Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model

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Despite treatment with therapeutic hypothermia, almost 50% of infants with neonatal encephalopathy still have adverse outcomes. Additional treatments are required to maximize neuroprotection. Melatonin is a naturally occurring hormone involved in physiological processes that also has neuroprotective actions against hypoxic–ischaemic brain injury in animal models. The objective of this study was to assess neuroprotective effects of combining melatonin with therapeutic hypothermia after transient hypoxia–ischaemia in a piglet model of perinatal asphyxia using clinically relevant magnetic resonance spectroscopy biomarkers supported by immunohistochemistry. After a quantified global hypoxic–ischaemic insult, 17 newborn piglets were randomized to the following: (i) therapeutic hypothermia (33.5°C from 2 to 26 h after resuscitation, n= 8) and (ii) therapeutic hypothermia plus intravenous melatonin (5 mg/kg/h over 6 h started at 10 min after resuscitation and repeated at 24 h, n= 9). Cortical white matter and deep grey matter voxel proton and whole brain 31P magnetic resonance spectroscopy were acquired before and during hypoxia–ischaemia, at 24 and 48 h after resuscitation. There was no difference in baseline variables, insult severity or any physiological or biochemical measure, including mean arterial blood pressure and inotrope use during the 48 h after hypoxia–ischaemia. Plasma levels of melatonin were 10 000 times higher in the hypothermia plus melatonin than hypothermia alone group. Melatonin-augmented hypothermia significantly reduced the hypoxic–ischaemic-induced increase in the area under the curve for proton magnetic resonance spectroscopy lactate/N-acetyl aspartate and lactate/total creatine ratios in the deep grey matter. Melatonin-augmented hypothermia increased levels of whole brain 31P magnetic resonance spectroscopy nucleotide triphosphate/exchangeable phosphate pool. Correlating with improved cerebral energy metabolism, TUNEL-positive nuclei were reduced in the hypothermia plus melatonin group compared with hypothermia alone in the thalamus, internal capsule, putamen and caudate, and there was reduced cleaved caspase 3 in the thalamus. Although total numbers of microglia were not decreased in grey or white matter, expression of the prototypical cytotoxic microglial activation marker CD86 was decreased in the cortex at 48 h after hypoxia–ischaemia. The safety and improved neuroprotection with a combination of melatonin with cooling support phase II clinical trials in infants with moderate and severe neonatal encephalopathy.
Introduction

Perinatal hypoxic–ischaemic brain injury of the term infant remains a significant problem throughout the world. After 2 decades of laboratory studies (Thoresen et al., 1995; Bona et al., 1998), clinical trials (Edwards et al., 2010) and recent endorsement from regulatory bodies (NICE, 2010), therapeutic hypothermia is becoming standard clinical care for moderate to severe neonatal encephalopathy in the developed world. Therapeutic hypothermia, however, is only partially effective, with almost 50% of treated infants having adverse outcomes (Edwards et al., 2010). Previous studies have shown that it may be possible to augment neuroprotection of the developing brain using a combination of adjunct drugs with cooling, and thus reduce the number of affected infants (Liu et al., 2004; Jatana et al., 2006).

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring hormone secreted by the pineal gland in response to environmental light–dark cycles (Lynch et al., 1975; Lewy et al., 1980). Melatonin has many other biological functions in different organs and tissues (Reiter et al., 2010) that include defence against oxidative stress (Tan et al., 2007), balancing energy metabolism (Leon et al., 2004), enhancement of immune function (Altun and Ugur-Altun, 2007) and retarding ageing (Bubenik and Konturek, 2011). Melatonin acts through several different pathways to modulate the physiology and molecular biology of cells. Many actions are mediated through the well-characterized G protein-coupled melatonin receptors in cellular membranes (Brzezinski, 1997; Luchetti et al., 2010); other actions of melatonin involve its interaction with orphan nuclear receptors and other molecules such as calmodulin mediated through the well-characterized G protein-coupled melatonin receptors in cellular membranes (Brzezinski, 1997; Luchetti et al., 2010); other actions of melatonin involve its interaction with orphan nuclear receptors and other molecules such as calmodulin mediated actions of melatonin and its metabolites relate to its ability to detoxify reactive oxygen species (Tan et al., 2002). Intriguingly, the synthesis of melatonin may be inducible as a result of oxidative stress after brain injury (Seifman et al., 2008).

Melatonin has neuroprotective actions against hypoxic–ischaemic brain injury in animal models. In adult stroke models, delayed intravenous administration of melatonin enhances electrophysiological and neurobehavioural recoveries (Lee et al., 2004), reduces cortical and striatal infarct sizes (Pei et al., 2002, 2003; Lee et al., 2005) and reduces the intracerebral inflammatory response (Lee et al., 2007). Neuroprotection was seen with both single and multiple 5-mg/kg injections when commenced within 2 h of middle cerebral artery occlusion in adult rats (Pei et al., 2003). In foetal sheep, direct infusion of melatonin reduced inflammation and cell death in white matter after umbilical cord occlusion (Welin et al., 2007); neuroprotection was also seen in sheep foetuses when melatonin was administered to the mother at the time of asphyxia (Miller et al., 2005). As therapeutic hypothermia is already a routine therapy for neonatal encephalopathy, it was important to determine whether melatonin augments hypothermic neuroprotection. Therefore, the aim of this study was to assess neuroprotective effects of melatonin-augmented hypothermia compared with hypothermia alone after a global hypoxic–ischaemic insult in a piglet model of perinatal asphyxia, using intravenous melatonin administered 10 min after resuscitation combined with cooling. The treatment effect was explored using the following clinically relevant biomarkers: in vivo proton (1H) magnetic resonance spectroscopy (MRS) for lactate, N-acetyl aspartate, creatine (Cheong et al., 2006; Thayyl et al., 2010), 31P-MRS for inorganic phosphate, phosphocreatine and nucleotide triphosphate (NTP) (Azzopardi et al., 1989) and serial EEG and amplitude-integrated EEG (Toet et al., 1999). At the end of the 48-h survival period, histological assessment to quantify cell death was performed using transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) and cleaved caspase 3-positive cells. Microglial ionized calcium-binding adaptor molecule 1 (IBAI) antibody staining for immunohistochemistry and quantitative PCR for inflammatory markers were performed to reveal the extent of microglial activation.

Materials and methods

Animal experiments and surgical preparation

All animal experiments were performed under UK Home Office Guidelines [Animals (Scientific procedures) Act, 1986]. Seventeen male piglets aged <24 h, with a mean (SD) weight of 1.71 (0.12) kg (Table 1), were anaesthetized and surgically prepared as described previously (Lorek et al., 1994). Briefly, piglets were sedated with intramuscular midazolam (0.2 mg/kg), and arterial O2 saturation was monitored (Nonin Medical). Isoflurane anaesthesia (4% v/v) was initially given through a face mask to facilitate tracheostomy and intubation and was maintained (3% during surgery and 2% otherwise). Piglets were mechanically ventilated; ventilator settings were adjusted to maintain partial pressure of oxygen (PaO2) and carbon dioxide (PaCO2) at 8–13 kPa and 4.5–6.5 kPa, respectively, allowing for temperature correction of the arterial blood sample.

An umbilical venous catheter was inserted for infusion of maintenance fluids (10% dextrose, 60 ml/kg/day), fentanyl (3–6 µg/kg/h) and antibiotics (benzylpenicillin 50 mg/kg and gentamicin 2.5 mg/kg, every 12 h). An umbilical arterial catheter was inserted for continuous monitoring of heart rate and arterial blood pressure, and intermittent blood sampling was used to measure PaO2, PaCO2, pH, electrolytes, glucose and lactate (Abbot Laboratories). Bolus infusions of colloid (Gelofusin, B Braun Medical Ltd.) and inotropes maintained mean arterial blood pressure >40 mm Hg. All animals received continuous physiological monitoring (SA instruments) and intensive life support throughout experimentation. Arterial lines were maintained by infusing 0.9% saline solution (Baxter, 1 ml/h); heparin sodium was added at a concentration of 1 IU/ml to prevent line blockage.

Keywords: hypoxia–ischaemia; therapeutic hypothermia; neuroprotection; melatonin; neonatal encephalopathy

Abbreviations: HypoT = hypothermia alone group; HypoT + Mel = hypothermia plus melatonin group; IBA1 = ionized calcium binding adaptor molecule 1; MRS = magnetic resonance spectroscopy; NTP = nucleotide triphosphate; TUNEL = transferase-mediated deoxyuridine triphosphate nick-end labelling
Both common carotid arteries were surgically isolated at the level of the fourth cervical vertebra and encircled by remotely controlled vascular occluders (OC2A, In Vivo Metric). After surgery, piglets were positioned prone in a plastic pod, and the head immobilized securely in a stereotactic frame.

**Cerebral hypoxia–ischaemia**

Two MRI surface coils were secured to the head, and the animal was positioned into the bore of a 9.4-T Varian spectrometer for serial 1H and 31P MRS data acquisition. Whilst in the MRS system, transient hypoxia–ischaemia was induced by remote occlusion of both common carotid arteries, using inflatable vascular occluders; the fractional inspired oxygen was also reduced to 12%. During transient hypoxia–ischaemia, cerebral energetic changes were observed every 2 min by 31P-MRS, and the \( \beta \)-nucleotide triphosphate (NTP; mainly ATP) peak height was continuously monitored. Once the \( \beta \)-NTP peak height had fallen to 40% of baseline, fraction of inspired O\(_2\) was titrated to maintain the \( \beta \)-NTP peak height at 40% baseline value for 12.5 min. At the end of this 12.5-min period, the occluders were deflated and fraction of inspired O\(_2\) was normalized. 31P magnetic resonance spectra were acquired for a further 1 h to monitor recovery from hypoxia–ischaemia.

**Table 1 Physiological variables for the piglets in each group**

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Hypothermia (33.5°C) mean (SD)</th>
<th>Hypothermia + melatonin (5 mg/kg/h during 6 h every 24 h) mean (SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-natal age (h)</td>
<td>24.1 (0.4)</td>
<td>24.7 (0.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>1713 (138)</td>
<td>1722 (135)</td>
<td>0.89</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>158 (25)</td>
<td>151 (19)</td>
<td>0.48</td>
</tr>
<tr>
<td>End of insult</td>
<td>186 (22)</td>
<td>164 (21)</td>
<td>0.05</td>
</tr>
<tr>
<td>2–3.5 h after time zero</td>
<td>121 (13)</td>
<td>125 (18)</td>
<td>0.69</td>
</tr>
<tr>
<td>3.5–26 h after time zero</td>
<td>114 (22)</td>
<td>124 (26)</td>
<td>0.38</td>
</tr>
<tr>
<td>26–48 h after time zero</td>
<td>145 (24)</td>
<td>146 (23)</td>
<td>0.97</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>55 (5)</td>
<td>55 (10)</td>
<td>0.99</td>
</tr>
<tr>
<td>End of insult</td>
<td>54 (7)</td>
<td>50 (9)</td>
<td>0.38</td>
</tr>
<tr>
<td>2–3.5 h after time zero</td>
<td>43 (9)</td>
<td>41 (8)</td>
<td>0.62</td>
</tr>
<tr>
<td>3.5–26 h after time zero</td>
<td>47 (3)</td>
<td>44 (8)</td>
<td>0.42</td>
</tr>
<tr>
<td>26–48 h after time zero</td>
<td>50 (6)</td>
<td>45 (8)</td>
<td>0.23</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>38.4 (0.6)</td>
<td>38.5 (0.2)</td>
<td>0.61</td>
</tr>
<tr>
<td>End of insult</td>
<td>38.5 (0.5)</td>
<td>38.6 (0.3)</td>
<td>0.69</td>
</tr>
<tr>
<td>2–3.5 h after time zero</td>
<td>35.3 (0.7)</td>
<td>35.2 (0.7)</td>
<td>0.62</td>
</tr>
<tr>
<td>6–26 h after time zero</td>
<td>33.5 (0.1)</td>
<td>33.5 (0.1)</td>
<td>0.84</td>
</tr>
<tr>
<td>26–48 h after time zero</td>
<td>37.1 (0.3)</td>
<td>37.1 (0.3)</td>
<td>0.92</td>
</tr>
<tr>
<td>PaO(_2) (kPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>8.8 (2.7)</td>
<td>8.4 (3.4)</td>
<td>0.79</td>
</tr>
<tr>
<td>Nadir of the insult</td>
<td>4.1 (2.2)</td>
<td>3.0 (0.6)</td>
<td>0.27</td>
</tr>
<tr>
<td>12 h after time zero</td>
<td>10.1 (2.2)</td>
<td>8.0 (1.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>24 h after time zero</td>
<td>12.0 (7.2)</td>
<td>10.2 (2.3)</td>
<td>0.54</td>
</tr>
<tr>
<td>48 h after time zero</td>
<td>12.0 (7.2)</td>
<td>10.2 (2.3)</td>
<td>0.54</td>
</tr>
<tr>
<td>PaCO(_2) (kPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>5.0 (0.9)</td>
<td>5.2 (1.1)</td>
<td>0.67</td>
</tr>
<tr>
<td>Nadir of the insult</td>
<td>3.9 (1.4)</td>
<td>4.6 (0.7)</td>
<td>0.31</td>
</tr>
<tr>
<td>12 h after time zero</td>
<td>4.4 (1.5)</td>
<td>4.8 (1.3)</td>
<td>0.60</td>
</tr>
<tr>
<td>24 h after time zero</td>
<td>5.6 (1.3)</td>
<td>5.6 (2.4)</td>
<td>0.95</td>
</tr>
<tr>
<td>48 h after time zero</td>
<td>5.7 (2.9)</td>
<td>4.8 (1.1)</td>
<td>0.47</td>
</tr>
<tr>
<td>Haematocrit (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>22.9 (5.0)</td>
<td>22.4 (5.1)</td>
<td>0.86</td>
</tr>
<tr>
<td>Nadir of the insult</td>
<td>22.4 (6.6)</td>
<td>21.7 (4.6)</td>
<td>0.83</td>
</tr>
<tr>
<td>12 h after time zero</td>
<td>23.3 (6.2)</td>
<td>23.4 (4.8)</td>
<td>0.98</td>
</tr>
<tr>
<td>24 h after time zero</td>
<td>22.9 (6.6)</td>
<td>24.9 (7.4)</td>
<td>0.56</td>
</tr>
<tr>
<td>48 h after time zero</td>
<td>22.3 (9.2)</td>
<td>15.2 (5.8)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Time zero was the time of resuscitation and the end of the baseline measurements. Mean (SD) values are presented for the two groups: (i) HypoT (\( n=8 \)) and (ii) HypoT + Mel (\( n=9 \)). Heart rate and blood pressure (mean arterial blood pressure) data are omitted for 30 min after a saline/Geloplasma bolus was administered or after cardiac arrest. Analysis using a Mann-Whitney test indicated that there was no evidence of a difference between the two groups for any of the outcomes examined at any of the time points.
hypoxia–ischaemia and the first 60 min of resuscitation gave the magnitude of acute energy depletion, as described previously (Faulkner et al., 2011).

Pilot studies exploring the effect of melatonin doses on mean arterial blood pressure during therapeutic hypothermia

Before the main randomized study, we explored the effect of three different doses of melatonin on mean arterial blood pressure in the piglet after a hypoxic–ischaemic insult. Intravenous melatonin (20, 10 and 5 mg/kg/h over 6 h) was administered 10 min after hypoxia–ischaemia over 6 h, with cooling started at 2 h after hypoxia–ischaemia (as described later in the text).

**Experimental groups**

Baseline data were acquired before transient hypoxia–ischaemia but after stabilization of the animal in the MRS system. After resuscitation, piglets were randomized into two groups with intervention from 2 to 26 h: (i) hypothermia (33.5°C), n = 8 (HypoT) and (ii) hypothermia (33.5°C) + melatonin infusion of 5 mg/kg over 6 h starting 10 min and 24 h after hypoxia–ischaemia, n = 9 (HypoT + Mel). Cooling was achieved using a water mattress; piglets were cooled for 90 min to reach the target rectal temperature of 33.5°C, which was maintained for between 2 and 26 h after hypoxia–ischaemia.

Melatonin (Sigma) was dissolved in ethanol (2.5% final v/v) and saline in the dark shortly before administration to HypoT + Mel animals. The HypoT animals received vehicle ethanol (2.5% final v/v) and saline during the same period. At 26 h after hypoxia–ischaemia, piglets were rewarmed to normothermia at 0.5°C/h. Blood was sampled at 6-h intervals after the hypoxic–ischaemic insult.

**Magnetic resonance spectroscopy**

Magnetic resonance spectra were acquired as described previously (Faulkner et al., 2011), using a combination of a 6.5 × 5.5-cm elliptical receive surface coil tuneable to 1H and a 150-mm (Rapid Biospec) transmit volume coil with a separate elliptical transmit–receive coil tuned to 31P resonance frequency. Whole brain 31P magnetic resonance spectra were acquired using single-pulse acquisition with a repetition time of 10 s. Baseline spectra were acquired before, during and for the first 60 min of recovery after hypoxia–ischaemia (31P only), and thereafter at 24 and 48 h (Fig. 1). Point-resolved (PRESS) hypoxia–ischaemia MRS was acquired from two positions: deep grey matter embedded in paraffin wax and sectioned to 5-mm thickness and stained with 2% paraformaldehyde for 7 days. Coronal slices (5 mm thick) of the right hemisphere, starting from anterior to the optic chiasma, were embedded in paraffin wax and sectioned to 5-μm thickness and stained for haematoxylin and eosin. To assess cell death and glial activation, additional brain sections were stained for nuclear DNA fragmentation for haematoxylin and eosin. To assess cell death and glial activation, additional brain sections were stained for nuclear DNA fragmentation.

**Magnetic resonance spectroscopy analysis**

1H magnetic resonance spectra were analysed using the LC Model (Provencher, 1993). For each spectrum, the relative signal amplitudes of choline, total creatine, N-acetyl aspartate and lactate were measured. The primary 1H MRS outcome measures nominated a priori were lactate/N-acetyl aspartate and lactate/creatine. Metabolite peak/area under the curve ratios were plotted from baseline to 48 h after hypoxia–ischaemia for each voxel in each subject, on a logarithmic scale and analysed according to the randomized group.

31P magnetic resonance spectra were analysed by AMARES (advanced method for accurate, robust and efficient spectral fitting with the use of prior knowledge) (Vanhamme et al., 1997). Signal amplitudes and chemical shifts were measured for phosphocreatine, inorganic phosphate (three independent components fitted) and α-, β- and γ-NTP; the equivalent signal amplitude for exchangeable phosphate pool was also calculated. Metabolite peak-area under the curve ratios were plotted from baseline to 48 h after hypoxia–ischaemia for each voxel in each subject on a logarithmic scale and analysed according to the randomized group.

**Electroencephalography and amplitude-integrated electroencephalography**

After surgical preparation, multichannel EEG (Nicolet) monitoring (six-lead) was acquired at baseline and during the period between the MRS data acquisitions i.e. between 2–20, 25–40 and at 48 h after hypoxia–ischaemia. Filtered amplitude-integrated EEG recordings were classified according to the pattern classification (Hellström-Westas, 2005). A score of 0 was flat trace; 1, continuous low voltage; 2, burst suppression; 3, discontinuous normal voltage and 4, continuous normal voltage, at baseline, 3, 6, 12 and then every 12 h after hypoxia–ischaemia, thereafter noting seizure activity and duration. Classification was omitted within 6 h of phenobarbitone treatment. The area under the curve of the amplitude-integrated EEG category was used for statistical comparison using a 2-tailed t-test. To further compare groups, a discrete threshold of 0.35 was used in a χ² test.

**Sample size**

The primary outcome, the change in area under the curve for lactate/N-acetyl aspartate from baseline to 48 h, was used to calculate the sample size. Previous work with our model suggested that the change in lactate/N-acetyl aspartate during 48 h varied between normo- and hypothermic groups by 1.0 U, with a standard deviation of 0.75 U (both log scale). Assuming similar magnitude of additional effect for melatonin-augmented hypothermia (versus hypothermia alone) and with 5% significance and 80% power, nine subjects would be required in each group.

**Brain histology**

At 48 h after hypoxia–ischaemia, piglets were euthanized with pentobarbital; the brain was fixed through cardiac perfusion with cold 4% paraformaldehyde in PBS, dissected out and post-fixed at 4°C in 2% paraformaldehyde for 7 days. Coronal slices (5 mm thick) of the right hemisphere, starting from anterior to the optic chiasma, were dehydrated in xylene (3 × 10 min) and rehydrated in graded
Figure 1 ¹H and ³¹P MRS shows that melatonin reduces metabolic changes after a hypoxic–ischaemic insult in the neonatal piglet. (A–F) Time course changes for the lactate/creatine (A and D), N-acetyl aspartate/creatine (B and E) and lactate/N-acetyl aspartate (C and F) ratios in the thalamic voxel, in animals treated with hypothermia alone (HypoT, A–C), and those cotreated with melatonin (HypoT + Mel, D–F) in the 48 h after a hypoxic–ischaemic insult (0 h is end of insult). The data points are plotted on a semi-logarithmic scale, and each trace of data points represents an individual animal. Note in the HypoT group the increase of lactate/creatine (A) and lactate/N-acetyl aspartate (C) ratios in four of eight animals, and a decrease in N-acetyl aspartate in three of eight animals (B). These changes were abrogated in the HypoT + Mel group. (G–L) ³¹P-MRS time course for inorganic phosphate/exchangeable phosphate pool (G and J), phosphocreatine/exchangeable phosphate pool (H and K) and NTP/exchangeable phosphate pool (I and L) in the whole forebrain voxel in HypoT (G–I), and HypoT + Mel piglets (J–L). In ³¹P-MRS, the HypoT group shows three of six animals had increasing inorganic phosphate/exchangeable phosphate pool (G) and reductions in phosphocreatine/exchangeable phosphate pool (H) and NTP/exchangeable phosphate pool (I). These changes were abrogated in the HypoT + Mel group. (M–O) Summary of the area under the curve data (mean ± SEM) for the thalamic ¹H (M), the white matter ¹H (N) and the ³¹P whole forebrain (O) metabolite ratio time courses. In the case of decreasing ratios (N-acetyl aspartate/creatine, phosphocreatine/exchangeable phosphate pool and NTP/exchangeable phosphate pool), the bars represent the area over the curve algorithm. Red bars show data for the HypoT group, blue bars for the HypoT and Mel group, mean ± SEM. *P < 0.05 in t-test. Cr = creatine; ePP = exchangeable phosphate pool; HI = hypoxia–ischaemia; Lac = lactate; NAA = N-acetyl aspartate; PCr = phosphocreatine; PI = inorganic phosphate; TH = thalamic; WM = white matter.
ethanol solutions (100–70%), followed by double-distilled water. For TUNEL, the sections were pretreated for 15 min in 3% H2O2 in methanol to remove endogenous peroxidase, followed by a 15-min peptidase predigestion with 20 μg/ml proteinase K (Promega) at 65°C, and then incubated at 37°C for 2 h with the TUNEL solution (Roche) containing biotinylated dUTP. For immunohistochemistry, the sections were processed for antigen retrieval (800-mW microwave irradiation in 0.1 M citrate buffer, 10 min), followed by overnight incubation with primary rabbit antibody against activated caspase 3 (1:500) (Abcam) or IBA1 (1:1000) (Wako), and then 2-h incubation with a biotinylated secondary goat anti-rabbit immunoglobulin antibody (1:100, Jackson Laboratory). The biotin residues were detected with the avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) and visualized with diaminobenzidine/H2O2 (Sigma), with CoCl2 and NiCl2 included to intensify TUNEL histochemistry. The sections were counterstained with haematoxylin, dehydrated in graded alcohol and xylene and mounted with Depex (VWR), or alternatively, mounted in mounting media (Vector Labs), to facilitate total cell number counts during analysis of Iba1 and activated caspase 3.

All analyses were made by an investigator blind to the treatment group. The regions studied are shown in Fig. 2. For each animal, section and region, TUNEL-positive nuclei were counted in three fields (at ×40 magnification, with an area of 0.76 mm²), and the average converted into counts per mm². The less numerous activated caspase 3 immunoreactive cells were counted in two fields of view at ×20 magnification, and cell number was adjusted to total DAPI-positive cell number. Activated caspase 3 was counted in the periventricular white matter, caudate nucleus, thalamus and in the parasagittal cortex and midtemporal cortex (insular region) containing the superficial, middle and deep cortical layers. The IBA1 counts were made in the periventricular white matter, caudate nucleus and thalamus at ×40 in two fields of view within each region and adjusted to total DAPI-positive cell number.

The threshold for statistical significance was P < 0.05. For statistical analysis in individual brain regions, original counts of TUNEL were normalized by log(x+1) algorithm conversion (Werner et al., 2001), and the differences between the two groups detected using 1-way ANOVA, followed by post hoc Tukey test. Cell number adjusted to total cell density for IBA1 and activated caspase 3 was assessed using a Student t-test. Trend analysis was performed across all seven forebrain regions, again using 1-way ANOVA followed by Tukey test. Statistical significance of the R² value in correlation plots was assessed using the F-test.

**Isoprostanates, neuroprostanates, isofluranes and neuroflurans**

Fresh cortical tissue was harvested from an area close to the right hemisphere periorlanel cortex through a burr hole just after termination, and 100-mg brain tissue was immediately frozen in liquid nitrogen and stored at −80°C until analysis. Isoprostanes, neuroprostanes, isofluranes and neurofurans were determined using gas chromatography/mass spectrometry with negative ion chemical ionization (NICI) technique (Milne et al., 2007). To allow extraction of lipids, the tissue was added to ice-cold Folch solution and then homogenized with a blade homogenizer. For sample purification, we used a C-18 and a Silica Sep-Pak® cartridge and thin-layer chromatography. For the gas chromatography/mass spectrometry, samples were injected into the preheated liner at 280°C; a helium carrier gas was used as a flux in a column of 1.8 mbar and housed in a thermostatic chamber at 190°C. For quantification purposes, we compared the derivatized IsoP (m/z 569), NeuroP (m/z 593), isoflurane (m/z 585) and neurofurane (m/z 609) with the height of the deuterated internal standard peak (m/z 573). Concentrations were expressed in ng/g of tissue.

**Quantitative reverse transcription polymerase chain reaction**

Fresh brain tissue collected for analysis of isoprostanes was used for qualitative reverse transcription polymerase reaction. Sample preparations, primer design and PCR protocol were similar to that previously described (Favrais et al., 2007). Primers were designed specifically using the Sus scrofa (white pig) Ensembl database, and primer sequences and accession numbers are given in Supplementary Table 2. The reference genes 14-3-3 protein zeta/delta (YWAHZ) and ribosomal protein L4 (Rpl4) were chosen to standardize all quantitative experiments (Cinar et al., 2012). For each duplicate sample, we calculated the specific ratio of the gene of interest/reference gene and averaged the duplicate values and adjusted to the average value observed in naïve piglets to give the final data point.
Plasma melatonin assay

Plasma melatonin concentrations were determined by radioimmunoassay (Claustrat et al., 1984). Melatonin was isolated from plasma using diethyl-ether extraction. Because the standard curve ranged from 3 to 2400 ng/l, the highest melatonin concentrations required dilution in a phosphate albumin buffer solution. The intra-assay coefficients of variation were <7% between 30 and 200 pg/ml, and the inter-assay coefficients of variation were 8.7 and 7.9% (n = 18) for melatonin concentrations of 55 and 115 pg/ml, respectively.

Results

Pilot study of melatonin doses combined with hypothermia

A dose of 5 mg/kg/h over 6 h was safe in a pilot study of three doses of melatonin combined with hypothermia. As the clinical use of therapeutic hypothermia in babies is associated with increased use of inotropes (Battin et al., 2009) and can potentially lead to hypotension in some animals (Kerenyi et al., 2012) we wanted to assess any interaction between the combination of hypothermia with melatonin. A melatonin dose of 10 mg/kg/h or higher was associated with hypotension and a requirement for saline boli and inotropic support to maintain the mean arterial blood pressure normal; 5 mg/kg/h melatonin was tolerated well in our model (see Supplementary material and Supplementary Fig. 1).

Physiological measures

There were no intergroup differences in body weight, post-natal age, baseline physiological (heart rate and mean arterial blood pressure) and biochemical measures (blood lactate, base excess and glucose) (Table 1 and Supplementary Table 1). Insult severity qualified with triphosphate levels and PaO2 and PaCO2 were similar between groups during hypoxia–ischaemia, and the time taken to reach target rectal temperature after hypoxia–ischaemia hypothermic induction was similar between the groups. Heart rate at the end of the insult was lower in the HypoT+Mel group than in the HypoT group (P = 0.05).

During the cooling induction (2–3.5 h), maintenance (3.5–26 h) and rewarming/normothermia (26–48 h) periods, mean heart rate, mean arterial blood pressure, blood chemistry and electrolytes (pH, lactate, base excess and glucose) were similar between the groups (Table 1). Median total (saline and Geloplasma volume (ml/kg) and dopamine use (µg/kg/min) were similar between groups. Inotropic support was required in both groups (dobutamine: 1/8 versus 2/9; adrenaline: 1/8 versus 3/9 9 for HypoT and HypoT+Mel groups, respectively); however, there were no significant differences between the groups (Table 2).

One piglet in the melatonin group suffered a cardiac arrest due to mechanical obstruction of the tracheal tube at 46 h but was resuscitated successfully; no piglets died prematurely.

Table 2 Average total fluid replacement and inotrope infusion for the piglets in each group during 48 h after insult

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Hypothermia (33.5 °C) median (IQR)</th>
<th>Hypothermia + melatonin (5 mg/kg/h during 6 h every 24 h) median (IQR)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline bolus ml/kg</td>
<td>0 (0, 17)</td>
<td>0 (0, 16)</td>
<td>0.65</td>
</tr>
<tr>
<td>Geloplasma bolus ml/kg</td>
<td>0 (0, 28)</td>
<td>30 (0, 34)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total ml/kg</td>
<td>17 (0, 37)</td>
<td>30 (17, 51)</td>
<td>0.26</td>
</tr>
<tr>
<td>Dopamine (µg/kg/min)</td>
<td>6.9 (2.5, 9.6)</td>
<td>5.3 (4.9, 6.0)</td>
<td>0.74</td>
</tr>
<tr>
<td>Dobutamine (µg/kg/min)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0.52</td>
</tr>
<tr>
<td>Adrenaline (ng/kg/min)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Median (IQR) values for saline and Geloplasma boluses and inotrope infusion are presented for the two groups. Analysis using a Mann-Whitney test revealed no evidence of a statistically significant difference between groups for fluid replacement or inotrope usage.
aspartate or a decrease in N-acetyl aspartate/creatine. All nine animals showed lactate/creatine and lactate/N-acetyl aspartate ratios of <1 (Fig. 1D and F, \( P < 0.05 \) in \( \chi^2 \) test comparison with the HypoT group), and N-acetyl aspartate/creatine ratios >0.8 (generally \( \sim 1.5 \)) (Fig. 1E, \( P = 0.055 \) in \( \chi^2 \) test). The choline/creatine ratios were unchanged (Supplementary Fig. 2I and J).

A similar divergence in the appearance of animals with lactate overshoot or drop in N-acetyl aspartate was also present in the \(^{1}H\)-white matter voxel (Supplementary Fig. 2A–F) and \(^{31}P\)-whole brain data (Fig. 1G–L). In the HypoT group, four of eight animals (piglets 148, 153, 161 and 162) showed an increase of >1 for lactate/creatine (Supplementary Fig. 2A) and lactate/N-acetyl aspartate (Supplementary Fig. 2C) and a drop <0.8 for N-acetyl aspartate/creatine (Supplementary Fig. 2B). In the HypoT + Mel group, none of the nine had an increase in lactate/creatine (Supplementary Fig. 2D), and one of nine had an increase in lactate/N-acetyl aspartate (Supplementary Fig. 2F) and a drop in N-acetyl aspartate/creatine (Supplementary Fig. 2E). The difference between the numbers for lactate/creatine and lactate/N-acetyl aspartate in the HypoT + Mel group was because of piglet 155, which showed a subthreshold increase in lactate/creatine but also a pronounced additional drop in N-acetyl aspartate/creatine levels in its white matter voxel.

In the case of \(^{31}P\)-MRS, three of six HypoT animals showed an increase of inorganic phosphate/exchangeable phosphate pool >75% (Fig. 1Q), and a drop of phosphocreatine and NTP <15% (Fig. 1H and I, respectively), compared with none of eight in the HypoT + Mel group (Fig. 1J–L, \( P = 0.024 \) in \( \chi^2 \) test). Piglet 155 again showed an increase in inorganic phosphate/exchangeable phosphate pool and a drop in phosphocreatine/exchangeable phosphate pool compared with its peer group, but these changes did not reach the thresholds described earlier in the text.

As a next step, the overall changes in biomarker outcome over 48 h were assessed using the area under the curve algorithm for increasing (lactate/creatine, lactate/N-acetyl aspartate, inorganic phosphate/exchangeable phosphate pool) and decreasing (N-acetyl aspartate/creatine, phosphocreatine/exchangeable phosphate pool, inorganic phosphate/exchangeable phosphate pool) biomarker ratios. In both cases, these outcome parameters increased with more pronounced brain metabolite change and showed a strong positive correlation with histological evidence of brain injury (Faulkner et al., 2011). A summary of all outcome areas under the curve and area over the curve parameters is shown for \(^{1}H\)-MRS in the thalamic (Fig. 1M) and white matter (Fig. 1N) voxels and for \(^{31}P\)-MRS in the whole forebrain voxel (Fig. 1O).

Compared with the HypoT group, combined treatment with HypoT + Mel was associated with a significant overall decrease in thalamic lactate/creatine and lactate/N-acetyl aspartate area under the curve (\( P < 0.05 \), t-test). All other hypoxia–ischaemia–affected outcome parameters (inorganic phosphate/exchangeable phosphate pool area under the curve, N-acetyl aspartate/creatine area over the curve, phosphocreatine/exchangeable phosphate pool area over the curve, NTP/exchangeable phosphate pool area over the curve) showed smaller values in the HypoT + Mel group, but these did not reach statistical significance.

Histology

The brain regions assessed are shown in Fig. 2. Representative haematoxylin and eosin histology, microglial IBA1 immunohistochemistry, TUNEL at low and high magnification and activated caspase-3 are shown in Fig. 3. In routine haematoxylin and eosin stains, compared with the naïve animals (Fig. 3A and D), the HypoT group revealed neural injury that was particularly pronounced in the caudatoputamen and thalamus, with extensive vacuolation of the neuropil (Fig. 3B) and, at high magnification, frequent pyknotic and karyorrhexis nuclear profiles (Fig. 3E). Quantification of the TUNEL-positive cells showed significant decreases in the HypoT + Mel group compared with the HypoT group for four forebrain regions—periventricular white matter, internal capsule, caudate nucleus and putaen (\( P < 0.05 \)) (Fig. 4A). Quantification of the number of activated caspase 3-positive cells per total (DAPI-positive) cell number revealed a reduced density of cells expressing this cell death marker in the thalamus (Fig. 4B). Immunostaining for IBA1 in brain microglia revealed a trend towards more microglial clustering (e.g. in the caudate nucleus in Fig. 3H), but there was no significant difference in the overall density of the IBA1-positive cell counts (Fig. 4C).

Gene expression analysis

In addition to direct counts of microglia (IBA1-positive), inflammatory mediators were assessed using quantitative PCR in seven Hypo and seven Hypo + Mel animals; expression of inducible nitric oxide synthase, CD86, insulin-like growth factor (IGF1), arginase 1 (ARG1), sphingosine kinase (SphK1) and suppressor of cytokines 3 (SOCS3) is shown in Fig. 4D. There was an increase in SphK1 gene expression in the HypoT + Mel group compared with the HypoT-only group (\( P < 0.05 \)). Expression of CD86 was decreased (\( P < 0.05 \), as was expression of SOCS3 (\( P < 0.05 \)), but expression of IGF1, ARG1 or inducible nitric oxide synthase was not altered.

Electroencephalography

At baseline, the raw EEG trace was active with an amplitude-integrated EEG bandwidth of 10–50 \( \mu \)V. The placement of EEG leads is shown in Fig. 5A. Typically, 3 h after hypoxia–ischaemia, cortical activity was suppressed (<5 \( \mu \)V) in all but a few animals (compare Fig. 5C at 3 h with Fig. 5B before insult). These were the same animals as those with adverse MRS bioenergetics (piglets 148, 153, 161 and 162). A score of 0 was given for a flat trace, 1 for continuous low voltage, 2 for burst suppression, 3 for discontinuous normal voltage and 4 for continuous normal voltage (Fig. 5B–E). As shown in Fig. 5G, most of the recovery took place before 36 h, with half maximal return occurring at \( \sim 20 \) h. The HypoT + Mel subgroup appeared to recover amplitude-integrated EEG somewhat more rapidly than the cooling-only group, but this did not reach statistical significance for any of the individual time points. Further analysis using the area under the curve parameter (Fig. 5H) for the HypoT group showed a dichotomy, with three animals revealing ‘good’ recovery (i.e. in the range of 2–3) (piglets 149, 150 and 160) and five with very poor recovery of <0.35 (piglets 145, 148, 153, 161 and 162). The HypoT + Mel subgroup demonstrated a more even distribution (Fig. 5H).
Figure 3 (A–R) Cotreatment with melatonin decreases histological damage, 48 h after hypoxia–ischaemia. For comparison, naïve tissue is shown in the left column. Each column shows representative sections from the same animal in the HypoT (middle column) and HypoT + Mel groups (right column) from the caudate nucleus (A–O) or thalamus (P–R). The tissue is stained for haematoxylin and eosin (H&E) histology at low (A–C) and high (D–F) magnification, immunohistochemistry for microglial IBA1 (G–I), TUNEL at low (J–L) and high (M–O) magnification and activated caspase 3 (P–R). In the HypoT animal, there is extensive vacuolation in the areas of the caudate nucleus near the ventricle (B), associated with pronounced nuclear pyknosis and karyorrhectic fragmentation (E), microglial activation and clustering in the caudate and the overlying internal capsule (H), a large number of TUNEL-positive cells (K and N) and numerous activated caspase 3 cells in the thalamus (Q). These changes are ameliorated in the HypoT + Mel animal. cdt = caudate nucleus; ic = internal capsule; v = cerebral ventricle. The blue arrows in E point to the karyorrhectic nuclear profiles, the asterisks to the cells shown at higher magnification in the inserts (rotated in D and E). Scale bars: A–C, G–L = 1 mm; D–F = 50 μm; D–F inserts = 10 μm; M–R = 0.2 mm.
**Isoprostanes, neuroprostanes, isofluranes and neurofurans**

Because one effect of melatonin treatment is to reduce cellular oxidative stress (Reiter et al., 2003; Welin et al., 2007), we assessed the effects on brain levels of isoprostanes, neuroprostanes, isofluranes and neurofurans as markers of this form of cellular stress. As shown in Fig. 6, however, there was no significant difference in the 48-h brain concentration of isoprostanes, neuroprostanes, isofluranes or neurofurans between groups.

**Pharmacokinetics**

Concentrations of melatonin in plasma were determined before surgical intervention (basal levels) and at 6-h intervals after the hypoxic–ischaemic insult. These melatonin data were available for
all nine piglets that were injected with melatonin, and for six of the eight treated with hypothermia alone. In both groups (HypoT and HypoT + Mel), the basal concentrations before surgical intervention were in the range of 0.05–0.8 μg/l, with no significant difference between the two groups (HypoT 0.10 ± 0.01 μg/l versus HypoT + Mel 0.18 ± 0.03 μg/l) (mean ± SEM). As shown in Fig. 7A and C, the HypoT + Mel piglet group (injected with melatonin twice, at 10 min and at 24 h) showed particularly high levels at 6 h and again at 30 h, ranging between 17 and 31 mg/l, with a gradual decline over the following 18 h.

Linear regression analysis of plasma levels for each individual piglet in the 6–24-h and in the 30–48-h intervals, using semi-logarithmic plot and least squares fit, revealed a decrease of −0.017 ± 0.005/h (mean ± SEM) for the first, and a very similar decrease of −0.016 ± 0.005/h (mean ± SEM) for the second interval.

Figure 5 Effect of melatonin cotreatment on amplitude-integrated EEG. (A) Position of recording amplitude-integrated EEG leads. (B–F) Amplitude-integrated EEG bandwidth analysis (top) and continuous EEG recordings (bottom) in case of continuous normal voltage (B), flat trace (C), continuous low voltage (D), burst suppression (E) and discontinuous normal voltage (F). (G) Amplitude-integrated EEG time course for the hypothermia alone (red dots) and hypothermia plus melatonin groups (blue dots) as mean ± SEM. Most recovery takes place before 36 h, in both groups. (H) Distribution of individual amplitude-integrated EEG responses using area under the curve parameter; each dot represents a single animal. Note the dichotomous distribution (three high, five very low) in the hypothermia alone group, and the appearance of intermediates in the group cotreated with melatonin. aEEG = amplitude-integrated EEG
HypoT = hypothermia group; HypoT + Mel = hypothermia plus melatonin group.
value of $-0.018 \pm 0.003/\text{h}$ for the second, later interval. The average decrease (first and second interval) was $-0.017 \pm 0.004/\text{h}$, corresponding to an average half-life for plasma melatonin of $21.6 \pm 3.3 \text{h}$.

In neonatal piglets treated with hypothermia alone (Fig. 7B), plasma melatonin values at every time point 6–48 h after hypoxia–ischaemia were on average 6–15 times higher than the basal levels, before surgical intervention ($P < 0.01$, paired $t$-test).

### Discussion

This study in newborn piglets demonstrates that 5 mg/kg/h melatonin administered intravenously 10 min after the end of transient hypoxia–ischaemia over 6 h and repeated at 24 h augments hypothermic neuroprotection based on improved cerebral energy metabolism using $^1\text{H}$ MRS biomarkers (deep grey matter lactate/$N$-acetyl aspartate and lactate/creatine). Melatonin-augmented hypothermia was also associated with increased levels of cerebral ATP (whole brain $^{31}\text{P}$ MRS NTP/exchangeable phosphate pool). Correlating with improved cerebral energy metabolism, TUNEL-positive nuclei were reduced in the melatonin-augmented hypothermia group compared with hypothermia alone in the thalamus, white matter, internal capsule, putamen and caudate, and there was reduced activated caspase 3 in the thalamus. The addition of 5 mg/kg/h over 6 h of intravenous melatonin to hypothermia at 10 min and 24 h after hypoxia–ischaemia did not alter the heart rate or mean arterial blood pressure, need for inotropes, blood electrolytes, or glucose and lactate levels during or after treatment.

We monitored brain metabolism using MRS biomarkers that are known to change after hypoxia–ischaemia in the piglet (Lorek et al., 1994; Penrice et al., 1997), serve as linking biomarkers in infants with neonatal encephalopathy (Robertson et al., 1999; Cheong et al., 2006), and are currently used as a surrogate outcome measure in clinical neuroprotection trials for neonatal encephalopathy (Azzopardi, 2011). Specifically, high levels of lactate/$N$-acetyl aspartate on thalamic MRS in neonates at 48 h are predictive of poorer 12–18-month neurodevelopmental outcome (Robertson et al., 1999; Cheong et al., 2006; Thayyl et al., 2010). As shown in Fig. 1, the addition of melatonin to hypothermia abrogated the MRS lactate/$N$-acetyl aspartate and lactate/creatine increases in the forebrain/thalamic voxel. This increase in MRS lactate/$N$-acetyl aspartate occurred in only 50% of HypoT-treated piglets; this is not surprising as therapeutic hypothermia itself compared with normothermia preserves energy metabolism after transient hypoxia–ischaemia in piglets (Penrice et al., 1997). We also observed a higher ATP level in the melatonin-augmented cooling group compared with the cooling-only group using $^{31}\text{P}$ MRS. Higher ATP on $^{31}\text{P}$ MRS in infants with hypoxic–ischaemic brain injury is associated with better long-term outcome in clinical studies (Azzopardi et al., 1989). We also used continuous EEG monitoring as another measure of cell bioenergetics and as this is also a predictor of outcome after hypoxia–ischaemia (Toet et al., 1999; Thoresen et al., 2010). We observed that melatonin decreased the number of piglets with a poor recovery after hypoxia–ischaemia (area under the curve $<0.35$), and increased those with an intermediate outcome (area under the curve $>0.35$).

Figure 6 (A–C) Effect of melatonin treatment on isoprostane (IP), neuroprostane (NP), neurofurans (NF) and isoflurane (IF) brain levels 48 h after insult. Mean $\pm$ SEM for naïve (green bars, $n = 2$), HypoT (red bars, $n = 8$) and HypoT + Mel (blue bars, $n = 7$) groups, respectively. None of the four neurochemicals affected by oxidative stress showed a significant difference between groups.

Figure 7 Melatonin pharmacokinetics. (A–C) Concentrations in blood plasma at the basal level (bsl) and 6–48 h after hypoxic–ischaemic insult. (A) Mean $\pm$ SEM levels for the HypoT alone (red circles, $n = 6$ animals) and HypoT + Mel (blue circles, $n = 9$ animals). (B and C) Time course data for individual HypoT (D) and HypoT + Mel (E) animals. Green arrows in A and C indicate intravenous melatonin injection. In B, the interruptions in individual traces were due to missing time points. Note that in B the $y$-axis is in $\mu\text{g}/\text{l}$ and in C the $y$-axis is in $\text{mg}/\text{l}$.

bsl = baseline; HI = hypoxia–ischaemia.
Melatonin treatment led to elevated plasma levels of melatonin for 48 h compared with baseline, and the average half-life for plasma melatonin in the piglets was 21.6 ± 3.3 h. This is considerably longer than reported in normothermic rodent studies (20–30 min) (Cheung et al., 2006); this is likely to be due to the effects of hypothermia to slow pharmacokinetic clearance of melatonin (van den Broek et al., 2010). An endogenous increase in plasma melatonin levels (6–15 times higher) was observed in the hypothermia-only group after hypoxia–ischaemia. This response to injury has also been reported in humans after adult traumatic brain injury (Seifman et al., 2008). It has been demonstrated that this melatonin release is a critical endogenous neuroprotective mechanism against hypoxia–ischaemia brain injury using pinealectomized and melatonin-supplemented adult rats (Manev et al., 1996; Joo et al., 1998).

The precise level of melatonin needed for neuroprotection is unknown; previous studies in other acute global or focal hypoxia–ischaemia models have shown neuroprotection with a wide range of melatonin doses (1.5–50 mg/kg) and timing (hours or days before or after hypoxia–ischaemia) (Reiter et al., 2004). We chose 5 mg/kg/h melatonin for safety reasons based on our pilot study using 5, 10 or 20 mg/kg/h over 6-h melatonin combined with cooling in the piglet hypoxia–ischaemia insult model (Supplementary material and Supplementary Fig. 1). A melatonin dose of 10 mg/kg/h or higher was associated with hypotension and a requirement for saline bolus and isotropic support to maintain a normal mean arterial blood pressure; 5 mg/kg/h over 6 h melatonin did not alter the need for support or any physiological variable. This dose (5 mg/kg/h over 6 h repeated at 24 h) is similar to that already used safely in sick newborn infants, where up to 100 mg of melatonin during 72 h has been administered safely (Gitto et al., 2001, 2004; Fulia et al., 2005). We administered melatonin at 10 min after hypoxia–ischaemia; further studies are needed to determine the therapeutic window for melatonin neuroprotection. However, rodent studies, which are more suited to exploring therapeutic time windows, have shown that the therapeutic window for a single melatonin dose is 4 h (Husson et al., 2002), and this increases to 24 h with five melatonin doses 24 h apart (P. Gressens, unpublished).

Receptor and non-receptor pathways underpin the multiple neuroprotective effects of melatonin that include supporting mitochondrial function and post-lesional plasticity and its anti-oxidant, anti-apoptotic and anti-inflammatory actions (Husson et al., 2002; Leon et al., 2004; Klici et al., 2005; Tan et al., 2007; Welin et al., 2007; Gressens et al., 2008). This study was designed to assess the preclinical safety and efficacy of melatonin to augment hypothermia and not specifically its mechanism of action. However, aspects of this study suggest melatonin may act to maintain cellular energetics and modulate neuroinflammation.

In the present study, cerebral ATP levels are protected by melatonin treatment, indicating a non-receptor-mediated effect of melatonin directly on the electron transport chain (Leon et al., 2004). Melatonin also prevented the increase in lactate/N-acetyl aspartate in the reperfusion phase (increased lactate/N-acetyl aspartate is indicative of compromised or damaged mitochondria) (Pennice et al., 1997; Kristián, 2004; Moffett et al., 2007); this effect may be due to melatonin’s actions to prevent compromise of the mitochondrial membranes (Andrabi et al., 2004; Wang, 2009). Such positive effects on the mitochondria and melatonin’s ability to increase expression of anti-apoptotic proteins (Ling et al., 1999) likely play a role in reducing the TUNEL and activated caspase 3 cell death in the melatonin-augmented hypothermia group.

Melatonin treatment in this study failed to reduce microglial cell number but decreased (to levels seen in naïve animals) expression of CD86, a prototypical marker of cytotoxic microglial activation, due to its co-stimulatory role with major histocompatibility complex Class II (Allison, 1994). Inducible nitric oxide synthase is also a marker of a cytotoxic microglial phenotype (Block et al., 2007), but we observed no change in its expression due to melatonin, as levels were already possibly reduced by hypothermia to levels seen in the naïve subjects. This is in agreement with the anti-inflammatory effects of hypothermia that have been previously reported (Lynch et al., 1975; Yenari and Han, 2012). Previous observations in normothermic hypoxia–ischaemia injury models have shown that melatonin treatment is anti-inflammatory (Welin et al., 2007; Koh, 2008; Villapol et al., 2011). Melatonin treatment increased expression of SphK1, which is produced (at least in part) by microglia suggested to be in a deactivated state (Colton, 2009). SphK1 activation is capable of decreasing proinflammatory cytokine production, increasing proliferation and decreasing cell death (Hughes et al., 2008). Melatonin-augmented hypothermia also altered expression of the immunomodulatory cytokine SOCS3 produced by microglia (Qin et al., 2007). In rodents, SOCS3 is increased after brain injury (Raghavendra Rao et al., 2003), and melatonin has previously been demonstrated to reduce SOCS3 expression (Tsai et al., 2011). Altogether these data suggest that melatonin-mediated neuroprotection may act in part by modulating microglial phenotype.

Melatonin-augmented hypothermia had no effect on brain isoprostanes at 48 h after hypoxia–ischaemia compared with hypothermia alone. This is in contrast to previous observations after melatonin treatment in normothermic hypoxia–ischaemia injury models where serum measurements of 8-isoprostanate were reduced at 6 h after hypoxia–ischaemia in those sheep foetuses administered melatonin (Welin et al., 2007). The absence of any effect of melatonin on isoprostanes in our study may be due to the rise in reactive oxygen species occurring at time points well before 48 h when our cerebral tissue was harvested or due to hypothermia already maximally reducing reactive oxygen species (Yenari and Han, 2012). Indeed, when comparing the hypothermia-only group with naïve brain, there was no difference (Fig. 6).

The piglet model of hypoxia–ischaemia used in the current study has strong preclinical value, as it allows hypothermia to be applied as it would in a clinical setting, and an intensive care level that is on par with a neonatal intensive care unit. This includes supported ventilation, maintenance of cardiac output, blood volume and electrolytes, cardiac resuscitation (if required) and continuous EEG monitoring. These studies have been designed in such a way that they are ‘proof of principle’, i.e. constructed to maximize
the demonstrable therapeutic effect. Thus, in the current study, piglets were subjected to a severe hypoxia–ischaemia insult, and intervention with melatonin was started at 10 min of completing hypoxia–ischaemia; this would be difficult to perform in the clinic. We initiated cooling 2 h after hypoxia–ischaemia, which is in line with recent suggested protocols for hypothermia (Kendall et al., 2010), although sooner than in randomized clinical trials (Gluckman et al., 2005; Shankaran et al., 2005; Azzopardi, 2010). Cooling for 24 h is shorter than the current clinical protocol of 72 h (Azzopardi et al., 2009), but the neuroprotective efficacy of this regime has previously been validated in this piglet model (O’Brien et al., 2006).

Melatonin’s multiple neuroprotective actions, its ability to penetrate the brain and organelles (Acuña-Castroviejo et al., 2001) and lack of toxicity in humans (Rybakowski et al., 1995) and animals (Jahnke et al., 1999) make it an attractive neuroprotective agent. Melatonin is relatively easy to administer by intravenous (Cheung et al., 2006), oral (Mistraletti et al., 2010) and rectal (Weishaupt et al., 2006) routes. In our study, melatonin-augmented hypothermia was safe and provided significant neuroprotection compared with hypothermia alone in this piglet perinatal asphyxia model. Augmented neuroprotection was demonstrated by improved cerebral energy metabolism on MRS biomarkers and reduced cell death across the brain. These data along with the safety profile of melatonin suggest the consideration of phase I and II clinical studies of melatonin-augmented therapeutic hypothermia for neonatal encephalopathy.

Acknowledgements

We thank Aron Kerenyi and Dorottya Kelen for their help with the experimental series, Kevin Broad for data analysis, Fabrizio Priettoi, Mariangela Longini and Giuseppe Buonocore (Sienna, Italy) for the isoprostane study, Mrs Odile Blanchard (INSERM, France) for her helpful technical assistance with the melatonin pharmacokinetic study and Sophie Lebon (INSERM, Paris) for the work on quantitative PCR for the inflammatory markers.

Funding

The study was funded by Wellbeing of Women, UK. This work was undertaken at University College Hospital/University College London, which received a proportion of funding from the UK Department of Health’s National Institute for Health Research Biomedical Research Centres funding scheme.

Supplementary material

Supplementary material is available at Brain online.

References


