MiR-132 Is Upregulated by Ischemic Preconditioning of Cultured Hippocampal Neurons and Protects them from Subsequent OGD Toxicity

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Abstract We explored the response of a panel of selected microRNAs (miRNAs) in neuroprotection produced by ischemic preconditioning. Hippocampal neuronal cultures were exposed to a 30-min oxygen–glucose deprivation (OGD). In our hands, this duration of OGD does not result in neuronal loss in vitro but significantly reduces neuronal death from a subsequent 'lethal' OGD insult. RT-qPCR was used to determine the expression of 16 miRNAs of interest at 1 and 24-h post-OGD. One miRNA (miR-98) was significantly decreased at 1-h post-OGD. Ten miRNAs (miR-9, miR-21, miR-29b, miR-30e, miR-101a, miR-101b, miR-124a, miR-132, miR-153, miR-204) were increased significantly at 24-h post-OGD. No miRNAs were decreased at 24-h. The increases observed in the 24-h group suggested that these miRNAs might play a role in preconditioning-induced neuroprotection. We selected the widely studied miR-132, a brain enriched, CREB regulated miRNA, to explore its role in simulated ischemic insults. We found that hippocampal neurons transduced with lentiviral vectors expressing miR-132 were protected from OGD and NMDA treatment, but not hydrogen peroxide. These findings add to the growing literature that targeting neuroprotective pathways controlled by miRNAs may represent a therapeutic strategy for the treatment of ischemic brain injury.

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Introduction

MicroRNAs (miRNAs) are potent regulators of gene expression involved in many biological processes. The role of these ~21 nucleotide non-coding RNA molecules in the brain remains an area of intense research efforts. The brain is miRNA-enriched with ~680 annotated miRNAs expressed and suggestions that there may be at least 1,000 (Jung et al. 2002; Berezikov et al. 2006; Bramham and Wells 2007; Hong et al. 2015). Studies have shown that miRNAs are involved in the control of fundamental CNS processes such as development (Nakazawa et al. 2003; Smirnova et al. 2005; Vo et al. 2005; Nakazawa et al. 2008), synaptic plasticity (Semenova et al. 2007; Scott et al. 2012b), cellular senescence (Zhao et al. 2007; Wagner et al. 2008) and endocytosis (Klein et al. 2007; Scott et al. 2012a) (see (Ballestar and Wolffe 2001; Kosik 2006; Bushati and Cohen 2007; Kim et al. 2009). Studies in this area have highlighted several key features of miRNA expression patterns. For example, not only do different neural cells (i.e. neurons versus glia) have different miRNA expression patterns, these patterns change through development (Miska et al. 2004; Smirnova et al. 2005; Kim et al. 2007; Chahrour et al. 2008). MiRNA enrichment within specific sub-cellular compartments of neuronal cells has also been described, hinting at differential effects at the compartmental level (Jung et al. 2002; Bramham and Wells 2007).

Altered miRNA expression has been linked with a variety of brain diseases and injury (Chen et al. 2003; Berezikov et al. 2006; Smith et al. 2011; Hong et al. 2015; Mushtaq et al. 2015; Kim et al. 2015). The role of miRNAs in ischemic brain damage has also been explored. These studies have shown

that blood and brain miRNA expression profiles are altered by ischemia, and that many individual miRNAs can be targeted to improve outcome. Jeyaseelan et al. (2008) reported significant alterations in miRNA expression profiles in brain and blood in rat model of focal cerebral ischemia (FCI) at 24- and 48-h post-insult. Dharap et al. (2009) followed this using a similar focal stroke model and demonstrated that blocking miR-145 expression decreased lesion size. Along similar lines, Yin et al. (2010) have shown that blocking miR-497 expression also leads to reduced lesion size in a mouse FCI model. Liu et al. (2010) compared brain and blood miRNA expression following focal stroke, intracerebral haemorrhage and kainite seizures. Their work suggests that some common miRNAs are altered following each of these injuries while other miRNAs are altered by specific injuries. Lusardi et al. (2010) reported that miR-132 was reduced in a preconditioning (PC) model of focal stroke, and that this was associated with improved outcome following a second more severe insult. Jimenez-Mateos et al. (2011) also recently reported that miR-132 expression was reduced in a model of epilepsy PC and that this too was associated with reduced neuronal damage following a second more severe insult.

In the present study we employed RT-PCR to explore the expression of 16 miRNAs of interest in a protective, PC model of oxygen-glucose deprivation (OGD) in vitro. In contrast to Lusardi et al. and Jimenez-Mateos et al., we found that miR-132 was significantly increased following PC. We generated lentiviral vectors expressing this highly studied, CREB-regulated miRNA and explored its neuroprotective effects in several in vitro models of simulated ischemia.

Experimental Protocols

All animal experiments in this study were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986

Hippocampal Neuronal Culturing and OGD

Neuron-rich hippocampal cultures were prepared from E18 Wistar rat pups (Kelly et al. 2002). At 11-days in vitro, neurons were subjected to OGD of 30–180 min (procedure described in (Kelly et al. 2004a). In brief, hippocampi were dissected from foetal rats and dissociated in Hanks balanced salt solution (HBSS) and Trypsin (2.5 g/l). Following isolation, cells were suspended in serum-free medium composed of Neurobasal (Gibco BRL), B27 supplement (2 %), L-glutamine and antibiotics. Cells were plated down in four-well plates (13-mm diameter wells) coated with poly-D-lysine (0.05 mg/ml) at 75,000 cells in a 50-μl spot. Twenty minutes later, cells were flooded with 450 μl of medium. At 24-h post-plating, neurons were treated with cytosine arabinoside

(5 μM)). After 12-days in vitro, cultures were washed with and then immersed in deoxygenated, glucose-free balanced salt solution (BSSo). Plates were placed in a hypoxia chamber (O_2 tension < 0.02 %) and returned to the incubator at 37 °C for the duration of the experimental procedure. OGD was ended by removing the plates from the hypoxia chamber, replacing BSSo with their own media and placing them back into the incubator. At 1 and 24 h after OGD, cultures were prepared for MTT assay and MAP2 staining to assess cell health ($n = 8$–12 wells per condition). Further cultures were washed with ice cold PBS and prepared for RNA extraction ($n = 8$–12 wells per condition). The ischemic preconditioning effects of 30-min OGD were also explored. In this experiment, hippocampal neurons were subjected to 30-min OGD at 10DIV, allowed to recover for 24-h and then subjected to 90-min OGD. At 24-h (i.e. 48-h after preconditioning), these cells were prepared for MTT assay ($n = 10$–12 wells per condition).

Extraction of miRNA from Brain and Neuronal Cultures

Total RNA (including miRNA) was extracted from hippocampal neuron cultures using the mirVana isolation kit from Ambion (Austin, TX, Lee et al. 2008). Briefly, cell cultures had their media removed and were washed in ice cold diethyl pyrocarbonate (Depc) 0.001 % treated PBS (Depc-PBS) before being exposed to lysis buffer (mirVana) for RNA isolation.

Construction of Lentiviral Vectors Expressing miR-132

MiR-132 expressing lentiviral vectors were produced using previously published approaches (see Scott et al. 2012a for details).

Northern Blotting

Total RNA (2 μg) was mixed with an equal volume of gel loading buffer II (Ambion). Samples were heated for 2 min at 95 °C and run on a denaturing 15 % poly acrylamide gel with the following composition; 8 M UREA (Sigma, St. Louis, MO, USA), 1× TBS (50 mM Tris.HCl and 150 mM NaCl), 15 % Acrylamide (40 % Acrylamide 19:1, Biorad), 0.05 % [v/v] ammonium persulfate (Sigma) and 15 l TEMED (Sigma) in 15 ml nuclease free water. The samples were run at 30–45 mA and stopped when bromophenol blue dye front reached the bottom of the gel. The gel was then bathed for 5 min in a 0.5 g/ml solution of ethidium bromide in 1× Tris/Borate/EDTA (TBE, 89 mM Tris base; 89 mM Borate, 10 mM EDTA, all Sigma). RNA was transferred to an uncharged nylon membrane (Hybond-N, GE Healthcare) by semi-dry technique for 60 min in 0.5× TBE at 400 mA. RNA was UV fixed to membranes at 254–302 nm for 1 min (Geldoc-it, UVP).

DNA oligonucleotides, complementary to mature miR-132 were obtained from MWG biotech. Probes were radio-labelled with 32P (PerkinElmer, Waltham, Ma, USA). Reactions were performed 10 pmol/l, 5 mM r-32P-ATP, 10 % [v/v] kinase buffer (Perkin-elmer), 10 % [v/v] T4 PNK (Ambion) in a 10-l reaction for 1 h at 37 °C. Reactions were stopped with the addition of 1 l EDTA (10 mM).

NMDA and H_2O_2 Toxicity Model

Hippocampal neurons maintained up to 11 days were used for NMDA toxicity experiments. Culture media was removed and 400 μl of conditioned media from parallel cultures was added to each well. NMDA (0.05 mM) or H_2O_2 (100 μM) was added to media for 1 h or 20 min, respectively under normal conditions (37 °C in 5%CO_2). Cells were washed with 1× PBS three times then stocked media returned to wells. Viability of the cultures was assessed after 24 h recovery.

miRNA Real-Time Quantitative PCR

MiRNA was reverse transcribed using Taqman miRNA Reverse Transcription Kit (Applied Biosystems, Leeds, West Yorkshire, UK) and miRNA-specific RT primers (Applied Biosystems). Real-time PCRs were performed in triplicate using miRNA TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and miRNA specific assay kits according to Applied Biosystems protocol. For each condition $n = 12$ wells of hippocampal neurons. The highly conserved RNU6B (Applied Biosystems) small nuclear non-coding RNA was used as an internal loading control.

Results

Characterization of Hippocampal OGD and Preconditioning Model

We exposed cultured hippocampal neurons to 30, 90 and 180-min of OGD at 11-days in vitro (DIV). MTT assays were used initially to assess cell injury at 24-h recovery. The data showed that following 90 and 180-min of OGD there were significant reductions in MTT activity, no reduction in MTT activity was seen following 30 min of OGD (Fig. 1a). Immunohistochemical staining with a MAP-2 antibody was then used to assess neuron viability following 30, 90 and 180-min OGD. There was no statistically significant difference in the number of MAP-2 positive neurons in control cultures and cultures exposed to 30-min OGD at 24-h recovery. There were however, significantly fewer MAP-2 positive neurons following 90 and 180-min OGD compared with control cultures (Fig. 1b, c). We next tested whether cultured neurons could be pre-conditioned (PC) and protected from a severe OGD

(90 m) 24 h later. Neurons subjected to a preconditioning dose of 30-min OGD 24-h prior to a second, otherwise lethal 90-min OGD survived significantly better compared with those subjected to 90-min OGD alone (Fig. 1D).

MicroRNA Expression Following Preconditioning OGD

We selected 16 miRNAs for analysis based on a number of factors including, their expression levels in brain, published and predicted gene targets and unpublished array data from experiments carried-out within the lab. RT-PCR was used to assess changes in miRNA expression following preconditioning with 30-min OGD. At 1-h following this insult only one miRNA, miR-98, was significantly altered (Fig. 2). At 24-h after PC OGD 10 of the 16 miRNAs studied were significantly increased (miR-9, miR-21, miR-29b, miR-30e, miR-101a, miR-101b, miR-124a, miR-132, miR-153, miR-204, Fig. 2). No miRNAs were significantly decreased at 24-h post-OGD.

Lentiviral Mediated miR-132 Expression Is Neuroprotective

We chose to further investigate the potential role of the CREB regulated brain enriched miR-132. Lentiviral vectors expressing miR-132 with EGFP and control vectors expressing EGFP alone transduced ~80 % of hippocampal neurons and the lentiviral-mediated expression of mature miR-132 was confirmed by Northern blot (see Schäbitz et al. 1997, 2000; Nakazawa et al. 2003, 2008; Smirnova et al. 2005; Vo et al. 2005; Scott et al. 2012b). Hippocampal neurons transduced with miR-132 were found to have significantly higher MTT activity (~20 %, $p < 0.001$) than controls following 90-min (Fig. 3a) and 180-min of OGD (~10 %, $p < 0.05$, Fig. 3b). OGD leads to neuronal damage via excitotoxicity (NMDA) and from reactive oxygen species. We this in mind, we examined whether miR-132 could provide protection from reactive oxygen species or excitotoxicity by exposing hippocampal neurons to H_2O_2 or NMDA. Intriguingly, cultured neurons were not protected from H_2O_2-mediated death (Fig. 3c) but survival was significantly increased (~10 % as assessed by MTT assays) in miR-132 transduced neurons following NMDA exposure ($P < 0.001$; Fig. 3d).

Discussion

In the present study, we set out to elucidate the expression of several miRNAs following ischemia in vitro. To this end, we performed OGD on hippocampal neurons to mimic ischemia in vitro. Of the 16 miRNAs explored, 10 were significantly increased at 24-h following PC OGD. The only other significant alteration was a decrease in the expression of miR-98 at 1-h. These data hint that
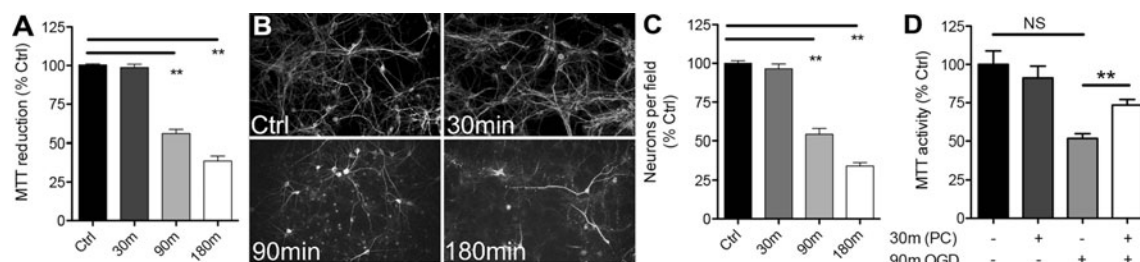
Fig. 1 PC protects neurons from OGD. **a** MTT assay highlighted that there was little change in cell activity between control (no insult) cultures and those subjected to 30 min of OGD (**a**). In contrast there is a marked decrease in activity following 90- and 180-min OGD. **b, c** This correlated with reduced numbers of neurons observed by MAP2 staining. **d** A 30-min OGD was used to stimulate pre-conditioning and protected neurons from a 90-min OGD performed 24 h later (**d**). *Bars* represent a mean of eight independent experiments with one-way ANOVA and Bonferroni post hoc test

increased expression of these miRNAs may be involved in the neuroprotective effects of ischemic PC. To explore this, we selected the widely studied miR-132 and examined its potential protective effects against lethal OGD, NMDA and H_2O_2. MiR-132 was selected for a number of reasons; it has been shown to influence a wide variety of neuronal functions including neurite outgrowth, synapse structure, inflammation and nutritional stress (Kelly et al. 2004b; Gogas 2006; Zhao et al. 2007; Wagner et al. 2008; Strum et al. 2009; Shaked et al. 2009; Edbauer et al. 2010; Magill et al. 2010). Intriguingly, miR-132 is regulated by CREB (Klein et al. 2007; Nudelman et al. 2009; Scott et al. 2012a), which is involved in ischemic PC neuroprotection (Ballestar and Wolffe 2001; Kelly et al. 2002; Glover et al. 2004; Meller et al. 2005; Kosik 2006; Bushati and Cohen 2007; Kim et al. 2009; Lin et al. 2009).

Several studies have attempted to elucidate the role of miRNAs after ischemia. Lusardi et al. (2010) used a 15-min

middle cerebral artery occlusion (MCAo) as a PC insult to induce ischemic tolerance to a subsequent MCAo delivered 24-h later. Lusardi et al. found that miR-132 was decreased at 24 h post MCAo mediated PC. Conversely, we found that miR-132 was upregulated after OGD-PC in vitro at 24 h. An important distinction between the two studies includes our use of neurons ex vivo. This allowed for observation of miRNA responses uniquely seen in the neurons, sans glia and other cell types. Second, major differences in neuron type can be found in the cortex and the hippocampus (from which our tissue was drawn). We also found that miR-132 expression was significantly reduced in a mouse model of ischemia. More recently, Hwang et al. (2014) observed that miR-132 was downregulated in neurons of the CA1 in the hippocampus and this effect was mediated by transcriptional repression through REST (Kelly et al. 2004a; Miska et al. 2004; Smirnova et al. 2005; Kim et al. 2007; Chahrour et al. 2008; Hwang et al. 2014). Crucially, lentiviral-mediated overexpression of miR-132 protected neurons from ischemia both in

Fig. 2 PC-OGD alters miRNA expression profile. MiRNA expression following 30-min OGD was assessed by qRT-PCR at 1 and 24 h after insult. Expression was normalized against the endogenous U6 snRNA then expressed relative to control conditions. *Bars* represent means of three independent experiments, Student's *t* test, *$p < 0.05$; **$p < 0.01$)
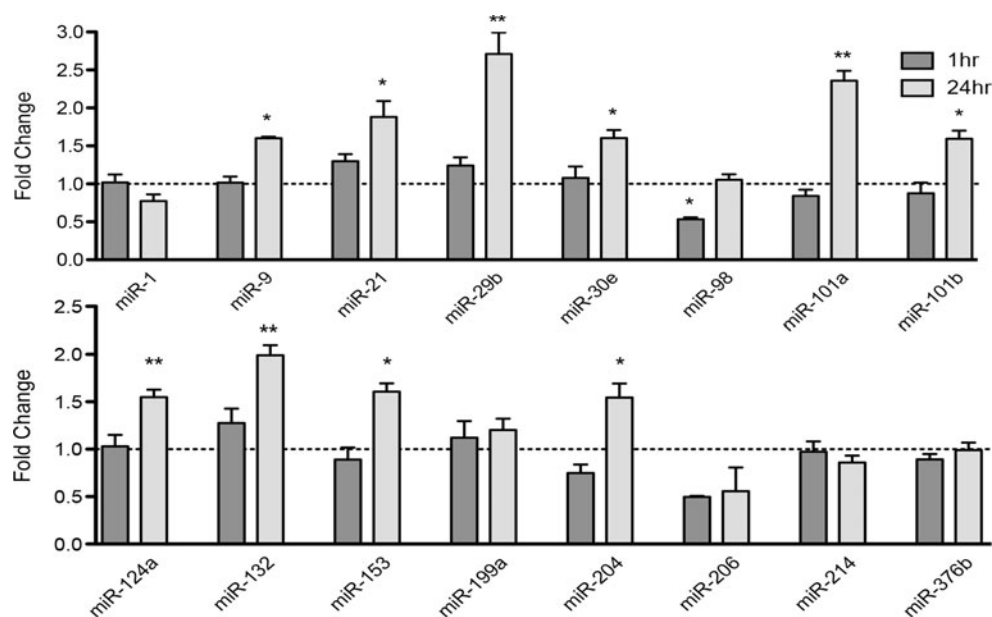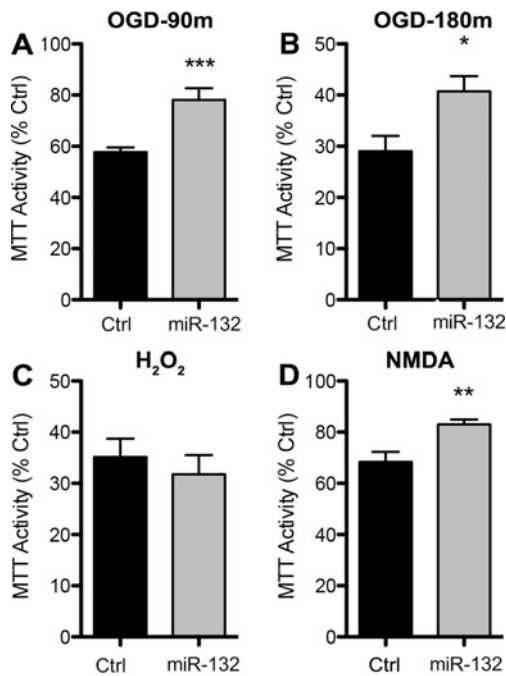
Fig. 3 MiR-132 reduces OGD and NMDA-induced neuronal death. **a** Hippocampal neurons transduced with miR-132-EGFP survived 90-min OGD or 180-min OGD (**b**) significantly better than EGFP neurons (Ctrl). **c** miR-132 did not protect neurons from H_2O_2-mediated death but did increase survival relative to controls following exposure to NMDA (**d**). *Columns* represent mean of 12 observations ± SEM, Student's *t* test with ***$p < 0.001$

vitro. Further, Hong et al. (2015) found that miR-132 delivery to cardiomyocytes reduced intracellular calcium increase and the presence of apoptotic bodies after hypoxic injury through targeting of the Na^+ Ca^{2+} exchanger (NCX1) (Hong et al. 2015).

Following a sub-lethal pre-conditioning OGD stress we found ten miRNAs (miR-9, miR-21, miR-29b, miR-30e, miR-101a, miR-101b, miR-124a, miR-153, miR-204, and the CREB-regulated miR-132) were increased significantly. Since, CREB-regulated miR-132 was observed to increase significantly following preconditioning (mild OGD) we investigated its function further. We found that the lentiviral-mediated expression of miR-132 protected neurons from OGD and NMDA toxicity suggesting that its actions may target elements of the excitotoxic pathway.

Studies have found that the Rho-GTPase, p250GAP, modulates the NMDA receptor (via an interaction with the NR2B subunit) and postsynaptic density-95 (PSD-95) function and that miR-132 suppresses p250GAP expression (Nakazawa et al. 2003, 2008; Vo et al. 2005). Because the NR2B subunit and PSD95 expression is increased following ischemia and are associated with poor outcome it has been suggested that p250GAP may play a key role in cell survival following ischaemic insult. This hypothesis is supported by data showing:

reduced p250GAP expression limits its role as a GTPase-activator of Cdc42 and RhoA hydrolysis; RhoA can mediate excitotoxic cell death via Ca^{2+}-dependent activation of the stress-activated protein kinase, p38α (Semenova et al. 2007). Knock-down of Cdc42 using antisense oligonucleotides attenuates apoptosis in hippocampal neurons via inhibition of c-jun-*N*-terminal kinase 3 (JNK3) cascade (Zhao et al. 2007).

MeCP2 (Methyl CpG binding protein 2) has also been highlighted as a miR-132-regulated gene (Klein et al. 2007). MeCP2 has generally been considered a global transcriptional repressor due to its methyl binding domain and transcriptional repressor domains (Ballestar and Wolffe 2001). A recent important study by Chahrour et al. (2008) however showed that MeCP2 actually activates the transcription of many genes (up to 85 %) as well as repressing transcription of others (Chahrour et al. 2008). Interestingly, MeCP2 expression in the hippocampal CA1 and CA3 fields is moderately upregulated at 24 h after forebrain ischemia with no increase associated with the dentate gyrus (Jung et al. 2002). MeCP2 exerts transcriptional repression of BDNF by binding the promoter region. Transcriptional inhibition is abolished upon membrane stimulation and subsequent phosphorylation (Chen et al. 2003). Hence, over-expression of miR-132 could also lead to protection of neurons via repression of MeCP2 translation and associated reduction in the regulation of BDNF. BDNF was shown to reduce infarct volume resulting from focal cerebral ischemia (Schäbitz et al. 1997, 2000). Moreover, accumulation of BDNF is thought to contribute to the protection provided by preconditioning insults in-vivo against subsequent ischemia (Yanamoto et al. 2000a, b). Together these results suggest that the significant neuroprotective effects associated with increased miR-132 expression reported in this study are due to important activators (p250GAP, Cdc42 and MecP2) of the excitotoxic pathway being targeted and repressed.

In summary, this study demonstrates that hippocampal miRNA expression is altered by mild OGD in vitro. We show that increased miR-132 expression following sublethal OGD may contribute to the protective effects of ischemic preconditioning and that virally driven expression of miR-132 protects neurons from severe OGD and NMDA toxicity. Two confirmed gene targets of miR-132, p250GAP and MeCP2 may underpin the observed protection. Though, several other predicted miR-132 targets including, BIM, GSK3β, cdc42 and Grin2B have been shown to protect neurons from ischemia (Kelly et al. 2004b; Gogas 2006; Zhao et al. 2007). Gaining further insight into the role of individual miRNAs in ischemic pathology may elucidate the mechanisms underlying neuronal cell death and also miRNA based therapies for neurodegenerative disorders.

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