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Gene expression changes after seizure preconditioning in the three major hippocampal cell layers

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Rodents experience hippocampal damage after status epilepticus (SE) mainly in pyramidal cells while sparing the dentate granule cell layer (DGCL). Hippocampal damage was prevented in rats that had been preconditioned by brief seizures on 2 consecutive days before SE. To identify neuroprotective genes and biochemical pathways changed after preconditioning we compared the effect of preconditioning on gene expression in the CA1 and CA3 pyramidal and DGCLs, harvested by laser capture microscopy. In the DGCL the expression of 632 genes was altered, compared to only 151 and 58 genes in CA1 and CA3 pyramidal cell layers. Most of the differentially expressed genes regulate tissue structure and intra- and extracellular signaling, including neurotransmission. A selective upregulation of energy metabolism transcripts occurred in CA1 pyramidal cells relative to the DGCL. These results reveal a broad transcriptional response of the DGCL to preconditioning, and suggest several mechanisms underlying the neuroprotective effect of preconditioning seizures.

Keywords: Kainate; Status epilepticus; Microarray; Hippocampus; Neuroprotection; Epilepsy; Rat; Pilocarpine; CA1; CA3

Introduction

It is well established that organ damage induced by hypoxia/ischemia can be reduced by a prior brief period of ischemic preconditioning. Ischemic preconditioning also protects the brain against prolonged ischemia in rodent models and may attenuate stroke severity in humans (e.g., Simon et al., 1993; Weih et al., 1999). In addition, several reports have shown that ischemic/hypoxic preconditioning can protect the brain from seizure-induced damage (Kitagawa et al., 1990; Emerson et al., 1999). Preconditioning against seizure-induced damage can also be accomplished by kindling or brief seizures (Kelly and McIntyre, 1994; Sasahira et al., 1995; El Bahh et al., 1997; Najm et al., 1998; Andre et al., 2000; Kondratyev et al., 2001; Zhang et al., 2002; Norwood and Sloviter, 2005). Najm et al. (1998) observed that the neuronal damage after kainate-induced prolonged status epilepticus (SE) was reduced by about 70% in the hilus and the hippocampal CA3 and CA1 areas if rats were conditioned the day before by 1 h of kainate-induced SE. Similarly, previous amygdala kindling reduced neuronal damage after lithium/pilocarpine-induced SE in rats in some brain areas, such as the hippocampal pyramidal cell layer, amygdala and piriform cortex, but not in the hilus (Andre et al., 2000). Optimum neuroprotection was achieved by administering two preconditioning episodes of kainate-induced seizures for 20 min each on 2 consecutive days (Zhang et al., 2002). These rats were reported to lack neurodegeneration after subsequent SE, induced either by kainate or pilocarpine. Here we confirmed the neuroprotective effect of this preconditioning protocol within the hippocampus. Our main goal was to identify genes that might underlie the neuroprotective effect of seizure preconditioning. To this end, we compared gene expression profiles in the vulnerable CA1 and CA3 pyramidal cell layers and the dentate granule cell layer (DGCL), which is typically resistant to seizure-induced damage, in preconditioned and control rats. Although energy metabolism transcripts were upregulated in CA1 compared to DGCL, by far the largest number of genes with expression changes was, surprisingly, found within the DGCL. Prominent functional categories of genes affected in the DGCL include signal transduction, neural transmission, and tissue structure.

Materials and methods

Animals

Adult male Sprague Dawley rats (200–270 g) were obtained from Charles River. Before treatment they were handled at least once. Kainate was obtained from Ocean Produce International and was dissolved at 3 mg/ml in phosphate-buffered salt solution (PBS, pH 7.4). For preconditioning, on 2 consecutive days rats received kainate (i.p.) and after 20 min of behavioral seizure activity

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seizures were stopped with pentobarbital (40 mg/kg, i.p.). Seizures were staged according to Racine (1972). Typically seizures began 40 to 70 min after kainate injection with immobility and staring and then progressed to forepaw clonus (stage 3), "wet dog shakes", rearing (stage 4) and rearing and falling (stage 5). Only rats that experienced at least one stage 3 seizure or wet dog shake within 20 min of seizure onset were used. The first kainate dose was 12–13 mg/kg, whereas on the second day rats received 13–14 mg/kg kainate to overcome a small reduction in sensitivity to kainate (Zhang et al., 2002). Control rats received PBS instead of kainate and were given all pentobarbital injections similar to the experimental rats. On the day after the second kainate or PBS injection rats were decapitated after isoflurane anesthesia, the brain was covered with Tissuetek (Fisher-Scientific) and frozen on dry ice for later sectioning and cell harvesting.

To investigate whether this seizure preconditioning protocol was neuroprotective, 17 preconditioned and 11 sham-conditioned rats were injected with kainate (15 mg/kg, i.p.) or pilocarpine (340 mg/kg, i.p.) to induce SE. To minimize peripheral side effects of pilocarpine, 4 mg/kg methylatropine was injected (i.p.) about 30 min before pilocarpine administration. Pilocarpine-induced SE was terminated after 90 to 120 min with pentobarbital (25 mg/kg, i.p.), whereas kainate-induced SE was not terminated but allowed to proceed for 4 to 5 h before SE spontaneously and slowly waned. Sham-conditioned rats were those that did not experience seizures in response to the first kainate-preconditioning injection. As for preconditioned rats, they were given pentobarbital (40 mg/kg, i.p.). On the second preconditioning day, these rats received saline instead of kainate, followed by pentobarbital. Rats were killed by decapitation under isoflurane anesthesia 1 to 3 days after SE to assess cell damage. Brains were removed from the skull and either frozen on dry ice or placed into 4% paraformaldehyde for at least 24 h.

Fluorojade staining and immunohistochemistry

Brains fixed in 4% paraformaldehyde were embedded in paraffin (Borges et al., 2003, 2006). Eight-μm sections were cut and deparaffinized in xylene. Fresh frozen brains were cut into 14-μm sections using a cryostat. Fluorojade staining (Schmued et al., 1997) was used to label degenerating cells according to the manufacturer (Histo-chem Inc., Jefferson, AR). Paraffin sections were immunolabeled for glial fibrillary acidic protein (GFAP) and neuropeptide Y (NPY) as previously described (Borges et al., 2003, 2006).

Tissue harvesting and RNA amplification

Fourteen-micrometer frozen sections were cut with a cryostat, melted onto RNAse-free microscope slides and immediately frozen on dry ice. Sections were fixed in 70% ethanol, stained briefly with cresyl violet and dehydrated to xylene. Within 24 h the three main cell layers of the hippocampal formation were harvested from two or three sections (3.5–4.5 mm behind bregma) using laser capture microscopy (Pixcell IIe system, Arcturus, CA) with the following parameters: spot size 30 μm, power 85 mW and duration 750–1200 μs. Total RNA was extracted using the Extractsure adaptor and the Picopure Isolation kit (Arcturus) with on-column DNAse digestion (Qiagen RNase-free DNase set) and underwent one round of amplification (Greene et al., 2005). Total RNA was reverse-transcribed using a T7-(dT)24 primer (Proligo, LLC, Boulder CO) and Superscript II reverse transcriptase (Invitrogen, CA). Second strand cDNA was then generated using Invitrogen second strand buffer, *E. coli* DNA ligase, *E. coli* DNA polymerase I, *E. coli* RNaseH and T4 DNA polymerase. The second strand cDNA was cleaned with Qiaquick PCR purification kit (Qiagen) and concentrated with a Micron YM-30 centrifugal filter device (Amicon). Amplified RNA (aRNA) was generated from double-stranded cDNA with the MegaScript T7 High Yield Transcription kit (Ambion). The aRNA was cleaned up using the Qiagen RNeasy cleanup protocol, concentrated again with a Micron YM-30 filter, subjected to a second round of first and second strand cDNA synthesis and finally concentrated with a Micron YM-30 filter. For quality control purposes, a small aliquot of second round single-stranded cDNA was removed and used for end point PCR with primers for neuronal enolase. A 200-bp band was found in all samples.

Microarray analysis

Sample labeling, microarray hybridization to the Affymetrix rat RAE230A chip and gene chip scanning were performed by the NINDS NIMH Microarray Consortium at the Translational Genomics Institute in Phoenix, AZ. Chips were developed, scanned and normalized by global scaling. We redefined the probe sets of the rat RAE230A chip according to Dai et al. (2005), which updated the original Affymetrix probe assignments to the newest Unigene build. In this process probe sets belonging to same gene were pooled into one gene identifier and probe sets targeting the non-coding strand were eliminated, which together with the new Unigene build reduced the number of unique transcripts represented on the chip from 15,876 to 10,179. Discrimination scores of the signal intensities for each spot on an individual chip were determined to be significantly different from background (i.e., present, marginally present, or absent) with a one-sided Wilcoxon's Sign Ranked test. We only accepted probe sets for further analysis that were flagged present in at least 65% of all rats examined within one area. We then eliminated all probe sets with expression changes less than 25% after preconditioning relative to the control expression. In order to identify transcripts with significant expression changes after preconditioning, for each cell population we used a *t*-test corrected for multiple comparisons (Hochberg and Benjamini, 1990). The false discovery rate (FDR) was set at 0.01.

The Unigene identifiers of all differentially expressed transcripts were confirmed by searching the NCBI databases using nucleotide–nucleotide Blast, Gene and Pubmed. In order to identify gene ontology categories, we used eGOn (http://nova2.idi.ntnu.no/egon/) to compare differentially expressed transcripts within the DGCL and the CA1 pyramidal cell layer to all transcripts expressed above-background in DGCL or CA1 (DGCL and CA1 master gene lists). Because eGOn does not yet recognize all genes, we manually assigned a biological meaning to each gene when possible using the Gene and Pubmed NCBI databases. To determine whether selected gene categories were over-represented among the differentially expressed genes in the three different hippocampal cell layers, a Chi-squared test on a large contingency table was performed (Motulsky, 1995).

Gene set enrichment analysis

GSEA (Subramanian et al., 2005) was used to provide an independent evaluation of cell layer-specific changes in gene