CO-PATHOLOGICAL STATES OF TAU PROTEINS IN A 3D MICROPATTERNED NEURAL CELL CULTURE

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ABSTRACT

In vitro studies of Alzheimer's disease are based on homogenously seeded neural cells. These studies reveal molecular pathway mechanism related to neurite degeneration, but they cannot analyze interactions between diseased and healthy cells. Furthermore, the cell environment in homogenous cell studies is mostly reduced to a two-dimensional surface. Here, we present a novel, microfluidic based cell culture method that combines (1) 3D micropatterning of neurons with (2) local pathological states of tau phosphorylation, a characteristic feature of Alzheimer diseased brains. Using this novel 3D Alzheimer model *in vitro* we can accelerate studies about propagation processes in future.

KEYWORDS: Co-pathology, Tauopathy, Microfluidics, 3D neural culture, Okadaic acid gradient

INTRODUCTION

A major characteristic of Alzheimer diseased brains is the progressive degeneration of the neurite network. One process leading to neurite degeneration is the modification of tau proteins into hyper-phosphorylated states. Tau is responsible for stabilizing the microtubules of neurites. In hyper-phosphorylated states, tau proteins are unable to carry out their stabilizing function [1]. The inhibition of the protein phosphatase leads to a hyper-phosphorylation of tau, so called tauopathy.

However, in today's cell culture methods controlling the 3D cell micro architecture and modifying proteins locally is missing. While *in vitro* neural cell cultures improved regarding cell compartmentalization and patterning of different cell types, neural cells stayed attached to 2D surfaces [2]. We present, therefore, a novel method to generate locally hyper-phosphorylated tau proteins in a 3D micropatterned neural cell culture.

THEORY

Micropatterning methods in combination with gradient devices allows the fabrication of diseased neural cells in co-pathology to healthy neurons (Fig. 1A). The 3D micropatterning method for primary cortical neurons has already been described [3]. The method is based on parallel laminar flow of liquid hydrogel solutions in a microfluidic channel.

Disease neural cells, here, are characterized by hyper-phosphorylated tau proteins and microtubule degeneration. Microtubule are proteins stabilizing the neural cytoskeleton in the axon. Tau proteins shape microtubule in their twisted way (Fig. 1A). When axons follow their guiding cues to build neurite networks, kinase and phosphatase are two reactions side constructing and reforming the cytoskeleton. In Alzheimer's disease these two reactions are disrupted leading into a strong hyper-phosphorylation of tau proteins. Hyper-phosphorylated tau cannot bind anymore to microtubule, which results into degeneration of the neurite network. Experimental models *in vitro*, which are mimicking Alzheimer characteristic lesions, commonly use okadaic acid. Okadaic acid inhibits protein phosphatase (P-1 and PP-2A), also of tau proteins [4]. Hence, i*n vitro* generation of characteristic Alzheimer lesions can be triggered through local okadaic acid treatment.

EXPERIMENTAL

Primary cortical neurons were immobilized in an agarose / alginate hydrogel matrix (0.5 % / 0.3 % w/v) and micropatterned in a microfluidic channel (Fig. 1B). Table 1 shows the composition of the hydrogel pattern. Neural cells are only injected in the left and right layers L1 and L4. The two hydrogel layer L2 and L3, in the middle of the culture channel were left cell free. Neural cells can then form a neurite network through neighboring hydrogel layers over a distance of ~320 µm.

We also enriched the middle hydrogel layers with B-27, a media supplement, as it has been demonstrated before, to stimulate and orient neurite outgrowth towards opposite layers [3]. After neurite network establishment (8 days in vitro), cell layers were locally exposed to an okadaic acid gradient using perfusion channels and device incorporated PDMS reservoirs for injection. The loading of reservoirs generates a pressure driven flow in both perfusion channels up to two hours. The perfusion generates a chemical gradient of okadaic acid through the four hydrogel layers in the culture channel. Maximum concentration of okadaic acid in the left perfusion channel was 600 nM. The experiment was performed for 1h, at 37°C.

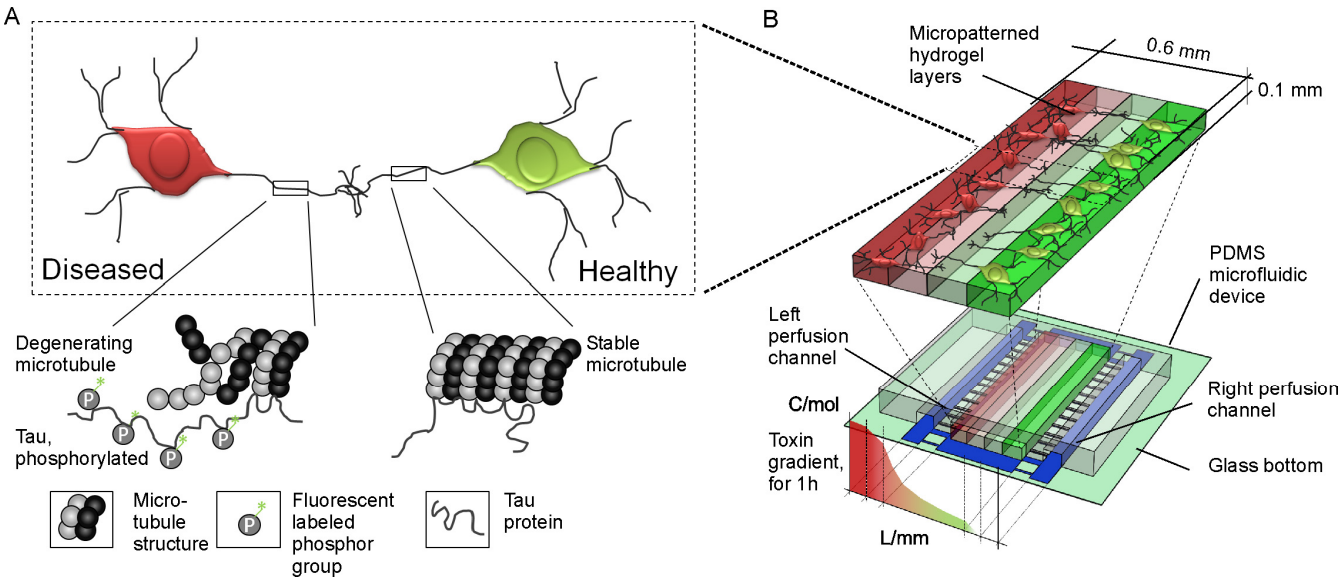

Figure 1: Generating co-pathological states of tau proteins in a 3D neural cell culture. (A) Opposite patterned neurons are locally treated with okadaic acid (600nM, 1h, 37°C), inhibiting de-phosphorylation of tau proteins = diseased cell state. Hyper-phosphorylated tau proteins de-attach from microtubule, which cause destabilizing of the microtubule structure and neurite network degeneration. (B) Microfluidic based 3D micropatterning of hydrogel and cell layers and gradient projection of toxins inducing locally diseased protein states in neurons.

Table 1. Micropatterned hydrogel layer and perfusion medium composition

Ingredients	**Channel positions**					
	PC-L	**L1**	**L2**	**L3**	**L4**	**PC-R**
Agarose (w/v %)	0	0.5	0.5	0.5	0.5	0
Alginate (w/v %)	0	0.3	0.3	0.3	0.3	0
Neurobasal (v/v %)	100	98	96	96	98	100
B27 (v/v %)	0	2	4	4	2	0
Okadaic acid (nM), 1h, after 8 DIV	600	420	370	250	180	0

PC-L: Perfusion channel left, PC-R: Perfusion channel right, L1 … L4: Layer notation, DIV: days *in vitro*

After okadaic acid treatment, 3D cell cultures were fixed with 4% paraformaldehyde and immunostained against phosphorylated tau (pS262, P-TAU), pre-synaptic units (synaptophysin, SYN) and cell nucleus (DAPI). Different phosphorylation states of tau were quantified from surface intensity plots of multiple z-stack confocal images. Intensity profiles were normalized on maximal intensity before averaging.

RESULTS AND DISCUSSION

The okadaic acid gradient induced local hyper-phosphorylated tau proteins (hyper p-tau) in hydrogel layer L1, see Figure 2 A. Figure 2 B presents the distribution of hyper p-tau states across the microchannel width, which confirms p-tau related co-pathological states (the co-existence of different pathological states). The intensity profile of p-tau, however, represents rather a step than a linear profile, although it was induced through a gradient.

Neurons in layer L4 were surrounded by few dots of p-tau proteins (Fig. 3A), which are significantly lower than in L1 (ANOVA, $P<0.001$). The comparison of p-tau states from L1 and L4 with non treated cells (Fig. 3B) showed that neural cells in L4 can be considered as healthy. Whereas neural cells in L1 presented four-fold and higher levels of hyper-phosphorylated tau states (Fig. 2C). Similar p-tau levels were also found in AD brains [1].

CONCLUSION

We demonstrated the local generation of hyper-phosphorylated tau proteins in 3D micropatterned primary neural cell cultures. The okadaic acid gradient induced four-fold higher levels of hyper-phosphorylated tau states in the diseased cell population, an equivalent lesions as *in vivo*. Using this novel 3D Alzheimer model will fasten the development of new therapies against neurodegeneration processes *in vitro*.

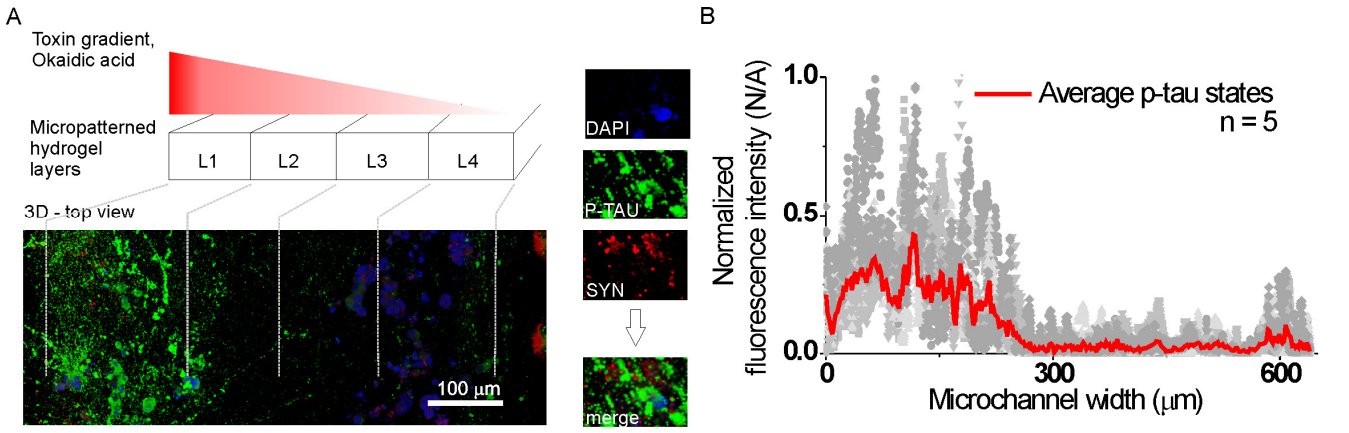

Figure 2: Connected co-pathological states of tau after okadaic acid treatment. (A) Confocal images of 17 merged top view stacks show cell nucleus (DAPI:blue) in layer L1 and L2, phosphorylated tau proteins (P-TAU:green) and pre synaptic units (SYN:red). (B) Surface plot of normalized green fluorescence signal demonstrates high amount of phosphorylated tau proteins in L1: 0 - 150 µm.

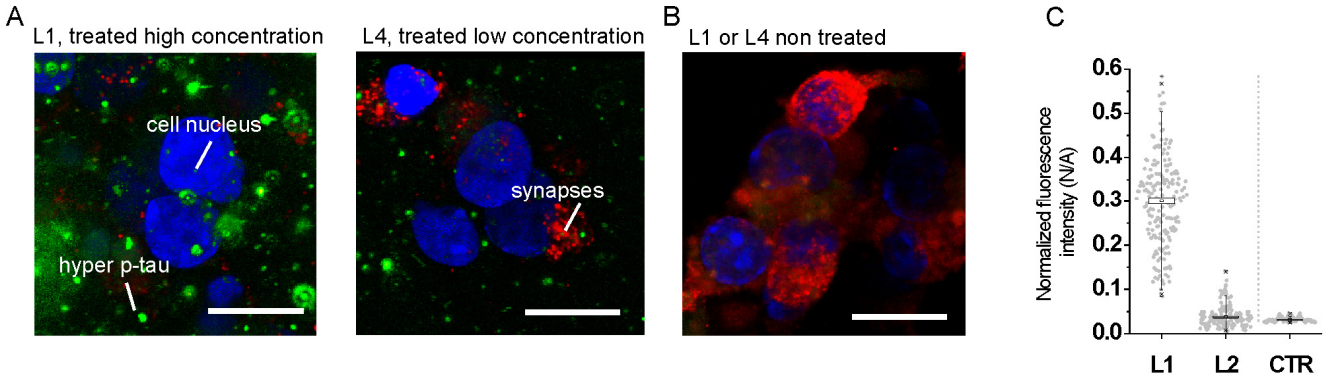

Figure 3: Morphology and quantification of phosphorylated tau (p-tau) states. (A) Neurons exposed to okadaic acid gradient. Layer L1 represents high and layer L4 low okadaic acid concentrations. Nucleus staining: DAPI- blue, Synapse staining: Synaptophysin-red, Hyper-phosphorylated tau: pS262-green. Bar = 10 µm (B) Neurons in healthy state taken from a control group (CTR). Bar = 10 µm. (C) Different p-tau states with SE (box) and CI=95% (line) for treated and non-treated neurons (CTR).

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