EFFECTS OF A LOW DOSE OF MELATONIN ON SLEEP IN CHILDREN WITH ANGELMAN SYNDROME

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Abstract

The effects of low dose melatonin therapy on sleep behavior and serum melatonin levels were studied in Angelman syndrome (AS) children suffering from insomnia. 24-hour motor activity was monitored in 13 AS children (age 2-1 0 years) in their home environments for 7 days prior to melatonin treatment and for 5 days during which a 0.3 mg dose of melatonin was administered daily 1/2 - 1 hour before the patient's habitual bedtime.

Blood samples were withdrawn at hourly intervals over two 21-hour periods in order to measure individual endogenous serum melatonin levels and the levels induced by melatonin treatment. Actigraphic recording of motor activity, confirmed by parents' reports, showed a significant improvement in the patients' nocturnal sleep pattern as a result of melatonin treatment. Analysis of the group data revealed a significant decrease in motor activity during the total sleep period following melatonin treatment, **and an increase in the duration of the totals sleep period**. Endogenous peak nocturnal melatonin values ranged from 19 to 177 pg/ml. The administration of melatonin elevated peak serum hormone levels to 128-2800 pg/ml in children of different ages and body mass. These data suggest that a moderate increase in circulating melatonin levels significantly reduces motor activity during the sleep period in Angelman syndrome children, and promotes sleep

1. Introduction

Angelman syndrome (AS) is a rare genetic disorder (incidence estimated to be approximately 1 in 20,000) characterized by severe mental retardation with absent speech; seizures; ataxia. characteristic facial features with easily provoked smiling and laughter; and disturbed sleep[1]. About 70% of AS individuals have a de novo deletion of chromosome 15 bands q11-13 on the chromosome inherited from their mother [2]. Three other etiologic types of AS include paternal uniparental disomy 15 (2-5%), imprinting mutations (2-5%) and AS cases that have no evidence of deletion, uniparental disomy, nor imprinting mutations (20-25%). In this last group, mutations in the UBE3A/E6-AP gene have recently been described [3,4]

The most common behavioral problems in AS children are hyperactivity attention deficit and difficulties initiating and maintaining sleep. Sleep problems are usually detectable at several months of age and persist for many years. Long latencies to sleep and prolonged awakenings at night lead to fatigue and sleepiness upon morning awakening and cause a chronic sleep debt which may potentiate other behavioral and neurologic problems.

Observations in work with human babies reveal a correlation between the timing of the consolidation of nocturnal sleep and the normal onset of rhythmic melatonin secretion, both of which occur when the infant is about 3 months old [5,6]. Likewise, the concurrent decline in melatonin secretion and sleep efficiency with age are thought to be related

phenomena; for example, middle-aged and elderly insomniacs reportedly exhibit lower melatonin production than do good sleepers of the same age [7,8] .

Initial studies regarding the acute effects of melatonin in humans revealed that pharmacological doses of the hormone induce sleepiness and sleep [9-11]. Recently it has been shown that low melatonin doses (0.1 - 0.3 mg), which induce serum hormone concentrations comparable to those typical of nocturnal melatonin levels in adults, are sufficient to facilitate sleep induction in healthy young males when the hormone is administered at different times of the day, from noon to 9 p.m.[12-14] . The clinical studies revealed that administration of pharmacological doses of melatonin (2.5 - 7.5 mg, p.o.) to multiply disabled children with severe sleep disorders, who had failed to respond to conventional management[15], or to children suffering from Rett syndrome[16] substantially improved their sleep patterns and increased sleep duration according their caregivers' reports, or decreased their latency to sleep onset as assessed actigraphically.

We tested the possibility that actigraphically registered motor activity during sleep in AS children would be diminished when their circulating melatonin levels were increased within the physiological or low pharmacological range. This was accomplished by the administration of a 0.3 mg dose of melatonin to the patients close to their bedtime.

2. Research design and methods

2.1 Subjects

Subjects were recruited from the population of AS children diagnosed or treated at Boston Children's Hospital, or whose parents responded to information about the study distributed by the “Angelman Syndrome Foundation”

The investigation protocol was approved by the Children's Hospital human research committee. Parents signed an informed consent form prior to their child's participation in the study. Thirteen
children, age 2-10 years at the time of entry into the study, participated in the protocol. Twelve subjects had typical 15 q 11 -q 13 deletions, confirmed by either FISH analysis or Southern blot analysis, and one (# 8) had paternal UPD 15 resulting from a 13; 15 Robertsonian translocation inherited along with a normal chromosome 15 from the father.

All patients showed typical clinical features of AS including severe developmental delay with absent or minimal speech, and seizures. Patient # 3 had been treated with melatonin (purchased in a health food store) 3 mg daily for approximately 4 months; this was discontinued 2 weeks prior to entering the study. Patient # 8 had been treated with 3-9 mg of melatonin each night for 4 months, and this too was discontinued 2 weeks prior to entry into the study. Patient # 4 had been treated with chloral hydrate, 500 mg each night, for approximately 3 years; this treatment also was discontinued 2 weeks before entering the study. Patients # 10 and 11 are monozygotic twins. All of the children's gender, weight, age, and initial medications are summarized in Table 1.

2.2

The subjects' sleep/wake schedules were monitored in their home environments for 7 days prior to melatonin treatment (baseline) and for 5 days during which a 0.3 mg dose of melatonin was administered 1/2 -1 hour before subject's habitual bedtime. The patient's parents maintained a sleep diary for their child, indicating bedtime, approximate times of falling asleep, and times of awakenings. Objective data on 24 hour motor activities were obtained using a portable body actigraph (Mini- logger 2000, Mini Mitter, Oregon), worn by the patient in a pocket on the back of a cloth vest. The recorder monitored the number of body movements per minute. On the first day and night after the blood withdrawal session the actigraphic recording was not obtained because the child's sleep was disturbed on the night the blood was drawn. That circumstance could provoke a rebound increase in sleep during the following night, thus, distorting the record of the treatment period. The sleep diary maintained by patient's parents described periods when the actigraph was not worn.

After seven days of a baseline motor activity and sleep/wake assessment, subjects were admitted to the Children's Hospital Clinical Research Center to evaluate their endogenous melatonin secretion patterns. Patients' rooms were maintained at 72 ^{0}F, and lights were dimmed at 19.00 h to <30 lux. Blood samples (2 ml each) were drawn hourly from 15.00 h to 10.00 h the next morning, through an intravenous catheter inserted into a forearm vein; a heparin lock was used to prevent clotting. Serum samples, separated by centrifugation, were stored at -20^{0}C until assayed for melatonin concentration. The patient's core body temperatures were monitored using a rectal probe (YSI, Yellow Springs, Ohio) and recorded by a Mini-logger 2000. Subjects received regular meals through the day which were similar to their habitual diet.

Starting on the second day after the initial Hospital admission, subjects received a 0.3 mg oral dose of melatonin on each of six consecutive evenings, 1/2 - 1 h prior to their habitual bedtime ranging from 19.30 to 20,30 h. The contents of a gelatin capsule (0.3 mg melatonin, Nestle, Co., Switzerland, and microcristalline cellulose 'Avicel') were mixed with a teaspoon of a semi-liquid food (e.g., pudding or applesauce) and added to the child's evening snack. Each patient's activity and sleep were assessed as described above. After six days of melatonin treatment, subjects were again admitted to the Clinical Research Center (CRC), and blood samples were drawn hourly from 17.00 to 1 1.00 h the following morning in order to assess the effects of erogenous melatonin (0.3 mg dose administered at 19.30 h) on their serum hormone profiles. The environment and procedures were held similar to those used during the first CRC admission.

Subjects' motor activities, recorded by actigraph were analyzed using a software algorithm developed at MIT. The total sleep period (TSP) within a bedtime period was defined as an interval elapsing between the first of ten consecutive minutes without movements, and the last minute of the last ten minute interval without movements. We chose not to use standard actigraphic algorithm criteria differentiating wakefulness and sleep within the sleep period since we lacked parallel polysomnographic data in work with this population. Because we did not have objective data on the timing, of lights out

in the home environment, we question the precision of our estimates of latency to sleep onset and did not include this parameter in our statistical analysis of the experimental data obtained. Thus, analysis of actigraphic data was limited to TSP and to the number of movements per hour during the TSP (M).

Melatonin concentrations were measured in 0.5 ml serum aliquots using a commercially available radioinununoassay kit (Bublmann Laboratories; Allschwil, Switzerland). Extraction was accomplished using C 18 columns. The limit of detection of the assay was 2.2 pmol/L (0. 5 pg/ml). The intra-assay coefficients of variation for control samples were 7.2% (3 8.8 pmol/L (9 pg/ml)) and 7.8 % (94. 8 pmol/L (22 pg/ml));

the corresponding inter-assay coefficients of variation were 12.6% and 16.1%. The parameters of interest were time of onset and offset of nocturnal melatonin secretion-area under the time-melatonin concentration curve within this period (AUC); and peak nighttime and minimum daytime hormone levels. The onset and offset of melatonin production were defined as the time points at which evening or morning serum melatonin reached a concentration two standard deviations above the mean daytime level. AUC was measured between the "onset" and “offset” time points. If, for technical reasons, blood withdrawal was interrupted close, but prior to an estimated “offset” time, as indicated in Table 2, AUC was measured within the period for which data were available.

Parents were given the option, as an extension of this study, to continue melatonin treatment of their AS children for up to one year, using either a 0.3 or a 0.2 mg dose of melatonin as a continuation of the described study. Twelve of the thirteen families chose to continue the treatment. In order to evaluate a possible shift in the phase of melatonin secretion, we repeated an overnight blood withdrawal in three children (# 1, 2 and 5) after several weeks of melatonin treatment.

Statistical analyses of the actigraph and melatonin concentration data involved repeated measures ANOVA to evaluate the differences between baseline levels of outcome variables and the levels on a 0.3 mg melatonin treatment. Due to scheduling or technical problems, not all of the subjects' data sets contained an equal number of baseline and/or treatment day recordings. Individual and group means represent four consecutive days of a baseline period and four consecutive days of a treatment period. In the cases where data were missing we chose to analyze any available four consecutive days, otherwise the last four days of the period were used for the analysis.

In four of the children (# 6, 9, 10, 11) who had to move from their homes to Boston for blood withdrawal sessions, we chose to analyze activity during four consecutive days of melatonin treatment while they were back at home, rather than the recordings obtained during their stay in a hotel.

3. Results

Endogenous serum melatonin profiles for the group of AS children studied were highly variable with respect to peak melatonin levels and areas under the time- concentration

curves (AUC, Table 2). Peak nocturnal values ranged from 81.9 pmol/L (19 pg/ml) to 762.9 pmol/L (177 pg/ml), the group mean value (±SE" was 387.9 ± 63.4 pmoVL (90 ± 14.72 pg/ml) (Fig. 1). Melatonin AUC values ranged from 607.7 pmol/L (141 pg/ml/h) to 4775 pmol/L (1108 pg/ml/h) in different subjects, group mean value (SEM) was 2745 ± 429.3 pmoYL (637± 99.61 pg/ml/h). In ten of the children the onset of nocturnal hormone secretion was consistent with their bedtime and their sleep onsets, however in three of the subjects (# 1, 2 and 13) the nocturnal surge was substantially delayed (Table 2). Prior to treatment, the sleep pattern in these three children did not correlate with the timing of melatonin secretion, i.e., their habitual bedtime and their sleep onset usually occurred in advance of the onset in nocturnal melatonin release. However, in two of them (# 1 and 13), daytime sleepiness until approximately noon was described by their parents, prior to melatonin administration.

Symptoms of sleep disturbance, as reported by parents, were variable among the subjects, including long periods of activity after the lights were out several prolonged awakenings at night with high motor activity, and early morning awakenings. Low sleep quantity and quality, reported by parents, was confirmed by actigraph data, collected prior to the treatment, showing a high number of movements during a sleep period (Table 3). In our age-diverse group of children we did not find a significant correlation between an individual's endogenous peak melatonin levels or AUC and the number of his or her movements per hour of TSP under baseline conditions.

Within an hour after ingestion of a capsule containing 0.3 mg of melatonin, circulating melatonin levels increased significantly, and remained above the daytime baseline levels for 12- 15 hours (Fig. 1). The administration of a uniform 0.3 mg dose of melatonin elevated peak serum hormone levels to from 552 to 12068 pmol/L (128 to 2800 pg/ml) in children of different ages and body mass (Table 2). Mean peak circulating melatonin levels after the treatment were 2701.2 ± 103 8 pmol/L (601.6 ± 223 pg/ml) (Fig. 1), significantly higher ($p<o. 001$) than the mean peak endogenous melatonin level. Highest peak levels following treatment were in the two year old identical twin patients (# 10 and 11), whose body masses were the lowest in the group studied (Table 1). The mean group AUC value was significantly increased ($p<0.001$) following the administration of a 0.3 mg dose of melatonin (11564 ± 3276 pmol/L (2683 ± 759.98 pg/ml/h); (Table. 2). Daytime melatonin levels were less than 21.6 pmol/L (5 pg/ml) for all the children studied and were not significantly changed after five days of melatonin treatment.

Administration of a 0.3 mg dose of melatonin substantially reduced the measured nighttime motor activity in eleven of the subjects studied. This finding was consistent with parents' reports regarding the children's sleep quality in response to treatment (Table 3). However, nocturnal motor activity did not change in two of the subjects (# 4 and 12) whose baseline activities did not exceed 30 movements per hour. Analysis of the group data revealed a significant decrease ($p<0.001$) in motor activity, M, during the total sleep period following melatonin treatment (Fig. 2); and a significant increase in the TSP ($p<0.05$). The magnitude of the observed effect on nocturnal motor activity did not significantly depend on the peak melatonin levels after the treatment, nor on the

difference between the endogenous level of the hormone and serum melatonin concentrations induced by the treatment.

During the one week period of melatonin administration at home, parents reported that subjects usually fell asleep within 15 to 60 minutes after melatonin administration, and that sleep onset occurred faster than it did prior to the treatment. In some cases, parents felt that children, after falling asleep, were not aroused by sounds that would have awakened them prior to starting treatment. Parents reported that children were alert during the day after melatonin administration, and that there was either no change in daytime behavior or that the subjects were more attentive and interactive. Core body temperature recordings were not accurate due to frequent displacement of the rectal temperature probe, and these measurement errors were highly provoked by the blood withdrawal procedures. Thus, analysis of these temperature recordings is not presented.

In patients # 1, 2 and 13 the pretreatment onset of nocturnal melatonin secretion was substantially delayed (Table 2). Administration of a low dose of the hormone an hour prior to bedtime for two weeks (subject # 1, Fig. 3, C) or four weeks (subject # 2) produced a 2 or 3 hour phase advance, respectively, in the onset of melatonin secretion. Fig. 3 illustrates the sleep patterns in patient # 1 during melatonin treatment (A) and after treatment was stopped (B). Withdrawal from treatment resulted in a delay in the sleep onset, which started to occur close to the time of the 'new', shifted, onset of her melatonin secretion around 23. 00 h. It is interesting to note that her typical pretreatment sleep pattern, according to her mother's report, was characterized by a 2-3 hour sleep period starting late in the evening, and a 2-3 hour sleep period early in the morning. Thus, while the subject's initial sleep schedule was not harmonized with her nocturnal melatonin secretion, melatonin treatment synchronized the two patterns. In contrast, administration of a 0.3 mg dose for 4 weeks followed by a 0.2 mg dose for 4 weeks to patient # 5, whose initial melatonin secretion onset was in phase with his habitual bedtime, caused no temporal or quantitative change in his nocturnal melatonin production (Fig. 4, C), in spite of a significant sleep promoting effect of the treatment (Fig. 4, B).

4. Discussion

The results of this study suggest that an induced moderate increase in circulating melatonin levels in children afflicted with Angelman syndrome promotes regularized and less interrupted sleep, Continuous actigraphic recording documented a significant decrease in motor activity during the sleep period while the children were treated with a daily low dose of melatonin. In addition, according to their parents' reports, most of the children showed signs of fatigue within 15 to 30 min after the treatment was administered, their latencies to sleep onset were shortened and their sleep was more consolidated.

Neurologically disabled and mentally retarded children frequently exhibit disrupted sleep/wake cycles and severe sleep disturbances[17, 18] which are difficult to treat using traditional sedatives. Insomnia in such a population may be related to abnormalities in brain development, which may include abnormal development or function of the

circadian system, a decrease in the expression of melatonin receptors, a reduction in melatonin production, or a change in the brain's sensitivity to the pineal hormone.

It has been shown that newborn infants do not display rhythmic melatonin secretion for the first two to three months [6] but rely on the hormone supplied via the mother's milk. Total melatonin production increases rapidly during the first year of life, and might be important for brain development and maturation of the circadian system, including sleep/wake mechanisms. The highest night-time melatonin levels have been observed in very young children, aged 1-3 years (329.5± 42 pg/ml)[19]. Melatonin levels start to fall around the time of the onset of puberty. Recent study [20] also reported a substantial inter-individual variability in circulating melatonin levels, which decline from 175 ± 109 pg/ml to 128 ± 44 pg/ml during the course of puberty in groups of children from 5 to 17 years of age. It was not possible for us to recruit a group of AS children of a homogenous age, nor a matched control group of children to study. Normative values for circulating melatonin concentrations have not been established for any age group., The majority of our patients, however, exhibited peak melatonin levels lower than the published mean blood melatonin values for children of comparable ages [20]. One of the possible explanations for lower melatonin levels in the population we studied is that most of our subjects received anti-seizure medications containing sodium valproate. This GABAergic compound has been shown to significantly suppress blood melatonin levels in humans [21]

Increased motor activity in sleep, a conventional sign of sleep disruption, appear to be inhibited by the substantial increases in serum melatonin levels that follow administration of a 0.3 mg dose of the hormone. The sleep-promoting effect of the hormone treatment in our patients did not show a dependency on either the endogenous peak melatonin levels nor on the duration of the nocturnal increase in melatonin secretion. This result might reflect an experimental limitation imposed by the different ages and weights of the children studied that did not allow us to adequately compare them in terms of endogenous melatonin levels and melatonin pharmacokinetics.

Among the AS children whose melatonin secretion was delayed, repeated melatonin treatment several hours prior to the onset of their own nocturnal melatonin secretion resulted in a phase advance of their circadian rhythm. However, in the patient who received erogenous melatonin for more than a month close to the time of his normal nocturnal increase in the hormone's secretion, the sleep promoting effect of the treatment was not accompanied by a detectable shift in the timing of his circadian rhythms. Thus, the repetitive, appropriately-timed administration of melatonin to AS children can phase-shift their circadian rhythms, but does not disturb the pattern if applied close to the time of onset of endogenous nocturnal melatonin release.

Our data suggest that an increase in circulating nocturnal melatonin levels within the physiologic range, or above it, is beneficial in establishing sleep integrity in the AS children. The mechanism of the acute sleep promoting effect of melatonin remains to be uncovered. It might be a result of a more robust endocrine signal for the homeostatic mechanisms of sleep initiation and sleep maintenance, a consequence of an acute

inhibiting effect of melatonin on the major site of the mammalian biological clock - suprachiasmatic nucleus of hypothalanius (SCN), or another unknown mechanism.

Our findings in those AS children that had a circadian phase delay prior to the treatment, also support the idea that melatonin has a dual role in the sleep/wake process: acute promotion of sleep, and the phase shifting of an underlying circadian oscillator. This dual action of melatonin suggests its potential as a treatment for patients whose insomnia is related to the hormone's deficiency and/or a circadian rhythm disorder. Melatonin's effects in humans are poorly studied. Since pharmacologic melatonin concentrations in humans have been consistently shown to decrease body temperature [22], and they are thought to have some effect on the development of the reproductive system [13] , a cautious attitude toward treatment with the hormone is indicated and, if treatment is initiated, a search for the minimum effective dose of the hormone for each individual is recommended.

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Figure legends:

Fig. 1 Mean (± SEM) peak serum melatonin levels in thirteen AS children: endogenous levels (0.0) and after the administration of a 0.3 mg dose.

Fig. 2 Mean (± SEM) number of body movements per hour of the sleep period in thirteen AS children before and during melatonin treatment.

Fig. 3 Sleep onset, judged from motor activity, in subject # 1 (A) during melatonin treatment, and (B) during melatonin withdrawal after 4 weeks of treatment; (C) timing of the onset of elevated scrum melatonin (▽) prior to treatment, (□) during treatment, and (○) after 4 weeks of melatonin treatment.

Fig. 4 Motor activity of subject # 5 during (A) four days of no treatment, and (B) during four days of melatonin treatment; and (C) the profiles of serum melatonin concentrations prior to treatment (▽), during treatment (□), and after 4 weeks of melatonin treatment (○).

Figure 1

Figure 2

Figure 3

Figure 4

Table 1 Subjects demographics and medication during the study

Subject #	Age	Gender	Weight	Medications
1	9	F	29.5 kg	Tranxene (375 rng qAM, 562 mg qhs) Vitamine E (400 lU qhs)
2	7	m	30.9 kg	Depakene (300 mg q AM, 250 mg q3PM, 250 mg qhs)
3	6	F	21.5 kg	Depakote (250 mg qAM, 125 mg q3PM, 250 mg qhs)
4	10	m	21.4 kg	Depakote (375 mg qAM, 375 mg q12N, 375 mg qhs) Desipramine(10 mg qAM, 30 mg qhs) Mebaral (50 mg qAM, 50 mg qhs)
5	7	m	23.9 kg	Neurontin (100 mg qAM, 200 mg qhs) Zarontin (125 mg qAM, 125 mg qhs)
6	7	F	24.6 kg	Kionopin (1 mg qAM, 1 mg q3PM, 1.5 mg qhs)
7	7	F	27.9 kg	Valium (2.5 mg qAM)
8	10	F	60.2 kg	Depakote (500 mg bid), Clonidine (0.1 mg bid)
9	4	F	17.9 kg	Mogadon (2.5 mg qhs), Depakote (500 mg bid)
10	2	F	9.8 kg	Valproic acid (245 mg tid)
1 1	2	F	10.1 kg	Valproic acid (245 mg tid)
12	4	F	15.8 kg	Depakote (250 mg qAM, 125 mg q3PM, 250 mg qhs)
13	10	m	30.9 kg	Depakote (312 mg tid), Phenobarbital (45 mg qhs)

Table 2 : Serum Melatonin patterns in AS children prior to treatment (0 mg) and as a result of Melatonin treatment (0.3 mg)

Subject #	Melatonin Secretion						
	Peak level 0 mg	pg/mi 0.3 mg	"Onset" 0 mg	Offset' 0 mg	Offset' 0.3 mg	AUC 0 mg	AUC 0.3 mg
1	177	300	24:00 h	***	11:00 h	888	***2611
2	19	165	24:00 h	11:00 h	12:00 h	141	1070
3	49	320	22:00 h	08.00 h	*	350	*
4	50	96	21:00 h	11:00 h	11:00 h	430	629
5	65	179	22:00 h	08:00 h	08:00 h	453	1117
6	129	197	22:00 h	09:00 h	10:00 h	885	1334
7	120	420	21:00 h	11:00 h	12:00 h	1023	2279
8	132	690	22:00 h	09:00 h	**	1008	**3966
9	54	276	21:00 h	10:00 h	11:00 h	492	1538
10	148	2800	20:00 h	09:00 h	11:00 h	1040	9807
1 1	148	1850	20:00 h	09:00 h	12:00 h	1108	5312
12	41	400	21:00 h	10:00 h	10:00 h	238	1693
13	38	128	01:00 h	11:00 h	10:00 h	228	845

*Samples not available after: * 03:00 h; ** 07:30 h; *** 11:00 h;

Table 3 : Actigraphically recorded total sleep period (TSP) and the number of movements per hour of TSP (M)

	No Treatment				0.3 mg Melatonin			
Subject#	TSP	SEM	M	SEM	TSP	SEM	M	SEM
1	587.2	79.9	143.9	45.7	614	49.3	9.3	1.8
2	524.5	104.6	60.4	20.7	697	53.3	15.9	6.7
3	558.5	21.2	66.2	20.8	575.7	30.3	9.4	2.1
4	539.7	19.8	28.2	19.2	572.2	13.6	20.4	7.4
5	450	25.5	64.2	19.1	643.7	32.1	2.5	1.53
6	485.7	27.2	374.3	130.9	593.7	18.5	11.4	3.5
7	617.2	39.8	72.7	21.5	550.5	23.1	9.4	1.9
8	524	54.2	301.2	86.2	680.7	17.9	58.6	14.1
9	471	32.2	89.1	35.9	528.7	44.1	50.3	24.5
10	706.2	35.5	237.6	78.9	608.2	33.2	25.6	6.9
11	644	29.9	81.6	38.6	580.2	34.6	12.6	2.6
12	475	31.3	17.9	11	540	18.7	10.5	6.3
13	628.7	40.61	113.6	15.75	587.5	11.55	8.2	1.57

* Missing baseline data was substituted using data collected after a 3 week melatonin withdrawal
SEM - Standard Error of the Mean