Neuroscience Letters 436 (2008) 201–204

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Vesicular acetylcholine transporter knock-down mice are more susceptible to pilocarpine induced status epilepticus

Patrícia A.M. Guidine[a], Gustavo H.S. Rezende[a], Cláudio M.T. Queiroz[d], Luiz Eugênio Mello[d], Vânia F. Prado[b], Marco A.M. Prado[c], Grace S. Pereira[a], Márcio F.D. Moraes[a,*]

[a] *Núcleo de Neurociências, Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, Avenida Antônio Carlos, 6627, CEP 31270-901, Campus Pampulha, Belo Horizonte, MG, Brazil*
[b] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. Avenida Antônio Carlos, 6627, Pampulha, (31270-901) Belo Horizonte, MG, Brazil*
[c] *Programa em Farmacologia Molecular, Departamento de Farmacologia; Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos, 6627, Pampulha, (31270-901) Belo Horizonte, MG, Brazil*
[d] *Departamento de Fisiologia, Universidade Federal de São Paulo, Rua Botucatu, 862-04023-062 São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 16 January 2008
Received in revised form 7 March 2008
Accepted 8 March 2008

Keywords:
Vesicular acetylcholine transporter
Pilocarpine
Seizures

ABSTRACT

The pilocarpine (PILO) animal model of Temporal Lobe Epilepsy (TLE) portrays the most common changes in hippocampal circuitry found in human TLE. The acute cholinergic insult induces *status epilepticus* (SE), which triggers an overwhelming set of plastic events that result on late spontaneous recurrent limbic seizures. It has been suggested that the cholinergic system plays an important role in the synchronization required for ictogenesis. We took advantage of a knock-down animal model for the vesicular acetylcholine transporter (VAChT KD) to investigate seizure genesis in a model of cholinergic dysfunction. We induced SE in VAChT KD and wild-type (WT) mice by a single intraperitoneal injection of PILO in order to evaluate susceptibility to seizures. Video-EEG recordings evaluated epileptiform activity and ictal behavior onset. The hypothesis tested is that innate cholinergic hypofunction could result in increased susceptibility to PILO. VAChT KD^{HOM} mice showed shorter latency for the first epileptiform discharge and for the first seizure episode, when compared to other groups. The duration of these seizure episodes, however, were not statistically different among experimental groups. On the other hand, VAChT KD^{HOM} had the shortest latency to isoelectric EEG, when compared to WT and KD^{HET}. Our results indicate that a reduction of brain VAChT protein to the levels found in VAChT KD^{HOM} mice alters the epileptic response to PILO. Thus, fine-tuning modulation of cholinergic tone can affect the susceptibility of epileptic responses to pilocarpine.

With a prevalence of 1–2% of the world population, epilepsies are complex neurobehavioral disorders resulting from increased excitability and synchronism of neurons in various brain regions [14]. Epilepsy is considered to be a syndrome characterized by unprovoked recurrent seizures in the absence of toxic-metabolic or febrile diseases. The understanding of what drives neural networks to dysfunctional hyperexcitable and hypersynchronic behavior is an important milestone in the field of epileptology. Muscarinic acetylcholine receptors are known to be involved in the control of many peripheral as well as central cholinergic responses [4], which, in turn, are associated to physiological processes such as learning and memory [3,10]. Muscarinic receptors are also involved in the generation of epileptic seizures [15]. Pilocarpine (PILO), a non-subtype-specific partial muscarinic agonist, induces recurrent chronic seizures after systemic injection which mimics human Temporal Lobe Epilepsy (TLE) [6,5]. In fact, the animal model produced by PILO portrays the most common changes in hippocampal circuitry found in human TLE. Simultaneous injection of the muscarinic antagonist atropine or pirenzepine with pilocarpine prevents the onset of seizures [16], but muscarinic antagonists have no effect on established seizures [9], indicating that mAChRs are involved in the initiation but not the maintenance of epileptic seizures. Thus, in the PILO animal model of epilepsy, the cholinergic system seems to play a major role in driving neural networks into the ictal state, ictogenesis, but is not essential for maintaining recurrent spontaneous seizures.

We have previously described a knock-down animal model of peripheral and central cholinergic hypofunction, mice deficient for the vesicular acetylcholine transporter (VAChT). Heterozygous and homozygous VAChT knock-down mice (VAChT KD) have a 45% and

* Corresponding author. Tel.: +55 31 3499 2930; fax: +55 31 3499 2924.
E-mail address: mfdm@icb.ufmg.br (M.F.D. Moraes).

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doi:10.1016/j.neulet.2008.03.020

65% decrease in VAChT protein expression, respectively [13]. VAChT KD^{HET} mice present a reduction in acetylcholine release in the striatum and frontal cortex and deficits in episodic-like and social memory, while KD^{HOM} demonstrate major neuromuscular deficits in addition to cognitive deficits [13]. These results point to a critical role for vesicular transport of ACh to maintain cholinergic tone and regulate physiological processes.

In this report, we induced SE in VAChT-KD and WT mice by a single intraperitoneal injection of pilocarpine hydrochloride in order to evaluate susceptibility to seizure. The hypothesis tested is that innate cholinergic hypofunction would result in receptor up-regulation and, consequently, increased susceptibility to PILO.

The engineering of VAChT knock-down mutant mice was described in detail elsewhere [13]. Heterozygous mutant VAChT mice were backcrossed with C57BL/6J animals for three generations (N3). Homozygous mutant VAChT mice (KD^{HOM}) were obtained by intercrossing N3 heterozygous (KD^{HET}) animals. Thus, all VAChT KD mice used in this study were N3. Wild-type (WT) littermate mice of the same sex and age were used as controls for the experiments. Mice were housed in groups of three to five animals per cage in a temperature-controlled room ($22 \pm 1\,^{\circ}C$) with a 12-h light:12-h dark cycles, food and water were provided ad libitum. Mouse colonies were maintained at the Federal University of Minas Gerais, Brazil, in accordance with NIH guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais (Protocol no. 073/03).

All three groups of mice were submitted to the same experimental procedure: WT, KD^{HET} and KD^{HOM}. Mice were anesthetized with 0.8–3% halothane (Cristalia, Itapira, Brazil) delivered via an inhalation mask with a flow rate of 1–2 L/min oxygen. Prophylactic treatment with antibiotics (enrofloxacin, 10 mg/kg s.c.; Fort Dodge Saúde Animal LTDA, Campinas, Brazil) was done in order to prevent post-surgical infections. The mice were submitted to surgery for EEG electrode implantation in the right and left parietal cortexes. The electrodes for superficial EEG recording were made with surgical screws (Fine Science Tools; model 19010-00; Foster City, CA, USA), previously soldered to Teflon-coated stainless-steel wires (A&M Systems; model 7916; Carlsborg, WA, USA) and introduced bilaterally in the parietal bones. A reference electrode, also made with surgical screws, was inserted in the nasal bone. All the electrodes were soldered to a common pin connector and anchored to the cranium with dental acrylic. Following surgery, animals were allowed to recover for at least 4 days before the injection of pilocarpine.

The *status epilepticus* (SE) were induced by a single intraperitoneal administration of pilocarpine hydrochloride (400 mg/kg; Sigma). Scopolamine methyl-nitrate (1 mg/kg, s.c.; Sigma) was injected 30 min before pilocarpine to reduce peripheral cholinergic effects. Following pilocarpine administration, mice were recorded until death or for a maximum period of 40 min.

The right and left cortexes bioelectrical activity was recorded with a video-EEG recording system (5:00–8:00 a.m.). The video-EEG setup is explained in detail elsewhere [11]. The EEG signal was amplified (5000–10,000 × gain), filtered (1 Hz high pass and 200 Hz low pass), sampled at 1 kHz in all channels and stored in a computer. The filtered signals were plotted in real time on the computer monitor along with the live image of the animal's behavior captured through a video camera. The integration of the EEG plots and the live video images was done using a video card (ATI Technologies; All-In Wonder Pro, Sunnyvale, CA, USA). The composite computer monitor images were converted to a VHS videocassette-recording format. The recording protocol consisted of 10 min before the pilocarpine injection (baseline recordings) immediately followed by 40 min of recording or until death.

The EEG records were analyzed according to the following parameters: (1) time of death, defined as total recording duration between the pilocarpine injection and the isoelectric EEG; (2) latency of the first epileptiform discharge in EEG; (3) latency for the beginning of the first seizure; (4) duration of the first seizure. The Kruskal–Wallis test, with Dunn's post hoc, was used to compare the evaluated parameters among the groups of animals. Results are expressed as median ± interquartile range.

Within few minutes after pilocarpine injection, piloerection, tremor, salivation, diarrhea, immobility, staring, facial automatisms, head nodding and uni/bilateral forelimb clonus was observed in all the animals which developed seizure ($n = 20$). Only three animals (one from the KD^{HET} group and two from WT group)

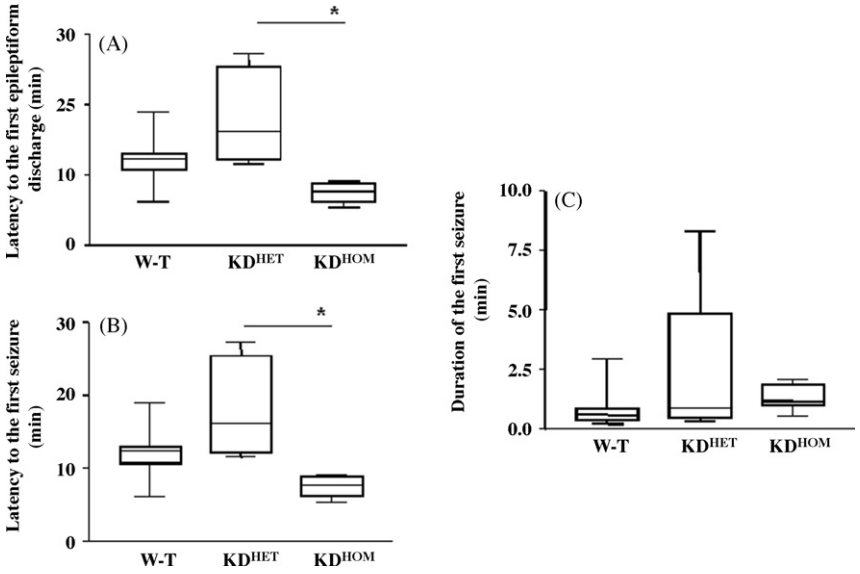

Fig. 1. EEG alterations of VAChT KD^{HET} and VAChT KD^{HOM}. (A) Latency to the first epileptiform discharge. Ordinates express median (interquartile range) latency to the firs epileptiform discharge, in minutes. Asterisk (*) indicates a significant difference from KD^{HOM}, compared with KD^{HET} ($^{*}p < 0.01$). Kruskal–Wallis, with Dunn's post hoc. (B) Latency to the first seizure. Ordinates express median (interquartile range) latency to the first seizure, in minutes. Asterisk (*) indicates a significant difference from KD^{HOM}, compared with KD^{HET} ($^{*}p < 0.01$). Kruskal–Wallis, with Dunn's post hoc. (C) Duration of the first seizure. Ordinates express median (interquartile range) duration of the first seizure, in minutes. Kruskal–Wallis, with Dunn's post hoc ($p < 0.05$).

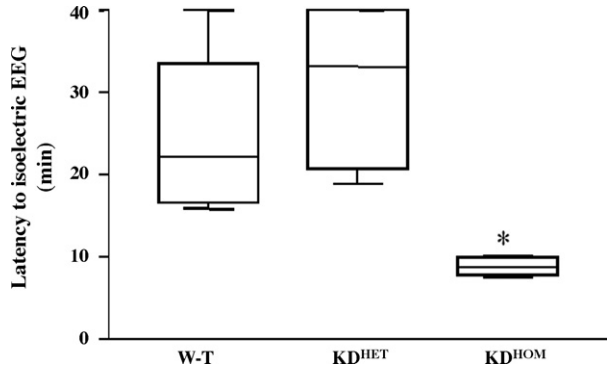

Fig. 2. Latency to isoelectric EEG (brain death) in both right and left parietal cortexes in VAChT KD^{HET}, KD^{HOM} and WT mice. Ordinates express median (interquartile range) latency to isoelectric EEG, in minutes. Asterisk (*) indicates a significant difference from KD^{HOM}, compared with WT ($^{*}p < 0.05$). Kruskal–Wallis, with Dunn's post hoc.

did not develop seizure after the pilocarpine injection, two of them remained without epileptiform alterations during the entire recording window. All remaining KD^{HET} animals went into SE. Fig. 1A depicts the latency for first epileptiform discharge, Fig. 1B the latency to first behavioral seizure and Fig. 1C the duration of first epileptiform discharge. The KD^{HOM} mice showed shorter latency for the first epileptiform discharge and for the first seizure episode, compared with WT and KD^{HET} mice groups. KD^{HET} in contrast did not differ from controls (WT) in any of these measures. In addition, we did not find any difference between the experimental groups regarding the duration of the first seizure. On the other hand (see Fig. 2), the KD^{HOM} had the shortest latency to isoelectric EEG ($8.6 \pm 7.8/9.4$ min), when compared with the other groups (KD^{HET}: $30.8 \pm 21.1/40$ min; WT: $25 \pm 17/30.2$ min).

The present study demonstrates that VAChT KD^{HOM} display a reduced seizure threshold after pilocarpine administration, thus suggesting higher susceptibility to cholinergic insults. There were good grounds for choosing to test the effects of pilocarpine in this animal model. Hamilton et al. [7] demonstrated that mice deficient of the M1-receptor gene are resistant to pilocarpine-induced seizures, but are susceptible to seizures caused by kainic acid. In a posterior study, Bymaster et al. [2] evaluated the seizures induced by pilocarpine (300 mg/kg) in muscarinic M1, M2, M3, M4 and M5-receptor knockout mice. They found that only M1 knockout mice are resistant to seizure and lethality induced by pilocarpine. The acetylcholinesterase mice knockouts are also insensitive to pilocarpine-induced seizures. Muscarinic receptor binding sites and proteins are downregulated, but no decrease in mRNA levels for these receptors was detected in AChE KO [8]. Altogether, these results suggest that M1-receptors are essential for pilocarpine-induced seizures. Additionally, if the acetylcholine levels were persistently increased, the M1-receptors will be downregulated and consequently the pilocarpine-induced seizures will be abolished.

VAChT KD^{HOM} provides a novel hypocholinergic model [13] and, in the present report, VAChT KD^{HOM} mice were more susceptible to the effects of pilocarpine. We postulate that this alteration results from an upregulation or over-activation of M1-receptors and this could be the cause of reduced seizure threshold in VAChT KD^{HOM}. In fact, upregulation of receptors is generally associated with increased sensitivity to agonists. Muscarinic M1-receptors are positively coupled to phosphoinositide (PI) hydrolysis by action of phospholipase Cβ [12]. In general, partial agonists like pilocarpine, can give robust responses only if there is sufficient receptor reserve, efficient coupling or signal amplification within the signal transduction pathway. Pilocarpine administered centrally or systemically produces a robust in vivo PI hydrolysis response in mouse hippocampus, suggesting that there is significant receptor reserve for the PI response in vivo. This response is predominantly mediated by the muscarinic M1-receptors [1]. Future studies are necessary to confirm if PI hydrolysis and consequently M1-receptor reserve is involved in exacerbate response of VAChT KD^{HOM} to pilocarpine injection.

Overall the data in this study support the conclusion that a moderate reduction in acetylcholine availability (VAChT KD^{HET}) did not induce alterations in: (1) the latency of the first epileptiform discharge, (2) the beginning of the SE and (3) the severity of the SE. However, VAChT KD^{HOM} mice were affected in all parameters cited above, indicating that a more drastic reduction of brain VAChT protein, by more than 65%, may induce cholinergic dependent plastic alterations in a way that is relevant for the induction of pilocarpine ictogenesis. Thus, a fine-tuning modulation of cholinergic tone can profoundly affect the susceptibility of pilocarpine.

Acknowledgements

We are grateful to José Eustáquio Oliveira, for technical assistance, and to CNPq, FAPEMIG-PRONEX, CAPES, for financial support. Márcio Flávio Dutra Moraes, Vânia Ferreira Prado, Danuza M. Diniz, Diogo A. Guimarães, Luiz Eugênio Mello and Marco Antônio M. Prado are recipients of CNPq research fellowships.

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