



Magnesium induces preconditioning of the neonatal brain via profound mitochondrial protection

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Abstract

Magnesium sulphate (MgSO₄) given to women in preterm labor reduces cerebral palsy in their offspring but the mechanism behind this protection is unclear, limiting its effective, safe clinical implementation. Previous studies suggest that MgSO₄ is not neuroprotective if administered during or after the insult, so we hypothesised that MgSO₄ induces preconditioning in the immature brain. Therefore, we administered MgSO₄ at various time-points before/after unilateral hypoxia-ischemia (HI) in seven-day-old rats. We found that MgSO₄ treatment administered as a bolus between 6 days and 12 h prior to HI markedly reduced the brain injury, with maximal protection achieved by 1.1 mg/g MgSO₄ administered 24 h before HI. As serum magnesium levels returned to baseline before the induction of HI, we ascribed this reduction in brain injury to preconditioning. Cerebral blood flow was unaffected, but mRNAs/miRNAs involved in mitochondrial function and metabolism were modulated by MgSO₄. Metabolomic analysis (H⁺-NMR) disclosed that MgSO₄ attenuated HI-induced increases in succinate and prevented depletion of high-energy phosphates. MgSO₄ pretreatment preserved mitochondrial respiration, reducing ROS production and inflammation after HI. Therefore, we propose that MgSO₄ evokes preconditioning via induction of mitochondrial resistance and attenuation of inflammation.

Keywords

Brain injury, neonatal, preconditioning, hypoxia-ischemia, magnesium

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Introduction

Perinatal brain injury in term and preterm infants remains a paramount clinical problem causing death and neurological disabilities.^{1,2} Approximately 30% of infants born preterm (<28 gestational weeks) will suffer from cerebral palsy (CP), cognitive impairment or neuropsychiatric disorders.^{3–5} CP is the most common cause of severe disability in children (2/1000 births) and a major cost to sufferers, their families and society,^{6,7} underscoring the need for development of treatment and preventive strategies.

Magnesium administered as magnesium sulphate (MgSO₄) has neuroprotective properties. Clinical cohort studies demonstrated that offspring of mothers treated with antenatal MgSO₄ (given to inhibit preterm labor or as seizure prophylaxis in preeclampsia) had

a lower risk of developing CP⁸ and intraventricular/periventricular hemorrhage.⁹ Randomized controlled trials have subsequently confirmed these results^{10–12} and a meta-analysis showed that the rate of CP or

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moderate/severe motor disability is reduced 30% by antenatal MgSO_4 .¹³ Additionally, recent discoveries demonstrate that the rates of echolucent/echodense brain lesions,¹⁴ as well as cerebellar hemorrhages,¹⁵ were attenuated. However, the mechanisms behind this protective effect remain obscure and the optimal dose for neuroprotection in preterm fetuses/neonates without serious side effects is unknown.

Preconditioning is a phenomenon whereby sub-threshold exposure or chemical agents confer resistance to a subsequent severe insult.¹⁶ The immature brain is responsive to a number of preconditioning exposures, e.g. sub-threshold hypoxia^{17,18} or pre-exposure to pharmacological agents such as xenon¹⁹ which reduce hypoxic-ischemic (HI) brain injury substantially. Clinically, MgSO_4 is given antenatally to mothers in preterm labor well in advance of the expected critical period for development of brain injury. Indeed, the issue of timing is critical as there is no evidence of efficacy when MgSO_4 is administered immediately before, during or after the insult.^{20–22} Here we investigate our hypothesis that MgSO_4 induces preconditioning, thereby providing tolerance to insults such as HI or excitotoxicity occurring during the early neonatal period, and define the mechanisms underlying its neuroprotective effect. A better understanding of the mechanisms behind the neuroprotective actions of MgSO_4 will optimize the efficacy and safety of treatment in preterms and widen its potential therapeutic applications in high-risk pregnancies to prevent asphyxial brain injury or prior to open heart surgery in neonates.

Materials and methods

Experimental design

Prior to data acquisition, a power analysis was performed for calculation of appropriate sample size for reliable measurements. Final endpoints/rules for stopping data collection were set in advance and all experiments, including potential outliers, were included. Pups were randomly allocated to different groups from a variety of litters to avoid litter-specific outcome and marked numerically rather than according to individual treatment. During analysis, samples for microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP), analyzed for serum Mg^{2+} content, proton nuclear magnetic resonance (^1H -NMR), mRNA/miRNA, mitochondrial respiration, reactive oxygen species (ROS) formation, Bio Plex and cerebral blood flow (CBF) measurements were completely blinded to the researchers through numerical sample-marking with the researchers unaware of group belonging in order to avoid bias. Male and female Wistar rat pups from in house breeding at the facility of Experimental

Biomedicine (EBM) were used along with Swiss mice from the Robert Debre hospital rodent facility. Animal experimental procedures conformed to guidelines established by the Swedish Board of Agriculture (SJVFS 2015: 38), were approved by the Gothenburg Animal Ethics Committee (ethical license 333-2012, 138-2013 and 01-2016) or by the Bichat-Robert Debre committee (ethical license 2011-14/646-048) and are reported in a manner consistent with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines. Animals were housed at EBM or at the Robert Debre hospital with access to food and water ad libitum. Mortality was low and not different between groups.

Preconditioning

Wistar rats were randomized into two groups and injected intraperitoneally (i.p.) with an MgSO_4 bolus (Magnesium sulphate, 1 mmol/ml, Addex; Fresenius Kabi, Halden, Norway) (1.1 mg/g ($n=245$), 0.55 mg/g ($n=46$) or 0.37 mg/g ($n=41$)) or as vehicle an equivalent volume of saline (Saline 9 mg/ml, B Braun Melsungen AG, Melsungen, Germany) prior to the induction of HI. The optimal dose (1.1 mg/g) was injected at different time-points prior to (six days ($n=43$), 72 h ($n=36$), 24 h ($n=41$), 12 h ($n=33$), 3 h ($n=35$) and 30 min ($n=33$)) or post (1 h ($n=31$)) HI. Brain injury was evaluated by macroscopic scoring and immunohistochemical staining seven days after HI.

Hypoxia-ischemia

At PND 7, pups were exposed to HI as previously described.^{23,24} Rats were anesthetized (isoflurane 1.5–5%) (IsoFlo vet 100%; Abbott Laboratories Ltd, Illinois, USA) in 1:1 oxygen-nitrogen mixture. The left common carotid artery was ligated with a 6.0 silk suture (Seide; Vömel, Germany), the incision closed and infiltrated with a local anesthetic (Xylocain 20 mg/ml, lidocain, hydrochloride; Astra Zeneca, Södertälje, Sweden). The total duration was <5 min. Pups were returned to their dams for recovery (1 h), then placed in a humidified chamber with air for 10 min, followed by 60 min hypoxia (8% oxygen in nitrogen), and 10 min in air (36°C).

Tissue collection and processing

On PND 14, rats were deeply anesthetized (0.1 ml of thiopental, Pentocur, Thiopental, 50 mg/ml i.p.; Abcur AB, Helsingborg, Sweden) and killed for histological processing; brains were fixed by transcardial perfusion (6% paraformaldehyde, Histofix; Histolab, Gothenburg, Sweden). Once dissected out, brains

were photographed and graded macroscopically according to a previously published protocol²⁵ (Table S1). After dehydration, brains were paraffin-embedded and cut (Meditome A550) into 7 μm thick sections at ~ 3.3 mm from bregma.²⁶

Immunohistochemistry

Sections were prepared for immunohistochemical staining by deparaffination in xylene followed by graded alcohol rehydration and boiling in 0.01 M citric acid buffer (pH 6.0) for antigen recovery followed by blocking of endogenous peroxidase/nonspecific binding (3% H_2O_2). Sections were incubated overnight with primary antibody: Mouse anti-MAP-2 (1:1000; M4403 Sigma-Aldrich, St. Louis, Missouri, USA) or Mouse anti-MBP (1:10,000; SMI-94 Covance, Princeton, New Jersey, USA), washed and incubated in appropriate secondary antibody. ABC elite was used for visualization of immunoreactivity followed by submersion into 0.5 mg/ml 3,3-diaminobenzidine, NiSO_4 .

Brain injury evaluation

Brain injury was quantified as area loss of MAP-2 immunoreactivity at the level of anterior hippocampus. Total MAP-2 positive area was measured in each hemisphere (MicroImage, 4.0, Olympus Optical) and tissue loss calculated as MAP-2 positive area in the ipsilateral hemisphere subtracted from the contralateral. Loss of MBP was used immunohistochemically to determine white matter injury. The MBP positive staining was measured (MicroImage, 4.0, Olympus Optical) in both hemispheres and the proportion of white matter injury was calculated by comparing the ipsilateral hemisphere to the contralateral.

Serum Mg^{2+} measurement

Pups (PND 6) were injected i.p. with MgSO_4 (1.1 mg/g ($n = 17$), 0.55 mg/g ($n = 13$), 0.37 mg/g ($n = 14$)) or vehicle ($n = 18$) and decapitated after 3 or 24 h followed by immediate blood-collection. Serum was stored (-80°C) until analysis for Mg^{2+} -content (Cobas 8000 Roche Diagnostics Scandinavia AB) by Centrallaboratoriet Klinisk Kemi, Sahlgrenska University Hospital, Gothenburg, Sweden.

RNA preparation

Pups (PND 6) were injected with MgSO_4 (1.1 mg/g) or vehicle and deeply anesthetized (0.1 ml of thiopental, sodium pentotal, 50 mg/ml i.p.) after 3 or 24 h ($n = 6$ /group), perfused with saline (0.9% Sodium Chloride Solution) and brains collected, snap-frozen and stored

in -80°C . Total RNA including miRNA was prepared from whole cortex samples using miRNeasy Mini Kit (Qiagen). The RNA quality, (RQI-scores) was 9–10 as checked with Experion RNA STDsense chip (Biorad). Purity and amount were determined using Nano Drop (260/280 ratio of 1.9–2.1).

GeneChip expression analysis

Array expression analysis was performed according to the Affymetrix expression analysis technical manual, Genomics Centre, King's College London. Briefly, double-stranded cDNA was synthesized from total RNA and an in vitro transcription reaction was performed to produce biotin-labeled cRNA which was fragmented and hybridized to Affy mRNA array Gene ST v2.0, APT v1.15 (36,685 genes) and Affy miRNA 4.0. CEL files using the robust multi-array average algorithm²⁷ performing three distinct operations: global background normalization, across array normalization, and \log_2 transformation of perfect match values (<http://stat-www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html>). The robust multi-array average analysis, data management, statistical analysis, and gene ontology was performed using GeneSifter (<http://login.genesifter.net/>) and IPA (<https://analysis.ingenuity.com>).

RT-PCR

cDNA was prepared from 1 μg RNA using QuantiTect Reverse Transcription Kit (Qiagen). Each PCR (20 μL) ((2 μL cDNA, 10 μL Quanti Fast SYBR Green PCR Master Mix (Qiagen) and 2 μL PCR primer (QuantiTect Primer Assay, Qiagen)), was run on a LightCycler 480 (Roche, Sweden) (Table S2). Melting curve analysis ensured that only one PCR product was obtained and a standard curve generated using increasing concentrations of cDNA ensured quantification/estimation amplification efficiency. Amplified transcripts were quantified using the relative standard curve and normalized against the reference gene *Ywhaz*. Results were expressed as ΔC_q ; the difference in the quantification cycle (C_q) values between the target and reference genes.²⁸ The difference in ΔC_q for the different samples was then directly compared.

Mitochondrial isolation

Pups (PND 6) were injected with MgSO_4 (1.1 mg/g) ($n = 34$) or vehicle ($n = 37$) and decapitated after 24 h (with or without HI). One hemisphere was immediately excised and homogenized in 3.8 ml 12% Percoll (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) in

ice-cold MIB [225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES–KOH, pH 7.2, 1 mg/mL fatty-acid-free bovine serum albumin (BSA)] using a Dounce Homogenizer (7 ml, #432-1271, VWR). Homogenate was pipetted onto 3.5 ml 26% Percoll laying on 3.5 ml 40% Percoll in centrifuge tubes, subsequently centrifuged at 30,000g for 10 min (4°C). The mitochondrial fraction was moved to a 2 ml tube containing 1500 µl MIB (ratio 1:4) and centrifuged at 15,000g for 5 min (4°C). Pellets were washed in 1 ml MIB, centrifuged at 15,000g for 5 min (4°C) and resuspended in 75 µl MIB containing BSA 1 mg/ml.

High-resolution respirometry and O2k-fluorometry

The Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) was used for measurements of respiration and combined with the Fluorescence-Sensor Green, O2k-Fluo LED2-Module for H₂O₂ measurement. A protocol modified from our published methods²⁹ was used for mitochondrial respiration measurements in the O2k. Measurements were performed on mitochondria (25°C) in 2.1 mL MIR05 respiration buffer [20 mM HEPES, 10 mM KH₂PO₄, 110 mM sucrose, 20 mM taurine, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 1 g/L BSA (fatty acid free)]. Pyruvate and malate (5 mM and 2.5 mM, respectively) or succinate (10 mM) with or without 0.5 µM Complex-I inhibitor rotenone (Rot) were used to determine Complex-I (CI) or Complex-II-(CII) linked LEAK respiration. ADP (f.c. 0.14 mM) was added which was saturating for oxygen flux to obtain oxidative phosphorylation (OXPHOS) capacity. FCCP (1 µM) was added to determine electron transfer system (ETS) capacity. Respiratory control ratio (RCR) was calculated as the ratio of the STATE 3 (ADP stimulated respiration of isolated coupled mitochondria in the presence of high ADP and P_i concentrations) to the resting respiration rate STATE 4 (respiratory state obtained in isolated mitochondria when ADP has been maximally phosphorylated to ATP).

Mitochondrial ROS formation can be detected with an H₂O₂ sensitive probe, here Amplex[®] UltraRed was used and a CII-linked protocol measuring respiration and H₂O₂ flux simultaneously in the O2k-Fluorometer was applied. For further details see *O2k-Fluorometry* in Supplementary Materials.

For ROS production measurements, mitochondria were stimulated with ADP followed by rotenone, blocking Complex-I mediated respiration, allowing for the study of ROS produced solely at Complex-II driven by succinate addition. To induce maximal respiration/ROS production, FCCP (1 µM) was added. Volume-specific H₂O₂ fluxes were calculated real-time

(DatLab software, OROBOROS INSTRUMENTS, Innsbruck Austria). The stable portions of the H₂O₂ fluxes were selected and artifacts induced by addition of substrates/chemicals were excluded. Values were normalized to total protein content.^{30–32}

Metabolomics

Brains were collected at 3 h (*n* = 10) or 24 h (*n* = 10) following injection (PND 6) with MgSO₄ (1.1 mg/g) or vehicle (*n* = 18); 24 h after injection with MgSO₄ (1.1 mg/g) (*n* = 9) or vehicle (*n* = 10), pups were exposed to HI (60 min) and brains collected immediately after hypoxia. Animals were decapitated and whole heads snap-frozen in –80°C isopentane. Portions (10–30 mg) of parietal cortex were dissected out (–20°C) and solubilized in 3 mol/L HClO₄³³ (–10°C). After centrifugation at 5000g for 10 min, one volume of the supernatant was neutralized with 2.5 volumes of KHCO₃ (2 mol/L) and centrifuged to remove precipitated KHCO₃. Blood was collected at decapitation and serum prepared as described above and saved in 2 ml Micro tubes (Sarstedt Aktiengesellschaft & Co, D-51588 Nümbrecht) (–80°C). For detailed description of H⁺-NMR protocol see *Metabolomics* in Supplementary Materials.

CBF

CBF was measured using the iodoantipyrine method³⁴ adapted for the immature rat.^{35,36} The measurements were performed in both hemispheres 24 h after MgSO₄ or vehicle administration without exposure to HI (no HI; vehicle *n* = 11, MgSO₄ *n* = 9), after 30 min of HI (30 min HI; vehicle *n* = 7, MgSO₄ *n* = 8) and after 60 min of HI (60 min HI; vehicle *n* = 9, MgSO₄ *n* = 8). In all animals, 10 µCi, 100 µl 4-Iodo(N-methyl¹⁴C) antipyrine (ARC 0126A, ARC; USA) was subcutaneously injected in the neck followed by decapitation after 60 s. Blood, 10 µl, was collected directly and the hemispheres were dissected out, dissolved in Solveble[™] (Perkin Elmer, Massachusetts; USA) and mixed with Ultima Gold[™] (Perkin Elmer, Massachusetts; USA). Samples were measured in a Beta-counter, Tricarb 3810tr (Perkin Elmer, Massachusetts, USA) and calculated as previously described.³⁷

BioPlex

Pups were exposed to HI 24 h after MgSO₄ injection (1.1 mg/g) (*n* = 20) or vehicle (*n* = 20). Brains were collected at 6 or 24 h following hypoxia, sonicated in PBS-buffer (5 mM EDTA, 1% Triton X-100, 1% Protease Inhibitor Cocktail (P8340, Sigma)) and centrifuged at 10,000g for 10 min (4°C). Supernatants were diluted in

Sample Diluent (Bio-Plex kit, Bio-Rad) and run on a Bio-Plex Pro™ Rat Cytokine 24-plex Assay (#171K1001MSP, Bio-Rad) according to the manufacturer's instruction.

Statistical measurements

All statistical analyses (except for gene expression and metabolomic analysis described above) were performed using Graph Pad Prism. Statistical significance was determined by nonparametric unpaired Mann–Whitney U-test, paired or unpaired two-tailed *t*-tests or ANOVA with or without Tukey's multiple comparisons test as specified in each figure legend (Mean ± SEM). *P* values < 0.05 were considered statistically significant. The number of sampled units, *n*, is stated for each experiment in the methodologic section.

Results

MgSO₄ pre-treatment reduces the extent of neonatal brain injury after HI

The effect of MgSO₄ on neonatal brain injury was first explored in a well-established model of HI in seven-day-old rats.²³ Macroscopic brain injury scoring (Table S1) showed that rats pre-treated with MgSO₄ (1.1 mg/g) 24 h prior to HI had markedly reduced brain damage 0.47 ± 0.14 (*n* = 16) compared with 2.30 ± 0.18 (*n* = 18) in pups pre-treated with saline (*p* < 0.0001) (Figure 1(a) and (c)). Significant protection, was also seen when MgSO₄ was administered six days prior to HI (0.9 ± 0.2 (*n* = 21) in MgSO₄-treated pups vs. 1.9 ± 0.3 (*n* = 20) in controls (*p* < 0.005)), three days prior to HI (1.7 ± 0.2 (*n* = 18) in MgSO₄-treated pups compared with 2.3 ± 0.2 (*n* = 18) in controls (*p* < 0.05)) and 12 h prior to HI: 1.1 ± 0.2 (*n* = 17) in MgSO₄-treated pups vs. 2.3 ± 0.1 (*n* = 16) in controls (*p* < 0.0001). The overall effect of MgSO₄ (1.1 mg/g) 6 days–12 h prior to HI is summarized in Figure 1(b) (*n* = 72/group). MgSO₄ had no significant effect on brain injury when administered 3 h or 30 min prior to or 1 h after HI (Figure 1(c)). We also assessed the effect of MgSO₄ pre-treatment on the loss of grey matter using immunoreactivity for MAP-2 and found a significant reduction in tissue loss when MgSO₄ was given 72 h prior to HI ($39.8 \pm 4.6\%$ (*n* = 17) vs. $57.5 \pm 4.3\%$ (*n* = 18) in controls (*p* = 0.01)), 24 h prior to HI ($18.3 \pm 4.4\%$ (*n* = 15) in MgSO₄-treated pups compared with $46.1 \pm 4.8\%$ (*n* = 19) in controls (*p* = 0.0003)) and at 12 h prior to HI ($48.9 \pm 3.7\%$ (*n* = 18) in MgSO₄-treated pups vs. $60.3 \pm 3.2\%$ (*n* = 17) in controls (*p* = 0.03); Figure 1(a) and (c)). For assessment of white matter loss, we estimated MBP immunoreactivity

and found significant differences in the groups pre-treated with MgSO₄ at 72 h prior to HI: loss of MBP positive immunoreactivity was $53.8 \pm 9.2\%$ (*n* = 18) in MgSO₄-treated pups and $78.0 \pm 5.3\%$ (*n* = 18) in controls (*p* = 0.03). This was maintained in treatments at 24 h prior to HI ($15.0 \pm 8.4\%$ (*n* = 15) in MgSO₄-treated pups vs. $70.4 \pm 7.4\%$ (*n* = 19) in controls (*p* < 0.0001)) and 12 h prior to HI ($34.8 \pm 9.3\%$ (*n* = 18) in MgSO₄-treated pups compared with $84.3 \pm 3.8\%$ (*n* = 17) in controls (*p* = 0.03); Figure 1(a) and (c)).

There are exposures other than HI that are believed to elicit preterm brain injury so we evaluated the neuroprotective effect of MgSO₄ in a well-characterized excitotoxic model of preterm brain injury.³⁸ MgSO₄ (0.92 mg/g) 24 h prior to ibotenate injection (10 μg) reduced both grey and white matter injury (Figure S1) supporting the concept that MgSO₄ offers efficacy in the treatment of immature brain injury regardless of its aetiology.

The neuroprotective dose of MgSO₄ induces preconditioning

As the optimal interval between MgSO₄ treatment and HI was 24 h, the next step was to identify the optimal dose required for maximal protection. We injected PND 6 rats with different doses of MgSO₄ (0.37 mg/g, 0.55 mg/g or 1.1 mg/g) 24 h prior to HI. Brains were analyzed as described above and we found that only a dose of 1.1 mg/g provided the animals with significant protection (Figure 2(a)). We measured the concentration of Mg²⁺ in serum samples from pups after receiving vehicle or MgSO₄ at 3 or 24 h after administration (Figure 2(b)). For all doses tested, serum Mg²⁺ levels increased transiently, peaking at 3 h after administration and returning to baseline within 24 h. Administration of the neuroprotective dose of MgSO₄ (1.1 mg/g) resulted in a peak Mg²⁺ concentration of 3.3 ± 0.2 mmol/L at 3 h later which is within the therapeutic window (2–4 mmol/L) when MgSO₄ is given antenatally to women for seizure prophylaxis in pre-eclampsia or for neuroprotection. As pre-exposure with MgSO₄ protects the brain in spite of the levels of magnesium being within the physiological range during/after HI, these data support our hypothesis that MgSO₄ induces CNS tolerance (i.e. confers preconditioning) rather than conferring direct protection during/after the insult.

Preconditioning with MgSO₄ does not change CBF

We then explored the possible mechanisms behind the profound preconditioning effect of MgSO₄. It is well-known that MgSO₄ has cerebrovascular effects,

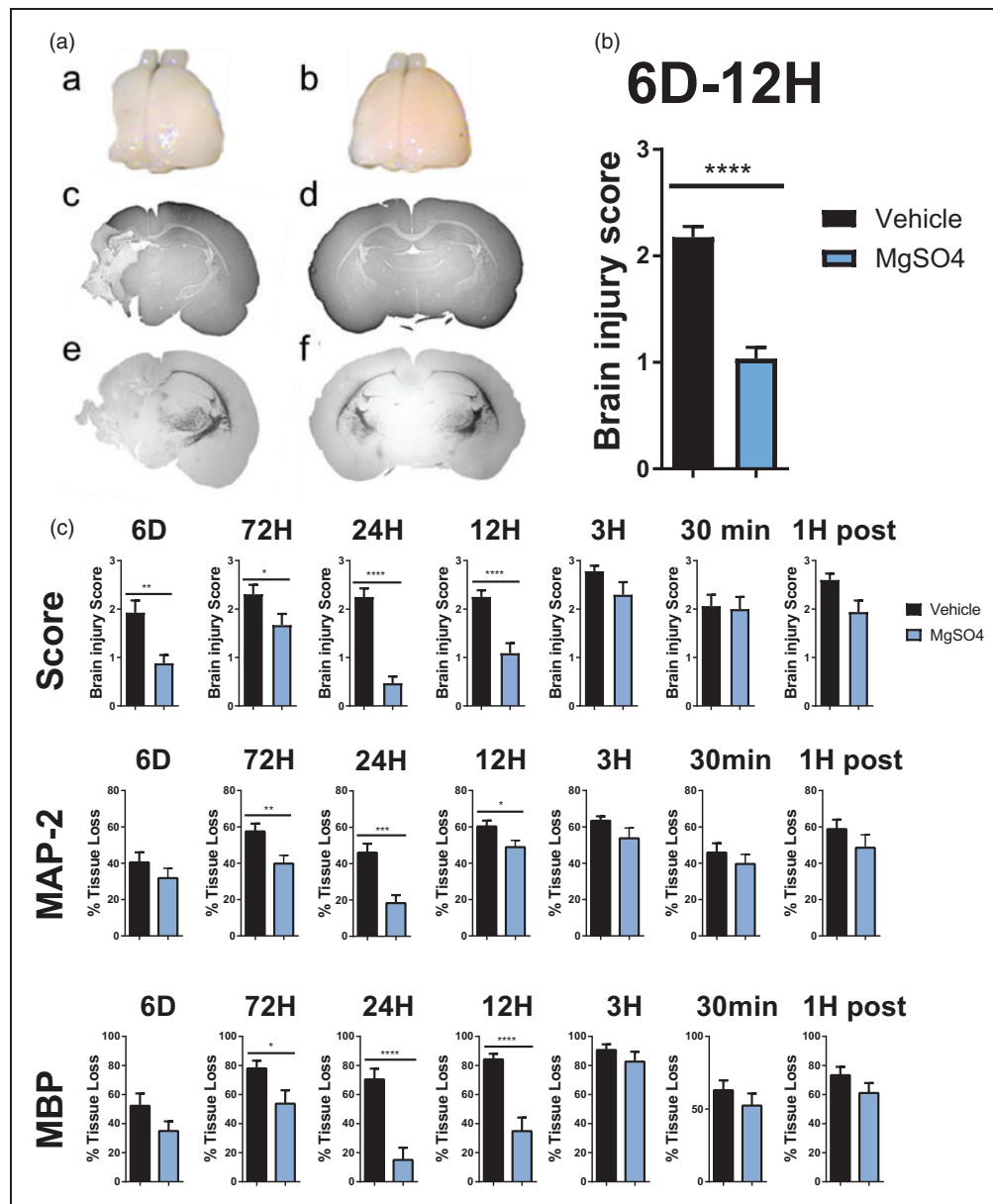


Figure 1. Evaluation of MgSO₄ preconditioning protection. (a) Macroscopic brain injury assessment – brains were collected from rat pups preconditioned with (a) vehicle ($n = 18$) or (b) MgSO₄ ($n = 17$) 24 h prior to HI, collected seven days post HI ($p < 0.0001$). Brain injury analyses were performed on MAP-2 stained sections (c and d) and MBP-stained sections (e and f) at the level of anterior hippocampus from brains shown in image (a) and (b). (B) Overall summary of macroscopic brain injury score at 6 days–12 h; $n = 72/$ condition ($p < 0.0001$) (Mann–Whitney U test, Mean \pm SEM). (c) Macroscopic brain injury score and measurement of grey (MAP-2) and white (MBP) matter injury at PND 14 after i.p. injection with NaCl (vehicle) or MgSO₄ at different time points prior to and post HI, ($p < 0.05$) (Mann–Whitney U test, Mean \pm SEM).

modulating the release of calcitonin gene-related peptide and nitric oxide,³⁹ and could theoretically improve CBF during HI, explaining its protective action. However, no significant difference in CBF could be detected between MgSO₄ and vehicle at 24 h after administration or during the subsequent period of HI (Figure 2(c)), implying that MgSO₄ does not provide protection by affecting CBF.

MgSO₄ regulates mitochondrial/metabolic mRNA and miRNA in the brain

The development of CNS injury resistance required >12 h delay after MgSO₄ injection (Figure 1). Previous studies have shown that preconditioning protection can depend on gene transcription and protein translation,¹⁶ therefore we analyzed the effect of

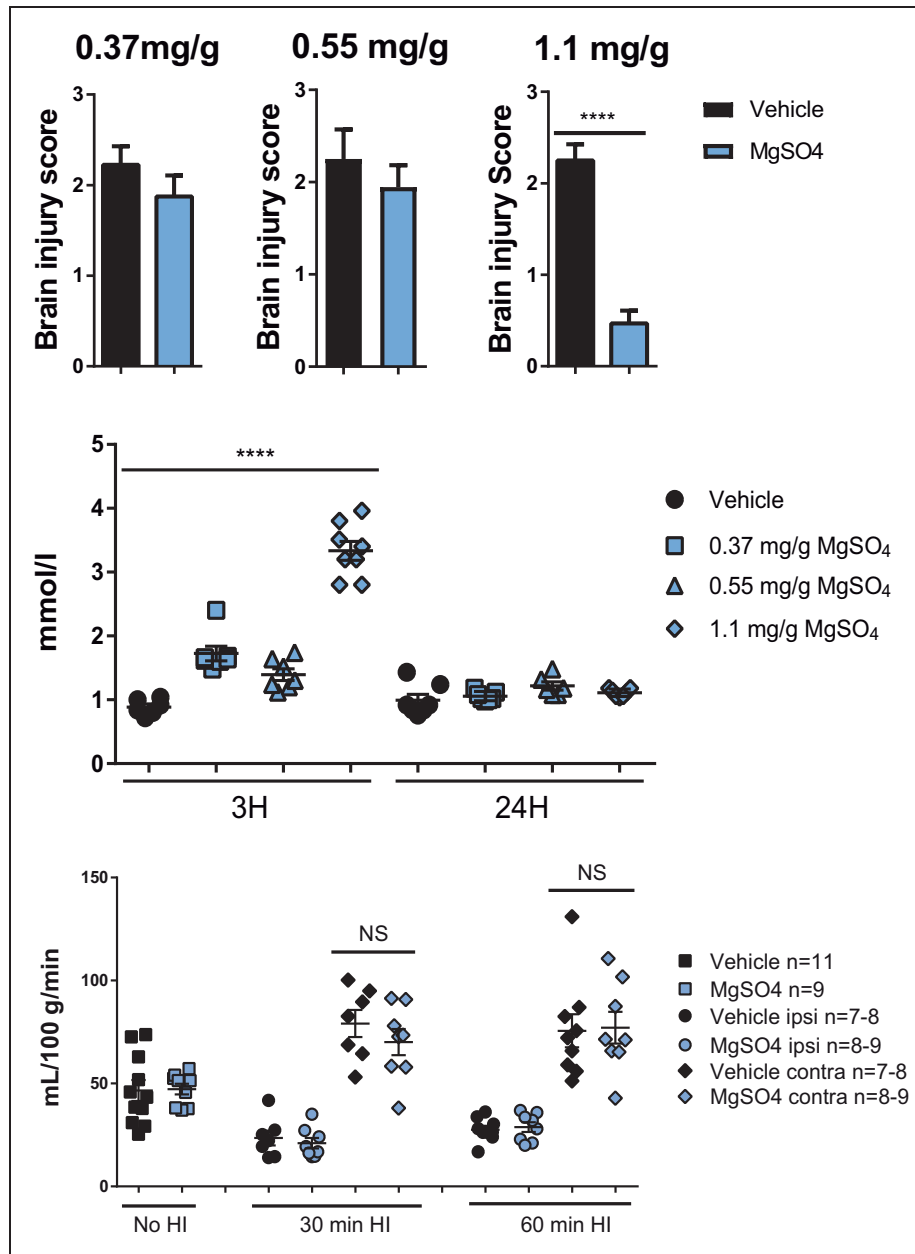


Figure 2. MgSO₄ dose-response, serum concentrations and effect on cerebral blood flow. (a) Preconditioning protection induced by different doses of MgSO₄; 0.37 mg/g (vehicle $n = 20$, MgSO₄ $n = 20$), 0.55 mg/g (vehicle $n = 18$, MgSO₄ $n = 28$) or 1.1 mg/g (vehicle $n = 18$, MgSO₄ $n = 16$) ($p < 0.0001$), unpaired t -test Mean \pm SEM. (b) Concentration of Mg²⁺ in serum samples collected at 3 h or 24 h after injection with one of the three doses of MgSO₄ (0.37 mg/g ($n = 14$), 0.55 mg/g ($n = 13$), or 1.1 mg/g ($n = 17$) or an equivalent volume of vehicle ($n = 18$) ($p < 0.0001$), unpaired t -test Mean \pm SEM. (c) Cerebral blood flow (CBF) was measured in ipsi- and contralateral hemispheres 24 h after MgSO₄ or vehicle administration in a group not subjected to HI, (no HI; vehicle $n = 11$, MgSO₄ $n = 9$) and in groups exposed to 30 min (vehicle $n = 7$, MgSO₄ $n = 8$) and 60 min (vehicle $n = 9$, MgSO₄ $n = 8$) of HI. There were no significant differences in CBF between MgSO₄ treated and vehicle groups ($p > 0.05$).

MgSO₄ on mRNA and miRNA expression in the cerebral cortex. Firstly, gene expression in MgSO₄ and vehicle-treated groups was analyzed by microarray and compared with ANOVA ($p < 0.05$) alone or combined with the requirement of ± 1.3 fold change. Multiple genes and miRNAs were altered

(Figure 3(a)) and several of them have been found to be regulated in other preconditioning paradigms (Tables S3 and S4). Selective RT-PCR was performed to confirm differences observed by microarray, establishing that MgSO₄ indeed modulates gene expression (Figure 3(b)).

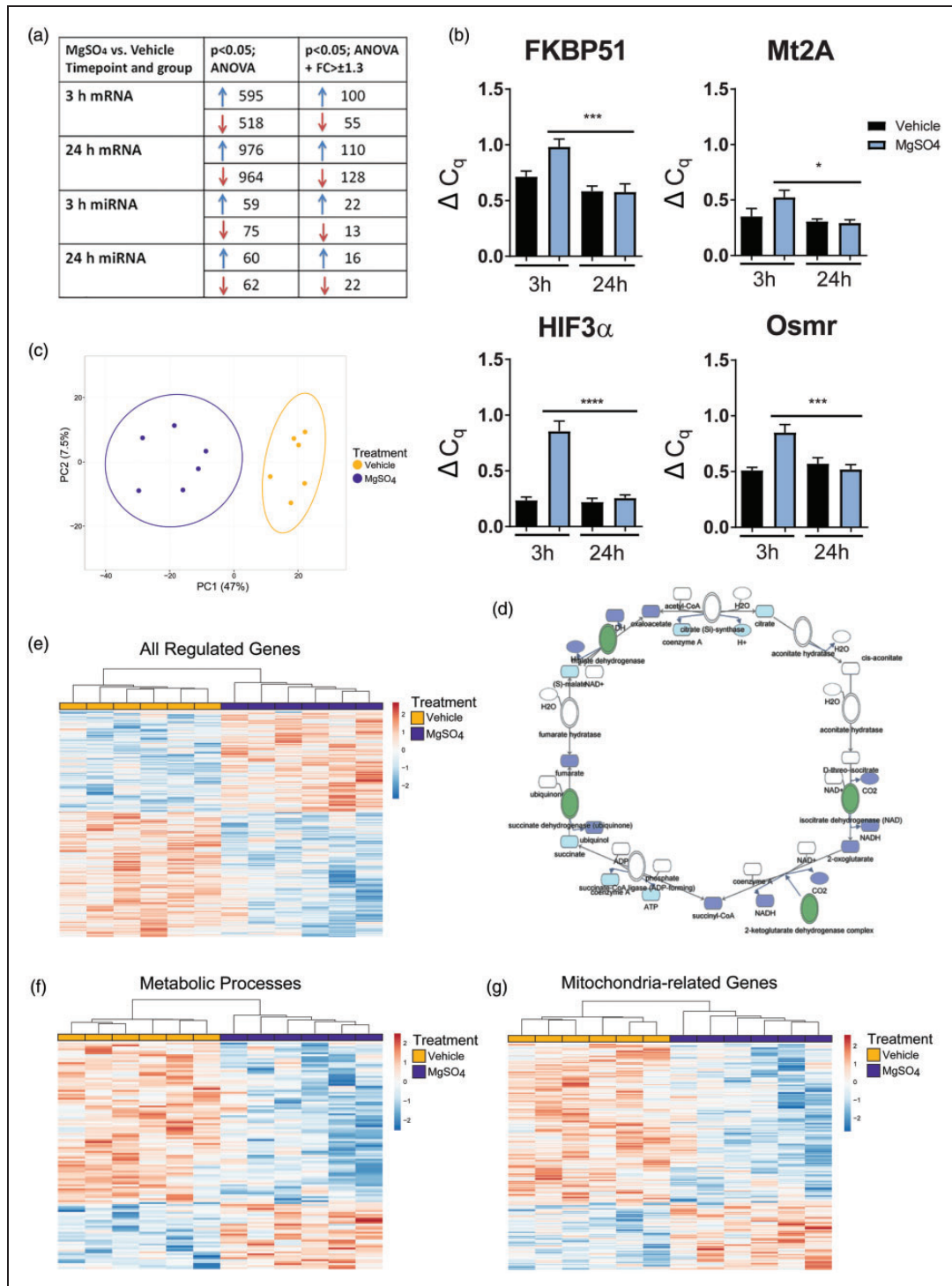


Figure 3. MgSO₄ PC causes regulatory changes in mRNA and miRNA and downregulation of metabolic and mitochondrial pathways. (a) Summary of mRNA and miRNA findings at 3 h and 24 h after administration of MgSO₄ or vehicle, ANOVA $p < 0.05$ with and without fold change $> \pm 1.3$. (b) RT-PCR results for a selection of genes upregulated at 3 h post injection with MgSO₄ or vehicle; FKBP51 (from $0.52 \pm 0.04 \Delta C_q$; $n = 6$ to $0.85 \pm 0.07 \Delta C_q$; $n = 6$) ($p = 0.0004$); Mt2A (from $0.29 \pm 0.03 \Delta C_q$; $n = 6$ to 0.52 ± 0.06 ; $n = 6$) ($p = 0.02$); HIF3 α (from $0.26 \pm 0.03 \Delta C_q$; $n = 6$ to $0.86 \pm 0.09 \Delta C_q$; $n = 6$) ($p < 0.0001$); Osmr (from $0.52 \pm 0.04 \Delta C_q$; $n = 6$ to $0.85 \pm 0.07 \Delta C_q$; $n = 6$) ($p = 0.0003$) (ANOVA, $p < 0.05$). (c) Principal component analysis plot of all regulated genes 24 h after MgSO₄/vehicle injection ($n = 6$ /group). (d) Network analysis demonstrates that several genes for enzymes in the citric acid cycle were downregulated (marked in green) after MgSO₄ injection, including succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and 2-ketoglutarate dehydrogenase complex at 24 h. (e) Heatmap cluster of all genes from (a). (f–g) Heatmaps visualizing the genes regulated in metabolic (f) and mitochondrial (g) pathways at 24 h post MgSO₄ vs. vehicle, respectively.

Using ClustVis, a PCA plot⁴⁰ was constructed using data from all 907 regulated genes at 24 h (Figure 3(c)) showing a clear separation between samples from MgSO₄ pre-treated animals and vehicle offering further evidence that MgSO₄ affects gene transcription in the brain. Many of the regulated genes relate to mitochondrial function and at 24 h after MgSO₄ administration, both succinate dehydrogenase (SDH) and malate dehydrogenase were significantly downregulated (Figure 3(d)). Furthermore, pathway analysis demonstrated that metabolic pathways were significantly downregulated at 24 h after MgSO₄ vs. vehicle (Z score: 2.98) which was further confirmed in the gene ontology analysis where 226/351 cellular metabolic process genes (Z score: 4.14) and 72/95 mitochondrial genes (Z score: 4.14) were downregulated (GeneSifter software, Geospiza). The gene data used for the PCA (above; 3(c)) was then used to create a heatmap showing up- or downregulation of multiple genes at 24 h after MgSO₄ treatment compared to vehicle (Figure 3(e)). Heatmap clusters were constructed from all genes found to be regulated in metabolic pathways visualizing that 65% of genes in cellular metabolic processes (Figure 3(f)) and 76% of mitochondria-related genes were downregulated (Figure 3(g)). We also performed analyses of corresponding proteins utilizing an experimental dataset from the proteomic study of about 7300 mitochondrial proteins from MitProNet^{40,41} (a functional linkage network of mitochondrial proteins generated by integrating genomic features encoded by a wide range of datasets including genomic context, gene expression profiles, protein-protein interactions, functional similarity and metabolic pathways). We superimposed our microarray data on MitoProtNet dataset and performed IPA analyses and found a significant down-regulation of genes corresponding to proteins in complexes I, II, III and IV of the ETC at 24 h by MgSO₄ (ANOVA; $p < 0.05$).

MgSO₄ targets pathological succinate accumulation in the immature brain

These results indicate that MgSO₄ modulates mitochondrial pathways, including succinate metabolism, which is interesting but not surprising as magnesium is involved in regulation of >600 enzymatic reactions including those involved in energy metabolism.^{39,42} However, recently Chouchani et al.⁴³ demonstrated that the extent of succinate increase during ischemia is critical as it triggers production of ROS and impairs mitochondrial function. Other groups⁴² suggest that succinate triggers a pro-inflammatory response which could be critical during post-HI recovery. Therefore, we explored whether MgSO₄ modulates the metabolic response before and during HI (Figure S2). The

analysis revealed that the MgSO₄-treated samples differ from vehicle in the 3 h group for cortex (Figure 4(a)), indicating that MgSO₄ itself has an effect on brain metabolism. Furthermore, MgSO₄ suppressed metabolic responses exacerbated in cerebral cortex during the HI insult. Succinate was significantly increased after HI in the vehicle-treated rats ($n=9$) (from 4.1 ± 0.5 a.u. to 11.6 ± 2.4 a.u.; $p < 0.01$), whereas no such increase was detected following MgSO₄ pre-treatment ($n=6$) (from 4.2 ± 0.5 a.u. to 6.6 ± 2.0 a.u.; NS) (Figure 4(b) and (c)). An increase in GABA in the cerebral cortex was detected in the vehicle-treated group ($n=9$) after 60 min of HI (from 2.3 ± 0.2 a.u. to 4.3 a.u.; $p < 0.0001$) whereas no increase was detected in the MgSO₄ group ($n=6$) (from 2.5 ± 0.3 a.u. to 2.5 ± 0.4 a.u.; NS). As expected, phosphocreatine and ATP/ADP levels decreased significantly in the vehicle group after HI ($p < 0.0004$ and $p < 0.0001$, respectively), whereas the levels were better maintained in the MgSO₄ group. Additionally, a significant difference in phosphocreatine ($p < 0.03$) and ATP/ADP ($p < 0.03$) concentration was detected between the vehicle and MgSO₄ pre-treated groups. Lactate was significantly increased in both the MgSO₄ (from 6.5 ± 0.6 a.u. to 30.2 ± 5.7 a.u.; $p < 0.0001$) ($n=6$) and vehicle group (from 6.1 ± 0.6 a.u. to 48.8 ± 8.0 a.u.; $p < 0.0001$) ($n=9$) although with a tendency towards being higher in the latter (NS). Glucose concentrations were lower after HI with no difference between groups (Figure 4(c)). These metabolic data indicate that the potentially toxic succinate accumulation is attenuated by MgSO₄ pre-treatment and this effect was paralleled by preservation of high-energy phosphates indicating protection of mitochondrial function.

MgSO₄ preserves brain mitochondrial respiratory capacity

The Complex-I-mediated respiratory capacity of isolated brain mitochondria was analyzed using high-resolution respirometry (O2k chamber). No significant difference in RCR, i.e. STATE 3/STATE 4 respiration, was detected between MgSO₄ and vehicle at 24 h after injection (Figure 5(a) and (c)). We found, however, that the RCR decreased from 5.3 ± 0.5 pmol/(s*mg); $n=7$ to 2.7 ± 0.4 pmol/(s*mg); $n=6$; $p < 0.002$ after 30 min HI in the vehicle group, whereas it remained unchanged at 30 min HI in the MgSO₄ group (4.5 ± 0.2 pmol/(s*mg); $n=6$ before HI and 4.4 ± 0.6 pmol/(s*mg) after 30 min HI; $n=7$) (Figure 5(b) and (c)). Hence, the RCR was significantly higher in the MgSO₄ group compared with vehicle at 30 min HI induced 24 h after administration ($p < 0.05$).

To further characterize the effect of MgSO₄ on mitochondrial function, we studied Complex-II-mediated

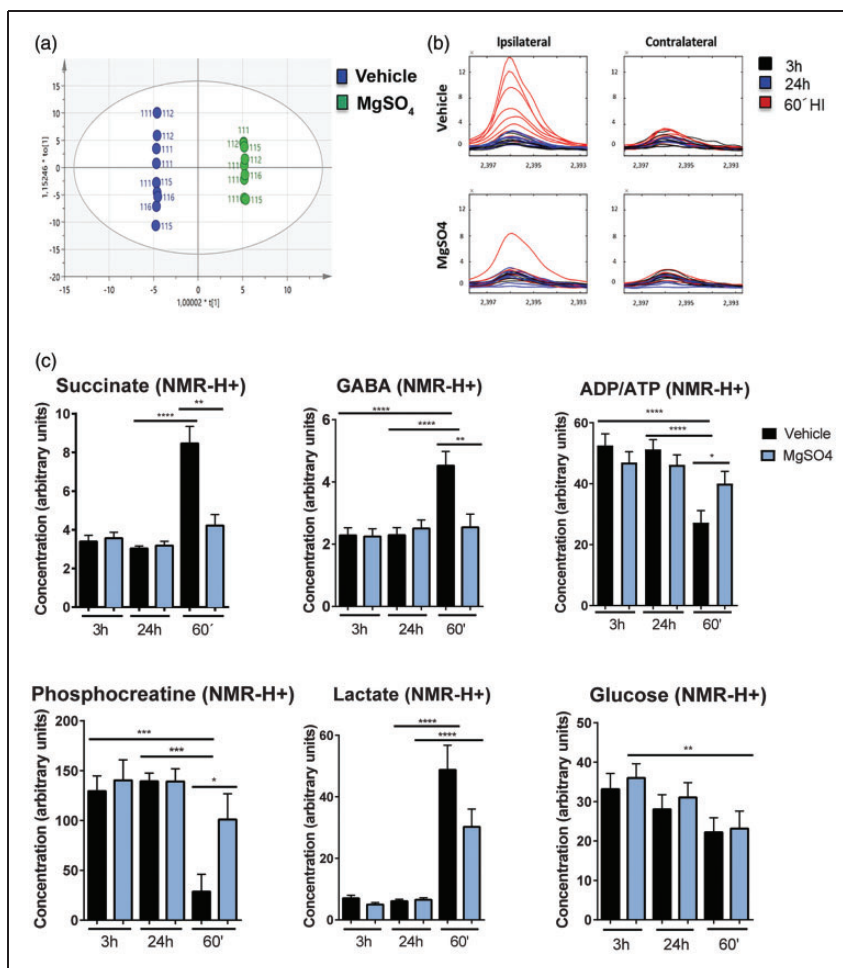


Figure 4. MgSO₄ preconditioning prevents succinate and GABA accumulation as well as high-energy phosphate depletion in the brain after HI. (a) Principal component analysis (OPLS-DA) of the metabolomic analysis of cortex samples (ipsi- and contralateral) collected 3 h after i.p. injection with vehicle ($n = 9$) as shown in blue or MgSO₄ ($n = 6$) shown in green. (b) Metabolic trace plots of succinate levels in cerebral cortex after 60 min of HI (red) in samples from rats injected with vehicle ($n = 9$) or MgSO₄ ($n = 6$) 24 h prior to HI in contra- and ipsilateral hemisphere and in animals exposed to treatment without HI 3 h or 24 h prior to sampling. (c) Cerebro-cortical metabolites 3 h and 24 h after administration of MgSO₄ or vehicle, and 24 h of MgSO₄ or vehicle + 60 min of HI (60'). There was an accumulation of succinate ($p < 0.05$) and GABA ($p < 0.001$) after 60 min of HI in the vehicle group but not in MgSO₄-treated group. Depletion of phosphocreatine ($p < 0.0002$) and ADP/ATP ($p < 0.02$) was attenuated during HI in the MgSO₄ group compared to vehicle-treated group. The lactate concentration increased to the same extent in MgSO₄ pre-treated animals ($p < 0.0001$) as in vehicle ($p < 0.0001$) after HI and glucose concentrations decreased in the cerebral cortex following HI in both groups ($n = 6-10$ /condition), (one-way ANOVA with Tukey's multiple comparisons test).

mitochondrial respiration using succinate and rotenone in the O2k. The results showed a significant increase in succinate-driven OXPHOS (S-OXPHOS) in the MgSO₄ pre-treated group compared to vehicle ($p < 0.005$) (Figure 5(e) and (f)) after 60 min HI. Further we analyzed the succinate driven ETS capacity (S-ETS) using the uncoupler FCCP to induce maximal Complex-II-mediated respiration, which was significantly higher in the MgSO₄ group ($p < 0.005$) (Figure 5(e) and (f)). No significant increase could be detected in the Complex-II-mediated respiratory capacity after the

addition of FCCP during these settings (compared to S-OXPHOS) which is in concordance with previous reports.⁴⁴ A significant difference was also detected in the basal mitochondrial respiration (STATE 1) between the MgSO₄ pre-treated group and vehicle ($p < 0.009$) as well as in STATE 2 respiration, (when ADP is added to mitochondria in the absence of reducing substrates) ($p < 0.005$, $n = 6$ /group) (Figure 5(d)). In summary, these data indicate that MgSO₄ preserves mitochondrial Complex-I and-II respiration after HI.

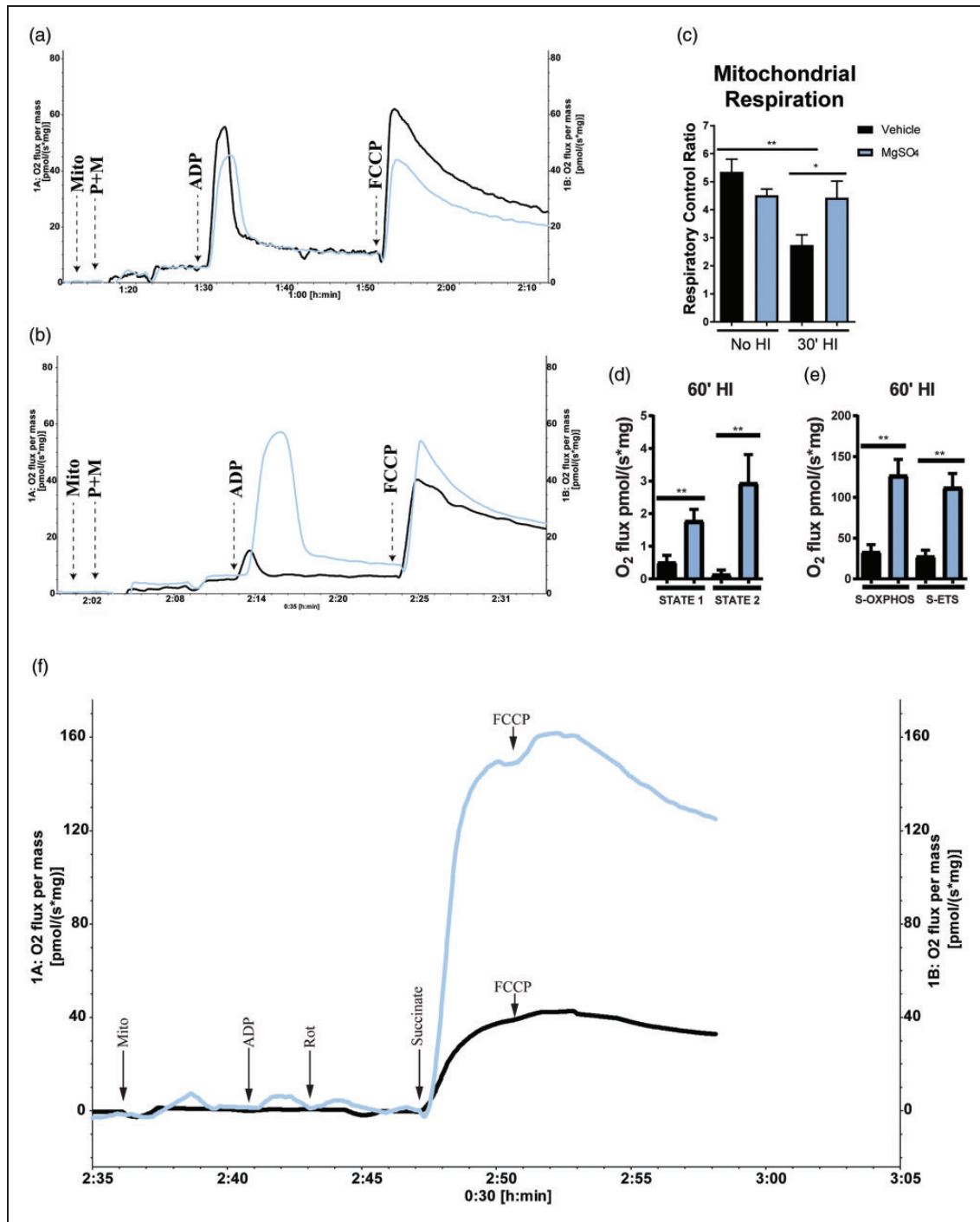


Figure 5. MgSO_4 preconditioning preserved brain mitochondrial respiration after HI. (a) Respirogram showing vehicle (black) and MgSO_4 -treated samples analyzed 24 h post injection. (b) Respirogram showing vehicle (black) and MgSO_4 -treated samples analyzed after 30 min of HI. Respiration was significantly higher in the MgSO_4 -treated group compared to vehicle ($p < 0.05$) (Unpaired t -test, Mean \pm SEM) ($n = 6\text{--}7$ /group). (c) Mitochondrial respiration. A significantly higher RCR was found in brain mitochondria isolated from animals pretreated with MgSO_4 compared to vehicle-treated animals after 30 min of HI (30' HI). No difference could be detected in RCR between MgSO_4 -treated animals with or without HI, whereas there was a $\sim 50\%$ drop in RCR in response to HI in the vehicle-treated group ($p < 0.002$), (ANOVA, $p < 0.05$). (d) Basal mitochondrial respiration (STATE 1) as well as respiration in the presence of added ADP but absence of reducing substrates (STATE 2) in vehicle (black) vs. MgSO_4 (blue; $n = 4$ /group) ($p < 0.03$). (e) Complex-II-mediated respiration driven by succinate as reducing substrate during blockage of Complex-I by Rotenone in MgSO_4 vs. vehicle ($p < 0.03$). Also, maximal ETS capacity induced by addition of FCCP ($p < 0.03$) ($n = 5$ /group), (Mann-Whitney U test, Mean \pm SEM). (f) Respirogram showing the respiratory activity of isolated mitochondria from MgSO_4 (blue) compared to vehicle (black) group. The time-point for addition of each substrate is indicated by arrows in the figure.

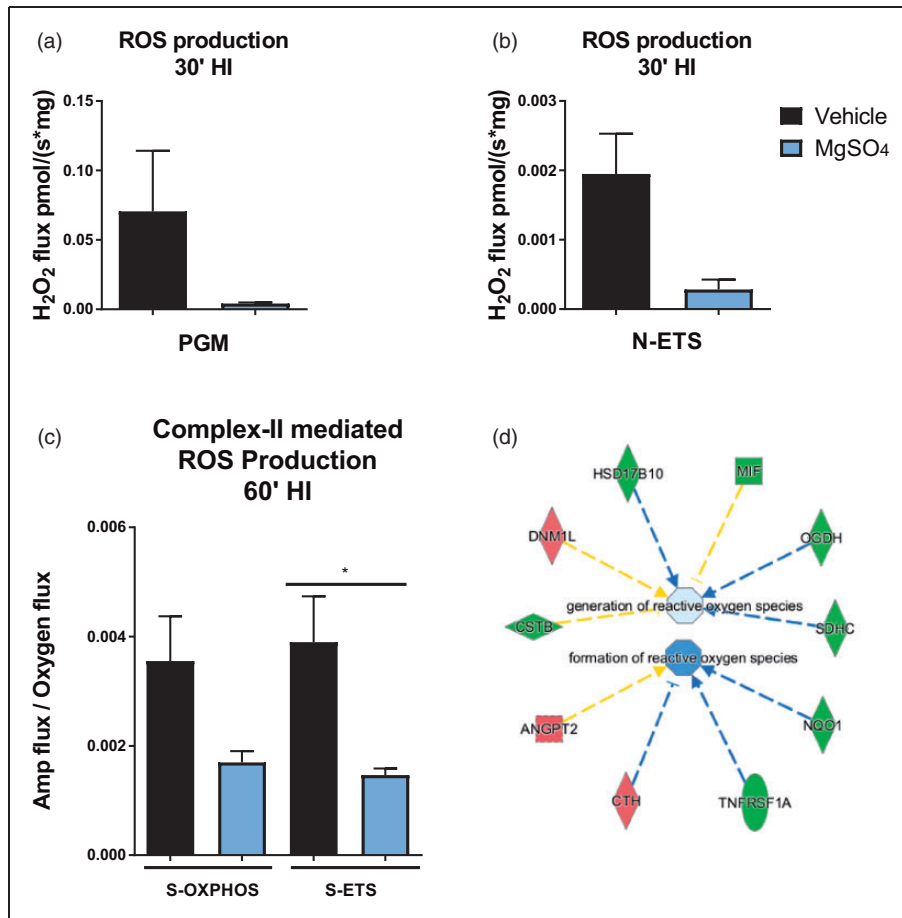


Figure 6. MgSO₄ preconditioning attenuates brain mitochondrial ROS following HI. (a) ROS production at 30 min HI in the presence of the NADH substrates pyruvate, glutamate and malate in MgSO₄ and vehicle group ($n = 4/\text{group}$). (b) ROS production at 30 min HI at maximal ETS induced by addition of FCCP ($n = 4/\text{group}$). (Mann–Whitney U test, Mean \pm SEM; NS). (c) ROS production in MgSO₄ vs. vehicle pre-treated samples collected at 60 min HI at the addition of succinate and FCCP ($n = 6/\text{group}$) controlled for level of mitochondrial respiration by dividing the total ROS production (the AMP-flux) by the mitochondrial respiration (O₂ flux) (one-way ANOVA with Tukey's multiple comparisons test, $p < 0.05$). (d) IPA prediction of decreased ROS generation and formation in the MgSO₄ group at 24 h after administration. Upregulated genes displayed in red and downregulated genes displayed in green. Downregulation as predicted by IPA is illustrated in blue.

MgSO₄ attenuates ROS production in the brain after HI

Next, we investigated whether this functional improvement was associated with alleviation of oxidative stress. Production of ROS in isolated brain mitochondria was measured using Amplex Red. We found that the Complex-I-linked production of ROS tended to decrease in the MgSO₄ samples ($n = 4$) at 30 min HI compared to vehicle ($n = 5$) in the presence of NADH generating substrates (pyruvate, glutamate and malate) (Figure 6(a)) as well as when FCCP had been added (N-ETS) (Figure 6(b)). Furthermore, the production of ROS was measured using the O₂k in the absence of exogenous NADH generating substrates

with the purpose of detecting endogenous ROS production only. When succinate was added to the isolated brain mitochondria stimulated with ADP, the detected production of ROS was 0.004 ± 0.001 pmol/(s*mg); $n = 6$ in vehicle vs. 0.002 ± 0.001 pmol/(s*mg) ($n = 6$) in the MgSO₄ group. Following the addition of FCCP, a significantly higher production of ROS was detected in the vehicle group (0.004 ± 0.001 pmol/(s*mg); $n = 6$) compared with the MgSO₄ group (0.002 ± 0.0001 pmol/(s*mg); $n = 6$) (Figure 6(c)) ($p = 0.02$). We also used IPA to analyze our microarray data and detected downregulation of several genes involved in the pathway of ROS production in the MgSO₄ group compared to vehicle, predicting inhibition of generation and formation of ROS

(Figure 6(d)) corresponding to our findings using the O2k equipment.

MgSO₄ pre-treatment attenuates the post HI accumulation of chemokines and pro-inflammatory cytokines

Previous studies suggest that succinate activates the succinate receptor 1 (SUCNR1) leading to activation of pro-inflammatory responses via p38, NFAT (nuclear factor of activated T-cells) and NFκB (nuclear factor kappa B) signaling.⁴² Therefore, we speculated that MgSO₄ could modify the cytokine/chemokine responses after HI due to the attenuation of succinate accumulation (above). Furthermore, mitochondrial ROS production has been suggested to enhance the pro-inflammatory response⁴² and as MgSO₄ reduced ROS production, we expected that the inflammatory response would be affected. We prepared whole brain homogenate at 6 and 24 h after HI comparing the MgSO₄ and vehicle groups. Indeed, pre-treatment with MgSO₄ significantly attenuated the accumulation of MCP-1 (CCL2), IL-1 α, IL-1 β, MIP-3α (CCL20), GRO/KC (CXCL1), MIP-1 α (CCL3), M-CSF and GM-CSF at 6-24 h after neonatal HI (Figure 7(a) to (h)), whereas the anti-inflammatory cytokines IL-4 and IL-10 were not different between the groups (vehicle no HI; *n* = 3, MgSO₄ no HI; *n* = 4, vehicle HI + 6 h; *n* = 10, MgSO₄ HI + 6 h; *n* = 11, vehicle HI + 24 h; *n* = 10, MgSO₄ HI + 24 h; *n* = 9) (Figure 7(i) to (j)).

Discussion

We have demonstrated for the first time that MgSO₄ conferred preconditioning to the immature rat brain if given 6 days–12 h prior to an HI or excitotoxic insult reducing brain injury in all areas, particularly cerebral cortex, by as much as 80%. Administration of MgSO₄ induced mRNA/miRNA changes in the cerebral cortex previously shown to be regulated in response to other forms of preconditioning and many of them related to mitochondria.^{45–48} Metabolic pathways were generally downregulated including mitochondrial network genes, especially those corresponding to proteins in the electron transport chain. The metabolome was significantly modulated in both serum and brain at 3 h after MgSO₄. Succinate accumulation, mitochondrial functional deterioration during HI and the increase of mitochondrial ROS production and tissue cytokines/chemokines after the insult were all attenuated by pre-treatment with MgSO₄.

MgSO₄ was given as an intraperitoneal bolus causing a temporary increase in serum magnesium concentration to ~3.3 mmol/L at 3 h but it is important to

point out that the levels were completely normalized at 24 h post injection, i.e. the concentration of magnesium was at physiological levels during and after the HI insult. According to previous studies, increases of serum MgSO₄ elevate magnesium concentrations in the brain tissue⁴⁹ as well as in the cerebrospinal fluid⁵⁰ suggesting that extracellular and intracellular magnesium concentrations increase at least transiently in the brain. Magnesium modulates calcium hemostasis and the activity of multiple enzymes⁵¹ possibly explaining why the metabolome is altered in blood and brain at 3 h after MgSO₄ administration. These metabolic/enzymatic changes may secondarily induce the subtle gene transcription changes detected in the brain at 3 and 24 h after treatment, probably contributing to development of mitochondrial and CNS resistance as well as the metabolism downregulation and attenuation of succinate accumulation in response to HI. Indeed, the gene ontology and pathway analysis indicating metabolic suppression by MgSO₄ are supported by a recent clinical study demonstrating a drop in cerebral oxygen consumption and cerebral oxygen extraction in newborns pre-exposed to MgSO₄ antenatally.⁵² These changes occurred in spite of unaffected CBF⁵² which agrees with our results showing that CBF was not different between the MgSO₄ and vehicle group at 24 h after administration or even during the subsequent HI, supporting that both groups were suffering from a similar degree of primary HI insult.

Succinate is reported to accumulate in tissues, including the immature and adult brain,^{53,54} in response to HI leading to succinate-driven excessive mitochondrial production of ROS during reperfusion and subsequent aggravation of injury.⁵¹ The cause of the succinate increase is reversal of SDH activity, resulting in the conversion of fumarate to succinate, driven by fumarate overflow from purine nucleotide breakdown and partial reversal of the malate/aspartate shuttle.⁵¹ The SDH inhibitor dimethyl malonate prevents accumulation of succinate and ROS, conferring significant neuroprotection.⁵³ Presently, we found that MgSO₄ pre-treatment prevented the accumulation of succinate and improved mitochondrial function in the neonatal brain. Interestingly, the mRNA analysis of the rat brain tissue demonstrated that the genes for SDH and malate dehydrogenase were downregulated by MgSO₄. When analyzing Complex-II-mediated succinate-driven respiration in isolated mitochondria, we detected an increase in the MgSO₄ pre-treated group, whereas no such increase was detected in the vehicle group, further confirming our data on endogenous succinate accumulation in the vehicle group. GABA accumulation during HI was also attenuated by MgSO₄. Speculatively, magnesium modulates both the reversal of SDH and the

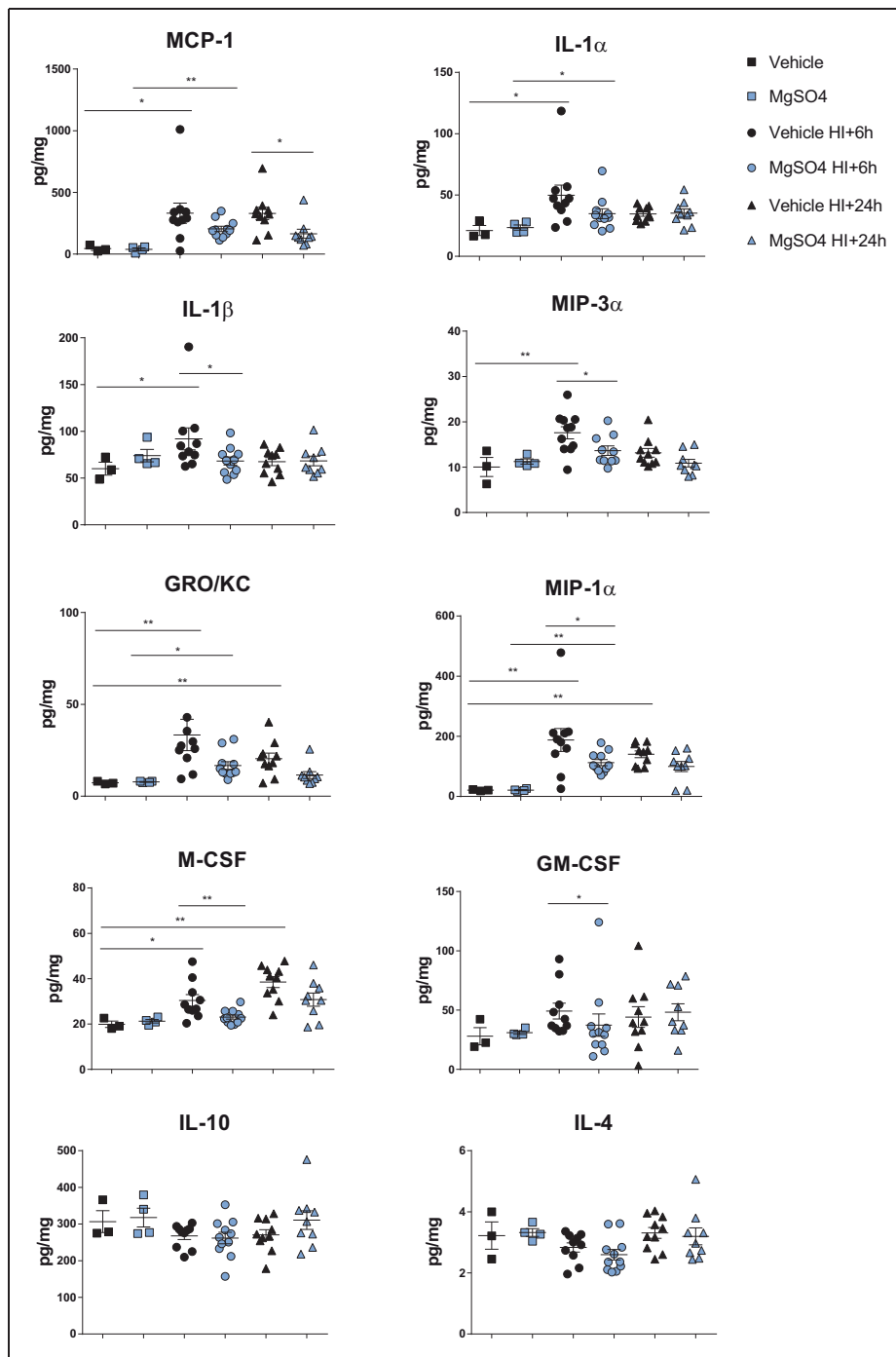


Figure 7. Attenuation of chemokines and pro-inflammatory cytokine accumulation in the brain by preconditioning with MgSO_4 . (a–h) Attenuation of MCP-1, IL-1 α , IL-1 β , MIP-3 α , GRO-KC, MIP-1 α , M-CSF and GM-CSF accumulation at 6 h and/or 24 h post HI in the MgSO_4 -treated group. No significant difference in IL-10 (i) or IL-4 (j) between the groups at either time-point. (one-way ANOVA with Tukey's multiple comparisons test, $p < 0.05$) (vehicle no HI; $n = 3$, MgSO_4 no HI; $n = 4$, vehicle HI + 6 h; $n = 10$, MgSO_4 HI + 6 h; $n = 11$, vehicle HI + 24 h; $n = 10$, MgSO_4 HI + 24 h; $n = 9$).

GABA-shunt, thereby preventing accumulation of toxic levels of succinate in the brain tissue after HI. Succinate accumulation during HI can also trigger a more profound inflammation^{42,55} during the

secondary phase after HI which may worsen brain injury.¹ In fact, several studies show that MgSO_4 attenuates the pro-inflammatory reaction in experimental models.^{56,57} Additional experiments are needed to

clarify the mechanisms behind succinate accumulation in the immature brain and to elucidate how these metabolic events and mitochondrial functions are modulated by magnesium preconditioning as well as their specific role in neuroprotection.

Many enzymes in intermediary metabolism are modulated by magnesium, affecting >600 biological reactions⁵¹ which could hypothetically shift the balance and attenuate succinate accumulation. Indeed, we found that genes related to proteins in the ETC were downregulated by MgSO₄ which has been shown to occur also in preconditioning of the heart in parallel to improvement of mitochondrial respiratory recovery.⁴⁸ Recently, it was discovered that when preventing degradation of components of the ETC, such as SDH, BAX dependent apoptotic cell death known to be critical in HI brain injury,⁵⁸ was attenuated.⁵⁹

This study offers mechanistic information indicating that succinate metabolism and mitochondrial function are involved in preconditioning by MgSO₄. However, more detailed information (using ¹H-NMR, ³¹P-NMR and ¹³C-NMR) on how the metabolic network is modulated by MgSO₄ treatment is needed, having to be addressed in the future.

Concerning clinical implications, the present finding that a transient increase of serum MgSO₄ induces tolerance in the developing brain rather than providing a direct neuroprotective effect during/after the insult is of great importance. Indeed, these data support the clinical findings that MgSO₄ is an effective neuroprophylaxis if treatment starts antenatally, some-time before the critical period for brain insults during delivery and the first few days of life. However, our results also indicate that it may be sufficient to offer a MgSO₄ bolus (high enough to reach a transient magnesium increase to >2.5 mmol/l which in most patients would correspond to 6 g of MgSO₄) rather than administration of MgSO₄ infusions during delivery up until birth. Today there is no consensus as to required MgSO₄ dose/duration for neuroprophylaxis as all published randomized trials have followed different dosing regimens.^{10–13} Some studies even indicate that long-lasting infusions of MgSO₄ may increase necrotizing colitis, gut perforations and perinatal mortality,^{60–62} problems that could be avoided by administration of only a bolus. In addition, MgSO₄ infusions require much more extensive monitoring of the mother than if only a bolus dose is provided which affects compliance for clinical implementation. Therefore, clinical studies are warranted to investigate whether providing only a bolus of MgSO₄ is an efficient mode of neuroprophylaxis with superior safety and compliance in women in preterm labor.

Furthermore, the understanding that MgSO₄ induced preconditioning protection of the immature CNS could lead to development of prophylactic treatment of other patients at high risk such as cases with high risk of intrapartum asphyxia or cases undergoing open heart surgery.

In conclusion, MgSO₄ induces strong preconditioning of the immature brain which provides resistance to HI- and excitotoxicity-mediated injury in rats and mice. Magnesium increases in the CNS and have previously been shown to modulate enzymatic activities, calcium homeostasis and NMDA receptor activity. These alterations are anticipated to provoke transcriptomal changes with modulation of neuroprotective and metabolic mRNAs and miRNAs. The metabolism will be downregulated and altered leading to reduced succinate toxicity, mitochondrial protection and slower loss of high energy phosphate reserves in the brain tissue after HI. Speculatively, these reactions contribute to attenuated ROS production and inflammation (Figure S3).

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Declaration of conflicting interests

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Authors' contributions

Gabriella Koning performed the animal experiments and tissue preparations, carried out the analyses, performed the statistical analysis and drafted the initial manuscript together with Henrik Hagberg. Anna-Lena Leverin participated in the design of the study, assisted in some of the experimental work, performed some of the tissue preparations, carried out analyses and reviewed and revised the manuscript. Syam Nair provided technical support and performed the measurements of mitochondrial respiration and ROS accumulation, assisted in interpretation of data, performed some of the bioinformatic evaluations and reviewed the manuscript. Leslie Schwendimann performed all experiments in the ibotenate model in PND 5 mice, participated in data interpretation and reviewed the manuscript. Joakim Ek interpreted data, performed some of the bioinformatic evaluations and reviewed the manuscript. Ylva Carlsson participated in data interpretation and reviewed and revised the manuscript. Pierre Gressens participated in data interpretation and reviewed and revised the manuscript. Claire Thornton participated in data interpretation, offered professional support in writing the manuscript and reviewed and revised it. Xiaoyang Wang participated in data interpretation and reviewed and revised the manuscript. Carina Mallard took part in designing the study, participated in data interpretation and reviewed and revised the manuscript. Henrik Hagberg conceptualized and designed the study, obtained funding, interpreted data and reviewed and revised the manuscript. All authors approved the final manuscript as submitted. None of the material included in this manuscript has been published or is under consideration elsewhere, including the Internet.

Supplementary material

Supplementary material for this paper can be found at the journal website: <http://journals.sagepub.com/home/jcb>

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