In Vitro Models of Brain Disorders

Joost le Feber

Abstract The brain is the most complex organ of the body, and many pathological processes underlying various brain disorders are poorly understood. Limited accessibility hinders observation of such processes in the in vivo brain, and experimental freedom is often insufficient to enable informative manipulations. In vitro preparations (brain slices or cultures of dissociated neurons) offer much better accessibility and reduced complexity and have yielded valuable new insights into various brain disorders. Both types of preparations have their advantages and limitations with regard to lifespan, preservation of in vivo brain structure, composition of cell types, and the link to behavioral outcome is often unclear in in vitro models. While these limitations hamper general usage of in vitro preparations to study, e.g., brain development, in vitro preparations are very useful to study neuronal and synaptic functioning under pathologic conditions. This chapter addresses several brain disorders, focusing on neuronal and synaptic functioning, as well as network aspects. Recent progress in the fields of brain circulation disorders, excitability disorders, and memory disorders will be discussed, as well as limitations of current in vitro models.

Keywords Brain disorders · In vitro model · Micro Electrode Array · Hypoxia · Excitability · Memory

1 Introduction

The brain is by far the most complex organ of the human body, and our understanding of brain physiology and pathology remains limited. Given the highly complex combination of physiological processes, various pathologies may result in a wide range of brain disorders. Due to the lack of understanding of the underlying

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mechanisms and difficulties to measure many of the relevant processes in patients, brain disorders are generally diagnosed and classified by the occurrence of clinical symptoms. Because different pathologies may result in similar symptoms, some brain disorders lead to spectrum diagnoses with no clear definition of the underlying pathology. Epilepsy, for example, is such a spectrum diagnosis that may have causes that range from channelopathies to traumatic brain injury or stroke (Bhalla et al. 2011; Shorvon 2011). On the other hand, different diagnoses may exist for disorders that to a certain extent share pathology. For example, the formation of Lewy bodies is strongly associated with Parkinson's Disease, but also with Dementia with Lewy bodies. Discrimination between these diagnoses is often based on the temporal evolution of clinical symptoms (Emre et al. 2007; McKeith et al. 2005).

Practical and ethical limitations severely hamper the investigation of brain disorders in situ, and new insights are obtained mainly from animal models. The poor correlation between clinical diagnosis of brain disorders and the underlying pathological mechanisms complicates the design of animal models to investigate brain disorders and to develop treatment. A primary criterion for animal models is often the ability to mimic clinical symptoms. However, these symptoms may arise from mechanisms that differ from that leading to the patient's disorder. Consequently, many approaches that seemed very promising in animal studies could not, or only partially, be translated to the clinic (van der Worp et al. 2010).

A possible improvement lies in the use of in vitro models of brain disorders. In vitro models can target a specific mechanism and investigate possible treatment. Adversely, in vitro models usually do not exhibit all symptoms that are clinically associated with the disorder. Whereas it may be difficult to distill a specific mechanism underlying a disorder for detailed investigation in an in vitro model, the translation of results to the clinic may also be obscured. Moreover, the high experimental freedom of in vitro models may lead to solutions that cannot be translated to treatment because clinical practice shows far less freedom. For example, drugs that worked well in in vitro models may not be able to pass the blood–brain barrier, or may appear to cause problems in systems that were not included in the in vitro model (de Lange et al. 2017). Still, in vitro models can be very useful to study brain disorders and for development of treatment, provided that relevant underlying mechanisms can be isolated in an in vitro model and care is taken that treatment may also be applicable in patients. Accordingly, currently available in vitro models concentrate on dysfunction at the cellular or synaptic level, or, more recently, at the level of small networks.

2 In Vitro Models

In vitro models exist for a range of brain disorders. The most prominent include stroke, epilepsy, and memory-related disorders. The main objectives to pursue through the model are investigation of disease pathophysiology, identification of novel biomarkers, options for mechanism-based treatment, or high-throughput drug

screening. In vitro models may be based on acute or organotypic brain slices, or on cultures of dissociated neurons. Acute slices are more frequently used than organotypic slices, and, after preparation, can typically be used for several hours, although recently methods have been developed that facilitate longer slice viability (Buskila et al. 2014). Animals are anaesthetized and decapitated. Then, the brain is removed and stored in cold artificial cerebrospinal fluid. After dissection of the relevant areas, usually hippocampus or cortex, ~300–500 μm thick slices are cut on a microtome and put in a dish with carbogen (a mixture of 95% O_2 and 5% CO_2) bubbled medium.

The use of brain slices allows the electrophysiological study of neurons, synapses, or neural circuits under controlled conditions, in isolation from the rest of the brain and body. It facilitates stimulating and/or recording from single or multiple neurons or axons and provides large experimental freedom. Brain slice experiments are faster and cheaper than in vivo studies and do not require anesthesia after the initial decapitation. Separation of the brain tissue from the body avoids muscle artifacts, as well as possible limitations imposed by the blood–brain barrier. Finally, brain slices maintain some of the structural connections that are present in vivo, but are lost in dissociated cell cultures. Drawbacks include the limited time window for experiments, the missing input and output connections as present in the whole brain, and, in particular, the high oxygen fraction in the gas mixture needed for perfusion. Maintaining brain slices in 95% O_2 may produce hyperoxia, oxidative stress, and increased cell death (D'Agostino et al. 2007). Furthermore, decapitation and extraction of the brain before the slice is placed in the recording solution may have effects on the tissue, and slicing of the brain damages the edges of preparations.

Organotypic slices combine an in vivo-like structure with a long time window for experimenting. However, this approach is technically more challenging because it generally requires thinner slices and sterility must be maintained throughout their life in vitro (Hutter-Schmid et al. 2015). Furthermore, organotypic slices are preferably obtained from a young donor, and undergo further development during their life in vitro. In vitro development may differ from regular in vivo development, which limits the usability of organotypic brain slices to model brain disorders that typically occur with aging (Humpel 2015).

An alternative approach uses neurons, usually obtained from embryonic or newborn rats or mice, which are dissociated, and plated on micro electrode arrays (MEAs). Also, the differentiation of induced pluripotent stem cells has become a compelling technique to acquire cells for plating on MEAs. After plating, neurons grow out dendrites and axons, and form new synapses. Newly formed synapses include glutamatergic, excitatory synapses as well as GABAergic ones, which are in principle inhibitory. During early development (up to ~10 days), however, GABAergic synapses exert a net excitatory effect (Ben-Ari 2002). After a maturation period of ~3 weeks, cultures show quasi stable firing patterns and are ready for experimenting. Cultures of dissociated neurons on MEAs offer easy access to many neurons, while cultures remain vital for up to several months. Whereas dissociated cultures lack typical in vivo brain structure, these models are

mainly applied to study basic physiological functioning of various types of neurons, synapses, and astrocytes. Relevant brain disorders include circulation disorders (Patel 2008), excitability disorders (Badawy et al. 2012; Holmes and Ben-Ari 2001), and memory disorders (Ashford 2008; Kopelman 2002), which will be addressed in the following sections.

3 Brain Circulation Disorders

3.1 Stroke

The most prominent disorder related to interrupted brain circulation is (ischemic) stroke. Taking into consideration that about 13 million people per year suffer a stroke worldwide, which is lethal in 30% of all patients and another third is left permanently disabled (Mackay and Mensah 2004), stroke poses a serious health problem, particularly to an aging population. The only effective treatment to improve outcome is acute recanalization by intravenous thrombolysis (Grond et al. 1998; Wardlaw et al. 1997) or intra-arterial thrombectomy (Goyal et al. 2016; Rodrigues et al. 2016). Treatment to promote recovery of ischemic cerebral damage is not available. Moreover, secondary damage of brain tissue occurs in approximately one third of patients during the first days after the infarct and leads to additional neurological impairment. For these patients, no therapy is available (Roger et al. 2011).

Occlusion of a brain artery typically results in an infarct core, with loss of neuronal functioning followed by irreversible brain damage and cell death within minutes. The core is often surrounded by a penumbral region, with some remaining, but significantly reduced perfusion through collateral arteries (Fig. 1). The ischemic penumbra is defined as an area of brain tissue with insufficient blood flow to maintain neuronal activity but adequate blood flow to preserve neuronal viability (Symon et al. 1977). Here, neuronal function is severely compromised although damage is initially reversible. During the first days, the penumbra may further deteriorate or recover. The underlying processes that determine either outcome remain ill understood. Whereas the infarct core must be regarded as lost, the penumbra offers opportunities for the development of treatment to promote recovery.

The restricted availability of oxygen and glucose in the penumbra significantly limits the mitochondrial production of adenosine tri phosphate (ATP), the major energy source in the brain. One of the early consequences of ATP depletion in the ischemic penumbra is large-scale synaptic failure (Bolay et al. 2002; Hofmeijer et al. 2014; Khazipov et al. 1995; le Feber et al. 2017). However, synapses initially remain intact, and if oxygen is restored in time, synaptic failure appears to be reversible (Somjen 1990). Impeded synaptic trafficking generally leads to strongly reduced neuronal activity in the penumbra. In stroke patients, a reduction in cerebral blood flow below 15–18 ml/100 g/min was found to cause immediate electrical silence,

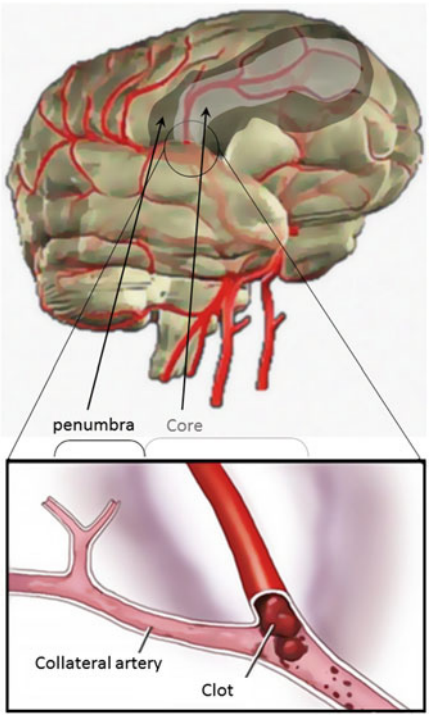

Fig. 1 The ischemic penumbra. In the core of a stroke (indicate as a light gray area), all perfusion is impeded and neurons rapidly progress to cell death. Often an area with some remaining collateral perfusion, the penumbra, lies around the core (indicated by the dark area). In the ischemic penumbra cells initially remain viable but silent, due to large-scale synaptic failure occurring in this area

as observed by flattening of the EEG (Yang et al. 2014). Although functionally silent, the penumbra is considered structurally intact and viable (Hofmeijer and van Putten 2012). Electrophysiological dysfunction is regarded as a key event in the pathogenesis of ischemic brain injury, but the following sequence of events is not well known. Further steps following the initial, reversible silence are difficult to determine in patients. This is where in vitro models can be exploited.

3.2 Postanoxic Encephalopathy

Another common disorder associated with failure of brain circulation is postanoxic encephalopathy (PAE), resulting from a period of low or absent cerebral perfusion after cardiac arrest or shock, severe respiratory distress, suffocation or near-drowning. In contrast to stroke, impeded circulation in PAE is transient. The duration, as well as the depth of ischemia, the "hypoxic burden", differs widely between patients, and is a key determinant of the neurological outcome. PAE after cardiac arrest has been widely studied. Annually, around 1 out of 1000 people in the western world experience a cardiac arrest (Berdowski et al. 2010; Rea et al. 2004). Around 80% of these patients remain comatose after restoration of

spontaneous circulation (Madl and Holzer 2004). Although on average 20–30% of these patients survive and regain consciousness (Zandbergen et al. 2003), most remain unconscious and evolve towards brain death or a persistent vegetative state (Kaye 2005). It is critical to restore circulation as soon as possible. While earlier reports suggested that mild therapeutic hypothermia may limit further brain damage (Arrich et al. 2010; Bernard et al. 2002; Hypothermia after Cardiac Arrest Study Group 2002), more recently it has been shown that prevention of fever is probably more relevant, motivating most centers to treat these patients accordingly (Nielsen et al. 2013). Other treatments have not shown substantial benefit (Moragas Garrido and Gascón Bayarri 2012).

Given the complexity and expenses of treatment, and the emotional burden for relatives, in combination with the relatively small fraction of patients with neurological recovery, early stage reliable prognosis for individual patients is invaluable. Studies on outcome prediction have focused mostly on neurological examination, clinical neurophysiological tests and biochemical parameters. Results for biochemical parameters and neuroimaging are inconclusive (Zandbergen 2008). Timing and development of abnormalities in continuous EEG recording reportedly provide better prognostic tools (Hofmeijer et al. 2015; Oh et al. 2015; Ruijter et al. 2017; Tjepkema-Cloostermans et al. 2015). While continuous EEG has been shown to allow reliable prognostication, underlying pathophysiological mechanisms remain unclear. Although the EEG reflects synaptic activity (Buzsaki et al. 2012), it is far from trivial, if not impossible, to deduce detailed characteristics of synaptic and neuronal functioning under postanoxic conditions in situ. In vitro models provide better accessibility to neurons and synapses and have been used to study mechanisms underlying PAE.

3.3 In Vitro Models of Oxygen/Glucose Deprivation

Regular cellular functioning requires ATP, which is normally produced by oxidizing glucose. Impeded blood circulation in the brain means that less glucose and oxygen, and therefore less ATP, become available to cells in the brain.

3.3.1 Brain Slices

Slices can be obtained from animals with induced stroke, but mostly hypoxia or transient anoxia are applied after preparation of the slices. For transient anoxia, carbogen perfusion is temporarily replaced by a 95% N_2/5% CO_2 mixture. For hypoxia, oxygen and nitrogen can be mixed in any ratio, and supplemented with 5% CO_2. Most slice models restrict oxygen, but not glucose. Reduction of glucose from the perfusion medium had similar effects as oxygen restriction although recovery of synaptic function occurred after longer periods of glucose lack than of oxygen lack (Schurr et al. 1989).

Brain slices have long been used to study the relationship between metabolism and activity (Lipton and Whittingham 1984). Neurons in acute brain slices usually survive periods of anoxia of several minutes, and they remain able to generate action potentials (Fujiwara et al. 1987). On this time scale, much stronger alterations were seen in synaptic functioning (Lipton and Whittingham 1982). Evoked inhibitory postsynaptic potentials (IPSPs) were abolished within a few minutes after the onset of hypoxia, while evoked excitatory postsynaptic potentials (EPSPs) were maintained five times longer (Fujiwara et al. 1987; Krnjević et al. 1991). This seemingly higher vulnerability of inhibitory hippocampal synapses, however, has been contradicted in later work that showed that inhibitory synaptic transmission is quite resistant to short (3 min or 4–6 min) lasting anoxia (Khazipov et al. 1993; Zhu and Krnjević 1994). Recordings from cortical slices did not reveal any differences in susceptibility for hypoxia between interneurons and pyramidal cells (Luhmann et al. 1993). Work by Khazipov et al. (1993) revealed that particularly excitatory synapses to inhibitory postsynaptic neurons appeared vulnerable to hypoxia. Administration of exogenous receptor agonists suggested that the suppression of EPSCs is due to presynaptic mechanisms (Khazipov et al. 1993, 1995; Sun et al. 2002). Suppression of IPSPs may also depend on presynaptic mechanisms (Khazipov et al. 1993; Krnjević et al. 1991) although later work suggested that evoked transmitter release from GABAergic terminals was not affected by anoxia (Khazipov et al. 1995).

Synaptic depression is in principle reversible, provided that the hypoxic burden, determined by depth and duration of hypoxia, is sufficiently mild. Lower oxygen levels during hypoxia, and longer duration were associated with a lower recovery rate of synaptic function upon restoration of oxygenation (Schurr et al. 1989). Excitatory synaptic transmission recovered immediately as oxygenation was reinitiated (Sun et al. 2002). After reoxygenation, inhibitory synaptic transmission (to pyramidal cells) recovered slowly, and not always completely (Krnjević et al. 1991). The hypoxia-induced reduction in excitatory and inhibitory synaptic transmission was significantly smaller in immature than in adult neocortical slices (Luhmann et al. 1993).

3.3.2 Cultures of Dissociated Neurons

While the use of acute brain slices has enabled the discovery of several consequences of exposure to hypoxia, one of the major limitations laid in the restricted duration of experiments. Recovery or further deterioration in the ischemic penumbra, as well as decisive development in postanoxic encephalopathy occurs at longer timescales. Therefore, other models have been developed, in particular based on cultures of dissociated neurons. Such cultures, plated on micro electrode arrays (MEAs), have been exposed to transient anoxia (Hofmeijer et al. 2014; Stoyanova et al. 2016) as an in vitro model of postanoxic encephalopathy, or to hypoxia of varying depth and duration (le Feber et al. 2016, 2017, 2018) to model the ischemic penumbra.

Hypoxia was achieved by regulation of the gas mixture above the culture medium bath, which contained air and N_2 in any desired ratio, supplemented with 5% CO_2. This resulted in partial oxygen pressures between 1% and 19% of atmospheric pressure, and facilitated variable duration of hypoxia. Although the composition of gas mixtures could be changed quite rapidly, slow diffusion in the medium bath significantly slowed down imposed changes. Consequently, the timing of changes observed under hypoxic conditions in dissociated cultures and acute slices cannot be directly compared.

Exposure to hypoxia rapidly decreased recorded spontaneous activity (Fig. 2), probably related to suppressed excitatory synaptic transmission (Hofmeijer et al. 2014; Segura et al. 2016). The extracellular recording technique enables the detection of action potentials, but does not show subthreshold fluctuations of the membrane potential. Traditional techniques, based on intracellular recordings, determine (changes in) synaptic efficacy by the observed changes in excitatory (inhibitory) postsynaptic potentials (EPSPs and IPSPs) or currents.

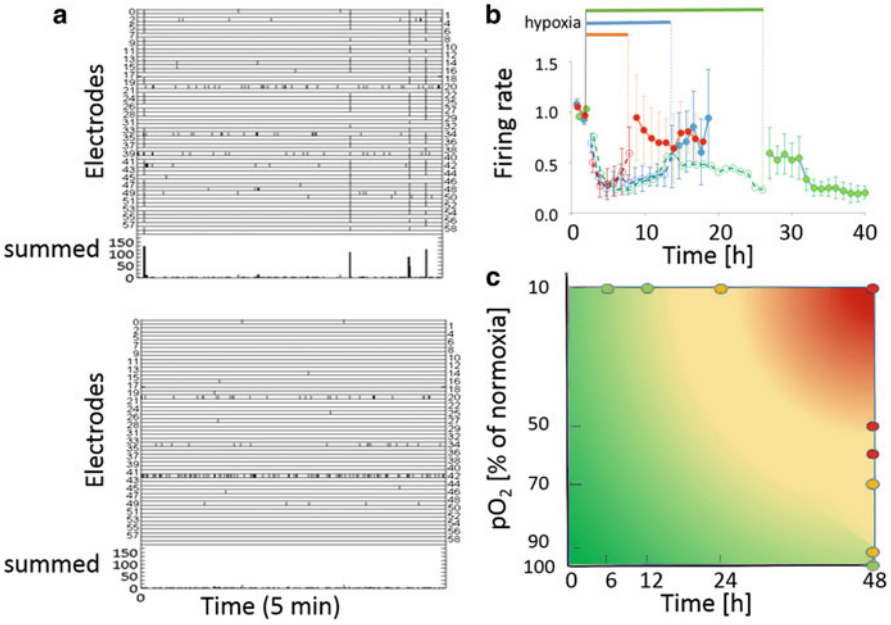

Fig. 2 Hypoxia affects network activity in cultures of dissociated cortical neurons. (**a**) shows the effect of severe hypoxia (10% of normoxia) on firing rate and pattern. During normoxic recording (upper panel), there is more activity and patterns show more frequent synchronized bursting than during hypoxia (lower panel). (**b**) quantifies network wide activity as recorded before, during, and after hypoxia at this depth (expressed as a fraction of baseline activity). Partial recovery of activity during hypoxia suggests the presence of activity homeostatic mechanisms that aim to compensate for the low activity. Further recovery of activity occurs if the culture is reoxygenated after 6 or 12 h. Upon reoxygenation after 24 h only partial recovery occurred. Recovery depended not only on the duration, but also on hypoxic depth (**c**). Figures based on le Feber et al. (2016, 2017, 2018)

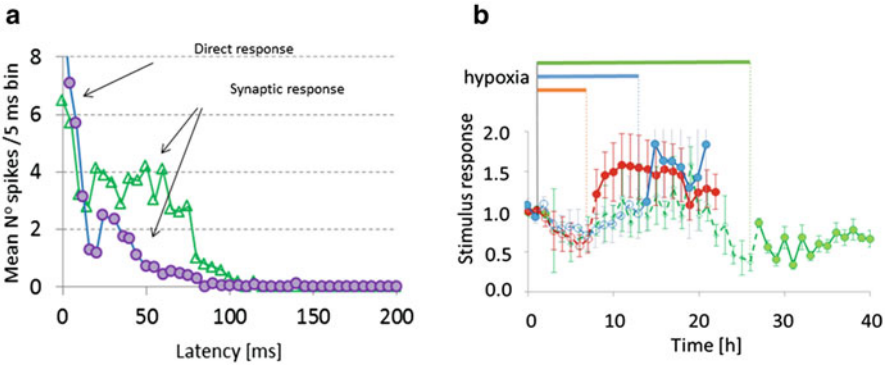

Fig. 3 Hypoxia affects stimulus responses. Responses to electrical stimulation through one of the electrodes typically contain a direct response dominated by directly induced action potentials with latencies up to 10–15 ms, followed by a synaptically mediated network response. (**a**) shows two examples of average responses to stimulation at two different electrodes in one culture when stimulated at $t = 0$. (**b**) quantifies the synaptic phase of stimulus responses before, during, and after hypoxia (10% of normoxia), and shows that stimulus responses become strongly potentiated if the culture is reoxygenated after 6 or 12 h, but not after 24 h (le Feber et al. 2015, 2018)

In MEA-based hypoxia models, synaptic functioning was assessed by the synaptically mediated phase of responses to electrical stimulation. These responses typically consist of two phases: a direct response and a synaptically mediated response (Fig. 3a). The direct response, with latencies up to ~15 ms is dominated by action potentials that are directly induced by the stimulation current. Consequently, this phase of the stimulus response reproduces relatively well, has low jitter, and persists during excitatory synaptic blockade (Marom and Shahaf 2002; Wagenaar et al. 2004), indicating that a substantial part of the response in this phase does not depend on synaptic transmission. The group of neurons that is synchronously activated in the first phase, in turn, often generates sufficient input to the rest of the network to induce a network response. This indirect response is abolished after synaptic blockade (Fedorovich et al. 2017) and represents the synaptically mediated network response.

Experimental results confirmed that synaptic failure occurs rapidly after the induction of hypoxia (Hofmeijer et al. 2014; le Feber et al. 2016), while neurons remain viable, and able to generate action potentials (le Feber et al. 2016; Segura et al. 2016). This is at least in part due to presynaptic mechanisms, including adenosine-mediated mechanisms (Khazipov et al. 1995; Sun et al. 2002), impeded phosphorylation of presynaptic proteins (Bolay et al. 2002), and impeded endocytosis and exocytosis of synaptic vesicles (Fedorovich et al. 2017). Synaptic failure leads to significant reduction of ongoing network activity (Hofmeijer et al. 2014; le Feber et al. 2016; Segura et al. 2016).

Low activity may jeopardize network viability because neuronal survival depends on regular calcium influx, which is promoted by electrical activity (Ghosh et al. 1994; Mao et al. 1999). Low activity has been shown to trigger compensatory

mechanisms aiming to maintain the total network activity within a certain (healthy) working range. Such homeostatic activity regulation can be achieved by up-regulation of excitatory synapses (Turrigiano 2008), and down-regulation inhibitory synapses (Kilman et al. 2002). At longer time scales, activity homeostasis may also be achieved by growth of axons (Schmitz et al. 2009) and dendrites (Wong and Ghosh 2002), and the formation of spines and boutons (Florence et al. 1998). Partial recovery of activity during hypoxia and potentiated stimulus responses upon return to normoxia (Fig. 3b), as well as a relative increase of the excitatory synapse density (le Feber et al. 2016, 2017, 2018) support the idea of activity homeostasis. As synaptic scaling has been shown to take place postsynaptically (Turrigiano et al. 1998), it may reflect postsynaptic compensation of presynaptic failure. This process requires ATP, which is scarce under hypoxic conditions, and the effectiveness is questionable. Furthermore, activity homeostatic processes may lead to network hyperexcitability, a phenomenon that is frequently observed in patients after stroke (Liepert et al. 2000; Manganotti et al. 2002; Swayne et al. 2008).

3.3.3 Limitations

For models of brain circulation disorders, it is important that the fraction of astrocytes in the cell population mirrors that in vivo. Astrocytes occupy a substantial amount of space in the in vivo brain (Azevedo et al. 2009; Magistretti and Pellerin 1999) and provide essential metabolic support to neurons during transient ischemia (Rossi et al. 2007; Takano et al. 2009). Experiments in hippocampus showed that during hypoxia astrocytes may reduce presynaptic transmitter release (Martín et al. 2007). Conversely, astrocytes are able to restore neuronal activity under conditions of glucose deprivation due to lactate provided by the astrocytes (Rouach et al. 2008).

A general limitation of slices as well as dissociated cultures lies in the interpretation of hypoxia/normoxia. Partial oxygen pressure during normoxia in the in vivo rat brain averages around $pO_2 \approx 30–35$ mmHg (Grote et al. 1996; Nair et al. 1987), much lower than normoxia as normally applied to slices or dissociated cultures. Neurons obtained from the striatum have been cultured under low oxygen conditions and were shown to survive. They showed larger mitochondrial networks, greater cytoplasmic fractions of mitochondria, and larger mitochondrial perimeters than those cultured at atmospheric oxygen levels (Tiede et al. 2011), illustrating that cells adapted to low oxygen, and that culturing under lower oxygen conditions from the time of plating may improve the resemblance between in vivo and in vitro.

4 Excitability Disorders/Epilepsy

Neuronal excitability at the cellular level can be described as the propensity of a neuron to generate an action potential in response to receiving a defined input signal. Excitability is a critical parameter for brain functioning and should not increase or

decrease beyond the boundaries of a certain healthy working range. Subthreshold excitability, for instance, may occur during anesthesia (Palmieri et al. 1999) or in disorders of consciousness (Lapitskaya et al. 2013), whereas the most prominent disorder associated with excessive excitability is epilepsy.

Epilepsy is a chronic condition, and the fourth most common neurological disorder in the USA (England et al. 2012). Affecting people of all ages, an estimated 70 million people suffer from epilepsy worldwide (Singh and Trevick 2016). Recurrent, unprovoked seizures form the hallmark of epilepsy, which can severely affect patients' safety, relationships, work, driving, and quality of life. Epilepsy is a spectrum condition with a wide range of seizure types, varying from person to person (Jensen 2011). For about one third of all patients with epilepsy, no adequate treatment is available. Despite significant efforts to develop new antiepileptic medications over the past decade, this percentage has remained relatively stable, possibly related to the unknown cause in ~60% of epilepsy cases (Epilepsy Foundation). Partial or focal seizures originate in a part of one hemisphere, whereas primary generalized seizures start in both hemispheres simultaneously. Further subdivision of seizures is based solely on clinical and electroencephalographic (EEG) descriptive data (Berg and Millichap 2013; Fisher et al. 2017), acknowledging that the events and mechanisms underlying different seizures remain largely unknown. Classification of epilepsy, on the other hand, is not solely based on clinical data, but also involves pathophysiologic mechanisms, anatomic substrates, and etiology.

Various models are available to study the underlying mechanisms of epilepsy and possible treatment. In vivo models are most suited to capture the behavioral outcome of epilepsy, however, underlying mechanisms often remain uncertain, as these are difficult to assess in vivo (although recent advances in optogenetics have facilitated such work (Paz et al. 2013)). As the underlying pathology may substantially determine the effectiveness of certain therapies, it is difficult to evaluate treatment in models that mimic behavioral outcome, but may build on different underlying mechanisms.

4.1 In Vitro Models of Epilepsy

As an alternative, in vitro models enable a more mechanistic approach of epilepsy. However, these models may not cover the behavioral aspects of epilepsy, which may complicate translation of results to clinical patient care. To provide a structure for in vitro research, different facets of epileptic disorders may be defined and modeled separately (Engel and Schwartzkroin 2006).

Epileptogenesis Acquired epilepsies often begin with an epileptogenic insult, which can occur at any stage in life. Alternatively, disrupting events like brain trauma or stroke may trigger epileptogenesis. Acquired epilepsies depend on plasticity-induced changes and require time to develop (Lopes da Silva and Gorter 2009).

The Interictal State Even if the brain is characterized by an epileptic condition, seizures are absent most of the time. In many patients with epilepsy, interictal activity may appear in the EEG, like spikes or spike-waves, or pathological high-frequency oscillations (HFOs). Results of numerous studies suggest that interictal spikes and HFOs reflect pathological network activity that leads to seizure generation (Levesque et al. 2017). The interictal state is particularly interesting to study the natural mechanisms that prevent or promote ictus generation (Avoli 2001).

Ictal Onset Alotaiby et al. reviewed electrophysiological techniques to detect and predict seizure onset minutes to hours before they occurred. In some conditions, the transition from the interictal state to ictal onset takes considerable time. Preictal EEG findings may reflect pathological development that slowly builds up to the ictus (Alotaiby et al. 2014; Engel and Schwartzkroin 2006).

Ictus and Ictus Termination Seizures can last from ~10 s (Hughes 2009) to more than 5 min, from which point it is defined as status epilepticus (Trinka et al. 2012). Seizure-like events lasting more than 10s have also been observed in vitro in most cortical and limbic structures (Armand et al. 1998; Dreier and Heinemann 1991). The vast majority of ictal events display an evolutional pattern which reflects a sequence of pathophysiologic disturbances (Antonio et al. 2016; Dietzel and Heinemann 1986; Lux et al. 1986). As a result, adjacent and distant anatomic structures are recruited in the epileptic process (Dreier and Heinemann 1991). Excessive synchrony is the feature that defines most seizure states. Mechanisms underlying this synchrony can be analyzed, potentially yielding insights into how to interfere with ongoing seizure activity (Uhlhaas and Singer 2006).

The Postictal Period Most seizures are followed by a period of neurologic deficit, often as a consequence of the natural mechanisms that act to terminate the seizure. Postictal deficits recover over time to a variable extent (Fisher and Engel 2010). Postictal disturbances can be more disabling than the seizures themselves (Sutula and Pitkänen 2002).

Long-Term Consequences Many studies have found that the occurrence of seizures may induce alteration in subsequent seizure manifestations, such as increased frequency and severity (Kadam et al. 2010; Williams et al. 2009)

4.1.1 Brain Slices

Acute slices combine preservation of certain circuitry with large experimental freedom and relative ease of preparation and have been used widely as in vitro models of epilepsy. They have yielded a wealth of new insights on neurobiological mechanisms responsible for the onset and termination of seizures (Librizzi et al. 2017; Motamedi et al. 2006; Weissinger et al. 2005), seizure control and prevention (Hongo et al. 2015), propagation of seizure activity (Losi et al. 2016; Weissinger et al. 2005), and seizure-induced cell death (Frantseva et al. 2000), as well as well-developed protocols to induce seizure-like activity (Harrison et al. 2004; Pal et al.

2001; Rutecki et al. 1987; Schwartzkroin and Prince 1978; Srinivas et al. 2007; Tancredi and Avoli 1987; Tancredi et al. 1990). However, acute slices can only be maintained healthy for several hours, and, as research in the epilepsy field is moving from a primary focus on controlling seizures to addressing disease pathophysiology (Pacico and Mingorance-Le Meur 2014), processes that occur on time scales beyond the lifespan of acute cultures become more relevant. Organotypic slices and cultures of dissociated neurons offer a much longer time span and may be preferred for pathophysiology studies. Organotypic slices generally require relatively thin slicing and a high degree of sterility, making this approach technically challenging. Furthermore, organotypic slices appear most viable when obtained from a young donor. However, many neural circuits relevant for epilepsy have not yet been fully developed in newborn animals. Consequently, not many papers have been published on intact functional adult organotypic slices (Humpel 2015). Cultures of dissociated cultures lack typical structure as found in vivo. However, certain processes that affect excitability at the cellular or network level may still be studied.

4.1.2 Cultures of Dissociated Neurons

In pioneering work, Furshpan and Potter showed that cultures of dissociated hippocampal neurons of neonatal rats that were chronically exposed to high Mg^{2+} and a glutamate receptor antagonist generated intense seizure-like activity, suggesting that such models allow seizure-related cellular mechanisms to be studied in long-term cell culture (Furshpan and Potter 1989). The observation that networks of dissociated cortical or hippocampal neurons develop activity patterns that are dominated by synchronous bursts that show remarkable resemblance to interictal spikes (Ramakers et al. 1990) has been confirmed in numerous later studies, see, e.g., (Chiappalone et al. 2007; Eckmann et al. 2008; Pasquale et al. 2008; van Pelt et al. 2004). In dissociated hippocampal cultures, AMPA antagonists were more effective to block synchronized bursts than NMDA antagonists, which agrees with reports involving comparison of AMPA and NMDA receptor antagonists in anticonvulsant therapy (Rogawski 2011). This indicates that developing network models may be useful for the study of mechanisms that govern pathological network activity in diseases such as epilepsy (Suresh et al. 2016).

Thus, without pharmacological manipulation, cultures of dissociated cortical or hippocampal neurons display characteristics of hyperexcitable networks. This increased excitability has been related to the absence of afferent input to these networks. It has been suggested that insufficient activity within neural networks leads to a very low average level of synaptic/neuronal depression (Eytan and Marom 2006; Steriade and Amzica 1999). Assuming that networks need a certain degree of synaptic depression to maintain homeostatic conditions, insufficient synaptic depression enhances recurrent excitation in strongly recurrent excitatory networks like cortex, and creates a hyperexcitable network (Fig. 4). Enhanced recurrent excitation has been described as one of the major causes of hyperexcitability (Paz and Huguenard 2015). Moreover, sustained activity deficiency induces homeostatic

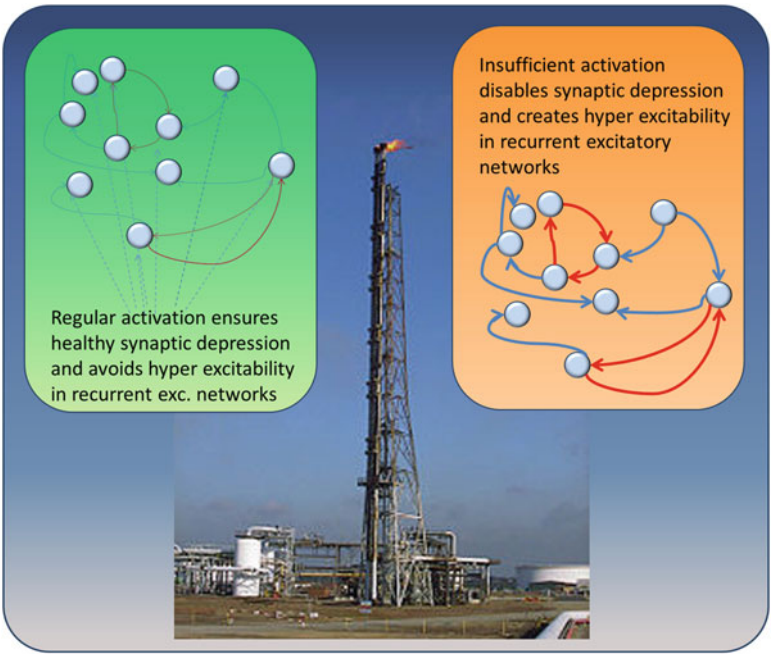

Fig. 4 The principle that regular firing may be necessary to avoid an "explosive" situation is not exclusive to neuronal networks, but also occurs in, e.g., a gas flare, used for burning off flammable gas released by plant equipment. Frequent burning off prevents the formation of dangerously explosive gas mixtures. However, a dangerously explosive gas mixture may develop during an extended period without burning off, and the same spark that was necessary for frequent burning off may trigger an explosion instead

up-regulation of excitability (Kilman et al. 2002; Turrigiano 2008; Turrigiano et al. 1998), thus reinforcing the hyperexcitability of networks.

Wagenaar et al. showed that providing input to cortical cultures by random electrical stimulation facilitated dispersed firing and impeded synchronized network bursts (Wagenaar et al. 2005). Also pharmacologically achieved mild excitation decreased network excitability (le Feber et al. 2014).

The transition to seizure-like activity in networks of dissociated neurons generally requires additional manipulation and may be achieved pharmacologically, e.g. using glutamate agonists (Kiese et al. 2017), or GABA antagonists like bicuculine (Colombi et al. 2013) or picrotoxin (Jewett et al. 2016). Also interference with the extracellular matrix formation early in development affects the establishment of balance between excitation and inhibition. A recent study suggested that decreasing expression of Hyaluronic acid (the backbone of the neural extracellular matrix) can be epileptogenic (Vedunova et al. 2013). Enzymatic removal of the ECM in mature cultures led to transient enhancement of neuronal activity, but prevented further disinhibition-induced hyperexcitability (Bikbaev et al. 2015).

Alternatively, directed genetic modifications that lead to lethal seizures in mice (i.e., mature microRNA-128 deficiency) can be reproduced in dissociated cultures and lead to significantly increased neuronal activity, burst rate, and burst duration, reflecting the increased excitability of these networks (McSweeney et al. 2016). Also cultures with mutant neuronal nicotinic acetylcholine receptors, which may cause a partial sleep-related epilepsy (autosomal dominant nocturnal frontal lobe epilepsy), were shown to become hyperexcitable and to represent an in vitro chronic model of spontaneous epileptiform activity, i.e., not requiring pre-treatment with convulsants (Gullo et al. 2014). These results support the utility of MEAs in developing in vitro models of neuroexcitability disorders, such as epilepsy.

In summary, cultures of dissociated neurons may be used to model the interictal period without any further manipulation. The transition to seizures and paroxysmal activity may be achieved by additional manipulations that affect the excitation—inhibition ratio, or genetic modifications. The development of such models facilitates the investigation of ictal onset, ictus and ictus termination, and is invaluable for pharmacological studies searching for anticonvulsant drugs. Recent advances in the differentiation of induced pluripotent stem cells provide the appealing opportunity to grow cultures that replicate patient-specific genetic deficits that may be crucial for the development of epilepsy.

4.1.3 Limitations

The link to behavioral outcome is not always clear in in vitro models. Different species may develop different "epilepsy" mechanisms and the in vitro spatiotemporal scale may differ from in vivo. It is important that in vitro models must survive long enough to observe processes of interest, which is especially true for slower biological processes, such as changes in gene expression and translation into proteins. This limits the use of acute brain slices in particular.

Seizure propagation cannot be studied as possibly relevant structures may not be included in slices. Schevon et al. (2012) showed that seizures may contain a core, showing intense hypersynchronous firing indicative of recruitment to the seizure, and adjacent territories where there is only low-level, unstructured firing (the "ictal penumbra"). Such processes, although possibly mechanistically crucial and useful, for example, for seizure prediction, may not be captured by slice models, and most likely not by dissociated neurons-based models. Cultures of dissociated neurons are relatively small, typically 1–2 mm in diameter, which does not facilitate investigation of seizure propagation.

In coupled networks, bursts were shown to propagate from one network to the other (Baruchi et al. 2008; Bisio et al. 2014). However, one of two connected cultures usually became dominant, initiating substantially more bursts than the other (Baruchi et al. 2008). This dominance was generally maintained during the entire monitored developmental frame, thus suggesting that the implementation of this hierarchy arose from early network development (Bisio et al. 2014). Dominance of one culture appeared more or less randomly, which hampered the construction

of engineered circuitry to mimic seizure propagation. Recent developments in patterned culturing based on surface micro patterning (Roth et al. 2012; Scott et al. 2012) or physical constraints (le Feber et al. 2015a; Pan et al. 2011; Renault et al. 2015) provide tools to incorporate certain circuitry in cultures of dissociated neurons, which may further facilitate the investigation of spreading seizures.

5 Memory Disorders

With aging, the risk of developing memory loss increases. Age-associated memory impairment is the mildest form, characterized by self-perception of memory loss and lower scores on a standardized memory test (Larrabee and Crook 1994). About 40% of people aged 65 or older have age-associated memory impairment, around 1% of these people develop dementia (Small 2002). In the Western world, prevalence doubles every 5 years beyond the age of 65 (Jorm and Jolley 1998), and averages 5–10% for people above that age (Hugo and Ganguli 2014). Globally, dementia affected about 46 million people in 2015 (Vos et al. 2016). About 10% of people develop the disorder at some point in their lives (Loy et al. 2014). Alzheimer's disease (AD) is the most common cause of late life dementia (Small 2002), followed by other causes like Dementia with Lewy Bodies (DLB), vascular disease, and Parkinson's Disease (PDD).

Patients diagnosed with dementia may be treated with cholinesterase inhibitors, but the benefit is generally small (Schneider et al. 2014). For milder forms of memory loss, no drug treatments is available (Small 2002). Despite tremendous efforts taken to investigate dementia, the underlying mechanisms are only partially understood, and may involve misfolded proteins, apoptosis, inflammatory responses, vascular deficiencies, mitochondrial impairment or synaptic damage, depending on the type of dementia. Whereas ischemia-induced malfunction seems a key aspect in vascular dementia, misfolding of specific proteins aggregation may be crucial in AD, DLB, or PDD. These different pathological etiologies, however, may share substantial common pathways (Raz et al. 2016).

5.1 Alzheimer's Disease

AD is characterized by the combined presence of extracellular amyloid-β (Aβ) plaques and intraneuronal neurofibrillary (tau) tangles (Bloom 2014). AD is associated with neurodegeneration, characterized by initial synaptic injury followed by neuronal loss, but the precise mechanisms leading to neurodegeneration are not completely clear (Crews and Masliah 2010). Animal models have relied on the utilization of genetic mutations associated with familial AD. The aggregation of both Aβ and tau has been faithfully reproduced in animal models, including aspects of memory impairment (Götz and Götz 2009), with cognitive deficits

appearing to occur earlier than extracellular plaques (LaFerla and Green 2012). Mechanisms, as determined from animal models, may involve impaired axonal transport, conceptually linked to oxidative stress, mitochondrial dysfunction, and widespread synaptic loss, in addition to inflammation and neuronal death (Götz and Götz 2009). A number of recent in vitro studies have investigated the interference of Aβ oligomers with synaptic function, for a review see (Crews and Masliah 2010).

5.1.1 Brain Slices

Aging organotypic brain slices have been shown to express beta-amyloid (Marksteiner and Humpel 2008). However, brain slices are usually obtained from neonatal brains, which may be inappropriate for studies on brain ageing and many age-related neuropsychiatric disorders (Jang et al. 2018). Furthermore, Aβ affects synaptic plasticity in the picomolar concentration range, and with aging the extracellular Aβ concentration decreases from the high picomolar to the low picomolar values. Some of the effects of Aβ may therefore be lost or altered after slice preparation (Waters 2010). Although the effect of Aβ exposure on synaptic functioning has been confirmed in hippocampal slice cultures (Ahuja et al. 2007), but differed between regions (Chong et al. 2011). Young age of the donor and limited duration of experiments remain restricting factors in the use of brain slice Alzheimer models.

5.1.2 Cultures of Dissociated Neurons

Aβ added to cultures of dissociated mouse hippocampal neurons on MEAs rapidly reduced their firing rate (Kuperstein et al. 2010), without significant cell death at low concentrations (Varghese et al. 2010). Reduced activity resulted from synaptic dysfunction, which could be reversed through use of curcumin, an inhibitor of Aβ oligomerization (Varghese et al. 2010). Recent work suggests that the sensitivity to detect early changes occurring after the addition of amyloid oligomers to the medium of in vitro electrophysiological recordings may be further enhanced by the use of high density electrode arrays (Amin et al. 2017). In vitro neuronal models using patient-derived stem cells are currently being developed, for a review see (Chinchalongporn et al. 2015)

5.2 Dementia with Lewy Bodies

Spherical inclusions of abnormal aggregates of (alpha-synuclein) protein in the somata (Lewy bodies) and elongated structures in the processes (Lewy neurites) are the neuropathological hallmark of Dementia with Lewy Bodies (DLB) (Goedert et al. 2013). It is not well understood whether and how these inclusions lead to cognitive impairment or dementia. Neurotoxin-based animal models are available,

as well as *disease gene-based* models (Bezard et al. 2013). Experimental results show that abnormal accumulation of α-synuclein in the hippocampus correlated with memory impairment and structural synaptic deficits (Lim et al. 2011). Power et al. (2017) showed mitochondrial and nuclear degradation in neurons with developing Lewy bodies. Lost integrity of mitochondria reduces the availability of ATP production and may thus form a link to mechanisms involved in vascular dementia. Accumulating evidence suggests that not cell death but rather α-synuclein aggregate-related synaptic dysfunction triggers DLB pathology (Calo et al. 2016; Colom-Cadena et al. 2017; Kramer and Schulz-Schaeffer 2007; Schulz-Schaeffer 2010; Sommer et al. 2000). More recently proposed models involve differentiation from human-induced pluripotent stem cells. Thus far, focus has mainly been on the differentiation of relevant cell types and the appearance of protein clusters, and not yet on the mechanisms of disease initiation and progression (Livesey 2014).

While animal models have been able to reproduce the most important clinical observations of misfolded proteins in combination with memory deficits, detailed insights into the mechanisms linking protein aggregation to memory loss remain hard to acquire, partly related to limitations in experimental control and accessibility of individual neurons and synapses. In vitro models have been developed to obtain detailed mechanistic insights.

5.2.1 Brain Slices

Excessive alpha synuclein was shown to affect cell morphology and synaptic plasticity. Viral overexpression of alpha-synuclein triggered the formation of distorted neurites, intraneuritic swellings, and granular perikaryal deposits in organotypic midbrain slice cultures (Zach et al. 2007). Hippocampal slices exposed to alpha-synuclein oligomers showed enhanced excitatory synaptic transmission within a few hours, driven by a receptor-mediated mechanism (Ferreira et al. 2017), which prevented further potentiation by physiological stimuli. (Diogenes et al. 2012). Fibrils or monomer did not disrupt long-term potentiation (Froula et al. 2018). The relatively short lifespan of these preparations impeded the investigation of changes on longer time scales.

5.2.2 Cultures of Dissociated Neurons

Volpicelli-Daley et al. (2011) showed that preformed alpha-synuclein fibrils added to the medium bath, enter primary neurons, leading to the formation of Lewy body-like inclusions, selective decreases in synaptic proteins, progressive impairments in neuronal excitability and connectivity, and, eventually, neuron death. Extracellular added monomers with or without low concentration fibril seeds, or rotenone also triggered the formation of intracellular alpha-synuclein inclusion bodies, with induction-dependent differences in morphology, location, and function (toxicity) (Raiss et al. 2016). Alpha-synuclein fibrils or oligomers added to the medium bath

of dissociated cortical cultures significantly reduced the mean firing rate and synchronicity (Peelaerts et al. 2015). Recent evidence shows that high concentrations of extracellularly added alpha-synuclein monomers may interfere with synaptic function, significantly preceding the formation of intracellular inclusion bodies, suggesting that these inclusions, although characterized as a pathological hallmark, may not be key in the pathology (Hassink et al. 2018). Rather, impeded activity may be an essential step as neuronal survival depends on regular calcium influx, which is promoted by electrical activity (Ghosh et al. 1994; Mao et al. 1999). This view is supported by the finding that alpha-synuclein was found mainly in excitatory neurons and synapses (Taguchi et al. 2014).

5.3 Memory In Vitro

Whereas animal models have been developed that provide quantification of memory performance in relation to pathologic protein clustering, this has been problematic in in vitro models. Recent progress, however, enables the evaluation of a kind of memory in networks of dissociated cortical neurons (le Feber et al. 2015b). The basic idea is that activity patterns are determined by connectivity and that connectivity, in turn, is affected by certain activity patterns through plasticity mechanisms like spike timing-dependent plasticity. The finding that input-deprived networks develop quasi stable activity patterns (Stegenga et al. 2008; van Pelt et al. 2004) and connectivity (le Feber et al. 2007) suggests that activity and connectivity are in equilibrium in these networks (le Feber et al. 2010). External input, in the form of electrical stimulation through one of the electrodes, may induce a new pattern, trigger connectivity changes, and drive the network out of the activity $\Longleftrightarrow$ connectivity equilibrium. Responses to electrical stimulation have been shown to rapidly activate "major burst leader" neurons (Eckmann et al. 2008) and to share great similarity beyond activation of a major leader neuron (Pasquale et al. 2017), suggesting that the driving forces behind connectivity changes occur in particular before activation of the major leader. Connectivity continues to change until a new balance between activity and connectivity has been established. The new equilibrium includes the response to the stimulus (le Feber et al. 2015b), and consequently, repeated application of this input induces no further connectivity changes. Thus, inability of a stimulus to alter network connectivity suggests that the network already memorized that stimulus. Stimulation at a different electrode was shown to still induce connectivity changes upon first application, but not when repeated multiple times. Switching back to the first electrode, electrical stimulation did not induce connectivity changes, indicating that the memory trace persisted (illustrated in Fig. 5). This work shows that (random) cortical networks are able to form memory traces of experienced inputs and shows that there is no direct relationship between the input and the memory trace. Rather, the formed memory trace depends on the input and the connectivity at the time of receiving the input. Memory retrieval might occur through stimuli that trigger the replay of the whole

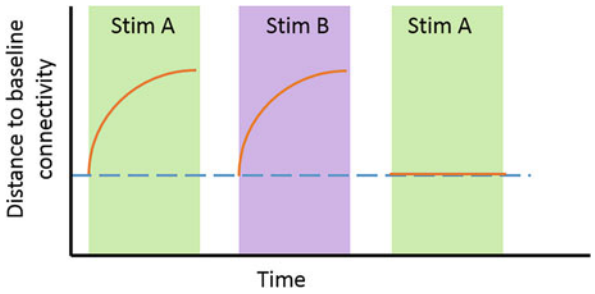

Fig. 5 Illustration of connectivity changes in random cortical networks upon stimulation (solid red lines), compared to unstimulated (dashed blue line), based on experimental data and modeling in (le Feber et al. 2015b). Vertical scale indicates connectivity differences with respect to connectivity before the first stimulus of that specific type (A or B). Without stimulation, connectivity fluctuates and the distance to the initial connectivity is not zero, but it does not increase. Stimulation through electrode A initially induces large connectivity changes, which rapidly decrease when the stimulation is repeated. Repeated stimulation at another electrode (B) yields a very similar pattern of connectivity changes. Return to stimulus A induces no connectivity changes that exceed random fluctuations. Green background indicates repeated stimulation at electrode A, purple: electrode B

trace. Recent work by Pasquale et al. (2017) showed strong similarity between spontaneous and induced activity patterns, but activity patterns evoked by the same stimulus were more similar to each other than to patterns evoked by other stimuli or spontaneous patterns.

In sum, cultures of dissociated neurons provide a platform that enables the induction of protein aggregates, evaluation of synaptic functioning and cell viability during and after the formation of aggregates, and associated memory performance. Thus, cultured neuronal networks seem very well suited to study the mechanisms underlying memory disorders, as well as possible therapeutic treatment.

6 Conclusions

Several models of brain disorders have been described in this section, which are exemplary to illustrate the power of MEA-based models of brain disorders. Depending on the research question, the cellular composition (fraction of inhibitory/excitatory neurons; ratio astrocytes: neurons, etc.) may be crucial, but this is not yet fully controlled in primary neuronal cultures. Recent techniques using forced differentiation of induced stem cells may help to solve this problem (Zhang et al. 2013). This provides a very strong platform for the development of new models of brain disorders, particularly in combination with newly developed tools to engineer-specific structures.

All presented models have their merits, but also drawbacks that should be solved to facilitate wider use. For example, it is not clear how hypoxia in cultures translates to in vivo oxygen levels, as physiological oxygen concentrations are

much lower than those commonly used to culture cells. Culturing cells under physiological oxygen pressure from the day of plating has been shown feasible and may solve future problems in the interpretation of normoxia and hypoxia. Also the interpretation of spontaneously occurring network bursts remains debated. They may reflect hyperexcitability of input-deprived networks, but may also play a role in information processing (Kepecs and Lisman 2003; Singer 1993), or to increase the reliability of communication between neurons and to avoid synaptic transmission failure (Chen et al. 2009). A crucial step, that still remains unclear in MEA-based memory disorder models, is memory retrieval. Discovery of this mechanism would not only be a major breakthrough in memory research, but would certainly facilitate widespread use of MEA-based models for memory disorders.

Whereas brain slices should be used when the in vivo connectivity is crucial, models based on cultures of dissociated neurons are well suited to investigate general functioning of neurons and synapses under pathological conditions. A major advantage of this approach is the longer lifespan, which allows for the investigation of processes that occur at time scales of days or weeks. Important new insights provided by MEA-based models include the finding that synaptic failure, and consequently neuronal silence, often precedes neuronal death under hypoxic conditions, or after exposure to excessive alpha-synuclein or beta-amyloid. This has brought forward that insufficient activity may be an important step in the evolution towards cell death. Regular activity appeared also crucial to maintain network excitability within boundaries. These are important new insights that could be obtained using the advantages of dissociated cultures, that emphasize the importance of activity homeostasis, and may open up new possibilities for treatment.

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