NEUROSCIENCE
INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION

RESEARCH ARTICLE

M. L. de Freitas et al. / Neuroscience xxx (2018) xxx–xxx

Na^+, K^+-ATPase Activating Antibody Displays *in vitro* and *in vivo* Beneficial Effects in the Pilocarpine Model of Epilepsy

Mayara Lütchemeyer de Freitas,[a] Clarissa Vasconcelos de Oliveira,[a] Fernanda Kulinski Mello,[a] Vinícius Rafael Funck,[a] Michele Rechia Fighera,[a] Luiz Fernando Freire Royes,[a] Ana Flávia Furian,[a] James W. Larrick[b] and Mauro Schneider Oliveira[a]*

[a] *Graduate Program in Pharmacology, Federal University of Santa Maria, Santa Maria, Brazil*

[b] *Panorama Research Institute, 1230 Bordeaux Dr, Sunnyvale, CA 94089, United States*

Abstract—Na^+, K^+-ATPase is an important regulator of brain excitability. Accordingly, compelling evidence indicates that impairment of Na^+, K^+-ATPase activity contributes to seizure activity in epileptic mice and human with epilepsy. In addition, this enzyme is crucial for plasma membrane transport of water, glucose and several chemical mediators, including glutamate, the major excitatory transmitter in the mammalian brain. Since glucose hypometabolism and increased glutamate levels occur in clinical and experimental epilepsy, we aimed the present study to investigate whether activation of Na^+, K^+-ATPase activity with specific antibody (DRRSAb) would improve glucose uptake and glutamate release in pilocarpine-treated mice. We found decreased uptake of the glucose fluorescent analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-il)amino]-2-desoxi-D-glucose (2-NBDG) in cerebral slices from pilocarpine-treated animals. Interestingly, decreased 2-NBDG uptake was not detected in DRRSAb-treated slices, suggesting a protective effect of the Na^+, K^+-ATPase activator. Moreover, DRRSAb prevented the increase in glutamate levels in the incubation media of slices from pilocarpine-treated mice. In addition, *in vivo* intrahippocampal injection of DRRSAb restored crossing activity of pilocarpine-treated mice in the open-field test. Overall, the present data further support the hypothesis that activation of the Na^+, K^+-ATPase is a promising therapeutic strategy for epilepsy.

Key words: sodium pump, epilepsy, pilocarpine, hypometabolism, excitotoxicity.

INTRODUCTION

The Na^+, K^+-ATPases constitute a family of plasma membrane proteins which have a crucial role in maintaining ionic homeostasis in virtually all mammalian cells (Aperia, 2012). By adjusting the gradient of Na^+ and K^+ across the plasma membrane, this enzyme indirectly modulates the intracellular concentration of other ions such as Ca^{2+}, Cl^- and H^+, as well as the transmembrane movement of water, glucose and several chemical mediators (Aperia, 2012). In the brain, the Na^+, K^+-ATPase is a major regulator of neuron excitability, being a primary contributor to the electrochemical gradient underlying neuronal after-hyperpolarization and resting potential (Gulledge et al., 2013). Moreover, Na^+-coupled secondary transport of glutamate, the major excitatory neurotransmitter in the mammalian brain, largely depends on the activity of the Na^+, K^+-ATPase. Furthermore, glutamate transporters and Na^+, K^+-ATPases are part of a macromolecular complex which regulates glutamatergic neurotransmission (Rose et al., 2009).

Many neurological disorders have been associated with changes in the activity of the Na^+, K^+-ATPases (Aperia, 2007; Benarroch, 2011; Holm et al., 2016). For instance, reduced Na^+, K^+-ATPase activity contributes to the initiation and/or spreading of seizures in epileptic mice and human epilepsy (Clapcote et al., 2009; Poulsen et al., 2010; Holm et al., 2016). Moreover, decreased Na^+, K^+-ATPase activity has been found in the epileptic human cerebral cortex (Rapport et al., 1975) and in the hippocampus of mice in the pilocarpine post-status epilepticus (SE) model of epilepsy (Funck et al., 2014). Altogether, these clinical and experimental findings may support Na^+, K^+-ATPase as an interesting target for epilepsy. In support of this point, we and others recently reported that intrahippocampal injection of DRRSAb, a site-specific antibody which activates the Na^+, K^+-ATPase (Zheng et al., 2011; Funck et al., 2015), decreased seizure susceptibility in post-SE mice (Funck et al., 2015).

*Corresponding author. Address: Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Departamento de Fisiologia e Farmacologia, Av. Roraima, n° 1000, Prédio 21, sala 5207, CEP 97105-900 Santa Maria, RS, Brazil. Fax: +55-55-3220-8241.
E-mail address: ms.oliveira@ufsm.br (M. S. Oliveira).

Abbreviations: 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-il)amino]-2-desoxi-D-glucose; aCSF, artificial cerebrospinal fluid; AMC, aminomethylcoumarin; FRAP, ferric-reducing antioxidant power; SE, status epilepticus; TLE, temporal lobe epilepsy.

https://doi.org/10.1016/j.neuroscience.2018.02.044
0306-4522/

Please cite this article in press as: de Freitas ML et al. Na^+, K^+-ATPase Activating Antibody Displays *in vitro* and *in vivo* Beneficial Effects in the Pilocarpine Model of Epilepsy. Neuroscience (2018), https://doi.org/10.1016/j.neuroscience.2018.02.044

Considering that glucose hypometabolism and increased glutamate levels are typically found in temporal lobe epilepsy (TLE) in humans (Cavus et al., 2005; Boling et al., 2008) and experimental models (Upreti et al., 2012; Zhang et al., 2015), and that these neurochemical parameters depend on the activity of the Na^+, K^+-ATPase, we sought to investigate the effect of DRRSAb antibody on glucose uptake and glutamate release in post-SE mice. In addition, since behavioral comorbidities are difficult to treat in many patients with epilepsy (Berg et al., 2017), and Na^+, K^+-ATPase activity modulates locomotor activity, anxiety- and depressive-like behavior (Moseley et al., 2007; Kirshenbaum et al., 2011), we evaluated the effect of DRRSAb antibody on behavioral tests in post-SE mice.

EXPERIMENTAL PROCEDURES

Animals

Ninety-three male C57BL/6 mice (20- to 25-g; 35- to 45-day-old) from the institutional vivarium of the Federal University of Santa Maria were used. Animals were kept under an artificial light–dark cycle (lights on from 7:00 to 19:00) and in a room with controlled temperature (22 ± 3 °C). Mice had free access to filtered water and rodent chow (Purotrato™, Santa Maria, RS, Brazil). Experimental design was planned to keep the number of animals to a minimum as well as to minimize their suffering. Studies were conducted in accordance with national and international legislation (guidelines of Brazilian Council of Animal Experimentation – CONCEA – and of U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria (protocol number 6262060616).

DRRSAb antibody

The polyclonal rabbit antibody DRRSAb binds to an activation site located at the ^{897}DVEDSYGQQWTYEQR^{911} (D-R) region of the α-subunit of the Na^+, K^+-ATPase (Zheng et al., 2011). Binding of DRRSAb alters Na^+, K^+-ATPase conformation and increases its Mg^{2+}/ATP affinity, augmenting Na^+/K^+ transport (Xu, 2005). The batch used in the present study was kindly provided by Dr. James W. Larrick (Panorama Research Inc., Sunnyvale, CA, USA). The concentration and schedule for DRRSAb incubation was based on the literature (Zheng et al., 2011; Funck et al., 2015) and on pilot experiments. Doses and schedule for *in vivo* injection of DRRSAb was based on our previous study (Funck et al., 2015).

Pilocarpine-induced SE

To induce SE we used a ramping protocol based on repetitive administration of low doses of pilocarpine (100 mg/kg, i.p.) (Groticke et al., 2007). Importantly, this approach has been shown to increase the number of mice displaying SE and to reduce mortality after SE. Briefly, thirty minutes before the injections of pilocarpine, mice received methylscopolamine (1 mg/kg, i.p.) to minimize peripheral cholinergic effects. Pilocarpine hydrochloride (Sigma–Aldrich, St. Louis, Missouri) was dissolved in 0.9% NaCl to 10 mg/mL and intraperitoneally administered every 20 min until onset of SE. Regarding this point, after injection of pilocarpine animals displayed a diverse behavioral repertoire ("limbic seizures") that included tremor or twitches involving head, limbs and/or whole body, forelimb and/or tail extensions (Winawer et al., 2011). In this context, SE was defined by occurrence of continuous limbic seizures starting after a generalized seizure. The total number of pilocarpine injections was limited to 6 injections per animal. After 60 min of SE diazepam (10 mg/kg, i.p.) was injected to quell SE severity (Gualtieri et al., 2012). Age-matched controls received methylscopolamine and diazepam, but received only 0.9% NaCl instead of pilocarpine. In attempt to maximize animal welfare after SE all mice received softened chow and fresh fruits (apples and bananas) and daily injections of Ringer's solution (containing 5% dextrose) for three days following the SE or control procedure.

Hippocampal slices

All *in vitro* experiments were carried out on hippocampal slices since decreased Na^+, K^+-ATPase activity has been found in this brain area after pilocarpine-induced SE (Funck et al., 2014). Horizontal slices (350-μm-thick) of hippocampi and associated entorhinal cortices were prepared (Upreti et al., 2012) from pilocarpine-treated and age-matched control mice 40–60 days after SE. Mice were decapitated and their brains were rapidly obtained and immersed in ice-cold sucrose-based cutting solution (in mM: 87 NaCl, 25 $NaHCO_3$, 10 glucose, 75 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$ and 4 $MgCl_2$). Slicing procedures were performed at 4 °C using a VT1000S vibratome (Leica). Slices were kept in cutting solution at 32 °C for 30 min, and then transferred to a holding chamber at room temperature containing artificial cerebrospinal fluid (aCSF; in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, 25 glucose and 10 sucrose). The solutions used for slice preparation and incubation were adjusted to pH 7.4, 290–310 mOsM and continuously aerated with carbogen (95% O_2/5% CO_2). Slices were allowed to rest and equilibrate for at least 60 min before starting an experiment. Slice viability was checked in every experiment by measuring LDH release in the incubation media (Oliveira et al., 2009).

Glucose uptake

Glucose uptake was measured in hippocampal slices using the glucose fluorescent analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-il)amino]-2-desoxi-D-glucose (2-NBDG) according to the methodology described by Itoh et al. (2004) with brief modifications. After a 60-min equilibration period the hippocampal slices from control and pilocarpine-treated animals were transferred to individual tubes (2–3 slices per treatment) and preincubated for 15 min in either aCSF (control) or DRRSAb (3 μM). After preincubation the medium of all tubes was replaced by aCSF containing 2-NBDG (30 μM) and incubation was

Please cite this article in press as: de Freitas ML et al. Na^+, K^+-ATPase Activating Antibody Displays *in vitro* and *in vivo* Beneficial Effects in the Pilocarpine Model of Epilepsy. Neuroscience (2018), https://doi.org/10.1016/j.neuroscience.2018.02.044