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José Luís Marques Ferreira

The Cholinergic Transmission in the Retrosplenial Cortex of the Epileptic Rat

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ABBREVIATIONS

Choline acetyltransferase (ChAT)

Electroconvulsive shock (ECS)

Hippocampal formation (HF)

Retrosplenial granular b cortex (Rgb)

Status epilepticus (SE)

Temporal lobe epilepsy (TLE)

Vesicular acetylcholine transporter (VAChT)

ABSTRACT

It has been previously hypothesized that changes in cholinergic neurotransmission can play an important role in epileptogenesis. The main purpose of this study was to address the issue whether prolonged seizures (status epilepticus, SE) and brief repeated seizures are associated with changes in the cholinergic transmission in the rat retrosplenial granular b cortex (Rgb), a cortical area strongly interconnected with other epilepsy-related limbic structures, including the hippocampal formation (HF). SE was induced by treating rats with pilocarpine (350 mg/kg). Brief seizures were induced by electroconvulsive shock (ECS). In this model, rats were given six ECS seizures, the first five of which were spaced by 24-h intervals, whilst the last two were 2h apart. Two months later, the brains of the animals were processed for immunostaining for vesicular acetylcholine transporter protein (VAChT) and the densities of fiber varicosities immunoreactive to VAChT were estimated. SE produced a statistically significant increase in the densities of cholinergic varicosities in Rgb layers I, II/III, IV and VI, but not in layer V. In ECS group there was a slight and non-significant increase in the densities of VAChT-positive varicosities in all layers of the Rgb cortex. These findings are consistent with the notion that changes in the activity of the cholinergic system in specific cortical areas of the limbic system can contribute to epileptogenesis.

INTRODUCTION

Epilepsies are complex neurobehavioral disorders caused by increased excitability and synchronism of neurons in various brain regions (Ampuero et al., 2007; Sharma et al., 2007; Guidine et al., 2008) and characterized by unprovoked recurrent seizures (Guidine et al., 2008). Temporal lobe epilepsy (TLE), the most common form of focal epilepsy in adults (Sharma et al., 2007; Bertram, 2009), is associated with partial seizures with or without secondary generalization (Sharma et al., 2007). It covers a variety of disorders that have the common feature of seizures arising from limbic structures in the temporal lobe (Englot et al., 2008; Bertram, 2009).

Human TLE and experimental models of this disease are associated with loss of neurons, atrophy, gliosis (Buckmaster and Dudek, 1997; Kotloski et al., 2002; Bertram, 2009), synaptic rearrangements and morphological alterations in the dendritic arbors (Ampuero et al., 2007; Cardoso et al., 2008a) in several limbic brain structures including the hippocampal formation (HF, including hippocampus proper and dentate gyrus) and adjacent parahippocampal cortical areas (Du et al., 1995; Schwarcz et al., 2000; Bertram, 2009). The entorhinal cortex-HF complex is believed to play a pivotal role in the initiation and propagation of seizures in the majority of patients with TLE (Ampuero et al., 2007; Sharma et al., 2007). Both these regions are enriched with cholinergic afferents which, under normal conditions, play an important role in the control of neuronal excitability (Friedman et al., 2007).

Status epilepticus (SE), one of the most serious manifestations of epilepsy (Marchi et al., 2009), is a state of continuous seizure activity for five minutes or longer (Sharma et al., 2007). It is thought that TLE is initiated by structural lesions and functional alterations secondary to insults like SE which, after a latency period of variable duration, generate

spontaneous motor seizures (Sharma et al., 2007). Neuronal damage induced by SE and other types of insults is thought to be responsible for the establishment of the epileptic network during the silent period, probably via mechanisms of neuroplasticity including formation of new synapses (MacNamara, 1999; André et al., 2000; Cardoso et al., 2008b).

Among the animal models developed to investigate the pathogenesis of TLE, post-SE models have received the greatest acceptance because they are characterized by a latency period, the development of spontaneous motor seizures, severe behavioral impairments (Turski et al., 1983; Buckmaster and Dudek, 1997; Kotloski et al., 2002; Kemppainen et al., 2006; Sharma et al., 2007; Cardoso et al., 2008b; Guidine et al., 2008) and a spectrum of structural lesions that mimic those found in TLE (Sharma et al., 2007), including mossy fibers sprouting in the dentate gyrus (Sharma et al., 2007) and neuronal loss in the entorhinal cortex, hilus of the dentate gyrus and CA3 and CA1 hippocampal pyramidal fields (Du et al., 1995; Buckmaster and Dudek, 1997; Schwarcz et al., 2000; Kotloski et al., 2002, Guidine et al., 2008). Pilocarpine, a partial muscarinic agonist (Guidine et al., 2008), is one of the most commonly chemoconvulsants used to create the SE models of TLE. The acute cholinergic insult induces SE which in turn triggers a set of plastic events that result in late spontaneous recurrent limbic seizures (Guidine et al., 2008).

Electroconvulsive shock (ECS) is widely used in animal studies as a model of brief generalized seizures (Cardoso et al., 2008a), causing subtle morphological alterations in the HF, such as enhanced neurogenesis of dentate gyrus granule cells and aberrant sprouting of their axons, the mossy fibers (Lukoyanov et al., 2004, Cardoso et al., 2008a). In addition, administration of ECS seizures in animals induces various metabolic and biochemical changes in the brain including increased blood flow, variations in the synthesis and expression of neuropeptides and changes in the levels of small-molecule

neurotransmitters (Cardoso et al., 2008a) and its receptors (André et al., 2000). It can also produce changes in the expression of factors involved in cellular stress and eventual death, as well as of those that are responsible for cell resistance (André et al., 2000). Although single or even repeated, but widely spaced, ECS seizures do not cause considerable brain damage, they do so when administered at shorter intervals (Cardoso et al., 2008a). In these conditions it has been observed that there are several seizure-vulnerable neuronal populations such as hilar cells of the dentate gyrus and neurons of the entorhinal cortex, in which occurs neuronal death (Cardoso et al., 2008a).

The cholinergic system is one of the major neurotransmiter systems of the brain which plays important roles in attention, learning and memory (Weckesser et al., 1997; Niewiadomska et al., 2002). Furthermore, its activity is crucial for neuronal plasticity and it exerts neurotrophic effects (Cardoso et al., 2006). The cholinergic neurons of the basal forebrain, including those of nucleus basalis magnocellularis and medial septal nuclei, are the major source of cholinergic innervations of the neocortex and HF, respectively (Niewiadomska et al., 2002; Cardoso et al., 2006). The basal forebrain cholinergic neurons are characterized by the presence of three proteins which are believed to define their phenotype: choline acetyltransferase (ChAT), high-affinity choline transporter and vesicular acetylcholine transporter (VAChT) (Gilmor et al., 1996). The latter is the proton-dependent transporter that packages acetylcholine, synthesized in the cytoplasm, into synaptic vesicles and belongs to a family of vesicular monoamine transporters which are believed to concentrate neurotransmitters in the synaptic vesicles through the exchange of protons for neurotransmitter (Gilmor et al., 1996). VAChT has been shown to be a reliable and specific marker of cholinergic neurons, fibers and synapses (Gilmor et al., 1996).

The effects of acetylcholine in Central Nervous System are mediated mainly by its muscarinic receptors (Friedman et al., 2007). In the neocortex, cholinergic boutons are

frequently observed in close apposition to asymmetrical (presumably excitatory) synapses on dendritic spines and shafts without forming recognizable synaptic specializations (Friedman et al., 2007). On the other hand, muscarinic receptors are found on postsynaptic elements apposed to symmetrical synapses, predominantly on dendritic shafts (Friedman et al., 2007). Muscarinic receptors are also found in pre- and postsynaptic elements of noncholinergic asymmetrical synapses, including those in dendritic spines (Friedman et al., 2007). Thus, acetylcholine influences synaptic integration both through activation of classical cholinergic synapses and through modulation of non-cholinergic synapses.

Furthermore, there is experimental evidence for alterations of the cholinergic system in epilepsy (Weckesser et al., 1997; Kaufer et al., 1998; Zimmerman et al., 2008). In effect, it has been suggested that changes in cholinergic modulation of neuronal excitability may initiate seizure events in epileptic cortex (Turski et al., 1989; Gloveli et al., 1999). Consistent with this hypothesis, it has been found that epilepsy can be associated with changes in the composition and/or levels of key muscarinic receptors involved in cholinergic neurotransmission (MacNamara, 1978; Dasheiff et al., 1981). In particular, it was found that part of the cholinergic hyperactivity in the epileptic brain can be attributed to altered functioning of M1 and M2 receptors (Mingo et al., 1997; Mingo et al., 1998). Studies on pilocarpine-induced focal seizures showed that M2 receptor antagonists potentiate seizures, while M1 antagonists prevent the induction of seizures (Friedman et al., 2007). In line with this, it has been reported that prolonged stimulation of M1 receptors can be epileptogenic (Cruikshank et al., 1994).

In humans, the retrosplenial cortex is a cytoarchitectonically distinct brain structure located in the posterior cingulate gyrus, bordering the splenium, precuneus and calcarine fissure, and forming Brodmann areas 29 and 30 (Kim et al., 2007). The retrosplenial granular b cortex (Rgb) of the rat occupies the anterodorsal part of the retrosplenial

granular area and is found ventrally to the retrosplenial dysgranular cortex and caudally to the anterior cingulated cortex (Vogt and Peters, 1981; Zilles and Wree, 1995). Rgb cortex is located within the transition zone between the three-layered hippocampal archicortex and neocortex and is known to be strongly interconnected with other limbic structures, including those implicated in epilepsy (Cardoso et al., 2008b). In particular, the Rgb cortex receives afferent input from and projects heavily to the hippocampal formation, mainly via the subicular complex (Wyss and Van Groen, 1992; Shibata, 1998; Van Groen and Wyss, 2003; Miyashita and Rockland, 2007). Additionally, Rgb area is reciprocally connected with anteroventral and anterodorsal thalamic nuclei, structures known to be specifically recruited in the propagation of limbic seizures within the Papez circuit (Wyss and Van Groen, 1992; Sherman et al., 1997; Van Groen and Wyss, 2003; Cardoso et al., 2008b). It is plausible that Rgb, being interposed between structures implicated in epilepsy like those listed above, may be a crucial neural hub involved in integrating thalamocortical activity during the initiation and/or propagation of generalized seizures (Cardoso et al., 2008b). Furthermore, the retrosplenial cortex may serve as a supplementary limbic pathway interconnecting the anterior thalamus and medial temporal lobe structures (Kim et al., 2007).

Previous studies in TLE patients with hippocampal sclerosis demonstrated that the extent of hippocampal atrophy significantly correlates with loss of cortical gray matter in the retrosplenial cortex. Consistent with this, studies in animal models of TLE revealed that generalized seizures produce a marked increase in blood oxygen level-dependent signal intensity in Rgb (Brevard et al., 2006) and that status epilepticus is associated with atrophic changes in dendrites of Rgb pyramidal neurons (Ampuero et al., 2007). Furthermore, it has been recently demonstrated that status epilepticus and repeated brief generalized seizures are accompanied by loss of neurons and volume reductions in this

cortical area (Cardoso et al., 2008b). Taken together, these data strongly support the hypothesis that Rgb cortex can be critically involved in limbic circuits that mediate seizure genesis and/or propagation.

Whereas it is well documented that seizure activity can lead to alterations in the cholinergic system of the hippocampus, an area well known to be involved in epilepsy, none work has evaluated the effects of epilepsy on cholinergic innervation of retrosplenial cortex. Thus, we hypothesized that if Rgb cortex is involved in seizure activity, as suggested by its connections with other limbic structures implicated in epilepsy, and if limbic seizures are indeed modulated by cortical cholinergic afferent, then the specific markers of cholinergic activity in this cortical area must be altered in epileptic subjects. To test this hypothesis we measured the densities of fiber varicosities immunoreactive to VAChT in the different layers of Rgb of rats treated with pilocarpine which developed spontaneous motor seizures, of rats treated with ECS and of sham-treated control rats.

EXPERIMENTAL PROCEDURES

Animals and treatments

A total of 26 male Wistar rats, obtained from Harlan Iberica (Barcelona, Spain), were used in this study. After arrival, they were maintained under standard laboratory conditions and had free access to food and water. At 2 months of age, the rats were randomly divided into three groups and submitted to the following protocols: pilocarpine-induced SE (group SE, n=8), repeated administration of ECS (group ECS, n=6), and two sham-treated control groups (6 rats in each). Following the respective treatments, the rats were daily observed for spontaneous behavioral seizures at random times between 08:00 h and 20:00 h. Six

animals in each group, selected at random, were killed at 4 months of age by transcardial perfusion and their brains were used for immunocytochemistry. The handling and care of the animals were conducted according to the European Communities Council guidelines in animal research (86/609/UE). All efforts were made to minimize the number of animals used and their suffering.

Pilocarpine model of SE

Animals in SE group were pretreated with scopolamine methyl bromide (1 mg/kg, s.c., Sigma) in order to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high dose of pilocarpine (350 mg/kg, i.p., Sigma) and were observed thereafter for signs of motor seizures. The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4-5 seizures on the Racine scale (1972), i.e. rearing, falling and generalized convulsions, which persisted for at least 2 minutes. SE onset was usually detected 30-60 min following the pilocarpine injection. It has been previously reported that pilocarpine-induced SE, if lasting for several hours, can be associated with high mortality rate which ranges between 15% and 50% depending on the dose of pilocarpine and other experimental conditions (Goodman, 1998; Glien et al., 2001; Williams et al., 2002; Gröticke et al., 2007). Therefore, because animal mortality is a prominent cause of bias in quantitative morphological evaluations (Herguido et al., 1999), special efforts were made in order to improve the survival rate of the animals in SE group. In particular, two hours after the beginning of SE, the rats were injected with diazepam (5 mg/kg, i.p.) in order to cease the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 3 hours after the onset of SE. Furthermore, the animals were periodically injected with

saline (s.c.) during the first 12 h of the recovery period. On the following days, the rat diet was supplemented with apples that were sliced and left at the bottom of the cage. Sham-treated control rats (group sham-SE) received handling and treatment identical to that of experimental rats, including injections of scopolamine and diazepam, but were not treated with pilocarpine.

ECS model

Animals in ECS group received a course of 5 ECS seizures, administered on a 24-h schedule as described by Lukoyanov (2004).

Each stimulation (50 Hz, 60 mA for 1 s) was delivered via ear-clip electrodes wired to a stimulus generator (model 215/IZ, Hugo-Sachs Elektronik, Germany). ECS stimulation produced full tonic-clonic seizures with hind-limb extension lasting for 5-10 s. Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. This protocol is based on the finding that repeated induction of five widely-spaced ECS seizures reduces the capacity of the brain amino acid reuptake system to maintain normal levels of glutamate for a minimum of 2 hr (Rowley et al., 1997), which renders neurons especially vulnerable to seizures elicited during this postictal period (Lukoyanov et al., 2004). Rats in the sham-treated control group (group sham-ECS) received handling identical to that of experimental rats, but were not stimulated.

Tissue preparation

General procedures

Animals were deeply anesthetized with pentobarbital and injected intracardially with 0.1 ml of a heparin solution, followed by 1 ml of 1% sodium nitrite in saline. Then, they were perfused transcardially with 150 ml of 0.1 M phosphate buffer (PB, pH 7.6) for

vascular rinse, followed by 250 ml of a fixative solution containing 4% paraformaldehyde in PB. The brains were removed from the skulls, immersed for 3 h in the fixative and infiltrated overnight in 10% sucrose solution at 4 °C. After trimming away the frontal poles, the blocks were mounted on a vibratome, sectioned in the coronal plane at 40 µm, and the sections were collected as free-floating. Only sections that were cut through the retrosplenial cortex, i.e. between coronal planes corresponding to the levels of approximately 1.6 mm and 7.8 mm posterior to the bregma (Paxinos & Watson, 1998), were collected. From each brain, two adjacent series of sections were separately collected in phosphate-buffered saline (PBS) to be used, respectively, for immunostaining for VAChT and for Nissl staining. The sections were stored until use at -20 °C in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.25 mM polyvinylpyrrolidone in PBS).

Immunostaining for VAChT

Sections were washed twice in PBS and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. The sections were immersed in a 5% solution of rabbit normal serum in PBS (Vector Laboratories), for 30 minutes at room temperature. Thereafter, the sections were incubated for 72h, at 4ºC, in a goat anti-VAChT polyclonal antibody (Chemicon, Temecula, CA, USA; 1:15000 dilution in PBS). Thereafter, the sections were washed twice and incubated with biotinylated anti-goat antibody (Vector Laboratories, Burlingame, CA, USA; 1:400 dilution in PBS). Sections were then treated with avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS). In the two last steps, the incubation was carried out for at least 1 h at room temperature. Following treatment with the peroxidase complex, sections were incubated for 10 min in 0.05% diaminobenzidine (Sigma) to which 0.01% H_2O_2 was

added. Sections were rinsed with PBS for at least 15 min between each step. To increase the tissue penetration, 0.5% Triton X-100 was added to PBS that was used in all immunoreactions and washes. Sections were then mounted on gelatin-coated slides and air-dried. They were dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%) and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA). All procedures were performed on a rocking table.

Nissl staining

Sections were mounted serially on gelatin-coated slides. After air-drying overnight at room temperature, they were stained with Giemsa (West et al., 1991), dehydrated, and coverslipped with Histomount.

Morphometric analysis

The cholinergic varicosities stained with VAChT were counted using a computer-assisted image analyzer (Leica QWin) fitted with a Leica axioplan microscope and a Sony Hyper HAD Digital color video camera. For each animal, approximately 10 VAChT-stained sections were analyzed. Adjacent Nissl-stained sections were used to delineate the boundaries of the Rgb cortex and its layers, which were consistently defined at all levels along the rostrocaudal axis of the brain as previously described in detail (Cardoso et al., 2008b) and using the rat brain atlas of Paxinos and Watson (1998). Measurements were performed separately in layers I, II/III, IV, V and VI at a final magnification of x2500. The varicosities were defined as darkly stained axonal dilations with size greater than 0.25 µm (Wong et al., 1999) (Fig. 1). A sample frame (3.82×10^5 μm^2) was laid over each field of view and the number of varicosities falling within it was counted. Within each cortical layer, three different placements of the frame, each time at a randomly selected position,

were used to obtain a mean count for that layer. The results were expressed as areal densities (number/mm^2).

Statistical analysis

The data were analysed using the Student's t-test. Results were expressed as the mean (SD). Differences were considered to be significant if $P < 0.05$.

RESULTS

Behavioral results

No behavioral alterations were detected in animals from the ECS-treated group and sham-treated control groups. Otherwise, following a latent period lasting 1-3 weeks, spontaneous motor seizures of stage 3 or greater on the Racine scale (1972) were repeatedly observed in all rats that have experienced SE.

Densities of VAChT-immunoreactive varicosities

Morphometric analysis of the VAChT-immunoreactive varicosities in the Rgb cortex of the control group revealed that their density (in number per mm^2) was higher in layers I (64 ± 21) and IV (67 ± 17) when compared to layers II/III (18.5 ± 7.8), V (32 ± 12) and VI (20 ± 5), (Fig. 2).

With respect to the treatment groups, there was a slight increase in the densities of cholinergic varicosities in all layers of the Rgb cortex in the ECS group compared to the control group (Fig. 2A, B and Fig. 3A). However, this effect was not statistically significant. There was a statistically significant increase in the densities of cholinergic

varicosities in the SE group compared to the control group in Rgb layers I (control – 64 ± 21, SE – 143 ± 74, $p < 0.05$), II/III (control – 18.5 ± 7.8, SE – 40 ± 13, $p < 0.05$), IV (control – 67 ± 17, SE – 113 ± 37, $p < 0.05$) and VI (control – 20 ± 5, SE – 36 ± 14, $p < 0.05$). In layer V, the increase in the densities of VAChT varicosities was not statistically significant (Fig. 2A, C and Fig. 3B).

DISCUSSION

The main findings of this study are: 1) pilocarpine-induced SE leads to a significant increase in the densities of cholinergic varicosities in all layers of Rgb cortex with the exception of layer V, 2) ECS administration does not produce any significant change in the densities of cholinergic varicosities in the Rgb cortex, at least, as measured two months after the termination of the treatment.

In the control group, the distribution of the densities of cholinergic varicosities between the layers of Rgb cortex matched well the data reported in a previous study (Gilmor et al., 1996). This previous study showed that in the neocortex the immunoreactivity for the cholinergic buttons is concentrated in layers I, IV and V, whereas layers II, III and VI show fewer staining. Our data are similar, except for the fact that we have found a low density of VAChT varicosities in layer V. This can be due to the fact that in this study we have studied specifically the Rgb cortex whilst the data from the previous study are concerned with the neocortex in general.

In the SE group we found a statistically significant increase in the densities of cholinergic varicosities in all layers of Rgb cortex except in layer V. These data suggest that the retrosplenial cortex can be affected in epilepsy, as it was predicted on the basis of

the strong connections that this cortical area establishes with other limbic areas involved in this disease, such the HF, subiculum and entorhinal cortex (Wyss and Van Groen, 1992; Van Groen and Wyss, 2003; Miyashita and Rockland, 2007; Cardoso et al., 2008b). The possibility that the Rgb cortex can be involved in epilepsy is supported by the results of another recent study, employing the same model pilocarpine-induced SE, which showed significant neuronal loss and volume reductions in Rgb cortex, suggesting that this cortical area is as vulnerable to seizures as the other epilepsy-related brain structures such as HF (Cardoso et al., 2008b). Interestingly, in one of the studies of TLE patients with hippocampal sclerosis, it has been found that hippocampal atrophy significantly correlates with loss of cortical gray matter in the retrosplenial cortex (Düzel et al., 2006). Our study is consistent with these previous results, having in common the findings which point to an involvement of Rgb in epilepsy. Furthermore, to the best of our knowledge the present study is the first to demonstrate that SE induces long-term dysfunction of the cholinergic neurotransmission system in the Rgb cortex. In fact, evidence for cholinergic dysfunction associated with epilepsy is restricted to data obtained in the HF (Friedman et al., 2007). Thus, the present results show that changes in the cholinergic transmission related to seizures occur not only in the hippocampal region, but also in the Rgb cortex which is an area of the neocortex. Although it is already well known that cholinergic functions are altered in the epileptic brain, the exact nature and role of these changes in the pathogenesis of the disease are not known. The results of a previous study point to a potential role of cholinergic mechanisms in epileptogenesis and generation of seizures, mainly in the entorhinal cortex and HF, which are believed to be the site of origin of seizure activity in the majority of patients with TLE (Friedman et al., 2007). The present study shows a more widespread distribution of cholinergic changes in the epileptic brain.

It has been previously reported that SE may be associated with changes in gene expression of key cholinergic proteins, including reduced expression of VAChT and ChAT and overexpression of acetylcholinesterase R isoform (Friedman et al., 2007). These data are in disagreement with our results, because we found that SE leads to an increase in the densities of the cholinergic buttons stained for VAChT. This discrepancy may be due to the fact that in this study we analyzed layer-specific changes in a certain cortical area (Rgb), while in the prior studies the SE-induced changes in gene expression were analyzed in the entire brain.

Interestingly, it has been also reported that the excessive muscarinic activation, which triggers seizures, induced by either agonist application or inhibition of acetylcholinesterase results in long-lasting alterations of gene expression and protein levels of key cholinergic proteins (Kaufer et al., 1998; Soreq and Seidman, 2001; Meshorer and Soreq, 2006). In the HF, such alterations in cholinergic transmission are associated with enhanced muscarinic receptor-mediated responses (Meshorer et al., 2002). At first glance, the increased density of cholinergic varicosities described in the present study might be a marker of an overall increase in the cholinergic transmission in epileptic brain. However, there is growing evidence that epileptiform activity is additionally related to changes in the expression of muscarinic receptors (Mingo et al., 1997; Mingo et al., 1998; Friedman et al., 2007). For example, a down-regulation of M1 receptors following induction of seizures was found in several SE models (Friedman et al., 2007). In contrast, in kindled animals a significant increase in both M1 and M3 receptors expression was found 28 days after kindling (Mingo et al., 1997; Mingo et al., 1998). Thus, taking into consideration these variations in the expression of muscarinic receptors in epileptic models, the resulting effect of the increase in the densities of cholinergic varicosities on the cholinergic transmission is difficult to assess. Moreover, the increase in the densities of cholinergic varicosities could

in fact be an adaptive response to the down-regulation of the muscarinic receptors caused by SE, or alternatively it may be one of the primary events of the epileptogenic process.

In addition to this mystery of whether the increase in the densities of Rgb cholinergic varicosities reflects an increase in the cholinergic transmission or its decrease, the picture is further complicated by the fact that acetylcholine can influence synaptic integration both through activation of classical cholinergic synapses and through modulation of noncholinergic synapses (Friedman et al., 2007). Considered together, the results of this and previous studies rise the question of whether observed changes in cholinergic markers are a cause or consequence of epilepsy, the question which is difficult to answer so far. Nevertheless, these findings are rather solid in what they clearly show that the cholinergic transmission is altered in the SE models of epilepsy. The changes in the cholinergic system may be a part of the plastic events which occur after SE and which lead to the establishment of epileptogenic neural networks and disease progression. This possibility is supported by the findings that acute cholinergic insult of pilocarpine injection triggers an overwhelming set of plastic events (Guidine et al., 2008). The plastic changes associated with prolonged seizures are known to include remodeling of neuritis and reorganization of synaptic connections in the dentate gyrus, hippocampus proper, entorhinal and subicular cortices (Ampuero et al., 2007; Cardoso et al., 2008a). This reorganization can also include the increase in the densities of cholinergic varicosities, as it is well known that the neuritic and synaptic plasticity is dependent, partly at least, on the activity of the cholinergic system (Cardoso et al., 2006).

We have found no increase in the densities of cholinergic varicosities in the ECS group, the model of brief generalized seizures. The lack of changes in this group can be related to the fact that, contrary to SE, ECS treatment is not epileptogenic as indicated by the absence of occurrence of recurrent unprovoked seizures in this model. In other words,

ECS seizures do not trigger the same plastic events which lead to the formation of epileptogenic neural networks and recurrent seizures as does SE. Another explanation for the difference between the two seizure models used in the present study is that the changes in VAChT staining found in the SE group may be related to the neurotoxic effects of the pilocarpine and not to its epileptogenic effect. Indeed, it has been reported that degenerative changes in the brain of pilocarpine-treated animals might be related to neurotoxic effects of this drug rather than to the seizures it induces (Cardoso et al., 2008b).

In conclusion, the present findings support the cholinergic hypothesis of epilepsy. For future experiments it would be interesting to answer the question if the SE-induced increase in the densities of cholinergic varicosities is also present in other cortical areas or if it is specific to the retrosplenial cortex due to its strong connections with limbic structures critically involved in epilepsy. Another fruitful experiment would be to assess the expression of muscarinic receptors in the retrosplenial cortex of epileptic rats in order to address the issue of whether the increase in the density of VAChT-positive varicosity corresponds to a real increase in the cholinergic transmission in the epileptic brain or it merely reflects an adaptive response to the loss of cholinergic receptors. In addition, it would be appealing to evaluate the state of the cellular bodies of cholinergic neurons of the basal forebrain, which are the origin of the cholinergic terminals that innervate the neocortex, to evaluate if the increase in the densities of cholinergic varicosities is associated or not with changes in the volume of those neurons.

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The author confirms that he has read the Journal's position on issues involved in ethical publication and affirms that this report is consistent with those guidelines. The author has no conflicts of interest to disclose.

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FIGURE LEGENDS

Fig. 1. Photomicrograph of a brain section of a sham-treated control rat that was cut through the retrosplenial granular b (Rgb) cortex and immunostained for VAChT. The image was taken from cortical layer V. VAChT-containing fiber varicosities used for counting purposes were defined as darkly stained axonal dilations with size greater than 0.25 μm^2. Scale bar = 25 µm.

Fig. 2. Representative photomicrographs of VAChT-immunostained brain sections cut through the retrosplenial granular b (Rgb) cortex of a sham-treated rat (**A**), an ECS-treated rat (**B**), and a pilocarpine-treated rat (**C**). Note that the density of VAChT-positive varicosities in the Rgb cortex of the pilocarpine-treated animal is markedly higher when compared to the control rat. In the ECS-treated rat, it also appeared somewhat increased relative to the control rat. Scale bar = 200 µm.

Fig. 3. Graphic representation of the areal density of VAChT-immunoreactive varicosities (number/mm^2) in layers I, II/III, IV, V and VI of the retrosplenial granular b cortex of ECS-treated rats (**A**) and rats which experienced status epilepticus (SE group, **B**). The data from the respective sham-treated control groups are also shown. Note that animals from the SE group had significantly more cholinergic fiber terminals than control rats in all cortical laminae, except in lamina V. The density of the cholinergic varicosities in the retrosplenial cortex of ECS-treated rats did not significantly differ from that of control rats. $*P < 0.05$ versus sham-treated control rats.

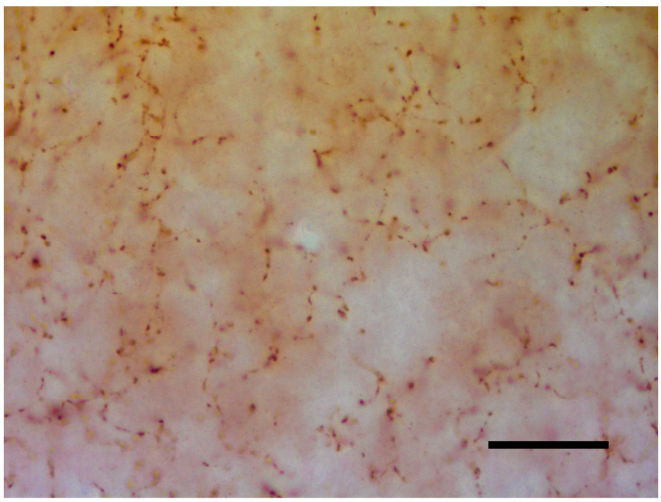


Figure 1

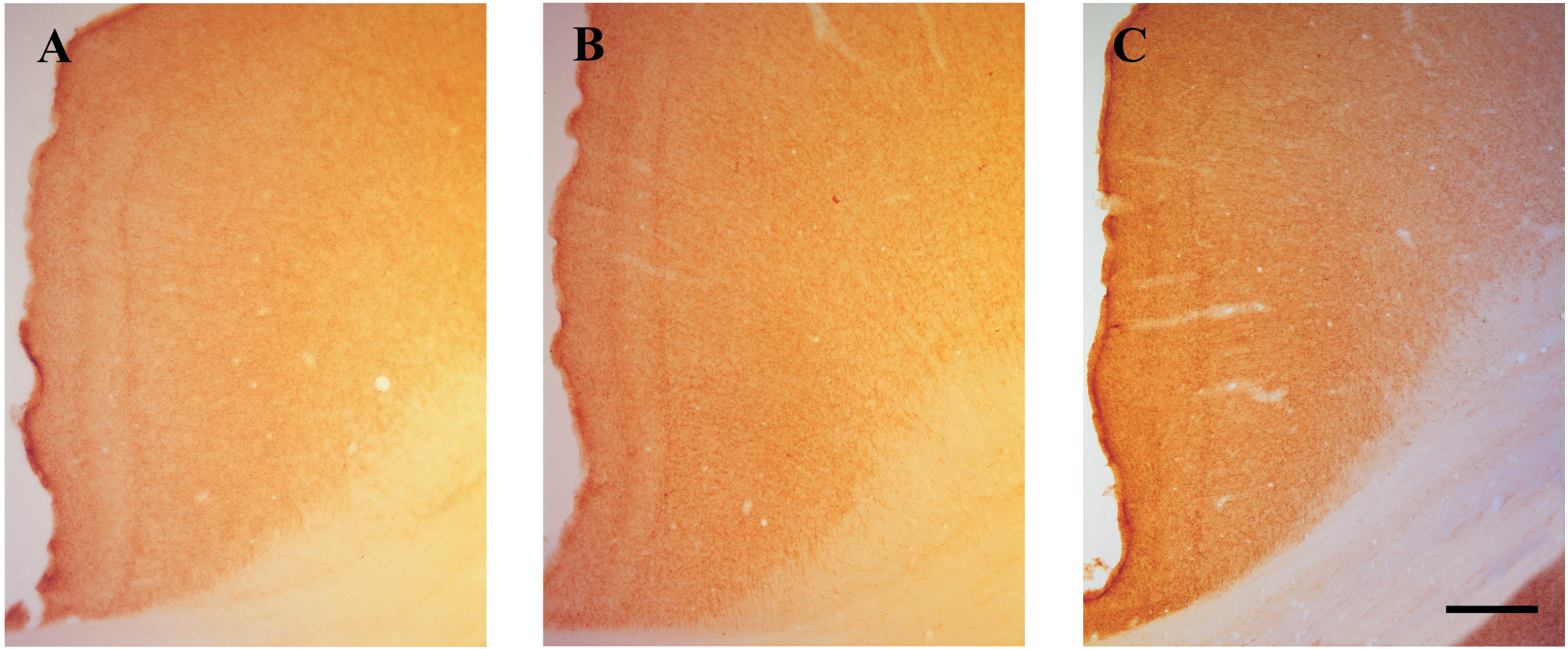
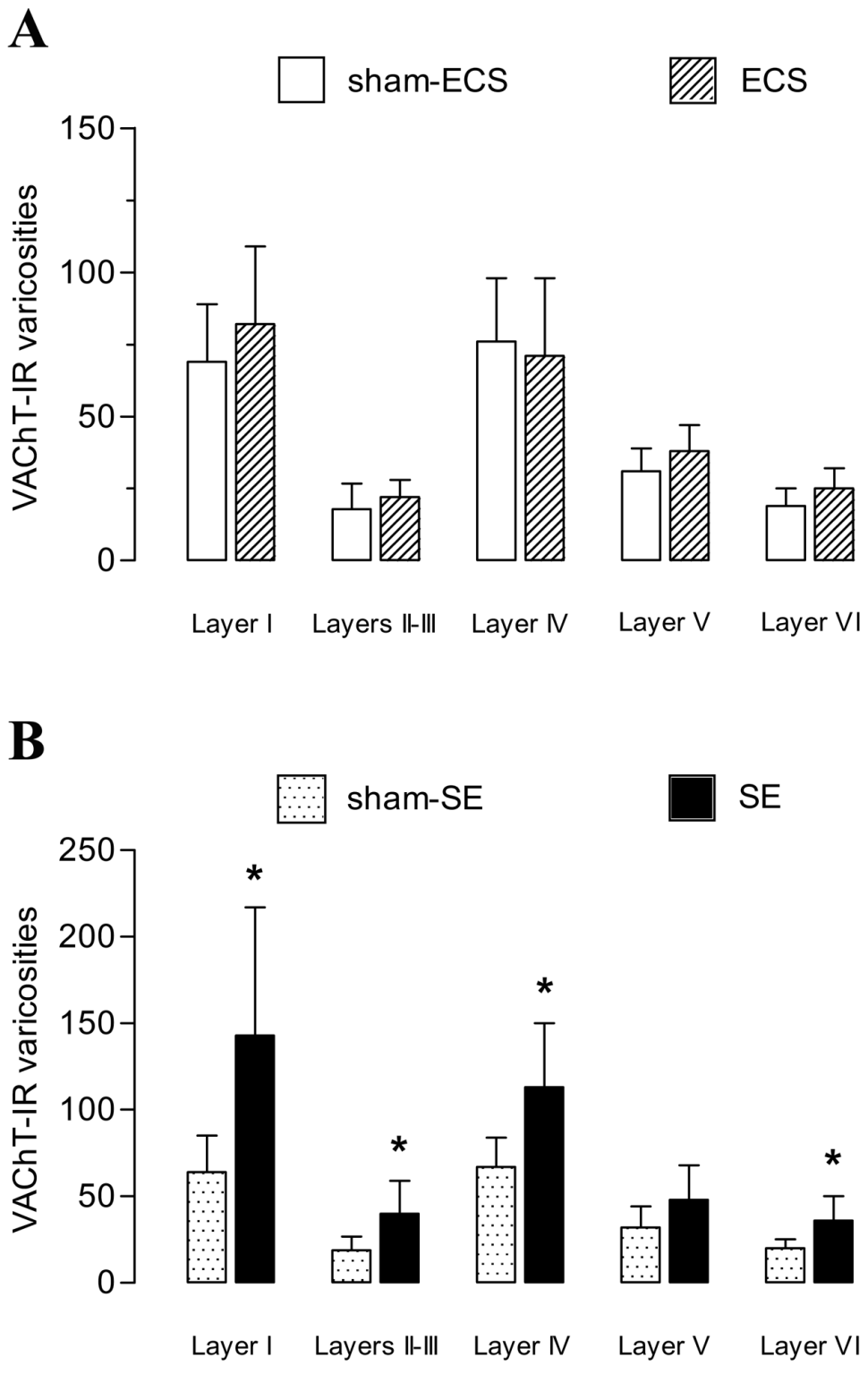


Figure 2


A
sham-ECS
ECS
VAChT-IR varicosities
150
100
50
0
Layer I
Layers II-III
Layer IV
Layer V
Layer VI
B
sham-SE
SE
VAChT-IR varicosities
250
200
150
100
50
0
*
*
*
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Layer I
Layers II-III
Layer IV
Layer V
Layer VI

Figure 3