Research Article

Melatonin receptor-mediated attenuation of excitotoxic cell death in cultured spinal cord slices

Azizul Haque[1], Donald C. Shields[2], Arabinda Das[2], Abhay Varma[2], Russel J. Reiter[3], Naren L. Banik[1,2,4*]

[1]Department of Microbiology and Immunology, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, USA
[2]Department of Neurosurgery, Medical University of South Carolina, 96 Jonathan Lucas St., Charleston, SC 29425, USA
[3]Department of Cell Systems and Anatomy, UT Health, Long School of Medicine, San Antonio, TX, USA
[4]Ralph H. Johnson Veterans Administration Medical Center, 109 Bee St, Charleston, SC 29401 Charleston, SC, USA
*Correspondence: baniknl@musc.edu, Tel: +1 843-792-7594

Running title: Melatonin receptor-mediated neuroprotection

Received: November 17, 2020, Accepted: April 17, 2021

ABSTRACT

Recent studies suggest *ex vivo* modeling of neuronal injury is a robust approach for the mechanistic study of neurodegeneration. Melatonin, an indolamine, is a versatile molecule with antioxidative, antiapoptotic, neuroprotective, and anti-inflammatory properties. While melatonin has been studied as a therapeutic agent for spinal cord injury (SCI) related neuronal cell loss, its actions in organotypic slice cultures approximating SCI effects are less well understood. The actions of melatonin were therefore examined following exposure of cultured rat spinal cord slices to glutamate excitotoxicity. Exposure to glutamate (500 μM) for 4 hours induced neuronal degeneration that was prevented by 0.5 μM melatonin (applied immediately or 4 hours following glutamate exposure). Decreased internucleosomal DNA fragmentation, Bax:Bcl-2 and calpain:calpastatin ratios, caspase 8, 9 and 3 activities in slice cultures were measured following melatonin treatment. Melatonin receptor (MTR1, MTR2) mRNA levels were increased in the melatonin treated spinal cord slices. To confirm melatonin receptor-mediated protection, slice cultures were treated with 10 or 25 μM luzindole (melatonin receptor antagonist) at 0 and 4 hours, respectively, after glutamate exposure. Luzindole significantly decreased the ability of melatonin to prevent cell death in the sliced culture model. These results suggest melatonin receptors may provide a pathway for therapeutic applications to prevent penumbral neuron loss following SCI.

Key words: apoptosis, calpain, glutamate, melatonin, motoneuron, slice culture

INTRODUCTION

Spinal cord injury (SCI) can cause severe disability, neurological incapacitation, and even death. Following the initial spinal cord parenchymal damage, secondary processes including inflammation, glial scar formation, ischemia and oxidative stress contribute to neuronal cell death in adjacent tissues (penumbra) just outside the primary injury site (umbra) hours to days after the initial insult (1, 2). Neuronal death in this setting is associated with glutamate excitotoxity and increases in free intracellular calcium levels (3, 4). Intracellular calcium levels are typically well controlled by the neuron, such that calcium fluxes during cell depolarization last only a few seconds to minutes, with no pernicious cellular effects. When intracellular calcium levels significantly exceed the normal range, pro-apoptotic proteases such as calcium activated neutral proteinase (calpain) and caspases are overactivated (5). Calpain (a non-lysosomal cysteine protease) selectively cleaves cytoskeletal proteins and myelin proteins without extensive degradation; it also activates pro-apoptotic factors including calcineurin, caspase-3, -8, -9 and Bax (6-10). The ubiquitous classic isoforms μ- and m-calpain require 3-50 μmol/L and 400-800 μmol/L calcium concentrations, respectively, for half-maximal activity *in vitro* (11). Mouse μ-calpain knockout models survive with defective platelet function, but the m-calpain knockout is lethal with disruption of embryogenesis (12). Moreover, in the presence of increased intracellular calcium levels, calpain activity is poorly regulated by the endogenous inhibitor, calpastatin (13). Neurons exposed to toxins *in vitro*, and those near the injury site in rodent SCI models, exhibit increased calpain activity; however, apoptosis in these neurons is significantly reduced in the presence of administered calpain inhibitors (5, 14-18).

To prevent these cascading events following such injuries, multiple calpain inhibitors and anti-inflammatory agents have been tested following SCI. One such agent, melatonin, reduces both inflammation and glutamate-mediated intracellular calcium influx (19-21). As an indoleamine, melatonin (N-acetyl-5-methoxytryptamine) is naturally produced from tryptophan in the pineal gland and other mammalian tissues. Beyond known effects on the sleep cycle, melatonin reduces lipid peroxidation under numerous environmental conditions. In addition to being a direct free radical scavenger, it also stimulates other antioxidants such as glutathione reductase, superoxide dismutase, peroxidase and catalase (19, 20, 22, 23). Likewise, melatonin has been found to reduce apoptosis in the central nervous system following injury (24). Melatonin's neuroprotective effects following traumatic SCI and ischemic injury likely involve, at least in part, an interaction with its membrane receptors since the neuroprotective effects are substantially decreased when these receptors are blocked.

The purpose of the present study was to determine whether melatonin is protective against glutamate toxicity, a commonly used agent that simulates the excitotoxicity and oxidative stress that occurs in ageing or injured neurons. Utilizing slice cultures of rodent spinal cord, we found melatonin to be protective against glutamate-induced cytotoxicity. Moreover, this protection was enhanced by inhibition of calpain and caspase activities. The use of a melatonin receptor antagonist, luzindole, blocked melatonin's protective effects. These data suggest that melatonin's membrane receptors play a central role in mediating neuroprotection in the central nervous system following cytotoxic injury.

2. MATERIALS AND METHODS

2.1. Slice culture preparation.

Spinal cord slices were prepared from 5-7-day old Wistar rats using a modified method as described (25). After anesthetizing the newborn rats with Vetbutal (pentobarbital; Sigma-Aldrich, St. Louis, MO), they were ice-cooled, placed into 70% alcohol solution and decapitated with scissors; the trunk was quickly removed and placed on the operating table. The vertebral column was cut from the dorsal side with a scalpel. The spinal canal was opened, and the limbs were fixed to the table with syringe needles. Using a scalpel blade, the spinal roots were carefully incised, and the spinal cord was dissected. The spinal cord was removed with two metal microspatulas, and it was immersed in ice-cold HBSS (Gibco, Dublin, Ireland). To obtain the slices, the cord was placed on the McIlwain tissue chopper and cut into 400 μm slices and 1.5-2.0 cm long slices. The slices were transferred onto the Millicell-CM (MilliporeSigma, Burlington, MA) membranes and three-four slices on each were used. The Millicell-CM membranes containing the spinal cord slices were maintained in 6-well plates that were preequilibrated with 1 mL of culture medium (pH 7.2, 50% DMEM, 10 mM HEPES, 25% HBSS), 25% horse serum (Gibco, Dublin, Ireland), 2 mmol/L L-glutamine, 5 mg/mL glucose, 1% amphotericine B, and 0.4% penicillin-streptomycin. The cultures were maintained in a regular, 25% horse serum medium to be gradually replaced (from DIV 4th until 7th) by SF, defined-solution-based medium. The SF medium contained DMEM/F12, 10 mM HEPES, 25% HBSS, 2 mmol/L L-glutamine, 5 mg/mL glucose, 1% amphotericin B and 0.4% penicillin-streptomycin, N2A (1 : 10; Gibco, Dublin, Ireland), and B27 (1 : 100; Gibco, Dublin, Ireland) supplements. The cultures were maintained in humid conditions with 5% CO_2 at 36°C for 4-5 weeks. Slices were exposed to: (a) 500 μM L-glutamic acid (LGA) plus 0.5 μM melatonin with or without 25 μM Luzindole for 4 hours, and then incubated in LGA-free medium for 48 hours; and (b) 500 μM L-glutamic acid (LGA) for 4 hours, and then incubated in LGA-free medium for 48 hours in the presence of 0.5 μM melatonin with or without 25 μM Luzindole to examine the biochemical features of cell death.

2.2. Analysis of DNA Fragmentation.

Genomic DNA fragmentation was then analyzed after treatments by agarose gel electrophoresis of DNA isolated from spinal cord tissue, as reported previously (26). Gels were stained with ethidium bromide (1 μg/ml), destained in water, and photographed on a UV (303 nm) transilluminator using Alpha Innotech (San Leandro, CA).

2.3. Analysis of mRNA Expression.

Extraction of total RNA, reverse transcription-polymerase chain reaction (RT-PCR), and agarose gel electrophoresis were performed as described previously (27). All primers for the RT-PCR experiments were designed using Oligo software (National Biosciences, Plymouth, Minn). The level of β-actin gene expression served as an internal control (Table 1).

2.4. Caspase-8, -9 and -3 Colorimetric Assays.

Measurement of caspase-3, caspase-8 and caspase-9 activity was performed using a commercially available Caspase Assay Kit (Sigma-Aldrich, St. Louis, MO).

2.5. Statistical Analysis.

Results obtained from different treatments were analyzed using StatView software (Abacus Concepts, Berkeley, Calif). Data were expressed as mean standard deviation (SD) of separate experiments (n=3) and compared by 1-way analysis of variance (ANOVA) followed by the Fisher post hoc test. Significant differences between control (Con) and LGA or luzindole treatments were indicated by * $P \leq 0.05$ or ** $P \leq 0.01$. Significant difference between LGA and melatonin post-treatment + LGA was indicated by #$P < 0.05$. Significant difference between control and melatonin treatments was indicated by • $P \leq 0.05$ or •• $P \leq 0.01$.

Table I. Sequences and sizes of primers used for reverse transcriptase polymerase chain reaction (RT–PCR).

Gene	Primer sequences	Product size (bp)
β-actin	F: 5′-TAC AAC CTC CTT GCA GCT CC-3′	630
	R: 5′-GGA TCT TCA TGA GGT AGT CTG TC-3′	
MT1	F: 5′-GAA GCT CAG GAA CTC AGG GAA-3′	251
	R: 5′-AAC GGT TCA TAG CGA TCC CC-3′	
MT2	F: 5′-TGC GGA ACG CAG GTA ATT TG-3′	167
	R: 5′-CCA ATG ACA CTC AGG CCC AT-3′	
m-calpain	F: 5′-GGG CAG ACC AAC ATC CAC CTC AGC AAA AAC-3′	404
	R: 5′-GTC TCG ATG CTG AAG CCA TCT GAC TTG AT-3′	
Calpastatin	F: 5′-AGT AGT TCT GGA CCC AAT G-3′	230
	R: 5′-CCC CAG TAG ACT TCT CTT TC-3′	
Bax	F: 5′-GCA GGG AGG ATG GCT GGG GAG A-3′	352
	R: 5′-TCC AGA CAA GCA GCC GCT CAC G-3′	
Bcl-2	F: 5′-GGA TGA CTT CTC TCG TCG CTA C-3′	255
	R: 5′-TGC AGA TGC CGG TTC AG-3′	

3. RESULTS

Slice cultures from rat spinal cords were evaluated after a 4-hour exposure to 500 μM of L-glutamate (LGA). Melatonin (0.5 μM) was applied immediately following LGA treatment or after 4 hours. Genomic DNA fragmentation was then analyzed 4 hours after LGA treatment by agarose gel electrophoresis of DNA isolated from spinal cord tissue (Figure 1). Slice cultures exposed to LGA showed significantly increased DNA fragmentation as compared to controls. LGA-induced DNA fragmentation appeared to be internucleosomal, and it was attenuated by treatment with melatonin. Interestingly, melatonin-mediated inhibition of DNA fragmentation was abrogated by melatonin receptor antagonist, luzindole (LGA+melatonin+luzindole), both at 0 and 4 hours post-

LGA. Taken together, LGA-induced DNA fragmentation in organotypic slice cultures is prevented by the addition of melatonin, and this action is mediated via melatonin membrane receptors.

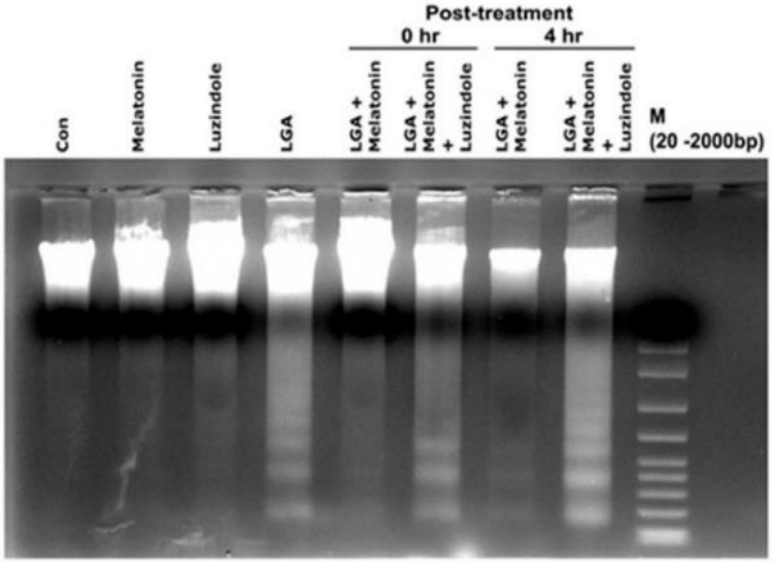

Fig. 1. Effects of melatonin on internucleosomal DNA fragmentation.

Genomic DNA fragmentation after melatonin, luzindole, and L-glutamate (LGA) treatment by agarose gel electrophoresis of DNA isolated from spinal cord tissue (shown from left to right after control (con) lane. At 0 and 4 hours after treatment, melatonin inhibited DNA fragmentation (LGA+melatonin); this was reversed by melatonin receptor antagonist, luzindole (LGA+melatonin+luzindole) at both time points.

Apoptotic cell death is an active and highly regulated process that produces a biochemical phenotype consisting of DNA fragmentation and the release of active caspases. While glutamate is an important CNS excitatory neurotransmitter involved in synaptic plasticity and the formation of neuronal networks during development, excessive glutamate is toxic to neurons and glia. Activities of caspases 3, 8, and 9 were significantly increased in LGA-treated organotypic cultures; these responses were attenuated by melatonin (Figure 2).

Melatonin receptor antagonist diminished the melatonin-mediated reduction of caspases, indicating that cellular apoptosis is blocked by melatonin. Of note, caspase activity was slightly increased in slice cultures exposed to LGA+melatonin 4 hours after LGA, suggesting a low-grade upregulation of caspases during the 4-hour period after LGA incubation.

Melatonin acts either directly or through the membrane melatonin receptor (MT1 and MT2) mechanisms. Slice cultures exposed to melatonin and LGA+melatonin showed significantly increased MT1/2 mRNA expression compared to controls (Figure 3). Blockade of melatonin membrane receptors with luzindole altered MT1/2 gene expression in slice cultures.

Oxidative stress and dysregulation of neuronal Ca^{2+} play important roles in the activation of calpains and caspases that may contribute to neuronal death (28). Thus, in addition to caspases, calpain and its inhibitor, calpastatin, were also evaluated in LGA/melatonin treated spinal cord slice cultures. Increased calpain gene expression was detected after LGA exposure; this rise was inhibited by melatonin (Figure 4). The effect of melatonin on inhibition of calpain was abrogated by luzindole, while calpastatin was diminished in these slice cultures. Bax mRNA was upregulated when exposed to LGA and LGA+melatonin+luzindole (0 and 4 hours post-LGA), suggesting the

calpain inhibitory effect of melatonin may have altered Bax/Bcl-2 molecules and neuronal survival in the LGA-treated spinal cord slice cultures.

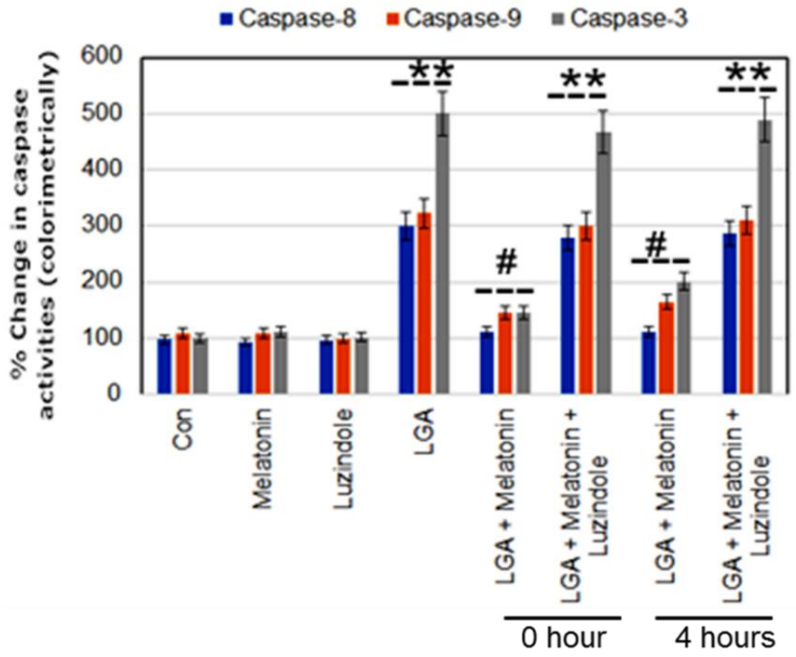

Fig. 2. Effects of melatonin on caspase activities.

*At 0 and 4 hours after treatment, the addition of melatonin receptor antagonist, luzindole, to slice cultures exposed to L-glutamate (LGA) and melatonin resulted in activities of all three caspases which approximated those exposed to LGA alone. (Significant difference between LGA and melatonin post-treatment + LGA indicated by #P<0.05; **P≤0.01 relative to controls).*

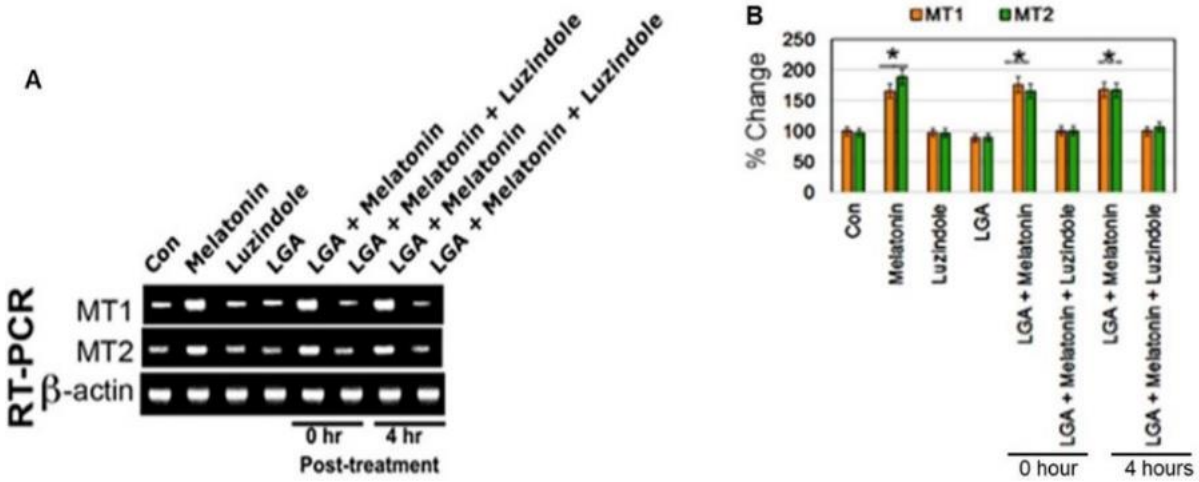

Fig. 3. Effects of melatonin on MT1/2 expression in cultured spinal cord slices.

*A: Analysis of MT1 and MT2 by RT-PCR. B: Graphical representation in percent change. At 0 and 4 hours after treatment, MT1/2 mRNA expression is not significantly changed from controls with the addition of luzindole (LGA + melatonin + luzindole) (*P≤0.05 relative to controls).*

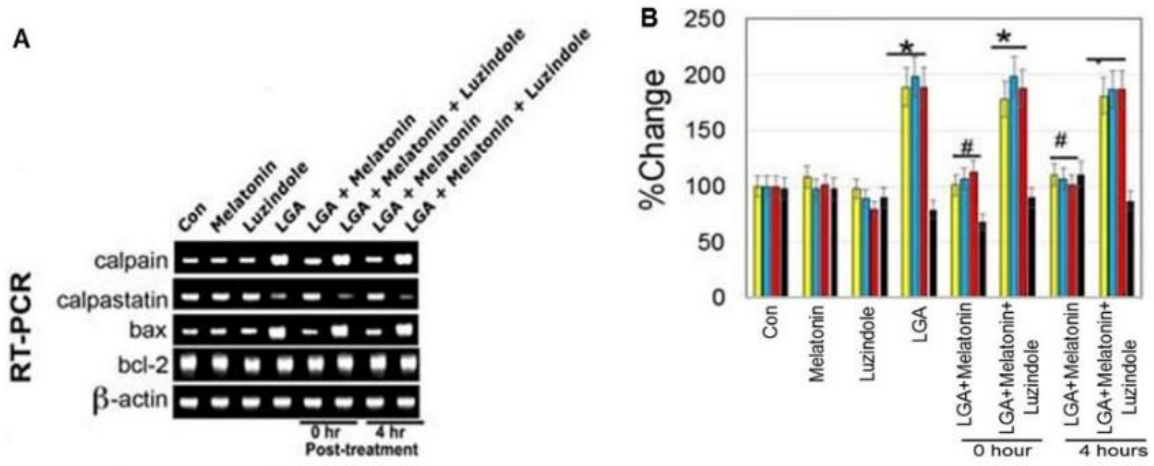

Fig. 4. Effects of melatonin on expressions of calpain, calpastatin, Bax and Bcl-2.

*A: Upregulation of calpain (yellow bar) and Bax (red) with down regulation of calpastatin (blue) after slice culture exposure to L-glutamate (LGA) as demonstrated by RT-PCR analysis. B: with graphical percentage change depicted. Melatonin attenuates these effects at 0 and 4 hours after treatment; however, the addition of melatonin receptor antagonist, luzindole, demonstrates these findings are largely melatonin receptor-mediated. (Significant difference between LGA and melatonin post-treatment + LGA indicated by #P<0.05; Significant difference between control (Con) and melatonin treatments were indicated by • P≤0.05; *P≤0.05 for LGA samples relative to controls).*

4. DISCUSSION

SCI causes secondary biochemical changes leading to widespread neurodegeneration and neuronal death. Thus, understanding the mechanisms underlying neuronal death in SCI and developing novel therapeutic approaches for this damage are critical for functional recovery. While a number of animal models of SCI are available for comparison with human SCI, *ex vivo* slice cultures are also useful in investigating cellular, biochemical, and molecular processes of the brain and spinal cord. This approach provides fewer experimental/ethical challenges and minimizes study time. *Ex vivo* brain slice culture models can also be easily used to test the effects of neurotoxic and neuroprotective molecules (e.g., growth factors or neuroactive drugs) (29, 30). Since glutamate toxicity is a major contributing factor to neuronal death in SCI, we evaluated neuronal viability *in vitro* when exposed to glutamate with or without melatonin. Previous *in vivo* SCI studies employing intraperitoneal melatonin administration have demonstrated reduction in neuronal death in the penumbra surrounding the injury site (31). Melatonin and melatonin metabolites are known free radical scavengers (32, 33). The present *in vitro* analysis thus allows for direct evaluation of melatonin interaction at the cellular level without the presence of the inflammatory cascade present in rodent SCI models. Melatonin has been shown to have autocrine, intracrine and paracrine effects in various mammalian cell types. Since melatonin is a known anti-inflammatory agent, this study was designed to separate the effects of melatonin on the immune system from the direct autocrine and intracrine effects of melatonin in neurons and glia.

One intracellular pathway which may contribute to melatonin-mediated cell viability during glutamate toxicity is upregulation of intracellular antioxidant enzymes such as superoxide

dismutase and glutathione reductase (22). Moreover, the downregulation of glutamine synthetase, an indirect measure of neuronal glutamate signaling and excitotoxicity may be modulated via the L-type voltage gated calcium channel (34, 35). Thus, melatonin may act indirectly to reduce glutamine synthetase production by decreasing glutamate release from nearby neurons. Melatonin agonists have also been used to stimulate brain-derived neurotrophic factor (BDNF) expression through binding with melatonin receptors (36). BDNF contributes to neuronal function, regeneration and survival (37). In addition, melatonin modulates multiple cell functions through calmodulin-mediated microtubule polymerization and cytoskeletal rearrangement; this interaction with calmodulin also inhibits NF-kB binding to nuclear DNA (pro-apoptotic during cell stress such as inflammation) (38).

Multiple anti-apoptotic actions of melatonin are localized in the mitochondria. In addition to acting as a scavenger for free radicals produced by mitochondrial electron transport mechanisms, melatonin promotes glutathione (GSH) dependent mitochondrial protection by enhancing expression of γ-glutamylcysteine synthase (rate-limiting enzyme in GSH production). During periods of oxidative stress, such as occur during glutamate toxicity, mitochondria respond to increased cellular energy requirements, but the resulting rise in reactive oxygen species contributes to the pro-oxidant loop which promotes mitochondrial damage, release of mitochondrial calcium stores, and ultimately apoptosis (39). Calpain is also activated in response to increased intracellular calcium levels. Both ubiquitous calpain isoforms are found in mitochondria (Arrington et al., 2006; Kar et al., 2010). Likewise, calpain 10 (an atypical mitochondrial calpain with an N-terminus mitochondrial targeting sequence) cleaves Complex I and ATP synthase (Arrington et al., 2006). Upregulated calpain 10 expression results in impaired mitochondrial respiration and subsequent cell death (Smith & Schnellmann, 2012). Ubiquitous calpain isoforms also degrade multiple apoptotic proteins including Bid, Bcl-xL, and caspase-3 (Bevers, Neumar, 2008; Smith & Schnellmann, 2012).

During the intrinsic apoptotic pathway, signals from the cell membrane trigger mitochondrial swelling or formation of mitochondrial membrane pores. Melatonin inhibits these signaling pathways by means of free radical reduction, inhibition of neuronal nitric oxide synthases, and interaction with transcriptional factors (40). Furthermore, melatonin reduces peroxidation of cardiolipin, an important mitochondrial inner membrane component. Cardiolipin peroxidation promotes apoptosis and impairs complex III/IV structural integrity (40). With respect to Bcl-2/Bax interactions, melatonin appears to promote upregulation of Bcl-2 during glutamatergic signaling. Bcl-2 prevents mitochondrial translocation of proapoptotic Bax, which triggers cytochrome *c* release from the intermembrane space for resulting caspase activation in apoptosis (39). Our *in vitro* results suggest melatonin interacts with the Bcl-2/Bax system through a receptor-mediated process, since the expression of Bax was increased (as seen with LGA exposure) when the receptor antagonist luzindole was added to the culture medium.

In addition to receptor-mediated signaling mechanisms, melatonin may also cross the cell membrane via glucose and oligopeptide transporters to interact directly with intracellular components such as cytoskeletal proteins, enzymes, and calcium-binding proteins (41). Moreover, the melatonin receptor (MT1) has been found on the mitochondrial membrane (42, 43) while presumptive nuclear receptors (retinoid Z receptor/retinoid acid receptor-related orphan receptors) have also been identified. Given this information, we evaluated whether the anti-apoptotic effects of melatonin are due to receptor signaling or receptor-independent pathways. The addition of luzindole to cells and slice cultures exposed to glutamate toxicity shows that apoptosis was largely receptor-mediated. Since luzindole also inhibits the extracellular signal-regulated kinase (ERK)

pathway (27, 44, 45), melatonin may preferentially alter the ERK-dependent signaling cascade, which then interferes with the anti-apoptotic program. Studies are underway to determine if melatonin operates through the ERK-dependent signaling pathway and protects neuronal cells. Future studies are also required to determine if the effects of melatonin are a result of its interaction with MT1/MT2 receptors on the cell membrane or via the MT1 receptor on the mitochondrial membrane. Nevertheless, our data suggests melatonin agonists may play a significant role in promoting viability of neurons at risk for apoptosis due to oxidative stress or adjacent tissue injury.

ACKNOWLEDGEMENTS

This work was supported in part by funding from Veterans Administration (1I01BX002349-01, 2I01 BX001262-05, 1I01 BX004269-01), and NIH-NINDS R01 NS62327, 1R21 NS118393-01); and the South Carolina State Spinal Cord Research Fund (SCIRF-0905, 2006-2008, SCIRF-2015P-01, SCIRF-2015P-04, SCIRF-2015-I-01, SCIRF-2016 I-03, and SCIRF #2018 I-01). Contents do not necessarily represent the policy of the SCIRF and do not imply endorsement by the funding agency. We thank Ms. Denise Matzelle for technical assistance.

AUTHORSHIP

Azizul Haque designed, wrote the manuscript, drew the figures, and also edited the manuscript. Donald Shields designed, wrote/edited the manuscript. Arabinda Das performed the experiments,wrote the manuscript, and drew the figures. Abhay Varma and Russel Reiter edited the manuscript. Naren Banik conceived, designed, and edited the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Fehlings MG, Hawryluk GW (2010) Scarring after spinal cord injury. *J. Neurosurg. Spine* **13**: 165-167; discussion 167-168 .
2. Fitch MT, Doller C, Combs CK, Landreth GE, Silver J (1999) Cellular and molecular mechanisms of glial scarring and progressive cavitation: in vivo and in vitro analysis of inflammation-induced secondary injury after CNS trauma. *J. Neurosci.* **19**: 8182-8198.
3. Dumont RJ, Okonkwo DO, Verma S, Hurlbert RJ, Boulos PT, Ellegala DB, Dumont AS (2001) Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clin. Neuropharmacol.* **24**: 254-264.
4. Xu GY, Hughes MG, Zhang L, Cain L, McAdoo DJ (2005) Administration of glutamate into the spinal cord at extracellular concentrations reached post-injury causes functional impairments. *Neurosci. lett.* **384**: 271-276.
5. Ray SK, Hogan EL, Banik NL (2003) Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. *Brain Res. Rev.* **42**: 169-185.

6. Wu HY, Tomizawa K, Oda Y, Wei FY, Lu YF, Matsushita M, Li ST, Moriwaki A, Matsui H (2004) Critical role of calpain-mediated cleavage of calcineurin in excitotoxic neurodegeneration. *J. Biol. Chem.* **279**: 4929-4940.
7. Blomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, Mallard C, Hagberg H (2001) Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis"? *J. Biol. Chem.* **276**: 10191-10198.
8. Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC, Newcomb EW (1998) Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* **17**: 1069-1078.
9. Ray SK, Neuberger TJ, Deadwyler G, Wilford G, DeVries GH, Banik NL (2002) Calpain and calpastatin expression in primary oligodendrocyte culture: preferential localization of membrane calpain in cell processes. *J. Neurosci. Res.* **70**: 561-569.
10. Nath R, Raser KJ, Stafford D, Hajimohammadreza I, Posner A, Allen H, Talanian RV, Yuen P, Gilbertsen RB, Wang KK (1996) Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. *Biochem. J.* **319 (Pt 3)**: 683-690.
11. Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. *Physiol. Rev.* **83**: 731-801.
12. Bevers MB, Neumar RW (2008) Neumar, Mechanistic role of calpains in postischemic neurodegeneration. *J. Cereb. Blood Flow Metab.* **28**:655-673.
13. Vosler PS, Brennan CS, Chen J (2008) Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Mol. Neurobiol.* **38**: 78-100.
14. Sribnick EA, Ray SK, Banik NL (2006) Banik, Estrogen prevents glutamate-induced apoptosis in C6 glioma cells by a receptor-mediated mechanism. *Neuroscience* **137**: 197-209.
15. Das A, Sribnick EA, Wingrave JM, Del Re AM, Woodward JJ, Appel SH, Banik NL, Ray SK (2005) Calpain activation in apoptosis of ventral spinal cord 4.1 (VSC4.1) motoneurons exposed to glutamate: calpain inhibition provides functional neuroprotection. *J. Neurosci. Res.* **81**: 551-562.
16. McCracken E, Hunter AJ, Patel S, Graham DI, Dewar D (1999) Calpain activation and cytoskeletal protein breakdown in the corpus callosum of head-injured patients. *J. Neurotrauma* **16**: 749-761.
17. Ray SK, Wilford GG, Crosby CV, Hogan EL, Banik NL (1999) Banik, Diverse stimuli induce calpain overexpression and apoptosis in C6 glioma cells. *Brain Res.* **829**: 18-27.
18. Ray SK, Matzelle DD, Wilford GG, Hogan EL, Banik NL (2001) Cell death in spinal cord injury (SCI) requires de novo protein synthesis. Calpain inhibitor E-64-d provides neuroprotection in SCI lesion and penumbra. *Ann. NY Acad. Sci.* **939**: 436-449.
19. Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA (2003) The chemistry of melatonin's interaction with reactive species. *J. Pineal Res.* **34**: 1-10.
20. Cuzzocrea S, Reiter RJ (2002) Pharmacological actions of melatonin in acute and chronic inflammation. *Curr. Top. Med. Chem.* **2**: 153-165.
21. Guerrero JM, Reiter RJ (2002) Melatonin-immune system relationships. *Curr. Top. Med. Chem.* **2**: 167-179.
22. Reiter RJ, Tan DX, Manchester LC, Qi W (2001) Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. *Cell. Biochem. Biophys.* **34**: 237-256.
23. Reiter RJ, Tan DX, Leon J, Kilic U, Kilic E (2005) When melatonin gets on your nerves: its beneficial actions in experimental models of stroke. *Exp. Biol. Med. (Maywood)* **230**: 104-117.

24. Jou MJ, Peng TI, Reiter RJ, Jou SB, Wu HY, Wen ST (2004) Visualization of the antioxidative effects of melatonin at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes. *J. Pineal Res.* **37**: 55-70.
25. Sugai F, Yamamoto Y, Sakoda S (2004) [Organotypic spinal cord culture using mice]. *Nihon Yakurigaku Zasshi* **124**: 19-23.
26. Das A, Belagodu A, Reiter RJ, Ray SK, Banik NL (2008) Cytoprotective effects of melatonin on C6 astroglial cells exposed to glutamate excitotoxicity and oxidative stress. *J. Pineal Res.* **45**: 117-124.
27. Das A, McDowell M, Pava MJ, Smith JA, Reiter RJ, Woodward JJ, Varma AK, Ray SK, Banik NL (2010) The inhibition of apoptosis by melatonin in VSC4.1 motoneurons exposed to oxidative stress, glutamate excitotoxicity, or TNF-alpha toxicity involves membrane melatonin receptors. *J. Pineal Res.* **48**: 157-169.
28. Tamtaji OR, Mirhosseini N, Reiter RJ, Azami A, Asemi Z (2019) Melatonin, a calpain inhibitor in the central nervous system: Current status and future perspectives. *J. Cell Physiol.* **234**: 1001-1007.
29. Drexler B, Hentschke H, Antkowiak B, Grasshoff C (2010) Organotypic cultures as tools for testing neuroactive drugs - link between in-vitro and in-vivo experiments. *Curr. Med. Chem.* **17**: 4538-4550.
30. Sundstrom L, Morrison B, 3rd, Bradley M, Pringle A (2005) Organotypic cultures as tools for functional screening in the CNS. *Drug Discov. Today* **10**: 993-1000.
31. Samantaray S, Sribnick EA, Das A, Knaryan VH, Matzelle DD, Yallapragada AV, Reiter RJ, Ray SK, Banik NL (2008) Melatonin attenuates calpain upregulation, axonal damage and neuronal death in spinal cord injury in rats. *J. Pineal Res.* **44**: 348-357.
32. Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, Vriend J, Tan DX, Reiter RJ (2015) Melatonin: an ancient molecule that makes oxygen metabolically tolerable. *J. Pineal Res.* **59**: 403-419.
33. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L (2016) Melatonin as an antioxidant: under promises but over delivers. *J. Pineal Res.* **61**: 253-278.
34. Benton RL, Ross CD, Miller KE (2000) Glutamine synthetase activities in spinal white and gray matter 7 days following spinal cord injury in rats. *Neurosci. Lett.* **291**: 1-4.
35. Sribnick EA, Del Re AM, Ray SK, Woodward JJ, Banik NL (2009) Estrogen attenuates glutamate-induced cell death by inhibiting Ca2+ influx through L-type voltage-gated Ca2+ channels. *Brain Res.* **1276**: 159-170.
36. Gumuslu E, Mutlu O, Sunnetci D, Ulak G, Celikyurt IK, Cine N, Akar F, Savli H, Erden F (2014) The Antidepressant agomelatine improves memory deterioration and upregulates CREB and BDNF gene expression levels in unpredictable chronic mild stress (UCMS)-exposed mice. *Drug Target Insights* **8**: 11-21.
37. Tardito D, Perez J, Tiraboschi E, Musazzi L, Racagni G, Popoli M (2006) Signaling pathways regulating gene expression, neuroplasticity, and neurotrophic mechanisms in the action of antidepressants: a critical overview. *Pharmacol. Rev.* **58**: 115-134.
38. Benitez-King G, Anton-Tay F (1993) Anton-Tay, Calmodulin mediates melatonin cytoskeletal effects. *Experientia* **49**: 635-641.
39. Luchetti F, Canonico B, Betti M, Arcangeletti M, Pilolli F, Piroddi M, Canesi L, Papa S, Galli F (2010) Melatonin signaling and cell protection function. *FASEB* **24**: 3603-3624.
40. Hardeland R (2017) Melatonin and the electron transport chain. *Cell. Mol. Life Sci.* **74**: 3883-3896.

41. Mayo JC, Sainz RM, Gonzalez-Menendez P, Hevia D, Cernuda-Cernuda R (2017) Cernuda-Cernuda, Melatonin transport into mitochondria. *Cell. Mol. Life Sci.* **74**: 3927-3940.
42. Suofu Y, Li W, Jean-Alphonse FG, Jia J, Khattar NK, Li J, Baranov SV, Leronni D, *et al.* (2017) Dual role of mitochondria in producing melatonin and driving GPCR signaling to block cytochrome c release. *Proc. Natl. Acad. Sci. USA* **114**: E7997-E8006.
43. Reiter RJ, Ma Q, Sharma R (2020) Melatonin in mitochondria: mitigating clear and present dangers. *Physiol.* **35**: 86-95.
44. Chen M, Cecon E, Karamitri A, Gao W, Gerbier R, Ahmad R, Jockers R (2020) Melatonin MT1 and MT2 receptor ERK signaling is differentially dependent on Gi/o and Gq/11 proteins. *J. Pineal Res.* **68**: e12641.
45. Zhang J, Wang L, Xie W, Hu S, Zhou H, Zhu P, Zhu H (2020) Melatonin attenuates ER stress and mitochondrial damage in septic cardiomyopathy: A new mechanism involving BAP31 upregulation and MAPK-ERK pathway. *J. Cell. Physiol.* **235**: 2847-2856.

Please cite this paper as:
Haque, A., Shields, D.C., Das, A., Varma, A., Reiter, R.J. and Banik, N.L. 2021. Melatonin receptor-mediated attenuation of excitotoxic cell death in cultured spinal cord slices. Melatonin Research. 4, 2 (Apr. 2021), 336-347. DOI:https://doi.org/https://doi.org/10.32794/mr11250098.