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Invited review

Building a zebrafish toolkit for investigating the pathobiology of epilepsy and identifying new treatments for epileptic seizures

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HIGHLIGHTS

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- New techniques for genome editing allow rapid generation of zebrafish genetic models of epilepsy.
- Powerful techniques for *in vivo* imaging of neuronal activity allow neural circuit activities to be visualised within the zebrafish Central Nervous System.
- Combining these novel technologies with classical electrophysiology and pharmacological screening approaches provides new opportunities for improving understanding of epileptogenesis, *in vivo* target validation and drug discovery.

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ABSTRACT

Recent advances in genomics and genome sequencing technologies provide a wealth of DNA sequence data that sheds new light on the causes of epilepsy. Animal models help to elucidate the biological significance of such disease-associated DNA sequence variation by enabling functional relationships between disease genotypes and phenotypes to be defined. Here I review the unique combination of attributes that is allowing the zebrafish to play increasingly prominent roles in investigating the mechanisms underlying epilepsy and in discovering new drugs to treat this condition. New techniques for genome editing now allow the zebrafish genome to be engineered to recapitulate key elements of the patterns of genomic variation that are observed in epilepsy patients. Moreover, a sophisticated range of imaging technologies enables spatio-temporal patterns of neural activity to be visualised in the intact zebrafish nervous system with single-cell levels of resolution. These technologies, together with refined techniques tor electrophysiological analysis and non-invasive modulation of specific neuronal circuit functions, allow the impacts of defined genetic variation on *in vivo* patterns of neural activity to be analysed in unprecedented depth. The pharmacological tractability of the zebrafish, and the amenability of its embryonic and larval stages to high throughput phenotype analysis, are also enabling advances in anti-epileptic drug discovery. Combining such pharmacological screening approaches with new tools for genome editing, live imaging, electrophysiology, conditional manipulation of circuit activity and behavioural analysis of zebrafish, could facilitate step changes in both understanding of epileptogenesis and *in vivo* discovery of new and improved anti-epileptic drugs.

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1. The need for new *in vivo* experimental models of epilepsy

Epilepsy is a disorder of the Central Nervous System (CNS) in which patients exhibit marked vulnerability to recurrent episodes of excessive neuronal activity in the brain, which are known as seizures. Repeated or prolonged seizures are seriously debilitating conditions and may cause additional pathological changes to the CNS, further affecting quality of life for patients and their carers. Around 1% of the general population suffer from epilepsy, but of these patients, around one third fails to derive significant benefit from the available treatments. There are therefore substantial imperatives to improve understanding of epilepsy mechanisms and to develop both new treatments for the currently untreatable forms, and more specific treatments for those forms of epilepsy which are accompanied by significant adverse side effects.

Epilepsy exhibits high heritability, implying that genetic variation confers elevated susceptibility to seizures. Indeed, genetic analysis of specific forms of inherited epilepsy, along with Genome-Wide Association Studies (GWAS) and exome sequencing studies of large cohorts of epilepsy patients and controls, have identified epilepsy-associated sequence variants in hundreds of genes with a wide variety of roles in nervous system development and function (Noebels, 2015). Many of these genes encode neuronal proteins that regulate membrane excitability, such as ion channels, neurotransmitter receptors and transporters (Deng et al., 2014), or factors controlling synaptic vesicle formation, release and trafficking (Casillas-Espinosa et al., 2012). Mutations in genes that regulate the differentiation, migration and connectivity of GABA-ergic inhibitory interneurons (Peñagarikano et al., 2011; Olivetti and Noebels, 2012), or the activity of intercellular signalling cascades (Rivière et al., 2013), are also implicated in epilepsy. Moreover, novel approaches, including the systems-genetics approach of combining GWAS and transcriptomic analysis of human epileptic brain tissue, have begun to uncover networks of transcriptionally co-regulated gene networks that are characteristically associated with epileptogenesis (Johnson et al., 2015). The ongoing studies to identify inherited or *de novo* mutations, or transcriptional changes which may have a genetic or epigenetic basis, will continue to provide a wealth of new genomic information that will be of growing importance for elucidating the molecular and cellular mechanisms underlying epilepsy. Whilst the genomic complexity that characterises epilepsy is challenging to understand, an emerging theme from these studies is that epileptogenic mechanisms frequently involve genes that normally functionalize inhibitory circuits (Noebels, 2015). Thus, greater focus on understanding of how this genetic heterogeneity engenders epileptic seizures through loss or attenuation of inhibitory neuronal activities will require a range of *in vivo* and *in vitro* approaches to elucidating the functions of the gene networks, signalling pathways and cell processes underpinning inhibitory circuit functions, and thus help to identify the most effective strategies for therapy.

Model organisms with experimental tractabilities that allow integrative *in vivo* studies of epilepsy phenotypes are likely to play increasingly prominent roles in elucidating the pathobiology of human epilepsy. Whilst the mouse has long been a genetically tractable mainstay of epilepsy research, the practical difficulties of accessing the rodent brain, together with the limitations of techniques for monitoring spatiotemporal patterns of neural activity within the brain, and the methodological challenges of administering drugs to the CNS and screening for new therapeutics, all emphasise the need to embrace new model organisms that are not constrained by such limitations. The availability of *in vivo* models which can be engineered to better approximate the genetic complexity of this disorder than is offered by monogenic murine epilepsy models will be of growing interest to epilepsy researchers in the years ahead.

2. The zebrafish as model organism for biomedical research and its suitability for research into epilepsy mechanisms and treatments for seizures

Within the last few years, the zebrafish has become an increasingly attractive model organism for epilepsy research. Originally employed by developmental biologists for its forward genetic tractability and its optically clear embryonic and larval stages, use of the zebrafish in biomedical research has widened extensively for studies of the pathobiology of a growing range of inherited and acquired disorders (Phillips and Westerfield, 2014). Its relatively short generation time, together with the large clutch sizes of offspring, the reliability with which germline mutagenesis can be achieved with chemical mutagens, and the ease with which large numbers of breeding adults can be maintained in small aquaria, have made this model vertebrate highly amenable to phenotype-based forward genetic screens. Moreover, antisense morpholino-based approaches to inhibiting specific gene expression are useful alternative tools for elucidating specific gene functions in embryos when mutant alleles are not available.

Recent advances in developing zebrafish genome-editing technologies now offer convenient, cost-effective and precise techniques for creating zebrafish with multiple knock-out and knock-in modifications to multiple genes within the same animal, which may be a particularly effective way of developing model genotypes that more closely resemble those of epilepsy patients. Targeted gene inactivation using TALEN- and CRISPR-Cas9-mediated DNA cleavage and repair by endogenous Non-Homologous End Joining approaches are efficient new routes to mutagenizing genes of interest (Bedell et al., 2013; Hwang et al., 2013). Moreover, refinements to the CRISPR-Cas9- based technique of genome editing have very recently been described which enable the precise, in-frame integration of exogenous DNA sequences into specific zebrafish genes, opening up exciting possibilities for adding additional reporter cassettes and regulatory elements into a predetermined expressible chromosomal locus, or even knocking-in specific gain-of-function mutations at particular genomic locations (Auer et al., 2014; Hisano et al., 2015). The application of successive rounds of genome editing using these types of approaches could enable reconfiguration of the zebrafish genome to contain multiple epileptogenic mutations at distinct loci, thus enabling the rapid development of experimental models that reliably recapitulate key constellations of human epileptogenic susceptibilities. The transparency of the accessible, externally developing embryos and larvae enables the use of powerful confocal, two-photon and light-sheet microscopes for *in vivo* visualisation of fluorescent transgenic markers such as Green Fluorescent Protein and its relatives, and the live imaging of excitable cell behaviour using activity reporters such as the GCaMP and pHluorin families of proteins (Akerboom et al., 2012, 2013; Broussard et al., 2014; Chen et al., 2013; Panier et al., 2013). This growing repertoire of new tools for visualising spatiotemporal changes in neuronal activities as integrated functional ensembles within the CNS, offers exciting opportunities to determine the systems properties of neural circuits in unprecedented detail. Moreover, the accessibility of zebrafish embryos and larvae also enables conventional patch-clamping for electrophysiological analysis of individual neurones and muscle cells, as well as extracellular field recording techniques to detect local circuit activities within brain regions (Drapeau et al., 1999; Baraban et al., 2005; Tong and McDearmid, 2012; Roy and Ali, 2013; Jay et al., 2015).

Combining techniques for genome editing at multiple loci, with live imaging of fluorescent transgenic reporters of neural activity within the larval CNS and *in vivo* electrophysiological measurements of synaptic and action potentials, could enable a step-change in the functional analysis of epilepsy mechanisms and facilitate the mapping of neural activity patterns in exquisite detail using this

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