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Melatonin: Neuritogenesis and neuroprotective effects in crustacean x-organ cells

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Abstract

Melatonin has both neuritogenic and neuroprotective effects in mammalian cell lines such as neuroblastoma cells. The mechanisms of action include receptor-coupled processes, direct binding and modulation of calmodulin and protein kinase C, and direct scavenging of free radicals. While melatonin is produced in invertebrates and has influences on their physiology and behavior, little is known about its mechanisms of action. We studied the influence of melatonin on neuritogenesis in well-differentiated, extensively-arborized crustacean x-organ neurosecretory neurons. Melatonin significantly increased neurite area in the first 24 h of culture. The more physiological concentrations, 1 nM and 1 pM, increased area at 48 h also, whereas the pharmacological 1μM concentration appeared to have desensitizing effects by this time. Luzindole, a vertebrate melatonin receptor antagonist, had surprising and significant agonist-like effects in these invertebrate cells. Melatonin receptors have not yet been studied in invertebrates. However, the presence of membrane-bound receptors in this population of crustacean neurons is indicated by this study. Melatonin also has significant neuroprotective effects, reversing the inhibition of neuritogenesis by 200 and 500 μM hydrogen peroxide. Because this is at least in part a direct action not requiring a receptor, melatonin's protection from oxidative stress is not surprisingly phylogenetically-conserved.

Keywords

Uca pugilator; crustacean; fiddler crab; melatonin; neuritogenesis; neuroprotection; oxidative stress; x-organ

1. Introduction

The vertebrate pineal hormone melatonin is involved in circadian and seasonal rhythmicity. It is produced during darkness and affects a variety of behavioral and physiological functions such as locomotion, thermoregulation, sleep-wake patterns, and reproduction (Cassone et al., 1993; Golombek et al., 1996; Hyde and Underwood, 1995; Reiter, 1991; Rowe and Kennaway, 1996; Stankov et al., 1991). Many of melatonin's influences occur through modulation of the nervous system. Some of these effects include the induction of morphological changes and growth in neurons and glial cells (Benítez-King et al., 1990;

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Bordt et al., 2001; Paulose et al., 2009). Other effects occur through modulation of the neurosecretory activity of the hypothalamic-pituitary axis (Falcón et al., 2007).

Melatonin has neuritogenic effect both in vitro (Benítez-King et al., 1990) and in vivo (Ramirez-Rodriguez et al., 2011) in mouse cell lines. In mouse MDCK and N1E-115 cells, microfilaments and microtubules are involved in development of dome formation and neurite outgrowth. Melatonin increases both processes through activation of cytoskeletal synthesis (Benítez-King et al., 1990; Benítez-King, 2000). This cytoskeletal rearrangement appears to be activated by multiple melatonin-interactive processes: 1) direct inhibition of calmodulin by melatonin (Antón-Tay et al., 1998a; Benítez-King and Antón-Tay, 1993, Benítez-King et al., 1993, 1996; Huerto-Delgadillo et al., 1994); 2) direct stimulation of protein kinase C by melatonin (Antón-Tay et al., 1998b; Bellon et al., 2007; Benítez-King et al., 2001); and 3) the activation of the MT1 melatonin receptor, which signals through two inhibitory G proteins that decrease adenylyl cyclase activity and a G protein that activates phospholipase C (Bordt et al., 2001; Brydon et al., 1999; Witt-Enderby et al., 2000). Melatonin has general and widespread antioxidant actions including the direct scavenging of free radicals and the activation of enzymes in antioxidant pathways (Reiter et al., 2007, 2008). Melatonin directly scavenges hydroxyl radicals, peroxyl radicals, peroxynitrite anions, and singlet oxygen (Reiter et al., 1999, 2007). Melatonin also regulates the activation and expression of antioxidant enzymes such as glutathione peroxidase, superoxide dismutases, and catalase, via the membrane and nuclear receptors previously described (García-Macia et al., 2011; Kotler et al., 1998; Limón-Pacheco and Gonsebatt, 2010; Rodriguez et al., 2004; Tomás-Zapico and Coto-Montes, 2005). Together, these neuroregenerative and neuroprotective effects suggest that melatonin may have therapeutic value in treating neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Benítez-King et al., 1990, 2003, 2005, 2010; Benítez-King, 2006; Dabbeni-Sala et al., 2001; González-Burgos et al., 2007; He et al., 2010; Matsubara et al., 2003; Sharma et al., 2006; Singhal et al., 2011; Weishaupt et al., 2006).

While melatonin has not been studied extensively in invertebrates, it has been detected in nearly every organism tested, including crustaceans (Balzer et al., 1997; Maciel et al., 2008; Markowska et al., 2009; Tilden et al., 1997, 2001b; Withyachumnarnkul et al., 1992, 1999). The eyestalks of crustaceans contain the optic lobes and the x-organ/sinus gland, a neuropeptide-secreting neurohemal structure that is analogous to the vertebrate hypothalamus-pituitary system in that its many hormone products regulate a variety of functions: molt-inhibiting hormone (MIH), the broadly-acting crustacean hyperglycemic hormone (CHH), red pigment concentrating hormone (RPCH), retinal light-adapting hormone (LAH) and dark-adapting hormone (DAH), crustacean cardioactive peptide (CCAP), methylfarnesoate-inhibiting peptide, and neurodepressing hormone (NDH) (for review: Christie et al., 2010). A variety of neurotransmitters have been shown to affect x-organ/sinus gland activity: The system has inhibitory responses to glutamate and GABA (Duan and Cooke, 2000), and excitatory responses to serotonin (Escamilla-Chimal et al., 2002; Lee et al., 2001), dopamine (Zou et al., 2003), and norepinephrine (Hsieh et al., 2006). Melatonin may interact with this system as well, since melatonin influences x-organ effector responses such as locomotion (Tilden et al., 2003b, a), glucose metabolism (Sainath and Reddy, 2010; Tilden et al., 2001a, 2003a), and limb regeneration (Tilden et al., 1997). Melatonin also influences crustacean ERG rhythms (Balzer et al., 1997; Solís-Chagoyán et al., 2008), potentially via MT2 receptor-type interaction (Mendoza-Vargas et al., 2009). Furthermore, exogenous melatonin has effects on the antioxidant system in crustacean locomotor muscles (Geihs et al., 2010) and gills (Maciel et al., 2010). However, the presence of melatonin receptors in invertebrates has not yet been determined. The x-organ/sinus gland system is a neurohemal organ consisting of approximately 200 somata (the x-organ) clustered on the periphery of the medulla terminalis whose axons terminate in

enlarged secretory endings (the sinus gland) on the distal side of the eyestalk. Two size categories of x-organ somata exist, small 15–25 μm and large 30–70 μm cells, which show immunohistological differences in neuropeptide content (Chang et al., 1987; Keller et al., 1985; Mangerich and Keller, 1988): small cells appear to produce RPCH, and large cells appear to produce CHH, MIH, and other peptides. When isolated and cultured, these somata show rapid outgrowth of neurites even under the most basic conditions, which may be due in part to their neurosecretory propensity for trafficking membrane and architectural elements (Cooke et al., 1989).

Crustacean x-organ cells are a potential model system for comparative studies of the role of melatonin in invertebrate neurophysiology. These cells may also serve as an excellent model system for the study of melatonin neuromodulation across phyla: they are functionally analogous to vertebrate neurosecretory hypothalamic cells and are similar in neuromodulatory responsiveness and neuritogenesis to vertebrate hippocampal cells. X-organ cells are functionally and morphologically distinct from the well-studied mouse neuroblastoma cells: neuroblastoma cells are relatively undifferentiated cells that produce approximately 2 to 4 primary neurites with little arborization. X-organ cells, on the other hand, are well-differentiated neurosecretory cells that maintain their neurosecretory function in culture; they are also multipolar with extensive arborization.

In the current study, we explored the influence of both physiological and pharmacological levels of melatonin and a vertebrate melatonin receptor antagonist on crustacean neurite area as a measure of neurite growth. We also studied the neuroprotective effects of melatonin against oxidative stress.

2. Materials and methods

2.1. Crab housing and care

Fiddler crabs (*Uca pugilator*) were purchased from Gulf Specimen Marine Laboratory (Panacea, FL) and were housed in clear plastic tanks with Coralife® artificial seawater such that both terrestrial and aquatic terrains were available. Crabs were acclimated to a 12L:12D photoperiod and 22 ± 1 °C for at least 2 weeks prior to experimentation; they were fed crushed dry Purina Cat Chow® every other day, and water was changed after every feeding.

2.2. Tissue dissection

Dissection procedures were adapted from Cooke et al. (Cooke et al., 1989). All supplies were purchased from Sigma-Aldrich except where otherwise noted. Eyestalks were removed, rinsed in 70% ethanol, and immediately placed in sterile crab saline (490 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 26 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.4) with antibiotics (100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.2 mg/mL neomycin). Incisions were made through the outer carapace on the lateral sides of each eyestalk up to the cornea, and the top portion of the carapace was lifted to reveal the underlying eyestalk muscular and neural tissue. The neural tissue was dissected away from the eyestalk and examined to locate the light blue, opalescent sinus gland on the medulla terminalis. X-organ-containing tissue on the opposite side of the medulla terminalis was removed and placed in Ca^{2+}- and Mg^{2+}-free saline (529 mM NaCl, 11 mM KCl, and 10 mM HEPES) with 0.1 % trypsin. X-organ tissue was gently stirred in the trypsin solution for 1 h and then rinsed 3 times in normal sterile crab saline.

2.3. Cell culture

Culture medium was adapted from Cooke et al (1989) and consisted of equal volumes of 1.16× concentrated crab saline and Liebovitz L-15 culture medium, with 20 mM HEPES,

0.1 mg/mL gentamicin, 120 mM glucose, and 2 mM L-glutamine. The final culture medium osmolality was 840 mM, equivalent to crab hemolymph osmolality. Each rinsed x-organ tissue was transferred to a 200 μL drop of culture medium in a glass-bottom culture dish, poly-L-lysine-coated, 35 mm, with 1.5 glass thickness (MatTek). The tissue was then triturated with a sterile glass pipette to dissociate cells. Cells were allowed to adhere to the glass-bottom portion of the culture dish for 45 min, and the volume was then brought to 3.6 mL with additional culture medium. The cultures were housed in darkness and high humidity at 22 ± °C. Culture medium was not replaced throughout the duration of an experiment. Cells usually survived for at least 7 days under these conditions.

2.4. Measurement of neurite area

Cells were viewed with a Zeiss Axiovert 200 microscope with phase contrast; images were collected and analyzed with Zeiss AxioVision software. In the neurite studies, images were collected from the first 10 cells observed in an ordered x-y scanning across each culture dish to provide the highest likelihood that we were measuring the same cells in successive 24-h measures. Cells that were within 200 μm of neighboring cells were excluded from analysis. With AxioVision software, we traced the perimeter of the neurite-encompassing area around each cell. To determine consistency of the neurite area measurement method, 4 people separately analyzed the same 8 unlabeled images. The standard deviation among the 4 observers was not greater than 3.84% of the mean area for any of the images, indicating acceptable precision of the measurement procedure.

2.5. Timecourse of neurite outgrowth

Cells were treated with 0 (control) or 1 μM melatonin (Sigma). Images were taken immediately after cells had adhered to the bottom of the culture dish (approximately 1 h after being placed in culture) and then every 24 h over 96 h. N = 10 cells per culture dish × 4 culture dishes per treatment.

2.6. Melatonin dose-response

Cells were treated with 0 (control), 1 pM, 1 nM, and 1 μM melatonin. Images were taken at 0, 24, and 48 h. N = 10 cells per culture dish × 4 culture dishes per treatment.

2.7. Influence of melatonin antagonist

Cells were treated with 1 μM melatonin, 1 μM melatonin plus 1 μM of the melatonin receptor antagonist luzindole, or no treatment. Images were taken at 0, 24, and 48 h. N = 10 cells per culture dish × 4 culture dishes per treatment.

2.8. Influence of hydrogen peroxide and melatonin

Cell cultures were treated at time zero with 100, 200, 500, and 1000 μM H_2O_2, with and without 1 μM melatonin (for 200 and 500 μM H_2O_2 only), versus untreated controls. Images were taken at 60 h. N = 10 cells per culture dish × 4 culture dishes per treatment.

2.9. Statistical analysis

We used a two-way ANOVA to determine differences among culture dishes and among treatments with the Holm-Sidak method for pairwise comparisons. Statistics were performed with SigmaStat software (SPSS, Inc.).

3. Results

3.1. Timecourse of neurite outgrowth

Figure 1 shows a typical x-organ cell with extensive 24-h arborization. Figure 2 shows neurite outgrowth area in microns^2 in melatonin-treated (1 μM) vs. control cells, measured every 24 h over 96 h. No cells had neurites at time = 0 h. Melatonin-treated neurite area was significantly greater than controls during the first 24 h of culture ($p = 0.007$) but was not significantly different from controls thereafter. Overall neurite area was greatest within the first 48 h, with no significant growth or retraction beyond this time over 96 h.

3.2. Melatonin dose-response

Figure 3 shows neurite growth with 0, 1 pM, 1 nM, and 1 μM melatonin, at 24 and 48 h (area was zero at time = 0). At 24 h, all melatonin concentrations demonstrated significantly greater neurite area than controls. At 48 h, cells treated with 1 nM and 1 pM melatonin showed significantly greater neurite area than controls ($p = 0.001$ and 0.008, respectively).

3.3 Influence of melatonin antagonist

Luzindole + melatonin-treated cells showed significantly greater neurite area than both controls and melatonin-treated cells, at both 24 and 48 h (Fig 4; $p < 0.001$ for 24 h, $p = 0.006$ for 48 h for luzindole vs. melatonin).

3.4. Influence of hydrogen peroxide and melatonin

Cells were incubated with 100, 200, 500, and 1000 μM H_2O_2; no effect on neurite area was seen at 100 μM H_2O_2, and cells did not survive at 1000 μM H_2O_2. Since 200 and 500 μM H_2O_2 had significant neurite-inhibiting effects ($p < 0.05$ for both) without causing cell death, these two concentrations were used for 1 μM melatonin treatment. H_2O_2 at 200 and 500 μM significantly reduced neurite outgrowth compared with control and melatonin-treated cells at 60 h (Fig. 5). Melatonin with H_2O_2 treatment significantly reversed the effects of H_2O_2 alone for both 200 and 500 μM H_2O_2 ($p < 0.01$ for both).

4. Discussion

Melatonin at 1 μM, a pharmacological concentration, caused a significant increase in neurite area within the first 24 h of culture; neurite area reached a maximum at 48 h in both controls and melatonin-treated cells and was sustained for 96 h in static cell cultures. Further growth may have occurred if cultures had been regularly perfused with fresh medium; furthermore, melatonin receptors – if present - were likely desensitized by pharmacological levels of melatonin beyond 24 h.

A dose-response study of melatonin showed that all tested concentrations of melatonin, from physiological (1 pM and 1 nM) to pharmacological (1 μM), increased neurite area in the first 24 h to an equal extent, nearly doubling area in comparison to controls. At 48 h, physiological concentrations had a greater effect on area than the higher pharmacological concentration, suggesting a receptor-mediated response of these cells to melatonin and further suggesting receptor desensitization by 48 h with higher concentrations of melatonin (Figs. 1 and 2).

The MT1 receptor was previously implicated in the neuritogenic effects of melatonin in mammalian cells (Bordt et al., 2001; Witt-Enderby et al., 2000). Luzindole is an MT1 and MT2 receptor antagonist but is more highly selective for the MT2 receptor. Treatment of crustacean x-organ cells with luzindole actually enhanced the effects of melatonin at both 24 and 48 h. Three hypotheses regarding this effect are: A) Crustaceans may have an as-yet

unidentified subtype of membrane-bound melatonin receptor for which luzindole acts as an agonist instead of an antagonist. B) Luzindole may prevent the desensitization of MT1 receptors by melatonin or may enhance MT1 expression. C) Luzindole may have a direct, non-receptor-mediated effect similar to melatonin's direct inhibition of calmodulin. Luzindole is structurally similar to melatonin (Dubocovich, 1988) and therefore may also be a ligand to calmodulin. A similar MT1-activating effect of luzindole was seen in cell lines expressing human MT1 receptors but with eventual receptor desensitization by melatonin (Kokkola et al., 2007). This agonist-type effect of luzindole was also seen in a mouse model of pain response (Ray et al., 2004). The results of the current study indicate that at least one type of membrane-bound melatonin receptor exists in crustacean x-organ cells. However, melatonin's actions are likely more complex, involving the previously-described direct calmodulin and protein kinase C interactions. A dose-response study of luzindole should be undertaken. Melatonin prevented the H_2O_2-induced inhibition of neuritogenesis, as seen in mouse N1E-115 cells (Benítez-King et al., 2005). We examined cells at 60 h, after the initial 24-h neuritogenesis-enhancing effects of melatonin had ceased, since those effects are separate from the receptor-independent neuroprotective effects of melatonin (Reiter et al., 1999). Thus some of the growth-enhancing effect of melatonin in this portion of the study was likely due to its direct and indirect protective effects against oxidative stress.

In conclusion, melatonin had neuritogenic and neuroprotective effects in well-differentiated, highly arborized crustacean neurosecretory cells that were similar to its effects in less differentiated mammalian neuroblastoma cells. We observed effects that may be due to both receptor interactions and direct intracellular regulation of enzymes and free radicals. Unusual receptor interactions were seen with the agonistic effects of luzindole, suggesting some receptor-level differences between mammalian and crustacean cells. The phylogenetically-conserved roles of melatonin have not been studied extensively; cellular-level studies of melatonin in invertebrates are very few, particularly of receptor-mediated activities. The ease of culture, prolific neurite outgrowth, and functional integrity of these cells make them a good comparative model system for the study of cellular-level growth in well-differentiated neuronal systems.

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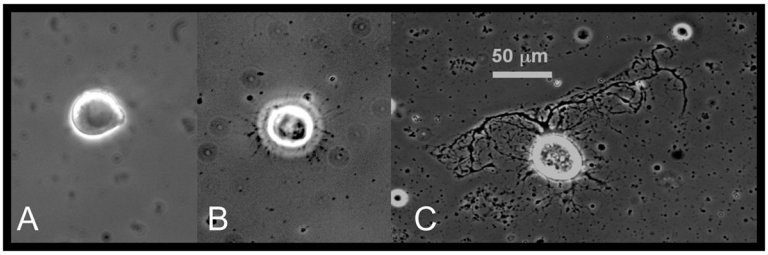

Figure 1.
Cultured crustacean (*Uca pugilator*) x-organ cell at 400× oil immersion with phase contrast (micron bar applies to all 3 images). Images taken A) within 1 h of culture, B) within 3 h of culture, and C) at 24 h in culture.

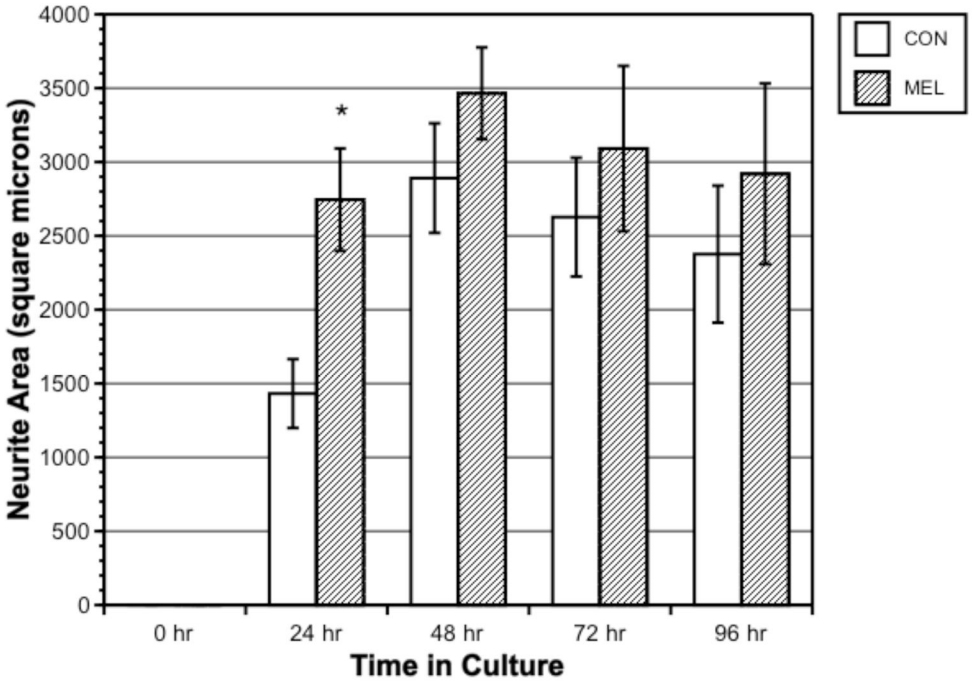

Figure 2.
Neurite area (microns^2) in control versus 1 μM melatonin-treated crustacean x-organ cells measured every 24 h over 96 h. Bars represent means ± SEM of 40 cells.

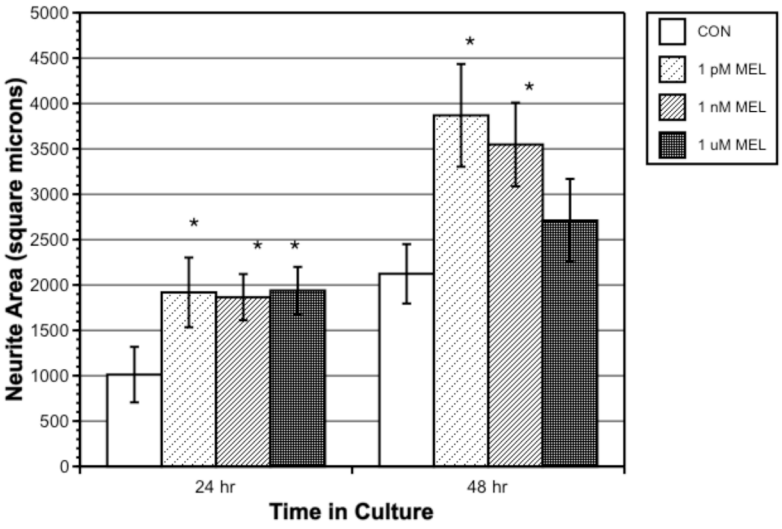

Figure 3.

Dose-response influence of melatonin on neurite area in crustacean x-organ cells measured at 24 and 48 h. Bars represent means ± SEM of 40 cells.

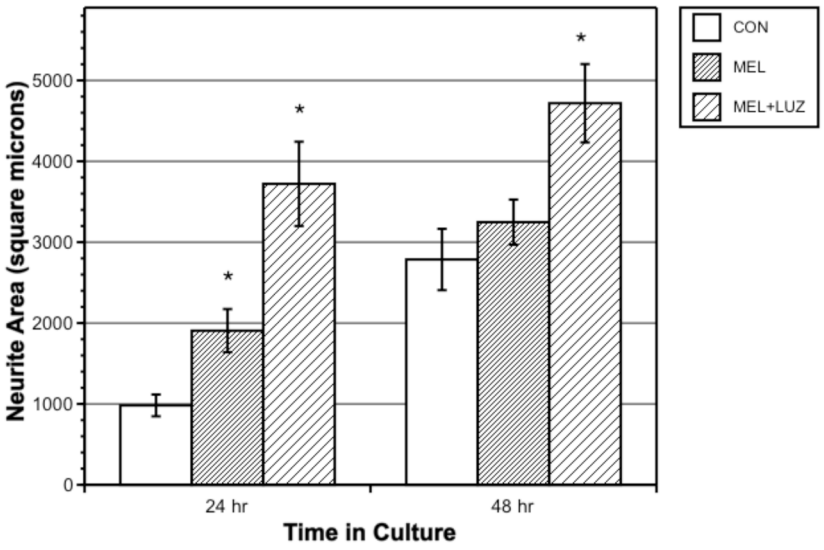

Figure 4.
Influence of melatonin or melatonin + luzindole on neurite area in crustacean x-organ cells measured at 24 and 48 h. Bars represent means ± SEM of 40 cells.

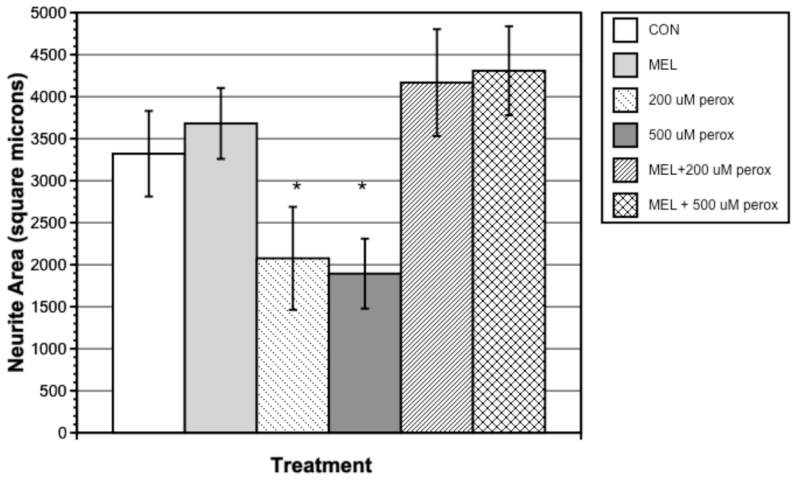

Figure 5.
Influence of peroxide or peroxide and 1 μM melatonin on neurite area in crustacean x-organ cells measured at 60 h. Bars represent means ± SEM of 40 cells.