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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

expression in preconditioned rats. This method does not require preselection of transcripts that meet statistical significance for differential expression, but rather considers all transcripts that are expressed above background in one or both of a pair of gene lists to be compared. For this reason GSEA can provide a somewhat more sensitive indication of functionally concerted expression changes than conventional statistical analysis of individual genes. Because CA1 and DGCL had the broadest expression changes after preconditioning (see below), we selected these two areas to compare the effect of preconditioning on expression in predefined functional groups of transcripts ("gene sets"). Transcripts were included in the analysis if a gene symbol could be identified and if the transcript was expressed above-background in >65% of rats in at least one of the four conditions (DGCL control and preconditioned, likewise for CA1). For each gene and each cell layer, the value in each preconditioned rat was expressed as a ratio to the average value in the 8–9 control rats. This process resulted in a 4592×16 array, with each column being a different rat (8 control and 8 preconditioned) and each row a different gene; the cell values reflect the fold change in expression ranging between ~0.1 and 10. GSEA was then run against 522 functional sets of genes (the "c2" set, Subramanian et al., 2005), with the constraint that at least 15 but no more than 100 genes could be represented in a given set in order to exclude small or very large gene sets. Out of 83 gene sets that survived this filter, several related to energy metabolism appeared high on the ranked list but none was statistically significant ($P > 0.05$), consistent with the low power of GSEA to confirm concerted expression differences (Kim and Volsky, 2005). Thirteen custom gene sets that consisted of highly ranked genes in functionally similar *c2* gene sets were then evaluated by GSEA, which led to the results presented here.

Results

Seizure preconditioning protects the hippocampus from SE-induced damage

Twenty-nine out of 79 rats (37%) were successfully preconditioned, i.e., they experienced 20 min of kainate-induced seizures on 2 consecutive days with less than 5% weight loss (Table 1). Eleven rats on day 1 and 7 rats on day 2 either died during kainate-induced seizures or lost >5% weight overnight. We noticed that rats with more than 5% weight loss after preconditioning showed neuronal damage typical for more prolonged SE (data not shown), indicating that weight loss is suggestive of seizures occurring during the first or second night. Therefore rats with >5% weight loss were eliminated from the study. In most of the remaining rats kainate did not induce behavioral seizures, namely in 21 out of 79 rats (27%) on day 1 and in 12 out of 47 rats (26%) on day 2 of preconditioning. These rats were used as sham-conditioned rats.

To determine whether preconditioning protects from SE-induced damage, preconditioned and sham-conditioned rats were subjected to prolonged SE induced either by kainate or pilocarpine. After injection of five preconditioned rats and seven sham-conditioned rats with kainate, three in each group developed typical SE and survived. After injection of 12 preconditioned rats and four sham-conditioned rats with pilocarpine, two and three rats developed SE and survived in each group, respectively. Fluorojade staining was used to evaluate neuronal damage following SE. In all five preconditioned rats, Fluorojade-staining was not detected within the hippocampus 1 to 3 days after ≥1.5 h of kainate- or pilocarpine-induced SE (Fig. 1), whereas all six sham-conditioned rats subjected to kainate- or pilocarpine-induced SE contained Fluorojade-positive cells within the hippocampus. Stained neurons were located mainly within the CA3 and CA1 pyramidal cell layers and the hilus. In other brain areas, including the thalamus, amygdala, piriform or neocortex, on the other hand, Fluorojade-positive cells were found in all but one of the five preconditioned rats, demonstrating that preconditioning does not protect all brain areas from SE-induced injury equally.

Genes with expression changes in hippocampal cell layers after seizure preconditioning

To identify genes and biochemical pathways that exhibit expression changes after preconditioning, we performed microarray analysis on the DGCL and the CA1 and CA3 pyramidal cell layers harvested using laser capture microscopy (Fig. 2) from control and preconditioned rats. The complete dataset can be found on the Gene Expression Omnibus (GEO) website http://www.ncbi.nlm.nih.gov/geo/dataset GDS1018. Table 2 shows the number of rats used and the number of probe sets at different stages of our analysis. About half of all 10,000 probe sets were flagged to be present in more than 65% of control and/or preconditioned rats. Plots of the mean signal intensities of these probe sets in preconditioned vs. control rats had correlation coefficients of 0.97–0.99 (Figs. 3A1, B1, C1), indicating that the expression level of most transcripts was unchanged. This was confirmed by constructing a frequency histogram of the number of transcripts against their log2 expression ratios (preconditioned vs. control) in each area, revealing normally distributed log2 gene expression ratios centered around a mean log2 expression ratio of 0.0 ± 0.1 (Figs. 3A2, B2, C2) for each cell population. For the statistical analysis we focused only on probe sets with ≥25% difference in signal intensities after preconditioning, which reduced the number of probe sets to be considered to between 651 and 1137 in the three cell layers (Table 2). Surprisingly, we found that expression of a high number of transcripts (632) was significantly changed (FDR < 0.01) within the DGCL, but many fewer within the CA1 and CA3 pyramidal cell layers (151 and 58 probe sets respectively,

Table 1
Success rates of inducing preconditioning and prolonged SE

Preconditioning	No. of rats injected	No. of rats with 20 min. seizures	No. of rats with weight loss or death	No. of non-responding rats
1. Preconditioning	79	47 (59%)	11 (14%)	21 (27%)
2. Preconditioning	47	29 (62%)	6 (13%)	12 (25%)
1. and 2. Preconditioning	79	29 (37%)		

The number of rats used for preconditioning and their response is shown. Successful treatment refers to rats that experienced 20 min of kainate-induced seizures with less than 5% weight loss overnight. Non-responding rats are those that did not experience behavioral seizures. They were used as sham-conditioned rats and were not protected against SE-induced damage.

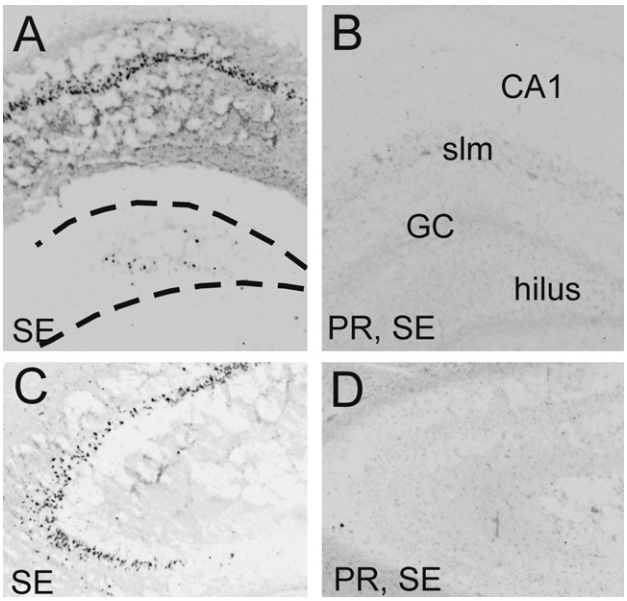

Fig. 1. Preconditioning protects the hippocampus from SE-induced cell damage. (A–D) Fluorojade stainings showing Fluorojade-positive cells (black) 3 days after kainate-induced SE within the CA1 (A) and CA3 (C) pyramidal cell layers and the hilus in sham-conditioned rats. (B, D) No Fluorojade-positive cells are found at the same time point if rats are preconditioned before prolonged kainate-induced SE.

Table 2). A Chi-squared test showed a significant difference in gene regulation among the three hippocampal cell layers ($P<0.001$). However, in all three cell layers transcript levels were more often increased than decreased (Chi-squared test $P<0.001$, Figs. 3A3, B3, C3). Fig. 4 shows the number of transcripts with significant expression changes in each area. Among the 16 transcripts with expression changes in all three areas, only two were downregulated, the other 14 being upregulated in all three areas (Table 3A). Four of these transcripts are involved in regulating signal transmission, including calpactin, NPY, the ion transport regulator 7 and the sodium channel β4 subunit, suggesting that some changes in signal transmission are induced throughout the hippocampus. Moreover, NPY is well-known to be upregulated after seizures (Gall et al., 1990; Vezzani et al., 1999). Using immunohistochemistry, we confirmed that NPY protein is upregulated 2 to 3 days after one ($n=4$ rats) or two preconditioning stimuli ($n=5$ rats) in the mossy fiber pathway (not shown).

Our original hypothesis was that preconditioning protects the pyramidal cell layers by inducing neuroprotective genes in these neurons. Surprisingly, we found only six commonly regulated

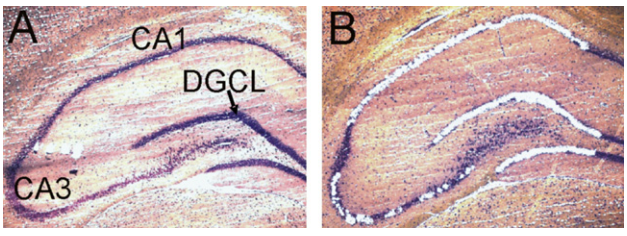

Fig. 2. Example of harvested cell layers for microarray analysis. (A) A cresyl violet-stained section is shown before cell harvesting. (B) Another section is shown after laser capture of the three main cell layers.

Table 2
Overview of microarray results

	DGCL	CA1 pyr	CA3 pyr
No. of rats examined	8 CON, 8 PR	9 CON, 8 PR	8 CON, 7 PR
No. of transcripts present in >65% of control or PR rats	5711	5596	5260
No. of transcripts with ≥25% change after PR	1137	726	651
No. of differentially expressed transcripts (FDR <0.01) (upregulated, downregulated)	632 (351, 281)	151 (101, 50)	58 (37, 21)

The number of control (CON, saline- and pentobarbital-injected rats) and preconditioned (PR) rats used for analysis of each cell layer is shown. An overview of the number of transcripts that survived different stages of our analysis is given. There were significantly more genes regulated in the DGCL than pyramidal cell layers (Chi-squared test, $p<0.001$), and more genes were up- than downregulated (Chi-squared test, $p<0.001$) in each cell layer.

genes in the pyramidal cell layers (Table 3B), including glutathione S-transferase, which is protective against oxidative stress. Moreover, phosphoprotein enriched in astrocytes 15 (PEA-15) is known to oppose apoptosis and may also modulate cell survival (Sharif et al., 2004). Interestingly, three of the commonly regulated genes are expressed in glial cells. PEA-15 is typically found in astrocytes and some neurons, insulin-like growth factor binding protein 2 in astrocytes and microglia (Chesik et al., 2004a,b), and diacylglycerol kinase alpha in oligodendrocytes (Goto et al., 1992).

Seizure-regulated genes in glial cells

As mentioned above, several genes expressed in glial cells were regulated by preconditioning (Tables 3, 4). This is not surprising because the pyramidal cell layers, the DGCL and the subgranular zone all contain GFAP-positive astrocytes (not shown) and oligodendrocyte precursor cells (Ong and Levine, 1999). Several markers of reactive astrocytes were upregulated, such as GFAP, vimentin and calcyclin. Interestingly calpactin mRNA levels were upregulated in all three cell layers as judged by microarray. Calpactin appears to stimulate GFAP assembly (Garbuglia et al., 1995) and may therefore be involved in astrogliosis. Heat shock protein 27 is upregulated, most likely mainly in astrocytes after stress, but also in some neurons (Akbar et al., 2001; Nishino and Nowak, 2004). Several astrocytic genes regulated by preconditioning encode important proteins involved in neuronal and glial signaling, including adenosine kinase (downregulated), glutamate transporter EAAT1/GLAST (upregulated), and the gap junction component, connexin 43 (upregulated). Other astrocytic genes upregulated by preconditioning encode proteins that regulate the composition of extracellular matrix, such as tissue inhibitor of metalloproteinase (Timp2), secreted acidic cysteine rich glycoprotein (Sparc), and cathepsin D. Moreover, genes influencing astrocyte proliferation and differentiation were regulated, such as CD81 (Kelic et al., 2001) and the interleukin 6 receptor (Marz et al., 1999). Finally, preconditioning regulated several genes expressed in oligodendrocytes and/or their precursor cells that are important in myelin formation, including the myelin and lymphocyte gene, *MAL* (Schaeren-Wiemers et al., 2004), tetraspanin 2 (Birling et al., 1999), and myelin oligodendrocyte

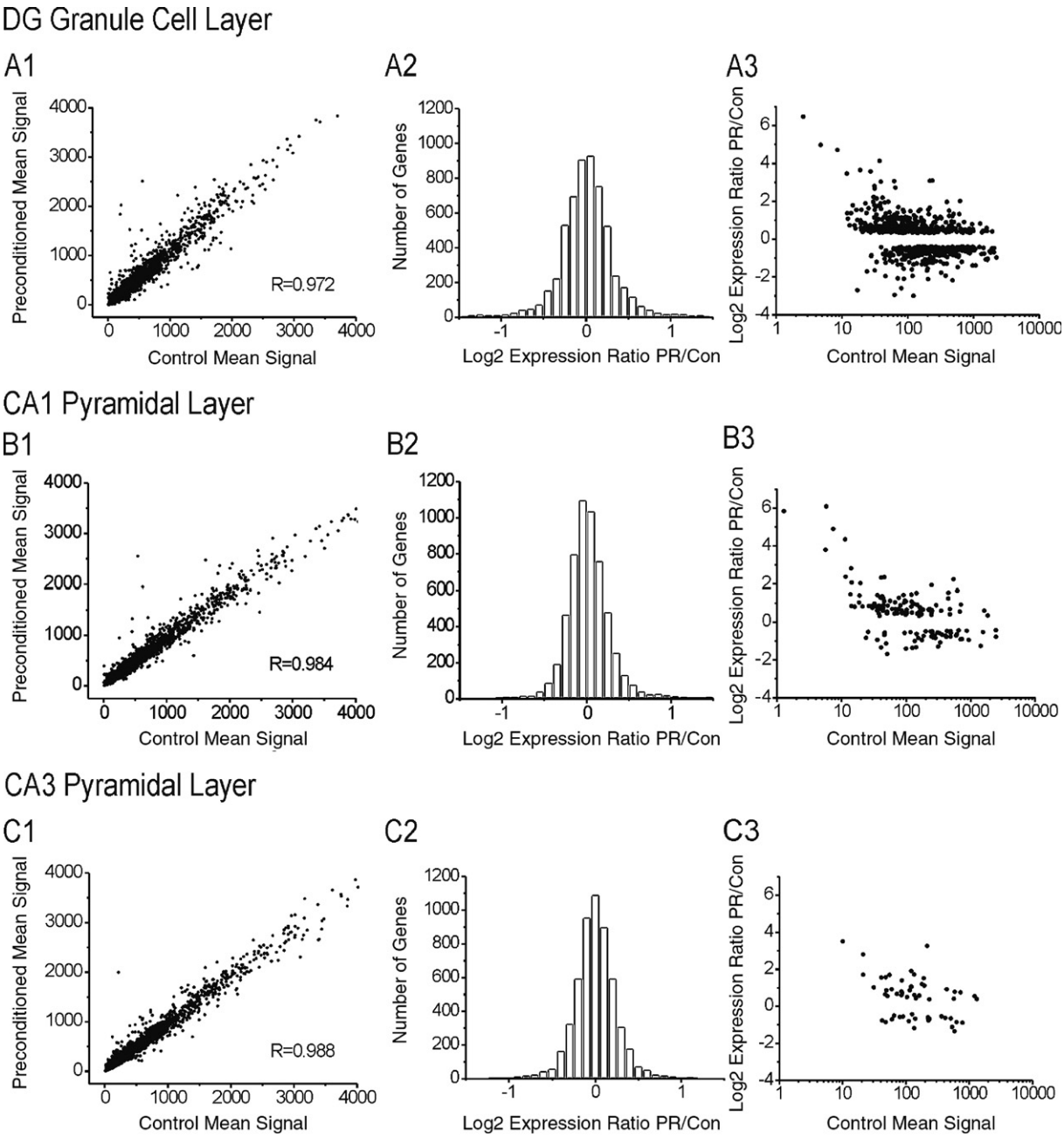

Fig. 3. Overview of microarray results. Panels A1, B1, C1 show the correlation of the average gene expression signals between control and preconditioned samples from the three different hippocampal cell layers as indicated. (B1, B2, B3) Frequency counts illustrate a normal distribution of the log2 expression ratio of preconditioned vs. control samples with an average of 0 in each area. (A3, B3, C3) For all genes with significantly changed expression level after preconditioning, the mean signal is graphed against the log2 expression ratio. These plots illustrate that more genes in all three areas were up- than downregulated, especially those genes with a low mean expression level in control rats.

glycoprotein, MOG (Li et al., 2002). Due to the importance of myelination and astrocytes in signal transduction, it is possible that these responses play a role in preconditioning.

Categorical expression changes within the DGCL after seizure preconditioning

Our analysis detected 632 transcripts with changed expression levels within the DGCL. In order to identify gene ontology categories that were coordinately regulated, we used the eGOn tool to compare the differentially expressed transcripts within the DGCL to all transcripts expressed in the DGCL with and without preconditioning (DGCL master gene list). This analysis revealed that certain molecular functions, biological processes or cellular components were represented in the differentially expressed genes ($P<0.05$) more frequently than would be expected simply from the relative abundance of transcripts in these categories in the DGCL master gene list. These functional categories include signal transduction, morphogenesis, peptide receptors and receptor binding, gliogenesis, defense, extracellular matrix and plasma membrane

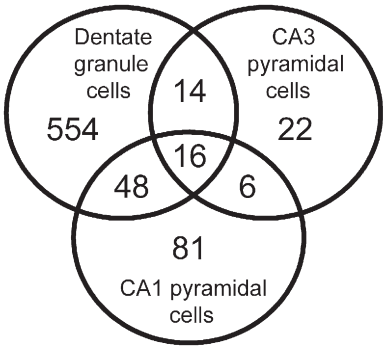

Fig. 4. Venn diagram illustrating the number of genes with statistically significant expression changes ($P<0.01$) after preconditioning in the different hippocampal cell layers.

proteins. This is consistent with the expectation that seizure preconditioning does not randomly change gene expression, but selectively regulates certain biological processes.

Because eGOn did not assign a function to all genes, or sometimes assigned several functions to one gene, we manually assigned one major biological meaning to each gene and then grouped the genes into our own categories (Table 5). Within the DGCL two major functional categories were upregulated, namely the signaling (147 transcripts) and tissue structure (81) categories. The signaling group consists of genes involved in intra- and extracellular signal transduction, including neurotransmission, growth factors and hormones. This suggests that major changes in neuronal and glial activity are induced at the transcriptional level within the DGCL. Genes in the tissue structure group include genes from the GO categories of morphogenesis, cytoskeleton and extracellular matrix, as well as genes involved in cell adhesion, pointing to possible physical changes of the tissue structure and circuitry after preconditioning.

Forty-eight transcripts in the reaction to injury category were also regulated in the DGCL (Table 5). This category includes genes involved in cell death, protection, stress or DNA repair, and defense genes. Transcripts in the defense category were coordinately upregulated after seizure preconditioning and include genes that are typically expressed by immune cells, suggesting expression of immune genes by brain tissue or infiltration and/or activation of immune cells, potentially including microglia. Relatively few genes involved in stress and/or protection (15) or cell death (5) were differentially expressed in the DGCL after preconditioning. eGOn analysis showed that the proportional representation of these genes is the same as that in the DGCL master gene list. The low number of differentially expressed genes in these categories also supports the histological finding that short preconditioning seizures do not induce damage.

Interestingly we found that nine out of 11 mitochondrial genes with expression changes in the DGCL after preconditioning were downregulated (Table 5), suggesting that energy metabolism in the DGCL might be downregulated 1 day after brief seizures. For the three remaining biological categories with changed gene expression – transcription, mRNA modification and turnover (43 genes), cell proliferation (32) and protein synthesis, modification and turnover (29) – the number of genes with expression changes simply reflected the proportion of expressed genes in these categories (Table 5). Supplementary Table 1 shows all differen-

Table 3
(A) Transcripts with expression changes in all three cell layers; (B) genes affected by preconditioning within the CA1 and the CA3 pyramidal cell layers, but not the DGCL

Gene name	Gene symbol	Accession #	DGCL PR/CON	CA1pyr PR/CON	CA3pyr PR/CON
A. Genes with changed expression in all three areas					
Transcribed locus		CB557812	**8.46**	**3.15**	**3.75**
Calpactin[G]	*S100a10*	AA900235	**4.43**	**1.48**	**3.29**
Glial fibrillary acidic protein[G]	*GFAP*	NM_017009	**4.03**	**4.73**	**9.59**
Similar to RECS1	*RECS1*	BC079087	**3.82**	**5.11**	**3.25**
Neuropeptide Y	*NPY*	CB712006	**3.16**	**2.95**	**2.87**
Musculoskeletal, embryonic nuclear protein 1	*Mustn1*	AY254906	**3.03**	**2.60**	**1.83**
Transcribed locus		BE116233	**2.95**	**2.15**	**2.06**
Latexin	*Lxn*	BQ779685	**2.62**	**1.91**	**2.64**
CD63 antigen	*Cd63*	CB327788	**2.40**	**2.91**	**1.50**
Glycoprotein 38, podoplanin	*Pdpn*	AA892329	**2.09**	**2.72**	**1.91**
Protein tyrosine phosphatase, non-receptor type 5	*Prpn5*	NM_019253	**1.88**	**1.53**	**2.30**
Corticotropin releasing hormone binding protein	*Crhbp*	NM_139183	**1.82**	**2.70**	**2.02**
Ubiquitin-conjugating enzyme E2L 6	*Upb216*	AI177729	**1.49**	**1.53**	**1.48**
Similar to hypothetical DEATH-like containing protein		AA866314	**1.46**	**1.38**	**1.41**
FXYD domain-containing ion transport regulator 7	*Fxyd7*	NM_022008	**0.60**	**0.40**	**0.39**
Sodium channel, voltage-gated, type IV, beta	*Scn4b*	AF544988	**0.58**	**0.54**	**0.46**
B. Genes changed within CA1 pyr and CA3 pyr only					
Insulin-like growth factor binding protein 2[G]	*Igfbp2*	AI599095	2.85	**5.00**	**2.97**
Calponin 3, acidic	*Cnn3*	AI103621	1.56	**2.75**	**2.11**
Deoxyhypusine synthase	*Dhps*	BM386265	0.94	**2.16**	**1.64**
Phosphoprotein enriched in astrocytes 15[G]	*Pea15*	BC085766	0.96	**1.95**	**2.17**
Diacylglycerol kinase, alpha (80 kDa)[G]	*Dgk*	NM_080787	1.28	**1.52**	**1.83**
Glutathione S-transferase omega 1	*Gsto1*	CK228201	1.16	**1.28**	**1.68**

The gene functions as well as the fold change in expression after preconditioning relative to control treatment are indicated. All bold numbers indicate that the fold change was significant. [G]Genes expressed by glial cells.

Table 4
Differentially expressed glial genes in different cell layers

Gene name	Gene symbol	Accession #	Preconditioned/control		
			DGCL	CA1pyr	CA3pyr
Heat shock protein 27	*Hspb1, Hsp27*	BI287717	**88.41**	**68.37**	46.06
Vimentin	*Vim*	AI599037	**26.21**	**29.80**	14.90
Calcyclin	*S100a6*	BQ079150	**2.92**	4.10	1.84
Connexin 43	*Gja1*	AY324140	**1.41**	**1.21**	1.18
CD 81 antigen	*Cd81*	AI013199	**1.35**	1.16	1.31
Glial high affinity glutamate transporter	*Slc1a3*	NM_019225	**1.28**	1.17	1.07
Adenosine kinase	*Adk*	BC081712	**0.72**	1.10	0.90
Interleukin 6 receptor	*Ilr6a*	AI171645	**0.16**	1.48	0.93
Tissue inhibitor of metalloproteinase 2	*Timp2*	AJ409332	**1.60**	1.31	1.13
Secreted acidic cysteine rich glycoprotein	*Sparc*	BC061777	**1.51**	1.75	1.69
Cathepsin D	*Ctsd*	AW917839	**1.30**	1.62	1.16
Tetraspan 2	*Tspan2*	AJ271442	**2.24**	1.66	1.06
Myelin oligodendrocyte glycoprotein	*Mog*	CA510729	**1.49**	1.27	1.18
Mal, T-cell differentiation protein 2	*Mal*	AA800666	**0.36**	0.97	1.05

Note that only the bold numbers represent significant changes.

tially expressed genes within the DGCL grouped into these categories.

Functional categories affected in pyramidal cell layers

For differentially expressed genes within the CA1 pyramidal cell layer, eGOn analysis revealed that the following categories contain significantly more genes than are expected from the CA1 master gene list (genes expressed in CA1 with or without preconditioning): morphogenesis, defense, cytoskeleton, and plasma membrane. The morphogenesis and plasma membrane gene ontology groups contain genes in our signaling or tissue structure categories, indicating that these categories are specifically affected by preconditioning. There were too few differentially expressed genes within the CA3 pyramidal layer to perform a meaningful eGOn analysis. However, we assigned our own gene categories to all differentially expressed genes within the pyramidal cell layers (Fig. 5). The gene categories were regulated to a similar degree as within the DGCL (Chi-squared test on large contingency table, $P>0.05$). The individual transcripts regulated by preconditioning can be accessed in Supplementary Tables 2 (CA1) and 3 (CA3). In summary, we found that in all three main hippocampal cell layers, the major processes affected by preconditioning were signaling, tissue structure and injurious processes.

Table 5
Biological processes of genes with expression changes after preconditioning within the DGCL and the number of genes belonging to each category

Biological processes	Total no. of genes	Individual processes	Total no. of genes	Upregulated	Downregulated
Signaling	147	Signal transduction[i]	62	38	24
		Neuronal activity[i]	59	32	27
		Hormones, growth factors[i]	26	13	13
Tissue structure	81	Cytoskeleton[i]	39	22	17
		Cell adhesion	20	8	12
		Extracellular matrix[i]	22	18	4
Injurious processes	48	Defense (immune genes)[i]	28	20	8
		Stress, protection	15	8	7
		Cell death	5	4	1
Metabolism sugar, lipid, amino acids, cofactor synthesis	49	Metabolism[d] (carbohydrates, amino acids, lipids, cofactors)	26	15	11
		Transporters, transporting molecules[d]	12	5	7
		Mitochondrial genes	11	2	9
Transcription, RNA modification	43	Transcription, RNA modification and turnover	43	23	20
Proliferation	32	Cell proliferation	19	16	3
		Metabolism of nucleotides	13	4	11
Protein synthesis, modification and turnover	29	Golgi, ER	14	9	5
		translation	7	2	5
		Ubiquitin, protease pathways	8	5	3
Miscellaneous	14		14	9	5
Unknown	189		189	98	91

Gene categories labeled with [i] or [d] showed larger [(i)] or smaller [(d)] than expected categorical changes in the eGOn analysis.

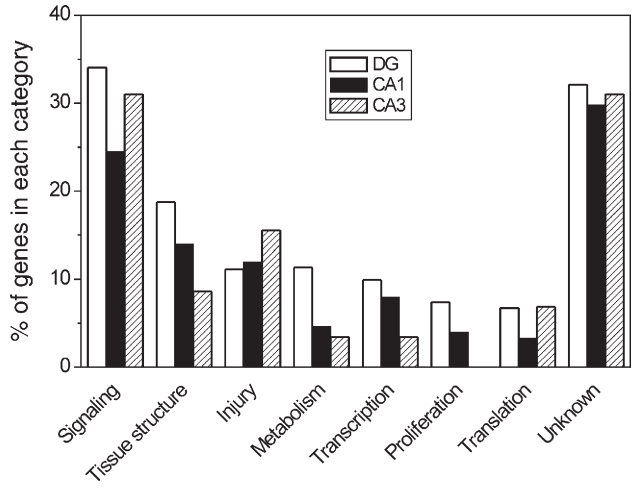

Fig. 5. The number of differentially expressed genes in each biological category, as a percent of the total number of expressed genes in that category, is shown for each of the three hippocampal cell layers. Note that the unknown category for the DGCL contains 2% of genes with miscellaneous function. There are no significant differences between the category representations in the different cell layers.

Gene set analysis reveals layer-specific regulation of energy metabolism genes

GSEA can identify relatively small but concerted changes in expression of functionally related transcripts and thus provides an alternate method of evaluating seizure-induced changes in gene expression. We applied this method to compare the effect of preconditioning on dentate granule and CA1 pyramidal cells. Layer-independent changes in expression (e.g., glial genes, genes in Table 3A) were purged by this analysis, leaving only those pathways and gene sets that mark layer-specific effects. A concerted upregulation of 55 energy metabolism genes in CA1 pyramidal cells relative to dentate granule cells was identified ($P<0.001$), 20 of which are shown in Fig. 6. Each cell in the heatmap represents the relative fold change in expression (red = high, blue = low). A separate analysis confirmed that these genes were upregulated in preconditioned CA1 compared to control CA1, and downregulated in preconditioned dentate vs. control dentate (not shown). Ten of these transcripts (* in Fig. 6) function in the mitochondrial electron transport chain. PYGB (glycogen phosphorylase) catalyzes the rate-limiting step in glycogen degradation, whereas IDH3B (a mitochondrial isocitrate dehydrogenase) is the rate-limiting enzyme of the tricarboxylic acid cycle. A similar comparison of preconditioned CA3 and dentate granule cells yielded similar although less striking results. Interestingly, the brain-specific creatine kinase (symbol CKB) transcript is selectively upregulated in CA3 after preconditioning (149% of control), as compared with either dentate (71%) or CA1 (85%). This enzyme interconverts the high energy phosphate of phosphocreatine and ATP, and supports the idea that at least one of the vulnerable cell layers (CA3) undergoes induction of this energy metabolism gene.

Discussion

The main findings of this study are: (1) preconditioning with 20-min bouts of kainate-induced SE on 2 consecutive days protected the hippocampus, but not all brain areas, from cell death induced by prolonged SE on day 3. (2) Preconditioning changed gene expression in distinct fashions within the three main hippocampal cell layers. The large majority of changes at the mRNA level occurred in the DGCL rather than the CA3 or CA1 pyramidal cell layers, indicating that preconditioning may involve

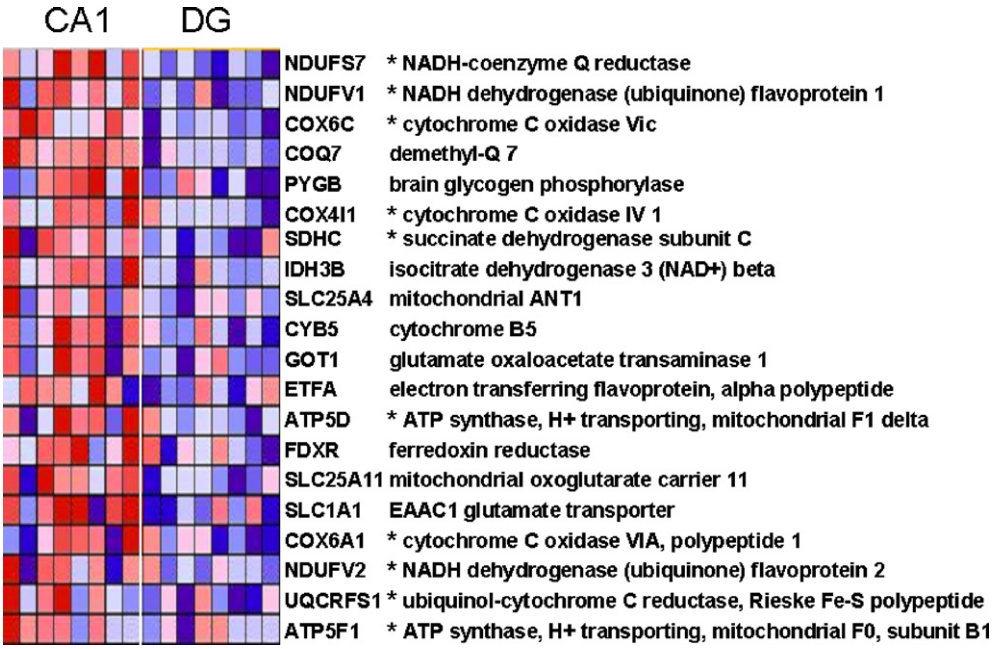

Fig. 6. Selective upregulation of energy metabolism genes in the CA1 pyramidal cell layer, relative to DGCL. Each cell in the heatmap represents the expression level of a gene (row) in a single preconditioned rat (column), normalized to the average expression of that transcript in control rats ($n=9$ controls for CA1 and $n=8$ for DGCL). Deep red reflects overexpression of the transcript in the preconditioned rats, whereas deep blue represents underexpression.

mechanisms other than induction of neuroprotective genes within the vulnerable pyramidal cell layers. The expression level of only 16 transcripts was changed in all three areas examined, indicating that each region responds differently to the preconditioning stimulus. (3) Within the DGCL and the pyramidal layers, preconditioning predominantly affected genes involved in energy metabolism, intracellular and extracellular signal transduction, tissue structure, and injurious processes, including cell stress and death as well as immune genes. Several glial genes were differentially expressed, reflecting the location of both astrocytes and oligodendrocyte precursors or oligodendrocytes within the principal cell layers. These findings are discussed below.

Broad transcriptional response in the dentate gyrus after seizure preconditioning

By far the most extensive changes in gene expression occurred within the DGCL, contrary to our expectation that the CA1 and CA3 pyramidal cells, which undergo extensive degeneration after prolonged SE, would show broader responses to neuroprotective preconditioning. Cells in each of the three harvested layers responded in a highly individual fashion to seizure preconditioning; there were very few differentially expressed genes in common between two or more of the cell layers.

Possible seizure preconditioning mechanisms

The categorical difference in expression of genes involved in energy metabolism after preconditioning seizures – upregulated in CA1 and CA3 pyramidal cells compared to dentate granule cells (Fig. 6) – is interesting given that mitochondrial complex I *deficiency* in the hippocampal CA3 pyramidal cell region is a biochemical hallmark of both human epilepsy (Kunz et al., 2000) and pilocarpine-treated rats (Kudin et al., 2002). This finding raises the possibility that preconditioning causes a transient improvement in energy metabolism in the vulnerable pyramidal cell populations, rendering them more resistant to a prolonged, ATP-depleting, bout of status epilepticus that would otherwise kill these neurons. Glutathione peroxidase was also upregulated in CA1 (Supplementary Table 1), which could neutralize the expected higher levels of reactive oxygen species that accompany elevated electron transport chain activity. Additional study is needed to pursue these proposals.

The unexpected result of large changes in gene expression in the dentate granule cell layer led us to search for alternative explanations for the neuroprotective effect of seizure preconditioning. One possible mechanism is that the preconditioned dentate gyrus may limit seizure spread to the hippocampus proper. The dentate gyrus is well known to dampen excitatory inputs to hippocampus (Stringer and Lothman, 1992; Patton and McNaughton, 1995; Behr et al., 1998), and showed the broadest transcriptional response to seizure preconditioning. If seizures do not invade the CA1 and CA3 pyramidal layers, these neurons are likely to be protected from damage. This suggestion recalls and adds weight to the observation that mossy fiber lesions protect CA3 pyramidal cells from kainate-induced neurodegeneration. The protective effect of mossy fiber lesions is specific for those hippocampal neurons deprived of mossy fiber innervation (Okazaki and Nadler, 1988).

Inspection of the functional categories represented by genes with individual statistically significant expression changes suggests additional potential mechanisms. Several expression changes within the DGCL are expected to be anticonvulsant or promote synaptic inhibition, such as upregulation of NPY and downregulation of adenosine kinase. The upregulation of NPY is well-described after seizures (reviewed by Gall et al., 1990; Vezzani et al., 1999), and expected to reduce glutamate release (Patrylo et al., 1999; Vezzani et al., 2000). NPY has also been proposed to mediate the neuroprotective effect of hypoxic preconditioning (Pohle and Rauca, 1994; Schwarzer et al., 1996). In the preconditioned hippocampus, NPY could limit the spread of ictal activity from the dentate gyrus to the hippocampus proper and thereby contribute to neuronal protection within the pyramidal layers.

Another neurotransmitter system within the signaling category is the GABA system, which showed downregulation of the α5 and δ $GABA_A$ receptor subunit mRNAs, the GABA transporter Scl6a1 (GAT1) and the GABA degrading enzyme 4-aminobutyrate aminotransferase. Downregulation of the latter two genes could result in increased extracellular GABA concentrations that might limit seizure spread within the hippocampal formation. Downregulation of the α5 and δ $GABA_A$ receptor subunit mRNAs was also found in the granule cell layer after kainate-induced SE by *in situ* hybridization (Tsunashima et al., 1997; Nishimura et al., 2005), confirming that expression of those subunits can be regulated by electrical activity. Interestingly the tonic GABA current in dentate granule cells is carried through δ-containing receptors (Stell et al., 2003). The effects of downregulation of the α5 and δ $GABA_A$ receptor subunits remain to be examined.

Preconditioning resulted in glial cell activation, indicated by the upregulation of GFAP, vimentin, calcyclin, and other glial genes. GFAP and vimentin are reported to be upregulated even after short non-injurious seizures (Stringer, 1996). The regulation of some glial genes may play a direct role in altering neurotransmission, e.g., the upregulation of the high affinity glutamate transporter, EAAT1 (Slc1a3, GLAST), and the downregulation of adenosine kinase. A major role of astrocytes is to take up glutamate, and EAAT1 is one of the two principal glial glutamate transporters. EAAT1 was also upregulated in the CA3 region and hilus by kainate-induced SE as judged by *in situ* hybridization (Nonaka et al., 1998). The upregulation of EAAT1 could result in lower extracellular glutamate concentrations. Adenosine kinase is the key regulator of the extracellular level of adenosine, which controls the spread of seizures (Gouder et al., 2004; Fedele et al., 2005). Downregulation of adenosine kinase could lead to a higher extracellular level of adenosine and could therefore limit seizure spread.

It is also likely that the neural circuitry is physically changed by preconditioning as many mRNAs encoding proteins involved in tissue structure were differentially expressed, including cell adhesion and extracellular matrix genes, as well as cytoskeletal genes. For example *GAP-43* is upregulated, a gene known to be involved in neuronal sprouting during development (Skene, 1989; Kruger et al., 1998) and associated with mossy fiber sprouting in rats (e.g., Bendotti et al., 1997; Cantallops and Routtenberg, 1999).

Upregulation of heat shock proteins is common after various stressors and has been implicated in enhancing cell survival. Both Hsp70 and Hsp27 are upregulated after prolonged kainate-induced SE and after certain ischemic preconditioning protocols (Kitagawa et al., 1990; Stenzel-Poore et al., 2003; Nishino and Nowak, 2004). Here, we found strong upregulation of Hsp27 but not Hsp70, suggesting that Hsp70 may not be required for neuroprotection by seizure preconditioning. These data are supported by the finding that not all ischemic preconditioning paradigms induce Hsp70

(Nishino and Nowak, 2004). Hsp27 overexpression in neurons could protect against cell damage induced by kainate-SE (Akbar et al., 2003; Kalwy et al., 2003), but it remains to be determined if Hsp27 is involved in the neuroprotective effect of seizure preconditioning.

The levels of cell proliferation transcripts tended to be coordinately upregulated in the dentate granule cell layer, consistent with seizure-induced proliferative activity in this area. It is likely that preconditioning induces proliferation of dentate neurogenic progenitor cells and astrocytes, as these cells show increased cell proliferation after short (Bengzon et al., 1997) and more prolonged seizures (e.g., Bengzon et al., 1997; Gage et al., 1998; Parent, 2003; Borges et al., 2006).

Seizure vs. ischemic preconditioning

Ischemic preconditioning has been established for many different organs, including brain. Short periods of ischemia/hypoxia can protect brain, including hippocampus, against damage caused by subsequent seizures or ischemia (Kitagawa et al., 1990; Pohle and Rauca, 1994; Emerson et al., 1999). To investigate whether seizure and ischemic preconditioning might involve similar pathways, we compared transcriptional changes after ischemic preconditioning in the cortex (Stenzel-Poore et al., 2003) to those found here after seizure preconditioning in the main hippocampal cell layers. The expression level of 80 assigned genes was changed by ischemic preconditioning according to Stenzel-Poore et al. (2003) and 64 of these genes were found on the rat 230A microarray chip used in our study. However, the levels of only four of these 64 genes, namely GFAP, calcyclin, groucho-related gene 1 and DNA-directed RNA polymerase II, were changed after seizure preconditioning within at least one of the major cell layers in the hippocampal formation. This meager overlap in transcriptional responses suggests that the genetic mechanisms of hippocampal seizure preconditioning and cortical ischemic preconditioning might be different. In contrast to seizure preconditioning, brief ischemia changed the transcript levels of very few tissue structure and extracellular signaling genes. It is likely that different mechanisms underlie preconditioning in different brain regions. Alternatively, multiple pathways may protect the hippocampus against SE-induced damage, because both ischemic and seizure preconditioning can protect this brain area. Taken together, our data suggest that the transcriptional responses underlying hippocampal seizure preconditioning and cortical ischemic preconditioning might be different.

In summary, our data show that preconditioning induces a much broader transcriptional response in the DGCL than in the pyramidal cell layers. The cellular functions of many of the differentially expressed genes are consistent with the notion that seizure preconditioning protects hippocampal pyramidal and dentate hilar neurons by increasing inhibition within the dentate gyrus and thereby limiting seizure spread into the hippocampus proper. Transcriptional changes in astrocytes as well as neurons appear to contribute to this process. Further study is needed to evaluate this hypothesis for the mechanism of preconditioning.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2006.12.001.

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