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Canalization of genetic and pharmacological perturbations in developing primary neuronal activity patterns

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ABSTRACT

The function of the nervous system depends on the integrity of synapses and the patterning of electrical activity in brain circuits. The rapid advances in genome sequencing reveal a large number of mutations disrupting synaptic proteins, which potentially result in diseases known as synaptopathies. However, it is also evident that every normal individual carries hundreds of potentially damaging mutations. Although genetic studies in several organisms show that mutations can be masked during development by a process known as canalization, it is unknown if this occurs in the development of the electrical activity in the brain. Using longitudinal recordings of primary cultured neurons on multi-electrode arrays from mice carrying knockout mutations we report evidence of canalization in development of spontaneous activity patterns. Phenotypes in the activity patterns in young cultures from mice lacking the Gria1 subunit of the AMPA receptor were ameliorated as cultures matured. Similarly, the effects of chronic pharmacological NMDA receptor blockade diminished as cultures matured. Moreover, disturbances in activity patterns by simultaneous disruption of Gria1 and NMDA receptors were also canalized by three weeks in culture. Additional mutations and genetic variations also appeared to be canalized to varying degrees. These findings indicate that neuronal network canalization is a form of nervous system plasticity that provides resilience to developmental disruption.

1. Introduction

Although large-scale human genome sequencing has identified hundreds of mutations causing brain disorders, all normal human individuals express a large number of highly damaging deleterious variants and disease-relevant mutations (Sulem et al., 2015; Xue et al., 2012). This raises the intriguing question: how does the brain maintain normal function in the presence of these mutations? Almost 70 years ago, Conrad Waddington recognised that mutations were masked during development and introduced the concept of 'canalization' to describe this inherent robustness (Waddington, 1942). Waddington developed the concept of canalization to describe the means by which developmental systems are channelled along a pathway or trajectory to their mature form. Deviations from this trajectory, caused by genetic or environmental perturbations, are diminished or canalized into developmental channels that lead to the final developed organism. Canalization not only allows organisms to develop and function normally in the presence of mutations but also hides genetic diversity in a population of phenotypically similar organisms, until it is unmasked by conditions of environmental stress and generates phenotypic diversity (Siegal and Bergman, 2006). Canalization is a self-organizing property of complex systems that is fundamentally different to homeostasis. Homeostasis maintains the stability of systems (using negative feedback) and canalization channels the

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system to a future and distinct set point. Although canalization has been recently studied in bacteria (Maisnier-Patin et al., 2005), yeast (Deutscher et al., 2006; Wagner, 2000) and *Drosophila* (Rutherford and Lindquist, 1998), little is known about its role in neural systems of vertebrates.

Cultured rodent primary neurons have been used extensively to study homeostatic mechanisms regulating neuronal excitability and firing patterns. It has been shown that networks of neurons stabilize their firing patterns in the face of environmental changes (Slomowitz et al., 2015). In hippocampus and cortex primary neuronal cultures from rodents, network synchrony can be perturbed by pharmacological manipulations, but typically returns over the course of hours (Kaufman et al., 2014).

To our knowledge there has been no direct experimental evidence showing canalization of mutation in the development of bursting and firing patterns in neural circuits. In the course of developing an *in vitro* assay for the purposes of studying the impact of disease-relevant mutations on neuronal circuits (MacLaren et al., 2011) we unexpectedly observed evidence for canalization. We monitored the activity of developing neural circuits, from birth to 4 weeks of age, in a tissue culture chamber where a 59 electrode array (MEA, Multi-electrode array) was overlaid with primary cultures of mouse hippocampal neurons. Using this approach, we have previously correlated longitudinal recordings of firing patterns and synchronization in neuronal networks with underlying changes in gene expression (Valor et al., 2007) and characterized differences in the network activity profiles of hippocampal versus cortical neurons (Charlesworth et al., 2015). Here we report experiments with mutations in these assays, using primary cultures from mice carrying knockout mutations in a glutamate receptor subunit, and multiple post-synaptic scaffolds and signalling proteins.

2. Materials and methods

2.1. Preparation of multi-electrode arrays

On the day of plating, planar multi-electrode arrays (59 titanium nitride electrodes, 30 μm-diameter, 200 μm-spacing, internal reference electrode; MultiChannel Systems, Fig. S1) were sterilized in a plasma cleaner (Diener Electronic). The central-most portion of the culture compartment was treated with an 8 μl drop of poly-D-lysine (Sigma) (0.5 mg/ml), washed with 8 μl sterile water, then coated with a 4 μl drop of ice-cold 1 mg/ml laminin (Invitrogen). 30 μl of full Neurobasal medium was dispensed round the perimeter of the culture compartment of the MEA prior to the laminin coating step. MEAs were fitted with a sterile, gas-permeable/water vapour-impermeable lid (Potter and DeMarse, 2001) and placed in an incubator (37 °C; 5%CO_2/95% air; humidified) until required for plating.

2.2. Primary neuronal cultures

Primary cultures of dissociated hippocampal neurons were prepared from embryonic day (E) 17–18 mice. Pregnant mice from timed matings were killed by cervical dislocation and embryos were removed and decapitated before dissecting hippocampi from embryonic brains, keeping tissues submerged in ice-cold Dulbecco's phosphate buffered saline (DPBS) (Invitrogen) containing 1% v/v penicillin/streptomycin solution (Invitrogen). For wild-type or null mutants, hippocampi from multiple embryonic brains were pooled. Following incubation at 37 °C in 10 units/ml papain (Worthington) for 22 min, hippocampal tissue was disaggregated by reverse pipetting in pre-warmed suspension medium (Dulbecco's Modified Eagle's Medium/F:12 (1:1) containing 10% v/v foetal bovine serum and 1% v/v penicillin/streptomycin solution). This cell suspension was centrifuged at 400 × *g* for 3.5 min, the pellet re-suspended in fresh suspension medium, and centrifuged a second time at 400 × *g* for 3.5 min. The final pellet was resuspended (100 μl/pair hippocampi) in pre-warmed full Neurobasal medium (Neurobasal, B27 supplement, 500 μM L-glutamine, 1% v/v penicillin/streptomycin solution). Cell yield was counted using a haemocytometer before seeding 2 × 10^5 cells (typically equating to around 30 μl of cell suspension) in the centre of multi-electrode arrays (prepared as described above) containing 600 μl full Neurobasal medium. Zero-evaporation lids were refitted and the MEAs housed in tissue culture incubators maintained humidified at 37 °C and 5% CO_2/95% air. At 3–4 DIV, cultures were fed by replacing 200 μl medium with pre-warmed fresh full Neurobasal medium. Cultures were subsequently fed using the same method after each recording, equating to a one third medium change twice per week. Where cultures were chronically treated with APV (50 μM), this was added when cultures were fed after recording at 7 DIV, and then maintained at this concentration with subsequent feeds.

24 h post-plating, MEAs were placed on an inverted microscope with heated stage (Axiovert 200; Zeiss) and photographed through a 32× phase objective at 5 different fields of view (Fig. S1b). To confirm similar numbers of adherent cells between preparations, automated cell counting of these images was performed using a pipeline designed in CellProfiler (Carpenter et al., 2006). After completion of time-courses, cells were trypsinated, pelleted as described above in DMEM/F-12/FBS and resuspended in Wizard SV lysis buffer (Promega) for confirmatory genotyping by PCR.

2.3. Mouse lines

All procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. The mouse lines used in this study were as follows:

Wild type

C57BL/6-*Tyr*$^{c\text{-}Brd}$ (C57; albino C57BL/6), 22 cultures, 76 MEA platings

129S5/SvEvBrd (129S5), 13 cultures, 53 MEA platings

Mutant lines (homozygous null breedings)

Gria1, (C57 background (Zamanillo et al., 1999)), 15 cultures, 68 MEA platings

Arhgap32, (C57 background), 3 cultures, 26 MEA platings

Dlg2, (C57 background (McGee et al., 2001)), 10 cultures, 71 MEA platings

Gnb1, (129 background), 3 cultures, 26 MEA platings

Dlg4, (129 background (Migaud et al., 1998)), 10 cultures, 40 MEA platings

Dlg3, (129 background (Cuthbert et al., 2007)), 9 cultures, 52 MEA platings

Sipa1l1, (129 background), 5 cultures, 41 MEA platings

We confirmed that the divergent genetic backgrounds of the two wild-type strains used in this study exerted no detectable influence on the electrophysiological parameters measured (see Fig. S6).

2.4. Multi-electrode array recording

Multi-electrode arrays and all data acquisition hardware and software were from MultiChannel Systems (Reutlingen, Germany). Pairs of MEAs were interfaced with duplex 60 channel amplifiers and 15 min recordings of spontaneous action potentials were made twice per week during the four weeks following plating. MEAs were heated and kept under a light flow of 5% CO2/95% air during recordings. Signals were digitized with a 128-channel analogue/digital converter card at a rate of 25 kHz and filtered (100 Hz High pass) to remove low frequency events and baseline fluctuations.

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