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MICROARRAY PROFILE OF SEIZURE DAMAGE-REFRACTORY HIPPOCAMPAL CA3 IN A MOUSE MODEL OF EPILEPTIC PRECONDITIONING

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Abstract—A neuroprotected state can be acquired by preconditioning brain with a stimulus that is subthreshold for damage (tolerance). Acquisition of tolerance involves coordinate, bi-directional changes to gene expression levels and the re-programmed phenotype is determined by the preconditioning stimulus. While best studied in ischemic brain there is evidence brief seizures can confer tolerance against prolonged seizures (status epilepticus). Presently, we developed a model of epileptic preconditioning in mice and used microarrays to gain insight into the transcriptional phenotype within the target hippocampus at the time tolerance had been acquired. Epileptic tolerance was induced by an episode of non-damaging seizures in adult C57Bl/6 mice using a systemic injection of kainic acid. Neuron and DNA damage-positive cell counts 24 h after status epilepticus induced by intraamygdala microinjection of kainic acid revealed preconditioning given 24 h prior reduced CA3 neuronal death by ~45% compared with non-tolerant seizure mice. Microarray analysis of over 39,000 transcripts (Affymetrix 430 2.0 chip) from microdissected CA3 subfields was undertaken at the point at which tolerance was acquired. Results revealed a unique profile of small numbers of equivalently up- and down-regulated genes with biological functions that included transport and localization, ubiquitin metabolism, apoptosis and cell cycle control. Select microarray findings were validated post hoc by real-time polymerase chain reaction and Western blotting. The present study defines a paradigm for inducing epileptic preconditioning in mice and first insight into the global transcriptome of the seizure-damage refractory brain.

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Abbreviations: Bok, Bcl-2-related ovarian killer protein; EEG, electroencephalogram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAHFDs, high amplitude high frequency discharges; KA, kainic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; Kif1a, kinesin family member 1A; Mapk8ip, mitogen-activated protein kinase 8 interacting protein; NeuN, neuronal nuclear protein (neuron-specific nuclear protein); PCR, polymerase chain reaction; rtPCR, real-time quantitative polymerase chain reaction; Stard4, StAR-related lipid transfer (START) domain containing 4; TUNEL, terminal deoxynucleotidyl dUTP nick end labeling.

Key words: epilepsy, transcriptome, kainic acid, tolerance, neuroprotection, apoptosis.

Stressful and potentially noxious insults that are subthreshold for damage are capable of rendering brain refractory to damage incurred by a subsequent, prolonged and otherwise harmful stressor (Dirnagl et al., 2003). This process, termed preconditioning, is a highly conserved endogenous mechanism by which brain can protect itself (tolerance) (Chen and Simon, 1997). The molecular mechanisms underlying this process may yield novel and potent neuroprotective strategies to mitigate the harmful effects of neurological insults such as ischemia, traumatic brain injury and prolonged seizures (status epilepticus) (Gidday, 2006).

Tolerance in brain was originally identified as a gene synthesis-dependent process that took 1–3 days to be acquired *in vivo* (Kitagawa et al., 1991; Simon et al., 1993; Chen et al., 1996). The process is highly conserved, being readily elicited in numerous rat and mouse models of ischemic brain injury (Dirnagl et al., 2003; Gidday, 2006; Stenzel-Poore et al., 2007). It may also have clinical relevance as evinced by more favorable outcomes in patients experiencing transient ischemic attacks prior to a large stroke (Weih et al., 1999). Preconditioning can also be induced by other brain insults including seizure (Sasahira et al., 1995; Najm et al., 1998; El Bahh et al., 2001; Borges et al., 2007) and certain chemicals/drugs (Rosenzweig et al., 2004), and cross-tolerance whereby ischemic and other paradigms are combined has also been reported in rodents (Plamondon et al., 1999; Towfighi et al., 1999).

Microarray technology has enabled large-scale understanding of global gene expression changes during complex, multi-factorial pathophysiological processes to be identified in a comprehensive and unbiased manner (Lockhart and Barlow, 2001). Microarray analysis has been applied to better understand the molecular mechanisms underlying the damage-refractory phenotype of the ischemia-preconditioned rodent brain (Stenzel-Poore et al., 2003, 2004, 2007). Preconditioning reprograms the brain's response to ischemia in a stimulus-specific manner via induction of novel pathways not previously implicated in neuroprotection (Stenzel-Poore et al., 2007). The major phenotype of the ischemia-preconditioned brain after prolonged ischemia is down-regulation of genes involved in heavily energy-dependent metabolic processes (Stenzel-Poore et al., 2007).

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Transcriptome commonalities are shared with organisms that hibernate, where prolonged periods of oxygen deprivation are tolerated (Frerichs et al., 1994; Lee and Hallenbeck, 2006).

Microarrays have been used to profile the transcriptome of seizure-damaged rat brain (Hunsberger et al., 2005), and rat brain during epileptogenesis (Elliott et al., 2003; Lukasiuk et al., 2003; Gorter et al., 2006). More recently, the transcriptome of epileptic preconditioning was reported in the major hippocampal subfields in a rat model (Borges et al., 2007) and microarrays have profiled hippocampus after electroshock seizure in rodents (French et al., 2001; Newton et al., 2003; Ploski et al., 2006); a potential preconditioning stimulus. Clinically, hippocampal levels of cell death-regulatory genes from patients with intractable temporal lobe epilepsy may share commonalities with expression profiles elicited by tolerance-conferring seizure paradigms (Shinoda et al., 2004b).

We recently developed a model of status epilepticus-induced CA3-dominant hippocampal injury in mice (Shinoda et al., 2004a). Presently, we sought to determine if seizure-preconditioning can generate a tolerant phenotype in this model, and then profile the transcriptome of the target CA3 at the time tolerance had been acquired.

EXPERIMENTAL PROCEDURES

Mouse model of epileptic preconditioning

Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and European Communities Council Directive (86/609/EEC) and were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, under license from the Department of Health, Dublin, Ireland. All efforts were made to minimize the number of animals used and their suffering.

Mice (9–10 weeks old, C57Bl/6 male, between 20 and 25 g) were obtained from Harlan UK Ltd. (Shaw's Farm, Bicester, Oxon, UK). Seizure preconditioning was produced by i.p. injection of kainic acid (KA) (7.5 or 15 mg/kg in 0.2 mL volume) (Ocean Produce International, Shelburne, Nova Scotia, Canada). Mice were behaviorally observed to verify mild seizure-related behavior had occurred. Control, littermate mice received i.p. injection of saline but were otherwise treated the same.

Induction of focally-evoked limbic status epilepticus by intraamygdala KA

Injurious seizures were produced by intraamygdala KA as previously described with modifications (Araki et al., 2002; Shinoda et al., 2004a). Briefly, 24 h following i.p. injection of KA or saline, mice were anesthetized using isoflurane (3–5%) and maintained normothermic by means of a feedback-controlled heat blanket (Harvard Apparatus, Edenbridge, Kent, UK). A catheter was inserted into the femoral vein for administration of anticonvulsant. Mice were next placed in a stereotaxic frame and following a midline scalp incision three partial craniectomies were performed and mice were affixed with cortical electrodes to record surface electroencephalogram (EEG). Electrodes were placed above dorsal hippocampus and a third across the forebrain area. EEG was recorded using a Grass Comet digital EEG (Medivent Ltd., Lucan, Ireland) with continuous Panorama® digital video. A guide cannula was affixed (coordinates from Bregma: AP=−0.94; L=−2.85 mm) (Franklin and Paxinos, 1997), anesthesia was discontinued and mice were placed in a clear Perspex recording chamber. Video-EEG recordings were commenced and then an injection cannula was lowered 8.7 mm below the dura for injection of KA (1 μg in 0.2 μL volume) into the basolateral amygdala nucleus. Non-seizure control mice received the same volume of intraamygdala vehicle. The duration of high amplitude, high frequency paroxysmal discharges (HAHFDs), which are responsible for damage in this model, was calculated for all groups as previously described (Shinoda et al., 2004a).

Mice were killed 24 h after preconditioning or prolonged seizures by pentobarbital overdose and perfused with saline to remove intravascular blood components. Brains for histology were flash-frozen in 2-methylbutane at −30 °C or microdissected on wet ice for isolation of hippocampal CA3 followed by processing as described below.

Histopathology

Brains were sectioned at 12 μm on a cryostat and stored at −80 °C until further use. Sections at the level of the dorsal hippocampus (−1.7 mm) (Franklin and Paxinos, 1997) were post-fixed (10% formalin), permeabilized, blocked in 5% goat serum and incubated with antibodies against neuronal nuclear protein (NeuN) (Chemicon, Temecula, CA, USA). Sections were washed in phosphate buffer and then incubated in rhodamine-conjugated goat anti-mouse secondary antibodies (Jackson Immunoresearch, Plymouth, PA, USA). Sections were washed again and then processed for detection of double-stranded DNA fragmentation using terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) as previously described (Shinoda et al., 2004b) (Roche Molecular Biochemicals, Indianapolis, IN, USA). Sections were washed again and mounted in 4′,6-diamidino-2-phenylindole (DAPI) –containing medium (Vector Laboratories Ltd., Peterborough, UK) and examined under 60× oil immersion using a Hamamatsu Orca 285 camera attached to a Nikon 2000s epifluorescence microscope (Micro-optica, Ireland) under Ex/Em wavelengths of 330–380/420 nm (blue), 472/520 nm (green) and 540–580/600-660 nm (red). Pseudocolor transforms from monochromatic Hamamatsu Orca 285 images were undertaken using Adobe® Photoshop® 6.0.

Sections were all scored by an observer blinded to experimental treatment. Surviving neurons were counted if retaining normal morphological appearance. Shrunken and condensed NeuN-positive hippocampal neurons were not included. Hippocampal CA3 NeuN and TUNEL counts were the mean of two adjacent sections as previously described (Shinoda et al., 2004a,b).

Hippocampal microdissection

RNA for microarray studies was obtained from the CA3-enriched portion of the hippocampus using a previously described microdissection procedure (Lein et al., 2004). Briefly, under RNase-free conditions the brain was immediately immersed in ice cold RNA later (Ambion Inc., Austin, TX, USA), the cerebellum was dissected away and the two hemispheres were separated. Under a dissecting microscope the hippocampus was separated en bloc from the cortex followed by dissection of the different subfields of the hippocampus. The boundaries between the CA1, DG and CA3 boundary were identified and were separated from each other intact. The CA3-enriched tissue portion was transferred to a tube and immersed in RNA later and place on ice. RNA from both hippocampi from the same control or preconditioned animal was pooled to yield ~5–10 ng RNA. Polymerase chain reaction (PCR) was routinely performed to ensure CA3 samples had minimal contamination of other hippocampal subfields. The marker for the CA3 was Bcl-2-related ovarian killer protein (*Bok*) mRNA and the marker for non-CA3 contamination was the dentate gyrus-en-