DRUG DISCOVERY TODAY TARGETS

Organotypic cultures as tools for functional screening in the CNS

Lars Sundstrom, Barclay Morrison III, Mark Bradley and Ashley Pringle

A major challenge for the pharmaceutical industry is the development of relevant model systems in which knowledge gained from high-throughput, genomic and proteomic approaches can be integrated to study function. Animal models are still the main choice for such studies but over the past few years powerful new *in vitro* systems have begun to emerge as useful tools to study function. Organotypic cultures made from slices of explanted tissue represent a complex multi-cellular *in vitro* environment with the potential to assess biological function and are uniquely placed to act as an important link between high-throughput approaches and animal models.

The discovery of new pharmaceutical leads has undergone a revolution in the past two decades. The advent of large-scale DNA sequencing and proteomics has provided a unique opportunity to understand biological systems to a level of detail that was hitherto unthinkable. Advances in genomics and proteomics allow us to approach the theoretical limits of defining biological systems completely. However, although it is now theoretically possible to know all the components that make up cells and organisms, the task of understanding the interactions between these components remains undiminished. One of the major challenges for the next phase of bio-pharmaceutical research must be to find ways of harnessing the power of such a reductionist analysis in a way that allows us to understand the biological relevance of the information produced.

Several recent developments have resulted in an explosion in the numbers of compounds that can be taken forward to the development phase as potential drug candidates. Advances in automation, genomics and proteomics have also considerably amplified the number of potentially interesting targets for drug manipulation. In addition, advances in synthetic chemistry such as combinatorial chemistry have generated an explosion in the number of molecules that can be screened against these targets. Clearly, the trend is towards an increase in the volume of information and although the system may be finite, and we may eventually have all the pieces to the puzzle, it is not clear how we are going to mine the information and use it to design better drugs. Many have recently argued that the current trend of simply increasing the number of hits will not automatically result in more new drugs on the market and that a return to more traditional empirical discovery methods is now called for [1]. In a recent review for *Drug Discovery Today: TARGETS*, Walker *et al.* argue that functional pharmacology has now become the main drug discovery bottleneck [2].

One of the main tasks in the near future is thus to develop relevant model systems in which the knowledge gained from high-throughput techniques can be applied to study function. Generally it can be stated that as a system becomes more complex, the throughput in terms of screening capacity decreases. Thus, while animal models are the most biologically relevant systems, they represent the most difficult system in which to study new targets and new leads. Testing new compounds in animal models is limited

Lars Sundstrom*
Ashley Pringle
Capsant Neurotechnologies Ltd.,
Biomedical Sciences Building,
Southampton SO16 7PX,
UK
Lars Sundstrom*
Ashley Pringle
Barclay Morrison III
Clinical Neurosciences,
University of Southampton,
Biomedical Sciences Building,
Southampton SO16 7PX,
UK
Mark Bradley
School of Chemistry,
University of Edinburgh,
Joseph Black Building,
The Kings Buildings,
West Mains Road,
Edinburgh EH9 3JJ,
UK
*e-mail:Lars@capsant.com

1359-6446/05/$ – see front matter PII: S1359-6446(05)03502-6

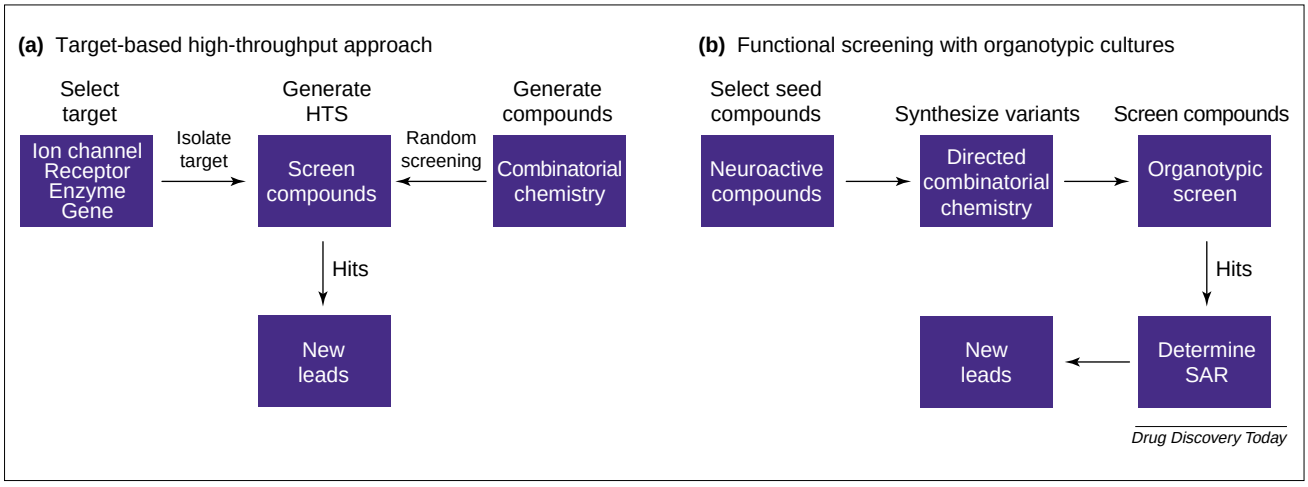


FIGURE 1

Target-based versus functional screening approaches. (a) The high-throughput approach relies upon the isolation of a target and random screening of compound librararies, for example those resulting from combinatorial chemistry programs. This method has value in that it allows great numbers of compounds to be analysed, but disadvantages include the inability to detect compounds acting on new mechanisms. **(b)** Functional screening using organotypic cultures selects leads on the basis of biological activity without the need for *a priori* knowledge of the target. By successively altering cardinal features of the molecules to be tested, it is possible to select compounds on the basis of structure-to-biological-activity relationships.

by the need for skilled personnel. In many cases they are also very time-consuming; mouse models of Alzheimer's disease, for example, can take several months to develop a disease phenotype. They are also expensive and increasingly unpopular on ethical grounds.

High-throughput screening is easiest to perform in cell-free systems; however, the likelihood of discovering molecules that act on novel targets is extremely low since one can only detect hits that interact with this isolated target or target system.

Clearly what is needed now is the development of intermediary cell-based systems that have sufficient throughput but that encapsulate enough biological complexity to enable pre-selection of hits for further *in vivo* studies. The closer *in vitro* models parallel *in vivo* models and reflect pathophysiological mechanisms related to human disease conditions, the more likely they are to be of value as interfaces between drug discovery and development [2].

High-throughput versus high-content screens

The trend across most of the pharmaceutical industry over the past decade has been to isolate potential targets and to generate high-throughput screens around these isolated targets (Figure 1a). The main goal of high-throughput screening has been to map chemical space onto target activity or binding. Although this approach allows a large number of compounds to be tested, it has major drawbacks with respect to selecting compounds that are suitable for further development. First, only compounds that act on known targets can be identified, and thus it is virtually impossible to discover compounds acting via novel biological mechanisms. In most cases the function of novel proteins identified from genomic and proteomic analyses is unknown and it will therefore prove difficult to assess the relevance of these targets using this approach on its own. The second major drawback is the sheer number of hits that can be generated in this way. It has been argued that increasing the number of hits will not necessarily lead to increased numbers of new drugs since it is not possible to test all of them in physiologically relevant systems. Thus this route alone is not suitable for selecting the best candidates for development.

The development of interface technologies that allow better selection of drug candidates for preclinical development is therefore of great importance. These systems should allow us to map the chemical space of new molecules onto biological function as a means to assess the importance of the findings. Over the last decade there has been a resurgence of interest in developing functional bioassays using tissue culture systems, sometimes referred to as 'high-content' or 'high-information-content' systems (HCS) [3]. Such systems traditionally rely on freshly isolated cell cultures or cell lines grown in multiwell plates and several readout platforms such as fluorescence imaging have been developed to enable automated functional screening. Parameters as diverse as ion channel function, intracellular translocation of proteins and cell proliferation have successfully been assessed in such high content systems and have proven useful in refining the selection of leads from high-throughput screens [4]. Most of the systems used to date have remained at the level of isolated cells or multi-cellular re-aggregated systems. The power of these systems in terms of their ability to predict *in vivo* efficacy depends essentially on two factors: first, the degree to which they model the biological system and disease state in question, and second, the degree to which explantation of the donor tissue into the test system alters its original characteristics. This is of particular importance in brain tissue, where the intrinsic connections between

nerve cells are an integral part of their function and where dissociation and re-aggregation of the tissue obviously impacts greatly on its original function. Since the original synaptic connections are lost during dissociation of the tissue, it may not be possible to regain the function of the original tissue that was established over a significant developmental period.

The level of complexity of high-content systems is steadily advancing and *in vitro* functional bioassays based on organ systems are rapidly emerging as an important new interface technology between discovery and development. These are referred to generally as organotypic models and have traditionally relied on acutely isolated tissue preparations such as liver or brain slices, which can be maintained for several hours ex-vivo. In CNS pharmacology, freshly isolated brain slices, termed 'acute slice preparations', have been used primarily for recording electrophysiological activity in two types of experiments. First, slices derived from animals exposed to CNS lesions (for example in *in vivo* models of epilepsy) have been employed to investigate the effects of the lesion and related pharmacological agents on synaptic activity [5]. Alternatively, the acute effects of lesions can be investigated on slices derived from naïve animals, a technique that has been extensively employed for both stroke and epilepsy models [6]. While both of these approaches have yielded valuable information, the technique is limited by the fact that slices can only be maintained *ex vivo* for several hours, excluding the possibility of discovering more chronic actions resulting from pharmacological intervention. Recently, however, it has become possible to grow slices of CNS tissue in tissue culture for several weeks enabling us for the first time to study the effects of compounds on CNS lesions in a complex *in vitro* system over a period of days to weeks in a context that reflects organ characteristics. In the past these have required extensive skill and time to manipulate, but methods to generate these cultures have become simpler and thus for the first time allow the application of such methodologies to the screening of novel compounds.

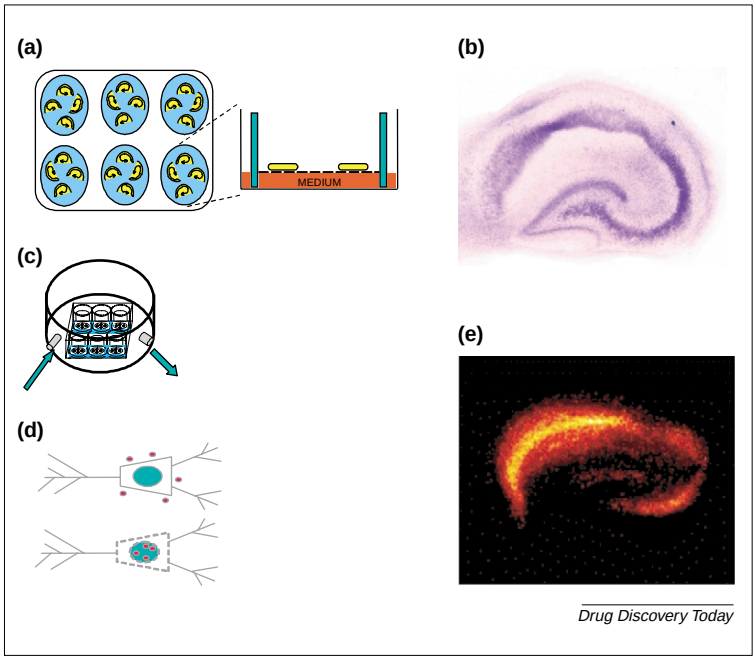


FIGURE 2

Modelling ischaemic neurodegeneration in organotypic slice cultures. **(a)** Organotypic cultures are grown on semiporous membranes at an air–liquid interface in six well plates (four cultures per insert). Tissue culture medium is located below the membrane and cultures are fed through the membrane by capillarity. Cultures can be maintained for several weeks, retain organotypic characteristics such as cellular connectivity and remain regionally differentiated. **(b)** Organotypic culture stained with thionine after two weeks of culture *in vitro* showing regional organization similar to rat hippocampus *in vivo*. **(c)** Ischaemic insults are generated by placing cultures in glucose-free medium saturated with 95%N_2/5%CO_2 in a modular incubator. To prevent equilibration of the medium with air, the atmosphere in the incubator is filled with 95%N_2/5%CO_2 by gassing through an inlet valve for 10 min before sealing the chamber and placing it into an incubator at 37°C for up to 3 h . **(d)** Schematic drawing of the detection of cell death using the fluorescent DNA intercalating dye propidium iodide, which is exluded from cells with an intact plasma membrane but can access the cell nucleus when the plasma membrane is disrupted. **(e)** An organotypic slice culture 24 h after a 60 min period of oxygen–glucose deprivation (OGD) demonstrating extensive propidium iodide fluorescence in the neuronal cell layers. Fluorescence intensity is highest in the CA1 region, indicating greater neuronal cell death there, a pattern of damage which reflects that observed in *in vivo* models of global forebrain ischaemia in rodents.

In this article we describe an illustrative example of the use of organotypic cultures of brain slices in the development of a new series of novel neuroprotective compounds based on synthetic polyamine molecules whose structure–activity relationships have been studied using this system.

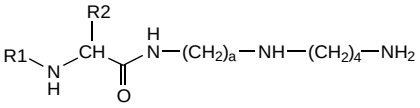
Ischaemic insults in organotypic hippocampal slice cultures

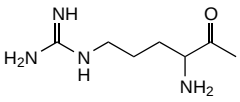
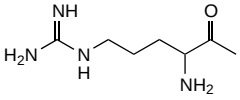
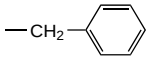
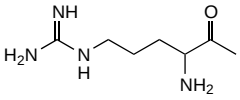
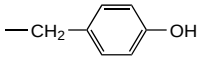
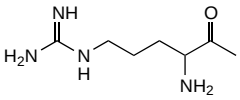
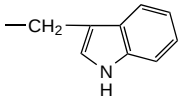
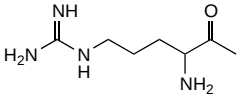
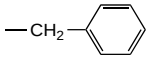
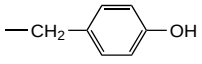
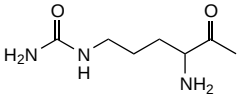
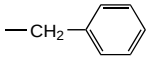
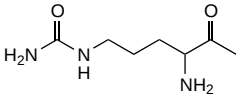
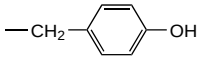
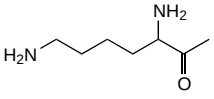
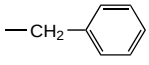
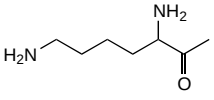
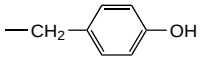
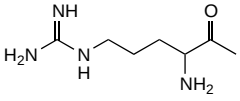
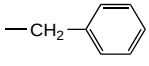
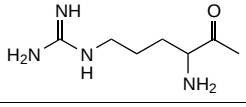
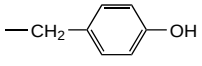
Since their introduction over two decades ago, organotypic cultures of rat brain slices have become a useful tool for studying drug effects *in vitro* (Figure 2). Originally introduced by Gahwiler in 1981 [7], cultured brain slices retain many essential organisational features of the host tissue, such as neuronal connectivity, relatively well preserved cellular stoichiometry and complex glial–neuronal interactions [8]. The introduction of a relatively simple method of culturing hippocampal slices on semiporous membranes by Stoppini *et al.* [9] has dramatically increased their use in the study of CNS function and dysfunction. In this system, explanted tissues are placed onto a culture insert containing a semiporous membrane and are kept alive in an air–liquid interface inside a small drop of fluid. The cultures are fed by medium on the underside of the membrane, as illustrated in Figure 2. Prior to this development it was necessary to grow cultures on coverslips in a chicken plasma clot inside test tubes placed in a slowly rotating drum that periodically immersed the cultures in tissue culture medium.

Over the last ten years, we and several other groups have developed *in vitro* models of acute and chronic neurodegenerative disorders in hippocampal slice cultures, which display many features that are found *in vivo*. For example, oxygen and glucose deprivation of brain slices *in vitro*

TABLE 1

An example of the use of organotypic culture for functional screening of neuroprotective compounds against oxygen deprivation (OGD). Combinatorial synthesis of a series of polyamine-based compounds was carried out with specific substitution of R1 and R2 with groups listed in columns 2 and 3. Several aspects of the molecule were studied including altering the length of the polyamine component, modifying the amino acid component, secondary amine and α-amino group. Neuroprotection is defined as the percentage reduction in propidium iodide fluorescence in the CA1 region observed 24 hours after a three-hour exposure to hypoxia. Significant neuroprotection is indicated by bold type. Data in this table is derived from a single concentration (300μM) administered 30 minutes prior to hypoxia, during hypoxia and during the post-hypoxia recovery period. In a typical assay, compounds which were active in this screen would then be assessed further in order to determine the EC50, and activity in other experimental paradigms (such as post-treatment only) before a choice of lead compound was made.



Compound	R1	R2	a	% Protection
ArgGly3,4		H	3	0
ArgPhe3,4			3	**60**
ArgTyr3,4			3	**71**
ArgTrp3,4			3	**72**
ArgPhg3,4			3	**64**
Phe3,4	H		3	7
Tyr3,4	H		3	3
CitPhe3,4			3	1.5
CitTyr3,4			3	11
LysPhe3,4			3	0
LysTyr3,4			3	0
ArgPhe4,4			4	19
ArgTyr4,4			4	0

induces cell death similar to that seen in global ischaemia models in the rat; the similarities include regionally selective cell death, delayed neurodegeneration and comparable pharmacology [10]. Such ischaemic insults can be generated by depriving the cultures of glucose and oxygen for 1 h by placing them in glucose-free medium saturated with 95% N_2, 5% CO_2. An alternative method to induce oxygen and glucose deprivation has been introduced recently by Frantseva *et al.* [11] that uses submersion, opening up the possibility of automating liquid handling in the assay (in this method the anoxic medium is place on top of the cultures rather than below as in the standard interface method).

Simple methods based on the exclusion of vital dyes have emerged, such as the use of the fluorescent DNA intercalating dye propidium iodide [12–14]. This dye is highly polar and cannot gain access to the cell nucleus unless the plasma membrane is compromised [15]. Fluorescence is not readily detectable until it is bound to DNA, resulting in very low background levels. In addition, propidium iodide fluorescence is retained after fixation, allowing the method to be used in combination with immunocytochemistry to study the phenotype of damaged cells [16]. Other methods such as lactate dehydrogenase release or fluoro-jade staining can also be used. [14].

Organotypic cultures as tools for discovering new neuroprotective compounds

In addition to the advantages outlined above, organotypic cultures allow us to develop functional screens in which the outcome measure is very closely related to the disease state. Although these screens currently have a relatively low throughput (typically tens of compounds per week), they have two major advantages over conventional HCS systems. First, the process of lead validation can be performed in a model more closely resembling animal models, thereby permitting a more robust selection of compounds for *in vivo* testing. Second, active compounds with an unidentified mode of action can be both identified and studied in a model that reflects the complex multicellular environment present *in vivo*. Therefore, it is not necessary to begin the drug discovery process with an identified target, a model of drug discovery that had a long history of success before HTS became popular (Figure 1b). We recently demonstrated how functional screening using organotypic cultures can be used for the discovery of new neuroprotective compounds for the treatment of acute and chronic neurodegenerative diseases. We used a directed combinatorial chemistry approach to synthesize several hundred variants of FTX, a naturally occurring toxin from the American funnel web spider *Agelenopsis aperta*. To determine how the altered chemical structure affected functional neuroprotective activity, propidium iodide uptake into organotypic cultures exposed to 3 h oxygen deprivation was used as a read-out [17]. By systematically altering cardinal features of the molecule, it was possible to determine the optimal structure for neuroprotection (Table 1). From the series of compounds illustrated in Table 1, eight were significantly neuroprotective and L-arginyl 3,4-spermidine was chosen as the lead compound for further validation (Figure 3a,b). This compound is of interest because it seems to lack the ion-channel-blocking activity and associated toxicity of the parent compound, FTX. Further characterisation in other organotypic models demonstrated that L-arginyl 3,4-spermidine interacts with the formation of free radicals, since it protected against toxicity from superoxide production induced by the uncoupling of electron transport using direct duroquinone application to the slice cultures. *In vivo*, the compound was highly protective in a four-vessel occlusion model of global cerebral ischaemia in the rat [18] (Figure 3c). Therefore, this functional screening approach was used to optimize the molecular structure of a neuroprotective compound without prior knowledge of the mechanism of action, which can be determined in the same system at a later time.

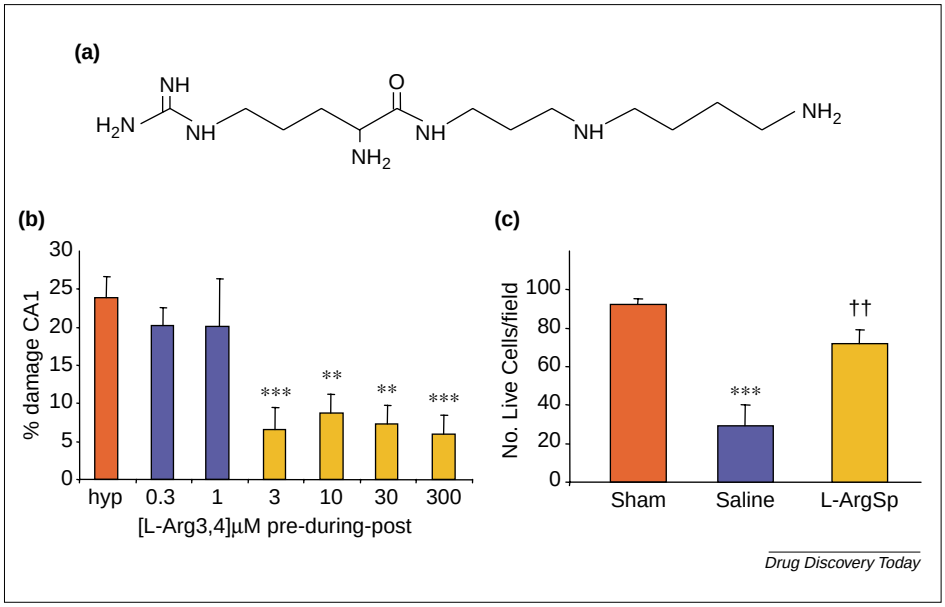


FIGURE 3

Characterization of a lead compound selected by functional screening. (a) Structure of L-arginyl-3,4-spermidine, which was selected on the basis of biological activity as a potential new lead for the treatment of neurodegeneative disease. **(b)** The compound was added 30 min before the three hour oxygen deprivation and was present during this time and the 24 h observation period. Under these conditions the compound gave ~50% neuroprotective activity in the low micromolar range (reproduced with permission from [17]). **(c)** The compound was further characterised and found to be effective in an *in vivo* model of global ischaemia and to interfere with free radical formation (reproduced with permission from [18]).

Organotypic brain slice cultures as an interface between high-throughput and *in vivo* screening

Organotypic culture technologies such as the brain slices described here offer great

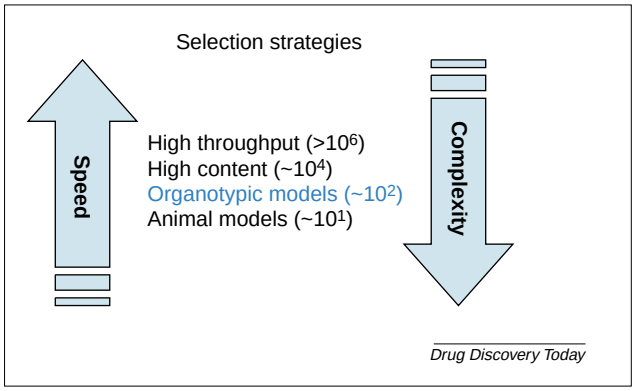


FIGURE 4

Organotypic cultures such as organotypic brain slice cultures are ideally placed to act as a companion to high-content and high-throughput screening approache, allowing preselection of molecules to be taken forward for testing in animal models.

potential as an interface between high-throughput screening and pre-clinical animal models. Currently, a major bottleneck exists between the numbers of compounds that are interesting to evaluate in animal models and the relatively limited throughput of these models. For example, we estimate that the testing of a compound in the four-vessel occlusion model of stroke requires approximately four weeks of a skilled operator's time to complete. In consequence, the evaluation of a typical high-throughput screen of several hundred compounds will be hugely costly and labour-intensive. By contrast, a skilled operator using organotypic brain slice cultures can easily generate over 100 data points, equivalent to 20 compounds tested at three concentrations with appropriate positive and negative controls, in the same period of time, representing a substantial increase in throughput and a very substantial reduction in costs (Figure 4). Also, given the considerable pressure to reduce the use of live animals in screening compounds, a very significant sparing of animals is achieved as ~30 slices can be produced from a single donor animal, allowing the generation of multiple data points from cultures derived from a single animal.

However, organotypic brain slice cultures are not without limitations. Brain slice cultures, for instance, can currently be produced only from juvenile donor animals (typically up to 12 days postnatal), and it is known that juvenile animals are more resistant to ischaemic damage than adults [19]. Recent reports have suggested that the production of slice cultures from older donors is theoretically possible [20]; however, the low yield, with only around 5–10% of cultures remaining viable for three to four weeks, currently makes their use in a screening system impractical [21]. Another limitation is that a skilled operator is required for the production of cultures from dissected brain material, representing a significant challenge to automation. Other forms of organotypic cultures (such as re-aggregated cultures, which are generated by allowing dissociated tissue to reform as spherical aggregates) can be produced in higher numbers and are more amenable to automated analysis [22]. The main disadvantage of re-aggregated systems compared with slice cultures is that the anatomical organization present in the original tissue is lost. Another disadvantage is that not all areas of the brain are amenable to culture. The organotypic method is ideal for brain regions with a lamellar structure that can be aligned parallel to the plane of slicing, such as the rat striatum, cerebellum, hippocampus and cortex as well as various brain nuclei. The production of slice cultures from other regions with significantly out-of-plane projections, such as the nigro-striatal pathway, remains challenging. Lastly, organotypic brain slice cultures do not have a functional vascular compartment. Therefore, the effects of drugs that act on vascular or systemic components may not be accurately modelled in these systems, although this can be used as an advantage when trying to dissociate direct actions on neuronal tissue from indirect actions on the cardiovascular system.

The future for organotypic cultures in target discovery and validation

Organotypic brain slice cultures occupy a unique niche in the drug discovery process and are a unique tool in the lead discovery and validation process. This review has focused on an illustrative application, notably the discovery of compounds with potential for the treatment of stroke, and this technology can be used for the evaluation of compounds that may be used to treat other brain disorders. Several other neurodegenerative parameters have been modelled in organotypic cultures, such as β-amyloid toxicity. In this case, the addition of toxic β-amyloid fragments triggers an acute neurodegenerative cascade that can be useful for the evaluation of new leads without the need for *in vivo* testing [23]. Similarly, traumatic brain injury can be modelled by growing cultures on deformable substrates such as silicone and stretching cultures using biomechanically relevant injury parameters [24]. In this latter case a very significant reduction in and refinement on animal testing is achieved as traumatic insults *in vivo* may take months to study. Even glioblastoma-induced toxicity has been modelled in organotypic systems by placing tumour cell lines on top of organotypic cultures [25]. In this case, multiple parameters including invasion and proliferation of the tumour and *in vitro* angiogenesis can be modelled, processes which are difficult and hugely time-consuming parameters to estimate using *in vivo* studies.

In each of these models a similar approach can been demonstrated to the one illustrated in this manuscript, as in all cases it has been demonstrated that reference compounds that have been effective *in vivo* show a similar response *in vitro* to that known to occur *in vivo*.

Given that connections between neurons are maintained throughout the culturing process, it is possible to use electrophysiological approaches to study the neural network activity in these brain slices to gain valuable

information on the structure–activity relationship with respect to function. Organotypic systems have often been used with multi-electrode arrays, where the cultures are simply placed on top of a grid of electrodes [26]. This eliminates the need for sharp electrode techniques and allows monitoring of the functional 'electrical' behaviour of the network in a manner not dissimilar to that used for analysing brain activity *in vivo*. Although this technique is not limited to cultured brain slices, organotypic cultures add the dimension of being able to monitor activity changes over longer time periods of up to several weeks. An example of this can be seen in the application of brain slice cultures in the study and screening of new molecules that affect repair and recovery processes. Stoppini *et al.* [27] have described a model in which they measured the functional reconnection of a transected pathway using multielectrode arrays over several days. Others have successfully studied the behaviour of endogenous and exogenously applied stem cells in an attempt to determine their role in recovery processes [28,29].

Because organotypic cultures encapsulate most of the features required for the development of brain damage in several pathological conditions, it seems reasonable to assume that they may play an increasingly important role in target discovery and validation. We previously demonstrated the validity of this approach using a series of antibodies that detected the activity of proteolytic calpain enzymes [16]. In this example, the effects of potential lead compounds on the target could be determined in an *in vitro* stroke model by studying the effects of the molecules on the formation of calpain cleavage products. It may soon be possible to extend this approach, for example by using proteomics-based methods to discover new targets that are modulated in specific disease conditions without the need for antibodies [30]. Several groups have taken advantage of the potential for growing organotypic cultures from transgenic mice, effectively producing a model knockout system for functional genomic studies [31]. Recent studies have also shown that brain slice cultures can be relatively easily transfected using either biolistic [32] or viral vectors [33] Although proof-of-concept is still lacking, there is no theoretical reason why RNAi should not be applicable to organotypic systems, opening up significant possibilities for target validation using functional genomic approaches.

Concluding remarks

In summary, organotypic slice cultures have the potential to become powerful tools in the arsenal of drug discovery technology, lying at the interface between high-throughput screening and clinically relevant animal disease models (Figure 4). Because the complex multicellular nature of organotypic cultures closely represents the tissue *in vivo*, and since it is possible to develop specific disease models, they have the potential to play a significant role in the discovery of new leads and targets and in the assessment of their biological importance in both normal and disease physiology.

We believe that they can act as a useful starting point for refining molecules for further *in vivo* analysis and reduce the number of potential leads one needs to evaluate in animals. In addition they can be used to functionally screen compounds in the absence of target information, as with the example described in this review.

References

1 Lansbury, P.T. (2004) Back to the future: the 'old-fashioned' way to new medications for neurodegeneration. *Nat. Med.* 10 Suppl., S51–S57

2 Walker, J.A. *et al.* (2004) Functional pharmacology: the drug discovery bottleneck? *Drug Discov. Today Targets*. 3, 208–215

3 Kapur, R. (2002) Fluorescence imaging and engineered biosensors: functional and activity-based sensing using high content screening. *Ann. N. Y. Acad. Sci.* 961, 196–197

4 Taylor, D.L. *et al.* (2001) Real-time molecular and cellular analysis: the new frontier of drug discovery. *Curr. Opin. Biotechnol.* 12, 75–81

5 Brener, K. *et al.* (1991) Chronic epileptic foci in neocortex: *in vivo* and *in vitro* effects of tetanus toxin. *Eur. J. Neurosci.* 3, 47–54

6 Sabelhau, C.F. *et al.* (2000) Neuroprotection against hypoxic/hypoglycaemic injury after the insult by group II metabotropic glutamate receptor agonist (R,S)-4-phosphonophenylglycine. *Br. J. Phramacol.* 131, 655–658

7 Gahwiler, B.H. (1981) Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Methods* 4, 329–342

8 Gahwiler, B.H. *et al.* (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* 20, 471–477

9 Stoppini, L. *et al.* (1991) A simple method for the organotypic culture of nervous tissue. *J. Neurosci. Methods* 37, 173–182

10 Pringle, A.K. *et al.* (1997) Neuroprotection by NMDA and non-NMDA receptor antagonists in *in vitro* ischaemia. *Brain Res.* 755, 36–46

11 Frantseva, M.V. *et al.* (1999) A submersion method to induce hypoxic damage in organotypic hippocampal cultures. *J. Neurosci. Methods* 89, 25–31

12 Rekling, J.C. (2003) Neuroprotective effects of anticonvulsants in rat hippocampal slice cultures exposed to oxygen/glucose deprivation. *Neurosci. Lett.* 335, 167–170

13 Vornov, J.J. *et al.* (1991) Direct observation of the agonist-specific regional vulnerability to glutamate, NMDA, and kainate neurotoxicity in organotypic hippocampal cultures. *Exp. Neurol.* 114, 11–22

14 Noraberg, J. *et al.* (1999) Markers for neuronal degeneration in organotypic slice cultures. *Brain Res. Brain Res. Protoc.* 3, 278–290

15 Macklis, J.D. and Madison, R.D. (1990) Progressive incorporation of propidium iodide in cultured mouse neurons correlates with declining electrophysiological status: a fluorescence scale of membrane integrity. *J. Neurosci. Methods* 31, 43–46

16 Brana, C. *et al.* (1999) Calpain activation and inhibition in organotypic rat hippocampal slice cultures deprived of oxygen and glucose. *Eur. J. Neurosci.* 11, 2375–2384

17 Pringle, A.K. *et al.* (2003) Characterisation of a novel class of polyamine-based neuroprotective compounds. *Naunyn Schmiedebergs Arch. Pharmacol.* 368, 216–224

18 Morrison, B., III *et al.* (2002) L-Arginyl-3,4-Spermidine is neuroprotective in several *in vitro* models of neurodegeneration and *in vivo* ischaemia without suppressing synaptic transmission. *Br. J. Pharmacol.* 137, 1255–1268

19 Towfighi, J. *et al.* (1997) Influence of age on the cerebral lesions in an imature rat model of cerebral hypoxi-ischemia: a light microscopic study. *Brain Res. Dev. Brain Res.* 100, 149–160

20 Xiang, Z. *et al.* (2000) Long-term maintenance of mature hippocampal slices *in vitro*. *J. Neurosci. Methods* 98, 145–154

21 Wilhelmi, E. *et al.* (2002) Organotypic brain-slice cultures from adult rats: approaches for a prolonged culture time. *Altern. Lab. Anim.* 30, 275–283

22 Atterwill, C.K. and Wing, M.G. (2002) *In vitro* preclinical lead optimisation technologies (PLOTs) in pharmaceutical development. *Toxicol. Lett.* 127, 143–151

23 Shimono, K. *et al.* (2002) Chronic multichannel recordings from organotypic hippocampal slice cultures: protection from excitotoxic effects of NMDA by non-competitive NMDA antagonists. *J. Neurosci. Methods* 120, 193–202

24 Bruce, A.J. *et al*. (1996) β-Amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic catalytic free radical scavenger. *Proc. Natl. Acad. Sci. U. S. A*. 93, 2312–2316
25 Morrison, B. 3rd *et al*. (2000) Dynamic mechanical stretch of organotypic brain slice cultures induces differential genomic expression: relationship to mechanical parameters. *J. Biomech. Eng*. 122, 224–230
26 de Bouard, S. *et al*. (2002) Invasion of human glioma biopsy specimens in cultures of rodent brain slices: a quantitative analysis. *J. Neurosurg*. 97, 169–176
27 Stoppini, L. *et al*. (1997) Sprouting and functional recovery in co-cultures between old and young hippocampal organotypic slices. *Neuroscience* 80, 1127–1136
28 Raineteau, O. *et al*. (2004) Neurogenesis in hippoampal slice cultures. *Mol. Cell. Neurosci*. 26, 241–250
29 Meyer, M. *et al*. (2000) Improved survival of embryonic porcine dopaminergic neurons in coculture with a conditionally immortalized GDNF-producing hippocampal cell line. *Exp. Neurol*. 164, 82–93
30 Gatherer, M. *et al*. (2002) Proteomics reveals altered protein expression after ischaemia in hippocampal organotypic cultures. *FENS Abstr*. Vol. 1, A150.10.
31 Duff, K. *et al*. (2002) Organotypic slice cultures from transgenic mice as disease model systems. *J. Mol. Neurosci*. 19, 317–320
32 Wirth, M.J. and Wahle, P. (2003) Biolistic transfection of organotypic cultures of rat visual cortex using a hand held device. *J. Neurosci. Methods* 125, 45–54
33 Glover, C.P. *et al*. (2002) Adenoviral-mediated, high-level, cell specific transgene expression: a SYN1-WPRE cassette mediates increased transcriptional expression with no loss of neuron specificity. *Mol. Ther*. 5, 509–516