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Melatonin Induction of Filamentous Structures in Non-Neuronal Cells That is Dependent on Expression of the Human mt1 Melatonin Receptor

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Melatonin has gained recent popularity as a treatment for insomnia and other sleep disorders; however, its cellular effects are unknown. We report the effects of melatonin on the cellular morphology of Chinese hamster ovary (CHO) cells transformed to express the human melatonin receptors, mt1 and MT2. Our results show that melatonin exerts a strong influence on cellular shape and cytoskeletal organization in a receptor-dependent and possibly subtype-selective manner. The cell shape change that we see after a 5-h treatment of these non-neuronal cells with a pharmacological concentration of melatonin consists of the formation of long filamentous outgrowths that are reminiscent of the neurite processes produced by differentiating nerve cells. This morphological change occurs exclusively in cells expressing the mt1 receptor. We find that the microtubule and microfilament organization within these outgrowths is similar to that of neurites. Microtubules are required for the shape change to occur as Colcemid added in combination with melatonin completely blocks outgrowth formation. We demonstrate that the number of cells showing the altered cell shape is dependent on melatonin concentration, constant exposure to melatonin and that outgrowth frequencies increase when protein kinase A (PKA) is inhibited. Concomitant melatonin-dependent increases in MEK 1/2 and ERK 1/2 phosphorylation are noted in mt1-CHO cells only. The production of filamentous outgrowths is dependent on the translation of new protein but not the transcription of new mRNA. Outgrowth number is not controlled by centrosomes but is instead controlled by the polymerization state of the actin cytoskeleton. The results of this work show that the organization of the cytoskeleton is affected by processes specifically mediated or regulated by the mt1 receptor and may represent a novel alternative mechanism for the stimulation of process formation. Cell Motil. Cytoskeleton 46:28–42, 2000.

Key words: cAMP; cytoskeletal organization; G-proteins; neurite outgrowth; protein kinase A; signal transduction

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INTRODUCTION

Melatonin, N-acetyl-5-methoxytryptamine, was first identified as a factor extractable from pineal glands that induced the aggregation of pigment granules in melanocytes and caused frog skin to lighten [Lerner et al., 1958]. It has subsequently been shown to be an important component in establishing circadian rhythms in several animal species, including humans. In animals and humans, it is produced and secreted into the bloodstream in response to darkness. The complexity of the function of melatonin within the body is becoming quite apparent. Melatonin influences physiological processes such as sleep induction [Brzezinski, 1997], entrainment of circadian rhythms [Cassone et al., 1993], dilation or constriction of blood vessels [Viswanathan et al., 1990, 1992; Krause et al., 1995], inhibition of the release of dopamine from the retina [Dubocovich et al., 1997], reproduction in animals and humans [Morgan et al., 1994; Yie et al., 1995; Murayama et al., 1997], and perhaps acts as an oncostatic agent [Brzezinski, 1997; Jones et al., 2000]. Melatonin has also been associated with neural changes [Vaughan, 1984; Lieberman, 1985]; however, the mechanisms underlying this and other effects are not known. Our work focuses on the effects of melatonin in cellular differentiation specifically by examining its effects on cellular morphology and signal transduction mechanisms.

Two human melatonin receptors have been cloned and classified by the International Union of Pharmacology as mt1 and MT2 [Reppert et al., 1994, 1995; Dubocovich et al., 1998]. These two receptors show differences in tissue distributions in the body [Dubocovich, 1995; Mazzucchelli et al., 1996]. Activation of endogenous mt1 receptor expressed in the suprachiasmatic nucleus of the hypothalamus [Niles and Hashemi, 1990] or in ovine pars tuberalis cells [Morgan et al., 1994] as well as in transfected Chinese hamster ovary (CHO) [Witt-Enderby and Dubocovich, 1996] or COS-7 cells [Reppert et al., 1994] by melatonin results in a reduction of cAMP formation via pertussis toxin sensitive and insensitive G-proteins [Morgan et al., 1994; Witt-Enderby and Dubocovich, 1996; Brydon et al., 1999]. This receptor is capable of stimulating the Ca^{2+}/IP_3 signaling pathway as well [Eison and Mullins, 1993; Ebisawa et al., 1994; Morgan et al., 1994; Popova and Dubocovich, 1994; McNulty et al., 1994; McArthur et al., 1997; Brydon et al., 1999; MacKenzie et al., submitted). Recent work has confirmed that the mt1 receptor signals through two inhibitory Gα proteins (G_{i2} and G_{i3}) that attenuate adenylyl cyclase activity and one stimulatory Gα protein ($G_{q/11}$) that increases phospholipase C activity [Brydon et al., 1999]. The possibility exists however, that the signaling actions of melatonin could also be due to receptor-independent processes that directly affect calmodulin [Poffenbarger and Fuller, 1976; Cardinali and Freire, 1975; Benitez-King et al., 1990] as melatonin is a small lipophilic molecule capable of traversing the plasma membrane.

To date, what is known about the effects of melatonin at the level of cellular and cytoskeletal structure is that treatment of MDCK, SK-N-SH, and N1E-115 cells with melatonin results in an increase in the number of cells with neurite processes (N1E-115 or SK-N-SH) or "domes" (MDCK) and in an increase in the number of microtubules within these cells [Benitez-King et al., 1990; Melendez et al., 1996; Cos et al., 1996]. Melatonin has also been shown to increase the levels of polymerized tubulin in N1E-115 cells [Melendez et al., 1996]. This increase in polymerized tubulin is attributed to the induction of microtubule-associated proteins, in particular MAP2 [Melendez et al., 1996].

Transfection of non-neuronal cells with the genes for MAP2 [LeClerc et al., 1996], τ [Knops et al., 1991; Barlow et al., 1994; LeClerc et al., 1996], and the motor protein CHO/MKLP1 [Sharp et al., 1996] all result in the formation of long processes by these cells. Transfection of non-neuronal cells with the gene encoding MAP4, a non-neuronal MAP, does not result in the production of outgrowths [Barlow et al., 1994]. This suggests that the simple presence of neuronal MAP isotypes can induce process formation.

Although studies of the effects of melatonin on selected cultured cell types demonstrate that shape changes occur, it is not known which subtype of melatonin receptor is being stimulated in these cells or whether multiple melatonin receptor subtypes are present. Our approach to investigating melatonin receptor functions and their influences on cytoskeletal organization was to use transfected cell models. We used CHO cells transfected with each of the cloned melatonin receptor subtypes (mt1 and MT2). Cells transfected with the neomycin resistance plasmid alone were used to assess the receptor-independent effects of melatonin. Because the transgenes are constitutively expressed, there is a greater receptor density in the transfected cells than what is found in native tissues (mt1 ~6.5×; MT2 ~100×) [Hazelrigg et al., 1993; Dubocovich, 1995; Witt-Enderby and Dubocovich, 1996; Dubocovich et al., 1997; Witt-Enderby et al., 1997]. However, the pharmacology, function and signaling events triggered in these transfected CHO cells have been shown to be nearly identical to what is seen in native tissues [Witt-Enderby and Dubocovich, 1995; Witt-Enderby et al., 1997] and (unpublished results).

Because so little is known about the events underlying the morphological changes induced by melatonin, we set out to determine (1) whether these events are

receptor-dependent and/or subtype-selective, and (2) how these events correlate with changes in the cytoskeleton, signal transduction cascades and/or protein-related processes. The results from our studies indicate that stimulation of the mt1 melatonin receptor exerts a strong influence on cytoskeletal organization and dynamics and that this receptor can regulate the signaling pathway that is closely associated with differentiation in neuronal cells.

MATERIALS AND METHODS

Cell Culture

The three cell lines used in this study were produced by co-transfecting CHO cells with the pSV2neo neomycin resistance plasmid (Clontech, Palo Alto, CA) (neo-CHO) and with a plasmid containing either the human mt1-melatonin receptor cDNA (human Mel_{1a} in pcDNAI [Reppert et al., 1994]) (mt1-CHO) or the human MT2 melatonin receptor cDNA (human Mel_{1b} in pcDNA3 [Reppert et al., 1995]) (MT2-CHO). The development and characterization of these cell lines are published elsewhere [Witt-Enderby and Dubocovich, 1996; Witt-Enderby et al., 1997]. Cells were maintained in F-12 media (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO-BRL) and 1% penicillin/streptomycin (GIBCO-BRL), at 37°C in a 5% CO_2 atmosphere. Cells were passed twice weekly and used for experiments up to passage number forty. At that time, new cells were grown from a frozen stock.

Treatment Conditions

After 2 days of growth, cells (neo-CHO, mt1-CHO, MT2-CHO) were treated as described in the results. Melatonin (Sigma Chemical Co., St. Louis, MO) solubilized in ethanol was added to achieve a final concentration of 1 μM. Colcemid (Calbiochem, San Diego, CA) or β-lumicolchicine (Sigma) solubilized in DMSO was added to a final concentration of 5 μM. Ultraviolet irradiation (366-nm light) of treated cultures to inactivate Colcemid was carried out for 2 min using a UVP (Upland, CA) UVL 56 ultraviolet (UV) lamp. The signal transduction activators or inhibitors used in this study were purchased from Calbiochem, and all were dissolved in DMSO. The compounds chosen have the property of being membrane permeable and were used at concentrations taken from published reports. The cells were treated with the specified activators of signaling in the absence of melatonin and included: forskolin (100 μM), an activator of adenylyl cyclase [Seamon and Daly, 1981], 8-bromo-adenosine-3′,5′-cyclic monophosphate (10 μM), which stimulates protein kinase A (PKA) [Hei et al., 1991], and the compound 1-oleolyl-2 acetyl-sn-glycerol (50 ng/ml), which activates protein kinase C (PKC) [Gilmore and Martin, 1983]. The cells were exposed to the specified inhibitors of signaling for 30 min. before the addition of melatonin to a concentration of 1 μM. The inhibitors included: KT5720 (0.01–0.12 μM), which preferentially inhibits PKA activity [Gadbois et al., 1992], U-73122 (5 and 10 μM) an inhibitor of phospholipase C (PLC) [Thompson et al., 1991] and W7 (35 and 70 μM) that inhibits calmodulin [Hidaka et al., 1981].

Control treatments consisted of the addition of the appropriate amounts of ethanol and/or DMSO vehicle alone. All treatments were carried out for 5 h at 37°C in a 5% CO_2 atmosphere.

Cell Shape Determination

Cells were grown to a subconfluent density in 60-mm tissue culture dishes (Nalge Nunc International, Copenhagen). F-12 media was replenished and the cells were subjected to treatment with melatonin with or without microtubule inhibitor or signal transduction activator/inhibitor. After the treatments, the cells were fixed with 0.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 0.62 mM KH_2PO_4, pH 7.4) for 5 min and washed 3 times with PBS. Cell shapes were observed and counted using a Nikon TMS inverted microscope equipped with phase-contrast optics. Images were digitally captured using a Scion Series 7 image capture system (Scion Corporation, Frederick, MD).

To quantify the morphological changes in the cells induced by melatonin treatment, populations of cells (100–500 cells/experimental group) were counted and classified by shape. Cells were scored as to whether they displayed a flat, round, spindle or thread-like cell shape. The shapes chosen were those that we presumed to represent the transition in form from a flattened to a bipolarized morphology. We specifically carried out these quantification experiments at subconfluent cell densities in order to avoid crowding-induced cell shapes. Cell counts were made by an observer who had no knowledge of the treatment that the cells had received.

Immunocytochemistry

Cells were grown to subconfluent densities on glass coverslips and were subjected to the aforementioned treatment conditions. After the treatment period, the cells were fixed for 20 min in 100% methanol at −20°C. Microtubules or microfilaments were localized with a mouse anti-chick brain β-tubulin, or a mouse anti-chicken gizzard actin primary antibody (1:100 dilution, Amersham Life Sciences, Arlington Heights, IL). Centrosomes and microtubule associated proteins were localized with mouse monoclonal antibodies (1:100 dilution,

20H5 [centrin], a gift of Dr. Jeffrey L. Salisbury; Tau5, a gift of Dr. Lester I. Binder; MAP2a,b,c and MAP 1b; LabVision, Fremont, CA). A Texas-Red conjugated anti-mouse secondary antibody (1:50 dilution, Amersham) and standard immunofluorescence techniques [Osborn and Weber, 1982; Melan and Sluder, 1992] were used for visualization of antibody binding. The coverslips were mounted onto slides with a polyvinyl alcohol mounting medium [Osborn and Weber, 1982]. Cells were viewed with a Nikon Eclipse 600 microscope equipped with fluorescence optics providing excitation wavelengths of light at 546 nm and emission filter at 660 nm. Photographs were taken with Kodak T-Max 400 film.

Concentration Dependence of Morphological Changes in mt1-CHO Cells

To determine whether outgrowth formation was a concentration-dependent phenomenon, mt1-CHO cells were grown to a subconfluent density on glass coverslips. Cells were replenished with F-12 media, containing various concentrations of melatonin (1 pM to 100 μM) and incubated for 5 h. After treatment, cells were fixed, washed, and mounted on slides. Cells were counted and classified by shape as described previously.

Removal of Melatonin

To determine whether the outgrowths induced after melatonin treatment were permanent or transient, cells were grown to a subconfluent density in 60-mm plates. Cultures were replenished with F-12 media containing either vehicle (control) or melatonin (1 μM) and incubated for 5 h. After the treatment, the control cells were washed three times with media and then incubated in F-12 media for an additional 24 h. For the cells exposed to melatonin for 5 h, one set of cells was washed three times and then incubated 24 h in F-12 medium without melatonin, and the other set of cells was washed and then re-exposed to 1 μM melatonin for an additional 24 h. After treatment, cells were fixed, washed, counted and classified by shape as described previously.

Effects of Transcriptional or Translational Inhibitors

To determine the contribution(s) of transcription and/or translation events to the induction of the outgrowths, mt1-CHO cells were grown for 2 days to a subconfluent density in 60-mm plates. Cells were replenished with F-12 media, containing melatonin with or without inhibitor and incubated for 5 h. Treatment compounds were solubilized in DMSO or H_2O and used at the following final concentrations: cycloheximide (10 μg/ml), actinomycin D (1 μg/ml), αamanatin (10 nM). After treatment, the cells were washed, fixed, rinsed, counted, and classified by shape as described previously.

Western Blot Analysis of MEK 1/2 and ERK 1/2 Phosphorylation

Samples of total cellular protein were collected by scraping cell monolayers into a lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 1 m EDTA, 1 mM EGTA and 1× Complete® protease inhibitors (Boehringer-Mannheim) [Mandai et al., 1999]. In this study, 40 μg of total protein were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). Blots were probed with primary antibodies against either MEK 1/2 total protein or phospho-specific antibodies that recognize phosphorylated MEK 1/2 (Calbiochem). Blots were also probed with primary antibodies against ERK 1/2 total protein or phospho-specific antibodies against diphosphorylated ERK 1/2 (Sigma). Secondary antibodies conjugated with horseradish peroxidase (HRP) (Amersham Life Sciences), enhanced chemiluminescent detection (ECL-Plus, Amersham) and exposure to Kodak BioMax Light film were used to visualize the protein bands.

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA), followed by the Newman-Keul post-hoc test or a two-tailed *t*-test (GraphPad Prism Software, GraphPad Prism, San Diego, CA). Significance was defined as $P < 0.05$.

RESULTS

The goal of this investigation was to determine whether the shape changes we saw in our preliminary observations occurred at a statistically significant frequency. We also wanted to determine the cellular mechanisms underlying these morphological changes, specifically those involved with and having influence on the cytoskeleton.

Cell Shape Changes

Observation of the three cell lines after 5 h of melatonin treatment showed that the majority of mt1-CHO cells displayed a bipolarized cell shape and that many possessed long filamentous outgrowths (Fig. 1E, arrows). The control cell line (neo-CHO) (Fig. 1D) and control treatment (0 μM melatonin) (Fig. 1A–C) showed cells whose morphology was typical of untransformed CHO cells (i.e., highly flattened fibroblasts). The MT2-CHO cell line showed a subtle shape change (Fig. 1F). Short projections were observed on a few cells, however, the frequencies of these changes were not statistically significant.

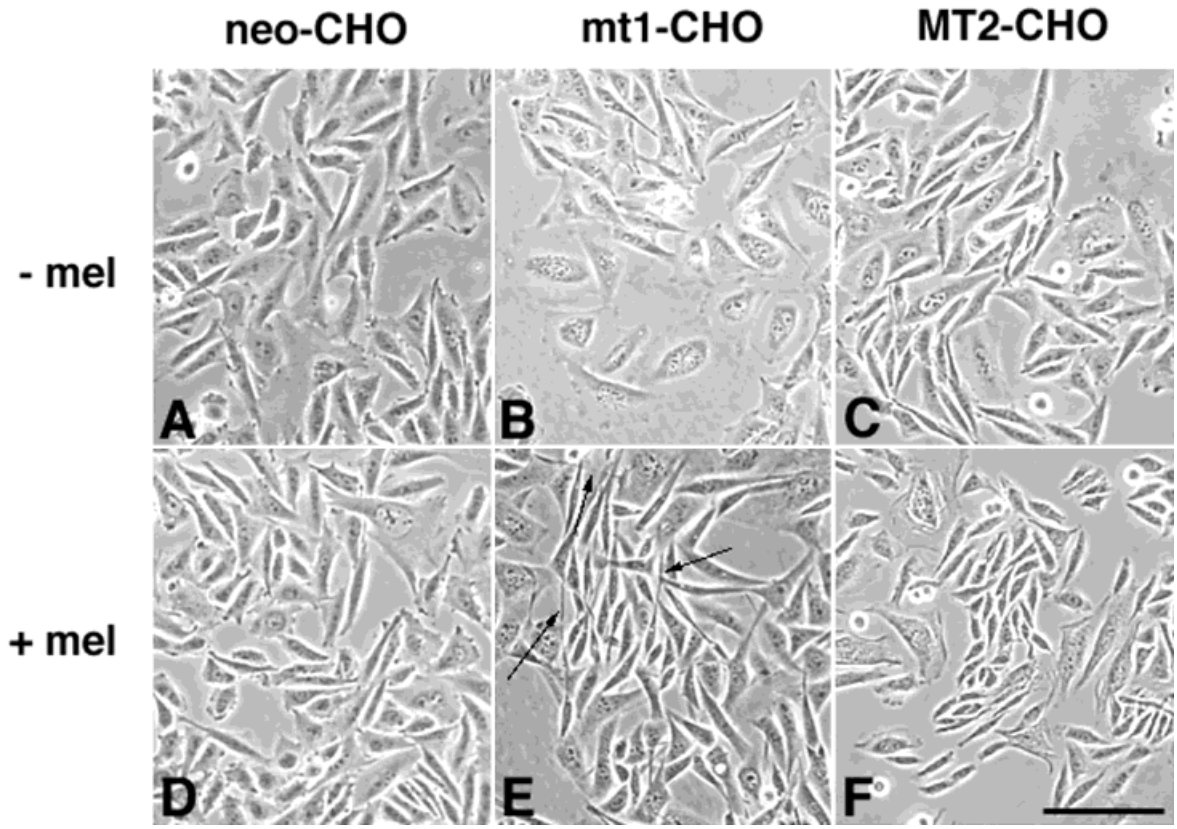

Fig. 1. Cell shapes after treatment with melatonin. Phase-contrast micrographs of neo-CHO cells (A,D), mt1-CHO cells (B,E), or MT2-CHO cells (C,F). **A–C:** Cell shapes after 5-h vehicle control treatment. **D–F:** Cell shapes after 5-h treatment with 1 μM melatonin. mt1-CHO cells treated with melatonin display a bipolarized shape (E) and the production of filamentous outgrowths (E, arrows). Melatonin-treated neo-CHO and MT2-CHO cells do not produce outgrowths (D,F). For vehicle treatments, all cells display a flattened morphology (A–C). Data shown are from one representative experiment repeated 2–6 times. Scale bar = 40 μm.

In melatonin-treated mt1-CHO cells, 46% of the cell population displayed a thread-like cell shape (filamentous outgrowths) (Table I). Comparison of the number mt1-CHO cells displaying a thread-like shape after melatonin treatment (46%) with mt1-CHO cells not exposed to melatonin (21%), reveals a statistically significant difference in the number of cells which display an altered shape after exposure to melatonin (ANOVA; $P < 0.05$) (Table I). Comparison of the number of these cells (mt1-CHO + melatonin) (46%) with the number of neo-CHO cells displaying a thread-like cell shape after melatonin treatment (5%) is also statistically significant (ANOVA; $P < 0.05$) (Table I). In addition, a significant difference in the number of melatonin-treated mt1-CHO cells displaying a flat shape (21%) is seen in comparison to neo-CHO cells after melatonin treatment (79%) (ANOVA; $P < 0.05$) (Table I).

When we first observed these changes in cell shape, we were concerned as to whether a true process of outgrowth was occurring (as opposed to retraction of the cell body). Measurements of cell lengths confirm that cells possessing outgrowths (thread-like shape) are significantly longer than cells displaying the flattened morphology. Flat cells have an average length of 44.7 ± 1.5 μm (n = 100), whereas thread-like cells have an average length of 98.4 ± 2.9 μm (n = 100). A two-tailed *t*-test confirms that this length difference is statistically significant ($P < 0.05$).

To further confirm that a true process of outgrowth was occurring, photographs of groups of cells were taken at 30-min intervals. Figure 2 shows the progressive elongation of one such cell (marked with asterisk above cell). The outgrowth process becomes noticeable after 1 h of melatonin treatment. In the example shown, the upper outgrowth proceeds faster than the lower outgrowth. Both processes show a loss of contact inhibition in that the outgrowths proceed over the surfaces of adjacent cells. Arrows in the first and last frames indicate the extremities of the lamellipodia and show that the cell has nearly doubled in length after 5 h.

TABLE I. Effects of Melatonin on Cell Morphology†

Cell line	Cell shape	Untreated	+Mel	+Mel/Colc	+Mel/β-lum
neo-CHO	Flat	78 ± 5	79 ± 4	95 ± 2	82 ± 8
	Round	5 ± 2	6 ± 3	3 ± 2	4 ± 2
	Spindle	11 ± 3	10 ± 2	2 ± 0.4	12 ± 7
	Thread	4 ± 1	5 ± 2	0.4 ± 0.2	2 ± 0.7
mt1-CHO	Flat	57 ± 13	21 ± 3**‡	79 ± 5	17 ± 5
	Round	18 ± 8	14 ± 5	14 ± 5	7 ± 4
	Spindle	18 ± 8	19 ± 5	5 ± 2	16 ± 4
	Thread	9 ± 2	46 ± 9*,**‡	1 ± 0.5	57 ± 10*,**,‡
MT2-CHO	Flat	81 ± 6	70 ± 5	94 ± 1	76 ± 14
	Round	5 ± 2	10 ± 4	3 ± 2	0
	Spindle	10 ± 3	16 ± 2	2 ± 1	8 ± 4
	Thread	4 ± 2	4 ± 1	0.9 ± 0.3	15 ± 14

†Classification of cells by morphology (flat, round, spindle, and thread) after a 5-h treatment with: F-12 media + vehicle (untreated); 1 μM melatonin (+Mel); 1 μM melatonin + 5 μM Colcemid (+Mel/Colc); or 1 μM melatonin + 5 μM β-lumicolchicine (+Mel/β-lum). Data are expressed as a percentage of total cells counted and represent the mean ±S.E.M. from 3–7 experiments.
*Significance from control (vehicle treated) cells, $P < 0.05$.
**Significance from neo-CHO cells, $P < 0.05$.
‡Significance from MT2-CHO cells, $P < 0.05$.

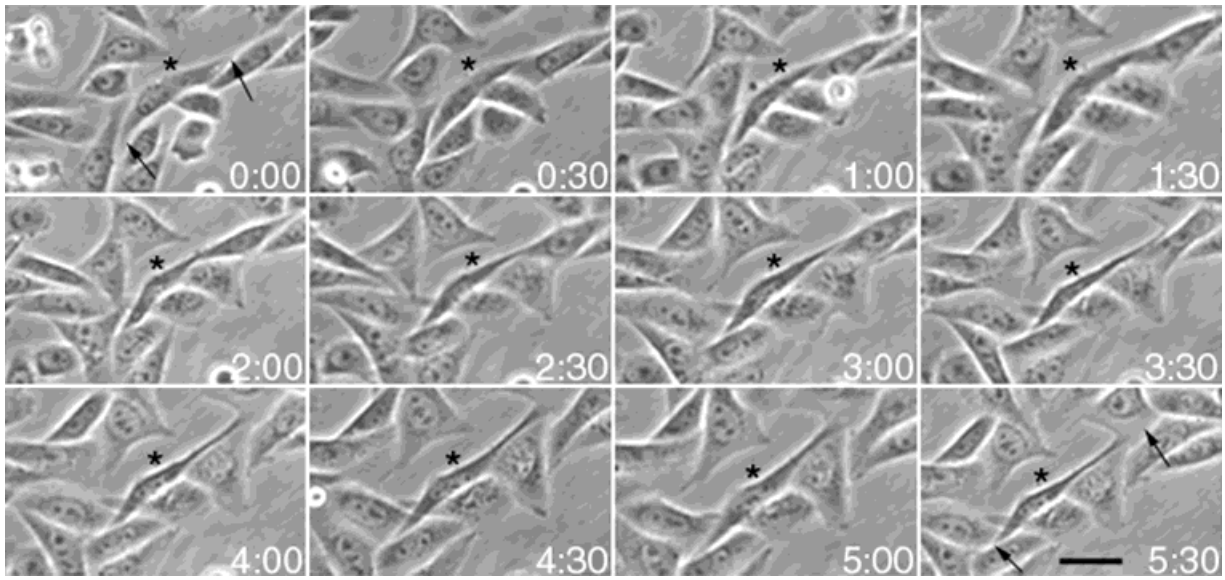

Fig. 2. Time-lapse series of outgrowth formation in mt1-CHO cells. Phase-contrast micrographs. Time in hours and minutes of application of 1 μM melatonin is shown in the lower right. Data shown are from one representative experiment repeated 10 times. Elongating cell is indicated with an asterisk above the cell. Arrows indicate the boundary of lamellipodial extension. Scale bar = 40 μm.

Microtubule and Microfilament Array Changes

The outgrowths we observed formed by the mt1-CHO cells were reminiscent of neurites formed by neuronal cells [Patterson, 1992a,b; Vale et al., 1992]. Because microtubules and microfilaments play a pivotal role in neurite formation [Drubin et al., 1985, 1988; Vale et al., 1992; Nathan et al., 1995], we examined the cytoskeletal arrangements in these cells, using immunofluorescence microscopy. In vehicle treated mt1-CHO cells, microtubules emanated from the centrosome and were randomly distributed throughout the cytoplasm (Fig. 3A). After 5-h treatment with 1 μM melatonin, the mt1-CHO cells displayed long bundles of microtubules extending into the outgrowths formed by the cells (Fig. 3B, arrow). No microtubule bundles were seen in either the neo-CHO or MT2-CHO cells with or without melatonin treatment (data not shown). The localization of actin filaments in mt1-CHO cells not exposed to mela-

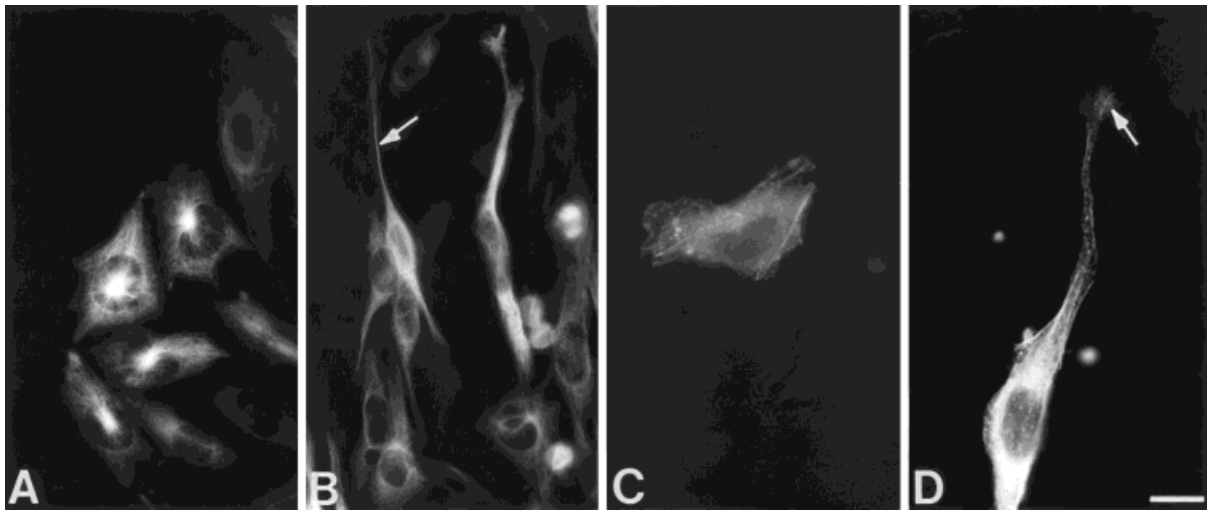

Fig. 3. Microtubule and microfilament arrangements in mt1-CHO cells after melatonin treatment. Fluorescence micrographs of microtubules (**A,B**) and actin (**C,D**). In the absence of melatonin, the cells show randomly oriented microtubules dispersed throughout the cytoplasm (A). After 5-h treatment with 1 μM melatonin, long bundles of microtubules are seen within the outgrowths (B, arrow). Microfilaments show a typical fibroblastic pattern in the absence of melatonin with large stress fibers in the cytoplasm (C). After melatonin treatment, microfilaments are found throughout the outgrowth and within the growth cone-like structure at the outgrowth terminus (D). Microtubules or microfilaments were localized with mouse anti-chick brain β-tubulin or mouse anti-chicken gizzard actin primary antibodies respectively. A goat anti-mouse IgG Texas-Red-conjugated secondary antibody was used for detection. Data shown are from one representative experiment repeated four times. Scale bar = 10 μm.

tonin showed a pattern typically seen in fibroblasts. Many actin filaments fill the cytoplasm with a thin layer of filaments at the cell periphery. Large stress fibers were seen as well, at presumed points of substrate adhesion (Fig. 3C). In cells treated with melatonin, actin filaments were seen throughout the outgrowth (Fig. 3D) and within the lamellipodium at the outgrowth terminus (Fig. 3D, arrow). No large stress fibers were noted within the lamellipodia.

Closer examination of the mt1-CHO cell outgrowths revealed that they possessed a lamellipodial structure at their leading edge reminiscent of a neuronal growth cone. Co-localization of actin filaments and microtubules within these structures showed that the arrangement of these cytoskeletal elements is similar to that noted for neuronal growth cones [Forscher and Smith, 1988; Vale et al., 1992]. Microtubules do not comprise the major formational component of these structures. Single microtubules extend into the area (Fig. 4A); however, the large bundles of microtubules do not extend into the flattened area itself (Fig. 4A, arrowheads). Actin filaments extend throughout the structure with a greater density noted in the region of the plasma membrane and within filopodia (Fig. 4B, arrowheads).

Previous studies have shown that increased expression or ectopic expression of microtubule associated proteins (MAPs) can cause cells to form long neurite extensions [Knops et al., 1991; Barlow et al., 1994; LeClerc et al., 1996; Melendez et al., 1996]. We examined melatonin treated mt1-CHO cells with immunofluorescence for the presence of the neuronal MAPs MAP2, MAP 1b, and τ. We did not detect the presence of these MAPs within the cells or outgrowths of the mt1-CHO cells (data not shown).

Dependence of Shape Changes on Assembly-Competent Tubulin

Because of the prominent microtubule bundles observed, we wanted to determine whether microtubules were required for the formation of mt1-CHO cell outgrowths as they are for neurite formation in neuroblastoma cell lines [Seeds et al., 1970]. To accomplish this, we treated the cells with a combination of 1 μM melatonin and 5 μM Colcemid for 5 h. Colcemid is a photoreversible analog of colchicine and causes the depolymerization of microtubules by binding to tubulin monomers preventing their assembly into polymers [Sluder, 1991; Alberts et al., 1994]. Quantification of the cell shapes after Colcemid treatment revealed several statistically significant results. The number of melatonin-treated mt1-CHO cells displaying a thread-like shape without Colcemid treatment (46%) (Table I; Fig. 1E) was significantly greater than the number of the same cells after treatment with a combination of melatonin and Colcemid (1%) (ANOVA; $P < 0.05$) (Table I; Fig. 5B). In addition, the number of mt1-CHO cells displaying a

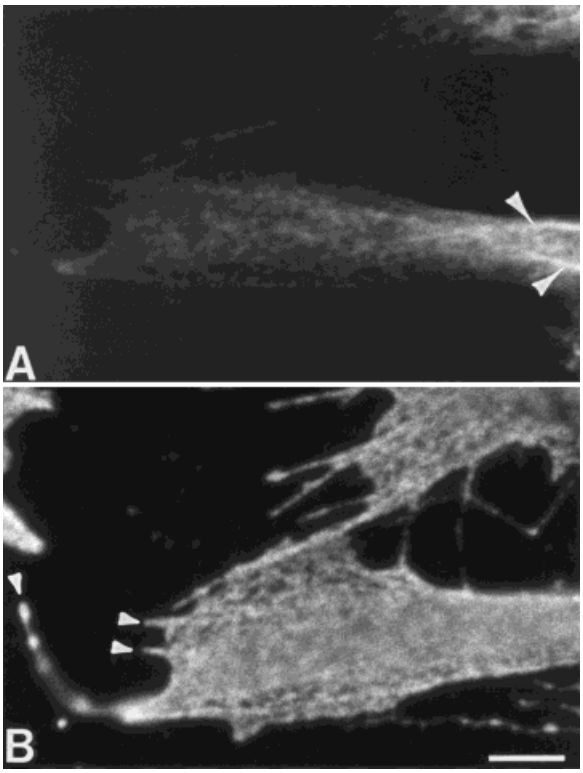

Fig. 4. Co-localization of microtubules and microfilaments within growth cone-like structures. **A:** Dispersed microtubules are found throughout the region; however, microtubule bundles are restricted to the outgrowth stalk (A, *arrowheads*). **B:** Microfilaments are distributed throughout the lamellipodial region and within filopodia (B, *arrowheads*). Data shown are from one representative experiment repeated three times. Scale bar = 5 μm.

flat shape after melatonin treatment (21%) was significantly less than the number of the same cells with a flat shape in the presence of Colcemid (79%) (ANOVA; $P < 0.05$) (Table I). Thus, we see that the microtubule inhibitor Colcemid effectively eliminated the formation of the filamentous outgrowths in mt1-CHO cells after melatonin pretreatment (Fig. 5B). No outgrowths were observed on neo-CHO or MT2-CHO cells in the presence of Colcemid (Fig. 5A,C).

Upon irradiation of Colcemid with 366 nm UV light, it is converted to its inactive isomer, β-lumicolchicine [Sluder, 1991]. Cells that had been treated for 5 h with combined melatonin and Colcemid were irradiated with 366 nm light for 2 min. We then allowed the cells to recover for an additional 5 h before observation. After Colcemid photoreversal, only mt1-CHO cells showed a reformation of outgrowths (Fig. 5E, arrows). These outgrowths, however, differed in appearance from those seen with melatonin treatment alone (Fig. 1E, arrows). The reformed outgrowths were monopolar, rather than bipolar. Neither the neo-CHO nor the MT2-CHO cells produced outgrowths after Colcemid photoreversal (Fig. 5D,F).

The prevention of microtubule assembly and outgrowth formation by Colcemid was not the result of nonspecific toxic effects. This was determined by directly treating cells with β-lumicolchicine, the inactive isomer of Colcemid. Treatment of cells with a combination of 1 μM melatonin and 5 μM β-lumicolchicine for 5 h had no effect on suppressing outgrowth formation in the mt1-CHO cells (Table I).

Dependence of Shape Changes on Melatonin Concentration and Exposure

The effect of melatonin on inducing outgrowths in mt1-CHO cells was concentration-dependent. Melatonin induced the formation of outgrowths over a range of melatonin concentrations from 1 pM to 100 μM. Outgrowth frequencies increased with melatonin concentration up to 1 mM (0 pM = 2.4 ± 0.68%, 1 pM = 5.7 ± 2.3%, 1 nM = 12.5 ± 1.6%, 30 nM = 13.7 ± 1.8%, 1 μM = 28.6 ± 3.7%; ANOVA, $P < 0.05$). The maximal shape change frequency occurred at the 1 μM melatonin concentration. Concentrations of melatonin higher than 1 μM showed a reduced percentage of cells displaying outgrowths (30 μM = 21.3 ± 6.9%, 100 μM = 13.0 ± 3.8%; ANOVA, $P < 0.05$) and is presumed to be due to receptor desensitization.

Extension and maintenance of nerve growth factor (NGF)-induced neurite outgrowths in PC12 [Drubin et al., 1988] and other neuronal cell types [Levi-Montalcini, 1987] require the constant presence of NGF. We wanted to know whether melatonin-induced outgrowths showed the same requirement for constant agonist exposure. The effects of melatonin on inducing the outgrowths in mt1-CHO cells were reversible. As shown in Figure 6 (wash), the frequency of thread-like outgrowths induced by melatonin decreased significantly after the removal of melatonin.

Contribution of Transcription and/or Translation to Changes in Cellular Morphology

The ectopic expression of neuronal MAPs was previously shown to induce neurite-like outgrowths in non-neuronal cells [Knops et al., 1991; Barlow et al., 1994; LeClerc et al., 1996; Sharp et al., 1996], prompting our interest in determining whether the formation of outgrowths in our cell line was brought about by gene expression and/or the synthesis of new protein. The effect of melatonin on inducing the outgrowths in mt1-CHO cells was dependent on protein synthesis but not on the transcription of new mRNA. As shown in Figure 6, treatment of cells with a combination of 10 μg/ml cycloheximide and 1 μM melatonin prevented the increase in outgrowth formation induced by melatonin alone. In

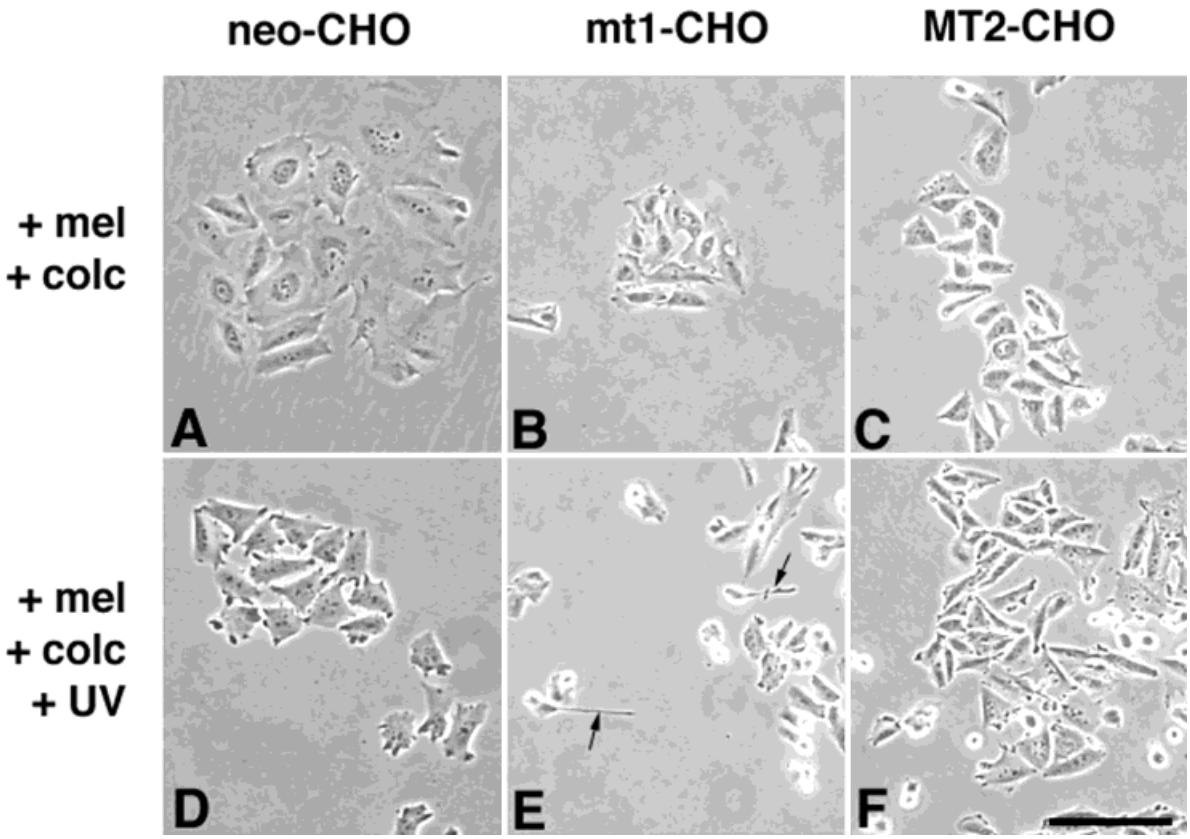

Fig. 5. Cell shapes after combined treatment with melatonin and Colcemid. **A–C:** Cell shapes after 5-h treatment with 1 μM melatonin + 5 μM Colcemid. **D–F:** Cell shapes after 5-h treatment 1 μM melatonin + 5 μM Colcemid, exposure to UV light (366 nm) for 2 min. and an additional 5-h incubation with 1 μM melatonin. Colcemid treatment blocks the melatonin-induced shape change in mt1-CHO cells (B). UV inactivation of Colcemid results in the re-formation of monopolar outgrowths in mt1-CHO cells (E, arrows). No outgrowths are produced in either neo-CHO or MT2-CHO cells. Data shown are from one representative experiment repeated 2-6 times. Scale bar = 40 μm.

contrast, treatment of the cells with a combination of 1 μM melatonin and 1 μg/ml actinomycin D, a steric inhibitor of RNA polymerase, or 10 nM α-amanatin, a specific inhibitor of RNA polymerase II, did not lower the number of cells displaying outgrowths (Fig. 6).

Changes in Shape Frequency After the Inhibition of PKA

In order to gain a more complete view of which signal transduction mechanisms were involved in the induction of outgrowth formation by melatonin, activators and inhibitors of specific signal transduction molecules in both the cAMP and Ca^{2+}/IP_3 pathways were tested. As shown in Figure 7, treatment of cells with the cAMP-dependent PKA inhibitor KT5720 in combination with 1 μM melatonin enhanced the frequency of outgrowth formation in mt1-CHO cells. No significant formation of outgrowths was seen for either neo-CHO or MT2-CHO cells (data not shown). This change in frequency was dependent on the concentration of the inhibitor with higher concentrations resulting in a greater number of outgrowths. Treatment of cells with 0.12 μM KT5720 alone resulted in the production of outgrowths at a frequency slightly less than that seen with melatonin treatment alone. Inhibition of calmodulin by W7 or phospholipase C by U-73122 did not enhance or suppress outgrowth formation in any of the cell lines. Similarly, activation of PKA by forskolin or 8-bromo cAMP or activation of phospholipase C by 1-oleolyl-2-acetyl-sn-glycerol had no effect on stimulating or inhibiting outgrowth formation in any of the cell lines tested (data not shown).

Increases in MEK 1/2 and ERK 1/2 Phosphorylation After Melatonin Treatment

Because previous investigators have shown that the MEK 1/2-ERK 1/2 pathway is an important component of neuronal differentiation [Fukuda et al., 1995; Creedon et al., 1996; Hashimoto et al., 2000], we examined two of the major members of this pathway to determine their activities in response to melatonin. We examined the

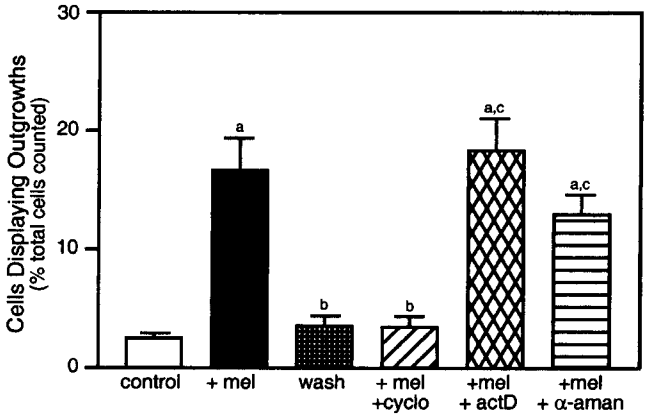

Fig. 6. Melatonin-induced outgrowth formation in mt1-CHO cells after melatonin removal or treatment with transcriptional or translational inhibitors. Graphical representation of the number of mt1-CHO cells displaying outgrowths after 5-h exposure to 1 μM melatonin and replacement with melatonin-free media (5 h) or 5-h treatment with 1 μM melatonin with or without cycloheximide (10 μg/ml), actinomycin D (1 μg/ml), or α-amanatin (10 nM). Removal of melatonin exposure or cycloheximide treatment prevents the formation of thread-like outgrowths. Actinomycin D and α-amanatin do not reduce outgrowth numbers. Control treatment consists of exposure to vehicle only. Data represent the mean ± S.E.M. of 3–6 individual experiments. [a] indicates significance from control (vehicle treated) cells, $P < 0.05$; [b] indicates significance from melatonin-treated cells, $P < 0.05$; [c] indicates significance from cycloheximide- treated cells, $P < 0.05$.

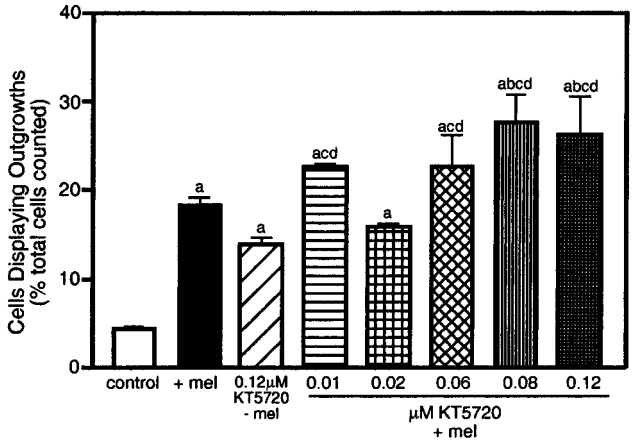

Fig. 7. Effects of inhibition of PKA by KT5720 in mt1-CHO cells. Graphical representation of the numbers of mt1-CHO cells displaying thread-like cell shapes after 5-h exposure to 1 μM melatonin in the absence or presence of increasing concentrations of KT5720 (0.01–0.12 μM) Control treatment consists of exposure to vehicle only. Data represent the mean ± S.E.M. of 3–6 individual experiments. [a] indicates significance from control (vehicle treated) cells, $P < 0.05$; [b] indicates significance from melatonin-treated cells, $P < 0.05$; [c] indicates significance from 0.12 μM KT5720-treated cells without melatonin, $P < 0.05$; [d] indicates significance from melatonin + 0.02 μM KT5720 -treated cells, $P < 0.05$.

neo-CHO, mt1-CHO and MT2-CHO cell lines for MEK 1/2 and ERK 1/2 phosphorylation using either antibodies directed against the total protein or phospho-specific antibodies to detect the activated protein form using Western analysis. Figure 8 shows that all cell lines possess the MEK 1/2 protein. Only mt1-CHO cells show the presence of phosphorylated MEK 1/2 and the amount of phosphorylated protein is increased after the 5-h melatonin treatment. Neither neo-CHO nor MT2-CHO cells show the presence of phospho-MEK. By the same token, all cells showed that they possess ERK 1/2 protein. However, only the mt1-CHO cell line showed a melatonin-dependent increase in the diphosphorylated form of ERK 1/2. Even though the neo-CHO cell line possesses a small amount of diphosphorylated ERK 1/2, there is no difference in the amounts of phosphorylated protein with or without melatonin treatment. MT2-CHO cells show that no phospho-ERK is present in the cells.

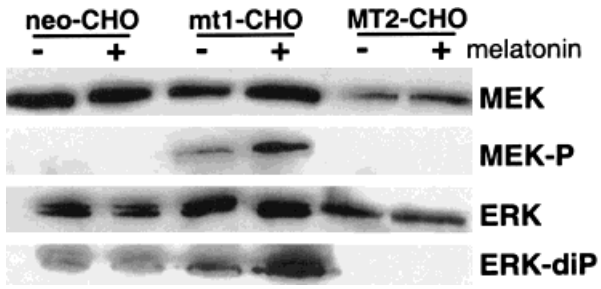

Fig. 8. Western analysis of MEK 1/2 and ERK 1/2 activation. neo-CHO, mt1-CHO or MT2-CHO cells were treated with either 1 μM melatonin or vehicle for 5 h. Total cellular protein extracts (40 μg/lane) were separated by SDS-PAGE and blotted onto PVDF membranes using standard techniques. Blots were probed with primary antibodies specific for MEK 1/2 or ERK 1/2 whole protein or activated phosphoprotein and detected with chemiluminescence. Data are from one representative experiment repeated three times. Melatonin-dependent increases in MEK 1/2 and ERK 1/2 phosphorylation are seen for mt1-CHO cells only

Control of Outgrowth Number by Cortical Actin, and Not Centrosomes

Because the cells with outgrowths generally formed only two processes (Figs. 1E, 2, 3B) and because centrosomal positioning has previously been correlated with process formation [Zmuda and Rivas, 1998], we hypothesized that cells in G_2 formed outgrowths nucleated by the two centrosomes present in the cells at this time. Immunostaining of melatonin-treated mt1-CHO cells with an anti-centrin antibody showed no correlation of centrosome number and/or position with the outgrowths formed (Fig. 9A). Most cells possessed a single centrosome located more or less equidistant from the two outgrowths, rather than at the outgrowth base(s) (Fig. 9B,

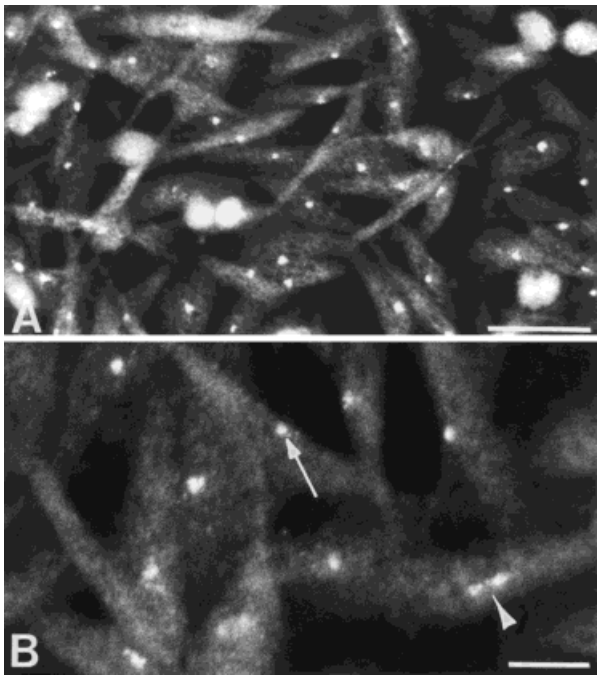

Fig. 9. Centrosome localization in mt1-CHO cells after melatonin treatment. Fluorescence micrographs of centrosomes show no correlation between centrosome number and position and the number or position of outgrowths (**A**). Nonspecific background staining shows the overall cell shape while centrosomes show a strong fluorescent signal. Higher magnification (**B**) shows that in cells with one (*arrow*) or two (*arrowhead*) centrosomes the centrosome is not located at the outgrowth base(s). Centrosomes were localized with a mouse anti-centrin primary antibody and a goat anti-mouse IgG Texas-Red conjugated secondary antibody. Data shown are from one representative experiment repeated two times. Scale bars = 30 μm in A; = 10 μm in B.

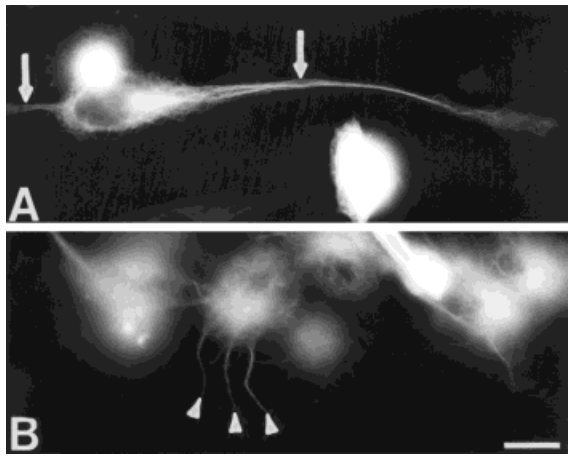

Fig. 10. Localization of microtubules in mt1-CHO cells with and without cytochalasin D treatment. Fluorescence micrographs of mt1-CHO cells treated with 1 μM melatonin alone (**A**) or 1 μM melatonin + 20 μM cytochalasin D (**B**). Cells that have intact actin cytoskeletons typically produce two outgrowths after melatonin treatment (A, *arrows*). Melatonin treatment in conjunction with actin depolymerization results in the formation of multiple outgrowths per cell (B, *arrowheads*). These cells, however, retain a bipolar character. Data shown are from one representative experiment repeated two times. Scale bar = 20 μm.

arrow) Outgrowth-bearing cells with two centrosomes similarly showed that the centrosomes were not located at the bases of the outgrowths (Fig. 9B, arrowhead).

Examination of cells treated with a combination of 1 μM melatonin and 20 μM cytochalasin D with phase-contrast optics showed cells that initially appeared to have a round morphology. However, when the microtubule arrays of these cells were examined, we noted that there were several bundles of microtubules emanating from the ends of the cells (Fig. 10B, arrowheads). These bundles were shorter than those noted for cells with intact actin arrays (Fig, 10A, arrows). The cells maintained their overall polarity with bundles of microtubules being produced in clusters at the ends of the cell (Fig. 10B, arrowheads). These outgrowths were barely visible under phase optics (data not shown) and are presumed to consist of microtubule bundles covered only by plasma membrane.

DISCUSSION

This study has demonstrated that treatment of non-neuronal cells expressing the human mt1 melatonin receptor with a pharmacological concentration (1 μM) of melatonin has the following effects: (1) it causes rapid cellular morphological changes consisting of the formation of long filamentous structures; 2) these morphological changes are receptor-dependent and possibly subtype selective, (3) the changes require the presence of assembly-competent tubulin, (4) the frequency of shape changes are concentration and protein synthesis-dependent but not dependent on transcriptional events, (5) the changes probably occur through inhibition of PKA with subsequent activation of MEK 1/2 and ERK 1/2, and (6) the number of outgrowths per cell is controlled by the actin cytoskeleton.

The effects of melatonin on cell morphology that we observe are consistent with and greatly extend the information reported in other studies. Cellular morphological changes have been noted for other cell types treated with melatonin. However, these changes occur at a much slower rate than what we observe in the mt1-CHO cells. After 24 h, MDCK cells treated with melatonin (10 pM to 100 nM) show an increase in the number of elongated cells [Benitez-King et al., 1990]. In addition, N1E-115 cells and SK-N-SH cells treated with melatonin for 48 and 96 h, respectively, show an increase in the number of cells with neurite processes [Benitez-

King et al., 1990; Cos et al., 1996]. Microtubule bundles were abundant in the N1E-115 neurite processes and a slight increase in the amount of tubulin correlated with the increase in neurites seen in these cells [Benitez-King et al., 1990]. The differentiation of N1E-115 cells due to changes in microtubule organization may be initiated by the expression of microtubule-associated proteins (MAPs) as an increase in MAP2 mRNA and protein levels are also seen in these cells after melatonin treatment [Benitez-King et al., 1990; Cos et al., 1996; Melendez et al., 1996].

Although melatonin is considered an exclusively neuronal hormone, melatonin receptors have been found in tissues of organs outside of the nervous system. In particular, the binding of labeled melatonin and the presence of melatonin receptors have been noted for the kidney [Song et al., 1997] and the ovary [Niles et al., 1999]. Interestingly, both organs possess cells that produce long outgrowths as part of their differentiation mechanism (i.e., renal glomerular podocytes and ovarian granulosa cells) [Mundel et al., 1997; Albertini and Anderson, 1974]. Specific localization of mt1 melatonin receptors at the cellular level has only been demonstrated for granulosa cells [Niles et al., 1999]; however, mt1 receptors have been localized in the glomerulus [Song et al., 1997]. The potential effects of melatonin on the differentiation of podocytes or granulosa cells has not yet been studied.

In this study, significant morphological changes were observed only in mt1-CHO cells. This demonstration of morphological changes occurring as a receptor-dependent and possibly subtype-selective event is novel. Previous studies showing melatonin involvement in the morphological changes of neuroblastoma cells have not determined whether these effects are specifically mediated through melatonin receptors and, if so, which subtype(s) of melatonin receptors are present and functioning [Benitez-King et al., 1990; Cos et al., 1996; Melendez et al., 1996]. Our results greatly clarify the observations of others in regard to cell shape changes. However, our results do not support the claim that melatonin causes cellular shape changes through receptor-independent actions [Benitez-King and Anton-Tay, 1993; Benitez-King et al., 1996], as we observed no discernible changes in the morphology of neo-CHO cells after melatonin treatment. In addition, we do not believe that calmodulin is inhibited in a receptor-independent manner as proposed by Benitez-King and co-workers [Benitez-King and Anton-Tay, 1993; Benitez-King et al., 1996] because inhibition of calmodulin in these cells by W7 did not promote the formation of outgrowths. The lack of a cellular response in MT2-CHO cells is intriguing and suggests subtype-selectivity. The MT2 receptor has been shown to attenuate adenylyl cyclase activity [Reppert et al., 1995; Jones et al., 2000] and has additionally been shown to be linked with the inhibition of guanylyl cyclase activity [Petit et al., 1999]. Further work is needed to firmly establish the functions of the MT2 receptor in CHO cells.

Examination of the actin filament and microtubule distributions within the mt1-CHO cells after melatonin receptor stimulation shows a change from the "normal" arrangement of these elements typically found in fibroblasts. After melatonin treatment, the microtubules rearrange into long bundles extending outward through the stalk of the outgrowth. Actin filaments extend through the outgrowth as well and are the major component of the expanded lamellipodial structure formed at the terminus of the outgrowth. The bundled microtubules do not extend into this area. This kind of cytoskeletal arrangement is similar to what is described for the growth cones of neuronal cells [Forscher and Smith, 1988; Vale et al., 1992], albeit on a much smaller scale.

This study has shown that tubulin, specifically assembly-competent tubulin, is needed to effect the shape change we observe. Colcemid treatment of mt1-CHO cells completely blocks the formation of the outgrowths. Depolymerization of microtubules has been shown to block neurite formation in N1E-115 cells [Seeds et al., 1970] and points out the indispensability of these cytoskeletal structures for the proper formation of these processes (Drubin et al., 1985, 1988; Vale et al., 1992; Nathan et al., 1995]. After the photoreversal of Colcemid, the mt1-CHO cells show a re-formation of outgrowths but with an altered, monopolar quality. We surmise that this difference in morphology is due to the dispersal of critical proteins (possibly dynein or kinesin-like proteins) during the time that the microtubules are disassembled. This suggests that the mechanism for establishing bipolarity is dependent on the presence of intact microtubules.

Transfection of SF9 cells, a non-neuronal cell line, with the neuronal MAPs MAP2, τ and CHO/MKLP1 results in the formation of cellular projections that closely resemble neurites [Knops et al., 1991; LeClerc et al., 1996, Sharp et al., 1996]. In the case of τ transfection, the processes formed are particularly long and thin and resemble axons [Knops et al., 1991]. Transfection of CHO cells with τ, however, results only in the formation of short spikes by these cells in contrast to the long projections seen for SF9 cells [Barlow et al., 1994]. This suggests that cytoskeletal changes in addition to MAP expression need to occur in these cells in order for long outgrowths to form. Edson et al. [1993] showed that depolymerization of portions of the actin cytoskeleton are needed to facilitate this kind of process formation. This is strongly supported by our finding that depolymerization of actin with cytochalasin D increases the number of outgrowths formed per cell. Removal of an actin

"barrier" with cytochalasin treatment has been shown to permit invasion of microtubule bundles into regions of the growth cones of *Aplysia* neurons from which they are normally restricted [Forscher and Smith, 1988]. It is most interesting to note that in the mt1-CHO cells treated with cytochalasin D, the cells retain their bipolar nature and produce outgrowths only from opposite ends of the cell. These outgrowths however, are shorter and thinner than those normally produced and suggests a critical role for actin in providing "substance" to the outgrowth structure.

CHO cells, a non-neuronal cell line, do not normally express neuronal MAP isoforms. However, it is possible that the signaling mechanism of the mt1 receptor could induce the production of these proteins. Interestingly, in our study, the production of filamentous outgrowths occurs after a much shorter time of treatment (5 h) than that noted for either neuroblastoma cells treated with melatonin or SF9 cells transfected to express neuronal MAPs or kinesin-like proteins (24–96 h). Our results with cycloheximide, actinomycin D, and α-amanatin show that the translation of mRNAs or activation of components (most likely via phosphorylation) already present in these cells accounts for the induction of outgrowths. We have also shown that τ, MAP2 and MAP1b are not present in cells possessing outgrowths. Future investigations will determine which specific cellular components are activated by the mt1 receptor.

The ability of melatonin to induce outgrowths in mt1-CHO cells occurred in a concentration-dependent manner up to a melatonin concentration of 1 μM. At concentrations greater than 1 μM, there was a decrease in the percentage of cells showing an altered shape. This kind of biphasic response has been shown for melatonin-mediated effects in other studies [Benitez-King et al., 1990; Molis et al., 1994; Cos et al., 1996]. The observed decreases of effect at higher melatonin concentrations may be due to the desensitization of the melatonin receptors and an attenuation of the signal transduction pathways involved [Jarzynka et al., manuscript in preparation).

We have shown that the outgrowths formed by mt1-CHO cells are maintained only in the presence of constant agonist exposure. A similar requirement is also noted for nerve growth factor (NGF)-induced neurite formation by PC12 [Drubin et al., 1998], dorsal root ganglion and other neuronal cell types [Levi-Montalcini, 1987]. For both melatonin-induced and NGF-induced processes, the outgrowths are retracted after removal of the agonist. This reversibility indicates that either continuous stimulation of signaling pathways is needed or that an independent mechanism is needed to stabilize these structures.

The finding that inhibition of PKA by KT5720 enhanced melatonin-induced outgrowth formation in mt1-CHO cells is contrary to what is reported for the induction of neurites in PC12 cells [Drubin et al., 1985, 1988]. In PC12 cells, increases in the amounts of cAMP correlate with increased neurite production. In our cells, stimulation of cAMP production by forskolin or treatment with 8-bromo cAMP did not stimulate outgrowth formation (data not shown). Nevertheless, increases in MEK 1/2 and ERK 1/2 phosphorylation have been noted in PC12 and other neuronal cells [Fukuda et al., 1995; Creedon et al., 1996; Hashimoto et al., 2000]. These findings along with our demonstration of a melatonin-dependent increase in MEK 1/2 and ERK 1/2 phosphorylation in mt1-CHO cells only, support the work of Boudewijn et al. [1995], who showed that PKA acts to inhibit Raf-1 or Raf-B, activators of MEK 1/2. We believe that stimulation of the mt1 receptor and its subsequent suppression of PKA activity leads to an activation of the MEK 1/2 - ERK 1/2 signaling pathway by relieving Raf inhibition. We additionally do not believe that cross-regulation of PLC-dependent pathways occurs for this outgrowth process because stimulation of PKC by 1-oleolyl-2-acetyl-sn-glycerol or inhibition of PLC by U-73122 had no effect on cell morphology (data not shown). Additional investigations are needed to resolve these apparent discrepancies in the involvement of cAMP in inducing differentiation.

The mechanisms that control and/or regulate cytoskeletal organization to bring about the formation of filamentous processes is not completely understood. Our study demonstrates a possible regulation via G-protein-coupled receptors. Thus, this study has provided novel insights into mt1 melatonin receptor function and has shown that this receptor can specifically regulate the organization of the cytoskeleton through mechanisms that involve proteins and mRNAs already present in non-neuronal cells. The most likely mechanism for this regulation is through the activation of the MEK 1/2 and ERK 1/2 signaling pathway. Activation of this pathway by a means other than the stimulation of receptor tyrosine kinases could represent a novel and alternative means for promoting cellular differentiation. The results of this and future work will lead to a greater understanding of the regulation of process formation in cells and could provide a means for stimulating cellular differentiation independent of receptor tyrosine kinase signaling.

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