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Proteomic profiling of epileptogenesis in a rat model: Focus on inflammation

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ABSTRACT

Detailed knowledge about the patterns of molecular alterations during epileptogenesis is a presupposition for identifying targets for preventive or disease-modifying approaches, as well as biomarkers of the disease. Large-scale differential proteome analysis can provide unique and novel perspectives based on comprehensive data sets informing about the complex regulation patterns in the disease proteome. Thus, we have completed an elaborate differential proteome analysis based on label-free LC–MS/MS in a rat model of epileptogenesis. Hippocampus and parahippocampal cortex tissues were sampled and analyzed separately at three key time points chosen for monitoring disease development following electrically-induced status epilepticus, namely, the early post-insult phase, the latency phase, and the chronic phase with spontaneous recurrent seizures.

We focused the bioinformatics analysis on proteins linked to immune and inflammatory responses, because of the emerging evidence of the specific pathogenic role of inflammatory signalings during epileptogenesis. In the early post-insult and the latency phases, pathway enrichment analysis revealed an extensive over-representation of *Toll-like receptor signaling*, *pro-inflammatory cytokines*, *heat shock protein regulation*, and *transforming growth factor beta signaling* and *leukocyte transendothelial migration*. The inflammatory response in the chronic phase proved to be more moderate with differential expression in the parahippocampal cortex exceeding that in the hippocampus.

The data sets provide novel information about numerous differentially expressed proteins, which serve as interaction partners or modulators in key disease-associated inflammatory signaling events. Noteworthy, a set of proteins which act as modulators of the ictogenic Toll-like receptor signaling proved to be differentially expressed. In addition, we report novel data demonstrating the regulation of different Toll-like receptor ligands during epileptogenesis.

Taken together, the findings deepen our understanding of modulation of inflammatory signaling during epileptogenesis providing an excellent and comprehensive basis for the identification of target and biomarker candidates.

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1. Introduction

Enhanced inflammatory signaling is considered as a key contributor to the development of epilepsy following initial brain insults (Vezzani et al., 2013). Inflammatory mediators can trigger molecular and cellular alterations resulting in hyperexcitability and decreased seizure threshold (Pitkänen and Sutula, 2002;

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Vezzani et al., 2008, 2011a; Vezzani and Viviani, 2015). Considering the putative key role of neuroinflammation during epileptogenesis, there is a major interest in the development and validation of anti-inflammatory, preventive or disease-modifying strategies (Vezzani, 2015). Moreover, up-regulation of specific inflammatory signalings is discussed as a basis for molecular imaging approaches or body fluids measurements aiming to identify biomarkers of the epileptogenic process (Vezzani and Friedman, 2011; Lukasiuk and Becker, 2014). Respective biomarkers are crucial to increase the throughput in preclinical assessment of antiepileptogenic strategies as well as to allow translational development for clinical testing. Both, the straight-forward development of preventive approaches and the identification of biomarkers are ideally based on comprehensive information about the complex molecular alterations of inflammation-associated proteins and their time course pattern during epileptogenesis.

Our current understanding of inflammatory signaling and its regulation during epileptogenesis is predominantly based on the analysis of selected inflammatory proteins and linked binding partners or signaling pathways by traditional focused protein detection and quantification techniques (Kolosowska et al., 2014; Russo et al., 2013). Functional genomic and transcriptomic profiling in models of epileptogenesis has rendered additional data sets including information about mRNAs linked with immune and inflammatory responses (Cacheaux et al., 2009; Okamoto et al., 2010; Wang et al., 2014). However, the translation of this information into protein expression patterns faces major limitations. Thus, our knowledge about the epileptogenesis-associated regulation of inflammatory signalings is still fragmentary and incomplete.

Large-scale proteomic profiling might help to fill this gap by providing comprehensive information about the sequential alterations in immune and inflammatory responses through the course of epileptogenesis. Bioinformatic analysis can provide an overview about the regulation of functional subcategories of proteins linked to immunity and inflammation and can point to yet unknown players in the disease-associated inflammatory interactome. In particular, hypothesis-driven and knowledge-based analysis of data sets can be used to identify co-regulated interaction partners of those inflammation-associated proteins, which are already known to be differentially expressed during epileptogenesis. The data sets might thereby provide novel target and biomarker candidates that can be further validated by focused expression analysis.

Here, we performed a focused bioinformatics analysis of expression patterns of proteins associated with inflammation and immune responses in a rat post-status epilepticus model. The investigations comprised tissue sampling at three key time points for monitoring disease progression, thus rendering proteomic profile information about the early post-insult phase, the latency phase, and the chronic phase with spontaneous recurrent seizures. Samples were subjected to a liquid chromatography tandem mass spectrometry (LC–MS/MS) proteomics profiling approach based on label-free quantifications.

The bioinformatics analysis of the comprehensive data sets identified novel differentially expressed proteins being co-regulated and serving as interaction partners or modulators in key disease-associated inflammatory signaling events. We also provide comprehensive information about the regulation pattern of several proteins modulating Toll-like receptor signaling.

2. Materials and methods

2.1. Animals

The investigation followed all relevant regulations and guidelines (European Communities Council Directive of 22 September 2010 (2010/63/EU) and the German Animal Welfare Act) and was approved by the Committees of the Government of Upper Bavaria (reference number 55.2-1-54-2532-94-11). Female Sprague Dawley rats (200–224 g; corresponding to an age range of 10–11 weeks) from Harlan Laboratories (Udine, Italy) were housed individually under controlled environmental conditions (20–24 °C, 45–65% humidity, 12-h dark/light cycle). Rats received nesting material and free access to standard food and tap water in their home cage. Before the experimental study, animals were allowed to acclimatize for at least one week. Every attempt was made to minimize the number of animals used in the study, and to avoid any pain or discomfort.

2.2. Electrode implantation

The electrode implantation was performed under general anesthesia using intraperitoneal injection of chloral hydrate (360 mg/kg). Combined stimulation and recording electrodes (Teflon-insulated bipolar stainless steel electrodes, diameter 0.45 mm) were stereotactically positioned in the right anterior basolateral nucleus of the amygdala (BLA). The atlas of Paxinos and Watson (1998) was used to determine the position of the electrodes (AP −2.2 mm, L +4.7 mm, DV −8.5 mm). Perioperative pain management included subcutaneous injections of the analgesic meloxicam (Metacam®, Boehringer-Ingelheim, Ingelheim, Germany; 1 mg/kg 30 min pre- and 24 h post-surgery) and local administration of bupivacaine (Bupivacaine 0.5%, Jenapharm, Jena, Germany) at the surgical site. Pre- and postoperatively, marbofloxacin (1 mg/kg, Marbocyl FD 1%, Vétoquinol, Ravensburg, Germany) was subcutaneously administered twice a day (starting one day before implantation until day seven post-surgery).

2.3. Electrical induction of a self-sustained status epilepticus

A status epilepticus (SE) was induced following a post-surgical phase of at least six weeks (corresponding to an age range of 17–18 weeks). Prior to electrical stimulation, a baseline electroencephalogram (EEG) was recorded via the BLA electrode from each rat to verify recordability. Subsequently, rats were continuously stimulated (intratrain pulse frequency of 50 Hz, 700 μA peak pulse intensity, 100 ms trains of 1 ms alternating positive and negative square-wave-pulses at a frequency of 2 Hz) in the BLA for 25 min according to a previously described protocol by Ongerth et al. (2014). Stimulation was performed using a stimulator (Accupulser, A310C) connected to a stimulus isolator (A365, World precision Instruments, Berlin, Germany). Unilateral electrical stimulation resulted in a self-sustained SE, which was confirmed by EEG recording. SE was interrupted by intraperitoneal diazepam injections (Diazepam-Ratiopharm, Ratiopharm, Ulm, Germany; 20 mg/kg) after a maximum duration of 4 h (including the 25 min of electrical BLA stimulation). Following diazepam injections, EEG was again recorded to confirm complete suppression of seizure activity. Behavioral seizure activity of the animals was visually observed for the duration of SE (4 h). According to Racine (1972), five stages of seizure severity can be observed: stage 1 was characterized by immobility and facial automatisms (eye closure, facial clonus), stage 2 by head nodding, associated with more severe facial and mouth clonus (mastication), stage 3 by unilateral forelimb clonus, stage 4 by rearing and bilateral forelimb clonus, and stage 5 by rearing and falling accompanied by generalized tonic–clonic seizures. Based on this rating scale, SE was divided into three different types: type 1, partial SE consisting of nonconvulsive seizure activity and stereotypies (e.g., sniffing); type 2, partial SE with generalized seizures which was characterized by predominantly partial seizures interrupted by occasional episodes of generalized convulsive (stage 4/5) seizures; and type 3, generalized convulsive SE which consisted of generalized seizure activity (Brandt et al., 2003). Only animals

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