Molecular and Cellular Neuroscience 47 (2011) 93–99

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Knockdown of mental disorder susceptibility genes disrupts neuronal network physiology *in vitro*

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ARTICLE INFO

Article history:
Received 9 August 2010
Revised 12 December 2010
Accepted 30 December 2010
Available online 30 March 2011

Keywords:
Multielectrode array
Neuronal culture
Tnik
Dlg2
Disc1
Dctn5

ABSTRACT

Schizophrenia and bipolar disorder are common diseases caused by multiple genes that disrupt brain circuits. While great progress has been made in identifying schizophrenia susceptibility genes, these studies have left two major unanswered mechanistic questions: is there a core biochemical mechanism that these genes regulate, and what are the electrophysiological consequences of the altered gene expression? Because clinical studies implicate abnormalities in neuronal networks, we developed a system for studying the neurophysiology of neuronal networks *in vitro* where the role of candidate disease genes can be rapidly assayed. Using this system we focused on three postsynaptic proteins *DISC1*, *TNIK* and *PSD-93*/*DLG2* each of which is encoded by a schizophrenia susceptibility gene. We also examined the utility of this assay system in bipolar disorder (BD), which has a strong genetic overlap with schizophrenia, by examining the bipolar disorder susceptibility gene *Dctn5*. The global neuronal network firing behavior of primary cultures of mouse hippocampus neurons was examined on multi-electrode arrays (MEAs) and genes of interest were knocked down using RNAi interference. Measurement of multiple neural network parameters demonstrated phenotypes for these genes compared with controls. Moreover, the different genes disrupted network properties and showed distinct and overlapping effects. These data show multiple susceptibility genes for complex psychiatric disorders, regulate neural network physiology and demonstrate a new assay system with wide application.

Introduction

Schizophrenia is a psychiatric disorder with a high heritability that is not caused by a common single gene mutation but rather by dozens or perhaps hundreds of mutations including both single nucleotide polymorphisms (SNPs) in coding and regulatory regions as well as copy number variants (CNVs) (Craddock et al., 2005, Stone et al., 2008). While this disparate set of mutations is involved in producing similar behavioral effects in patients, the identity of the genes has not uncovered a single family or category of molecules but rather has broadly implicated biological processes such as neurodevelopment and synaptic transmission. While it may be implied that these genes all impinge on some common aspect of neuronal and brain function, there is still a lack of neurophysiological methods for testing and comparing many genes in many different functional classes which would be required to tease out the differential roles each susceptibility gene plays in the etiology of schizophrenia.

There is evidence to support the hypothesis that schizophrenia is a disorder of brain circuits (Arnold et al., 2005) and recordings from patients with schizophrenia, bipolar disorder (BD) and autism show abnormalities in patterns of neural activity (Adler et al., 2006; Ford et al., 2007; Uhlhaas and Singer, 2007). More recently, synchrony in the activity between neurons of the hippocampus and prefrontal cortex was found to be impaired in a model of schizophrenia in which a human microdelection in chromosome 22 (22q11.2) was reconstituted in a mouse (Sigurdsson et al., 2010). This study demonstrated a predictive correlation between the degree of neuronal synchrony and the ability of the animal to perform a learning task. Although none of the genes examined in this work is in the 22q11.2 region, this study suggests the possibility that *in vitro* cellular deficits can be a proxy, or indicator, for *in vivo* behavioral phenotypes.

Primary cultures of hippocampal neurons from embryonic mice grown on multielectrode arrays demonstrate behavioral characteristics such as bursting and synchronized firing, and could provide a model system for monitoring and measuring the effects of genetic manipulations on the development of neural networks. More specifically, these cultures form networks that spontaneously generate action potentials (spikes), rapid bursts of spikes and synchronized firing, which become increasingly coordinated in spatial and temporal dimensions as the cultures mature (Chiappalone et al., 2006). This network activity can be readily recorded using MEAs (Valor et al., 2007) which allow the network-level effects of any manipulation of the cultures to be observed. Since many candidate mutations for schizophrenia and related psychiatric disorders involve a loss of the gene product, either through a deletion of some or all of the exons or a

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doi:10.1016/j.mcn.2010.12.014

translocation event that interrupts the genomic structure of the gene, RNA interference (RNAi) offers a simple, rapid and flexible method of modeling the effect of these mutations directly in primary cultures. Previous studies have documented the effects of pharmacological interventions on the network properties of cultured neurons (Arnold et al., 2005; Gramowski et al., 2004), however the contributions of targeted gene knockdown, including disease gene candidates, to these phenotypes have not yet been reported.

In order to determine if common network deficits were created by schizophrenia-related genes, we studied the impact of gene knockdown on neuronal networks grown on MEAs using siRNAs and targeted three genes associated with schizophrenia: TRAF2 and NCK interacting kinase (*Tnik*), Discs Large Homolog 2 (*Dlg2*/*PSD-93*), and Disrupted in Schizophrenia 1 (*Disc1*), as well as a candidate gene for bipolar disorder (BD), Dynactin 5 (*Dctn5*). All of these genes have either been localized to the post-synaptic density (PSD) directly or, as in the case of *Dctn5*, are part of a multiprotein complex that has been found at the PSD (DeGiorgis et al., 2006; Kirkpatrick et al., 2006; Kwinter et al., 2009). *Tnik* (TNIK) is a kinase that localizes to the post-synaptic density and interacts with other proteins strongly associated with schizophrenia including NMDA receptors and DISC1 (Wang et al., 2010). *Dlg2* (PSD-93/Chapsyn-110) is a scaffolding protein of the post-synaptic density deleted in schizophrenia in a study of Genome-Wide Copy Number Variation (Walsh et al., 2008) and shows a reduction in protein expression in post-mortem brain samples from schizophrenics (Kristiansen et al., 2006). Moreover, mouse knockouts of Dlg2 show hypofunction of NMDA receptor signaling (Carlisle et al., 2008), a process implicated in schizophrenia (Javitt, 2007; Mohn et al., 1999). A hemizygous translocation that disrupts the *Disc1* gene segregates with schizophrenia and other psychiatric disorders in a Scottish pedigree (Millar et al., 2000). Finally, schizophrenia and BD likely share common genetic mechanisms (Purcell et al., 2009) and so we also tested a BD susceptibility gene, Dynactin 5. *Dctn5* (p25) encodes a subunit of the dynactin/dynein motor complex, which is known to be important for retrograde dendritic transport in neurons, and lies within a genomic region that has been identified by a Genome-Wide Association Study (GWAS) in BD (Burton et al., 2007). Furthermore, components of the dynactin complex have previously been associated with motor neuron degeneration and familial dementia in animals and humans (Laird et al., 2008; Munch et al., 2005).

Results

Hippocampal neurons from E17.5 mouse embryos were cultured on MEAs (Fig. 1a) and transfected after 4 days *in vitro* (DIV 4). The four target genes were tested using three different conditions: transfection of siRNAs targeting the gene of interest and two negative controls, untransfected controls and transfection of non-targeting siRNA (NTC). The network activity of the neurons was recorded for 15 min daily beginning on DIV 5 and continuing until DIV 12 (Fig. 1b). Knockdown for all genes was assayed at DIV 7 (72 h post-transfection) and ranged from 60 to 84% compared with control cultures (Fig. 1d), which is in the range relevant to the levels of expression occurring in the human hemizygous mutations (Kristiansen et al., 2006; Millar et al., 2005). Because the mRNA and protein were harvested from the entire culture, and are derived both from neurons transfected with the siRNAs and untransfected cells, the level of expression knockdown can be taken as a lower limit of the transfection efficiency. That is, if the siRNA pool targeting *Dctn5* reduces mRNA expression by 85% overall in the culture, 85% of the cells in that culture have been transfected at a minimum. Thus, the siRNAs used to target the genes in this study are transfected into the majority of the cells in the cultures.

During the first 12 days in control cultures, a dramatic transition from silent neurons to active synchronized cultures was observed as an increase in both the number of spikes recorded each day as well as an increase in the number of electrodes recording bursts (Fig. 1c). The MEA recordings allowed quantitation of the following seven parameters of neural network activity that were used to observe the phenotypic effects of the gene knockdowns: i) total spikes, ii) % of spikes in bursts, iii) burst rate, iv) burst duration, v) burst pattern, vi) network size and vii) correlation index (summarized in Fig. 1a). These seven parameters have a high degree of information content describing the activity of the developing neuronal networks, and allow the effect of an siRNA to be determined in multiple dimensions and therefore facilitate the direct comparison of genes with disparate cellular functions. A summary "barcode" of the knockdown results is presented in Fig. 3 showing parameters that are significantly increased or decreased by the knockdown of each gene at each DIV measured. In addition to this summary barcode, the detailed measurements for each parameter are reported for each of the five timepoints in culture (Supplementary Tables 2–5).

In order to check for evidence of significant toxicity of the siRNA in the cultures, the total spike parameter was examined first. The total number of spikes detected on MEAs depends, in part, on the number of cells in close proximity to the electrodes (Wagenaar et al., 2006) as well as the synapse density in the culture (Brewer et al., 2009). Total spikes can therefore be used as an indirect measure of toxicity with a decrease in the overall number of spikes suggesting a toxic effect of the siRNAs. There was no significant difference in total spikes, neither an increase nor decrease, recorded between transfected and non-transfected cultures under any of the conditions examined ($p>0.05$) (Supplementary Fig. 1), nor any detectable loss of neurons on visual inspection. In the absence of obvious cytotoxic effects, the bursting behavior in the siRNA treated networks was next examined in more detail.

The network effects of knocking down *Tnik* in these cultures were the most dramatic as shown in Fig. 2. The effect was dramatic enough to be observed by raster plot of the firing frequency recorded by the electrodes of the MEAs (Fig. 2a). The significantly altered parameters included: increased percentage of spikes in bursts, decreased duration of bursts and increased coordination of bursting activity across neurons in the network as reflected by the decreased burst pattern and increased correlation index parameters. All quantitative data is shown in Supplementary Table 2. The burst correlation in particular is of interest because of the connection between schizophrenia and neural network synchrony. In all cases the effect observed becomes significant only after 7 DIV, indicating that the networks remained normal for at least three days after application of the siRNA, and remained significant through the 12th day of culture. The delayed onset of effect after the application of the siRNA could be due to the time it takes for protein to turnover in the cell after new translation has stopped. The 11–12 DIV timepoint is past the expected efficacy of siRNA treatment, and could also suggest the protein turnover "lag" between translation and function. However, it may also indicate irreversible effects to the development of neuronal networks that cannot be compensated at a later time.

In parallel experiments, *Dlg2* mRNA levels were reduced in the neuronal cultures by $69\pm3\%$ ($p<0.01$) with siRNA treatment and, most strikingly, caused an increase in the observed bursting rate (Fig. 3c and Supplementary Table 4). We also observed an increase in the average duration of bursts, in contrast to the *Tnik* results. These differences in burst rate and duration disappeared by one week post-transfection (DIV 12), returning to the same levels as the controls (Fig. 3c). This suggests a transient effect of the siRNA from which the cultures later recover, a marked difference from the *Tnik* results. Thus *Dlg2* was also required for normal neuronal network activity.

RNAi knockdown of *Disc1* resulted in minimal phenotypic effects compared with the other two genes, with only an increase burst duration reaching statistical significance at DIV 12 (Fig. 3d and Supplementary Table 5). While it remains possible that the $60\pm19\%$ ($p<0.05$) reduction in mRNA was insufficient to produce a more robust phenotype, this decrease is similar to the 40–50% reduction of mRNA reported in a human study (Millar et al., 2005).