

# Circadian rhythm abnormalities of melatonin in Smith-Magenis syndrome

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## Abstract

**Background**—Smith-Magenis syndrome (SMS) is a multiple congenital anomalies/mental retardation syndrome associated with a hemizygous deletion of chromosome 17, band p11.2. Characteristic features include neurobehavioural abnormalities such as aggressive and self-injurious behaviour and significant sleep disturbances. The majority of patients have a common deletion characterised at the molecular level. Physical mapping studies indicate that all patients with the common deletion are haploinsufficient for subunit 3 of the COP9 signalosome (*COPS3*), which is conserved from plants to humans, and in the plant *Arabidopsis thaliana* regulates gene transcription in response to light. Haploinsufficiency of this gene is hypothesised to be potentially involved in the sleep disturbances seen in these patients. Melatonin is a hormone secreted by the pineal gland. SMS patients are reported to have fewer sleep disturbances when given a night time dose of this sleep inducing hormone. **Methods**—Urinary excretion of 6-sulphatoxymelatonin (aMT6s), the major hepatic metabolite of melatonin, in 19 SMS patients were measured in conjunction with 24 hour sleep studies in 28 SMS patients. Five of the 28 patients did not have the common SMS deletion. To investigate a potential correlation of *COPS3* haploinsufficiency and disturbed melatonin excretion, we performed fluorescence in situ hybridisation (FISH) using two BACs containing coding exons of *COPS3*.

**Results**—All SMS patients show significant sleep disturbances when assessed by objective criteria. Abnormalities in the circadian rhythm of aMT6s were observed in all but one SMS patient. Interestingly this patient did not have the common deletion. All patients studied, including the one patient with a normal melatonin rhythm, were haploinsufficient for *COPS3*.

**Conclusions**—Our data indicate a disturbed circadian rhythm in melatonin and document the disturbed sleep pattern in Smith-Magenis syndrome. Our findings suggest that the abnormalities in the circadian rhythm of melatonin and altered sleep patterns could be secondary to aberrations in the production, secretion, distribution, or metabolism of melatonin; however, a direct role for *COPS3* could not

## be established.

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Smith-Magenis syndrome (SMS) is a clinically recognisable disorder associated with del(17)(p11.2p11.2). SMS is considered a contiguous gene deletion syndrome<sup>1</sup> and 95% of patients harbour a common deletion of approximately 5 Mb. This deletion is molecularly defined by the identification of a patient specific junction fragment by Southern analysis following pulsed field gel electrophoresis (PFGE).<sup>2</sup> SMS patients with smaller and larger deletions have also been reported<sup>3</sup> and are considered to have atypical sized deletions. The neurobehavioural features of SMS are perhaps the most distinctive characteristic of this microdeletion syndrome and include self-injurious and aggressive behaviour and significant sleep disturbances.<sup>4–6</sup> The sleep abnormalities include daytime sleepiness, difficulty falling asleep at night, nocturnal awakening, decreased sleep time, and abnormalities in the percentage of rapid eye movement (REM) sleep.<sup>6,7</sup> We have previously reported abnormalities in REM sleep in SMS.<sup>6</sup> In this report an additional cohort of SMS patients was studied more extensively to define the spectrum of sleep abnormalities further.

Melatonin is the principal hormone secreted by the pineal gland and is implicated in the bioregulation of circadian rhythms, sleep, mood, reproduction, tumour growth, and ageing.<sup>8–11</sup> It is normally produced at night, in the absence of light.<sup>12</sup> Although the circadian rhythm of melatonin secretion is endogenous, the natural light/dark cycle entrains this rhythm.<sup>13</sup> The production of melatonin is under the regulation of a multisynaptic pathway involving retinal photoreceptors, the hypothalamic suprachiasmatic nucleus, and the sympathetic superior cervical ganglia.<sup>14,15</sup> Serum concentrations of melatonin peak between 2 am and 4 am and vary with the age of the person. Highest peak levels are found in children between the ages of 1 and 3 years, then gradually decline.<sup>8</sup> Melatonin is enzymatically metabolised in the liver and secreted in the urine as a stable compound, 6 sulphatoxymelatonin (aMT6s).<sup>16,17</sup> Urinary aMT6s is a major metabolite of melatonin in humans, and its levels reflect the cyclic production and secretion of melatonin from the pineal gland.<sup>18</sup> Melatonin has been used to treat the sleep disturbance in SMS, and although there have

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been no controlled studies documenting an effect, many parents report an improvement of their child's sleep with treatment. Given the clinical phenotype of SMS and the anecdotal response to treatment with melatonin, we investigated the urinary metabolite of this hormone in SMS patients. We previously reported 24 hour polysomnography results and aMT6s levels in a pilot study of six SMS patients<sup>19</sup> and showed an abnormal diurnal rhythm of aMT6s. Similar findings were also recently reported by De Leersnyder *et al.*<sup>20</sup> The present study includes our preliminary data and investigates abnormalities of sleep in 28 SMS patients and abnormalities in the diurnal patterns of melatonin in 18 of 19 SMS patients tested. This study also shows haploinsufficiency of *COPS3* by FISH in all SMS patients studied.

### Materials and methods

#### PATIENT ASCERTAINMENT

Twenty eight subjects (12 females, 16 males) with SMS and one with the reciprocal 17p11.2 duplication<sup>21</sup> were enrolled in the multidisciplinary clinical study of SMS through the General Clinical Research Center for Children at the Texas Children's Hospital in Houston, from December 1996 to September 1999, under a protocol approved by the Baylor College of Medicine Institutional Review Board. Informed consent was obtained from the patient's parent or legal guardian. As part of the comprehensive clinical protocol, a sleep history was obtained and all patients underwent a physical examination and polysomnographic studies. In addition, a cytogenetic evaluation and molecular analysis using pulsed field gel electrophoresis (PFGE) was performed on each patient to determine the nature and size of the 17p11.2 deletion (or 17p11.2 duplication). None of the patients was being treated with melatonin and none had ingested this substance within six weeks of the sleep study. Twenty eight SMS patients and the patient with duplication 17p11.2 completed the 24 hour sleep study and urine collection.

#### CYTOGENETIC ANALYSIS

Chromosome analysis was performed by Giemsa trypsin banding (GTG banding). FISH analysis with probes for genes within the SMS region was used to determine the size of the deletion. Probes for the *PMP22* locus in the CMT1A region of 17p12<sup>22</sup> and *FLI*,<sup>23</sup> *COPS3*,<sup>24</sup> *ZNF179*,<sup>25</sup> and *MFAP*<sup>26</sup> within the SMS region of 17p11.2 were used. For *COPS3*, human BAC clones 41C11 and 96019 from the RPCI-11 library (Roswell Park Cancer Institute) were independently studied. Two colour FISH was performed as previously described.<sup>27</sup>

#### PFGE ANALYSIS

High molecular weight DNA was isolated in agarose plugs from peripheral blood samples and Epstein-Barr virus transformed lymphoblastoid cell lines established from controls and patients as previously described.<sup>2, 28</sup> The SMS patient specific junction fragment and the

dup(17)(p11.2) junction fragment were identified with a probe to the flanking repeat gene cluster SMS-REP.<sup>2, 21</sup>

#### SLEEP LABORATORY PROCEDURES

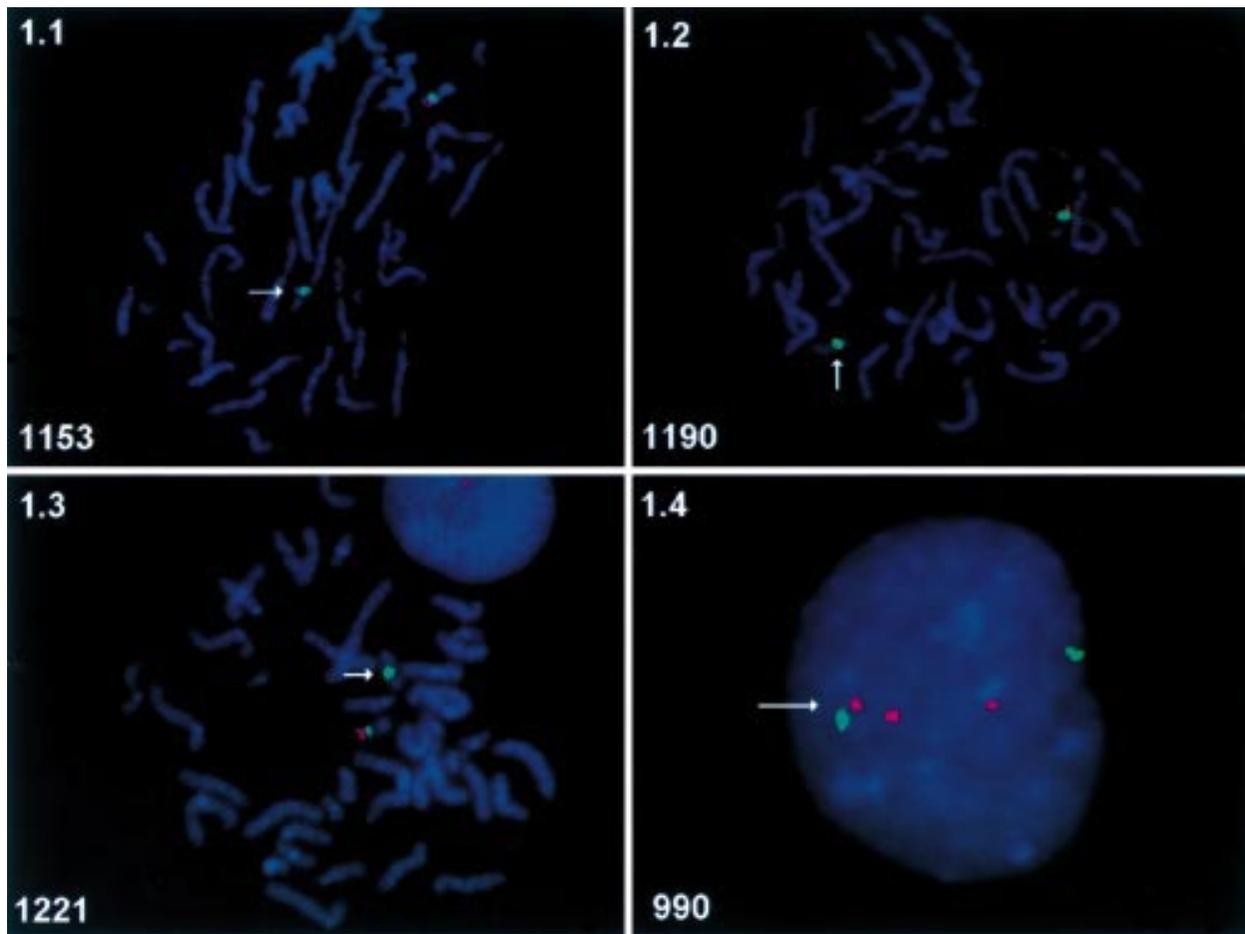
All patients were evaluated in the sleep laboratory at the Texas Children's Hospital for one night in sound attenuated, light and temperature controlled rooms. The patients were monitored continuously during the nocturnal session with a 21 channel polygraph (Nihon-Kohden model 4421K EEG recorder, Tokyo, Japan). The following parameters were recorded continuously: electroencephalogram (EEG), six channels (FP1-C3, FP2-C4, C3-01, C4-02, F7-T3, F8-T4) from surface electrodes placed according to the 10-20 international system; electro-oculogram (EOG), two channels from surface electrodes placed over the left/right outer canthus and forehead; electromyogram (EMG), one channel from submental surface electrode; electrocardiogram (ECG), one channel recorded from surface electrodes; respiratory effort-thoracic and abdominal strain gauges; respiratory airflow, one channel from oral and nasal thermocouples; oxygen saturation, one channel from pulse oximeter on finger, toe, or ear; end tidal pCO<sub>2</sub>, one channel from catheter placed at the nares or mouth; leg movement, one channel from triaxial accelerometer; behavioural observations, technologist log and time synchronised video monitoring. Sleep staging was determined by standard criteria.<sup>29</sup> Sleep onset latency was calculated as elapsed time from lights out to the first epoch of sleep. Following the nocturnal session, patients were monitored during five scheduled nap sessions at two hour intervals (Multiple Sleep Latency Test, MSLT) and given the opportunity to fall asleep. The technologist awakened the patient 15 minutes after onset of any sleep period during this session or the nap session was terminated at 20 minutes if sleep was not achieved. A sleep latency of less than 10 minutes was considered to suggest moderate sleepiness, while a sleep latency of less than five minutes was considered to suggest severe sleepiness.

#### URINE COLLECTIONS

The patient's spontaneously voided urine was collected by the nursing staff during the 24 hour period of the sleep study. The total volume and time voided of each sample were recorded and three 1 ml aliquots were frozen at 0°C for between one and 24 hours, then frozen at -80°C until assayed.

#### 6-SULPHATOXYMELATONIN DETERMINATION

Before assay, the urine samples were thawed and centrifuged. An aliquot of the supernatant was taken for measurement of aMT6s and estimated using the radioimmunoassay of Arendt *et al.*<sup>30</sup> as modified by Aldhous and Arendt.<sup>16</sup> Briefly, after thawing, urine samples were diluted 1:250 with assay buffer. The diluted samples (500 µl) were incubated with a specific anti-sheep antiserum to aMT6s (Stockgrand, Surrey, England) and trace amounts of radioiodinated aMT6s<sup>31</sup> were then added. Free and



**Figure 1** Representative results of the FISH analyses. 1.1, patient 1153; 1.2, patient 1190; 1.3, patient 1221. The *COPS3* locus (red) is deleted on one chromosome 17 (*del 17*). The chromosome 17 centromeric locus (green) is present on both homologues. 1.4, patient 990. Interphase FISH shows a duplication of *COPS3* on one homologue (*dup 17*). The *COPS3* BACs were labelled with digoxigenin and detected with anti-digoxigenin conjugated to rhodamine, which fluoresced red. The centromeric probe was purchased labelled with biotin (Vysis Inc, Downers Grove, IL) and detected with avidin conjugated to FITC, which fluoresced green.

antibody bound fractions of aMT6s were separated using a dextran coated charcoal suspension. The free aMT6s fraction was precipitated with the charcoal by centrifugation and the radioactivity was counted in a Wallac 1970 Wizard gamma counter. A standard curve was constructed using standards prepared with charcoal stripped urine. The normal pattern of urinary aMT6s in a 20 year old male<sup>17</sup> was used as a representative control.

### Results

The 28 SMS patients in this study varied in age from 2 years 8 months to 31 years (average 9 years 4 months). Sixteen of the patients were males. All patients were deleted for the *FLI* locus within 17p11.2 (data not shown). Twenty three patients (82%) had the common deletion junction fragment by PFGE (data not shown), which results from homologous recombination between flanking repeat gene clusters,<sup>2</sup> while five patients (1221, 1190, 1456, 1153, 1354) had different sized deletions and therefore lacked this junction fragment. Patient 1221 had a complex, de novo chromosomal rearrangement which resulted in the deletion of the SMS critical region.<sup>32</sup> Patients 1190 and 1456 had smaller deletions and were not deleted for the *ZNF179* and *MFAP* loci by FISH. Patients

1153 and 1354 were deleted for all FISH probes used in the SMS region but lacked the SMS patient specific junction fragment.<sup>2</sup> We performed FISH with the *COPS3* BAC probes on all patients without the common deletion and in two patients with the common deletion. All patients harboured a heterozygous deletion of *COPS3*, including patient 1153 who had a normal melatonin circadian rhythm (data not shown) (fig 1). Patient 990 had a tandem duplication of the SMS region<sup>21</sup> and was shown by FISH in this study to have a duplication of *COPS3* (fig 1).

### SLEEP STUDIES

Table 1 summarises the objective measures of sleep disturbances in relation to the nature of the SMS deletion. The Epworth Sleepiness Scale (EES), modified for children, reflects the tendency for daytime sleepiness based on parental report and suggested a significant tendency for daytime sleepiness (score greater than 10) in 65% (17/26) of SMS patients. Findings during the nocturnal session of the sleep study include decreased total sleep time (less than seven hours) in 43% (12/28), decreased percentage of REM sleep in 39% (11/28) (normal 18-25%), increased percentage of REM sleep in 25% (7/28), and multiple

Table 1 Objective measures of sleep in Smith-Magenis syndrome

	Common deletion	Uncommon deletion
	Number with finding/total tested*	Number with finding/total tested*
Epworth sleepiness scale (score >10)	13/21 (62)	4/5 (80)
Total sleep time (<7 hours)	8/23 (35)	4/5 (80)
Percentage REM sleep	13/23 (57)	5/5 (100)
Decreased (<18%)	8/23 (35)	3/5 (60)
Increased (>25%)	5/23 (22)	2/5 (40)
Spontaneous awakenings ( $\geq 10$ )	20/23 (87)	5/5 (100)
Multiple sleep latency test (average latency <10 minutes)	10/21 (48)	3/5 (60)
6-Sulphatoxymelatonin (inversion of normal rhythm)	16/16 (100)	2/3 (67)

\*Percentage given in parentheses.

spontaneous awakenings (range 3-29, 16 patients with  $\geq 10$  arousals). Patient 990, who is duplicated for the region deleted in SMS, had no unusual sleep behaviour by clinical history or the ESS. His nocturnal session was normal except for a minimally decreased percentage of REM sleep (17%). The Multiple Sleep Latency Test (MSLT), conducted on the day following the nocturnal session, was used as an objective measure of excessive daytime sleepiness. The average MSLT sleep latencies were significantly shortened (<10 minutes) in 50% (13/26) of SMS patients and normal (19.5 minutes) in patient 990.

All patients had deviations from expected normal values in one or more of the above measures and, on average, three of the five parameters were altered in each patient (table 1). Thus, all SMS patients showed significant sleep disturbances when assessed by objective criteria. There was no correlation with age or gender between total sleep time, altered

percentage of REM sleep, spontaneous awakenings, or abnormalities on MSLT. Of the 23 patients with the SMS common deletion who were evaluated objectively for sleep disturbance, there were differences in the measured parameters suggesting variability of the clinical phenotype in SMS patients in the face of one specific deletion mutation (table 1).

#### URINARY aMT6S

The pattern of urinary aMT6s has been established in normal subjects and persons with Down syndrome.<sup>17</sup> The previously published data of the normal values are used here. In the present study, abnormalities in the circadian rhythm of aMT6s were observed in all but one SMS patient. Fig 2 contrasts the normal diurnal rhythms of aMT6s in an unaffected control, patient 990, and patient 1153 with the inversion of the normal circadian pattern observed in the other SMS patients studied (including a representative patient with an atypical deletion (1221)). An inversion of the normal circadian pattern was observed in all but one SMS patients (1153), in that the peak values are found before sleep onset and the trough value is at or soon after the first morning void (fig 3). Only one SMS patient (1153) has a normal aMT6s rhythm. This patient is presumed to have an uncommon deletion because of the absence of an SMS patient specific junction fragment on PFGE. The pattern in other SMS patients with uncommon deletions, including an SMS patient with a smaller deletion also showed an inversion of the normal aMT6s rhythm.

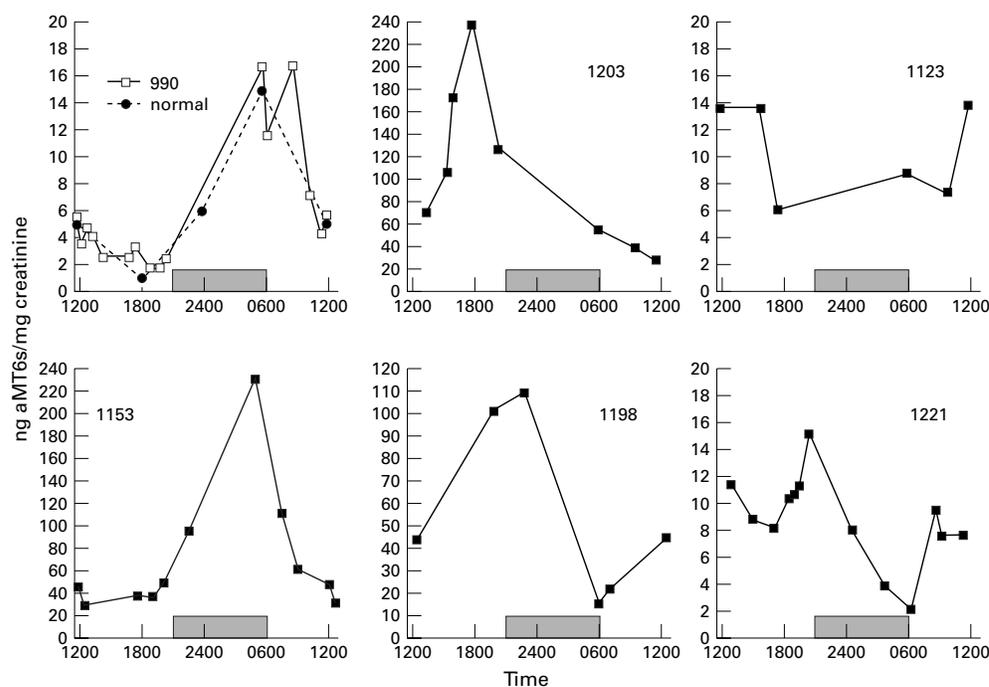
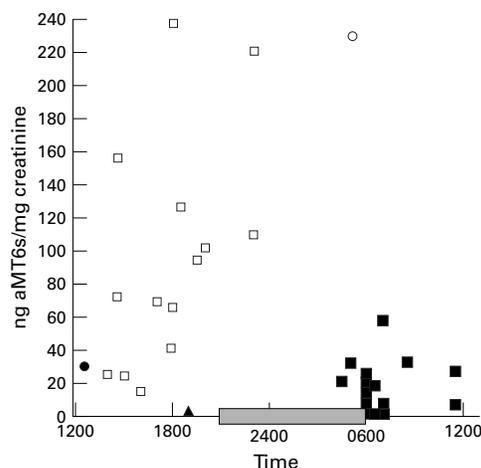


Figure 2 Levels of urinary 6-sulphatoxymelatonin (aMT6s) per mg creatinine plotted against time of collection. The shaded box represents the period of darkness. Left upper panel: an unaffected 20 year old male (normal control) (closed circles) and a subject (990) with duplication of the SMS region (dup(17)(p11.2p11.2)) (open squares). Normally, the highest concentration of aMT6s is found in the first morning sample and reflects the normal rise of serum melatonin during the night. The duplication patient exhibited a normal diurnal rhythm of aMT6s. Left lower panel: patient 1153 (5 year 2 month old female) exhibited a normal diurnal rhythm of aMT6s. The remaining panels illustrate the inverted melatonin rhythm in three SMS patients with the common deletion (1203, a 2 year 8 month old male; 1198, a 5 year 8 month old female; 1123, a 31 year old male) and one SMS patient with an atypical deletion (1221, a 12 year 10 month old male).



**Figure 3** Peak (open squares) and trough (closed squares) values of urinary 6-sulphatoxymelatonin (aMT6s) per mg creatinine plotted against time of collection in SMS patients. Values for patient 1153, who had a normal rhythm (see fig 2), are depicted by open (peak) and closed (trough) circles. Values for patient 990, who also had a normal rhythm (see fig 2) are depicted by an open triangle (peak value of 17 ng aMT6s/mg creatinine is obscured by other data on graph) and a closed (trough) triangle. The shaded box represents the approximate period of darkness for all patients. The trough values of aMT6s for SMS patients are abnormally clustered in the morning hours.

### Discussion

Smith-Magenis syndrome is a complex disorder caused by a heterozygous deletion of chromosome 17 band p11.2. All patients with SMS have significant sleep disturbances which have been documented by abnormalities on polysomnography in this and previous clinical studies.<sup>5-6</sup> There are no published reports on the circadian pattern of aMT6s in SMS although preliminary data from our study<sup>19</sup> and others<sup>20</sup> have shown an inversion of the aMT6s pattern in SMS patients. Our current data show an inversion of the normal circadian rhythm of melatonin secretion in all SMS patients with the common deletion, and all but one SMS patient with different size deletions. These data correlate with the patients having abnormally high serum melatonin levels during the day and low serum melatonin levels during the night and are consistent with the role of melatonin as a regulator of sleep. Our data suggest there is aberrant melatonin biosynthesis and/or degradation in SMS, and that this abnormality may be the cause of the sleep disturbances in SMS.

The biochemical and neurophysiological pathways involving melatonin production are complex. At present the only known gene within the SMS critical region which was thought to have a potential impact on these pathways is the gene which encodes subunit 3 of the COP9 signal transduction complex, *COPS3*.<sup>24-33</sup> The COP9 signalosome is closely related to the 26S proteasome regulatory complex which is implicated in the control of the metabolism of *N*-acetyltransferase,<sup>34</sup> the rate limiting enzyme in melatonin production. In the present study we show that *COPS3* is deleted in all SMS patients, including patient 1153, a 5 year 2 month old, with a normal melatonin rhythm. Furthermore, we find that

*COPS3* is duplicated in patient 990 who also has a normal melatonin rhythm and no history of sleep disturbances.

Our study documents disturbed circadian rhythm of melatonin metabolism and sleep patterns in SMS and illustrates the need for controlled clinical trials in conjunction with frequent melatonin analysis to determine the efficacy of melatonin treatment in this disorder. Whether or not exogenous melatonin treatment should be administered in conjunction with pharmacological agents to suppress the disturbed endogenous circadian rhythm of melatonin release is something to be considered.<sup>20</sup> The observation of apparently normal melatonin circadian rhythm in one 5 year 2 month old SMS patient who does not have the common deletion but is deleted for *COPS3* suggests that *COPS3* haploinsufficiency alone may not be directly responsible for the disturbed melatonin circadian rhythm. Alternatively, there may be an age dependent penetrance or variability of expression of this phenotype. Regardless, the hypothesis that *COPS3* haploinsufficiency may cause disturbed melatonin circadian rhythm in mammals can be tested directly in *Cops3* heterozygous and homozygous knockout mouse models. Further characterisation of the deletion breakpoints in patient 1153 may define a critical region for abnormal melatonin metabolism, and hence implicate other dosage sensitive gene(s) within the SMS region wherein haploinsufficiency may cause or contribute to this phenotype.

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