AMPK-regulated miRNA-210-3p is activated during ischaemic neuronal injury and modulates PI3K-p70S6K signalling

Shona Pfeiffer1,2 | Anna Tomašcová1,3 | Uta Mamrak4 | Stefan J. Haunsberger2 | Niamh M. C. Connolly1,2 | Alexa Resler2 | Heiko Düßmann1,2 | Petronela Weisová1 | Elisabeth Jirström1,2,5 | Beatrice D’Orsi1,6 | Gang Chen1 | Mattia Cremona2,7 | Bryan T. Hennessy2,7,8 | Nikolaus Plesnila4,9 | Jochen H. M. Prehn1,2,5

1Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland
2Centre for Systems Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland
3Biomedical Centre Martin, Comenius University in Bratislava, Bratislava, Slovakia
4Institute for Stroke and Dementia Research (ISD), Munich, Germany
5FutureNeuro SFI Research Center, Royal College of Surgeons in Ireland, Dublin, Ireland
6Institute of Neuroscience, Italian National Research Council (CNR), Pisa, Italy
7Dept of Molecular Medicine (Medical Oncology group), Royal College of Surgeons in Ireland, Dublin, Ireland
8Department of Medical Oncology, Beaumont Hospital, Dublin, Ireland
9Munich Cluster of Systems Neurology (Synergy), Munich, Germany

Correspondence
Shona Pfeiffer, Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St. Stephen’s Green, Dublin, Ireland.
Email: shonapfeiffer@rcsi.ie.

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Abstract
Progressive neuronal injury following ischaemic stroke is associated with glutamate-induced depolarization, energetic stress and activation of AMP-activated protein kinase (AMPK). We here identify a molecular signature associated with neuronal AMPK activation, as a critical regulator of cellular response to energetic stress following ischaemia. We report a robust induction of microRNA miR-210-3p both in vitro in primary cortical neurons in response to acute AMPK activation and following ischaemic stroke in vivo. Bioinformatics and reverse phase protein array analysis of neuronal protein expression changes in vivo following administration of a miR-210-3p mimic revealed altered expression of phosphatase and tensin homolog (PTEN), 3-phosphoinositide-dependent protein kinase 1 (PDK1), ribosomal protein S6 kinase (p70S6K) and ribosomal protein S6 (RPS6) signalling in response to increasing miR-210-3p. In vivo, we observed a corresponding reduction in p70S6K activity following ischaemic stroke. Utilizing models of glutamate receptor over-activation in primary neurons, we demonstrated that induction of miR-210-3p was accompanied by sustained suppression of p70S6K activity and that this effect was reversed by miR-210-3p inhibition. Collectively, these results provide new molecular insight into the regulation of cell signalling during ischaemic injury, and suggest a novel mechanism whereby AMPK regulates miR-210-3p to control p70S6K activity in ischaemic stroke and excitotoxic injury.

KEYWORDS
AMPK, ischaemia, microRNA, neuronal injury, stroke

Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; ER, endoplasmic reticulum; FC, fold change; HIF1α, hypoxia-inducible factor 1-α; LNA, locked nucleic acid; miRNA, microRNA; MTI, miRNA-target interactions; NMDA, N-methyl-D-aspartate; OGD, oxygen-glucose deprivation; p70S6K, ribosomal protein S6 kinase; PDK1, 3-phosphoinositide dependent protein kinase 1; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; RPS6, ribosomal Protein S6; RRID, Research Resource Identifier; TF, transcription factor; tMCAO, transient focal cerebral ischaemia; WT, wild type.

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INTRODUCTION

The ischaemic penumbra represents a window for therapeutic intervention to limit the progressive damage and death in the compromised tissue and any potentially detrimental effects of reperfusion contributing to ischaemic injury (Green & Shuaib, 2006; Kalogeris et al., 2012). To effectively utilize this opportunity in the treatment of acute brain injury, the development of neuroprotective agents targeting multiple pathobiological pathways may be required, thereby widening the therapeutic window, improving outcome and facilitating brain recovery (Green, 2004). Cerebral ischaemia triggers a complex series of physiological, biochemical and gene expression changes precipitating the onset of neuronal injury and cell death (Dnrngl et al., 1999). Despite an increasing wealth of knowledge, the heterogeneous nature of the biochemical consequences of ischaemic stroke has resulted in halting progress in the development and translation for neuroprotective agents into the clinical setting (Majid, 2014). To reduce the devastating impact of stroke, there is an urgent need for a greater understanding of the molecular mechanisms mediating neuroprotective and neurotoxic events during acute stroke for novel approaches in the field of neuroprotection and better success in translational efficacy (Dnrngl et al., 1999; Liou et al., 2003).

Progressive neuronal injury in response to ischaemia is associated with a series of biochemical cascades, many of which result from impaired energetics and the collapse of ion gradients, precipitating neuronal cell death (Dnrngl et al., 1999). Glutamate receptor over-activation, in particular N-methyl-D-aspartate (NMDA) receptors, is believed to be a central process in the spreading of neuronal injury during ischaemic stroke. Ischaemia-induced excitotoxic neuronal injury caused by excessive glutamate release and aberrant Ca^{2+} levels have been suggested to play a key role in peri-infarct depolarization, cell death activation, inflammation and oedema formation, all of which may contribute to secondary decompensation within the penumbra (Belov Kirdajova et al., 2020; Dnrngl et al., 1999).

AMP-activated protein kinase (AMPK) is a critical energy sensor and central regulator of energy homeostasis, with the ability to sense compromised availability of cellular energy (Carling, 2004; Hardie et al., 1999; Kahn et al., 2005). Rapid energy depletion in acute stroke results in the activation of AMPK in response to impaired cellular bioenergetics, functioning to maintain energy balance within the cells by promoting processes leading to ATP production and restoration (Marsin et al., 2002; Wu & Wei, 2012), while simultaneously inhibiting anabolic growth-promoting processes, such as protein synthesis, proliferation and cell cycle progression (Carling, 2004; Hardie, 2011; Hardie et al., 2012; Jones et al., 2005; Li & McCullough, 2010). The direct effects of AMPK activation have conflicting implications in neuronal outcome however, paradoxically promoting decisions in cell survival and death signalling (Culmsee et al., 2001; Li & McCullough, 2010). The extent and duration of AMPK activation, in combination with the duration and nature of the metabolic stressor and extent of ATP and bioenergetic recovery, are pivotal in determining the downstream effects and cellular response to insult (Davila et al., 2012). Transient increases in AMPK activity in neurons prior to ischaemic-like challenge have been shown to enhance cell survival, promoting an adaptive, neuroprotective response that reduces the impact of subsequent more severe stimuli, whereas chronic sustained AMPK activation may adversely exacerbate injury (Anilkumar et al., 2013; Culmsee et al., 2001). To this end, elucidation of the molecular signatures associated with AMPK activation, determining cellular fate in the balance of survival signalling and susceptibility towards cell death stimuli, warrants consideration in the setting of ischaemia.

Given the complex nature of the ischaemic cascade, identification of clinically useful biochemical targets for intervention has been challenging and as such, a single target of neuroprotection may be ineffective (Maas & Furie, 2009; Sharp et al., 2011). Endogenous microRNAs (miRNAs) are potent modulators of gene function, regulating the expression of multiple target genes at a post-transcriptional level, with crucial roles as regulators of signalling pathways involved in pathophysiology and progression of ischaemia–reperfusion injury (Dharap et al., 2009; Khoshnam et al., 2017). Furthermore, the rapid induction of an ischaemic miRNA profile can be detected before the induction of protein markers (Lee et al., 2010; Sharp et al., 2011). The dysregulation of miRNA profiles in disease, impacting on and contributing to pathology development and outcome, combined with their ability to regulate multiple genes in similar pathways, leave them uniquely poised as ideal biomarkers and therapeutic targets. Therefore, we aimed to explore AMPK-regulated miRNA as modulators of key downstream pathways in the response to progressive neuronal injury and provide mechanistic insights into the regulation of neuronal fate in ischaemic stroke.

MATERIALS AND METHODS

2.1 | Animals

All animal experiments were carried out under license from the Department of Health and Children (Ireland) and in accordance with the European Communities Council Directive (86/609/EEC). All procedures were reviewed and approved by the RCSI Research Ethics Committee and reported following the ARRIVE guidelines. The study was not pre-registered. Wild type (WT) C57BL/6 mice (Charles River Laboratories; RRID:IMSR_CRL:27) were housed (minimum two, maximum five animals / cage) in a controlled environment with 12 hr light–dark cycles (7a.m.–7p.m.), with standard laboratory animal chow and water ad libitum. Animals were monitored daily as part of standard husbandry and each individual cage checked daily. Mice were used for breeding from 6 to 8 weeks of age and were closely matched for age when paired for breeding. Additional bedding is made available to pregnant females 3–4 days before delivery. A total of 109 adult (7–8 weeks) WT C57BL/6 mice were used (14 for in vitro experiments, 95 for in vivo experiments). Male mice 7- to 8-weeks old (20–24 g) were arbitrarily assigned to sham/experimental groups; no exclusion criteria were pre-determined and no...
randomization was performed. All surgical procedures were carried out in the morning (9:00 ± 1 hr) and post-procedural animals were monitored carefully after surgery for signs of discomfort or adverse effects. All researchers were blinded to experimental groups and analysis was carried out by researchers independent of the experimenter, also blinded to the experimental groups. Timeline of experimental design and analysis points is illustrated in Figure 1.

2.2 | Preparation of primary neurons

Primary murine neocortical neurons were prepared and cultured from WT C57BL/6 mice as described previously (Concannon et al., 2010; Pfeiffer et al., 2014). Cells were plated on poly-d-lysine-coated plates at 2 × 10^5 cells/cm² and maintained at 37°C in a humidified atmosphere of 5% CO₂. All in vitro experiments were carried out on mature untreated cultures or cultures pre-treated with 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Cell Signalling; 9944) to stimulate AMPK activation. ER stress was induced by exposing neurons to tunicamycin (3 μM) or thapsigargin (1 μM) (Sigma Aldrich; T9033, T7765) for various time points.

2.3 | Plasmids and transfection

Primary cortical neurons (DIV 6) were transfected using NeuroMag™ Transfection Reagent (OZ Biosciences; NM50500) as per manufacturer’s instructions. For inhibition or over-expression of miR-210-3p, neurons were transfected with 5’FAM-labelled locked nucleic acid (LNA) probes containing either a sequence-specific antisense miR-CURY LNA Power Inhibitor targeting miR-210-3p; a miR-CURY LNA miR-210-3p mimic or non-targeting negative control (Exiqon). Cells were used for experiments 48 hr after transfection.

2.4 | NMDA toxicity

Primary neurons cultured at DIV 8–9 were sham-treated or subjected to excitotoxic injury induced by NMDA receptor over-activation (D’Orsi et al., 2012). Cells were exposed to NMDA/glycine (100 μM/10 μM) for 5 min (Sigma Aldrich; M3262, G8790), washed twice in experimental buffer containing (in mM): 120 NaCl, 3.5 KCl, 0.4 KH₂PO₄, 5 NaHCO₃, 20 HEPES, 1.2 Na₂SO₄, 1.2 CaCl₂ and 15 glucose, pH 7.4, supplemented with high Mg²⁺ (1.2 mM) and returned to preconditioned media.

2.5 | Oxygen–glucose deprivation (OGD)

Healthy cortical neurons at DIV 8–9 were transferred to a hypoxic chamber (COY Lab Products) with an atmosphere comprising 1.5% O₂, 5% CO₂ and 85% N₂, maintained at 35°C. Feeding medium was removed from cultures and replaced with pre-equilibrated, deoxygenated OGD medium consisting of (mM): 0.3 CaCl₂, 70 NaCl, 5.25 NaHCO₃, 70 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 10 sucrose, pH 6.8. After 45 min of OGD, cultures were returned to oxygenated
feeding medium and allowed to recover for 24 hr under normoxic conditions (21% O$_2$, 5% CO$_2$). Sham-treated cultures were maintained under normoxic conditions (Pfeiffer et al., 2014; Rytter et al., 2003).

### 2.6 MicroRNA array profiling

miRNA expression profiling was carried out by Exiqon Services, Denmark using the miRCURY LNA Array platform under standard operating procedures (Supplementary methods). After normalization, fold change (FC) values and $p$-values of expression changes were calculated using the Limma package in R/Bioconductor. Statistical significance was determined using two-way ANOVA, post hoc Tukey’s test; $p$-values were adjusted for multiple comparisons with Benjamini–Hochberg correction. Results were subjected to unsupervised hierarchical clustering and differentially expressed miRNAs identified through volcano plot filtering.

### 2.7 Reverse transcription and real-time quantitative PCR

Total RNA enriched with miRNAs was isolated using miRNeasy kits (Qiagen, 217004) according to manufacturer’s instructions. RNA concentration and purity were assessed by Nanodrop 2000 spectrophotometer. Validation of selected miRNAs of interest was performed with 200 ng total RNA using TaqMan miRNA Reverse Transcription kits (Applied Biosystems, 4366596) with miRNA-specific TaqMan primers for hsa-miR-210-3p (ID_000512) and hsa-miR-124 (ID_002197) (Applied Biosystems; 4427975). U6 (ID_001973) was used for miRNA normalization (Jimenez-Mateos et al., 2011). cDNA for gene expression was generated using SuperScript VILO cDNA Synthesis kit (ThermoFisher Scientific, 11754050) and amplified using specific TaqMan Gene Expression Assays Rps6kb1, Mm01310033_m1; Actb, Mm00607939_s1 (Applied Biosystems, 4331182). Quantitative real-time PCR (qPCR) was performed using TaqMan Universal PCR Master Mix according to manufacturer’s instructions (Applied Biosystems, 4324018) and cycled using the StepOnePlus Real-Time PCR System (Applied Biosystems; RRID:SCR_015805). Cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All PCR amplification reactions were carried out in triplicate using specific TaqMan assays. A negative control was included for each assay.

### 2.8 Digital PCR

Digital PCR (dPCR) amplifications were carried out to quantify miRNA copy number using the QuantStudio™ 3D Digital System (Applied Biosystems, A291544). Briefly, total RNA was reverse transcribed using TaqMan miRNA-specific RT primers as described for miRNA RT-qPCR. An input volume of 2.3 µl RT product diluted 1:10 was amplified in a total reaction volume of 14.5 µl containing QuantStudio™ 3D Digital PCR Master Mix (Applied Biosystems, A26358) and miRNA-specific TaqMan assay and loaded onto a 20,000 nanoscale reaction well QuantStudio™ 3D Digital PCR 20K Chip (Applied Biosystems, A26316). Chips were cycled on the ProFlex 2X Flat PCR System (Applied Biosystems, 4484078) as follows: 96°C for 10 min followed by 39 cycles of 60°C for 2 min and 98°C for 30 s, and 60°C for 2 min. Chips were read and analysed on the QuantStudio™ 3D Digital PCR instrument to obtain the number of wells with an amplified target (FAM positive) and number of empty wells (FAM negative) and raw data were analysed using QuantStudio™ 3D AnalysisSuite to determine miRNA copies/µl.

### 2.9 Protein extraction and western blotting

Tissue and cell pellets were lysed in ice-cold lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate) to obtain whole lysates. Protein concentration was determined with micro BCA (bicinchoninic acid) assay (Thermo Scientific, 23225). Equal amounts of protein were supplemented with Laemmli buffer, denatured at 95°C for 5 min and separated on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and blocked in 5% non-fat dry milk in Tris-buffered saline (15 mM Tris-HCl, pH 7.5, 200 mM NaCl) containing 0.05% Tween 20 (TBS-T) with for 1 hr at room temperature (RT, 20-22°C). Membranes were then probed with primary antibodies in 5% milk/dH2O overnight: p70S6K (Thr389) (1:1000; Cell Signaling Technology, Cat. #9205, RRID:AB_90456) horseradish peroxidase-conjugated (HRP) secondary antibodies in 5% milk/dH2O overnight; p70S6K (Thr389) (1:1000; Cell Signaling Technology, Cat. #9205, RRID:AB_90456) horseradish peroxidase-conjugated (HRP) secondary antibodies in 5% milk/dH2O overnight; p70S6K (Thr389) (1:1000; Cell Signaling Technology, Cat. #9205, RRID:AB_90456) horseradish peroxidase-conjugated (HRP) secondary antibodies in 5% milk/dH2O overnight; p70S6K (Thr389) (1:1000; Cell Signaling Technology, Cat. #9205, RRID:AB_90456) horseradish peroxidase-conjugated (HRP) secondary antibodies in 5% milk/dH2O overnight. Membranes were washed and incubated with rabbit (1:5000; Millipore, Cat. #AP124P, RRID:AB_90264) or mouse (1:5000; Millipore, Cat. #AP124P, RRID:AB_90264) horseradish peroxidase-conjugated (HRP) secondary antibodies in 5% milk/dH2O overnight and 70% air throughout the procedure. Densitometry was performed using ImageJ and normalized to actin or tubulin.

### 2.10 Focal cerebral ischaemia model

Induction of transient focal cerebral ischaemia (tMCAO) was performed in 75 male C57BL/6 mice (20–24 g) aged 7–8 weeks using the monofilament suture method as described (Culmsee et al., 2005; Plesnila et al., 2004). Briefly, mice were anaesthetized with 5% isoflurane, 30% O$_2$ and 70% air and maintained with 2% isoflurane, 30% O$_2$ and 70% air throughout the procedure. Isoflurane inhalation anaesthesia was employed because of its rapid induction and recovery time, greater safety, lesser cardiovascular depression and steady maintenance of anaesthetic depth.
Body temperature was maintained normothermic at 36.8-37.4°C via a feedback-controlled heat blanket. A silicone-coated monofilament (701956PK5Re; Doccol Corporation, USA) was introduced into the left internal carotid artery and advanced past the carotid artery bifurcation to occlude the middle cerebral artery. After 60 min the suture was removed to allow reperfusion. To control for effects of occlusion, sham-treated mice underwent the same surgical procedure, but the filament was not advanced to occlude the vessel. Effective occlusion and assessment of microcirculatory function was monitored by LDF (Laser Doppler Flowmetry) with a probe fixed to the exposed left parietal skull for continuous monitoring of regional cerebral blood flow (Perimed 5001 Master, Perimed) (Table S1). Mice were killed via cervical dislocation at various time points after reperfusion and brains processed for analysis.

**2.11 | Determination of neurological deficit and ischemic infarct volume**

Infarct volume, neurological deficit and hemispheric swelling were assessed 24 hr after ischemia. Neurological deficit scores were assessed 1 hr post-MCAO and 24 hr following reperfusion on a four-point scale (0 = no deficit; 1 = weakness of the contralateral forepaw; 2 = circling; 3 = loss of righting reflex; 4 = no motor activity) (Plesenila et al., 2001). Coronal sections (10 μm) from each brain were cut by CryoStar NX70 Cryostat (Thermo Scientific). Sections (750 μm intervals) were stained with Cresyl violet/Nissl, and infarct area and hemispherical swelling were evaluated quantitatively using an image analysis system (Axiovision 4.8, Zeiss).

Hemisphere and ischemic infarct volumes (mm$^3$) were calculated by dividing the total volume of ipsilateral hemisphere by the total volume of contralateral hemisphere and expressed as the percentage of contralateral hemisphere to correct for differences in the brain size and oedema.

**2.12 | In situ hybridization**

Following tMCAO, mice were anaesthetized with pentobarbital sodium (200 mg/ml Dolethal) and perfused with ice-cold paraformaldehyde (PFA, 4%) and 12 μm thick sections cut by cryostat (Leica, Germany) and mounted on SuperFrost-Plus slides (VWR, MNJ-700010N). In situ hybridization was carried out as previously described (Jimenez-Mateos et al., 2012). Signals for miR-210-3p, non-targeting negative control or U6 were localized in sections using 5' and 3'-digoxigenin (DIG)-labelled miRCURY LNA detection probes (Exiqon, 612239-360) and secondary detection of probe binding was detected using polyclonal anti-DIG antibody (Anti-DIG-AP, Fab fragment 1:1000; Roche, Cat. # 11093274910, RRID:AB_2313640). Tyramide Signal Amplification (TSA) Plus Fluorescence kit (Perkin Elmer, NEL741001KT) was used to amplify probe-target miRNA labelling and detect fluorescent signals.

**2.13 | Imaging of whole-brain sections**

Imaging was performed in a tile imaging mode using an inverted microscope equipped with a 20X 0.5NA objective and hardware autofocus (AxioObserver) with motorized stage (Prior, UK), a scientific CMOS camera (Orca Flash 4, Hamamatsu, UK) and controlled by MetaMorph software (Molecular Devices, UK). Images were taken with 10% overlap, background corrected and stitched using the Fiji/ImageJ software (Wayne Rasband, NIH) with the stitching plugin written by Stephan Preibisch (Preibisch et al., 2009).

**2.14 | In vivo LNA oligonucleotide delivery**

To over-express miR-210-3p we employed a synthetic miRCURY LNA miRNA-210-3p mimic or non-targeting negative control sequence (Exiqon). Twenty WT C57BL/6 mice were anaesthetized with isoflurane and received an infusion of 0.5, 5 or 50 pmol LNA miRNA-210-3p mimic or non-targeting negative control sequence complexed in Invivofectamine 2.0 (Life technologies, 1377501) or vehicle alone (Invivofectamine). Two microlitres of complex was delivered by intracerebroventricular (i.c.v.) injection. Coordinates from Bregma were anteroposterior, −1.0 mm; mediolateral = −1.0 mm; and dorsoventral = −3.0 mm. Dosing studies were carried out by a researcher blinded to the treatment groups and analysed by an independent researcher also blinded to the treatment groups.

**2.15 | Reverse Phase Protein Array (RPPA) analysis**

WT C57BL/6 mice were killed 4 hr following in vivo i.c.v. infusion of 0.5, 5 or 50 pmol miRCURY LNA miRNA-210-3p mimic oligonucleotide, non-targeting negative control or vehicle (Invivofectamine) and brain tissue processed for protein expression analysis (Figure 5a). Protein was extracted and RPPA performed as previously described (Hennessy et al., 2010). Briefly, lysates were two-fold serial diluted and arrayed on nitrocellulose-coated slides and each slide was probed with a single primary antibody using the Catalysed Signal Amplification (CSA) System (DAKO, USA, K1500) and visualized by DAB colorimetric reaction using biotin-conjugated secondary antibodies. Slides were scanned on a flatbed scanner to produce 16-bit TIFF images. Spots from TIFF images were identified and the density was quantified using MicroVigene software (VigeneTech) to obtain spot signal intensities. All data points were normalized for protein loading and transformed to linear values. Repeated measures ANOVA was used to test whether mean protein levels independently differed by treatment. Protein levels were treated as continuous linear variables and treatment was treated as a categorical variable, with the following categories: Invivofectamine control;
non-targeting negative control (50 pmol); miR-210-3p mimic (0.5, 5, 50 pmol). Statistical significance was determined using false discovery rate (FDR) p-values to correct for multiple comparisons based on p-values from repeated measures ANOVA F tests. Tukey’s honest significant difference test was used as a post hoc test to examine whether there were differences in mean protein levels between treatment categories. Mean protein levels within each treatment category and 95% confidence intervals were computed using the Loftus and Masson (1994) method.

2.16 | Pathway analyses and bioinformatics

After verifying that the sequence of miR-210-3p is conserved across human (hsa), mouse (mmu) and rat (rno) (miRbase V22) (Kozomara et al., 2019), mRNA targets of miR-210-3p were extracted. Predicted targets of hsa-miR-210-3p were identified from miRDIP V4.1 (Tokar et al., 2018) and experimentally validated targets of miR-21-3p0 (hsa, mmu, rno) were identified from miTarBase V7.0 (Chou et al., 2018) and TarBase V8 (Karagkouni et al., 2018). To identify high confidence interactions, we retained only those either predicted with Very High (top 1% of interactions) or High (top 5%, excluding top 1%) scores, or validated with strong experimental evidence (Western Blot, PCR, Luciferase assay). These 2009 ‘high confidence’ miRNA-mRNA target interactions are listed in Table S2. Reactome pathway enrichment analysis was performed and visualized using the ReactomePA package (Yu & He, 2016). An adjusted p-value (Benjamini–Hochberg) of 0.05 was considered significant. These analyses were performed in RStudio (Version 3.6.3 (Team 2018)) utilizing the htr, dplyr, stringr, tidyr, plyr, (Wickham, 2011) and clusterProfiler (Yu et al., 2012) packages. An R markdown file is available at https://github.com/niamhconno/Pfeiffer-et-al-2020. Transcription factors and their targets were identified using the manually curated databases TRRUST V2.0 (https://www.gnavpedia.org/trrust/) and TRANSFAC (http://amp.pharm.mssm.edu/Harmonizome/dataset/TRANSFAC+Curated+Transcription+Factor+Targets), and the high-throughput ENCODE (http://amp.pharm.mssm.edu/Harmonizome/dataset/ENCODE+Transcription+Factor+Targets) and ChEA (http://amp.pharm.mssm.edu/Harmonizome/dataset/ChEA+Transcription+Factor+Targets).

2.17 | Statistical analysis

Statistics were carried out on IBM SPSS 25.0 software (IBM). No statistical method was employed for exploratory in vivo dosing studies; sample size was determined based on previous experience of the group to minimize the number of animals required (Pfeiffer et al., 2015; Plesnila et al., 2001). No animals were excluded from analysis. No test for outliers was conducted. Normality was confirmed by the Shapiro–Wilk test for each individual dataset and normally distributed data were analysed by analysis of variance (ANOVA) and post hoc test or Student’s t-test; please refer to figure legends for statistical tests used. p-values <0.05 were considered to be statistically significant; p-values were adjusted for multiple comparisons with Benjamini–Hochberg correction. Data are presented as mean ± SEM unless otherwise indicated.

3 | RESULTS

3.1 | Activation of AMPK with AICAR shows robust induction of miR-210-3p

We have previously demonstrated robust activation of AMPK (Thr172) following AICAR (2.5 mM) treatment in primary neurons (Concannon et al., 2010; Davila et al., 2012; Weisova et al., 2009, 2011). Similarly, rapid and sustained increases in AMPK (Thr172) activity occur in response to ischaemia in models of tMCAO over 24 hr following reperfusion (McCullough et al., 2005; Venna et al., 2012). To examine the molecular mediators of the effects of neuronal AMPK activation, we performed miRNA array profiling using miR-CURY LNA microRNA array on primary cortical neurons treated with 2.5 mM AICAR to activate AMPK and identified a profile of AMPK-regulated miRNAs. We observed robust induction of miR-210-3p (miR-210) (log2(FC) 0.38; adj. p = 4.85E-06), increased expression of miR-3103-5p (log2(FC) 0.199, adj. p = 4.94E-02) and a reduction of miR-124-5p (miR-124) (log2(FC) −0.275, adj. p = 1.17E-03) in neurons treated with 2.5mM AICAR for 1 hr compared to vehicle control (Figure 2a). Quantitative real-time and digital PCR validation of differentially expressed miRNAs subsequently identified log fold change expression of miR-124 (log2(FC) −0.29, p = .51) (Figure S1a) and miR-210-3p in primary neurons, confirming significantly elevated levels of miR-210-3p in response to AMPK activation with 2.5 mM AICAR for 1 hr (988.6 ± 66.3 copies /µl) compared to vehicle-treated controls (420.9 ± 26.9 copies /µl, p =.001) (Figure 2b and c).

3.2 | miR-210-3p is up-regulated in the cortex following transient focal ischaemia with reperfusion

Next, we examined the expression of differentially expressed miRNAs in vivo in the cortex of WT mice following 60 min tMCAO with reperfusion. Levels of miR-124 were detectable by PCR following 60 min tMCAO with reperfusion but no significant difference was observed in expression levels at 3 hr (log2FC −0.19, p =.53) and 24 hr (log2(FC) 0.1, p =.98) compared with sham-treated controls (Figure S1b). Quantitative real-time and digital PCR confirmed a robust induction of miR-210-3p following ischaemic stroke in vivo at 24 hr (2.597.4 ± 92.9 copies /µl) compared to 3 hr (1951.8 ± 106.9 copies /µl) reperfusion and sham control (1816.2 ± 62.6 copies /µl) (Figure 3a). This substantial increase of miR-210-3p was observed 24 hr following 60 min tMCAO, at which time the establishment of ischaemic infarct is clearly evident (Figure 3b and c). In situ hybridization performed on sections following tMCAO confirmed induction
of miR-210-3p in the cortex at 3 hr following focal cerebral ischemia with reperfusion, with increased expression detected at 24 hr (Figure 3d to h).

### 3.3 Identification of miR-210-3p targets and pathway enrichment analysis

To elucidate putative molecular mechanisms of miR-210-3p induction, we assessed miRNA-target interactions (MTIs) of miR-210 (miR-210-3p is the mature sequence of miR-210 (Kozomara et al., 2019)). We identified 2009 high confidence predicted and experimentally validated miR-210-3p mRNA targets (Table S2). Of these, miR-210-3p is predicted to target \( \textit{PRKAA1} \) (AMPK catalytic subunit \( \alpha_1 \)), representing a potential feedback mechanism in the regulation of AMPK activity. Of particular interest, \( \textit{PDPK1} \) (3-phosphoinositide dependent protein kinase 1; PDK1) is both a validated (Li et al., 2017) and predicted target of miR-210-3p. PDK1 is known to phosphorylate ribosomal protein S6 kinase (p70S6K) at Thr389/412 as part of the phosphatidylinositol 3-kinase (PI3K) pathway (Balendran et al., 1999; Templeton, 2001). miR-210-3p is also predicted to target PI3K catalytic subunits \( \textit{PIK3CG} \) (Class I) and \( \textit{PIK3C2A} \) (Class II) and regulatory subunits \( \textit{PIK3R5}, \textit{PIK3R1} \) (Class I), with important signalling roles in the control of cellular growth and survival, autophagy and cell cycle progression (Vanhaesebroeck et al., 2012). Also within the PI3K signalling pathway, miR-210-3p is predicted to target \( \textit{PTEN} \) (phosphatidylinositol 3-kinase), a functional antagonist of PI3K activity. Reactome pathway enrichment analysis of these high confidence targets identified 23 significantly enriched Reactome pathways, including several signal transduction networks, such as Receptor Tyrosine Kinases (NRTK) and NOTCH signalling (Figure 4a).

miR-210-3p could alternatively regulate PI3K/PTEN/PDK1 signalling and downstream effectors indirectly, through transcription factor (TF)-mediated signalling. To explore this, we identified TFs targeted by miR-210-3p that themselves target \( \textit{PTEN}, \textit{RPS6KB1} \) (p70S6K), and/or downstream target \( \textit{RPS6} \) (Ribosomal Protein S6; RPS6), as downstream effectors regulating cellular proliferation and growth, contributing critically to cell survival (Iwanami et al., 2009). Of these TFs, 10 are predicted targets of miR-210-3p with very high confidence (Table S3, Figure 4b). Such analyses reveal the complexity of miRNA downstream signalling and highlight multiple mechanisms whereby miR-210-3p could regulate cell growth and active translation through PI3K signalling, mediating a central role in cell survival.

### 3.4 Increased miR-210-3p expression alters p70S6K signalling in vivo

To further investigate the mechanistic involvement of miR-210-3p in these critical signalling pathways and validate MTI predictions, we employed RPPA to examine the effects of increased miR-210-3p...
on expression levels of target proteins in vivo (Figure 5a). Dose-dependent increase of miR-210-3p expression in brain tissue was confirmed by digital PCR 4 hr following i.c.v. administration of a LNA miR-210-3p mimic at 0.5 pmol (1895.18 ± 565.1 copies/µl), 5 pmol (8,714.4 ± 4,646.6 copies/µl) and 50 pmol (34,392.7 ± 10,757.8 copies/µl) compared to non-targeting control (545.1 ± 113.3 copies/µl) and vehicle (605 ± 121.8 copies/µl) controls (Figure 5b). PDK1 demonstrated a significant reduction following 0.5 pmol miR-210-3p (mean (95% CI), 4.23 (2.56–5.91)) compared to vehicle control (8.19 (6.51–9.86), $p = .04$ repeated measures ANOVA, post
As suggested by bioinformatics analysis, increasing doses of LNA miR-210-3p mimic resulted in reduced PTEN expression (ANOVA F, \( p = 0.001 \); adj. \( p = 0.036 \)), (Figure 5d). Of note, reduced protein levels of p70S6K1 (ANOVA F, \( p = 0.0001 \); adj. \( p = 0.006 \)), (Figure 5e) and target protein RPS6 at both Ser240/244 (ANOVA F, \( p = 0.019 \); adj. \( p = 0.167 \)), (Figure 5f) and Ser235/236 (ANOVA F, \( p = 0.0002 \); adj. \( p = 0.006 \)), (Figure 5g) were observed in response to increasing miR-210-3p. Analysis of protein expression changes in WT mice after 60 min tMCAO demonstrated a rapid and sustained decrease in p70S6K (Thr389) activity at 3 and 24 hr reperfusion, concomitant with our observed increase in miR-210-3p following ischaemia in vivo and down-regulation of p70S6K in response to increased miR-210-3p (Figure 6a).

### 3.5 miR-210-3p modulates p70S6K activity in response to NMDA-mediated excitotoxicity

To elucidate the potential role played by miR-210-3p during ischaemic stroke in a more controlled environment, we examined miR-210-3p activity in response to excitotoxic neuronal injury associated with ischaemic insult in primary neurons. Following our identification of potential targeting of p70S6K, supported by the down-regulation of its downstream target RPS6, we confirmed a rapid and progressive decrease of p70S6K (Thr389) in vitro in primary cortical neurons 2–24 hr following NMDA receptor over-activation (Figure 6b). Analysis of miR-210-3p expression identified a robust increase of miR-210-3p (1,190.9 ± 75.2 copies/µl) 2 hr following NMDA-induced excitotoxicity compared to control (516 ± 39.1 copies/µl); no increases were observed in miR-210-3p in tunicamycin- (622.2 ± 28.9 copies/µl) and thapsigargin- induced (488.4 ± 8.9 copies/µl) models of endoplasmic reticulum (ER) stress (Figure 6c–e).

To further examine the effects of miR-210-3p modulation on p70S6K activity, primary cortical neurons were transfected with synthetic LNA miR-210-3p mimic (5 nM), power inhibitor (10 nM), non-targeting negative control (10 nM) or vehicle for 48 hr and subsequently exposed to NMDA-mediated excitotoxic injury or conditions of OGD, followed by 24 hr recovery. TaqMan miRNA assays confirmed significant up-regulation of miR-210-3p expression following transfection with 5 and 50 nM with synthetic LNA miR-210-3p mimic (log2(FC) 7.44, 10.19); no evidence of changes in miR-210-3p expression in neurons treated with non-targeting control was observed. A decrease in miR-210-3p was observed in neurons treated with low-dose miR-210-3p inhibitor (log2(FC) -2.23) (Figure S2). Analysis of p70S6K (Thr389) expression revealed a significant attenuation of p70S6K (Thr389), and higher molecular weight identical nuclear p85S6K (Thr412) isoform, in response to miR-210-3p over-expression and NMDA-mediated excitotoxicity. This effect was reversed in inhibitor-treated cultures.
FIGURE 5 miR-210-3p manipulation alters neuronal PI3K-p70S6K signalling in vivo. WT mice were killed 4 hr following i.c.v. administration of increasing doses of LNA miR-210-3p mimic (0.5, 5, 50 pmol), non-targeting negative control (Ctrl, 50 pmol) or vehicle and brains processed for RNA and protein expression analysis. (a) Schematic overview of RPPA workflow. (b) Dose–response validation of miR-210-3p expression by digital PCR. Data presented as mean ± SEM, n = 4 for each group, *p < .001 compared with non-targeting control (ANOVA, post hoc Tukey’s test). (c–g) Statistically significant differences in mean protein levels by treatment (mean, 95% CI). Statistical significance was determined using FDR p-values with Benjamini–Hochberg correction to correct for multiple comparisons based on p-values from repeated measures ANOVA F tests, post hoc Tukey’s test, n = 4 per group.
subjected to NMDA-receptor over-activation under conditions of miR-210-3p suppression (Figure 6i). Consistent with our previous observations demonstrating down-regulation of p70S6K (Thr389) in response to excitotoxic injury, reduced levels of p70S6K (Thr389) were observed in non-targeting control-treated neurons following NMDA insult. Interestingly, under conditions of OGD, we did not identify a difference in the levels of p70S6K (Thr389) and nuclear p85S6K (Thr412) in non-targeting control-treated cultures and neurons over-expressing miR-210-3p following OGD (Figure 6j).

3.6 | Effect of pre-treatment with exogenous miR-210-3p on focal ischaemic brain injury in vivo

To investigate the potential contributions of miR-210-3p signalling to central nervous system (CNS) ischaemic injury, we examined the effects of pre-treatment with miR-210-3p mimic in a setting of ischaemia–reperfusion in vivo. We observed no attenuation of infarct volume or hemispheric swelling in response to pre-treatment with 1.5 pmol miR-210-3p mimic (Figure 7(a–c)); however, there was a significant improvement in neurological deficit score in the treated group at 24 hr when compared with 1 hr post-MCAO, whereas the control group showed no significant improvement at 24 hr (Figure 7d. Pre-treatment with 0.5 and 5 pmol doses also demonstrated no significant effect on infarct volume, oedema or functional outcome (Figure 7e–h). Nevertheless, analysis of neurological deficit following removal of one animal that displayed stroke-related mortality in the 0.5 pmol treatment group identifies a significant reduction in neurological deficit score in the 0.5 pmol treated group at 24 hr (1.39) when compared with 1 hr post-MCAO (2.0; adj. p = .02). Furthermore, a subgroup analysis revealed a significant treatment effect in the 5 pmol group on hemispheric swelling (125.4%, n = 5; 103.8%, n = 5; p < .0001) and infarct volume (135 ± 6.5 mm³, n = 5; 26.8 ± 12.6 mm³, n = 5; p < .0001) and compared to control-treated group (84.5 ± 6.8 mm³) and 0.5 pmol treated group (88.6 ± 10.1 mm³).

4 | DISCUSSION

Increased levels of neuronal AMPK activation are observed rapidly following ischaemic injury both in vitro and in vivo within the ischaemic penumbra and contralateral hemisphere (Concannon et al., 2010; McCullough et al., 2005; Venna et al., 2012; Weisova et al., 2009, 2011). We identified differential down-regulation of miR-124 and up-regulation of miR-210-3p in response to neuronal AMPK activation, potentially targeting downstream molecular pathways associated with ischaemia. Our validation of miR-210-3p expression demonstrated robust increases of miR-210-3p following AMPK activation with AICAR in primary cortical neurons and in the cortex of WT mice following 60 min tMCAO. In situ signal readily confirmed induction of miR-210-3p in the cortex following ischaemia at the same time points, and we demonstrated a rapid and specific increase in expression in response to NMDA excitotoxicity, as a central mechanism of neuronal injury following ischaemic stroke (Lai et al., 2014). miR-210-3p has previously been shown to be expressed in ischaemic cells and tissues, playing a key role in cellular adaptation to low oxygen environments such as tumourigenesis and ischaemia (Chan & Loscalzo, 2010). However, the mechanistic involvement of miR-210-3p in mediating cellular response to cerebral ischaemia is yet to be understood and studies to date have focused on ischaemic stroke in the subacute phase (Ren et al., 2016; Vijayan & Reddy, 2016; Zeng et al., 2011).

To elucidate the potential involvement of miR-210-3p in the regulation of downstream pathways we carried out pathway enrichment analysis. We report significant over-representation of several signal transduction networks with a pivotal role in neuronal survival, proliferation and differentiation, notably Receptor Tyrosine Kinase (RTK) signalling, with enrichment in signalling by Neurotrophin Receptors (NTRKs) and NTRK1 (TRKA). TRK signalling modulates neuronal proliferation, differentiation and metabolism through activation of multiple downstream signalling pathways including RAS/ MAPK, PI3K/PDK1/Akt and PLCγ signalling (Jiang et al., 2020; Molloy et al., 2011). We also identified significant enrichment of signalling by Notch1 and Notch3. Notch signalling is activated under conditions of cerebral ischaemia and hypoxia, and in addition to its role in proliferation and differentiation, suppression of Notch activation in vivo has been reported to attenuate the development of ischaemic infarct development and neurological deficits (Arunagam et al., 2006, 2018). Furthermore, Notch1 mediates excitotoxic neuronal injury through negative regulation of PTEN and activation of PI3K/Akt signalling via direct interaction with PI3K catalytic subunit p110γ (PI3Kγ) (Marathe et al., 2015). Of particular relevance, we predicted targeting of both PTEN and PI3Kγ (PI3KCG) by miR-210-3p. PI3Kγ is induced by excitotoxic NMDA receptor activation, and the strong interaction of Notch1 with this catalytic subunit, along with inhibition of PTEN, induces Notch-dependent activation of Akt (Brennan-Minnella et al., 2013; Kim et al., 2011; Marathe et al., 2015). This reciprocal crosstalk between Notch and PI3K/Akt signalling in the promotion of cell survival and proliferation is well reported as a promising targeted therapeutic strategy in the treatment of cancer (Calzavara et al., 2008; Saito et al., 2019). We also predicted targeting of Class I PI3K regulatory subunit p85α (PI3Kδ1), which plays a critical regulatory role in PI3K/PTEN signalling through direct interaction with both PI3Kγ and PTEN, inhibiting catalytic PI3Kγ activity and positively regulating PTEN activity (Chagpar et al., 2010; Chen et al., 2018).

We identified PI3K downstream effector PDK1 (PDPK1) as both a predicted and validated target of miR-210-3p and confirmed altered expression of PTEN, along with PDK1, p70S6K and target RP56 signalling in response to increased miR-210-3p in vivo, further supporting a multi-targeting role for miR-210-3p in PI3K/PTEN/PDK1 signalling. PDK1 has previously been reported as a direct target of miR-210-3p, regulating miR-210-3p-induced apoptosis via inhibition of PI3K/Akt signalling, which is reversed upon PDK1 over-expression (Li et al., 2017). PDK1 functions as a constitutively active 'master
kinase and is a required activator of p70S6K, phosphorylating the activation loop at Thr389/412 (Thr389) and Thr229/252 (Thr229) in vitro and in vivo, both of which must be phosphorylated for substantial kinase activation (Alessi et al., 1998; Balendran et al., 1999; Mora et al., 2004; Templeton, 2001). Phosphorylation of p70S6K at Thr389 is required for the subsequent selective phosphorylation of Thr229 by PDK1; catalytically inactive PDK1 has been shown to prevent p70S6K activation and phosphorylation at both Thr389 and Thr229, whereas p70S6K was found to be completely inactive, with no detectable phosphorylation at Thr389 in PDK1-knockout cells (Williams et al., 2000). Furthermore, PDK1 over-expression induces increased phosphorylation at Thr389 in unstimulated cells (Alessi et al., 1998; Balendran et al., 1999). Our analysis found a significant reduction in PDK1 following low-dose miR-210-3p, inversely correlating with regulation of PTEN expression. Loss of PTEN activity has been shown to result in the activation of PI3K-dependent PDK1 activity in vivo (Grego-Bessa et al., 2016). PTENP1, a pseudogene of PTEN, has been widely reported as a competing endogenous RNA (ceRNA), acting as a ‘miRNA sponge’ for PTEN-targeting miRNAs to regulate PTEN expression (Gao et al., 2019; Zhang et al., 2017). Sequestering of miR-210-3p by PTENP1 at lower concentrations presents one possible explanation for the effect of increasing doses of miR-210-3p impacting on PTEN regulation, and correlating disinhibition on downstream PDK1. The multiple targeting of PI3K-PTEN-PDK1 pathway components may also result in a dilution of the impact of miR-210-3p-mediated repression on particular targets. Moreover, the impact of miR-210-3p expression may function contextually as a regulator of particular targets in response to environmental conditions such as energetic stress and ischaemia (van Rooij et al., 2007; Wilczynska & Bushell, 2015). Others studies have shown that miR-210-3p has regulatory roles in cell cycle, angiogenesis, DNA damage repair and immune response (Lu et al., 2019; Pasculli et al., 2019).

To further support the potential modulation of PI3K-p70S6K signalling by miR-210-3p in settings of ischaemic stroke, we report that induction of miR-210-3p is accompanied by a rapid and sustained decrease in p70S6K (Thr389) activity following tMCAO in vivo and following glutamate receptor (NMDA) over-activation in primary neurons. This effect was reversed by miR-210-3p inhibition, resulting in restoration of p70S6K activity. In contrast, no induction of miR-210-3p was observed following tunicamycin- and thapsigargin-induced ER stress, indicating miR-210-3p induction and modulation of p70S6K activity as a specific response to NMDA receptor-mediated excitotoxicity (Concannon et al., 2008).

Both PI3K/PTEN and MEK/ERK signalling pathways converge on p70S6K to regulate its activity as a central molecule in the control of cell proliferation and growth through active protein translation and cell cycle progression (Lehman & Gomez-Cambreron, 2002). The high energy cost of protein translation is tightly controlled by p70S6K through its ability to regulate the multiple phosphorylation of major downstream target, RPS6 and decreases in p70S6K (Thr389) and RPS6 (Ser235/236) phosphorylation have been observed following MCAO injury in vivo (Biever et al., 2015; Koh, 2013). RPS6 phosphorylation occurs sequentially on four highly conserved residues, Ser235/236 and Ser240/244 (Hutchinson et al., 2011). The critical role of p70S6K in the regulation of RPS6 phosphorylation is reflected in its ability to phosphorylate RPS6 at all residues, modulating mRNA translation in response to multiple signalling pathways (Biever et al., 2015). We detected decreased phosphorylation of RPS6 on Ser235/236 and Ser240/244 in response to increased miR-210-3p and decreased p70S6K in vivo. Inhibition of PI3K signalling during reperfusion has been shown to induce ERK-p70S6K activation, demonstrating a well-characterized ‘cross-talk’ ensuring robust signalling response (Hausenloy et al., 2004). While Ser240/244 is only phosphorylated by p70S6K, RPS6 phosphorylation at Ser235/236 can be regulated independently of p70S6K downstream of ERK through p90S6 kinases RSK1 and RSK2, representing an additional regulatory mechanism for RPS6 phosphorylation to play a role in the regulation of integrated signalling outcomes that occur between PI3K–p70S6K and MEK/ERK–p70S6K cascades following reperfusion (Hausenloy et al., 2004; Jensen et al., 1999). Finally, our analysis also highlighted the potential for indirect regulation of PTEN, p70S6K and RPS6 via TF-mediated signalling, presenting an integrated picture of the multifaceted dynamics of miRNA-mediated gene regulation.

Rapid phosphorylation of AMPK downstream targets results in the down-regulation of energy-consuming processes, such as protein translation via inhibition of p70S6K, allowing for the restoration of energy homeostasis. Catalytic subunit AMPKα1 is a direct target of miR-210-3p, raising the interesting possibility that miR-210-3p regulates mature miRNA levels, presenting a potential feedback.
mechanism in the regulation of AMPK activity which may in part explain our findings. Notably, in the context of our findings, activation of AMPK\(\alpha\) has been shown to play a central role in mediating downregulation of PDK1 expression (Hann et al., 2014).

The dual nature of many biochemical and gene expression responses to ischaemia, promoting both susceptibility and resistance to neuronal injury, presents a complex challenge in approaches to the regulation of cell signalling during ischaemic injury, impacting on the development of neuroprotective strategies. While we reported significant effects of miR-210-3p modulation on p70S6K activity through the use of a miR-210-3p mimic and inhibitor following NMDA excitotoxicity, we did not observe the same effect in response to OGD. Conditions of OGD are known to affect multiple other signalling pathways, including ER stress response and hypoxia-inducible factor 1-\(\alpha\) (HIF1\(\alpha\)) induction, with integral roles in adaptation to low-oxygen and/or glucose conditions. While NMDA-induced excitotoxicity is not associated with significant modulation of ER stress signalling, conditions of OGD have been shown to increase the expression of ER stress response genes (Concannon et al., 2008). HIF1\(\alpha\) plays a crucial role in hypoxia-sensing and adaptation through...
transcription of hypoxia-responsive genes involved in angiogenesis, cell proliferation and energy metabolism. miR-210-3p is a direct transcriptional target of HIF1α; HIF-1α binds to the miR-210-3p promoter and up-regulates miR-210-3p in response to low-oxygen conditions, and HIF1α is itself an established target of miR-210-3p, showing a mutually regulated feedback mechanism between miR-210-3p and HIF1α (Dang & Myers, 2015; Lu et al., 2019).

While not further investigated in our study, miR-124 is well established as a highly abundant brain-specific miRNA and its dysregulation is implicated in many CNS disorders (Sun et al., 2015). Suppression of neuronal miR-124 has been shown to regulate AMPK/mTOR signalling, significantly increasing p-AMPK activity (Gong et al., 2016). Although we did not confirm a significant decrease in miR-124, we observed a biological effect, decreasing following AMPK activation in vitro and 3 hr following ischaemia in vivo. Increased plasma levels of miR-124 have been reported in rat MCAO models 24 – 48 hr following occlusion and circulating levels positively correlated with tissue damage (Vuokila et al., 2020; Weng et al., 2011); disruption of brain tissue and increased blood-brain barrier permeability allowing for increased release may contribute to circulating miRNA levels not reflected in neuronal tissue. While we initially identified a decrease in miR-124 expression following 1 hr AICAR treatment in primary neurons, neuronal miR-124 levels were not measured in vivo until 3 hr post-ischaemia, potentially highlighting the importance of the time point of miRNA measurement in an evolving profile.

Our findings highlight the concerted action of multi-targeting modulation in ensuring physiological regulation of signalling pathways converging on specific protein activity. Elucidation of triggers and mediators of ischaemic cell death within the penumbra is particularly worthy of investigation given the complexity of interlinking physiological events impacting on brain injury maturation and outcome following stroke. However, for these reasons, artificial in vivo manipulation of miRNA may be associated with difficult-to-predict off-target effects. We assessed the impact of pre-treatment with exogenous miR-210-3p in an in vitro setting. While we report no significant impact on cerebral injury, we identified a reduction in neurological deficit in low dose-treated groups; however, low level of expression may not be sufficient to have a substantial effect on cerebral injury. A subgroup analysis at higher doses reveals a dichotomous effect that is likely attributable to toxicity associated with the use of miRNA mimics at higher therapeutic levels in vivo. Off-target effects associated with the multi-targeting actions of miRNAs, based on seed complement frequencies in the transcriptome, may cause potential toxicities, off-setting potential for therapeutic efficacy. This study is limited by the small sample size of in vivo exploratory studies carried out; these effects may be more pronounced in pathway analyses carried out in larger validation cohorts. Furthermore, the complex multi-targeting profile of miR-210-3p carries implications for other intracellular cascades not identified in our RPPA profiling. While not the purpose of this mechanistic study, future studies should address the therapeutic relevance of these findings on injury and functional outcome; miRNA mimics are a well-established method for characterization of miRNA function, however, success of in vivo therapeutic applications is limited as there are considerable implications associated with dose-dependent efficacy and toxicity at therapeutic concentrations.

The secretion of miRNAs, their high specificity, stability and easy detection in the circulation also places miR-210-3p as a functionally relevant blood biomarker in the diagnosis and management of ischaemic stroke and associated outcomes. Such a robust, non-invasive diagnostic and prognostic biomarker would contribute valuable and timely information necessary for prompt patient management decisions in the acute setting, enabling more effective determination of appropriate therapeutic intervention and monitoring of treatment response. Collectively, these results provide new molecular insight into the multi-targeting regulation of cell signalling in response to ischaemia, and suggest a novel mechanism whereby AMPK regulates miR-210-3p to control p70S6K activity in response to excitotoxic neuronal injury.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTIONS
S.P. and J.H.M.P. involved in conception and design of the article. S.P., A.T., U.M., H.D., P.W., E.J., B.D., G.C., M.C., A.R., N.M.C. and S.H. carried out research and data acquisition. N.M.C., S.H.; RPPA: M.C., A.R. and B.H. involved in bioinformatics. S.P., H.D., A.R., N.M.C., S.H., N.P. and J.H.M.P. carried out analysis and interpretation of data. S.P. also carried out writing of the manuscript. All authors reviewed and approved the manuscript prior to submission.

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ORCID
Shona Pfeiffer https://orcid.org/0000-0002-8728-1620
Niamh M. C. Connolly https://orcid.org/0000-0002-6005-1307

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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