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The immunoproteasome β5i subunit is a key contributor to ictogenesis in a rat model of chronic epilepsy

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ABSTRACT

The proteasome is the core of the ubiquitin proteasome system and is involved in synaptic protein metabolism. The incorporation of three inducible immuno-subunits into the proteasome results in the generation of the so-called immunoproteasome, which is endowed of pathophysiological functions related to immunity and inflammation. In healthy human brain, the expression of the key catalytic β5i subunit of the immunoproteasome is almost absent, while it is induced in the epileptogenic *foci* surgically resected from patients with pharmaco-resistant seizures, including temporal lobe epilepsy.

We show here that the β5i immuno-subunit is induced in experimental epilepsy, and its selective pharmacological inhibition significantly prevents, or delays, 4-aminopyridine-induced seizure-like events in acute rat hippocampal/entorhinal cortex slices. These effects are stronger in slices from epileptic *vs* normal rats, likely due to the more prominent β5i subunit expression in neurons and glia cells of diseased tissue. β5i subunit is transcriptionally induced in epileptogenic tissue likely by Toll-like receptor 4 signaling activation, and independently on promoter methylation.

The recent availability of selective β5i subunit inhibitors opens up novel therapeutic opportunities for seizure inhibition in drug-resistant epilepsies.

1. Introduction

Epilepsy is a CNS disorder characterized by recurrent seizures often associated with emotional and cognitive dysfunctions. Up to 40% of people with epilepsy experience seizures that are not controlled by currently available drugs, thus prompting the search of new targets offering novel therapeutic options (Weaver and Pohlmann-Eden 2013). In this context, clinical and experimental findings support a key role of immune and inflammatory processes in seizure pathogenesis in drug-resistant forms of epilepsy (Vezzani et al., 2011a). Notably, specific pro-inflammatory molecules and immune pathways contribute to seizure generation and epileptogenesis in experimental models (Pitkanen and Lukasiuk, 2011; Ravizza et al., 2011; Vezzani et al., 2013). We showed recently that the inducible and proteolytically active β1i and β5i immuno-subunits of the proteasome are induced in neurons and glia in surgically resected temporal lobe epilepsy (TLE) hippocampi and in focal cortical dysplasia, whereas they are barely expressed in healthy human brain (Mishto et al., 2006a; Mishto

Abbreviations: 4-AP, 4-aminopyridine; ACSF, artificial cerebrospinal fluid; CNS, central nervous system; i-proteasome, immunoproteasome; HMGB1, High Mobility Group Box-1; LPS, lipopolysaccharide; MCA, 7-amino-4-methylcoumarin; s-proteasome, standard proteasome; SE, status epilepticus; SLE, seizure-like event; TLE, temporal lobe epilepsy; TLR, Toll-like receptor-4; UPS, ubiquitin–proteasome system.

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et al., 2010; Mishto et al., 2011). Similarly, these immuno-subunits are induced in brain cells in chronic neurodegenerative diseases where they can assemble in intermediate-type- or immuno-proteasome (i-proteasome) (Bellavista et al., 2013; Bellavista et al., 2014). This evidence suggests that i-proteasome is part of the pathologic inflammatory cascade described in some brain diseases.

I-proteasome varies from the standard (s)-proteasome (which includes the standard catalytic subunits β1, β2 and β5) with respect to proteolytic properties and for its peculiar regulation of cytokine-mediated inflammation (Groettrup et al., 2010; Bellavista et al., 2013; Mishto et al., 2014). In the CNS, the proteasome (the core of the ubiquitin–proteasome system, UPS) is involved in various metabolic processes, and regulates pre- and post-synaptic plasticity (Tai and Schuman, 2008), including long-term potentiation (Fonseca et al., 2006; Cai et al., 2010; Hamilton et al., 2012; Dong et al., 2014). The pathophysiological consequences of i-proteasome expression in CNS are mostly unexplored, although it was reported that modulation of its activity alters disease development, or its progression, in murine models of autoimmune diseases, such as multiple sclerosis (Bellavista et al., 2013; Basler et al., 2014).

We show here that selective inhibition of the β5i subunit activity using a recently developed drug, prevents or delays 4-aminopyridine (4-AP) induced seizure-like events (SLEs) in combined hippocampal/entorhinal cortex slices from chronic epileptic rats. This effect is greater in epileptic than control rats, likely due to specific induction of β5i subunit in diseased tissue. Activation of specific inflammatory pathways leading to transcriptional induction of the β5i subunit gene may account for protein over-expression in epileptic *foci*.

2. Materials and methods

2.1. Animals

Male Wistar rats were housed at constant temperature (23 ± 1 °C) and relative humidity (60 ± 5%) with free access to food and water and fixed 12 h light/dark cycle. Procedures involving animals and their care were conducted in conformity with institutions guidelines that are in compliance with national (German Tierschutzgesetz, Dec. 9, 2010) and international laws and policies.

2.2. Status epilepticus (SE) induction

Rats received methylscopolamine (1 mg/kg) subcutaneously 30 min before pilocarpine (340 mg/kg intraperitoneally, i.p.; $n = 56$) or 0.9% NaCl (vehicle) in the control group (labeled as *normal*; $n = 20$). SE was behaviorally observed in ~70% of pilocarpine-treated rats (labeled as *PS*; 17 out of 39 PS rats were used in this study) and was attenuated by diazepam (10 mg/kg, i.p., 90 min from SE onset). Two to three months post-SE, rats were continuously video-recorded for 3 days to detect generalized motor seizures. Animals were killed for *ex vivo* electrophysiology after experiencing at least 3 stage 4–5 seizures (Racine, 1972). Seventeen pilocarpine-treated rats that did not develop either SE or spontaneous tonic–clonic seizures (labeled as PNS) were included in this study.

2.3. Electrophysiology

We used a well established *in vitro* model of epileptiform activity (*i.e.*, SLEs), induced by perfusion of 4-AP onto hippocampal/entorhinal cortex slices. 4-AP does not directly interfere with GABA, glutamate receptor channels or Na^+ currents but blocks presynaptic voltage dependent K^+ channels of the Kv1 and Kv3 family, thus causing enhanced release of ACh in the periphery and of glutamate in the brain (Schubert and Heinemann, 1988). The electrophysiological properties of 4-AP- induced SLEs are very similar to *in vivo* evoked seizures (Avoli et al., 1996; Barna et al., 2000; Louvel et al., 2001; Zahn et al., 2008; Uva et al., 2013) and are more stable than those induced by other convulsants such as low Mg. 4-AP induces SLEs both in normal brain slices and in slices from chronic epileptic brain tissue (Louvel et al., 2001). SLEs are characterized by tonic-like and subsequent clonic-like activity (Fueta and Avoli, 1992; Barbarosie et al., 1994; Buchheim et al., 2000) which are readily induced in entorhinal cortex slices; the response of epileptiform activity to standard antiepileptic drugs has validated this *in vitro* model for testing new drugs against difficult-to-treat seizures (Bruckner and Heinemann, 2000).

Rats (normal, $n = 14$; PNS, $n = 10$ and PS, $n = 13$) were decapitated under deep isoflurane anesthesia and their brains rapidly removed and transferred into ice-cold carbogenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (129), $NaHCO_3$ (21), KCl (3), $CaCl_2$ (1.6), $MgSO_4$ (1.8), NaH_2PO_4 (1.25) and glucose (10). Horizontal 400 μm-thick hippocampal/entorhinal cortex slices were cut (bregma 5.6–8.1 mm, plates 55–61; (Paxinos et al., 1985) on a vibrating microtome (Leica Microsystems) and immediately transferred to an interface chamber perfused with carbogenated ACSF at 36 ± 0.5 °C (flow rate: ~1.8 ml/min, pH 7.4, osmolarity: 300 ± 3 mosmol/l). Slices were left in ACSF for 3 h to recover before field potential recordings were started from layer V of the medial entorhinal cortex using glass microelectrodes (5–10 MΩ) filled with ACSF (Sandow et al., 2009). Signals were amplified during the reference input of custom-made two channel amplifiers equipped with negative capacitance compensation and input resistance larger than 10^{11} Ω for one channel and 10^{13} Ω for the other channel following a previously described design (Neher and Lux, 1973; Heinemann and Lux, 1977; Gabriel et al., 2004). Data were stored into a PC for offline analysis using spike software ver. 6.09.

Six slices (three for each hemisphere) were used from each rat brain and randomly divided among those exposed to 100 μM 4-AP + vehicle (control, $n = 74$) or 4-AP + 2 or 10 μM inhibitor (ONX-0914, $n = 44$/dose; PR-825, $n = 30$/dose) dissolved in 0.1% DMSO in ACSF (vehicle). Slices were pre-incubated for 1 h either with ONX-0914, the β5i-specific inhibitor, or with PR825, the β5-specific inhibitor, both covalently binding the respective proteasome subunits. Then, the medium was removed and replaced by ACSF containing 4-AP for 1 h to evoke SLEs (Zahn et al., 2008; Zahn et al., 2012). Outcome measures were the latency to SLEs onset (Fig. 4) and SLE maximal amplitude and duration (*data not shown*).

2.4. Tissue preparation for biochemical and histological analysis

At the end of the electrophysiology experiment, slices in each experimental group were washed in cold ACSF, then either rapidly frozen for western blot assay and proteasome functional analysis, or post-fixed for immunohistochemistry. Before cutting the hippocampal/entorhinal cortex slices for electrophysiology (see Section 2.3), the most temporal part of the entorhinal cortex was dissected out bilaterally from 1 mm-thick slice in normal ($n = 7$), PNS ($n = 6$) or PS ($n = 9$) rats. In each rat, the homotypic areas from both hemispheres were pooled, then divided into two horizontal specimens, rapidly frozen and kept at −80 °C until assay. The two tissue samples in each rat were randomly assigned to gene expression or methylation analysis (Fig. 2).

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