

PET Imaging and Neurohistochemistry Reveal that Curcumin Attenuates Brain Hypometabolism and Hippocampal Damage Induced by Status Epilepticus in Rats

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ABSTRACT

Numerous preclinical studies provide evidence that curcumin, a polyphenolic phytochemical extracted from *Curcuma longa* (turmeric) has neuroprotective, anti-inflammatory and antioxidant properties against various neurological disorders. Curcumin neuroprotective effects have been reported in different animal models of epilepsy, but its potential effect attenuating brain glucose hypometabolism, considered as an early marker of epileptogenesis that occurs during the silent period following status epilepticus (SE), still has not been addressed. To this end, we used the lithium-pilocarpine rat model to induce SE. Curcumin was administered orally (300 mg/kg/day, for 17 days). Brain glucose metabolism was evaluated *in vivo* by 2-deoxy-2-[¹⁸F]Fluoro-D-Glucose ([¹⁸F]FDG) positron emission tomography (PET). In addition, hippocampal integrity, neurodegeneration, microglia-mediated neuroinflammation, and reactive astrogliosis were evaluated as markers of brain damage. SE resulted in brain glucose hypometabolism accompanied by body weight (BW) loss, hippocampal neuronal damage, and neuroinflammation. Curcumin did not reduce the latency time to the SE onset, nor the mortality rate associated with SE. Nevertheless, it reduced the number of seizures, and in the surviving rats, curcumin protected BW and attenuated the short-term glucose brain hypometabolism as well as the signs of neuronal damage and neuroinflammation induced by the SE. Overall, our results support the potential adaptogen-like effects of curcumin attenuating key features of SE-induced brain damage.

Introduction

Curcumin is a natural polyphenolic yellow pigment extracted from turmeric rhizome (*Curcuma longa* L., Zingiberaceae) used for millennia in traditional Indian ayurvedic medicine [1,2]. Curcumin is a subject of interest in scientific research due to its pleiotropic therapeutic effects on diseases affecting nearly every system of

the body. Thus, multiple conditions have been claimed to be improved by curcumin, such as cancer, diabetes, hyperlipidemia, osteoarthritis, myocardial infarction, different types of infections, traumatic brain injury, mood disorders, aging, and neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's diseases, and epilepsy [3–5].

ABBREVIATIONS

[¹⁸ F]FDG	2-deoxy-2-[¹⁸ F]Fluoro-D-Glucose
BW	Body Weight
CT	Computed Tomography
FITC	Fluorescein Isothiocyanate
GFAP	Glial Fibrillary Acidic Protein
MRI	Magnetic Resonance Imaging
O. D.	Optical Density
PET	Positron Emission Tomography
SE	Status Epilepticus
SUV	Standardized Uptake Value
TLE	Temporal Lobe Epilepsy
TRITC	Tetramethyl-Rhodamine Isothiocyanate
TSPO	18 kDa Translocator Protein

Epilepsy is a neurological disorder that affects more than 50 million people worldwide. According to the World Health Organization, nearly 80% of epilepsy patients live in low- and middle-income countries and it is estimated that 70% of them could live seizure-free if properly diagnosed and treated (<https://www.who.int/news-room/fact-sheets/detail/epilepsy>). Among the different types of epilepsy, the temporal lobe epilepsy (TLE) is the most predominant form of focal epilepsy in adults [6]. TLE is often accompanied by hippocampal sclerosis [7] and it is highly refractory to the available pharmacological treatments [8]. Coherently, finding safe, effective, and affordable antiepileptic therapies should be a main purpose to prevent and/or to counteract the symptoms therefore reducing the burden associated to this condition [6, 8].

Preclinical studies in different animal models of epilepsy have revealed that curcumin has anti-seizure and neuroprotective effects, also reducing cognitive impairment [9, 10]. Thus, antiepileptic effects have been reported: (i) delaying the onset of kainic acid-induced seizures and reducing hippocampal neuronal death [11]; (ii) preventing iron-induced epileptogenesis [12]; (iii) increasing the threshold current in the electroshock model [13] and (iv) protecting and slowing down the epileptogenic process in both the amygdala and the pentylenetetrazole kindling models [13, 14].

Pilocarpine, an alkaloid obtained from the leaves of different species from genus *Pilocarpus*, is often administered intraperitoneally to trigger status epilepticus (SE), resulting in an animal model of epileptogenesis that resembles many, but not all the behavioral, electrographic, proteomic, and neuropathological features in human TLE [15–17], being a suitable tool to study the potential antiseizure, antiepileptic and neuroprotective drugs.

The epileptogenic process in the pilocarpine model, as well as in its variant lithium-pilocarpine model, is characterized first by the rapid manifestation of the SE, followed by a latent silent period without spontaneous seizures. This silent stage is also accompanied by brain metabolic dysfunction, reflected by a generalized hypometabolism measurable by 2-deoxy-2-[¹⁸F]Fluoro-D-Glucose ([¹⁸F]FDG) positron emission tomography (PET) [18]. Brain glucose hypometabolism is concurrent with severe neurodegenera-

tion and neuronal death, neuroinflammation and intense reactive gliosis, affecting both astroglia and microglia [19, 20]. Interestingly, in epilepsy patients, glucose hypometabolism during the interictal period measured by [¹⁸F]FDG PET has proven to be very sensitive allowing for the localization of the epileptogenic focus and its consequences as well as a minimally invasive procedure [21]. Even though the data regarding the effects of curcumin on the pilocarpine models are scarce, overall they support its anticonvulsant and neuroprotective properties [22–25].

Though curcumin can impact a diverse range of molecular targets and signaling pathways [5], most of the studies attribute its broad therapeutic benefits primarily to its antioxidant and anti-inflammatory properties [3, 26]. Because oxidative stress takes part in neuronal damage in epilepsy and seizures [10], multiple endogenous and exogenous antioxidants have been proposed as add-on therapy. In fact, it is believed that the antioxidant effect of curcumin is responsible for protection from the pilocarpine-induced SE [24, 27, 28].

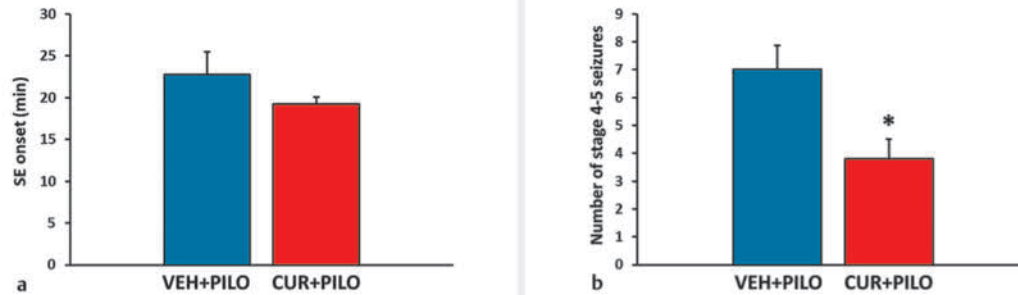
While numerous *in vitro* and *in vivo* preclinical studies support the potential therapeutic spectrum of curcumin as well as its safety and tolerability in humans [4, 5], its clinical effectiveness and indication remains to be conclusively confirmed by randomized, placebo-controlled clinical trials [10, 29].

To our knowledge, no studies have been conducted in the lithium-pilocarpine SE model to evaluate the effect of curcumin on the brain metabolic impairment occurring shortly after the SE, considered as an early marker of epileptogenesis. Accordingly, herein we used [¹⁸F]FDG PET neuroimaging to evaluate brain glucose metabolism during the early phase of the latent period of epileptogenesis. Besides, behavioral variables related with SE and different neurohistochemical assays were performed to evaluate the potential anti-seizure, neuroprotective and anti-inflammatory properties of curcumin.

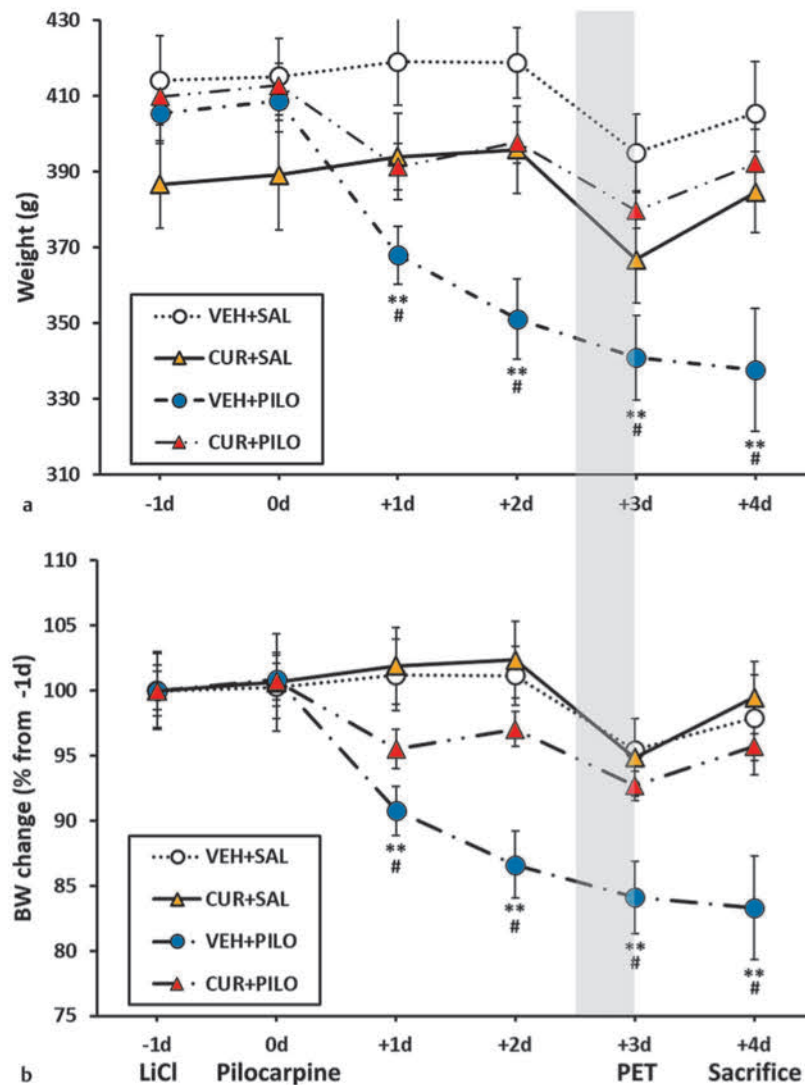
Results

Curcumin did not affect the latency time to reach SE (VEH+PILO: 22.8 ± 2.7 min vs. CUR+PILO: 19.9 ± 0.6 min, $p = 0.275$, ► **Fig. 1 a**). However, curcumin treatment significantly reduced the number of SE (level 4–5 in the Racine scale) that occurred during the 45 min of observation after the pilocarpine insult (VEH+PILO: 7.0 ± 0.87 vs. CUR+PILO: 3.80 ± 0.70; $p = 0.01$, ► **Fig. 1 b**). Death rate reached 50% in VEH+PILO and 58% in CUR+PILO ($p = 0.987$) reflecting that curcumin had no effect on the mortality associated to the SE. Body weight (BW) changes are shown in ► **Fig. 2 a** and **b**. Compared with their respective controls, VEH+PILO rats lost a 10% of their BW in the 24 h after the SE ($p < 0.01$) and this effect remained until the end of the experiment (4 d) resulting in a total BW loss of 17.4% (d0 to d4). By contrast, CUR+PILO rats did not show a statistically significant BW loss 24 h after the SE and furthermore, they were able to defend their BW, maintaining it throughout the experiment (CUR+PILO vs. VEH+PILO, $p < 0.05$).

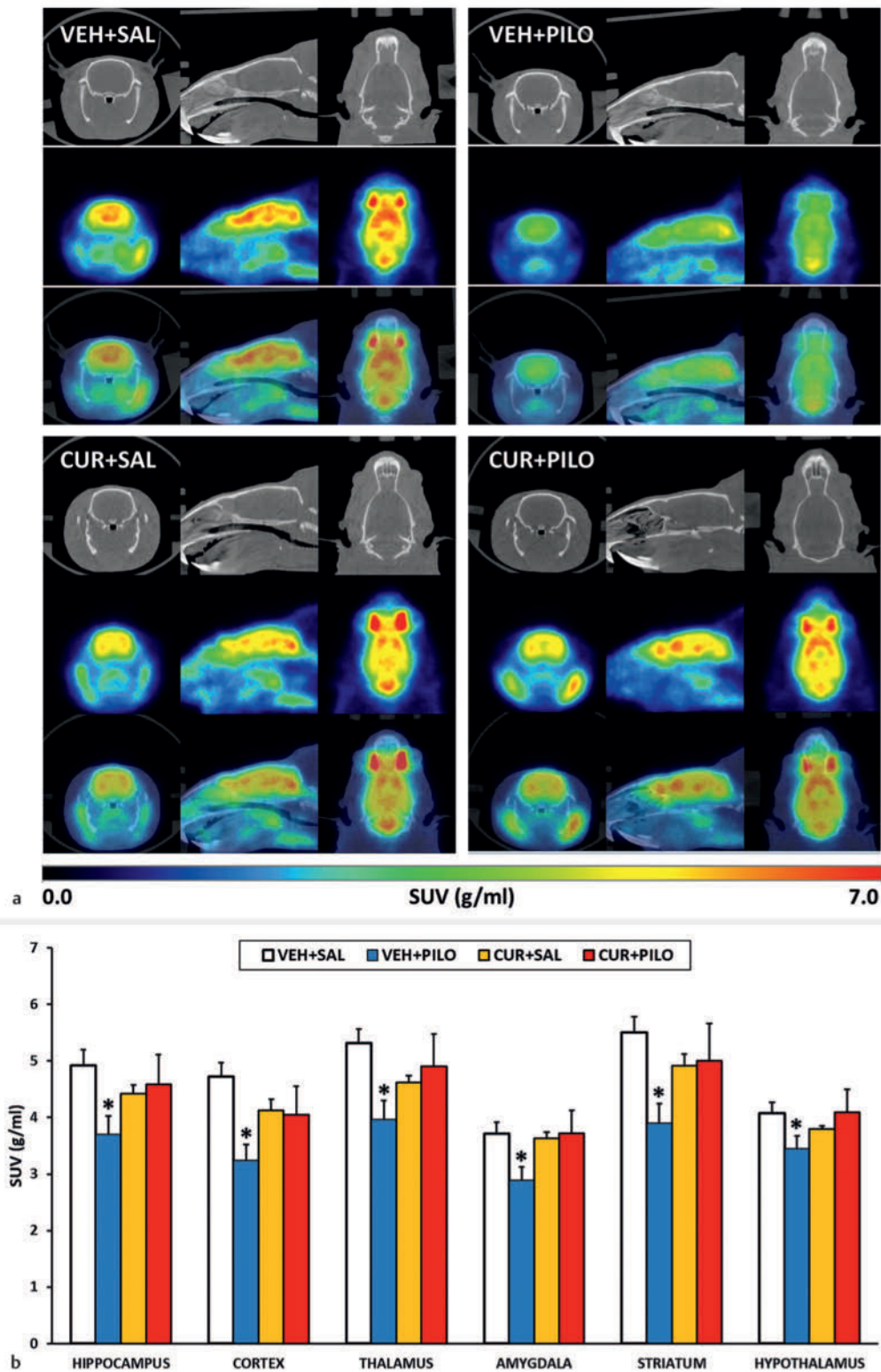
An intense hypometabolism in epilepsy-related brain areas was evident 3 days after the induction of SE (► **Fig. 3 a**) as measured by SUV. Compared to VEH+SAL, VEH+PILO showed a reduction that ranged from 15.4% in hypothalamus to 31.3% in cortex ($p < 0.01$; ► **Fig. 3 b**). Specifically in hippocampus this decrease reached a



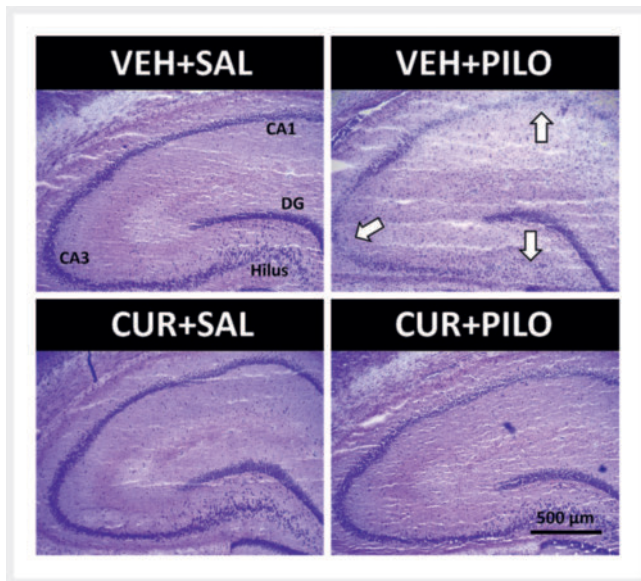
► **Fig. 1** Curcumin treatment in adult male rats did not delay the onset of SE triggered by pilocarpine, but it significantly reduced the number of seizures after SE. **a** Latency time to the onset of SE. **b** Number of Racine stage 4–5 seizures in VEH+PILO and CUR+PILO. Data are shown as mean \pm SEM ($n = 8–10$ rats/group); * $p < 0.05$, t-tests



► **Fig. 2** SE in adult male rats resulted in a significant BW loss that was prevented by chronic oral administration of curcumin. **a** BW data from the day of LiCl administration (-1 d) to the day of sacrifice ($+4$ d). **b** BW changes calculated as percentage of BW on day -1 . Shaded area indicates the 12 h fasting period before PET acquisitions. Data are shown as mean \pm SEM ($n = 3–7$ rats/group, rats that survived the experimental procedure). ** $p < 0.01$ VEH+PILO vs. VEH+SAL; # $p < 0.05$ VEH+PILO vs. CUR+PILO; two-way ANOVA followed by post-hoc Tukey tests



► **Fig. 3** SE induced by pilocarpine in adult male rats led to a significant hypometabolism measured as SUV in key areas known to be involved in epileptogenesis, an effect that was ameliorated by curcumin. Regional brain glucose metabolism was evaluated by [^{18}F]FDG PET 3 days after the SE. **a** Representative CT (upper row), [^{18}F]FDG PET (mid row) and [^{18}F]FDG PET/CT fused images (bottom row) in coronal, sagittal and trans-axial views scaled to SUV of the 4 experimental groups. **b** Regional brain uptake in the 4 experimental groups is shown as SUV units (mean \pm SEM, $n = 3-7$ rats/group, rats that survived the experimental procedure). * $p < 0.05$ VEH+PILO vs. VEH+SAL and vs. CUR+PILO; two-way ANOVA followed by post-hoc Tukey tests

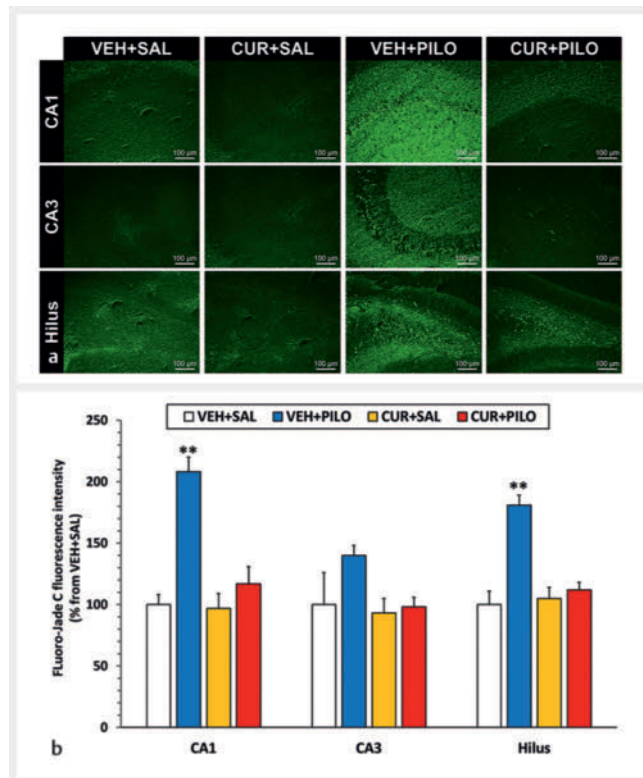


► **Fig. 4** Nissl (cresyl violet) staining micrographs from adult male rat brains illustrate, in a qualitative manner, the damage induced by SE on the anterior hippocampus 3 days after the insult. It can be observed that this effect is reduced by curcumin administration. White arrows indicate the hippocampal areas affected by pilocarpine (CA1, CA3 and hilus).

24.8% ($p < 0.01$; ► **Fig. 3b**). Curcumin alone (CUR+SAL) had no significant effect on glucose brain metabolism. However, curcumin fully prevented SE-induced hypometabolism (► **Fig. 3b**). Despite the differences in BW, there were not statistical differences among groups regarding blood glucose concentrations after 12 h of fasting (measured immediately before PET acquisitions). Thus, the values in mg/dL were as follows: VEH+SAL: 103.3 ± 3.2 ; VEH+PILO: 108.0 ± 2.9 ; CUR+SAL: 105.8 ± 6.3 and CUR+PILO: 104.2 ± 1.9 . When brain metabolism was analyzed as [^{18}F]FDG uptake as percentage of the dose injected and corrected by pre-scan whole blood glucose concentrations ($\%ID_{\text{WBglc}}$), the brain hypometabolism induced by SE was no longer detected (data not shown).

Cresyl violet stainings illustrating the qualitative effects of SE reflected an apparent reduction in hippocampal neurons at the CA1, CA3 and hilus. Curcumin alone (CUR+SAL) had no effect, but it seemed to attenuate the effects of SE (CUR+PILO) (► **Fig. 4**). These results were in line with and support those obtained from Fluoro-Jade C fluorescence labeling. Thus, and as expected, Fluoro-Jade C fluorescence labeling revealed no signs of neurodegeneration in VEH+SAL or CUR+SAL rats. Instead, SE in VEH+PILO rats triggered neurodegeneration in CA1 and hilus ($p < 0.01$; ► **Fig. 5a–b**). Curcumin by itself had no effects but it significantly ameliorated the increase of Fluoro-Jade C labeling in CUR+PILO rats (► **Fig. 5a–b**; $p < 0.05$).

In the VEH+PILO group, the SE resulted in an approximately 254% increase in fluorescence GFAP signal in the hippocampal hilus (vs. VEH+SAL group, $p = 0.003$; ► **Fig. 6a–b**), pointing towards SE-induced astroglia activation. Compared to VEH+PILO rats, curcumin reduced the astrocytic activation in response to SE. In this way, the quantitative data revealed that the fluorescence signal



► **Fig. 5** Hippocampal neurodegeneration induced by SE is ameliorated by chronic curcumin treatment in adult male rats. **a** Representative images at the level of the CA1 (top row), CA3 (mid row) and dentate gyrus/hilus (bottom row) of the 4 experimental groups. The images show degenerating neurons in VEH+PILO rats 3 days after the SE over an increased FITC fluorescence background signal, and the protective effect of curcumin (CUR+PILO). **b** Bar plot corresponding to quantitative data from Fluoro-Jade C fluorescence intensity values as marker of neurodegeneration. Data are expressed as percentage of the signal obtained in the VEH+SAL group and shown as mean \pm SEM ($n = 3–7$ rats/group; rats that survived the experimental procedure). ** $p < 0.01$ VEH+PILO vs. VEH+SAL and vs. CUR+PILO; two-way ANOVA followed by post-hoc Tukey tests

found in CUR+PILO in response to SE was approximately a 31% lesser than in VEH+PILO group ($p = 0.039$). Besides, and as it can be observed in ► **Fig. 6c**, the astroglia activation was accompanied by a qualitative apparent thickening of astrocyte bodies and processes.

We also performed [^3H]PK11 195 autoradiography as a marker of neuroinflammation at the level of the anterior and posterior hippocampus (► **Fig. 7a**). Curcumin by itself had no effects on [^3H]PK11 195 binding. Regarding the effects of pilocarpine-triggered SE and comparing it to VEH+SAL, VEH+PILO rats showed an approximately 100% increase in the optical density (O.D.) in all the regions studied ($p < 0.01$; ► **Fig. 7b**). This difference in signal was also found between VEH+PILO and CUR+PILO groups ($p < 0.01$; ► **Fig. 7a–b**). These effects were also significant when measured and analyzed in the hippocampal CA1, CA3 and hilus subregions (► **Fig. 7c**).

Discussion

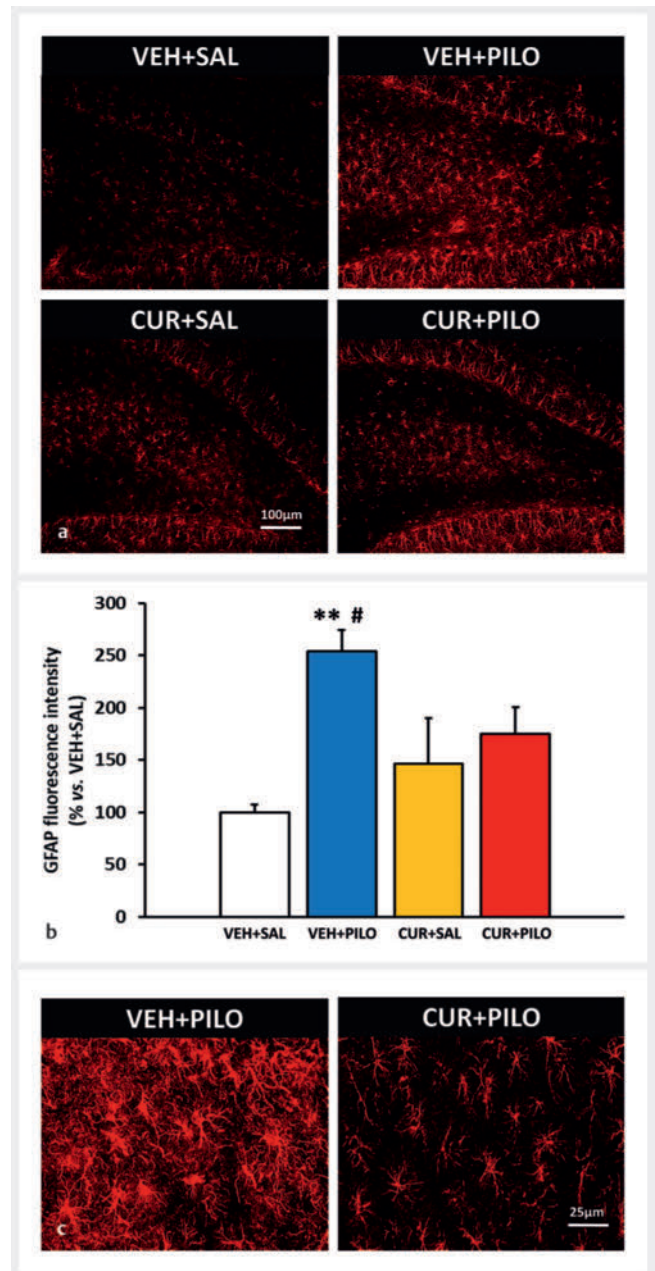
In the current work, we have explored the effects of chronic oral administration of curcumin on brain glucose metabolic dysfunction, hippocampal neurodegeneration, and neuroinflammation, typical features of the brain damage associated to the SE in the rat lithium-pilocarpine model [19]. We have also studied the effects of curcumin on latency to SE, number of seizures, mortality, and BW change. Overall, our results show that curcumin did not affect either latency time to SE or mortality rate. However, curcumin treatment significantly reduced the number of stage 4–5 seizures and ameliorated signs of brain damage associated with the SE also having a marked protective effect on BW in the rats that survived the insult.

Curcumin is a polyphenol extracted from the rhizomes of *Curcuma longa* (family Zingiberaceae). Commonly known as turmeric and referred to as the “golden spice” and “spice of life”, curcumin has been traditionally employed as a dietary component as an herb, a spice, as a cosmetic product, and as a natural medicinal agent in Asia, particularly in Ayurveda medicine [1, 2]. In fact, numerous *in vitro* and *in vivo* preclinical and clinical studies on curcumin have put forth pleiotropic beneficial effects dealing with its anti-cancer, anti-diabetic, antimicrobial, antioxidant, and anti-inflammatory properties [30]. Furthermore, curcumin has been reported to have neuroprotective and cognitive-improving properties that may delay or prevent many of the deleterious processes occurring in most neurodegenerative and neurological diseases, including epilepsy [31].

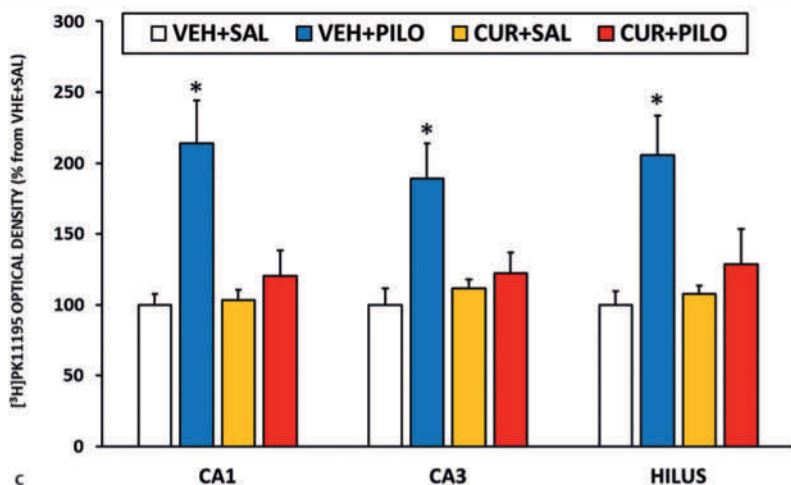
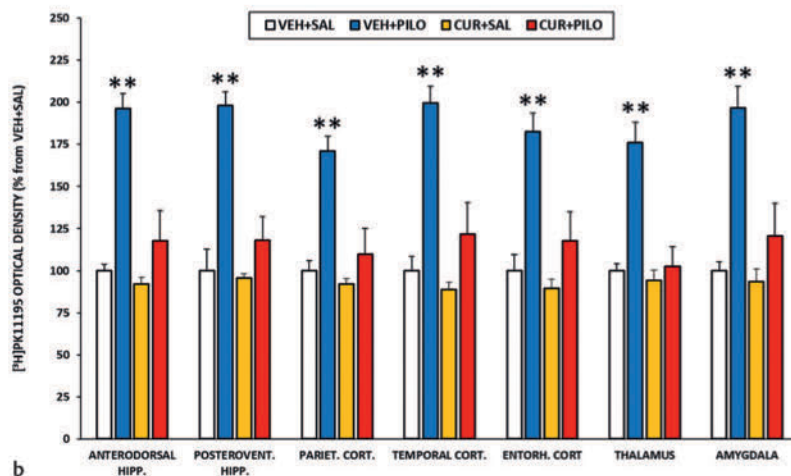
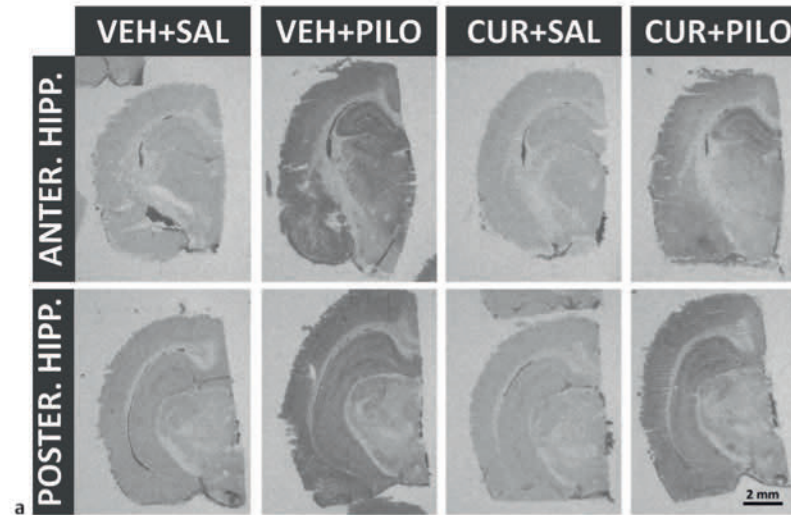
Despite these alleged beneficial effects, it is important to mention that recent studies have called into question the real *in vivo* effectiveness of curcumin. One of the main limitations is the poor physical-chemical and pharmacokinetic properties of the curcumin molecule characterized by low aqueous solubility, gut absorption, and limited entry to the CNS through the blood brain barrier, as well as rapid metabolism and systemic elimination [9, 29, 31, 32].

In keeping with the controversy, curcumin, like many other natural compounds, has been labeled as both PAINS (pan assay interference compounds) and IMPS (invalid metabolic panaceas) compounds [29]. Thus, curcumin might interfere with many *in vitro* and *ex vivo* biochemical assays used to evaluate multiple biological activities, leading to erroneous claims for a non-existent therapeutic effect. Yet, despite these caveats, the overwhelming amount of wide-range experimental evidence regarding the beneficial effects of curcumin, including its potential therapeutic role in epilepsy, cannot be set aside.

In the present study, and to tackle the poor oral bioavailability of curcumin, we used 10% Cremophor EL (10 mL/kg) as vehicle [25,33]. Cremophor EL is a non-ionic solvent for hydrophobic compounds that has been shown to improve solubility [34] and oral bioavailability of curcumin [35]. Even though Cremophor EL is a non-inert relatively nontoxic solvent, it is important to mention that several reports suggest that it can induce serious complications such as anaphylactoid-hypersensitivity and cellular toxicity, especially when administered intravenously [36]. Nevertheless, observational follow-up of our rats, including BW changes,



► **Fig. 6** Curcumin reduced hippocampal astrogliosis in response of the SE triggered by pilocarpine in adult male rats. **a** Images showing representative GFAP immunofluorescence micrographs at the hilus/dentate gyrus area of the 4 experimental groups. **b** Bar plot corresponding to quantitative data from GFAP immunofluorescence intensity as marker of astrogliosis on the hilus. Data are expressed as percentage of the signal obtained in the VEH+SAL group and shown as mean \pm SEM ($n = 3-7$ rats/group; rats that survived the experimental procedure). ** $p < 0.01$ VEH+PILO vs. VEH+SAL and # $p < 0.05$ VEH+PILO vs. CUR+PILO; two-way ANOVA followed by post-hoc Tukey tests. **c** Magnified micrographs illustrate the apparent morphological changes (hypertrophy of astrocyte bodies and processes) induced by SE and the effect of curcumin.



► **Fig. 7** Chronic curcumin treatment reduced the neuroinflammation induced by SE in adult male rats as measured by the [^3H]PK11195 binding in major brain regions involved in epileptogenesis. **a** Representative [^3H]PK11195 autoradiograms corresponding to the 4 experimental groups obtained from both anterior and posterior hippocampus. **b** [^3H]PK11195 binding expressed in O. D. in various brain regions involved epileptogenesis. **c** [^3H]PK11195 binding expressed in O. D. in the hippocampal regions CA1, CA3 and hilus. Data is shown as mean \pm SEM ($n = 3\text{--}7$ rats/group; rats that survived the experimental procedure). * $p < 0.05$ and ** $p < 0.01$ VEH+PILO vs. VEH+SAL and vs. CUR+PILO; two-way ANOVA followed by post-hoc Tukey test

throughout the whole duration of our study, indicated neither digestive nor other signs of toxicity.

As previously mentioned, the SE induced by lithium-pilocarpine is an animal model of epileptogenesis that resembles many, but not all, pathological features of human TLE [15–17]. In so far, to date few studies have tackled the therapeutic efficacy of curcumin (administrated either as a single dose or repeatedly) on the pilocarpine model of SE in rats [24,25,27,28,37]. Epileptogenesis is associated, among others, with neurochemical imbalances, neurodegeneration, neuroinflammation, and reactive gliosis, as well as synapsis modification and reorganization of specific brain areas [19,38]. Many of these alterations are present in the rodent pilocarpine and lithium-pilocarpine SE models [6,16,17]. In these models, pilocarpine administration results in a SE that is followed by a silent latency period during which generalized glucose hypometabolism occurs concomitantly with severe neurodegeneration and neuronal death, neuroinflammation, and intense reactive gliosis, affecting both astroglia and microglia [18–20], and ultimately leading to a chronic epileptic state characterized by spontaneous recurrent seizures. Therefore, it is in this period that [^{18}F]FDG PET acquisitions and neurohistochemical assessments were carried out.

In our study, curcumin treatment neither delayed the latency time to the SE nor reduced the mortality rate consequence of the severity of SE induced by pilocarpine. The mortality rate in our current study, being around 50%, is within the range for this model. In fact, the death rate described for the rat lithium-pilocarpine model can be as high as 95%. Furthermore, high intra-strain, inter-strains and sub-strains variability have been linked to different pilocarpine sensitivity. Even more, the commercial providers and the time of purchase of animals seem to be factors contributing to the variability on mortality [39]. However, curcumin significantly reduced the number of stage 4–5 seizures that occurred during the 45 min after the beginning of the SE. Unfortunately, we did not measure the duration of the seizures. Nevertheless, our results might point towards an anticonvulsant effect of curcumin that would be in line with previous reports [28,37]. Likewise, anticonvulsant effects of oral curcumin have been also reported in other animal models of epilepsy and seizures such as the iron-induced experimental model of epileptogenesis in rats [12] and in the pentylenetetrazole-kindled rat model of epilepsy [40,41]. Therefore, it is likely that the effects of curcumin reducing the number of seizures might be one the factors contributing to the control of further spontaneous seizures and to the neuroprotective effects observed in the rats that survived the SE [41,42]. Nonetheless, lack of long-lasting anti-epileptogenic, neuroprotective and anti-inflammatory effects of intracerebroventricular administration of curcumin have been also reported in a kindling rat model [43].

BW change is widely accepted as a marker of the overall animal well-being. In rats that did not undergo SE, oral curcumin administration did not affect BW change throughout the 17 days of experimental procedure. By contrast, curcumin significantly reduced the effects of SE inducing BW loss (► Fig. 2). Thus, curcumin treatment allowed rats to defend their BW in the face of the SE. It is likely that the effect of curcumin enhancing the ability of the rats to defend their BW is both a reflection of the reciprocal

central and peripheral protective effects of curcumin that, could ultimately contribute to set in motion a more adaptive response to the metabolic demands imposed by the SE. In this line of reasoning, other studies in rats have shown that curcumin treatment protected from BW loss promoting resilience to chronic social defeat stress [44], and reduced BW loss in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin administration [45]. Interestingly, curcumin also has shown beneficial effects on BW reduction and energy metabolism in rodent models of obesity, nowadays accepted as a pro-inflammatory disease [46].

The interictal temporal lobe glucose hypometabolism is one of the early biomarkers identified by [^{18}F]FDG PET neuroimaging in TLE patients, providing even better results than magnetic resonance imaging (MRI) [47,48]. The hypometabolism has been attributed, among others, to neuronal death, altered neuronal excitability, and/or reduced brain blood flow in the epileptic focus. Importantly, brain glucose hypometabolism has been repeatedly reproduced in many animal models, including the pilocarpine model [19,49–51]. Our current study corroborates that the SE induced by pilocarpine results in glucose hypometabolism (measured by SUV) in epilepsy-related brain areas (► Fig. 3).

More remarkably, glucose hypometabolism was fully prevented by curcumin treatment (► Fig. 3). As far as we know, this is the first time that functional neuroimaging PET has been implemented to assess the effect of curcumin on glucose hypometabolism induced by SE.

Considering that brain glucose hypometabolism seems to be an early biomarker of epileptogenesis in different animal models of epilepsy [52], our results might point towards an antiepileptogenic effect of curcumin in this model. Nevertheless, because we have not evaluated the long-term effects, we cannot state that the effect of curcumin preventing SE-triggered brain glucose hypometabolism is necessarily associated with the alleged antiepileptic effect.

Nonetheless, we want to notice that the characteristic brain glucose hypometabolism in response to SE was not observed when quantified as percentages of the injected dose and corrected by the pre-scan whole blood glucose values (%ID_{WBglc}). It is known that SUV and %ID need to be corrected for glucose, especially when the fasting period is shorter than 12 h [53]. Moreover, not considering the rapid BW loss in response to SE (see ► Fig. 2) might result in underestimation in hypometabolism quantification [53,54].

In agreement with previous reported studies, pilocarpine-triggered SE resulted in hippocampal neuronal death and neurodegeneration [19]. The results obtained regarding hippocampal integrity based on the visual observation of Nissl staining, and neurodegeneration based on the quantitation of Fluoro-Jade-C fluorescence [55,56], support a neuroprotective effect of curcumin treatment. Thus, curcumin contributed to preserve neuronal integrity in the CA1 and hilus, hippocampal areas where the apparent neuronal loss (► Fig. 4) and marked neurodegeneration (► Fig. 5) occurred in response to pilocarpine. Those results support the neuroprotective effects that have been attributed to curcumin, mainly based on its antioxidant and anti-inflammatory properties [14,57]. Similarly, a previous study in the lithium-pilocarpine model has reported that curcumin protected hippocam-

pal neurons through induction of autophagy and inhibition of necroptosis [25].

Neuroinflammation is an adaptive physiological response to brain cell damage or loss and, gliosis affecting both astrocytes and microglia have been reported in epileptic disorders. We and others have consistently reported that neuroinflammation is also a feature of the pilocarpine rodent model [19,20] as well as of other animal models [49,58]. Accordingly, our current results show that SE triggered reactive astrogliosis and microglia-mediated neuroinflammation. Furthermore, our results reveal that curcumin significantly ameliorated both hippocampal astrogliosis (evaluated by GFAP immunofluorescence; ► **Fig. 6**) and brain microglia-mediated neuroinflammation (evaluated by [³H]PK11195 autoradiography). PK11195 is a ligand of the mitochondrial 18 kDa translocator protein (TSPO), which is mainly, but not exclusively, present in microglia. Thus, TSPO is expressed in vascular endothelial cells and astrocytes. Brain TSPO expression is low under non-pathological conditions, but it is upregulated in response to neuroinflammation, being considered as a marker of neuroinflammation in many neurological diseases [59]. In our study, [³H]PK11195 autoradiography did not include non-specific binding, as such. It has been reported that curcumin can interact directly with low micromolar affinity with TSPO [60]. This might point towards a potential direct anti-inflammatory mechanism of action for curcumin. Nevertheless, because the binding was performed in brains collected 2 days after the last curcumin administration, it is most likely that the 48 h washout period, added to the poor pharmacokinetic properties of curcumin, may have significantly reduced these eventual binding interferences in all curcumin-treated rats. Herein, we show that SE results in an increase in [³H]PK11195 binding signal (► **Fig. 7**). This increase is in line with previous studies reporting increased [¹⁸F]GE180 (a TSPO PET tracer) signal when neuronal damage occurs as consequence of seizures and epileptogenesis [19,49]. Altogether, these results point towards an anti-inflammatory and neuroprotective effect of oral curcumin.

Numerous and redundant are the mechanisms attributed to curcumin neuroprotective properties, including its ability to reduce oxidative stress and to regulate anti-inflammatory and pro-inflammatory pathways [61]. Despite its low bioavailability and the questionable blood brain barrier-crossing previously mentioned [29], various mechanisms might partially explain the central effects of curcumin in brain. Among them, we can mention the implication of the gut-brain axis, involving the metabolic role of microbiota [31] as well as upregulation of epithelial enzymes with antioxidant and anti-inflammatory effects [62]. Besides, it is interesting to mention that curcumin improves ghrelin expression [63,64]. Furthermore, in the pilocarpine rat model of SE, ghrelin and ghrelin analogs have shown to be neuroprotective without anticonvulsant effects [65].

To summarize, chronic (17 d) oral curcumin treatment neither delayed the SE nor reduced the mortality rate associated with our experimental model. However, curcumin seemed to have a certain anti-seizure effect that, in the survival rats, might have contributed to the amelioration of the damage induced by SE. Actually, BW loss, brain glucose hypometabolism, neurodegeneration, and neuroinflammation were reduced by curcumin treat-

ment. Furthermore, and as far as we know, this is the first time that functional neuroimaging PET has been implemented to assess the effect of curcumin on brain glucose hypometabolism induced by SE. Although the exact direct and/or indirect mechanisms need further enquiry, and despite the controversy regarding the actual bioavailability and effectiveness of oral curcumin, our overall results would support the claimed neuroprotective and anti-inflammatory properties of this phytochemical. Altogether, taking into consideration that curcumin in the absence of injury had no effect, it is likely that curcumin has adaptogen-like properties, enabling the animals to withstand and to adaptatively respond to the demands imposed by insults of various nature.

Materials and Methods

Animals and drug treatment protocol

Adult male Sprague-Dawley rats (Charles River Laboratories) weighing 326.9 ± 3.5 g at the beginning of the experiment were used. Rats were housed in standard rat cages (2 rats/cage), on a ventilated rack (Tecniplast) under controlled temperature ($22 \pm 2^\circ\text{C}$) and a 12 h light/dark cycle (8:00 AM-8:00 PM). Throughout the study, rats had free access to standard rodent chow and tap water, were weighed daily at morning and BW registered as a marker of overall welfare. Food was removed for the 12 h before the [¹⁸F]FDG PET acquisitions to reduce competition between plasma glucose and the radiotracer for the glucose transporters. The study was approved on the 10th of August of 2015 by both the Animal Research Ethical Committee of the Universidad Complutense de Madrid and by the Autonomous Community of Madrid (PROEX 238/15), being carried out in accordance with regulations of the European Union (2010/63/UE) and Spain (RD53/2013) regarding animal welfare.

Four experimental groups were used in the present study: (1) control naïve group: rats received Cremophor EL daily as vehicle instead of curcumin (14 + 3 days) and saline instead of lithium-pilocarpine, therefore they never underwent SE (VEH+SAL); (2) rats that received Cremophor EL daily as vehicle instead of curcumin (14 + 3 days) and underwent lithium-pilocarpine-induced SE group (VEH+PILO); (3) rats that received curcumin (14 + 3 days) and were not exposed to SE (CUR+SAL) and finally, (4) rats that chronically received curcumin and underwent the lithium-pilocarpine insult (CUR+PILO). Besides all rats were exposed to the same procedures required for PET studies.

SE induction

The lithium-pilocarpine model of SE followed in the current study has been described in previous studies [19]. Briefly, lithium chloride (127 mg/kg i. p., Sigma-Aldrich) was administered 18–20 h before SE induction. The next day, and following the daily protocol, either curcumin or vehicle were administered at morning. Approximately 2 h later, methyl-scopolamine (2 mg/kg, i. p.) was administered to reduce the pilocarpine-induced muscarinic peripheral effects. Thirty min later, pilocarpine was injected (25 mg/kg, i. p.; Sigma-Aldrich). The onset of SE was considered when the animal reached the stage 4 according to the Racine scale [66] and showed continuous seizure activity. The seizure activity was

ended by injecting pentobarbital (25 mg/kg, i. p.) 45 min after the SE onset. The rats that did not undergo SE (naïve- and curcumin-control groups) were administered with the same drug regime (including lithium chloride, methyl-scopolamine, and pentobarbital) but saline solution was administered instead of pilocarpine.

Curcumin treatment

Because the relevance of preclinical studies mainly rely on their prospective eventual translation into the clinical setting, we administered curcumin by oral route, by intragastric gavage. To improve bioavailability, curcumin was suspended in 10% Cremophor EL (Sigma-Aldrich) [25, 33]. Curcumin was administered once daily in the morning (300 mg/kg/10 mL, p. o.) for 14 days before the SE, the day of the SE induction and for the 2 following days. The dosing regime was chosen based on a previously reported study administering either 200 or 300 mg/kg of curcumin for 14 days, by intragastric gavage, to adult Sprague-Dawley male rats in the lithium-pilocarpine model of SE [25]. To prevent any eventual degradation of curcumin, the suspension was freshly prepared every morning and the container wrapped with aluminum foil to protect it from light.

According to the 3Rs principles and the ARRIVE guidelines (<https://arriveguidelines.org>), the number of animals was selected considering the mortality associated with the severity of the model. Besides, considering that: (i) studies do not support for relevant effects of curcumin under baseline or control conditions; (ii) main effects of curcumin have been reported when animals were exposed to psychological, physical, or chemical insults and, (iii) our current main objective was to study the potential effects of curcumin in the face of the insult triggered by SE, the number of animals in the control groups was reduced. The initial experimental sample size was then as follows: (1) VEH+SAL, n = 3; (2) VEH+PILO, n = 14; (3) CUR+SAL, n = 4; and (4) CUR+PILO, n = 14. After the mortality caused by the pilocarpine treatment the final sample size was: (1) VEH+SAL; n = 3; (2) VEH+PILO; n = 7; (3) CUR+SAL; n = 4; and (4) CUR+PILO; n = 6.

[¹⁸F]FDG PET neuroimaging

PET scans were carried out 3 days after the SE. To this aim, a hybrid PET/CT (computed tomography) scanner (Albira scanner, Bruker NMI) was used. The protocols have been previously reported [19, 49]. Briefly, the rats were fasted (12 h) before scanning. [¹⁸F]FDG was injected into the tail vein (approximately 13 MBq – 350 μ Ci- in 0.2 mL of 0.9%; Curium Pharma) and 30 min later, PET and CT acquisitions were consecutively carried out under isoflurane anesthesia. After reconstruction of the tomographic images, the metabolic activity was quantified using PMOD 3.6 software (PMOD Technologies Ltd.). As index of metabolic activity, the standardized uptake value (SUV) for each region was then calculated, taking into account the rat weight, the dose injected, and the [¹⁸F]FDG uptake decay-corrected to the scan start time. Nevertheless, and because the effects of pilocarpine and/or curcumin on brain glucose metabolism might be indirectly reflecting changes in BW and/or glucose metabolism, we also report the regional brain [¹⁸F]FDG uptake data as %ID_{WBglc} [53, 54].

Neurohistochemical assessments

Rats were sacrificed by decapitation the day after the PET acquisition procedure. Brains were dissected, cut longitudinally in two halves, quickly frozen on dry ice, and stored at – 80 °C. Brain slices (30 μ m-thickness) from the left hemibrain were obtained using a cryostat (Leica CM1850, Leica Biosystems). The brain sections containing the hippocampus (6 slices/glass slide) were thaw-mounted onto Superfrost Plus slides (Thermo Scientific), dried on a hot plate (36 °C) and stored into slide boxes at – 80 °C until the day of the assays. The histochemical assays were as follows:

1. *Neuronal viability and disruption of hippocampal integrity* was evaluated by Nissl staining as previously described [19, 49]. Briefly, the slices were fixed in 4% formaldehyde in phosphate buffer pH 7.4 (10 min), washed, and incubated for 30 min in 0.5% cresyl violet acetate solution. Afterwards, the sections were washed and dehydrated in graded ethanol series (70%, 95% and 100%). Finally, the slices were cleared in xylene and cover-slipped with DPX mounting medium (Fluka). The histological images of the hippocampus were captured with a digital camera (Leica DFC425, Leica) coupled to a microscope (Leica DM 2000 LED, Leica). The histological images are presented as a visual aid reflecting the qualitative changes induced by SE and the potential effect of curcumin on the hippocampus.
2. *Hippocampal neurodegeneration* was evaluated by Fluoro-Jade C staining, as previously reported [19, 49, 56]. Briefly, after fixing in 4% formaldehyde for 10 min, the samples were rinsed in basic alcohol, 100% ethanol, distilled water, 0.06% potassium permanganate, 0.1% acetic acid solution containing 0.0001% Fluoro-Jade C (Millipore), distilled water, and xylene. Next, the slides were cover-slipped with DPX (Fluka). The fluorescence images were captured with a digital camera (Leica DFC3000G) coupled to a microscope (Leica DM 2000 LED) by using the FITC filter. At the hippocampal CA1, CA3 and hilus, the fluorescence signal was measured using ImageJ 1.46 r software. The average value for each rat was calculated. The results are expressed as percentage vs. the control group (VEH+SAL).
3. *Reactive astrogliosis* was evaluated by glial fibrillary acidic protein (GFAP) one-step immunofluorescence as previously reported [19, 49]. Briefly, after fixing and washing, the slices were blocked and permeabilized with 3% BSA, 0.1% triton X-100 in TBS for 60 min. The slides were then incubated overnight with anti-GFAP-Cy3 antibody (1:500, Sigma Aldrich) in 1% BSA in TBS at 4 °C. Afterwards, the slides were washed in 0.1% Tween 20 dissolved in Tween for 3 times (5 min each) and cover-slipped with Mowiol. The images were captured and examined using the same optical systems used for Fluoro-Jade C with the TRITC filter. For each brain section (4 sections/rat) containing the CA1, CA3 and hilus areas within the anterior (dorsal) hippocampus were selected and the fluorescence intensity was measured (ImageJ 1.46 r software). The average value for each rat was calculated. The results are expressed as percentage vs. the control group (VEH+SAL).
4. *Neuroinflammation* was studied by [³H]PK11 195 autoradiography with minor modifications [67]. [³H]PK11 195 is a specific ligand of the 8 kDa translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor. TSPO is pre-

dominantly, but not exclusively, expressed in microglia, and increases in conditions of neuroinflammation [59]. Slides were dried at 37 °C (10 min) and preincubated with 50 mM Tris-HCl pH 7.4 at RT (15 min). The samples were then incubated with 1 nM [³H]PK11195 (Perkin Elmer) in preincubation buffer (60 min). Afterwards, the samples were washed in ice cold preincubation buffer (2 × 5 min) and dipped in ice-cold distilled water. Once dry, the slides were exposed to Kodak BioMax MR autoradiography film (Carestream) inside an exposure cassette for 2 months. The developed film was placed onto a light box (Kaiser Prolite 5000, Kaiser Fototechnik) and the images from each section were captured with a camera (Leica DFC425) coupled to a stereomicroscope (Leica MZ6). Within each brain section, the O.D. was measured in the selected regions and in the background. The O.D. values obtained after subtracting the background were expressed as percentage of the VEH +SAL group and used as index of neuroinflammation.

Statistical analyses

Analyses were performed with SigmaPlot 11.0 software (Systat Software Inc.). Behavioral markers of SE onset (latency), number of seizures, and mortality rate were only analyzed in the lithium-pilocarpine-treated rats (VEH+PILO vs. CUR+PILO) by unpaired Student t-test and z-test for rates and proportions, respectively. BW, PET, and histochemical data were analyzed by two-way analysis of variance (ANOVA) with curcumin and pilocarpine treatments as the two main factors. When interaction between factors was significant, further post hoc Tukey tests were performed. In all cases, statistical significance was considered when $p < 0.05$. Data are shown as mean ± SEM.

Contributors' Statement

Data collection: L. García-García, M. Delgado, R. Fernández de la Rosa, K. Slowing Design of the study: L. García-García, F. Gomez, M. Delgado, K. Slowing Statistical analysis: L. García-García, M. Delgado, N. Hernández-Martín Analysis and interpretation of the data: F. Gomez, L. García-García Drafting of the manuscript: F. Gomez, L. García-García Critical revision: M. A. Pozo

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Conflict of Interest

The authors declare that they have no conflict of interest.

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