Drug Discovery Today • Volume 00, Number 00 • October 2017 PERSPECTIVE

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3D nerve cell cultures and complex physiological relevance

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The field of tissue engineering has not yet provided knowledge on which a consensus for the complex physiological relevance (CPR) of neuronal cultures could be established. The CPR of 3D neuronal cultures can have a profound impact on the drug discovery process through the validation of *in vitro* models for the study of neuropsychiatric and degenerative diseases, as well as screening for neurotoxicity during drug development. Herein, we assemble evidence in support of the potential of $[Ca^{2+}]_i$ oscillation frequency as a CPR outcome that can demonstrate the *in vivo*-like behavior of 3D cultures and differentiate them from 2D monolayers. We demonstrate that $[Ca^{2+}]_i$ oscillation frequencies in 2D cultures are significantly higher than those found in 3D cultures, and provide a possible molecular explanation.

Introduction

The exponentially increasing number of publications over the past decade in the area of 3D cultures has been driven by the belief that monolayer (2D) cultures poorly represent the natural *in vivo* microenvironment and, as such, the results obtained with these 2D cultures in, for example, cell-based preclinical drug screening, are less optimal [1]. Although most of the evidence in support of this belief comes mainly from differences in structure and/or function between the two types of culture, with limited *in vivo* validation, a few well-controlled landmark studies have been published. For example, the well-known *in vivo* cell adhesion-mediated drug resistance of EMT6 tumor cells was fully recapitulated by 3D tumor spheroids of the same cells, but not observed in 2D cultures [2]. Similarly, the amoebic migration of HT-1080 cells through narrow 3D collagen matrix gaps as a compensation strategy to counterbalance the loss of pericellular proteolysis, in the presence matrix metalloproteinases inhibitors, is not possible in monolayer cultures [3]. Such structure and/or function mimicking *in vivo* and in 3D but not in 2D cultures, is collectively described by CPR [4].

Complex physiological relevance

The microenvironment factors (MEFs) that affect a cell at all levels, and hypothesized to give rise to CPR, are organized into four general categories: (i) chemical or biochemical composition; (ii) spatial (geometric); (iii) temporal; and (iv) force/substrate physical properties [4]. For any cell type, knowledge of the minimum MEFs (quality and/or quantity) required to support CPR outcomes is desirable. However, before such studies are conducted, the CPR of a cell for a given application must be defined. For 3D liver cells, a CPR outcome can comprise tight junctions between cells and the formation of microvilli-lined bile canaliculi-like structures that are absent in monolayer cultures [5]. In addition, albumin production, generally recognized as an indicator of liver-specific activity [6] is observed in 3D, such as in rat hepatocyte spheroids [7], but can be entirely lacking in 2D formats [8]. Another example of CPR is the beat frequency and contraction force exhibited by cardiac tissue-derived cells. In a study by Kelm *et al*. [9], neonatal rat cardiomyocytes, cultured as spheroids in a hanging drop, exhibited rhythmic contractions at a frequency of 60 beats per min (bpm). Similar beat frequencies (43 ± 21 bpm) were observed when these cells were transplanted into adult rat and formed microtissues [10], compared with 2D culture bpm values of 83.4 ± 4.5 [11] and 85.6 ± 9.3 [12]. These results suggest that a contraction frequency in the *in vivo* heart beat range is a suitable CPR outcome for muscle cells. However, *in vivo* contractile force levels have not yet been achieved in 3D cultures.

A CPR outcome for nerve tissue-derived cells has been elusive, yet the physiological relevance of these cells is crucial in various biomedical applications. CPR exhibited by 3D cultures could

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2017.10.014

Please cite this article in press as: Cheng, X. et al. 3D nerve cell cultures and complex physiological relevance, Drug Discov Today (2017), doi:10.1016/j.drudis.2017.10.014

impact the field of drug discovery in two ways: (i) during screening for neurotoxicity, the model has to be physiologically relevant or emulate the *in vivo* situation to better predict the toxicity of the compounds being tested; and (ii) target validation and discovery against neurodevelopmental, psychiatric, and/or degenerative diseases would require a physiologically relevant model to be perturbed to exhibit the diseased state. Exploration of intracellular calcium ($[Ca^{2+}]_i$) oscillation frequency as a potential CPR outcome for nerve tissue-derived cells has been inspired by the muscle cell beat frequency, described above, which is partly controlled by $[Ca^{2+}]_i$ dynamics and 2D/3D comparative studies that have reported lower $[Ca^{2+}]_i$ transients in 3D cultures, in response to depolarization-induced voltage-gated calcium channel (VGCC) gating [13]. The 2D versus 3D difference in depolarization-induced $[Ca^{2+}]_i$ transients has been attributed to differences in membrane structure, as suggested by differences in the co-localization of VGCCs with caveolin-1 [14], a widely recognized lipid raft marker protein [15].

Lipid rafts

The cell membrane that 'holds' macromolecules, such as VGCCs and NMDA receptors, has classically been viewed as 'a sea of lipids through which proteins drift with little or no organization' [XX]. This classic model of the plasma membrane was first questioned a little more than a decade ago by Simons and Ikonen [16], who proposed that glycosphingolipids can self-assemble *in vivo*, forming specific membrane microdomains that serve as a sorting device for the selective transport of proteins and lipids to the epical membrane. Given the indirect means of the concept assessment, questions of fact or artifact were raised. More recently, various advanced biophotonics approaches (e.g., Ref. [17]) have been used to tackle the problem at higher spatiotemporal resolutions within living cells, which has provided compelling evidence for the existence of lipid rafts *in vivo* [18]. Currently, lipid rafts are viewed as dynamic nano-scale assemblies enriched in sphingolipid, cholesterol, and glycosylphosphatidylinositol-anchored proteins [19]. The plasma membrane can comprise more than 2000 species of lipids [20], which separate into distinct populations within the bilayer, forming a patchwork of different lipid environments at the cell surface.

Based on differences in lipid and protein composition, multiple rafts are likely to exist [21]. However, the only morphologically identifiable raft-like domain is a caveola, associated with the cholesterol-binding protein caveolin [22]. Caveolin causes the formation of small (50–100 nm) flask-shaped invaginations of the surface membrane, which are clearly visible in scanning or transmission electron microscopy images [23].

Evidence suggesting that lipid rafts and/or caveolae associate with ionic channels and/or regulate their function has come from studies that: (i) isolate the channels and their associated protein from the bulk of the bilayer as solubilization-resistant components in response to cold non-ionic detergents extraction; (ii) directly visualize the rafts; and (ii) destroy the domains and assess the effects on channel activity. Using the solubilization-resistant component approach, VGCCs have been shown to concentrate in cholesterol-rich lipid raft microdomains [24]. A wealth of calcium signaling proteins in addition to ion channels have also been found to accumulate in rafts, and their activity is modulated by mechanosensing by the lipid rafts [15].

Potential of spontaneous $[Ca^{2+}]_i$ oscillation frequency to serve as nerve cell CPR

$[Ca^{2+}]_i$ oscillation has been a research focal area since its first discovery by Ringer [25], which marked the initiation of calcium-signaling studies. For over a century, many shapes of calcium signaling have been found in many cell types. Generally, calcium signals can be classified as transient, sustained, or oscillatory. With infinite shapes resulting from variation in frequencies and amplitudes, $[Ca^{2+}]_i$ oscillations make perfect information carriers in cells [26]. For example, high-frequency $[Ca^{2+}]_i$ oscillations regulate fast responses, such as synaptic transmission and secretion, whereas low-frequency oscillations regulate slow processes, such as fertilization and gene transcription [26].

Here, we assemble evidence from the literature in support of the potential of $[Ca^{2+}]_i$ oscillation frequency as a CPR outcome. Our search was limited to spontaneously generated oscillations, as opposed to externally induced, and to the nine most-recent 2D culture studies (there are many 2D culture reports). Of the few pertinent 3D culture studies, only five were found to be usable. Some studies were excluded because of the experimental conditions used. For example, one of the excluded studies used a buffer lacking Mg^{2+}, which caused seizure-like rather than spontaneous oscillations. The key experimental details of the included studies are provided in Table 1 and the frequency results are

TABLE 1

Summary of key experimental details of the studies represented in Fig. 1[a]

Source reference	Cell/tissue type	Age of source animal	Days in culture	Cell culture format	Buffer (Mg^{2+} concentration)
[38]	Rat striatum	P12–P18 Rat	0	3D (BS)	ACSF (1.2 mM)
[39]	Rat cerebral cortices	E17–E19 (spheroids); P20 host animal	7–14-day spheroids, 8 days after transplantation	3D (SN)	HBSS/HEPES (0 mM)
[27]	Immature rat cortex	P1–P2 rat	0	3D (BS)	(1 mM)
[40]	Immature mice cortex and hippocampus	P0–P2 mice	0	3D (BS)	Low Mg ACSF/ACSF (1.3 mM)
[41]	Hippocampal cells	P14–P32 rat	0	3D (BS)	ACSF (1.4–2.0 mM)
[34]	Hippocampal neurons	E18 rat	5 days	2D	(0.4 mM)
[42]	Hippocampus cell	E17–E19 rat	9–11 days	2D	KRH (1 mM)
[43]	Hippocampal neurons	E18 rat	>7 days	2D	KRH (1 mM)
[44]	Hippocampal cells (transgenic)	E17 rat	12–14 days	2D	HBSS/HEPES (0.9 mM)
[45]	Cortical neurons	E18 rat	7 days	2D	KRH (1.2 mM)
[46]	Cortical neurons	E17–E18 rat	13 days	2D	KRH (1.2 mM)
[47]	Hippocampal neurons	18-day fetal rat	9–11 days	2D	KRH (1 mM)
[35]	Neocortical neurons	E 16–E17 rat	9–13 days	2D	Locke's/HEPES (1 mM)
[36]	Hippocampal cells	E19 rat	17–18 days	2D	KRH (0.9 mM)

[a] Abbreviations: ACSF, artificial cerebrospinal fluid; BS, brain slices; KRH, Krebs–Ringer–Hepes buffer; SN, spheroid networks.

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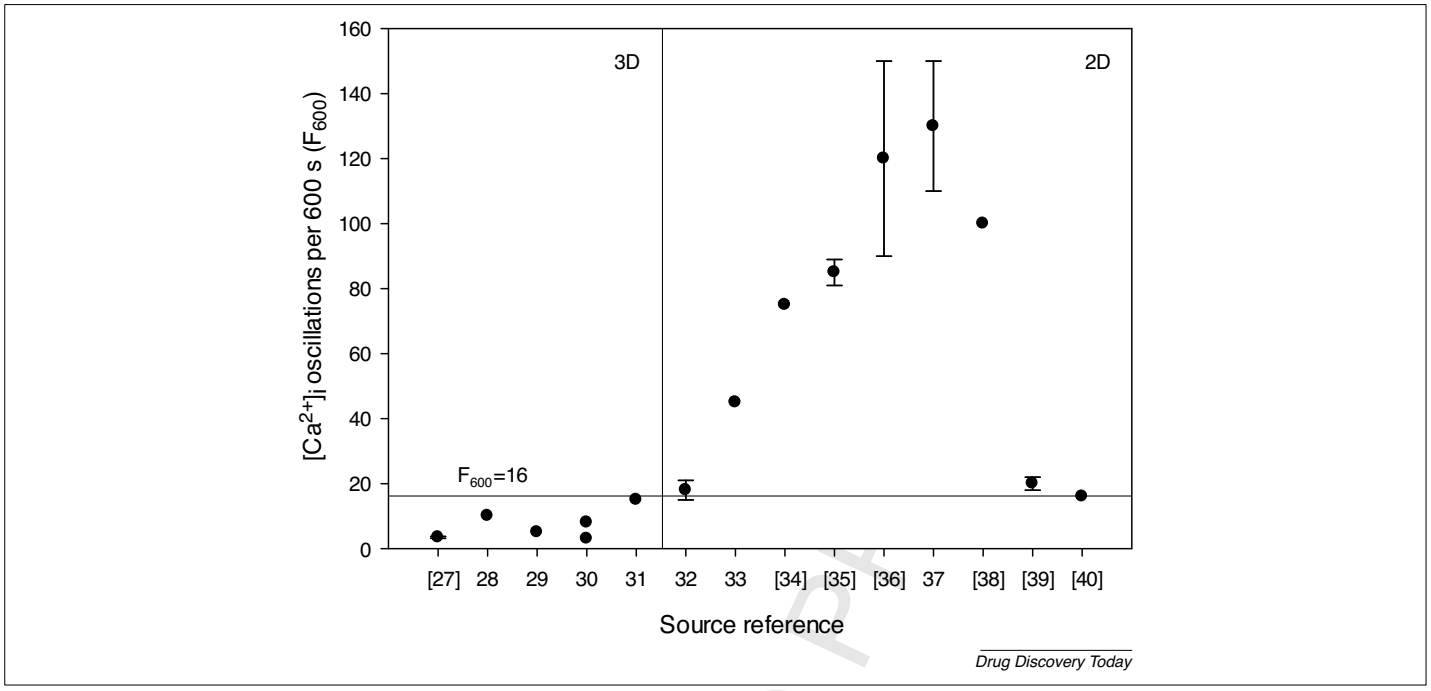

FIGURE 1

Scatter plot of $[Ca^{2+}]_i$ oscillation frequency. The numbers on the x-axis refer to the source references, with the same numbers in Table 1 and the reference list. With a few exceptions, the spontaneous calcium frequency is lower in 3D compared with 2D cultures.

presented in Fig. 1. The cells from all the aforementioned studies were of neural origin (striatum, hippocampus, or cerebral cortex). As shown, 2D cultures' $[Ca^{2+}]_i$ oscillation frequencies (number of 'spikes' or 'transients' in 600 s, F_{600}) were significantly higher than those in 3D cultures (Mann-Whitney U test: 2D group was statistically distinct from 3D group, $p = 0.003$). The 2D/3D cut off of $F_{600} = 16$ was chosen arbitrarily.

Q5 The high F600 value of 192 reported in [XX] is easily explainable. Recently, large neuronal networks in slices of newborn rats revealed synchronized $[Ca^{2+}]_i$ oscillations that were mediated by AMPA and NMDA glutamate receptors and were maintained until the developmental transition of the GABAergic transmission from depolarization to hyperpolarization [27]. NMDA receptors are ligand-gated channels that are inhibited by the physiological concentration of extracellular Mg^{2+} at resting membrane potential [28]. These receptors are coincident detectors, which means that their signaling depends on agonist binding as well as on membrane depolarization. Therefore, removal of Mg^{2+} from the extracellular environment or membrane depolarization to −50 mV or more induces spontaneous $[Ca^{2+}]_i$ oscillations in neurons because of Ca^{2+} influx through NMDA receptors and L-type VGCC [23]. Accordingly, it has been reported that NMDA receptors were necessary for the generation of $[Ca^{2+}]_i$ oscillations in the absence of extracellular Mg^{2+} [29] in hippocampal [30], neocortical [31], and immature cortical neurons [32]. This was further substantiated by the fact that Cd^{2+}, a voltage-dependent Ca^{2+} channel blocker, reduced $[Ca^{2+}]_i$ oscillations, while APV (a NMDA receptor inhibitor) blocked it [33]. However, removal of Mg^{2+} was detrimental to neuronal survival and led to excitotoxic cell death in cortical neurons because of excessive NMDA receptor activation [31]. Also, removal of Mg^{2+} from the extracellular media created a non-physiological condition and was associated with the occurrence of very high $[Ca^{2+}]_i$ oscillations dependent on NMDA receptors. As shown in Table 1, the buffer used in the high-frequency case [XX] contained no Mg^{2+}. In the remaining studies, Mg^{2+} in the buffers used was in physiological range and, as such, not a factor affecting the $[Ca^{2+}]_i$ frequencies observed.

The two 2D culture F_{600} values that were well into or close to the 3D range [34–36] are interestingly the cases where the cells were in culture the shortest (5 days) or the longest (17–18 days). According to Bacci *et al.* [37], after 12–14 days in culture, a network of synaptic contacts can be established and neurons can show lower spontaneous activity. These observations point to the importance of time in culture (or temporal MEF in general) for future comparative studies of 2D versus 3D, freshly dissected or *in vivo* constructs.

Concluding remarks

The fact that F_{600} values from 3D cultures under different experimental conditions (e.g., spheroids, brain slices, or age of animal cell source) are significantly lower than those from 2D culture counterparts lends credibility to the idea of $[Ca^{2+}]_i$ oscillation frequency as a potential functional marker for better *in vivo* emulation. Detailed studies to understand the molecular basis for the differences in frequency are needed. Differences in lipid raft quantity and/or structure offer not only an ideal starting point for functional CPR studies, but might also provide a robust structural CPR for nerve tissue-derived cells. Q6

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