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A role for Ca_V1 and calcineurin signaling in depolarization-induced changes in neuronal DNA methylation

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ABSTRACT

Direct manipulations of neuronal activity have been shown to induce changes in DNA methylation (DNAm), although little is known about the cellular signaling pathways involved. Using reduced representation bisulfite sequencing, we identify DNAm changes associated with moderate chronic depolarization in dissociated rat hippocampal cultures. Consistent with previous findings, these changes occurred primarily in the vicinity of loci implicated in neuronal function, being enriched in intergenic regions and underrepresented in CpG-rich promoter regulatory regions. We subsequently used 2 pharmacological interventions (nifedipine and FK-506) to test whether the identified changes depended on 2 interrelated signaling pathways known to mediate multiple forms of neuronal plasticity. Both pharmacological manipulations had notable effects on the extent and magnitude of depolarization-induced DNAm changes indicating that a high proportion of activity-induced changes are likely to be mediated by calcium entry through L-type Ca_V1 channels and/or downstream signaling via the calcium-dependent phosphatase calcineurin.

1. Introduction

Epigenetic processes act to dynamically control gene expression independently of DNA sequence variation and are known to regulate key neurobiological and cognitive processes including brain development (Numata et al., 2012; Lister et al., 2013; Spiers et al., in press), circadian processes (Azzi et al., 2014), synaptic function (Nelson et al., 2008; Feng et al., 2010), and memory formation (Day and Sweatt, 2010; Zovkic et al., 2013). Interindividual variation in epigenetic modifications in the brain is associated with a number of neuropsychiatric and neurodegenerative disorders (De Jager et al., 2014; Lunnon et al., 2014; Numata et al., 2014; Pidsley et al., 2014). DNA methylation (DNAm), typically in the context of palindromic 5′-CpG-3′ dinucleotides and more rarely in a non-CpG context, is the most extensively studied epigenetic modification, playing a role in many important genomic regulatory processes. The covalently attached methyl groups project into the major groove of DNA where they can inhibit transcription by blocking the binding of transcription factors and by recruiting methyl-CpG binding proteins such as MECP2, which remodel chromatin into a condensed heterochromatic state.

Although dynamic changes in DNAm were believed to only occur in dividing cells, recent studies support a role for active methylation (and demethylation) in postmitotic neurons. Direct manipulations of neuronal activity in vitro and in vivo have been shown to induce significant de novo DNAm and/or active demethylation across the genome (Nelson et al., 2008; Martinowich et al., 2003; Ma et al., 2009; Guo et al., 2011a,b), whereas significant neuronal DNAm changes in the brain have been associated with a wide range of learning outcomes and behavioral modifications (eg, Weaver et al., 2004; Lubin et al., 2008; McGowan et al., 2009; Murgatroyd et al., 2009). Little is currently known, however, about the cellular signaling pathways linking changes in electrical activity to alterations in DNAm. Here we test the contribution to activity-dependent DNAm of 2 highly interrelated pathways known to mediate multiple forms of neuronal plasticity: calcium entry via voltage-gated L-type Ca_V1 channels and downstream signaling via the calcium-dependent phosphatase calcineurin (Zeng et al., 2001; Deisseroth et al., 2003; Misonou et al., 2004; Greer and Greenberg, 2008; Schwartz et al., 2009; Schonewille et al., 2010; Wheeler et al., 2012; Evans et al., 2013). We use dissociated hippocampal cultures to identify DNAm changes associated with

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moderate chronic depolarization, using pharmacological interventions to demonstrate that a notable subset of the identified changes depend on signaling through these 2 pathways.

2. Materials and methods

2.1. Dissociated culture, depolarization and DNA extraction

We dissected hippocampi from embryonic day (E) 18 Wistar rat embryos of either sex (Charles River, UK) into Hank's Balanced Salt Solution. Tissue was digested with trypsin (Worthington, 0.5 mg/mL; 15 minutes at 37°C) before trituration and subsequent plating at 90,000 cells/well directly into plastic 12-well plates precoated with poly-L-lysine (50 μg/mL; Sigma, Gillingham, UK) and laminin (40 μg/mL). Neurons were cultured at 37°C with 5% CO_2 in neurobasal medium supplemented with 1% B27, 1% fetal calf serum, and 500 μmol/L Glutamax. At 4 and 7 days in vitro (DIV), half of the media was changed with media supplemented with 2% B27 and 500 μmol/L Glutamax. Unless otherwise stated, all culture reagents were from Invitrogen (Paisley, UK). For chronic depolarization, we treated cultures at 10 DIV for 24 hours with +10 mmol/L KCl or +10 mmol/L NaCl as an osmolarity control. Pharmacological agents were stored in stock solutions in Dimethyl Sulfoxide (DMSO) and then added at previously described effective final working concentrations (1 μmol/L nifedipine in 0.001% DMSO; 1 μmol/L FK506 in 0.1% DMSO) at least 30 minutes before control or depolarizing treatment. Cells were washed in Hank's Balanced Salt Solution and then exposed to 0.15% trypsin for 1 minute at 37°C. Cells were subsequently scraped and aspirated into collection tubes containing neurobasal medium with 2% fetal calf serum for trypsin deactivation. After centrifugation at 1700 rpm for 5 minutes, DNA was extracted using the Qiagen AllPrep kit and eluted in RNAse-free water. DNA samples were checked for purity and degradation, before storage at −80°C.

2.2. Reduced represented bisulfite sequencing

Reduced represented bisulfite sequencing (RRBS) was undertaken using a standard published protocol (Gu et al., 2011), and the 8 individual libraries were sequenced on an Illumina HiSeq2500 sequencer. An average of 40 million high-quality 49-bp paired end reads was obtained for each individual sample. The quality of all RRBS reads were visualized and assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) with all samples being deemed of satisfactory quality (Phred score >30) to be included in this study (see Supplementary Fig. 1). *Trim galore* (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to remove any low-quality called bases at the end of sequences. Bismark (Krueger and Andrews, 2011) was used to in silico bisulfite convert the rat reference genome (rn4). On average 59% (SD, 3.77%) could be uniquely aligned to the bisulfite-converted rat genome using custom scripts developed for RRBS data. DNA methylation at all cytosine bases were quantified using the *–comprehensive*, *–CX*, and *–no-overlap* flags.

2.3. Statistical analysis

Cytosine sites were filtered to those with a minimum coverage of 10 reads in each of the 8 RRBS data sets. Cytosine sites showing consistent changes after depolarization were identified from 2 experimental replicates, which both had a change in DNAm of greater than 20% in the same direction. These changes were then defined as "blocked" if the addition of either FK506 or nifedipine meant that a change of at least 20% (in the same direction) was not observed between the unstimulated drug-naive sample and stimulated drug-treated sample. Gene annotation files for rn4 were downloaded from University of California, Santa Cruz (UCSC) and were used to annotate whether sites were found in exons, introns, transcription start sites (defined as 5-kb upstream), or transcription end sites (defined as 5-kb downstream). Any remaining sites not annotated to one of these categories were classed as intergenic and the nearest gene within 10 kb identified. In addition, CpG island annotation was downloaded from UCSC and used to identify which sites were found in CpG islands or shores. Ontology pathways were downloaded from the Gene Ontology website (http://geneontology.org/). All sites located within 10 kb of a gene annotated to at least 1 ontology pathway were mapped to pathways, including all parent terms. Overrepresentation for any pathway, with at least 10 genes, in the sites with consistent large epigenetic changes was tested with a Fisher exact test compared to the number of sites passing quality control filtering annotated to each pathway in the same manner. All statistical analyses were performed in the R statistical language.

2.4. Calcium imaging

Calcium imaging was carried out on hippocampal cultures plated onto 18-mm glass coverslips precoated with poly-D-lysine (50 μg/mL; Sigma) and laminin (40 μg/mL) at 90,000 cells/well. We loaded cells with the ratiometric calcium indicator fura-2-AM (5 μmol/L; Invitrogen) in a HEPES-buffered saline solution containing, in millimoles per liter: 136 NaCl, 2.5 KCl, 10 HEPES, 10 D-glucose, 2 $CaCl_2$, and 1.3 $MgCl_2$ (30 minutes at room temperature). After washing, cells were incubated in phenol red-free neurobasal medium (Invitrogen) with or without 1 μmol/L nifedipine (30 minutes at 37°C). Cells were then maintained in a steady gravity-fed flow of phenol red-free neurobasal medium (34°C-36°C; maintained with an in-line heater SH-27B, Harvard Apparatus) and were allowed to equilibrate for 10 minutes before an imaging protocol began. Single *z*-axis images were captured for both 340- and 380-nm excitation wavelengths at 0.5 Hz using an inverted Olympus IX71 microscope, an Olympus oil immersion objective ×40 and a Charge-coupled device (CCD) camera coupled to Slidebook software (2 × 2 pixel binning). Baseline fluorescence was established during 5 minutes of perfusion before a +10 mmol/L KCl stimulus was washed in. Fura-2 340/380 ratios were then calculated before and during +10 mmol/L KCl wash-in, using fluorescence intensities averaged across neuronal cell body regions of interest and normalized to background fluorescence.

3. Results and discussion

To elevate neuronal activity in dissociated hippocampal cultures, we used a well-characterized manipulation involving chronic depolarization with moderately increased extracellular potassium. A +10 mmol/L KCl stimulus is known to depolarize hippocampal neurons by ~15 mV, producing a significant and sustained elevation in intracellular calcium levels that has been previously associated with a range of structural and functional plastic neuronal changes (Evans et al., 2013; Grubb and Burrone, 2010a). Although this is certainly not a strictly physiological manipulation, it is an appropriate and useful one—plastic changes induced by this stimulus have also been produced in response to more naturalistic temporal patterns of spike activity (Evans et al., 2013; Grubb and Burrone, 2010a; Mistry et al., 2011). It is also considerably less extreme than the chronic depolarizing stimuli commonly used to initiate activity-dependent signaling in cultured neurons, which often reach 50 mmol/L KCl or higher (Redmond et al., 2002; Flavell et al., 2008). Here, we used a moderate (+10 mmol/L) KCl stimulus for 24 hours from 10 DIV, alongside a +10 mmol/L NaCl treatment as a control for osmotic changes, to investigate the DNAm changes brought about by sustained depolarization. We first used RRBS (Gu et al., 2011) to quantify DNAm in DNA extracted from replicate control (NaCl) and depolarized (KCl) hippocampal samples (Supplementary Table 1). Raw RRBS reads underwent stringent quality control, were aligned to the rat reference genome (rn4), and filtered so that all potentially methylated cytosines (at both CpG and non-CpG sites) included in subsequent analyses had a minimum read depth of 10 reads in all samples (see Materials and methods). In total 1,552,276 CpG sites with a median minimum read depth of 33 (SD, 59.06) and 5,889,893 non-CpG sites with a median minimum read depth of 27 (SD, 51.61) were included