Protective Effects of Melatonin in Corpus Callosum Astrocytes During Ischemia in Transgenic Mice

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ABSTRACT

Objective: Stroke is a leading cause of adult death and disability worldwide. Around 80% of all stroke cases are classed as ischemic stroke, which affects both gray and white matter brain regions. During ischemia, reactive oxygen species are generated and initiate multiple damaging processes such as lipid peroxidation, mitochondrial dysfunction, blood–brain barrier damage and brain edema. Melatonin is a potent antioxidant which can act as a direct oxygen species scavenger. Melatonin can also act at receptors (melatonin receptor 1 and 2), which are expressed in the nervous system cells including astrocytes.

Methods: Using live cell imaging of transgenic mice, the viability of astrocytes in the corpus callosum of adult brain sections was monitored during a standard 60 minutes period of modeled ischemia (oxygen-glucose deprivation) and 30 minutes of reperfusion in 8 groups; Control, artificial cerebrospinal fluid + Melatonin, oxygen-glucose deprivation, oxygen-glucose deprivation + Melatonin, oxygen-glucose deprivation + Melatonin + Luzindole (a nonselective melatonin receptor antagonist), oxygen-glucose deprivation + Luzindole, oxygen-glucose deprivation + Melatonin + propionamidotetralin (a selective melatonin receptor 2 antagonist), and oxygen-glucose deprivation + propionamidotetralin.

Results: Addition of melatonin significantly reduced the level of astrocyte death during oxygen-glucose deprivation from 71.94% ± 1.45 to 37.84% ± 1.9; p < 0.0001. This protective effect was blocked by luzindole or propionamidotetralin. Following known scoring categories for glial injury, ultrastructural morphology confirmed the protective effect of melatonin against acute ischemic injury in the white matter glial cells.

Conclusion: Melatonin is a promising neuroprotective agent during white matter ischemia.

Keywords
Melatonin; Ischemia; Astrocyte.
INTRODUCTION

Ischemic stroke remains the leading cause of disability and the second most common cause of death in the world[1,3]. Ischemic stroke affects both the white and gray matter of the central nervous system (CNS). In white matter, ischemic lesions can damage both axons and glial cells, including astrocyte, oligodendrocyte, and microglial cells[4,5]. Any failure of cerebral blood supply, the underlying pathogenic origin of stroke, causes energy deprivation and dysregulation of membrane potential and ionic homeostasis[6]. This results in excitotoxicity, wherein the excitatory neurotransmitter glutamate is over-secreted, producing a cytotoxic influx of Ca²⁺, Na⁺, and K⁺ mediated by N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors[6,7]. As a result, acute cell death and oxidative stress ensue[6]. The brain is highly sensitive to damage by oxidative stress and reactive oxygen species (ROS) due to its high oxygen consumption, high fat content, and relatively lower level of antioxidant enzymes[7]. Melatonin is a neurohormone secreted from the pineal gland and plays a wide range of regulatory roles in the mammalian body such as the regulation of circadian rhythm[8]. It is also reported that melatonin is a potent antioxidant agent and has shown promising results as a therapeutic intervention in ischemic studies[3,6,7,9,10]. Melatonin receptors (MT1 and MT2) are widely distributed in the CNS[6]. Melatonin receptors (MT1 and MT2) are strongly expressed on astrocytes[11,12]. Astrocytes play a crucial role in ischemic brain injury, regulating ions, neurotransmitters, and blood flow; they also aid in synaptic remodeling following transformation into reactive astrocytes[13]. Targeting astrocytes in the penumbra region, area around the ischemic core, is of great potential as a therapeutic intervention for stroke patients and may limit infarct size and further damage. Corpus callosum involvement in the infarcted area of stroke has been reported in several clinical studies[14,15]. The aim of the current study is to examine the effect of melatonin on adult corpus callosum astrocytes during an oxygen glucose deprivation (OGD) model of ischemia in transgenic mice, focusing on the role of MT1 and MT2 receptors.

MATERIALS AND METHODS

Cell Imaging

A total of 38 Transgenic (green fluorescent protein (GFP)/glial fibrillary acid protein (GFAP)) adult mice (~3 months old and ~90g in weight) were used following Plymouth University Animal Welfare and Ethical Review Board and UK home office rules and regulations. Mice were divided into 8 groups; Control, aCSF + Melatonin, OGD, OGD + Melatonin, OGD + Melatonin + Luzindole, OGD + Luzindole, OGD + Melatonin + PDOT, and OGD + PDOT. Time-scale of different experimental conditions are clarified in Table 1.

After sacrificing and dissection, the brain was washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) holding artificial cerebrospinal fluid (aCSF) solution and immediately immersed in 2% agarose. The slicing chamber of a Leica VT1200S vibrating blade microtome (Leica Microsystems GmbH, Wetzlar, Germany) was filled with cold HEPES holding aCSF solution and surrounded with dry ice. Sections of brain were taken with a thickness of 200 μm. Brain slices were carefully transferred using a plastic pipet into warmed (~37°C) oxygen-bubbling HEPES holding aCSF solution in a brain slice keeper for a 1-hour recovery period, which helped to prevent tissue edema (See Table 1). The brain sections were then transferred to the chamber and held in place by an anchor and cell imaging was started.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Color Code</th>
<th>0-10 Min of Experiment</th>
<th>11-70 Min of Experiment</th>
<th>71–100 Min of Experiment</th>
<th>Drug Application</th>
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<tr>
<td>Control</td>
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<td>OGD</td>
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<td>OGD + Melatonin</td>
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<td>OGD + Melatonin + Luzindole</td>
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* 10 μM Melatonin is applied to all stages.
# 10 μM Luzindole is applied to all stages.
● 10 μM 4-PDOT is applied to all stages.

using MetaMorph® Microscopy Automation and Image Analysis Software. Only one brain section was used from each mouse. Temperature was carefully controlled (~37°C) throughout the experiment via a fluid flow controller (Warner Instrument Corp., Hamden, CT) and an objective lens controller (Bioptechs Inc., Butler, PA). Brain slices were continuously perfused with aCSF at a rate of 2 ml/min and the gas flow rate at 1 l/min. During the first 10 min, aCSF, 95% O₂/5%CO₂ was perfused followed by 60 min of either aCSF, 95% O₂/5%CO₂, or OGD, 95% N₂/5%CO₂, followed by 30 minutes reperfusion, aCSF, 95% O₂/5%CO₂, at the end, for a total of 100 min per experiment. Images were collected every 1 minute of the experiment by Olympus inverted confocal microscope with a 60x oil-immersion objective and cells in any area of corpus callosum were counted by ImageJ. All materials were supplied from Sigma-Aldrich Corp., (St. Louis, MO USA) unless stated otherwise.

**Solutions**

All solutions (HEPES, aCSF, and OGD) were bubbled for at least 1 hour before use to ensure adequate pH buffering and carbogen saturation⁴, ¹⁶. The pH (~7.4) and osmolarity (~308) were calibrated via pH meter and osmometer, respectively.

**Electron Microscope (EM)**

After the 100-minute procedure described above, brain sections were carefully cut, leaving only the corpus callosum, which was immediately immersed in 3% glutaraldehyde at pH 7.4. This step was followed by several steps of washing, dehydration, and finally embedding in resin⁴, ¹⁶-¹⁸. Three grids of different brain sections were used to collect the micrographs of different glial cells. Following the well-established scoring system for glial used to collect the micrographs of diff erent glial cells.

**Statistical Analysis**

GraphPad Prism v7.04 was used to analyse the data. Data were presented as Mean ± SEM. One-way ANOVA and post hoc Tukey test were used to confirm statistical differences between groups in which p < 0.05 were considered significant.

**RESULTS**

**Cell Imaging of GFAP-GFP Astrocytes in Adult Mouse Corpus Callosum**

The viability of astrocytes was monitored via live cell imaging of GFP-expressing astrocytes in the adult mouse corpus callosum brain sections. Typical representative images of GFP-expressing astrocytes are shown in Figure 1. GFP(+) astrocytes in perfused adult mouse corpus callosum brain sections were imaged at 20-min intervals over a 100-min period. Control experiment (only aCSF) show relatively stable astrocytes morphology throughout the 100 minutes of the experiment (Fig. 1 A). Similar results are seen in the presence of melatonin with no decline in GFP(+) astrocyte viability (Fig. 1 B). In contrast to the control aCSF perfusion, OGD evoked severe progressive decline in the number of GFP(+) astrocyte present in the slice, indicative of cell lysis and loss of the GFP into the extracellular space¹⁹ (Fig. 1 C). GFP(+) astrocyte loss during OGD was relatively less when melatonin was added (Fig. 1 D).

**Ischemic Injury to Astrocytes in Adult Corpus Callosum Brain Sections**

A quantitative analysis of the degree of astrocyte death from live-cell imaging of GFP-expressing astrocytes showed a significant increase in astrocyte death after 60 minutes of OGD and 30 minutes of reperfusion (71.94% ± 1.45, N = 6 brain sections) compared with the control (3.13% ± 3.12, N = 4 brain sections; p < 0.0001; Fig. 2).

Astrocytes started to die during the first 10 minutes of OGD and continued to die until the end of reperfusion (Fig. 3). Control experiments show a limited degree of late onset astrocyte lysis, starting 90 min into the experiment.

**Melatonin Decreases Astrocyte Lysis During OGD through MT1 and MT2 Receptors**

Addition of 10 μM melatonin to the aCSF during the 100-min perfusion did not significantly change the percentage of astrocyte lysis compared to the control (2.78% ± 2.8, N = 4 brain sections; Fig. 2). However, 10 μM melatonin significantly decreased the total percentage of astrocyte lysis during OGD (37.84% ± 1.9, N = 6 brain sections; p < 0.0001; Fig. 2), a 34.1% reduction compared to OGD with no melatonin. Application of 10 μM luzindole, a nonsel ective melatonin receptor (MT1/MT2) antagonist²⁰,²¹, with and without melatonin during OGD significantly increases the percentage of astrocyte death (67.85% ± 1.45, N = 5 brain sections; 71.19% ± 1.88, N = 4; p < 0.0001, respectively) compared to OGD with melatonin (Fig. 2).

Addition of 10 μM PDOT (4-phenyl-2-propionamidotetralin), a selective MT2 melatonin receptor antagonist²⁰,²¹, with and without melatonin during OGD significantly increased the percentage of astrocyte death (69.45% ± 3.1, N = 5; 67.3% ± 2.06, N = 4; p < 0.0001, respectively) compared to OGD with melatonin (Fig. 2).

Focusing on the time-scale of astrocyte lysis during the experiment, we found that astrocyte death during OGD in the presence of melatonin followed the OGD-only pattern during the 60 minutes of ischemia, but continuing in a plateau during the 30 minutes of reperfusion (Fig. 3). Both antagonists show a similar pattern of astrocyte death onset as seen in the OGD-only experiments (Fig. 3).
FIGURE 1.
Live GFP imaging of corpus callosum astrocytes. Four series of images at successive 20 min collected from adult GFAP-GFP mouse corpus callosum sections. The time series are arranged in descending order. (A) aCSF; in the control images, a number of astrocytes (dotted red circles) can be seen at 0 min and remain unchanged over the 100 minutes time course of the tracking. A similar finding is apparent in (B) aCSF + 10 μM melatonin. In the OGD series (C), astrocytes apparent at 0 min disappear over time as they lyse and release GFP into the extracellular space (arrows). During OGD + 10 μM melatonin (D) there is a reduced level of astrocyte death with time compared to OGD. Scale bar = 25 μm.
FIGURE 2.
Percentage of astrocyte death in adult GFAP-GFP mouse corpus callosum sections exposed to various conditions (minimum of four sections per condition, n = astrocyte number). A significant astrocyte death is seen in OGD vs. control; OGD + melatonin vs. OGD; and all OGD + melatonin receptor antagonists vs. OGD + melatonin (* p < 0.0001).

FIGURE 3.
Time-scale of astrocyte lysis onset under various conditions plotted at 5-minute intervals (minimum of four brain sections per condition). Ischemia (red line at the top of the graph) is applied to all experiments (except control and aCSF + melatonin) from 10 min to 70 min of the total experiment time.
FIGURE 4.
Electron-ultramicrographs demonstrating the ultrastructural morphology and injury scoring of a glial cell in adult GFAP-GFP mouse corpus callosum. (A, B) This glial cell shows healthy intracellular organelles; nucleus (N), mitochondria (arrowheads), and Golgi apparatus (bold arrow). The white box in (A) is magnified in (B). The glial cell has an injury score of “0”.

(C, D) This glial cell shows appearance of small intracellular vacuoles (arrows) and normal intracellular organelles; nucleus (N) and mitochondria (arrowheads). The white box in (C) is magnified in (D). The glial cell has an injury score of “1”.

(E, F) This glial cell shows appearance of small intracellular vacuoles (arrows) and swollen intracellular mitochondria (arrowheads). Note that cell membrane is still intact. The white box in (E) is magnified in (F). The glial cell has an injury score of “2”.

(G, H) This glial cell shows destroyed cell membrane and swollen mitochondria (arrowheads) around the nucleus (N). The white box in (G) is magnified in (H). The glial cell has score “3". Scale bar = (A, G) 5 μm, (B, C, E, H) 2 μm, and (D, F) 1 μm.
**Melatonin Improves Ischemic Scoring System of Glial Injury**

The ultrastructural morphology of OGD-mediated injury was studied in the corpus callosum of adult GFP-GFAP mice. Following the glial scoring system of ischemic injury described by Constantinou and Fern[17], glial cells were classified into four scores. Score 0 indicates glial cells that look healthy, with intact cell membrane and intracellular organelles and no signs of injury (Fig. 4A, 4B). Score 1 is characterized by the presence of small intracellular vacuoles with intact intracellular organelles (Fig. 4C, 4D). Score 2 is characterized by the appearance of either large cytoplasmic vacuoles or swollen intracellular organelles or both (Fig. 4E, 4F). Score 3 is characterized by cell membrane destruction, which can be produced by either large vacuoles or frank necrosis (Fig. 4G, 4H).

Corpus callosum glial cells (astrocytes, oligodendrocytes, and microglia) in control, OGD, aCSF and melatonin, and OGD and melatonin conditions were examined, which revealed different levels of glial injuries (Fig. 5A). In the OGD experiment, 60 minutes of ischemia followed by 30 minutes of reperfusion was found to significantly increase the mean injury score of all glial cells in adult mouse corpus callosum sections.

**FIGURE 5.**

(A) Mean injury scores of glial cells in electron-ultramicrographs of control, aCSF + 10 μM melatonin, OGD, and OGD + 10 μM melatonin conditions. *p < 0.0001 vs. control and † p < 0.01 vs. OGD; n = number of cells. (B) Electron-ultramicrographs scoring for ischemic glial injury in control, aCSF + 10 μM melatonin, OGD, and OGD + 10 μM melatonin.
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(2.44 ± 0.12, n = 32 cells; p < 0.0001) compared with the control (0.87 ± 0.12, n = 49 cells) (Fig. 5 A). More precisely, OGD produced glial injury at different levels of approximately 0% (score 0), 12.5% (score 1), 31.25% (score 2), and 56.25% (score 3) compared to the control, which produced glial injury of around 38.78% for score 0, 40% for score 1, 14.28% for score 2, and only 6.12% for score 3 (Fig. 5 B). Addition of melatonin did not significantly alter the mean injury score of glial cells in adult mouse corpus callosum (0.97 ± 0.17, n = 41 cells) compared to the control (Fig. 5 A). Glial injury in aCSF with melatonin was found to be approximately 48.78% for score 0, 19.51% for score 1, 17.07% for score 2, and 14.63% for score 3 (Fig. 5 B). In contrast, addition of melatonin to the OGD condition significantly reduced the mean injury score of glial cells in adult mouse corpus callosum (1.72 ± 0.16, n = 40; p < 0.01). More precisely, melatonin with OGD produced glial injury of around 15.38% for score 0, 28.20% for score 1, 28.20% for score 2, and 30.76% for score 3.

**DISCUSSION**

Stroke is a leading cause of adult death and disability worldwide; around 80% of the cases are ischemic stroke[1-24]. During ischemia, excess ROS are generated and contribute to injury[25-28]. Melatonin is a potent antioxidant molecule that has been reported to have a protective effect during ischemia[9,10,29]. Melatonin receptors (mainly MT1 and MT2) are widely distributed in the mammalian CNS, hippocampus, caudate putamen, suprachiasmatic nucleus (SCN), paraventricular nucleus, periventricular nucleus, supraoptic nucleus, substantia nigra, mamillary bodies, retinas, nucleus basalis of Meynert, nucleus accumbens, and tuberomammillary nucleus[30-32]. Both MT1 and MT2 are found on neurons and glial cells[33-35]. These receptors are strongly expressed on astrocytes[36-37]. Acute ischemia evoked rapid and irreversible astrocyte injury[38-40]. White matter astrocytes are reported to be markedly more vulnerable to ischemic injury than gray matter astrocytes[41]. Astrocytes are pivotal responders during traumatic brain injury, playing important roles in neuronal tissue repair and synaptic functions[42]. Thus, astrocytes protection may significantly improve function outcomes in stroke.

**Protective Effect of Melatonin on Adult Mouse White Matter Astrocytes**

The current study focused on the acute ischemic injury / perfusion effect on adult mouse white matter corpus callosum brain section GFP(+) astrocytes. A significant increase in astrocyte death is shown during OGD compared to the control. During OGD, astrocytes started to die as early as 10 minutes of ischemia, followed by continuous gradual increase throughout the 60 minutes of ischemia and 30 minutes of reperfusion. This is consistent with previous work showing a progressive decrease in astrocyte viability over the course of ischemia[43]. Melatonin significantly reduced the rate of astrocyte death during the 60 minutes of OGD and 30 minutes of reperfusion. These results support those of previous work showing that melatonin is protective to glial cells in general during middle cerebral artery occlusion (MCAO) in a mouse model[44]. On the other hand, another study reported ineffectiveness of melatonin in astrocyte culture during OGD[45]. However, it is clearly reported that in vitro model of ischemia is not the optimal model to study ischemia due to uncontrollable extracellular space[34]. In the current study, astrocyte death plateaus during the 30-minute reperfusion period (Fig. 3). This highlights the protective effect of melatonin especially during the reperfusion period after ischemia. It is known that there is an increase in ROS production during reperfusion, which exacerbates ischemic injury[39]. It is widely accepted that melatonin has a direct free radical scavenger effect[46]. This effect explains the plateau phase of astrocyte cell death during reperfusion and highlights the potent effect of melatonin during the reperfusion period after stroke. The protective effect of melatonin is blocked by luzindole, nonselective melatonin receptor (MT1/MT2) antagonist, and PDOT, a selective MT2 melatonin receptor antagonist. This provides further evidence of the protective effect of melatonin on adult mouse white matter astrocytes through both MT1 and MT2 receptors.

**Melatonin Protects Glial Cells at the Ultrastructural Level**

Sixty minutes of OGD followed by 30 minutes of reperfusion significantly increased the mean injury score of glial cells in adult mouse white matter compared to the control. However, melatonin significantly protects glial cells and decreased the mean injury score of glial cells in adult mouse white matter compared to OGD. However, in an attempt to study the effect of melatonin on astrocyte ultrastructure, melatonin non-significantly decreased the mean astrocyte injury. This may have been due to the difficulty of identifying all astrocytes in stage 3 frank necrosis as no cytoplasm is left to identify glial filaments of astrocytes.

**CONCLUSION**

These findings provide compelling evidence of the protective effect of melatonin, especially during reperfusion periods after ischemia. This highlights the importance of combining melatonin at the time of thrombolysis or thrombectomy when used as an interventional procedure to remove blood vessel obstruction during stroke.

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**Conflict of Interest**

The author has no conflict of interest.
Disclosed: The author did not receive any type of commercial support either in forms of compensation or financial for this study. The author has no financial interest in any of the products or devices, or drugs mentioned in this article.

Ethical Approval
Obtained.

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MT1 and MT2 are decreased in Alzheimer’s disease. Eur J Histochem. 2006; 50(4): 311-316.


التأثيرات الوقائية للميلاتونين في الخلايا النجمية في الكوربس كالوزم خلال نقص التروية في الفناران المعدلة وراثيا

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المستخلص.

الأهداف: السكتة الدماغية هي السبب الرئيسي لوفيات البالغين والإعاقة في جميع أنحاء العالم، وتصنف حوالي 80% من جميع حالات السكتة الدماغية على شكل سكتة إقفارية، مما يؤثر على مناطق الدماغ في المادة الرمادية والبيضاء، وخلال نقص التروية يتم توليد أنواع الأكسجين التفاعلية والشروع في عمليات متعددة ضارة مثل بروكسيد الدهون، وخلال في الميتوكيندريا وضرر في الحاجز الدموي الدماغي وثورم الدماغ، و الميلاتونين هو أحد مضادات الأكسيدة القوية التي يمكن أن تكون بمثابة المخلص المباشر للأكسجين، كما يمكن أن يؤثر الميلاتونين أيضاً في المستقبلات (مستقبلات الميلاتونين 1 و 2)، والتي تتواجد في خلايا الجهاز العصبي بما في ذلك الخلايا النجمية.

الطريقة: باستخدام تصوير الخلايا الحية من الفناران المعدلة وراثيا، تم رصد بقاء الخلايا النجمية في الكوربس كالوزم في الدم خلال فترة 60 دقيقة من نقص التروية (حرمان الجلوكوز والأكسجين) و 30 دقيقة من التروية. تم تجهيز مجموعات وهي: المجموعة المرجعية، السائل المحيطي الشوكيك + ميلاتونين، نفس الجلوكوز والأكسجين، نفس الميلاتونين، نفس الجلوكوز والأكسجين + ميلاتونين، نفس الجلوكوز والأكسجين + لوتيند، نفس الجلوكوز والأكسجين + لوتيند + أوروبروبيوميدوتيرالين، نفس الجلوكوز والأكسجين + لوتيند + أوروبروبيوميدوتيرالين + ميلاتونين.

النتائج: إضافة الميلاتونين أدى إلى خفض كبير في مستوى موت الخلايا النجمية خلال الحرمان من الجلوكوز والأكسجين من 0.71% إلى 0.13% بالمائة، والقيمة الإحتمالية = 0.001. تم اقتحام هذا التأثير الوظيفي بواسطة لوتيند، وهو مضاد عام لمستقبلات الميلاتونين، أوروبروبيوميدوتيرالين، وهو مضاد لمستقبلات الميلاتونين 2 وباستخدام تصنيف معروف تقييم الخلايا حسب درجة الضرر، أدى هذا التصنيف التأثير الوقائي للميلاتونين ضد الإصابة الإقفارية الحادة في خلايا المادة البيضاء بالدماغ.

الخلاصة: الميلاتونين هو عامل واعد للوقاية أثناء نقص التروية في المادة البيضاء في الدماغ.