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Impedance spectroscopy based measurement system for quantitative and label-free real-time monitoring of tauopathy in hippocampal slice cultures

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ABSTRACT

Alzheimer's disease (AD) and other tauopathies comprise death of cell bodies, synapses and neurites but there is surprising little knowledge of the temporal sequence and the causal relationships among these events. Here, we present a novel biosensoric approach to monitor retrograde neurite degeneration before cell death occurs. We induced tau hyperphosphorylation in organotypic hippocampal slice cultures (OHSC) and applied marker-independent real-time electrical impedance spectroscopy (EIS) for cellular real-time pathology monitoring. Using this approach, we were able to define two distinct phases of neurite degeneration, first a rapid swelling of axonal processes that manifests itself in relative impedance above control levels followed by a slower phase of collapse and subsequent fragmentation indicated by decreased relative impedance below control levels. Initial axon swelling is strictly dose-dependent and swelling intensity correlates with second phase impedance decrease implicating a causative link between both degenerative mechanisms. Moreover, suppressing tau hyperphosphorylation by kinase inhibition nearly prevented both phases of axon degeneration. Our findings demonstrate that the temporal sequence of tau-triggered neurite degeneration can be directly visualized by EIS-based, non-invasive and label-free monitoring. We therefore suggest this approach as a powerful extension of high content applications to study mechanisms of neurite degeneration and to exploit therapeutic options against AD and tau-related disorders.

1. Introduction

A progressing loss of neuronal cell bodies is observed in different adult-onset neurodegenerative disorders, including Alzheimer's (AD) and Parkinson's disease. In the affected brain regions, neuritic projections may degenerate in a distal-to-proximal mode over years or even decades. Although synaptic dysfunction has been proposed as initial locus of AD progression (Selkoe, 2002), it is surprisingly difficult to determine the spatiotemporal sequence and underlying mechanisms of chronic neurodegeneration (Conforti et al., 2007). Neurite degeneration has been observed in several transgenic animal models of AD and other dementias (Gotz et al., 2006). Unfortunately, *in vivo* models that appropriately recapitulate these pathologies are incompatible with high-content and high-throughput application, which in turn are required for efficient drug development programs. To overcome these limitations, organotypic hippocampal slice cultures (OHSC) may provide a comprehensive *ex vivo* solution (Cho et al., 2007; Sundstrom, 2007). Given the recent progress in modeling AD pathology in OHSC (Alberdi et al., 2010; Bruce et al., 1996; Hubinger et al., 2008), investigations of neurite degeneration have become possible in selected AD-vulnerable brain tissues, employing both electrophysiological parameters (Shipton et al., 2011) and biochemical markers (Hinners et al., 2008).

To examine neurite degeneration directly, we developed a label-free and non-destructive real-time monitoring of cellular morphology based on EIS to monitor and quantify AD and tauopathy-related neurite degeneration in a non-invasive manner. In contrast to monitoring neuritic monolayer cell lines (Jahnke et al., 2009) or even simple aggregated 3D-cultures (Krinke et al., 2010), OHSC comprises a high degree of structural complexity and an *in vivo* like neuronal network structure as well as activity. Moreover, the hippocampus is one of the initial affected brain regions by AD, favoring this tissue as *ex vivo* model system for AD related neurite degeneration investigations and active pharmaceutical testing (API). To overcome the bottleneck of sensitive and feasible quantitative pathology monitoring of OHSC EIS is a suitable technique

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that was previously used in a wide range of immunosensors (Ciani et al., 2012; Steude et al., 2011), for quantification of specific cellular events like apoptosis/necrosis, cell adhesion/motility (Qiu et al., 2008), ion channel activity (Panke et al., 2011) as well as the activation of distinct signaling cascades (Wolf et al., 2008) in single cell, monolayer (Haas et al., 2010; Krinke et al., 2009), and 3D-cultures (Kloss et al., 2008; Krinke et al., 2010). In this context, EIS is a promising technique for monitoring of tau hyperphosphorylation-dependent degeneration in OHSC and may facilitate the study of tau-related pathologies like AD and advance the identification of better intervention strategies.

2. Material and methods

2.1. Preparation and culturing of hippocampal slices

All of the animal use procedures were approved by the Committee of Animal Care and use of the relevant local governmental body. OHSC were prepared from one to six day old sprague dawley rat pups as previously described (Stoppini et al., 1991) and cultivated on 0.4 μm Millicell-CM filters (Millipore, USA) (SI Section 2 for details).

2.2. Induction of hyperphosphorylation and compound treatment

After at least five days of *in vitro* cultivation the hippocampal slices were treated with okadaic acid (Sigma Aldrich, Germany) to induce hyperphosphorylation of the tau protein and SRN-003-556 (KeyNeuro Tek AG, Germany) to attenuate hyperphosphorylation of the tau protein. After an appropriate incubation time (1 h, 5 h or 8 h) the OHSC were proceeded as described in the immunohistochemistry and western blot section, respectively.

2.3. Live/dead staining

To label dead cells OHSC were incubated with 1 μg/ml propidium iodide (VWR, Germany) supplemented medium for two hours at 37 °C. 15 min prior to imaging Calcein-AM (Invitrogen, USA) was added at a final concentration of 2.5 μM. Afterwards stained hippocampal slices were imaged by a Nikon Eclipse TE 2000-U inverse microscope, equipped with a mercury arc source and fluorescence filter sets for Cy2 (Calcein-AM) and Cy3 (propidium iodide).

2.4. Immunohistochemistry

For immunohistochemical staining OHSC were fixed with 4% formaldehyde for one hour. After permeabilization, OHSC were stained against NF200 and NF-L, respectively with primary antibody and appropriate fluorescence labeled secondary antibodies and imaged by confocal microscopy (SI Section 2 for details).

2.5. Electrochemical impedance spectroscopy (EIS)

After at least five days of *in vitro* culturing, the OHSC were transferred to the multielectrode arrays (MEA). Therefore, the MEAs (MultiChannelSystems, Germany) with 60 nanocolumnar structured titanium nitride electrodes, 30 μm in diameter and arranged in an 8 × 8 grid, were coated with nitrocellulose (0.1 cm^2 nitrocellulose was solved in 5 ml methanol). After placing OHSC on the electrodes culture medium was removed and MEAs were heated at 37 °C for one minute before the culture chamber was filled with 500 μl culture medium. Then, MEAs were closed with a lid, placed in our self-developed impedance measurement station and pre-monitored for one hour at 37 °C in a humidified atmosphere. For each MEA at least 30 electrodes were recorded. These electrodes were chosen by microscopic assessment of those electrodes that were fully covered by an OHSC. The impedance spectra were recorded as previously described (Haas et al., 2010). Briefly, impedance spectra were recorded every 15 min by applying an alternating voltage of 10 mV in a frequency range from 500 Hz to 5 MHz (Agilent 4294A; Agilent Technologies, USA) with equidistant spacing in a logarithmic scale and 51 frequency points overall. The contribution of individual OHSC to the total impedance signal (relative impedance $|Z|_{rel}$ (%)) was calculated by the self-developed software IDAT v3.6.5 (Impedance Data Analyzing Tool) after the formula $|Z|_{rel}\,(\%) = ((|Z|_{covered} - |Z|_{cell\text{-}free})/|Z|_{cell\text{-}free} \times 100)$. The software removed automatically those electrodes without detectable or to low (below 30% relative impedance) OHSC signal. At least 20 electrodes per MEA were required, otherwise the MEA was considered as not analyzable. The frequency of maximum relative impedance were determined automatically and traced over time followed by normalization (0 h) for statistical comparison. Finally, from each MEA the mean values of all measured electrodes were averaged. For all presented datasets, either four (OA treatment) or six (OA + SRN treatment) independent experiments using different OHSC preparations were performed and statistically analyzed.

2.6. Equivalent circuit modeling

To get further information of the nature of the detected effects the experimental data was analyzed using the simplified electronic equivalent circuit model for the tissue–electrode interface (see Fig. 3A). The culture medium resistance was considered as constant (70 Ohm). The parameters of the equivalent circuit were determined in a two step optimization procedure. First, the bare electrodes (R_{MEA} and CPE_{EL}) were calculated using a reduced equivalent circuit without the tissue specific parameters C_{Tissue} and R_{Tissue} using the acquired spectra of tissue free MEAs. Second, the tissue covered electrodes were analyzed by applying the entire equivalent circuit. At this step, the system and electrode dependent parameters (obtained from first step) were kept constant and only C_{Tissue} and R_{Tissue} were determined by the fitting procedure. For the fittings the self-developed impedance data analysis software IDAT v3.6.5 was used.

2.7. Statistics

All statistical analyses were done using Graphpad Prism 5. In general, all values are given as means ± s.e.m. until described differentially. Multiple group comparisons were analyzed by two-way ANOVA and Bonferroni post hoc test. Comparisons between two groups were analyzed by *t*-test. Differences between two means with $p < 0.05$ were considered significant, $p < 0.01$ very significant and $p < 0.001$ extremely significant.

3. Results

3.1. Optimization of OHSC-based taupathology model for on chip monitoring

First, we optimized preparation and culture conditions of rat hippocampal slices for long-term culturing and use in EIS based monitoring on multielectrode arrays (MEAs). In line with previous observations (Mielke et al., 2005), we verified post-natal day six as an optimum starting point for subsequent long-term cultivation. At day six, OHSC comprised an authentic neuronal network under *ex vivo* conditions, characterized by appropriate slice size (Fig. S1A and B) and increased neurofilament-L mRNA expression levels (Fig. S1C). In the most prevalent dementia,