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# Expression profiling the microRNA response to epileptic preconditioning identifies miR-184 as a modulator of seizure-induced neuronal death

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ABSTRACT

Brief seizures (epileptic/seizure preconditioning) are capable of activating endogenous protective pathways in the brain which can temporarily generate a damage-refractory state against subsequent and otherwise harmful episodes of prolonged seizures (tolerance). Altered expression of microRNAs, a class of non-coding RNAs that function post-transcriptionally to regulate mRNA translation has recently been implicated in the molecular mechanism of epileptic tolerance. Here we characterized the effect of seizure preconditioning induced by low-dose systemic kainic acid on microRNA expression in the hippocampus of mice. Seizure preconditioning resulted in up-regulation of 25 mature microRNAs in the CA3 subfield of the mouse hippocampus, with the highest levels detected for miR-184. This finding was supported by real time PCR and *in situ* hybridization showing increased neuronal miR-184 levels and a reduction in protein levels of a miR-184 target. Inhibiting miR-184 expression *in vivo* resulted in the emergence of neuronal death after preconditioning seizures and increased seizure-induced neuronal death following status epilepticus in previously preconditioned animals, without altered electrographic seizure durations. The present study suggests miRNA up-regulation after preconditioning may contribute to development of epileptic tolerance and identifies miR-184 as a novel contributor to neuronal survival following both mild and severe seizures.



## Introduction

Exposure of the brain to a stimulus or stressor that is sub-threshold for injury (preconditioning) activates endogenous programs of neuroprotection resulting in a damage-refractory phenotype (tolerance) (Chen and Simon, 1997; Dirnagl et al., 2003; Gidday, 2006). Brain damage caused by prolonged seizures (status epilepticus, SE) can be substantially reduced when preceded by seizure preconditioning, resulting in epileptic tolerance. This has been demonstrated for several preconditioning stimuli including kindling, electroshocks and low doses of chemoconvulsants such as kainic acid (KA) (Jimenez-Mateos and Henshall, 2009).

Although the molecular mechanisms that underlie tolerance are incompletely understood, microarray profiling shows that epileptic tolerance features, like ischemic tolerance (Stenzel-Poore et al., 2003), large-scale down-regulation of protein-coding genes (Jimenez-Mateos et al., 2008). Among the pathways most impacted are those associated with neuronal excitation and excitotoxicity, which may contribute to protection against neuronal death and post-SE spontaneous seizures (Jimenez-Mateos et al., 2008, 2010). Work has since focused on the mechanism of suppressed transcription, identifying contributions from DNA methylation (Miller-Delaney et al., 2012), polycomb group proteins (Stapels et al., 2010) and microRNAs (miRNAs) (Saugstad, 2010).

MiRNAs are a class of small (~22 nt), non-coding RNAs which mediate post-transcriptional regulation of gene expression. They are predicted to regulate protein levels of about half of all protein-coding genes in mammals (Bartel, 2009; Krol et al., 2010), with over 1000 miRNAs now identified in humans and over 700 in mice. MiRNAs mainly mediate their effects via imperfect base-pairing with the 3′ untranslated region (UTR) of their target mRNAs, resulting in mRNA degradation or inhibition of translation (Bartel, 2004; Krol et al., 2010). Their biogenesis begins with transcription from independent genes or from introns of protein-coding genes (Krol et al., 2010). The primary transcript folds into a hairpin structure that is processed by the Drosha microprocessor complex to generate a pre-miRNA. In the cytoplasm, Dicer generates a ~20-bp miRNA/miRNA* duplex and the mature strand is guided to the RNA-induced silencing complex (RISC) where Argonaute (Ago) proteins mediate translational

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repression or mRNA decay (Peters and Meister, 2007). miRNAs are critical for normal brain development and function (Fiore et al., 2008) and loss of miRNA production results in synaptic dysfunction and neuronal apoptosis (Davis et al., 2008; Schaefer et al., 2007; Tao et al., 2011). Dysregulation of miRNAs may contribute to neurologic, psychiatric and neurodegenerative diseases including ischemic and epileptic brain injury (Eacker et al., 2009; Hebert and De Strooper, 2009; Jimenez-Mateos et al., 2011; McKiernan et al., in press; Saugstad, 2010).

Several recent studies reported expression profiles for miRNAs following SE (Hu et al., 2011; Liu et al., 2010b; Pichardo-Casas et al., 2012). MiRNA profiling has also been undertaken for ischemic tolerance (Lusardi et al., 2010), ischemic preconditioning (Dharap and Vemuganti, 2010; Lee et al., 2010; Lusardi et al., 2010) and electroshock (Eacker et al., 2011), which can model seizure preconditioning (Jimenez-Mateos and Henshall, 2009). We recently contrasted the response of mature miRNAs after SE with the profile in tolerant animals that had been exposed to seizure preconditioning before SE, revealing epileptic tolerance was associated with subdued and occasionally bi-directional changes for miRNAs (Jimenez-Mateos et al., 2011). The effect of seizure preconditioning alone has not been reported. Since many protein-coding genes are down-regulated after SE in preconditioned animals we postulated seizure preconditioning would, broadly, increase miRNA expression. Here we profiled the miRNA response to seizure preconditioning and identify miR-184 as contributing to neuronal survival in this model.

## Materials and methods

### *Animal procedures*

Animal experiments were carried out as previously described (Hatazaki et al., 2007) in accordance with the 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. Adult male C57BL/6 mice (20–25 g) were obtained from Harlan (Oxon, Bicester, U.K.) and housed in a vivarium on a 12 h light/dark cycle with access to food and water *ad libitum*.

Seizure preconditioning was induced by a single intraperitoneal (i.p.) injection of KA (15 mg/kg, Ascent Scientific Ltd, Bristol, U.K. in 0.2 ml volume). Control mice received a single i.p. injection of 0.2 ml phosphate buffered saline (PBS) as sham-preconditioning.

For epileptic tolerance, mice were prepared as described (Hatazaki et al., 2007; Jimenez-Mateos et al., 2008). Briefly, mice received seizure preconditioning on day 1 followed by intra-amygdala KA (1 μg in 0.2 μl) 24 h later. For the intra-amygdala injections, mice were anesthetized using isoflurane (5% induction, 1–2% maintenance) and fixed into a stereotaxic frame and maintained normothermic with a heat pad (Harvard Apparatus, Kent, U.K.). Three partial craniectomies were performed for skull-mounted recordings (Bilaney Consultants, Sevenoaks, Kent, U.K.) and craniectomy for fixation of a guide cannula (coordinates from Bregma (Paxinos and Franklin, 2001) [AP = −0.94 mm, L = −2.85 mm]). The assembly was fixed with dental cement and mice were allowed to recover and move freely in a Perspex chamber. Baseline EEG activity (Grass Comet XL lab-based EEG) was recorded and then an internal injection cannula lowered for injection of KA or vehicle into the basolateral amygdala. Lorazepam (6 mg/kg, i.p.) was administered to all animals 40 min later to reduce morbidity and mortality and animals were removed to a warmed recovery chamber.

All mice were euthanized by pentobarbital overdose and perfused with ice-cold saline to remove intravascular blood components. Brains were then either frozen whole or microdissected to obtain CA1, CA3 and DG enriched portions from the hippocampus, or neocortex, as described (Hatazaki et al., 2007).

### *EEG analysis*

Using TWin® software, the duration of high amplitude, high frequency discharges (HAHFDs), was recorded for 40 min after KA administration as described (Jimenez-Mateos et al., 2011). Additional analysis of frequency and amplitude parameters was performed using LabChart Pro v7 software (ADInstruments Ltd, Oxford, U.K.).

### *Antagomir injections*

For intracerebroventricular (i.c.v.) administration of antagomir, mice were affixed with an additional cannula (Coordinates from Bregma: AP = −0.3 mm, L = −1.0 mm, V = −2.0 mm) ipsilateral to the side of KA injection, as described (Jimenez-Mateos et al., 2011). Scrambled or miRNA-184 antagomirs (Exiqon, LNA- and 3′-cholesterol modified oligonucleotides) were infused in a volume of 2 μl artificial cerebrospinal fluid (aCSF) (Harvard Apparatus) and mice underwent seizure-preconditioning or epileptic tolerance, as above.

### *Behavioral analysis*

Behavioral analysis of mice subject to seizure preconditioning was performed using a modified ethogram (Clifford et al., 2000). Briefly, mice were observed continuously for 1 h following i.p. injections and the duration of time spent conducting various behaviors scored for exploring, immobility, grooming, socially interacting and twitching.

### *MiRNA extraction and expression profiling*

The two CA3 subfields from each control or seizure preconditioned animal were combined to generate individual samples ($n = 4$ per group). Total RNA was extracted using a miRNA easy kit (Qiagen, West Sussex, U.K.) as previously described (Jimenez-Mateos et al., 2011). The quality and quantity of RNA yield were determined using a Nanodrop Spectrophotometer (Thermoscientific, Loughborough, U.K.) and RNA concentrations were normalized in nuclease-free water. Reverse transcription of 100 ng of miRNA was carried out using stem-loop Multiplex primer pools (Applied Biosystems), allowing reverse transcription of 48 different miRNAs in each of eight RT pools. The miRNA screen was carried out using a 7900HT Fast Realtime System and TaqMan Low-Density Arrays (TLDA) (TaqMan TLDA MicroRNA Assays v1.0 containing 382 human microRNAs assays; Applied Biosystems). A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). miRNAs were called present when amplified within 35 cycles in 3 out of 4 samples. Data for each miRNA were normalized to the average Ct value for each mouse, as reported (Bray et al., 2009; Jimenez-Mateos et al., 2011). A threshold of ≥1.5 fold was considered either increased or decreased relative to control.

### *Stem-loop reverse transcription and real time qPCR of individual miRNAs*

Reverse transcription of 100 ng of total RNA from hippocampal CA1 or CA3 was carried out for individual qPCRs using the High-Capacity Reverse Transcription Kit (Applied Biosystems). RT specific primers for miR-184 and miR-204 (Applied Biosystems) were used. Individual qPCRs were carried out on the 7900HT Fast Realtime System (Applied Biosystems) using the complementary Taqman miRNA assay probes (Applied Biosystems). The endogenous control, RNU6B, was used for normalization. Potential miR-184 targets were identified using microrna.org as previously described (Sano et al., 2012).

### *Western blotting*

Western blotting was performed as previously described (Hatazaki et al., 2007). Hippocampal subfields from individual mice were