

Evaluation of *in vitro* neuronal networks for the study of spontaneous activity

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ABSTRACT

In the absence of external stimuli, the nervous system exhibits a spontaneous electrical activity whose functions are not fully understood, and that represents the background noise of brain operations. Spontaneous activity has been proven to arise not only *in vivo*, but in *in vitro* neuronal networks as well, following some stereotypical patterns that reproduce the time course of development of the mammalian nervous system. This review provides an overview of *in vitro* models for the study of spontaneous network activity, discussing their ability to reproduce *in vivo* - like dynamics and the main findings obtained with each particular model. While explanted brain slices are able to reproduce the neuronal oscillations typically observed in anaesthetized animals, dissociated cultures allow the use of patient-derived neurons and limit the number of animals used for sample preparation. Moreover, dissociated neurons can be cultured on three-dimensional (3D) substrates that mimic the extra-cellular matrix of the brain. Depending on the material used, 3D substrates are able to increase neuronal connectivity and synaptic strength.

Keywords: Cortical oscillations · Up states · Calcium transients · Neuronal cultures · Brain slices

1. Introduction

Spontaneous neural oscillations have been observed by researchers as early as the 1930s, when the electroencephalography (EEG) machine was invented (1). Specifically, slow oscillations in the frequency of *delta* waves (0.4 - 5 Hz) have first been observed in the cortex of sleeping patients by W Gray Walter (2). Subsequently, slow oscillations in the same frequency range were recorded in anesthetized mammals as well (3). Nevertheless, their function remained one of the most mysterious questions in neuroscience.

A first characterization of neuronal spontaneous oscillations was provided by Steriade *et al.* in 1993. Measuring the electrical activity of cortical and thalamic neurons in anesthetized cats, they observed oscillations composed of a slow depolarizing phase (0.8 - 1.5 s) followed by a long-lasting hyperpolarization. These oscillations had an even lower frequency than the one of *delta* waves, i.e. less than 1 Hz. Therefore, they were referred to as slow oscillations (4). The duration of the depolarizing phase was reduced by administration of the

N-methyl-D-aspartate (NMDA) blocker ketamine.

In the hippocampus of anesthetized rats, instead, neuronal oscillations have been observed mainly in the higher, *theta* frequency range (4 - 8 Hz). Hippocampal *theta* waves are generated by the entorhinal input and by CA3 collaterals, through the activation of voltage-dependent Ca²⁺ currents in pyramidal cells. GABAergic and cholinergic neurons contribute as well to the generation of *theta* waves by feed-forward disinhibition of CA1 pyramidal cells by the interneurons, and cholinergic activation of CA3 (5).

Subsequent studies revealed the mechanisms of neuronal oscillations at the cellular level. In particular, some *in vivo* intracellular recordings of neostriatal spiny neurons showed fluctuations between a depolarized and an hyperpolarized membrane potential that were referred to as Up and Down states, respectively (6). Subsequently, Constantinople and Bruno demonstrated that this two-state behavior observed in anesthetized rats was eliminated during wakefulness. In addition, they showed that the dynamics of the awake state and under anesthesia were unaltered even after elimination of afferent thalamic input, suggesting an internal cortical neuromodulation (7). In fact, it was subsequently proven that - in both humans and rodents - the large majority of excitatory synapses onto cortical excitatory neurons come from other cortical excitatory neurons, giving the cortex a vast recurrent connectivity (8).

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Synchronized Up and Down states are the cellular equivalent of the slow waves observed - at the network level - from human EEG data (9) to anesthetized animals, and even in *in vitro* brain slices isolated from the rest of the brain (10) (**Figure 1**). Since they arise in such different systems, Up and Down states have been proposed to be a default activity pattern resulting from the basic architecture of the network. Nevertheless, they may still have a functional role. From experimental observations of their pathways, Up and Down states are supposed to transfer information across brain regions and to consolidate memories. A current hypothesis is that cortical Up states travel through the entorhinal and visual cortex to the hippocampus phasing the hippocampal rhythm, which in turn transfers its activity pattern - acquired during learning - back to the cortical networks for memory consolidation (5,11). Moreover, the hippocampal *theta* rhythm is believed to be critical for temporal coding/decoding of active neuronal ensembles and the modification of synaptic weights. Down states, as periods of low synaptic inputs, are thought to be necessary for metabolic restoration (see for review 12).

Spontaneous oscillations have been observed also in developing neuronal networks. An example is represented by synchronous plateau assemblies (SPAs) and giant depolarizing potentials (GDPs) in the immature hippocampus. Driven by synchronized GABAergic neurons, GDPs are thought to shape synaptic currents in the immature network (13,14).

In the regulation of this rhythmic activity, a key role is played by inhibitory interneurons. In fact, interneurons were proven to synchronize the spiking of hippocampal pyramidal cells, thus maintaining homeostatic levels of activity in the brain (15). Moreover, spike-triggered local field potentials measured in humans and monkeys were proven to be composed by inhibitory neurons for the largest part, suggesting that the onset of Up states is more driven by a decrease in inhibition rather than an increase

in excitation (16).

Not only Up and Down states, but also spontaneous calcium signals were frequently observed in neuronal networks. In fact, intracellular calcium ions increase as a consequence of action potentials and membrane depolarization, entering the cytosol through voltage-gated calcium channels. Spontaneous calcium transients can be considered as indicators of Up and Down states since they follow parallel dynamics, despite being slower (17) (see **Figure 1** for a representation). Calcium imaging techniques have become a diffused method for studying network dynamics, since they allow the simultaneous recording of a large number of neurons.

Many of these findings were obtained *in vitro*. In this review, I discuss the commonly used methods for preparing neuronal samples, the patterns of spontaneous activity that typically arise from each one of these networks and their similarity with *in vivo* neuronal oscillations. Network disorders are at the basis of several pathologies starting from epilepsy, where an increased synchronization of excitatory neurons is causing seizures. Altered network dynamics are also reported in sleep disorders such as narcolepsy, where a specific neuronal population (hypocretin neurons) is reduced in number, and the EEG rhythms normally observed during sleep are modified (18). Moreover, an impaired connectivity of resting-state networks was observed in schizophrenia by functional magnetic resonance imaging (19). *In vitro* experimental models can be a powerful tool for a deeper understanding of network disorders, and for drug screening of new neuroactive compounds.

2. “Traditional” dissociated cultures – Methods, applications and spontaneous activity

Neurons preserve the ability to generate spontaneous electrical pulses even when disrupted from their original connectivity and cultured *in vitro*. In fact, primary dissociated cultures, obtained from embryonic or postnatal

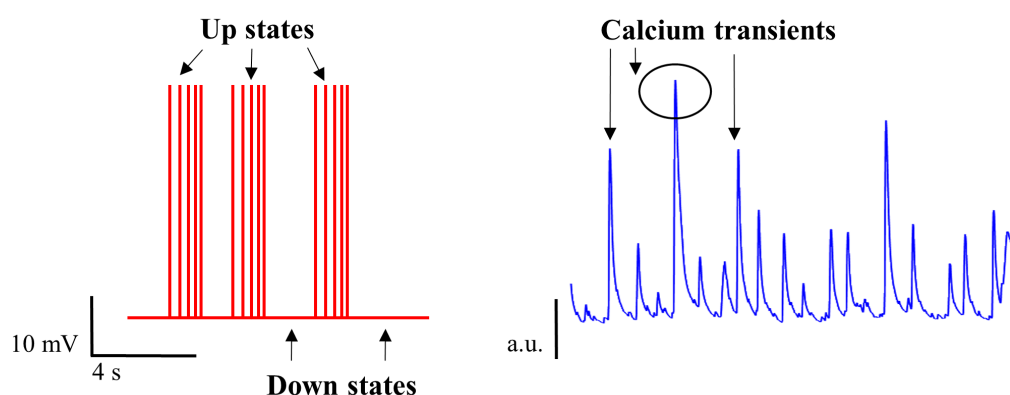


Figure 1. Schematic representation of spontaneous Up and Down states and calcium transients. The red trace on the left represents an example of a typical intracellular recording from a neuron in spontaneous conditions. Up and Down states are visible as rhythmic oscillations between membrane depolarization and hyperpolarization. The blue trace on the right depicts some typical calcium transients recorded from a single neuron in spontaneous condition. The time scale is the same for both traces. Note the increased duration of a single calcium transient comparing to a single Up state event.

rodent brain regions such as the hippocampus or the cortex, represent a well-established *in vitro* model for studying neuronal networks, both on a short (network formation) and a long term (differentiation, maturation) scale (20,21). When cells are enzymatically dissociated, plated on suitable substrates and cultured in appropriate media, they grow processes and form *ex-novo* a functional network. Dissociated cell cultures are traditionally plated on glass-made flat surfaces, allowing a fine space resolution for morphological investigation of small cellular structures, like neurites and growth cones. Neurons attach to surfaces through their anionic phospholipids (e.g. phosphatidylserine) (22), therefore the surface needs to be positively charged. Previous coating with polyaminoacids such as polylysine and polyornithine have since long been used for this purpose, together with the addition of components mimicking the extracellular matrix *in situ* (i.e. Matrigel® (23)). The morphology and development of dissociated neurons as well as their network activity are strongly influenced by the culturing conditions and, in particular, the culturing medium used (24). Different chemical compositions have been tested and are commercially available for neuronal cell cultures, all based on a physiological solution added with a particular supplier of nutrients and growth factors. Fetal bovine serum, for example, provides neurons with sufficient factors for short-term cultures. As any animal-derived product, however, it is prone to batch-to-batch variability (25) and, most of all, it results in a low differentiation of neuronal types (26). For this reason, serum-free media were tested with different additives. A very commonly used one is B27 Supplement®, aimed at obtaining almost pure neuronal cultures (27). Another option is to use glial cells, specifically astrocytes, as the intrinsic source of growth factors in the neuronal culture. For this purpose, it is possible to plate neurons on a glial feeder layer (28) or, in order to avoid the spatial interference of astrocytes while maintaining the soluble factors released by them, to grow neurons in an astrocyte-conditioned medium (ACM). ACM is supposed to contain lipids, thrombospondins and Brain Derived Neurotrophic Factor (BDNF), that are essential components for neuronal growth and survival (29). In fact, it was demonstrated to increase the life span of dissociated cultures up to several months (24,30).

Once established the optimal plating and growing conditions for the specific experimental purposes, it is possible to explore the properties of dissociated networks with a fine, single cell resolution. A common application is to analyze different neuronal populations optically labeled. A comparison of spike parameters between the GABAergic and glutamatergic neurons has been possible, in fact, because of the labelling of the former with a green fluorescent protein (31,32).

Network (i.e. synchronized) activity in dissociated neurons emerges at 3 - 7 days *in-vitro* (DIV) and matures over the following several weeks in culture, as shown by electrophysiological and imaging measurements (33,34). Synchronized neuronal activity has been proven to be mediated by the NMDA receptor, by serotonin

and acetylcholine (35). Network activity is also strongly dependent on the initial plating density of neurons: neurons plated at high density present higher rates of less synchronized activity comparing to sparse networks (28). In fact, there is an inverse relationship between the density of the network and its synaptic strength, such as sparse neurons establish less but stronger synaptic connections, as shown also by the longer duration of their bursts. Interestingly, dense neuronal cultures present a peak in synchronicity at DIV15-16, followed by a decrease (36) that resembles the one observed in the intact cortex during development (37).

Recent studies showed some differences in spontaneous, single-unit spike activity between dissociated neurons and awake animals: *in vitro*, neurons were more likely to fire in synchronized bursts; while *in vivo* the firing events were mainly composed by single spikes. Moreover, a greater variability was observed *in vitro* when quantifying the frequency of firing (38). However, as previously mentioned, the variability in spontaneous activity of dissociated neurons can be minimized by adopting a consistent culturing method and, most of all, the same plating density among different preparations.

Dissociated neurons served as a model for studying the progression of neuronal disorders. In a recent study (39), neurons dissociated from a mouse model of Fragile X syndrome (Fmr1 KO) were recorded by means of microelectrode arrays (MEAs) from 7 to 35 DIV, and exhibited an abnormally increased synchronization of spontaneous firing events at mature stages. Moreover, they showed oscillations in the *beta* frequency range, which were not observed in controls. These results are in line with previous studies showing network hyperactivity and neuronal hyperconnectivity in the intact cortex of Fmr1 KO mice (40,41).

Finally, dissociated cultures represent the only method for growing human-induced pluripotent stem cells (hiPSCs). These precursors can be differentiated into neurons, allowing the investigation of their molecular and electrophysiological properties (42,43). Most of all, they can be derived from patients for the purpose of specific drug development or cell therapies. Using appropriate differentiation methods, hiPSCs can generate three-dimensional (3D) brain organoids composed by neurons and glial cell types (44).

3. 3D neuronal cultures – methods, applications and spontaneous activity

Traditional, 2D neuronal cultures have been replaced in many studies by more recent 3D architectures for several reasons. First, neurons cultured on glass coverslips are prone to artifacts due to the flat condition in which they grow. Glass coverslips are in fact distant from the physiological extra-cellular environment of the brain, which is 3D and soft (less than 1 kPa in Young modulus, while the stiffness of the glass is in the order of GPa). Neurites forced to grow in a planar region present indeed an abnormal excitability (45). Moreover, using plastic materials instead of glass allows for directing the

differentiation of stem cells towards bone, muscle and neuronal cell types by progressively decreasing the stiffness of the substrate (46). Therefore, 3D substrates with appropriate stiffness were developed for neuronal cultures, and called neuroscaffolds.

An interesting example is represented by the carbon-based neuroscaffolds, engineered for interfacing the cell membrane structures. Carbon-based materials have since long raised the interest of neuroscientists because of the electrical conductivity of the material, that might find its most obvious application in restoring/improving neuronal connectivity (47,48). These materials can be assembled in the form of wrapped graphite sheets, and therefore called carbon nanotubes (CNTs), or in the form of graphene foams. Both cases were proven to be biocompatible and conductive scaffolds not only for culturing primary neurons, but also for neural stem cell differentiation (49,50).

Interestingly, primary hippocampal neurons plated on 3D graphene showed a more frequent and synchronized spontaneous activity comparing to both 2D graphene and traditional 2D glass substrates (51). These results suggest a specific role of dimensionality in network dynamics that is independent from the material where neurons grow. However, a recent study demonstrated that a single layer of graphene modifies the ionic concentration at the interface with neurons so that they will exhibit stronger potassium currents and will shift from adapting to tonically firing. This effect was not observed when neurons were plated on different conductive materials (52). Taken together, these results suggest that both the 3D structure and the electrical properties of the substrate can increase neuronal firing and connectivity. In fact, adding CNTs as a coating to a 3D substrate was proven to boost the synaptic activity of dissociated neurons, as shown by calcium imaging experiments (53). For these reasons, carbon-based materials have been tested in *in vivo* implants for restoring nerve mechanical or chemical injury (54,55) as well as for deep brain stimulation and monitoring (56,57).

Polydimethylsiloxane (PDMS) and other plastic materials represent another interesting category for neuronal studies: while not electrically conductive, these materials have the advantage of being highly ductile and with a Young modulus closer to the one of brain tissues. The fabrication of these types of scaffolds usually requires photolithography techniques combined with micro/nano-patterning (58). Some preliminary treatments - such as plasma cleaning and polylysine/polyornithine coating - are necessary to reduce the hydrophobicity of the plastic and ensure neuronal attachment. Plastic micropillars have often been used as substrates for cell cultures in order to decrease the stiffness of the original material: while a flat surface of PDMS has a Young modulus of around 1 MPa, PDMS micropillars have a stiffness of 5 - 20 kPa depending on their diameter and height (59). For these reasons, micropillars are useful for determining the mechanical responses and forces exerted by cells (60). Plastic

materials can also be molded at the nanoscale: the use of nanopillars with different dimensions and stiffness has been proven to control the adhesion and differentiation of neuronal precursors (61). Moreover, the addition of plastic nanofibers made of poly (lactic-co-glycolic acid) to PDMS micropillars was proven to increase the frequency and amplitude of spontaneous calcium transients in primary hippocampal neurons (62). Due to their biocompatibility, PDMS-based microchannel electrodes have successfully been implanted in rodents for monitoring neural activity over time and for providing a mechanical support for peripheral nerve regeneration (55).

In addition, scaffold-free 3D cultures can be derived from hiPSCs to generate brain organoids. HiPSC-derived organoids have been used for drug screening and modeling of central nervous system diseases (63).

In summary, 3D neuronal cultures recapitulate several aspects of *in vivo* brain tissue, from synaptic properties of individual neurons to network synchronization. Moreover, comparing to 2D cultures, they offer the unique advantage of recreating organoids with neuronal and glial types interconnected in a cyto-architecture that is able to reproduce the one of the brain.

4. Explanted brain slices – methods, applications and spontaneous activity

Slices of the brain or spinal cord can be isolated from the region of interest and directly used for electrophysiological measurements and imaging (17,61). These preparations are referred to as acute slices and, in contrast to dissociated cell cultures, they preserve the native cyto-architecture of the tissue. Acute cortical slices are able to reproduce the most characteristic feature of the cerebral cortex, i.e. slow oscillations between Up and Down states, as shown by the work of Sanchez-Vives starting from the 2000s (65,66). In this work, intracellular recordings from ferret visual and prefrontal cortical slices were performed using microelectrode arrays. While no spontaneous activity was recorded in the “traditional” slice bathing medium (2 mM Ca^{2+} , 1 mM Mg^{2+} , 2.5 mM K^+), some rhythmic oscillations were observed after changing the bath solution to a composition more similar to the brain interstitial fluid (1.0 or 1.2 mM Ca^{2+} , 1 mM Mg^{2+} and 3.5 mM K^+). These oscillations were initiated by pyramidal neurons of layer V, had a periodicity of once every 3.5 seconds and were stable for the life of the slice (12 hours). The cortical slices were prepared from 3 to 8 month-old ferrets and showed a close similarity to those observed in adult, anesthetized cats. In fact, the spontaneous activity of slices was studied as the most likely *in vitro* equivalent of the activity of the brain in the absence of external stimuli, i.e. during quiescent sleep or under anesthesia. In a more recent study from the same group, variable propagation modes of Up states were observed, suggesting a memoryless generation of slow oscillations (10).

Slices of tissue cultured *in vitro* for several days are instead referred to as organotypic slice cultures. These

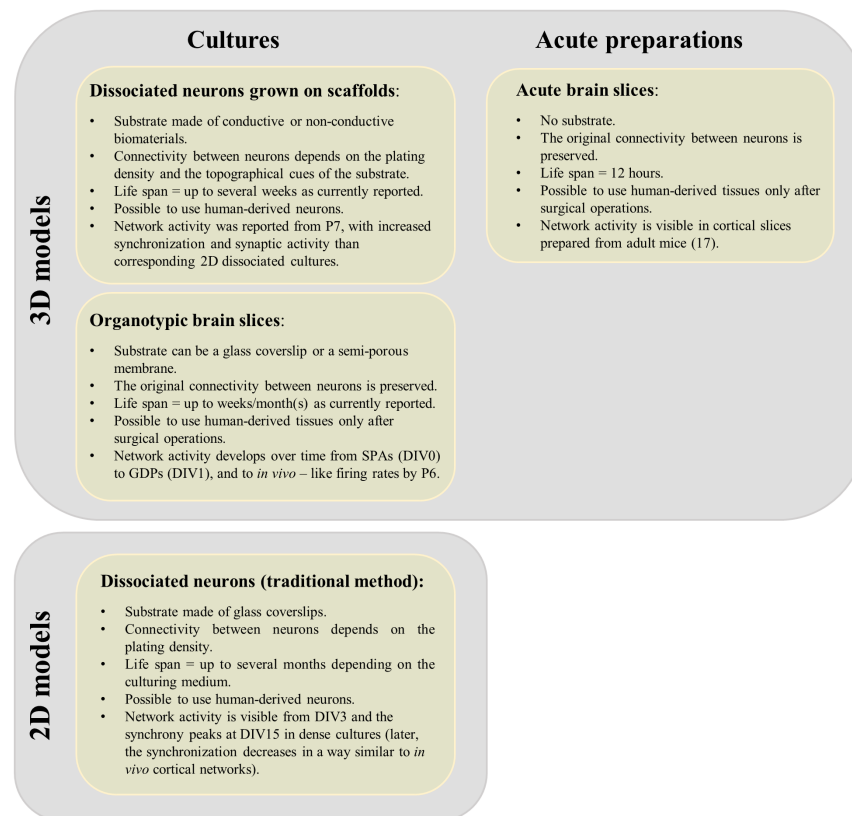


Figure 2. Summary and comparison of in vitro neuronal network models. 3D models include brain slices and dissociated neurons grown on scaffolds. 2D models are represented by dissociated neurons grown on glass (traditional method). The only preparation that does not require culturing is represented by acute brain slices.

preparations were proven to regrow synaptic processes and to restore the functions potentially impaired during the cutting procedure. There are two commonly used methods for preparing organotypic cultures from nervous tissues. In the Gähwiler method (67), slices are cut and attached on glass coverslips by coagulating chicken plasma with thrombin; the coverslips are then inserted into plastic tubes and kept in serum-added culturing medium on a roller drum (ensuring balanced time of oxygenation) at 37 °C. The Stoppini method (68) follows a similar procedure, but the slices are placed on sterile, transparent semiporous membranes in Petri or multiwell dishes, and kept at the interface between air and culturing medium at 37 °C. In this case, it is possible to culture the slices in Neurobasal medium added with B27 supplement, with no addition of animal serum. However, organotypic slices grown in the absence of serum have been reported to develop spontaneous epileptiform activity (69).

The use of organotypic cultures allows to perform chronic multineuron imaging. For example, daily morphological analyses and calcium imaging in hippocampal slices revealed that GABAergic interneurons are involved in the generation of SPAs at DIV0; starting from DIV1, the spontaneous activity of the slice switches to giant depolarizing potentials (70), as in line with previous findings on the development of hippocampal

networks (71).

Interestingly, in 2014, Okamoto *et al.* demonstrated that organotypic hippocampal slices exhibit firing rates and excitatory synaptic activity more similar to those observed *in vivo* comparing to acute slices (72). In fact, cutting several neurites during the preparation might affect the network activity of acute slices, which do not have time to regrow these processes.

Without the technical issues presented by *in vivo* measurements (i.e. background noise, the interference of anesthetics and the need of complex surgeries), experiments on slices have been useful to address questions like how inhibitory neurons coordinate synchrony in the network. In a study of 1995 (73), GABAergic neurons were anatomically identified from hippocampal slices and depolarized to produce fast inhibitory postsynaptic potentials: the excitatory, pyramidal neurons synaptically connected to them exhibited some sub-threshold oscillations in the *theta* frequency range, demonstrating that these spontaneous oscillations in the hippocampus are tuned by the inhibitory activity of GABAergic neurons. Subsequently, a reconstruction of functional connectivity in acute hippocampal slices was performed by Bonifazi *et al.*, combining electrophysiological recordings with calcium imaging: connections in between neurons were assumed

on the base of the temporal correlations of their activation. Stimulating some specific, highly connected neurons in the network, they observed a higher occurrence of spontaneous GDPs in the network, suggesting the existence of functional hubs that were identified as a subpopulation of GABAergic neurons (64). Results obtained in cortical slices also showed that networks of fast spiking, electrically coupled interneurons coordinate the spontaneous activity (74).

These results are in line with subsequent studies in behaving mice, characterizing the membrane potential dynamics of fast-spiking inhibitory neurons vs excitatory ones (75), and also in human and monkeys during sleep, when the local field potential of the neocortex was measured with MEAs (16). Taken together, these results confirm that brain slices are a suitable *in vitro* model for investigating network dynamics. They are prepared typically from animal models. However, the use of human brain slices is possible from resected or postmortem tissues (76,77).

5. Conclusions

Both dissociated and organotypic cultures exhibit a spontaneous neuronal firing with *in vivo*-like patterns in their course of development (quantified by days *in vitro*). Acute and organotypic slices were used for the most remarkable studies on spontaneous neuronal oscillations, given the fact that they preserve the original cyto-architecture of the region of interest. On the other side, 3D substrates made of carbon-based or plastic materials can compensate for the lack of architecture in traditional neuronal cultures and boost the synaptic activity of dissociated neurons, opening the way for neural prostheses and cell culture implants in the central and peripheral nervous system. However, there is no strong evidence that differentiated neurons plated on neuroscaffolds can reproduce the time course of development of intact neuronal networks. HiPSCs, as opposite, are able to reproduce some aspects of human brain development when differentiated into scaffold-free, 3D organoids. HiPSCs offer in fact a powerful model for drug screening and customized therapy. Finally, the use of dissociated neurons and hiPSCs can diminish the number of experimental animals used, making it a more ethical tool for scientific studies and even a pre-clinical test platform for screening of new neuroactive compounds.

For a summary and comparison of the properties of the *in vitro* models discussed within this review, see **Figure 2**.

Conflict of interest

The authors declare that they have no conflict of interest.

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