampal formation volume (F (1, 8) = 0.9792, $p = 0.3514$) (Fig. 3a). However, when the volume of each region was analyzed, a significant difference was observed.

The volume of CA3 and CA1 regions was higher in the Pilo-Vehicle and Pilo-siRNA groups compared to their Saline groups (CA3 Bonferroni: Saline-Vehicle, $p < 0.0001$, 91%; Saline-siRNA, $p = 0.0040$, 19%; CA1 Bonferroni: Saline-Vehicle, $p < 0.0001$, 110%; Saline-siRNA, $p = 0.0035$, 26%). A lower volume in these regions was observed in the

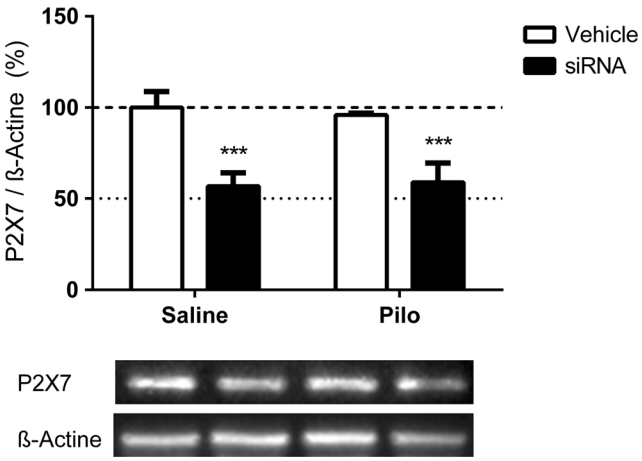

Fig. 1 Effect of siRNA in decreasing P2X7R levels, as measured by western blotting. Percentages of P2X7R protein level of Saline-Vehicle, Saline-siRNA, Pilo-Vehicle, and Pilo-siRNA groups. *Bars* represent the mean ± standard deviation for each group ($N = 5$/group). Data normalized to β-actin. *** $p < 0.001$

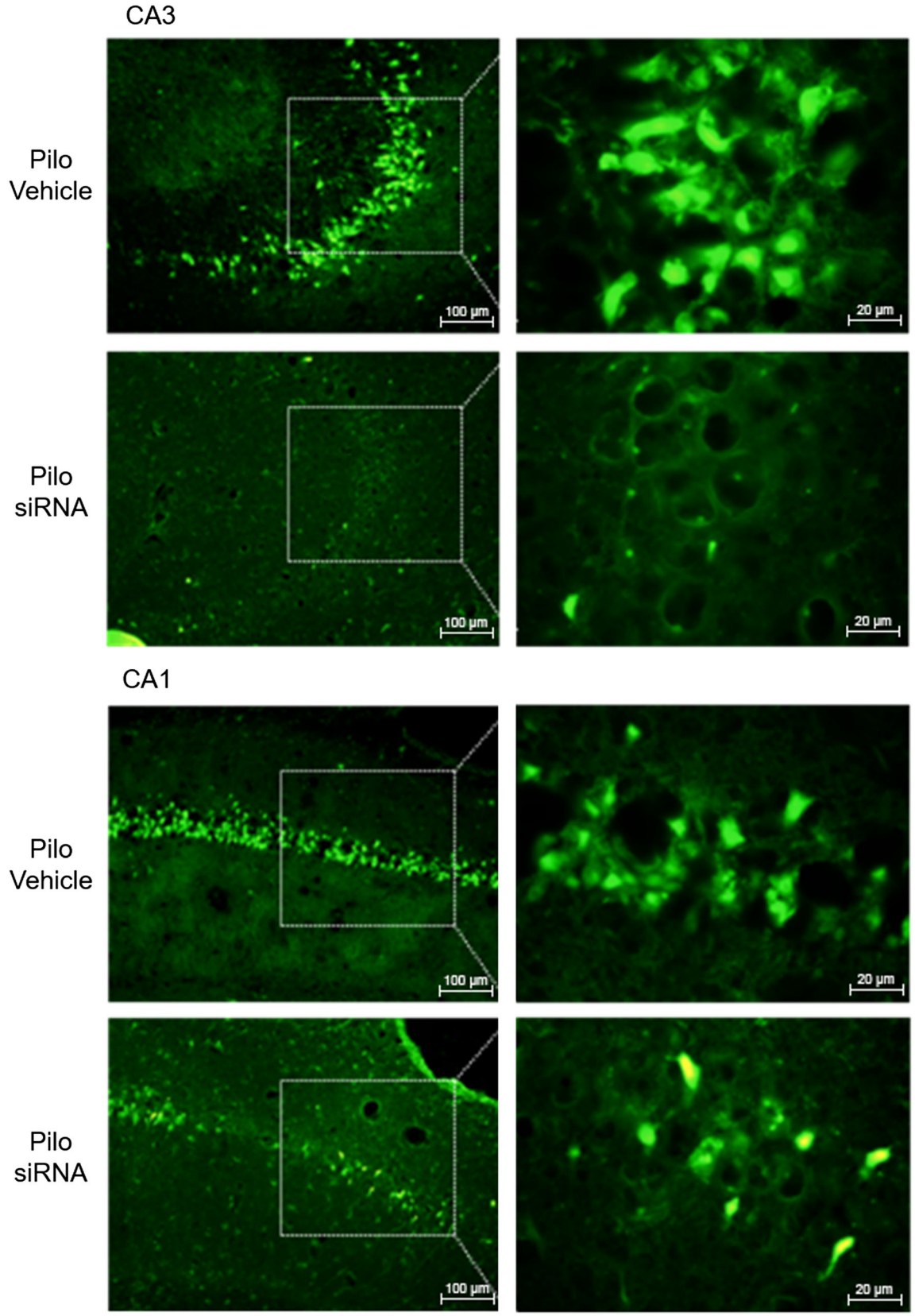

Fig. 2 Pilocarpine-induced neuronal death in the rat hippocampus, an effect partially protected by P2X7R knockdown. FJ-B staining in CA1 and CA3 of rats injected with pilocarpine after intracerebroventricular P2X7R siRNA (Pilo-siRNA) or vehicle (Pilo-Vehicle) administration. *Scale bars* 100 and 20 μm

Pilo-siRNA group compared to the Pilo-Vehicle group (Bonferroni: $p < 0.0001$, CA3 −26%, CA1 −34%). The volume of CA3 was higher in the Saline-siRNA group compared to the Saline-Vehicle group (Bonferroni: $p = 0.0331$, 19%) (Fig. 3b, c).

The volume of suprapyramidal region of dentate gyrus of the Pilo-Vehicle group was higher when compared to the Saline-Vehicle group (Bonferroni: $p = 0.0009$, 62%) and was lower in the Pilo-siRNA group compared to the Pilo-Vehicle group (Bonferroni: $p = 0.0308$, −22%) (Fig. 3d). Nevertheless, no change was observed in infrapyramidal dentate gyrus comparing to all groups (F (1, 8) = 0.1792, $p = 0.6832$) (Fig. 3e). The volume of the hilus was lower in the Pilo-siRNA group compared to the Saline-siRNA group (Bonferroni: $p = 0.0306$, −24%) and to the Pilo-Vehicle group (Bonferroni: $p = 0.0225$, −26%) (Fig. 3f).

Behavioral data

Mortality rate in the Pilo-siRNA group following SE was 63% lower than that in the Pilo-Vehicle group (38%). The latency period to the appearance of the first spontaneous seizure following pilocarpine administration was significantly increased in the Pilo-siRNA group when compared to the Pilo-Vehicle ($t_{5,238} = 4.027$, $p = 0.0009$) (Fig. 4a). The number of spontaneous seizures (Racine's scale 3–5) was significantly lower in Pilo-siRNA group than that in the Pilo-Vehicle ($t_{7,884} = 6.233$, $p = 0.0002$) (Fig. 4b). Seizure severity was classified according to Racine's scale 3–5. Seizure severity was decreased in the Pilo-siRNA group when compared to the Pilo-Vehicle (Pilo-siRNA versus Pilo-Vehicle, F (1, 36) = 59.3322, $p < 0.0001$; Bonferroni: stage 3, $p < 0.0001$; stage 4, $p < 0.0001$; stage 5, $p = 0.0089$), but no difference was

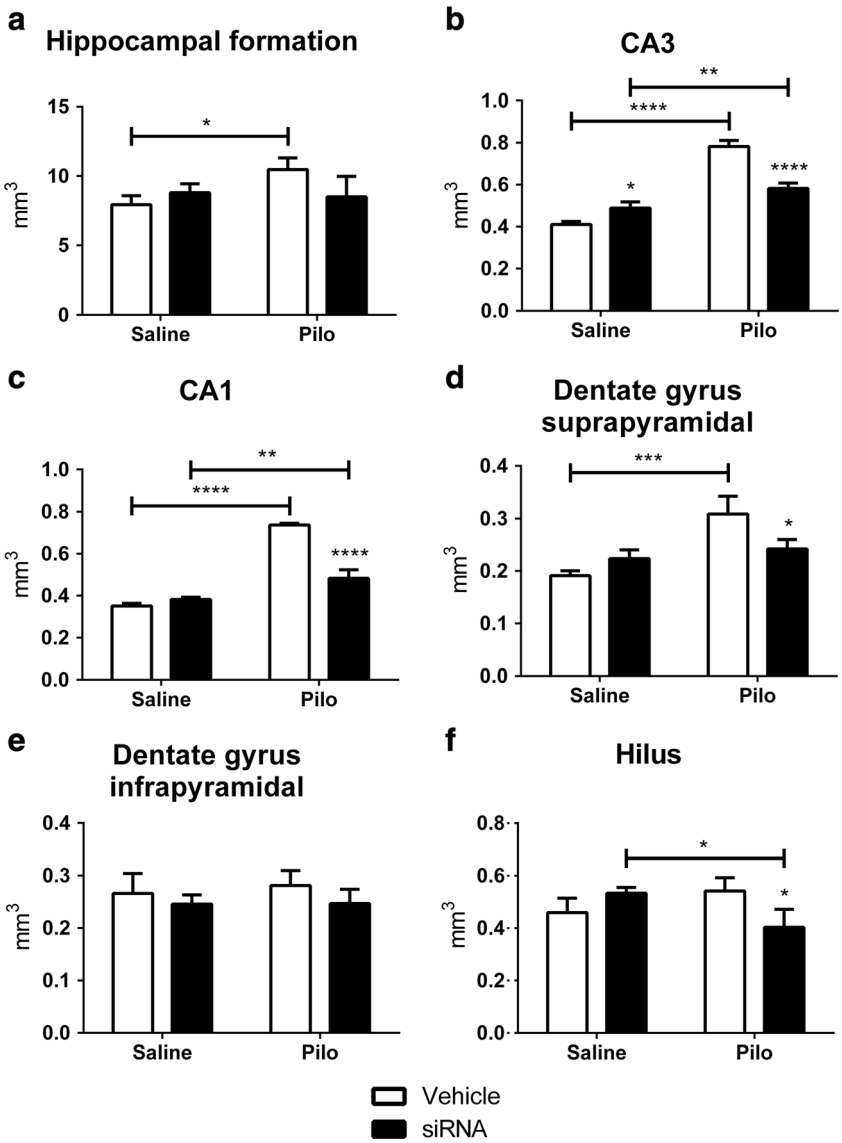

Fig. 3 Changes in hippocampal volume induced by pilocarpine and P2X7R siRNA. **a** Hippocampal formation, **b** CA3, **c** CA1, **d** dentate gyrus suprapyramidal, **e** dentate gyrus infrapyramidal, and **f** hilus of Saline-Vehicle, Saline-siRNA, Pilo-Vehicle, and Pilo-siRNA groups. *Bars* represent the mean ± standard deviation for each group ($N = 3$/group). Interactions: **a*** $p < 0.05$; **b**, **c****** $p < 0.0001$; **d**, **f**** $p < 0.01$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

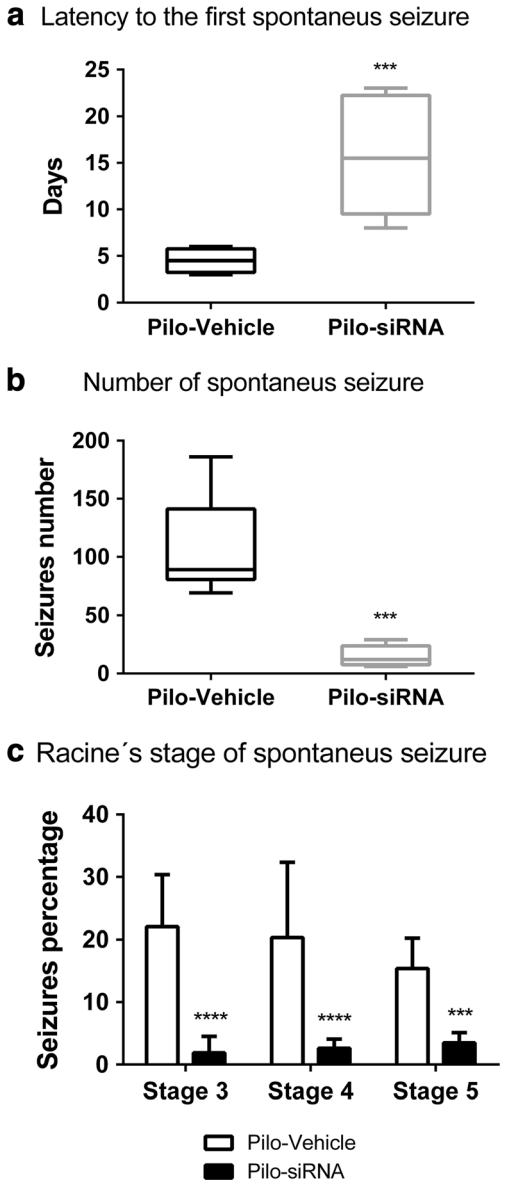

Fig. 4 **a** Latency period to the appearance of the first spontaneous seizure of Pilo-Vehicle and Pilo-siRNA groups. **b** Number of spontaneous seizures during 60 days following pilocarpine administration in Pilo-Vehicle and Pilo-siRNA groups. *Box plot* represents the median, first and third quartiles, and maximum and minimum values of each group. **c** Percentage of spontaneous seizure stages 3–5 during 60 days in Pilo-Vehicle and Pilo-siRNA groups. *Bars* represent the mean ± standard deviation for each group (Pilo-Vehicle—$N = 8$ and Pilo-siRNA—$N = 6$). *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$

observed between the stages (stages 3 versus 4 versus 5, $F(2, 36) = 0.5134$, $p = 0.6028$) (Fig. 4c).

Discussion

This study shows that the application of siRNA against P2X7R, 6 h after the onset of status epilepticus (SE), is able to reduce by 40% the expression of P2X7R in the rat hippocampus at 48 h later. This effect resulted in hippocampal neuroprotection, increase in the latency for the appearance of the first spontaneous seizures, and decrease in the mortality rate post SE, frequency and severity of the seizures, and protection from hippocampal volume changes, due to SE-induced edema. These data reinforce the participation of P2X7R in mechanisms underlying the increase in hyperexcitability, edema, and cell death triggered by pilocarpine-induced SE.

Previous studies performed in our group showed an increase in P2X7R expression in the hippocampus of rats submitted to pilocarpine model of SE, during acute and chronic phases of the model [12, 37]. The differential expression of P2X7R during acute (12 h) or chronic phases (90 days), indicate different roles in the progression of the epileptogenic process. During the acute phase, P2X7R were mainly located in glial cells, modulating the inflammatory process and hyperexcitability, while in chronic phase, they were mainly located in synaptic terminals modulating neurotransmitter release as glutamate and GABA [12]. Besides, a decreased level of P2X4R expression in CA1, CA2, CA3, hilus, and dentate gyrus during chronic phase of Pilo model was also observed, reflecting neuronal loss and functional alteration of reminiscent neurons following brain insult (SE) as a compensatory response to ineffective GABAergic neurotransmission [12]. Normal level of P2X7R was detected during the latent period (7 days following SE) located mainly in nerve terminals in CA3 and the dentate gyrus [12].

The pilot study performed to determine a time curve of P2X7R knockdown showed reduced expression of P2X7R 96 h following siRNA application, but the peak of the blockade was 48 h. Based on this study, the time of 48 h was chosen for the analysis following siRNA application. Considering that P2X7R increases significantly in glial cells at 12 and 24 h after SE [12], we can suppose that the knockdown induced by siRNA was sufficient to block microglial and astrocytic activation resulting in less release of cytokines in the hippocampus which consequently contributed to the neuroprotection as well as the late benefits observed in the Pilo-siRNA group.

Several studies have shown that P2X7R activation in neurons is associated with increased intracellular calcium (Ca^{2+}) and with the facilitation of glutamate release, promoting excitotoxicity and cell death [7, 12–14, 19, 23]. In microglia, P2X7R activation is associated with the inflammatory response in the central nervous system through cytokines production and release, especially interleukin-1β (IL-1β), interleukin-18 (IL-18), tumor necrosis factor (TNF-α), signaling through nuclear factor kappa B (NF-kB), nitric oxide synthase (NOS) activation, free radical production, and proapoptotic transcription factor formation [6, 7, 9, 10, 14, 15, 17–19, 21, 57]. The P2X7R activation in astrocytes has been related to inflammatory response and facilitation of glutamate release [7, 9, 12, 13, 15, 18, 19, 21, 23, 57, 58].

Neuronal death in pilocarpine model occurs by different mechanisms [59]. The increase in intracellular Ca^{2+} resulting from cholinergic activation triggers a cascade of reactions involving proteases, lipases, and nucleases activations, and generation of free radicals as intermediate products, which in turn can potentiate the release of glutamate and inflammatory mediators [50, 59, 60].

Studies have shown that the P2X7R antagonists Brilliant Blue G (BBG) and A-438079 are neuroprotective against damage caused by kainic acid, by reducing neuronal death and microgliosis in the hippocampus and neocortex [41, 42]. These data corroborate other studies that also showed reduction in astrocytes loss in the molecular layer of the dentate gyrus and frontoparietal cortex following P2X7R antagonists oxidized ATP (OxATP) and BBG in pilocarpine model [40]. In contrast, some authors showed increased cell death in CA3 layer by using P2X7R antagonists OxATP, A-438079, and A-740003 following pilocarpine [39], and that inhibition of microglial activation by the P2X7R antagonists may not be sufficient to protect neurons of the excitotoxicity caused by SE [43]. We have shown that pilocarpine-induced SE caused an increase in FJ-B positive cells in accordance with previous studies [61, 62] and that the reduction in the expression of P2X7R by siRNA was neuroprotective, mainly in CA1 and CA3 hippocampal layers.

There are many reports in the literature showing that SE can cause brain edema that can by itself, contribute to the epileptogenic process [63–65], although the mechanisms underlying this process are unknown. Edema resulting from seizures in human or experimental model can be of two types, cytotoxic edema and vasogenic edema [63–69]. In the cytotoxic edema, the glutamate hyperstimulation causes intracellular Ca^{2+} increase and promotes cytotoxicity in neurons and glial cells [64, 70]. Conversely, in the vasogenic edema, the cellular signaling triggered by SE can induce pro-inflammatory cytokines release and increases the production of kinins [71–78]. The kinins along with the cytokines may affect the junction of blood vessel epithelial cells and reduce the integrity of the tight junctions in endothelial cells walls, leading to a dysfunction of the blood brain barrier and consequently increasing the vascular permeability and accumulation of extracellular fluid [63, 70, 79, 80]. There are a number of studies showing robust evidence that IL-1β released following seizures may be pro-convulsant in experimental models of epilepsy [6, 14, 15, 76, 81]. According to our results, P2X7R can modulate the mechanisms involved in edema since the reduction in the expression of these receptors by siRNA prevented the edema in hippocampal subareas as hilus, dentate gyrus suprapyramidal, CA1, and CA3 of rats underwent to pilocarpine-induced SE. Although the mechanisms are not elucidated, the edema processes have been associated with cell death, particularly neuronal death, and hippocampal atrophy [82, 83]. A positive relationship between cerebral edema and the occurrence of spontaneous recurrent seizures has been shown in kainic acid model, strengthening the association of edema to epileptogenesis [65]. Knowing that the P2X7R are involved in the inflammatory activation following seizures, reduction in their expression by siRNA may have caused decrease in the release of inflammatory mediators preventing edema and cell death, and decreasing seizure expression.

Previous studies have shown that P2X7R antagonists trigger anticonvulsant effect, reducing electrographic and behavioral seizures, microglia activation, decrease IL-1β production and prevent cell damage resulting from seizures [14, 15, 33, 38, 41, 42]. Studies using P2X7R (Pfizer) [46] knockout mice also show reduction in the severity of seizures compared to wild-type mice [33].

Despite the evidence that P2X7R blockade by antagonists triggers protective mechanisms following seizures, in apparent contradiction, recent studies by us in collaboration with other group demonstrate a neuroprotective effect of P2X7R antagonists BBG and AZ10606120 resulting in increased expression of spontaneous seizures in rat underwent to pilocarpine-induced SE [84, 85]. According to the authors, the blockade of P2X7R by the negative allosteric modulator AZ10606120 may have caused hilar neuroprotection and favored the survival, ectopic migration, and differentiation of neuronal progenitor cells (NPCs) expressing P2Y1 and P2X7 receptors, which in turn integrate abnormal circuits in the hippocampus, contributing with excitability [85]. Similar hypothesis that neurogenesis contributes to worsen seizures was presented previously [86]. P2X7R have been shown to modulate mechanisms involved with apoptosis/necrosis and neuronal differentiation of NPCs [87–90], while P2Y1 receptors modulate proliferation and migration of NPCs [91–93]. Klaft et al. [94] also found that P2X7R antagonists had a minor antiepileptic effect in medial entorhinal cortex on rats subjected to pilocarpine-induced epilepsy.

The heterogeneity between pharmacological studies using antagonists may occur due to a number of factors including specificity, dose, and variability of the pharmacological agents tested; the use of different experimental models of epilepsy; route of administration; and temporal window in which studies are made. However, our pharmacological data using the P2X7 antagonist AZ10606120 are in opposite with the present data using siRNA to knockdown P2X7R in pilocarpine model. The results are intriguing and require further studies to elucidate molecular mechanisms involved with P2X7R blockade in epileptic process. We also do not have data about the participation of other P2 receptors in the changes obtained with the P2X7R knockdown, being a subject to be studied in the future.

As stated in this work, there are many studies showing beneficial effect of P2X7R blockade on epilepsy, either by pharmacological antagonists or by genetic manipulation

(transgenic, knockout, or RNAi) and attenuation of inflammatory cascades are among the mechanisms involved. However, there are few studies elucidating the differences between mechanisms triggered by one or other methodological condition and it is very important to increase the knowledge of the role of this receptor in epilepsy.

The main finding of this study was that knockdown of P2X7R by the administration of siRNA 6 h after SE onset reduced the mortality rate, resulted in little or no hippocampal edema, increased the latency to the onset of the first spontaneous seizure, and reduced the number and severity of spontaneous seizures in a later period. In addition, knockdown of P2X7R by siRNA induced important neuroprotection in the hippocampus of rats underwent to the pilocarpine model of SE. Further studies are required to elucidate the mechanisms associated with the knockdown of P2X7R during SE induced by pilocarpine resulting in attenuation of changes triggered by seizures.

Conclusion

Collectively, our data shows that the P2X7R may have an important role in the pathophysiology of pilocarpine-induced epilepsy, since the inhibition of the expression of these receptors in vivo, improved edema, and had neuroprotective. These data may have clinical relevance and highlight the therapeutic potential of adjunct treatment of epilepsy with P2X7R expression modulators.

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Compliance with ethical standards All experimental procedures were performed under the supervision and with the approval of our internal Ethics Committee (Federal University of São Paulo, CEP N. 0961/10). Animal protocols were conducted in accordance with national and international legislation (Guidelines of the Brazilian College of Animal Experimentation, COBEA; NIH Guide for Care and Use of Laboratory Animals) and the experiments followed the principles outlined in the Basel Declaration [45].

Conflict of interest Rebeca Padrão Amorim declares that she has no conflict of interest.

Michelle Gasparetti Leão Araújo declares that she has no conflict of interest.

Jorge Valero declares that he has no conflict of interest.

Iscia Lopes-Cendes declares that she has no conflict of interest.

Vinicius Davila Bitencourt Pascoal declares that he has no conflict of interest.

João Oliveira Malva declares that he has no conflict of interest.

Maria José da Silva Fernandes declares that she has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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